

Gene expression in visceral and subcutaneous adipose tissue in overweight women

Mirko Korsic¹, Kristina Gotovac², Matea Nikolac³, Tina Dusek¹, Mate Skegro⁴, Dorotea Muck-Seler³, Fran Borovecki², Nela Pivac³

¹Department of Endocrinology and Metabolic Disorders, Clinical Hospital Center Zagreb, University Hospital Center Zagreb, Zagreb, Croatia, ²Department for Functional Genomics, Center for Translational and Clinical Research, University of Zagreb School of Medicine, and University Hospital Center Zagreb, Zagreb, Croatia, ³Division of Molecular Medicine, Rudjer Boskovic Institute, Zagreb, Croatia, ⁴Department of Hepato-Biliar Surgery and Transplantation of Abdominal Organs, Clinical Hospital Center Zagreb, University Hospital Center Zagreb, Zagreb, Croatia

TABLE OF CONTENTS

1. Abstract
2. Introduction
3. Materials and methods
 - 3.1. Patients
 - 3.2. Gene expression profiling of subcutaneous and visceral fat tissue
 - 3.3. Gene expression analysis by real-time PCR
 - 3.4. Statistical analysis
4. Results
5. Discussion
6. Acknowledgment
7. References

1. ABSTRACT

Excess weight and obesity are common health problems with multifactorial and polygenic causes. Abdominal or visceral obesity is associated with a higher risk of obesity related complications. The aim of the study was to evaluate differentially expressed genes in visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) of 10 overweight women undergoing elective laparoscopic cholecystectomy. Following expression profiling using microarrays, a set of 294 genes that exhibited differential expression between VAT and SAT was further analyzed to test the functional correlation of gene sets using the Gene Set Enrichment Analysis method. To confirm the functional pathways involved in differential expression between SAT and VAT, additional pathway analysis was done using the GeneGo MetaCore software and the Ingenuity Pathway Analysis. Ten differentially expressed genes were selected according to the microarray data, with seven exhibiting significant differential expression in the RT-PCR experiments. The data from this preliminary study suggest enrichment of inflammation and oxidative stress related pathways in VAT, while insulin homeostasis pathways as well as pathways pertaining to several growth factors are enriched in SAT.

2. INTRODUCTION

Overweight and obesity are becoming major health problems worldwide, especially since they significantly contribute to the risk for a number of chronic diseases. Accumulation of VAT, but not SAT is associated with a higher risk of common medical obesity-related complications, such as cardiovascular disease, atherosclerosis, hyperlipidemia, hypertension and type 2 diabetes. Mechanisms responsible for such functional and metabolic differences between these two fat compartments are still unknown. The evidence suggests that VAT is more lipolytically active, resulting in the increased release of the free fatty acids into the portal vein, which are then delivered directly to the liver (1). Visceral fat is also an important source of proinflammatory cytokines, complement factors and components of the coagulation and fibrinolysis pathways (2). These characteristics of VAT might be responsible for its association with the insulin resistance. Accumulation of the visceral fat remains a cardiovascular risk factor, despite the normal values of the body mass index (3), pointing out to the fact that visceral adiposity is important for the individual health risk profile.

Visceral adipose depot represents only 10-20% of total body fat, but, due to its metabolically harmful profile (4), VAT may be responsible for a higher risk of developing metabolic disorders. Obese individuals with fat stored predominantly in visceral adipose depots suffer greater obesity-associated metabolic consequences than those with fat stored predominantly in subcutaneous fat areas (5). Depot-specific differences, resulting from different gene expression patterns of visceral and subcutaneous adipocytes, could be responsible for the obesity-related complications. There is evidence of a different gene expression profile between VAT and SAT involving genes included in cell cycle, transcription, cell to cell interaction, metabolism, signal transduction and cytokine/receptor secretion (6).

The aim of the study was to analyze differences in gene expression profiles between VAT and SAT, and to identify functional pathways that might be involved in the regulation of this differential expression. The hypothesis was that these newly-identified genes sets may contribute to the functional and metabolic differences between these two fat compartments, and that they might offer us a new approach in prevention and treatment of obesity. In comparison to previously published studies (6,7), our study for the first time utilized advanced statistical methods to ascertain enrichment of functional gene sets and signaling pathways in an effort to identify differential activation of pathways specific for the two fat compartments.

3. MATERIALS AND METHODS

3.1. Patients

The study group was comprised of 10 postmenopausal women undergoing elective laparoscopic cholecystectomy at University Hospital Center 'Zagreb' (Zagreb, Croatia). All study participants underwent careful clinical examination including measurement of body weight, height, waist and hip circumferences, and resting blood pressure. None of the patients had any identified chronic diseases including type 2 diabetes, hypertension (defined as blood pressure higher than 140/90 mmHg or taking antihypertensive therapy), and dyslipidemia (defined as lipid values higher than average normal according to the local laboratory reference range: total cholesterol levels <5.0 mmol/L; low-density lipoprotein cholesterol <3.9 mmol/L; high-density lipoprotein cholesterol >1.2 mmol/L; triglycerides <1.7 mmol/L). None of the patients received statin therapy, or any chronic medication besides occasional therapy with spasmolytics and non-steroidal antirheumatic agents (none of the medications was taken one week before the surgical procedure). None of the subjects had been involved in a weight reduction program in the last 6 months, and their body weight was stable at the time of study. Subjects had fasted overnight before the surgical procedure. General balanced anesthesia was induced by a short-acting barbiturate and maintained by fentanyl and a mixture of oxygen and nitrous oxide. Adipose tissue samples were obtained within 30 minutes from the beginning of the surgery. First sample

representing SAT was taken from the abdominal subcutaneous wall (close to the umbilicus) and the second sample representing VAT was taken from the greater omentum. The size of the adipose tissue samples was about 1 cm³. All study participants signed informed consent before entering the study. The study was approved by the local Ethics Committee.

3.2. Gene expression profiling of subcutaneous and visceral fat tissue

RNA was isolated from SAT and VAT samples collected from 10 patients using the RNeasy kit (Qiagen) according to the manufacturer's protocol. All samples were treated with the RNase-free DNase set (Qiagen). Total RNA quality was analyzed by RNA 6000 Nano LabChip kit on a 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). For the gene expression profiling experiments, SAT and VAT samples from 5 patients were analyzed using the Human Genome U133 PLUS 2.0 Arrays containing 54,675 25-mer probes. The RNA was processed using the standard One-Cycle cDNA Synthesis Affymetrix protocol. All arrays were run at the Department for Functional Genomics, School of Medicine, and University of Zagreb.

3.3. Gene expression analysis by real-time PCR

Quantitative RT-PCR was performed using 2 µg of total RNA isolated from SAT and VAT from 10 patients. The RNA was transcribed using the Superscript First-Strand Synthesis System (Invitrogen, Carlsbad, California, United States of America) with random hexamer primers according to the manufacture's protocol. Gene expressions of interest were measured using LightCycler FastStart DNA Master SYBR Green kit (Roche Diagnostics, Mannheim, Germany) in the LightCycler instrument (Roche Diagnostics, Mannheim, Germany) as described previously (8). Results were represented as fold change of comparative expression level. Sequences of primers are shown in Table 1.

3.4. Statistical analysis

The statistical analysis of the clinical characteristics of subjects, expressed as means ± SD, was conducted with Sigmaplot 3.5 (Jandel Scientific Corp. San Raphael, California, USA). The gene expression profiling data were filtered using the two-sided Student *t* test with *P* <0.05 and an expression ratio of average visceral/average subcutaneous tissue >1.4 or <0.7 as cut-off values.

Furthermore, in an effort to test the functional correlation of gene sets that might be systemically altered in VAT when compared to SAT, we applied a statistical method entitled Gene Set Enrichment Analysis. We analyzed complete microarray data set to calculate the enrichment scores of predefined gene sets (MSigDB2, Broad Institute, Boston, USA) containing genes involved in specific metabolic and signaling pathways. Estimated statistical significance (nominal *P* value) of the enrichment scores was also calculated using an empirical phenotype-based permutation test procedure that preserves the complex correlation structure of the gene expression data. A nominal *P* value ofin the analysis.

Gene expression profiling of fat depots in women

Table 1. Sequences of primers used in QRT-PCR gene expression analysis

Gene	5'→3' Forward
	5'→3' Reverse
ALDH1A3	GCATGAGCCCATTTGGTGTCT
	CGCAGGCTTCAGGACCAT
CDH1	TGAAGGTGACAGAGCCTCTGGAT
	TGGGTGAATTCGGGCTTGTT
EGFL6	CGGGTGTGTAGCATCGGCA
	ATGTAGCTTCACAGACTCCCT
FGF9	ATGGCTCCCTTAGGTGAAGTT
	AGGTGGTCACTTAACAAAACCG
FGFR2	CTTGCCTTGAGAATGGTCGTC
	AGTCTGGGGAAGCTGTAATCT
GDF10	TCGACCAGAAGGCCGTGTAT
	AGAAGTGAAAGTGCCGTAAG
HOXC10	CTATCCGCTCTACCTCTCGCA
	ACATGCAGCAGACATTCTCT
PTGFR	GCACATTGATGGGCAACTAGAA
	GCACCTATCATTTGGCATGTAGCT
WNT5A	AGAAGAACTGTGCCACTTGTATCAG
	CCTTCGATGTCCGGAATTGATACT
WT1	GCTGTCCCACTTACAGATGCA
	TCAAAGCGCCAGCTGGAGTTT
β-ACTIN	GTGGGCCGCTCTAGGCACCAA
	CTCTTTGATGTACGCACGATTTC

Table 2. Clinical characteristics of the 10 women undergoing elective laparoscopic cholecystectomy

Clinical characteristics	Mean ± SD	Range
Age (years)	61.50 ± 7.42	45 - 69
Body weight (kg)	78.40 ± 5.78	70 - 90
Height (cm)	164.50 ± 3.95	159 - 170
BMI (kg/m ²)	28.97 ± 1.76	26.1 - 31.9
Waist circumference (cm)	92.80 ± 5.92	82 - 100
Hip circumference (cm)	102.10 ± 4.73	94 - 110
Waist to hip ratio	0.909 ± 0.033	0.86 - 0.98

Finally, in an effort to ascertain the functional pathways which may be regulating differential expression in SAT and VAT, we performed pathway analysis using the GeneGo MetaCore software package (GeneGo bioinformatics software, Inc.) and the Ingenuity Pathway Analysis-IPA (Ingenuity® Systems, www.ingenuity.com). In the GeneGo analysis the Affymetrix ID number, *P* value and fold change data were uploaded into MetaCore and a workflow data analysis report performed, resulting in the list of most significantly enriched pathways. The Ingenuity Pathway Analysis was performed by uploading the aforementioned microarray data into the commercially available IPA software v.7.6 for biologic function and pathway analysis. IPA is built upon a very large manually-curated and up-to-date database of genes, proteins, functions, interactions/networks, and pathways. The IPA “Core Analysis” was performed using the Ingenuity Knowledge Base (Genes only) as the reference set, using direct and indirect relationships for network analysis, and data from mammal species, and all tissues, cell lines and data sources.

4. RESULTS

Clinical characteristics of patient demographic data, expressed as means ± SD, are shown in Table 2. Microarray analysis of VAT and SAT revealed 294 differentially expressed genes using the statistical cut-off values of *P* <0.05 and an expression ratio of average

visceral/average subcutaneous tissue sample >1.4 or <0.7. Among the differentially expressed genes, 158 exhibited increased and 136 decreased expression in visceral compared to subcutaneous fat depot. Within the group of genes with increased differential expression in VAT were those pertaining to the cell cycle, classical complement activation, wntless (WNT) signaling, mitogen activated protein kinase (MAPK) cascade and eicosanoid synthesis, while down-regulated genes belonged to the integrin-mediated cell adhesion, glycogen metabolism and specificity protein 1 signaling pathways.

Gene set enrichment analysis revealed 67 gene sets significantly enriched in VAT and 23 gene sets significantly enriched in SAT. The gene sets enriched in VAT samples included, among others, the phosphatidylinositol signaling system, chemotaxis, calcineurin pathway, heat shock protein 27 pathway, inducible nitric oxide synthase pathway, oxidative stress pathway, WNT signaling, cell adhesion receptor activity and reactive oxygen species. On the other hand, the gene sets enriched in SAT samples included genes involved in the insulin-like growth factor (IGF)-1 pathway, insulin pathway, integrin pathway, as well as interleukin (IL)-6 and IL-3 and tumor necrosis factors (TNF) pathways.

Finally, in concordance with the previous results, pathway analysis using the MetaCore GeneGo software package revealed increased enrichment of pathways belonging to increased inflammatory activation in VAT, namely through complement activation (classical complement activation, lectin induced complement pathway and alternative complement pathway) as shown in Figure 1, and cell adhesion (cell adhesion – extracellular matrix remodeling pathway and cell adhesion – urokinase-type plasminogen activator signaling). Additionally, pathways involved in the epithelial-mesenchymal transition were also enriched: hypoxia induced epithelial-mesenchymal transition, transforming growth factor (TGF)-beta-dependent induction of epithelial-mesenchymal transition via MAPK. Furthermore, several WNT associated signaling pathways were significantly enriched, in particular WNT signaling pathway and wntless homolog WNT5A signaling pathway (Figure 2). Finally, within the SAT pathways, several growth factors and hormones exhibited significant activation, namely the fibroblast growth factor (FGF)-family signaling, TGF signaling, as well as leptin signaling via janus kinase/signal transducers and activators (STAT) and MAPK cascades and androgen receptor nuclear signaling.

To correlate expression findings with relevant biological processes and pathways, we performed pathway analysis using the manually-curated literature-based Ingenuity database. The top ten canonical pathways over-represented among our differentially expressed genes were complement system (6 molecules), oncostatin M signaling (5 molecules), ascorbate and aldarate metabolism (3 molecules), CDK5 signaling (7 molecules), CNTF signaling (5 molecules), bile acid biosynthesis (5 molecules), 14-3-3-mediated signaling (8 molecules), acute phase response signaling (10 molecules), HER-2 signaling

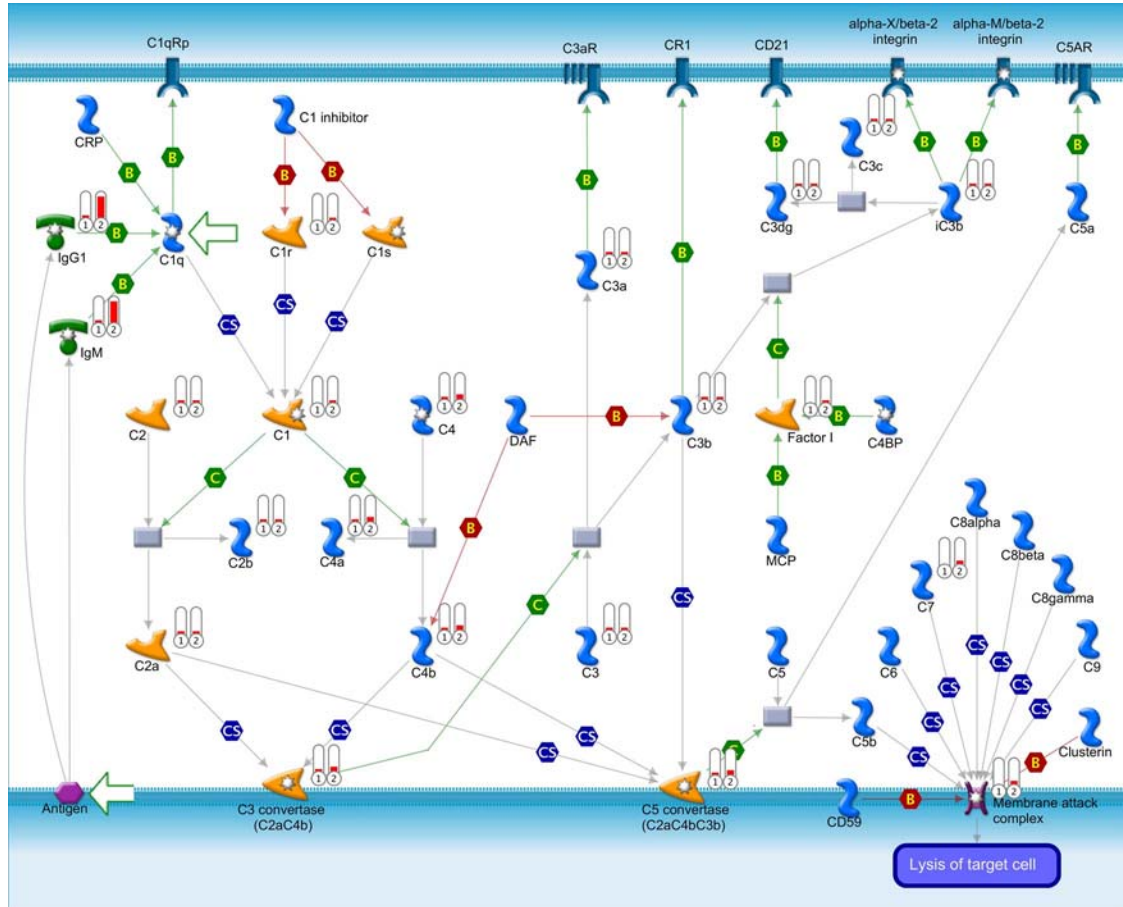


Figure 1. Pathway analysis of the Classical complement activation cascade. Analysis of the Classical complement activation pathway exhibits significant activation of the complement cascade. Statistically significantly expressed genes are designated by a double bar. Bar number 1 represents the *P* value, while the bar number 2 represents fold change.

in breast cancer (6 molecules), and thrombopoietin signaling (5 molecules). Among the top 5 networks activated among the differentially expressed genes were Infectious Disease, Connective Tissue Disorders, Immunological Disease (Figure 3), containing a large number of complement related genes and Cell-To-Cell Signaling and Interaction network indicating the central role of WNT5A overexpression.

In order to confirm the differential expression observed in microarray experiments, we performed QRT-PCR analysis of ten representative genes: epidermal growth factor (EGF)-like domain, multiple 6 (EGFL6), FGF receptor 2 (FGFR2), prostaglandin F receptor (PTGFR), WNT5A, growth differentiation factor 10 (GDF-10), FGF9, aldehyde dehydrogenase 1 family, member A3 (ALDH1A3), Wilms' tumor suppressor 1 (WT1), cadherin 1, type 1 (CDH1) and homeobox C10 protein (HOXC10). The genes were selected according to individual statistical significance of differential expression, but they were also chosen to represent significantly enriched gene-sets and pathways derived from subsequent statistical analyses. These pathways included eicosanoid synthesis, cell adhesion, growth factor signaling (EGF, FGF, and TGF),

WNT signaling and energy metabolism. Of the ten genes, all exhibited differential expression in concordance with that observed in microarray experiments, with seven (WT1, CDH1, ALDH1A3, FGFR2, GDF10, EGFL6, HOXC10) showing statistically significant differential mRNA expression (Table 3).

5. DISCUSSION

The results of the microarray analysis exhibited differential expression of numerous large numbers of genes between VAT and SAT in overweight/obese female subjects. Differentially expressed genes included those involved in cytokine/receptor secretion, cell adhesion, cell cycle, transcription, signal transduction, transport, cell growth and morphogenesis, cytoskeleton, lipid metabolism, prostaglandin metabolic processes and others. Differences between VAT and SAT were confirmed by the Gene Set Enrichment Analysis that revealed 23 gene sets enriched in SAT. These were genes involved in IGF-1 pathway, insulin pathway, integrin pathway, and IL-6, IL-3 and TNF pathways. On the other hand, VAT related enriched gene sets included the phosphatidylinositol signaling system, chemotaxis, calcineurin pathway, heat shock protein 27

Table 3. Differential expression in a subset of genes selected for validation measured by real-time PCR

Symbol	Accession No.	Description	Fold Change	P value
WT1	NM 000378.4	Wilms tumor 1	3.98	0.04
CDH1	NM 004360.3	E-cadherin	2.85	0.05
ALDH1A3	NM 000693.2	aldehyde dehydrogenase 1 family, member A3	2.55	0.01
FGF9	NM 002010.2	fibroblast growth factor 9	1.68	0.45
WNT5A	NM 003392.3	wingless-type MMTV integration site family, member 5A	1.20	0.86
PTGFR	NM 000959.3	prostaglandin F receptor	-2.70	0.07
FGFR2	NM 000141.4	fibroblast growth factor receptor 2	-4.00	0.03
GDF10	NM 004962.3	growth differentiation factor 10	-4.76	0.03
EGFL6	NM 001167890.1	EGF-like-domain, multiple 6	-8.33	0.02
HOXC10	NM 017409.3	homeobox C10	-11.11	0.02

pathway, inducible nitric oxide synthase pathway, oxidative stress pathway, WNT signaling, cell adhesion receptor activity and reactive oxygen species. Furthermore, these finding were confirmed using the GeneGo software package which showed increased enrichment of the complement system, cell adhesion and WNT signaling pathways in VAT, while SAT samples exhibited increased enrichment of growth factor and leptin related pathways.

Therefore, our study provided additional evidence of the significant differences in the expression patterns between VAT and SAT, since VAT had a significantly higher expression of genes involved in inflammation- and oxidative stress-related pathways, while SAT included higher expression of the genes related to insulin homeostasis pathways (IGF-1, insulin, integrin, IL-6 and IL-3 and TNF). Furthermore, in comparison to previously published studies (6,7), our study for the first time utilized advanced statistical methods to ascertain enrichment of functional gene sets and signaling pathways in an effort to identify differential activation of pathways specific for the two fat compartments.

Adipose tissue is an active endocrine organ involved in energy homeostasis (2). Adipose cells and immune cells, which are recruited to adipose tissue as a result of the increased fat mass, produce inflammatory cytokines and chemokines (9). When the activation of proinflammatory pathways exceeds the effect of antiinflammatory adipokines, it results in impaired triglyceride storage and increased release of free fatty acids, all leading to induced insulin resistance and altered glucose and lipid metabolism (9). The chronic inflammation, which has been associated with obesity (9), might be linked to VAT, since this fat depot expands in response to chronic positive energy balance (10). Our data suggest increased activation of the inflammatory signaling pathways in visceral fat depot, namely through complement activation (Figure 1) and cell adhesion functional pathways. High expression of complement-related genes in intra-abdominal adipose tissue was found, suggesting that the complement system might be involved in the development of visceral adiposity and associated with the metabolic complications of visceral obesity (11). The expression of the components of the alternative pathways, such as C3, factor D/adipsin and factor B, was shown to be dysregulated in obesity and insulin resistance (12). The lectin pathway was also associated with obesity and insulin resistance (13). Zhang and colleagues (14) demonstrated the obesity-related excessive activation of the classical pathway of complement.

Furthermore, oxidative stress pertaining genes also exhibited increased expression in visceral tissue samples within our study. Oxidative stress, obesity and obesity-related diseases are inter-related. There are several possible sources of oxidative stress in obesity: hyperglycemia, increased muscle activity required for carrying excessive body weight, elevated tissue lipid levels, inadequate antioxidant defenses, chronic inflammation, endothelial reactive oxygen species production and hyperleptinemia (15). Inflammation and oxidative stress in adipose tissue affect adipocyte gene expression, thus modulating adipocyte lipid metabolism and triglyceride content (16), resulting in the systemic increase in circulating inflammatory cytokines and reactive oxygen species, that impact systemic insulin action and substrate metabolism (17,18). These results were further confirmed in our QRT-PCR experiments, namely CDH1, a calcium-dependent cell-cell adhesion molecule expressed in adherent junctions between epithelial cells (19) which exhibited higher expression in VAT. This calcium-dependant transmembrane glycoprotein is required for the maintenance of solid tissues and it plays an important role in cell recognition and cell sorting during development. The zinc-finger transcription factor snail homolog 2 represses CDH1 expression and induces epithelial-mesenchymal transition (20), the first step towards preadipocyte differentiation from epithelial cells.

Different expression pattern between VAT and SAT also included energy metabolism enzymes like aldehyde dehydrogenase isozymes. We have detected a higher expression of ALDH1A3 in VAT. This enzyme is involved in detoxification of aldehydes and products of oxygen free radical metabolism (21). It also catalyzes the oxidation of retinal to retinoic acid, which then regulates the transcription of genes important for morphological development, proper cellular differentiation and growth control. In preadipocytes retinoic acid acts through activating the nuclear retinoic acid receptor and cellular retinoic acid-binding protein 2 whose repression is critical for allowing adipogenesis to proceed (22).

Our collective data also suggested increased WNT signaling and enriched hormone regulation pathways (mostly through leptin and androgen signaling) in VAT. WNT family members are secreted glycoproteins that regulate cell proliferation, survival, fate and behavior through paracrine and autocrine signaling. Canonical WNT (WNT/beta-catenin) signaling cascade has been implicated in the regulation of adipocyte differentiation (23). Activation of WNT/beta-catenin signaling results in



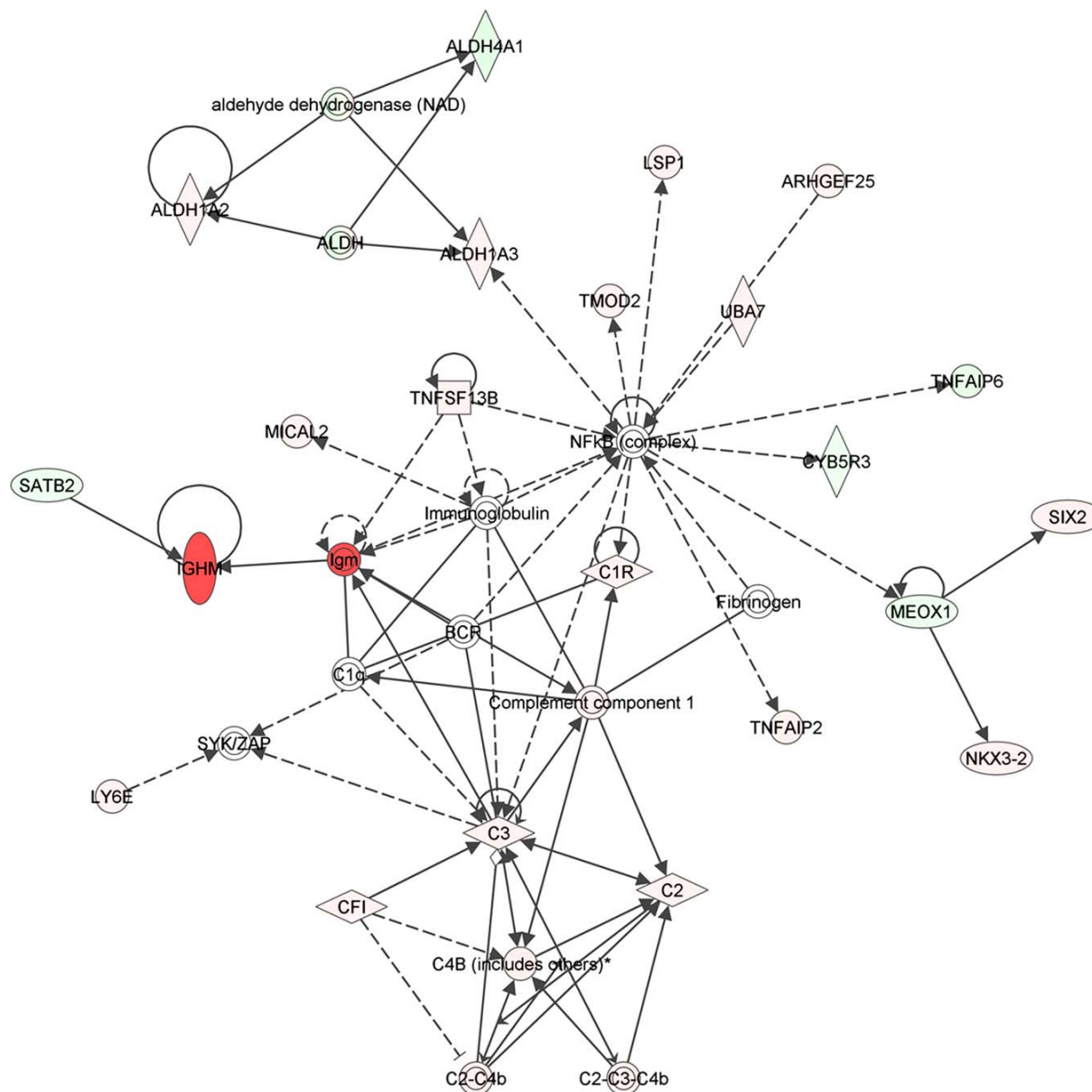


Figure 3. IPA analysis of the Immune response related genes. A graphical representation of the molecular relationships between genes involved in the immune response. Molecules are represented as nodes, and the biological relationship between two nodes is represented as a line. All edges are supported by at least one reference from the literature, from a textbook, or from canonical information stored in the Ingenuity Knowledge Base. The intensity of the node color indicates the degree of up- (red) or down- (green) regulation. Nodes are displayed using various shapes that represent the functional class of the gene product.

hypophosphorylation of beta-catenin which then translocates to the nucleus, binds to transcription factors and activates WNT target genes thus inhibiting adipogenesis (23). The exact role of non-canonical WNT signaling is still unknown but there are indications that its role might be just the opposite to the role of the canonical pathway. Namely, it has been demonstrated that WNT members like WNT5A, which are involved in non-canonical WNT pathway, play a role in promoting differentiation in the early stage of adipogenesis (24). The

members of the non-canonical WNT signaling pathway promote adipogenesis probably by antagonizing the canonical pathway (23). The balance between WNT5A and secreted frizzled-related protein 5 is important for the control of the degree of inflammation and insulin sensitivity in adipocytes. In obese people the adipocyte volume increases, and they secrete more WNT5A and less secreted frizzled-related protein 5 which leads to an increase in the inflammatory signaling and insulin insensitivity (25). There have been suggestions that WNT

signaling pathway is more active and/or responsive in VAT (6).

On the other hand, within the SAT our results indicated a higher expression of the genes related to insulin homeostasis pathways. Among its well-known functions, insulin also plays an important role in modulating adipose tissue growth and differentiation by controlling transcriptional factors like sterol regulatory element-binding protein 1 and peroxisome proliferator-activated receptor, gamma (26). Visceral fat is less sensitive to antilipolytic action of insulin than subcutaneous fat, suggesting that visceral depot has a less efficient intracellular insulin signaling system (26). The QRT-PCR analysis of ten representative genes, among others, showed a higher *WT1* expression in VAT. *WT1* activates genes included in differentiation, cell cycle and apoptosis pathways and it is critical in the formation of the kidney and gonads. The *WT1* factor inhibits the expression of certain growth factors such as IGF-2 and its type 1 IGF receptor, which is constitutively expressed by most body tissues and plays a significant role in regulating cell proliferation (27). There is evidence of the possible role of IGF-2 in adipose tissue development (28).

Adipose tissue plays an important role in energy homeostasis by secreting leptin, adiponectin, angiotensinogen, TNF-alpha, IL-6, adipsin and other factors. The studies have showed that subcutaneous fat depot is the major source of leptin (29). Leptin levels generally increase with the size of adipose tissue and, since it has the ability to cross the blood-brain barrier, leptin functions as a fasting as well as a satiety signal (30). Leptin signal is transmitted through janus kinase/STAT and MAPK signaling cascades. Interaction between leptin and STAT3 has a certain role in energy balance, reproduction, growth and glucose regulation (31). In adipose tissue, leptin increases STAT1 and STAT3 phosphorylation and, to a lesser degree, activates MAPK and phosphoinositide 3-kinases (32). There are indications of a possible cross-talk between leptin and insulin signaling pathways, resulting in an opposite effect of leptin and insulin on lipolysis (33).

There are a lot of indications that sex hormones participate in the adipose tissue metabolism (34). Sex hormones affect the expression of adipogenic transcription factors; modulate the adipocyte proliferation, glucose metabolism and the expression of several adipocyte hormones. Androgen receptors deficiency was associated with chronic positive energy balance and obesity (35). Differences in the androgen-modulated adipokines could be explained by a higher responsiveness of visceral fat to androgens compared to subcutaneous fat, resulting in a higher lipolytic activity of visceral depot (36).

Additionally, growth factors, such as members of FGF and TGF family, also seem to be included in the regulation of adipose tissue. FGFs are a large family of growth and differentiation factors playing a diverse role in development, regulating cell proliferation, migration, survival and differentiation. In adult organisms, FGFs are important in tissue and organ repair, but also in regulating neuronal signal transduction, neuroprotection, learning and

memory. Signaling through FGFR2b is crucial for both the proliferation and differentiation of pre-adipocytes in white adipose tissue (37), and signaling through FGFR2c plays a role in the hypertrophy of mature adipocytes at postnatal stages (38), indicating an important role of FGF signaling in the white adipose tissue development.

TGF-beta is a protein that exists in three isoforms known as TGF-beta₁, TGF-beta₂ and TGF-beta₃, which are involved in the control of proliferation, cellular differentiation, and other cell functions. Enhanced release of TGF-beta₁, that was associated with obesity, might be a response to an enhanced release of inflammatory cytokines such as interleukin 8 in obesity (39). TGF-beta₁ is an anti-inflammatory cytokine that inhibits differentiation of pre-adipocytes and is also associated with increased insulin resistance in obesity. The QRT-PCR analysis within our study revealed that the growth factor GDF-10, which is member of TGF-beta superfamily and closely related to bone morphogenetic protein 3, also showed a higher expression in SAT. Stewart and al. (40) demonstrated that bone morphogenetic protein 3 stimulates mesenchymal stem cell proliferation through TGF-beta/activin pathway raising the possibility of implicating GDF-10 in mechanisms of adipogenesis.

The EGFL6, a member of the EGF repeat superfamily, was also differentially expressed, with higher expression rate in SAT. The *EGFL6* gene product contains a signal peptide and displays characteristics of an extracellular protein believed to have an adhesive function. This gene showed an increased expression in adipose tissue of obese patients, and these findings identified EGFL6 as a paracrine/autocrine growth factor responsible for increased proliferation of preadipocytes, stimulating further adipose tissue expansion in obesity through hyperplasia (41).

There are a few limitations of our study that should be taken in consideration. Our expression analysis was limited to mRNA expression, since we didn't analyze the expression at the protein level. Also, the limits are the small sample size and a possibility that some of the differentially expressed genes were isolated from cells other than adipocytes, since we used whole adipose tissue samples.

The data from this preliminary study show increased enrichment of inflammation and oxidative stress related pathways in VAT, while insulin homeostasis pathways as well as pathways pertaining to several growth factors seem to be enriched exclusively in SAT. These data, confirmed by the use of advanced statistical methods that ascertained enrichment of functional gene sets and signaling pathways, suggest the existence of a possible bi-directional relationship between systemic inflammation, oxidative stress, overweight and abdominal obesity. In addition to prevention strategies such as change in sedentary lifestyle, calorie restriction and increase of physical activity and exercise, we might speculate that further therapeutic strategies aimed to treat obesity should be focused also on reducing inflammatory and oxidative stress activities.

6. ACKNOWLEDGMENTS

This work was supported by grants from the Croatian Ministry of Sciences, Education and Sports (no. 108-0000000-0162; 098-0982522-2455; 098-0982522-2457; and 108-1081874-1923). Fran Borovecki and Nela Pivac contributed equally to this work.

7. REFERENCES

1. Vanessa van Harmelen, Andrea Dicker, Mikael Ryden, Hans Hauner, Fredrik Lonnqvist, Erik Naslund, Peter Arner: Increased lipolysis and decreased leptin production by human omental as compared with subcutaneous preadipocytes. *Diabetes* 51, 2029-2036 (2002)
2. Rexford S. Ahima, Jeffery S Flier: Adipose tissue as an endocrine organ. *Trends Endocrinol Metab* 11, 327-332 (2000)
3. Clinical Guidelines on the Identification, Evaluation, and Treatment of Overweight and Obesity in Adults--The Evidence Report. National Institutes of Health. *Obes Res* 6 Suppl 2, 51S-209S (1998)
4. Delma J Nieves, Miriam Cnop, Barbara Retzlaff, Carolyn E Walden, John D Brunzell, Robert H Knopp, Steven E Kahn: The atherogenic lipoprotein profile associated with obesity and insulin resistance is largely attributable to intra-abdominal fat. *Diabetes* 52, 172-179 (2003)
5. Carl T Montague, Stephen O'Rahilly: The perils of portliness: causes and consequences of visceral adiposity. *Diabetes* 49, 883-888 (2000)
6. Marie-Claude Vohl, Rou Sladek, Julie Robitaille, Scott Gurd, Picard Marceau, Denis Richard, Thomas J Hudson, Andre Tchernof: A survey of genes differentially expressed in subcutaneous and visceral adipose tissue in men. *Obes Res* 12, 1217-1222 (2004)
7. Kristina Linder, Peter Arner, Amilcar Flores-Morales, Petra Tollet-Egnell, Gunnar Norstedt: Differentially expressed genes in visceral or subcutaneous adipose tissue of obese men and women. *J Lipid Res* 45, 148-154 (2004)
8. Kenneth J Livak, Thomas D Schmittgen: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods* 25, 402-408 (2001)
9. Haiyan Xu, Glenn T Barnes, Qing Yang, Guo Tan, Daseng Yang, Chieh J Chou, Jason Sole, Andrew Nichols, Jeffrey S Ross, Louis A Tartaglia, Hong Chen: Chronic inflammation in fat plays a crucial role in the development of obesity-related insulin resistance. *J Clin Invest* 112, 1821-1830 (2003)
10. Claude Bouchard, Angelo Tremblay, Jean-Pierre Despres, Andre Nadeau, Paul J Lupien, Germain Theriault, Jean Dussault, Sital Moorjani, Sylvie Pinault, Guy Fournier: The response to long-term overfeeding in identical twins. *N Engl J Med* 322, 1477-1482 (1990)
11. Britt G Gabrielsson, Jenny M Johansson, Malin Lonn, Margareta Jernas, Torsten Olbers, Markku Peltonen, Ingrid Larsson, Lars Lonn, Lars Sjostrom, Bjorn Carlsson, Lena M. Carlsson: High expression of complement components in omental adipose tissue in obese men. *Obes Res* 11, 699-708 (2003)
12. Lisa N Choy, Bruce M Spiegelman: Regulation of alternative pathway activation and C3a production by adipose cells. *Obes Res* 4, 521-532 (1996)
13. Jose Manuel Fernandez-Real, Marek Strackowski, Joan Vendrell, Federico Soriguer, Sofia Perez Del Pulgar, Luis Gallart, Abel Lopez-Bermejo, Irina Kowalska, Melania Manco, Fernando Cardona, Maria M Garcia-Gil, Geltrude Mingrone, Cristobal Richart, Wifredo Ricart, Antonio Zorzano: Protection from inflammatory disease in insulin resistance: the role of mannan-binding lectin. *Diabetologia* 49, 2402-2411 (2006)
14. Jinhui Zhang, Wendy Wright, David A Bernlohr, Samuel W Cushman, Xiaoli Chen: Alterations of the classic pathway of complement in adipose tissue of obesity and insulin resistance. *Am J Physiol Endocrinol Metab* 292, E1433-1440 (2007)
15. Heather K Vincent, Ann G Taylor: Biomarkers and potential mechanisms of obesity-induced oxidant stress in humans. *Int J Obes (Lond)* 30, 400-418 (2006)
16. Doris Joy Espiritu, Theodore Mazzone: Oxidative stress regulates adipocyte apolipoprotein e and suppresses its expression in obesity. *Diabetes* 57, 2992-2998 (2008)
17. Erin E Kershaw, Jeffrey S Flier: Adipose tissue as an endocrine organ. *J Clin Endocrinol Metab* 89, 2548-2556 (2004)
18. Hideki Urakawa, Akira Katsuki, Yasuhiro Sumida, Esteban C Gabazza, Shuichi Murashima, Kohei Morioka, Noriko Maruyama, Nagako Kitagawa, Takashi Tanaka, Yasuko Hori, Kaname Nakatani, Yutaka Yano, Yukihiko Adachi: Oxidative stress is associated with adiposity and insulin resistance in men. *J Clin Endocrinol Metab* 88, 4673-4676 (2003)
19. Nives Pecina-Slaus: Tumor suppressor gene E-cadherin and its role in normal and malignant cells. *Cancer Cell International* 3, 17 (2003)
20. Victoria Bolos, Hector Peinado, Mirna A Perez-Moreno, Mario F Fraga, Manel Esteller, Amparo Cano: The transcription factor Slug represses E-cadherin expression and induces epithelial to mesenchymal transitions: a comparison with Snail and E47 repressors. *J Cell Sci* 116, 499-511 (2003)
21. Lily C Hsu, Wen-Chung Chang, Lea Hiraoka, Chih-Lin Hsieh: Molecular cloning, genomic organization, and

Gene expression profiling of fat depots in women

chromosomal localization of an additional human aldehyde dehydrogenase gene, ALDH6. *Genomics* 24, 333-341 (1994)

22. Daniel C Berry, Hooman Soltanian, Noa Noy: Repression of cellular retinoic acid-binding protein II during adipocyte differentiation. *J Biol Chem* 285, 15324-15332 (2010)

23. Sarah E Ross, Nahid Hemati, Kenneth A Longo, Christina N Bennett, Peter C Lucas, Robin L Erickson, Ormond A MacDougald: Inhibition of adipogenesis by Wnt signaling. *Science* 289, 950-953 (2000)

24. Makoto Nishizuka, Akiko Koyanagi, Shigehiro Osada, Masayoshi Imagawa: Wnt4 and Wnt5a promote adipocyte differentiation. *FEBS Lett* 582, 3201-3205 (2008)

25. Noriyuki Ouchi, Akiko Higuchi, Koji Ohashi, Yuichi Oshima, Noyan Gokce, Rei Shibata, Yuichi Akasaki, Akihiko Shimono, Kenneth Walsh: Sfrp5 is an anti-inflammatory adipokine that modulates metabolic dysfunction in obesity. *Science* 329, 454-457 (2010)

26. Luigi Laviola, Sebastio Perrini, Angelo Cignarelli, Annalisa Natalicchio, Anna Leonardini, Francesca De Stefano, Marilena Cuscito, Michele De Fazio, Vincenzo Memeo, Vincenzo Neri, Mauro Cignarelli, Riccardo Giorgino, Francesco Giorgino: Insulin signaling in human visceral and subcutaneous adipose tissue *in vivo*. *Diabetes* 55, 952-961 (2006)

27. Gangyi Dong, Roopmathy Rajah, Thanh Vu, Andrew R Hoffman, Ron G Rosenfeld, Charles T Roberts, Jr, Donna M Peehl, Pinchas Cohen: Decreased expression of Wilms' tumor gene WT-1 and elevated expression of insulin growth factor-II (IGF-II) and type 1 IGF receptor genes in prostatic stromal cells from patients with benign prostatic hyperplasia. *J Clin Endocrinol Metab* 82, 2198-2203 (1997)

28. Carine Nezer, Laurence Moreau, Benoit Brouwers, Wouter Coppieters, Johann Dettelleux, Roger Hanset, Latifa Karim, Alex Kvasz, Pascal Leroy, Michael Georges: An imprinted QTL with major effect on muscle mass and fat deposition maps to the IGF2 locus in pigs. *Nat Genet* 21, 155-156 (1999)

29. Vanessa Van Harmelen, Signy Reynisdottir, Per Eriksson, Anders Thorne, Johan Hoffstedt, Fredrik Lonnqvist, Peter Arner: Leptin secretion from subcutaneous and visceral adipose tissue in women. *Diabetes* 47, 913-917 (1998)

30. William A Banks, Christopher R DiPalma, Catherine L Farrell: Impaired transport of leptin across the blood-brain barrier in obesity. *Peptides* 20, 1341-1345 (1999)

31. Sarah H Bates, Walter H Stearns, Trevor A Dundon, Markus Schubert, Annette W K Tso, Yongping Wang, Alexander S Banks, Hugh J Lavery, Asma K Haq, Eleftheria Maratos-Flier, Benjamin G Neel, Michael W

Schwartz, Martin G Myers, Jr: STAT3 signalling is required for leptin regulation of energy balance but not reproduction. *Nature* 421, 856-859 (2003)

32. Christian Bjorbaek, Barbara B Kahn: Leptin signaling in the central nervous system and the periphery. *Recent Prog Horm Res* 59, 305-331 (2004)

33. Young-Bum Kim, Shigeo Uotani, Dominique D Pierroz, Jeffrey S Flier, Barbara B Kahn: *In vivo* administration of leptin activates signal transduction directly in insulin-sensitive tissues: overlapping but distinct pathways from insulin. *Endocrinology* 141, 2328-2339 (2000)

34. Marta Monjo, Ana M Rodriguez, Andreu Palou, Pilar Roca: Direct effects of testosterone, 17 beta-estradiol, and progesterone on adrenergic regulation in cultured brown adipocytes: potential mechanism for gender-dependent thermogenesis. *Endocrinology* 144, 4923-4930 (2003)

35. WuQiang Fan, Toshihiko Yanase, Masatoshi Nomura, Taijiro Okabe, Kiminobu Goto, Takashi Sato, Hirotaka Kawano, Shigeaki Kato, Hajime Nawata: Androgen receptor null male mice develop late-onset obesity caused by decreased energy expenditure and lipolytic activity but show normal insulin sensitivity with high adiponectin secretion. *Diabetes* 54, 1000-1008 (2005)

36. Sergio Rodriguez-Cuenca, Marta Monjo, Ana M Proenza, Pilar Roca: Depot differences in steroid receptor expression in adipose tissue: possible role of the local steroid milieu. *Am J Physiol Endocrinol Metab* 288, E200-207 (2005)

37. Morichika Konishi, Toshiyuki Asaki, Naomi Koike, Hiroyuki Miwa, Ayumi Miyake, Nobuyuki Itoh: Role of Fgf10 in cell proliferation in white adipose tissue. *Mol Cell Endocrinol* 249, 71-77 (2006)

38. Morichika Konishi, Hirotoshi Nakamura, Hiroyuki Miwa, Pierre Chambon, David M Ornitz, Nobuyuki Itoh: Role of Fgf receptor 2c in adipocyte hypertrophy in mesenteric white adipose tissue. *Mol Cell Endocrinol* 287, 13-19 (2008)

39. John N Fain, David S Tichansky, Atul K Madan: Transforming growth factor beta1 release by human adipose tissue is enhanced in obesity. *Metabolism* 54, 1546-1551 (2005)

40. Aaron Stewart, Haiyan Guan, Kaiping Yang: BMP-3 promotes mesenchymal stem cell proliferation through the TGF-beta/activin signaling pathway. *J Cell Physiol* 223, 658-666 (2010)

41. Rupert Oberauer, Wolfgang Rist, Martin C Lenter, Bradford S Hamilton, Heike Neubauer: EGFL6 is increasingly expressed in human obesity and promotes proliferation of adipose tissue-derived stromal vascular cells. *Mol Cell Biochem* 343, 257-269 (2010)

Gene expression profiling of fat depots in women

Abbreviations: SAT: subcutaneous adipose tissue; VAT: visceral adipose tissue; WNT: wingless; MAPK: mitogen activated protein kinase; IGF: insulin-like growth factor; IL: interleukin; TNF: tumor necrosis factors; TGF: transforming growth factor; FGF: fibroblast growth factor; FGFR: FGF receptor; EGF: epidermal growth factor; EGFL: EGF-like domain; STAT: signal transducers and activators; PTGFR: prostaglandin F receptor; GDF-10: growth differentiation factor 10; ALDH1A3: aldehyde dehydrogenase 1 family, member A3; WT1: Wilms' tumor suppressor 1; CDH1: cadherin 1, type 1; HOXC10: homeobox C10 protein

Key Words: Visceral adipose tissue, Subcutaneous adipose tissue, Gene expression, Insulin homeostasis, Inflammation pathway

Send correspondence to: Nela Pivac, Division of Molecular Medicine, Rudjer Boskovic Institute, Zagreb, Croatia, Tel: 38514571207, Fax: 38514561010, E-mail: npivac@irb.hr