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Intra- and interindividual variation in gene expression in human adipose tissue

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Abstract Adipose tissue is a highly plastic tissue with an important endocrine and metabolic function. To understand its role in human health and disease, it is necessary to understand the extent of variation and the specific differences within and between different depots and subjects. We employed cDNA microarray analysis to investigate this in human subjects ranging from lean to mildly obese. We observe (1) high similarity between different samples of one adipose depot, (2) only small differences between the subcutaneous and visceral adipose tissue depot and (3) larger differences in gene expression between different individuals (per depot). The major variation within adipose depots can be attributed to differences in the non-adipocyte component of adipose tissue. Using only non-obese subjects, we identified genes that were consistently differentially expressed between subcutaneous and omental adipose tissue, despite the variation in gene expression between these subjects. Using quantitative real time polymerase chain reaction (PCR), fatty acid binding protein 4 (FABP4), vimentin (Vim), four and a half LIMs domains (FHL1), CD36 (all higher in subcutaneous adipose tissue) and Matrix Gla protein (MGP; lower in subcutaneous adipose tissue) were confirmed to be significantly differentially expressed between depots.

Keywords Omental · Subcutaneous · Adipose depot · Microarray · FABP4 · FHL1 · VIM · CD36 · MGP

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Introduction

Adipose tissue is the primary place in the human body for long-term energy storage. Until a few years ago, this was the only important function attributed to adipose tissue. Following the discovery of leptin [23], a satiety hormone excreted by adipose tissue, adipose tissue is now also recognized as an important endocrine organ. Indeed, not only do adipocytes store fat, and as such, have a role in energy homeostasis, but they also secrete a large number of peptides, hormones and other signalling molecules. These adipokines have a functional role in a large number of diverse processes, including glucose homeostasis, lipid homeostasis, appetite regulation, immune function, hormonal processes, angiogenesis and blood pressure control [54], establishing adipose tissue as an organ with important regulatory functions. Adipose tissue can be divided in different depots [13]. In humans, the subcutaneous and omental adipose depot can be distinguished. Different functions are attributed to these depots, as studies have shown that the size of the omental, but not the subcutaneous, adipose tissue is related to the relative risk for the development of insulin resistance [6, 32]. This is the case not only for obese persons, but also for persons with a healthy body mass index (BMI), where excess of omental adipose tissue can lead to insulin resistance [48].

Elegant overfeeding studies in non-obese twins suggest that the distribution of adipose tissue in the body is, to a large extent, determined by genetic constitution [11]. The ability of adipocytes to transdifferentiate into myocytes [1] or secretory epithelial cells [44] suggests that within the context of their genetic programming, (pre-)adipocytes can adapt to their surroundings through stimulation by specific factors such as hormones, cytokines or neuro-endocrine factors. The observed functional differences between the subcutaneous and omental adipose depots may be explained by adaptation of (pre-)adipocytes to their surroundings imposed by the location in the body.

Gene expression differences between subcutaneous and visceral adipose tissue have been shown in the past. In studies primarily performed on adipose tissue of extreme obese individuals using several different techniques, including microarray technology [18, 46, 57], representational display analysis [40] and quantitative polymerase chain reaction (PCR) [14, 24, 43], Leptin [14, 24, 43, 50, 57], calcyclin, adipsin [40, 63] and perilipin [2] were shown to be more highly expressed in the subcutaneous depot, while thrombospondin and carboxypeptidase E [46] were found to be more highly expressed in the omental depot. This confirmed the existence of functional differences between different depots. In addition, studies have been performed to study the differences in gene expression between lean and obese subjects in omental adipose tissue [20]. However, studies focussing on the differences in gene expression between subcutaneous and omental adipose tissue in lean individuals are limited [39]. Furthermore, no information is available on the variation in gene expression within depots.

In this study, we assess the homogeneity of the subcutaneous and omental adipose depots of lean to mildly obese subjects to determine the extent of variation between these depots and between individuals. Furthermore, we aim to obtain insight in the physiological differences between the depots in non-obese individuals.

Materials and methods

Adipose tissue sampling

Written permission for adipose biopsy sampling was obtained from the Medical Ethical Committee of Gelderse Vallei Hospital and the Maastricht University Hospital and consent was given by the subjects. From each of three subjects (person A: female, age 55, BMI 21.9; person B: male, age 54, BMI 28.4; person C: female, age 49, BMI 22.0) undergoing abdominal surgery, three subcutaneous and three omental adipose tissue biopsies were collected, approximately 5 cm apart from each other. From a fourth subject, three adipose tissue needle biopsies were taken from the left side, the right side and the middle of the anterior abdominal area from the subcutaneous adipose depot (person D: male, age 51, BMI 35.5). The fat biopsies were snap frozen and stored in liquid nitrogen until RNA isolation. From five additional subjects, three men and two women (age 68.8±10.6; BMI 27.5±3.2, mean±SD), a single adipose tissue biopsy was taken from both the subcutaneous and the omental depot. The gene expression data of four of the latter subjects were exclusively used for the selection of genes with a depot-specific expression pattern. From one subject, not enough RNA could be isolated for microarray hybridization, and therefore, this material was only used for quantitative real-time reverse transcriptase (RT)-PCR.

RNA isolation

RNA was isolated using TRIzol reagent (Invitrogen, Breda, the Netherlands). Briefly, frozen adipose tissue was ground in liquid nitrogen. TRIzol reagent was added and RNA was isolated according to the protocol of the supplier with the following exception: after obtaining the water phase, an additional phenol:chloroform:isoamylalcohol (25:24:1) extraction and chloroform extraction were performed. The amount of RNA and its integrity were determined by spectrophotometry and electrophoresis on a 1% agarose/TBE gel containing ethidium bromide.

RNA labelling

RNA labelling was performed as described previously [45]. Twenty micrograms total RNA was labelled by incorporation of aminoallyl dUTP in the cDNA followed by chemical coupling to Cy5 monofunctional dye. A standard reference sample, consisting of a pool of total RNA from all biopsies was used and coupled to Cy3 monofunctional dye. All arrays were hybridized with 1:1 mixture (v/v) of a Cy5-labeled sample cDNA and a Cy3-labeled reference cDNA.

Microarray construction, hybridization and scanning

A cDNA microarray was constructed, containing sequences obtained from a cDNA library enriched for adipose tissue transcripts, known genes selected for functional relevance and control genes to check cDNA synthesis efficiency. The cDNA library was constructed using RNA obtained from subcutaneous adipose tissue and lung RNA as starting material for subtractive hybridization [55]. The adipose tissue was a pooled sample of biopsies that were obtained from eight obese women undergoing gastro-plastic surgery. Full-length PCR products of the inserts of the resulting cDNAs were printed on silylated slides (CELAssociates, Houston, Texas, USA), using a PixSys 7500 arrayer

(Cartesian Technologies, Durham, NC, USA) as described [8]. The array consisted of a total of 3,136 spots comprising 2,433 sequences originated from the adipose tissue enriched cDNA library, 47 control (5′, middle and 3′) luciferase fragments to check for cDNA synthesis efficiency, 280 preselected cDNAs spotted in duplicate and 94 empty spots. Before hybridization, the arrays were dried at room temperature for at least 3 days after which the free aldehyde groups were blocked according to the method of Schena et al. [49]. Nearly all cDNAs were sequenced (unpublished results) and major redundancy was removed resulting in 926 sequences that were used for data analysis. Pre-hybridization of the arrays was performed as described previously [8]. A gene frame, enlarged to 55 μl (Westburg, Leusden, the Netherlands) was used during an overnight hybridization at 42°C in a humid hybridization chamber. All labeled RNA samples were hybridised in duplicate. After hybridization, the arrays were washed and dried as described before [8]. Scans with a pixel resolution of 10 μm, a laser power of 90%, PMT voltage of 92% for Cy3 scans and 80% for Cy5 scans, were obtained using a confocal laser scanner Scan Array 3000 (General scanning, Watertown MA, USA). ArrayVision (version 7.0; Imaging Research, Ontario, Canada) was used for image analysis. Density values of each spot, multiplied by the area and the background values (measured between individual spots) were collected.

Data analysis

The data normalisation was performed exactly as described previously [45]. For analysis of the variation in gene expression calculated by the Intra Class Correlation Coefficient (ICC), technical outliers of duplicate slides caused by stains were removed by omitting values that showed a twofold or greater difference at the same spot. The (ICC) was calculated as described before [45], using SPSS 10.0 for Windows.

Before the analysis of differentially expressed genes within and between adipose depots, all normalised signal values lower than 10 (corresponding to approximately three times background) were omitted from the dataset. This resulted in a dataset with 498 spots per array. Cluster analysis and bootstrapping was performed in Genemath (version 2.0, Applied Maths, St Marthens-Lathem, Belgium). For identification of genes differentially expressed between subcutaneous and omental adipose tissue, discriminant analysis, a form of principal component analysis that allows selection between pre-identified groups, was performed using Genemath software.

Genes showing differential expression within depots in three or more subjects or depots were removed before further analysis. Genes were selected based on their importance for discrimination between the two groups (discrimination grade). The 1 to 10% most discriminating genes were selected and arranged by discrimination grade. A paired Student's *t*-test was performed using Excel, and genes with a *p*-value higher than 0.05 were disregarded.

Q-PCR

Quantitative real-time RT-PCR (Q-PCR) was used to verify microarray results. From one microgram of total RNA, cDNA was synthesized using oligo dT and Super Script First Strand Synthesis (Invitrogen, Breda, the Netherlands) according to the protocol of the supplier. Q-PCR was performed for one housekeeping gene, beta-2 microglobulin (β2M), and seven target genes. Four and a Half LIMs domains (FHL1) cDNA levels were determined relative to β2M using the LightCycler (Roche Diagnostics Nederland, Almere, the Netherlands). The reaction contained 0.5 μM primer, 4 mM $MgCl_2$, 2 μl light Cycler-FastStart DNA Master SYBR Green I (Roche) and 2 μl of the cDNA template in a final volume of 20 μl. The amplification procedure consisted of a pre-incubation step for activation of the Taq DNA polymerase (10 min at 95°C), followed by 45 cycles consisting of a denaturation step (10 s at 95°C), annealing (10 s at 55°C) and extension step (18 s at 72°C). The other target genes, fatty acid binding protein 4 (FABP4), vimentin (VIM), Matrix Gla Protein (MGP), CD36, annexin A5 (ANXA5) cDNA levels were determined relative to β2M using MyiQ real-time PCR detection system (Biorad, Veenendaal, the Netherlands). Each reaction contained 0.5 μM primer, 10 μl IQ SYBR green supermix (Biorad, Veenendaal, the Netherlands) and 2 μl of the cDNA template in a final volume of 20 μl. The two-step amplification procedure consisted of a pre-incubation step for activation of the Taq DNA polymerase (3 min at 95°C), followed by 45 cycles consisting of a denaturation step (10 s at 95°C) followed by an annealing and extension step (45 s at 57.5°C for FABP4, B2M, MGP, VIM and CD36 and 45s at 60°C for ANXA5). For all measurements, fluorescence was quantified during the extension step. A melting curve analysis was performed to evaluate efficiency and primer function.

The following intron spanning primers were used: for FLH1: 5′-CTG CGT GGA TTG CTA CA-3′ (forward) and 5′-GCA CAG TCG GGA CAA TA-3′ (reverse), for B2M: 5′-CCT GAA TTG CTA TGT GTC TGG GTT TC-3′ (forward), 5′-CTC CAT GAT GCT GCT TAC ATG TCT CG-3′ (reverse), for FABP4: 5′-AAG TCA AGA GCA CCA TAA CCT TAG ATG-3′ (forward), 5′-CTC TCT CAT AAA CTC TCG TGG AAG TG-3′ (reverse), for CD36: 5′-TGC CTA TTC TTT GGC TTA ATG AG-3′ (forward) and 5′-TTA CTT GAC TTC TGA ACA TGT TTG C-3′ (reverse), for secreted protein, acidic, cysteine-rich, also

known as osteonectin (SPARC): 5′-TTT GAT GATG GTG CAG AGG A-3′ (forward) and 5′-GTG GTT CTG GCA GGG ATT T-3′ (reverse) and for ANXA5: 5′-AGC ATC CTG ACT CTG TTG ACA TCC-3′ (forward) and 5′-TCA TAC TCC TGC TTG CTG ATC CAC-3′ (reverse). For MGP and VIM, primerbank sequences ID 4505179a1 and ID 4507895a2, respectively, were used [60].

All samples were analysed in triplicate and a negative control was included. The standard curve method was used for calculation. Data analyses were performed with the LightCycler analysis software version 3.5 (Roche) and MyiQ system software version 1.0.410 (Biorad, Veenendaal, the Netherlands) respectively. The obtained efficiencies of all PCRs were B2M: 99.0%, FABP4: 95.4%, VIM: 99.0%, FHL1: 98.0%, CD36: 98.2%, ANXA5: 101.2%, SPARC: 97.4% and MGP: 100.2%.

Results

Variation in adipose gene expression was assessed using DNA microarrays. For this, three biopsies were obtained from both the subcutaneous and the omental adipose depot at three different positions from three individuals (subjects A, B and C). In addition, three subcutaneous biopsies at three different positions in the abdominal area (left and right lateral side and medial) were obtained from a healthy volunteer (subject D), using needle biopsying, a procedure that can easily be used during intervention studies. Each experimental sample was hybridised in duplicate, together with the reference sample, to a microarray containing cDNA clones from an adipose tissue enriched cDNA library. Fluorescence intensities were determined and were used for the visualisation and calculation of variation.

To visualise the variation in gene expression, samples were plotted against each other in scatter plots. In Fig. 1,

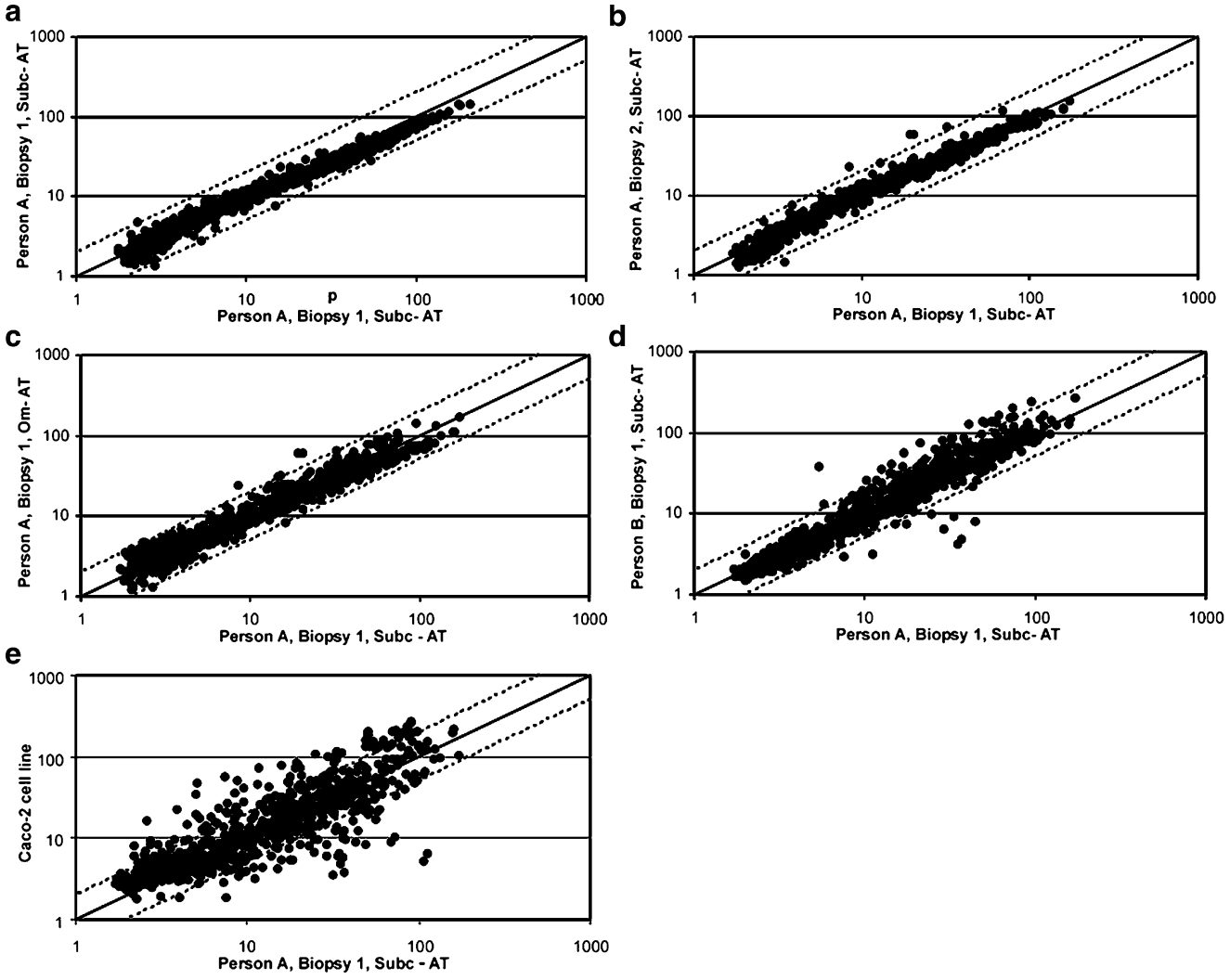

Fig. 1 Scatter plots of DNA microarray hybridization signals. **a** Technical duplicate of subcutaneous adipose tissue. **b** Two biopsies from the same adipose depot and person. **c** A subcutaneous and an omental adipose biopsy from one subject. **d** Two subcutaneous adipose tissue biopsies from two different subjects. **e** A subcutaneous adipose tissue biopsy and the CaCo-2 cell line

this is illustrated with typical examples. First, the hybridization signals of one sample (person A, subcutaneous, biopsy 1) is compared with its technical duplicate (Fig. 1a), then with another sample (person A, subcutaneous, biopsy 2) from the same subcutaneous depot (Fig. 1b), with a sample from the omental depot of the same person (person A, omental, biopsy 1) (Fig. 1c), and with a sample of the subcutaneous depot of another person (person B, subcutaneous, biopsy 1) (Fig. 1d). To illustrate the extent of the observed differences, the expression pattern of the adipose tissue samples was compared to that of the human intestinal colon cancer cell line CaCo-2 (Fig. 1e). Clearly, the adipose tissue samples are comparable but different from that of CaCo-2. In addition to the scatter plots, the rate of variation was determined by calculating the Intra Class Correlation Coefficient (ICC). An ICC of 1 indicates no variation. The resulting ICC values are stated in Table 1.

First, the homogeneity of the subcutaneous and omental adipose depots, the within depot variation in gene expression, was assessed by comparing the biological variation to the technical variation, the variation between duplicate slides. The biological variation between different positions within the same adipose depots $(\mathrm{ICC} = 0.970 \pm 0.027)$ was found to be approximately equal to the technical variation $(\mathrm{ICC} = 0.965 \pm 0.042)$, i.e., the ICC calculated between the duplicate slides (Table 1). This indicates that the gene expression patterns of biopsies taken from one depot are highly similar to each other, and in fact, can be seen as identical.

To determine the variation in gene expression between adipose depots (the between depot variation) the average value for each adipose depot was used. As shown in Table 1, the ICC for the 'between depot variation' within an individual $(\mathrm{ICC} = 0.970 \pm 0.0240)$ is equal to the 'within depot variation' of an individual $(\mathrm{ICC} = 0.970 \pm 0.027)$, indicating that overall there is little variation between the expression of genes in the subcutaneous and omental adipose depots (Student's t-test, $p=0.98$). Calculation of the 'between subject variation' (0.915 ± 0.005) showed that this was more variable than between the depots within one subject (0.970 ± 0.0240) (Table 1) (Student's t-test, $p=0.05$).

Although the within depot variation was small, we were interested to see if we could identify the nature of this variation. For this, we calculated the average values from the biopsies and identified cDNA clones on the array showing a twofold or greater differential expression. cDNA clones representing three different annotations showed differential expression within a depot in three subjects in both subcutaneous and omental adipose tissue. These are the cytochrome c oxidase subunit 1 (MT-CO1), hemoglobin and core promoter element binding protein (COPEB). These genes most likely indicate variation in the amount of non-adipocyte cells in the fat tissue samples. In the needle biopsy samples, also L-apoferritin, Annexin A5 and

Table 1 Intra-class correlation coefficient as a measure of variation

Subject	Adipose depot	Biopsy nr	Technical variation	Within depot variation	Between depot variation	Between person variation, omental depot	Between person variation, subcutaneous depot
A	S	1	0.9866	0.9877	0.9879		
		2	0.9875				
		3	0.9796				
	O	1	0.9678	0.9694			
		2	0.9839				
		3	0.9816				
B	S	1	0.8217[a]	0.9520	0.9413	0.9250	0.9085
		2	0.9562				
		3	0.9347[a]				
	O	1	0.7943[a]	0.9754			
		2	0.9815				
		3	0.9534				
C	S	1	0.9853	0.9879	0.9825		
		2	0.9886				
		3	0.9781				
	O	1	0.9788	0.9827			
		2	0.9734				
		3	0.9749				
D	S	1	0.9837	0.9860	–	–	
		2	0.9773				
		3	0.9776				

S Subcutaneous adipose depot, *O* omental adipose depot
[a]Arrays are not taken into account for analysis due to insufficient quality

ATPase show intra-depot variation in gene expression (data not shown). The greater variation observed when using needle biopsies may be the result of the sampling procedure leading to a higher level with non-adipocyte material, and in particular, with blood in the biopsy.

The genes exhibiting differential expression within a depot in three subjects in both subcutaneous and omental adipose tissue, were considered unreliable. After removal of these genes, cytochrome c oxidase subunit 1 (MT-CO1), hemoglobin and COPEB, reanalysis of the data using ICC (data not shown) and using cluster analysis (UPGMA and Euclidian distance) confirmed the results obtained on inter- and intra-individual variation (Fig. 2). Subjects cluster together, which is consistent with the observation that differences in gene expression are larger between subjects than between the adipose depots. Furthermore, within an individual, expression pattern of the three biopsies of one depot always group together. In other words, samples belonging to the same depot cluster together within each subject.

The ICC can also be used to calculate how many subjects are needed to study gene expression between adipose depots despite the large interindividual variation [45]. We used the ICC in this way to calculate the number of subjects that are needed for a study into gene expression differences between subcutaneous and omental adipose tissue. A theoretical number of five subjects gave an ICC_5 equal to the technical variation, and from this, it was concluded that six subjects should be enough for analysis of depot specific gene expression.

To identify differentially expressed genes between subcutaneous and omental adipose tissue, and in concordance with the calculation of the number of subjects needed, the dataset was enlarged with four additional subjects. Of these four subjects, only one biopsy per depot was available, which were analysed in a separate microarray experiment. This resulted in a dataset consisting of seven non-obese subjects. Principal component analysis with predefined groups (Discriminant analysis) was used to select the 10% most discriminating genes among those that were 54% above background. In addition, a paired *t*-test was done and those with a *p*-value higher than 0.05 were considered unreliable and discarded (50%). Table 2 shows the annotations that are differentially expressed between the subcutaneous and omental depot arranged by discrimination grade. Of the 12 identified genes, nine are more highly expressed in the subcutaneous depot (ANXA5, SPARC, FHL1, PLIN, FABP4, CDK2AP1, CD36, MGP, CRYAB, VIM, APOD), while two are higher in the omental depot (RPL23a and MGP). The leptin gene was expressed at background levels, and was therefore not included in the analysis.

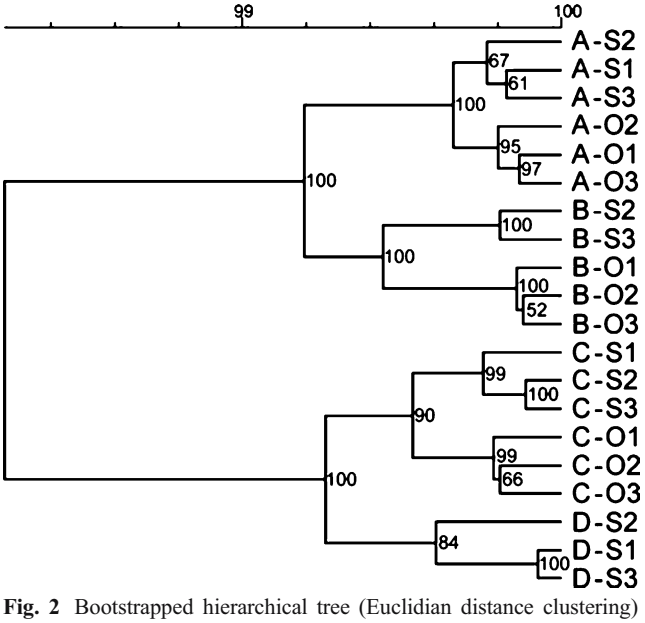

Fig. 2 Bootstrapped hierarchical tree (Euclidian distance clustering) of subcutaneous and omental adipose tissue biopsies from four subjects. **A**–**D**, subject; *S*, Subcutaneous adipose depot; *O*, omental adipose depot; *1*–*3*, biopsy number

Seven of the 12 genes identified in this study were chosen for confirmation using Q-PCR. B2M was used as a reference gene, because it is an accepted house keeping gene [15] and was used in a previous study on expression differences between subcutaneous and omental adipose tissue [46]. FABP4 (p=0.02, Fig 3a), VIM (p=0.006; Fig 3b), FHL1 (p=0.006; Fig. 3c), CD36 (CD36 antigen, collagen type I receptor; p=0.03; Fig. 3d), ANXA5 (0.08; Fig. 3e); MGP (p=0.013; Fig. 3f) and SPARC (p=0.08; data not shown) and were found to be differentially expressed between the depots, although for SPARC and ANXA5 this was not significant. No gender differences were observed for all genes tested with Q-PCR. In a separate microarray experiment (data not shown), we found that all genes that were tested with Q-PCR were expressed in isolated adipocytes, and are therefore valid adipose expressed genes.

Discussion

Adipose tissue is a highly plastic tissue and consists of different depots in the human body [12, 38, 53]. In this paper, we analysed gene expression of two different human adipose tissue depots using a microarray with cDNAs derived from and enriched for adipose tissue mRNA. Our findings that gene expression within any adipose depot is highly homogenous, that there is some variation between depots and that there is substantial variation between people, are consistent with the idea that the characteristics of the adipose depots are both genetically and environmentally determined.

Within one depot, genetic and local environmental contributions are identical, nevertheless, some differences

Table 2 Genes differentially expressed between subcutaneous and omental adipose tissue

Name	Short name	Accession number	Higher in depot	Discrimination grade (%)	Mean fold difference	*P* value[a]
Annexin A5	ANXA5 [b]	NM_001154	S	1	1.8	0.013
Osteonectin	SPARC [b]	NM_003118	S	1	1,5	0.026
Four and a half LIM domains 1	FHL1 [b]	NM_001449	S	2	1.5	0.007
Perilipin	PLIN	NM_002666	S	2	1.3	0.021
Fatty acid binding protein 4, adipocyte	FABP4 [b]	NM_001442	S	3	1.5	0.005
Apolipoprotein D gene	APOD	M16696	S	4	1.4	0.029
CDK2 associated protein 1	CDK2AP1[b]	NM_004642	S	5	1.2	0.004
CD36 antigen	CD36 [b]	NM_001001548	S	6	1.2	0.040
Ribosomal protein L23a		U43701	O	7	−1.2	0.045
Matrix Gla protein	MGP [b]	NM_000900	O	8	−1.2	0.015
Crystallin, alpha B	CRYAB	NM_001885	S	10	1.2	0.042
Vimentin	VIM [b]	X56134	S	10	1.3	0.047

The differentially expressed genes were identified using discriminant analysis and listed by discrimination grade.
S Subcutaneous adipose depot, *O* omental adipose depot
[a]Genes with a *p*-value (paired Student's *t*-test) higher than 0.05 were removed from this table.
[b]These genes were selected for quantitative RT-PCR

in gene expression were observed. The differences consisted primarily of hemoglobin, mitochondrial expressed genes (MT-CO1) and core promoter element binding protein (COPEB). Therefore, it is most likely that the differences found between the three biopsies within one depot are caused by the presence of non-adipose tissue, such as blood, lymph or connective tissue. This is supported by the observation that differential expression of these genes was consistently found for either all or none of these genes. Physiologically, it can be explained as follows. Hemoglobin is virtually exclusively present in red blood cells [10]. Consequently variation in blood content in the biopsy will result in apparent expression differences. Blood cells, mainly reticulocytes, are often a contaminant in genomic studies [9].

Mitochondrial content differs greatly between tissues and contamination with other tissues will result in apparent expression differences in mitochondrial related genes. COPEB is expressed in pre-adipocytes [26] and is one of the many immediate early genes following adipogenic stimulation. However, it is also expressed in large number of other cell types such as endothelial cells [33], neurophils [36] and fibroblasts [64], allowing for differences in expression due to differences in sample constitution. We conclude that adipose samples within one depot are highly homogenous and that analysis of one biopsy will provide representative data for a single depot. On the basis of our data, correction for the non-adipocyte component of adipose tissue is necessary and can be done by taking two biopsies of a limited number of subjects to identify contributors to this source of variation. The necessity for this is exemplified by COPEB, which has been identified as being differentially expressed between depots [57], but our results suggest that this may be due to the presence of non-adipocyte components of adipose tissue.

Differences in adipose gene expression between people can be the result of differences in genetic constitution [61], gender [46, 61] and in environmental factors, such as diet and exercise [30, 56]. Based on this, it can be expected that this results in greater variation in adipose gene expression between people than within people. Indeed, this notion is substantiated by the ICC (Table 2) and cluster analysis (Fig. 2). The set of genes that was used in this analysis is adipose derived, which may have contributed to the distinct results that have been obtained. Furthermore, the fact that non-obese to mildly obese individuals of different gender were studied may have contributed to this result, which may have been less clear if only very obese males or females would have been studied.

Based on the observed large variation between subjects and the small differences between depots, it could be expected that identification of discriminating transcripts would be difficult. Despite these reservations, it was possible to identify consistently differential expressed mRNAs in non-obese subjects between the omental and the subcutaneous adipose depot. This substantiates the functional differences between these depots. As individuals have an identical genetic constitution and external environmental exposure, it is most likely that these differences are due to local environmental or metabolic interactions within subjects.

The differences in expression levels of the identified differentially expressed genes are, although significant, rather low. This small difference is in agreement with

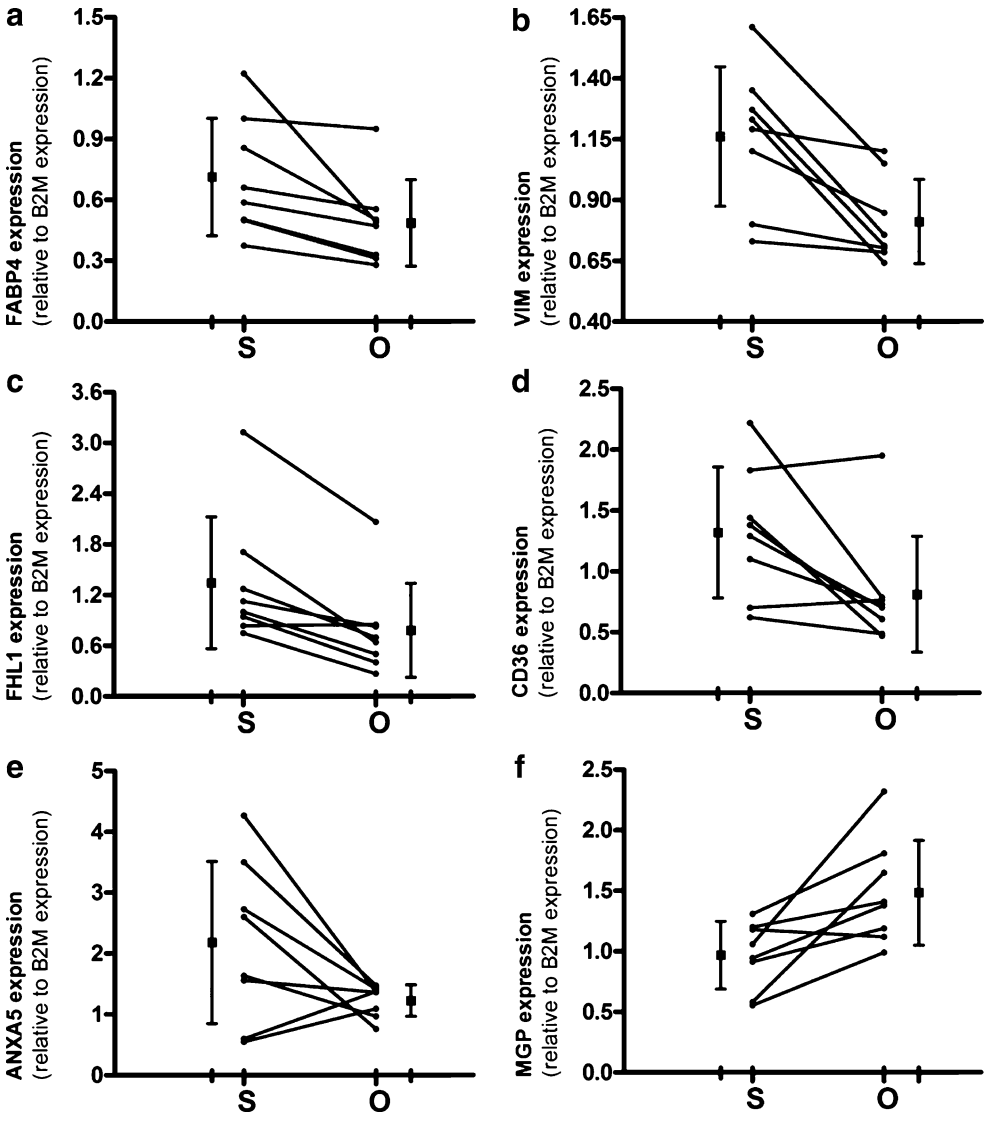

Fig. 3 Quantitative RT-PCR results of target gene expression relative to B2M expression in subcutaneous and omental adipose tissue plotted per subject: *filled circles* (•) and average expression: *filled squares* (▪). **a** FABP4 expression, $p=0.02$ (paired Student's *t*-test). **b** Vimentin expression, $p=0.006$ (paired Student's *t*-test). **c** FHL1 expression, $p=0.003$ (paired Student's *t*-test). **d** CD36 expression, $p=0.03$ (paired Student's *t*-test). **e** ANXA5 expression, $p=0.08$ (paired Student's *t*-test), f MGP expression, $p=0.013$ (paired Student's *t*-test)

another microarray study that analyses the differences between depots, in that case, in obese individuals [40]. Most of the differentially identified expressed genes that are more highly expressed in the subcutaneous depot are associated with adipocyte maturation. This is most clearly so for FABP4, which is a well-known marker of adipocyte differentiation [25], and for perilipin (PLIN) and VIM, which are structural proteins associated with lipid droplets [7, 16, 58]. For CDK2AP1 and CRYAB, this functional relationship is less clear. CDK2 associated protein CDK2AP1 was first identified as down regulated in ovarian cancer (DOC1) [42], but little is known about its physiological function. Crystallin alpha-B belongs to the small heat shock protein family and is associated with correct assembly of structural proteins in other tissues [17, 59]. Vimentin, CDK2AP1 and CRYAB have not been identified previously as differing between depots, which may be due to the focus of other studies on very obese subjects. FHL1 is another newly identified gene differing between the subcutaneous and omental depot. FHL1 is a LIM only protein and consists of four LIM domains. Proteins containing LIM domains have been discovered to play important roles in many biological processes, including cytoskeleton organization, cell lineage specification and organ development [4]. FHL1 is involved in myocyte differentiation, and we speculate that it may have a similar role in adipocyte differentiation [4] and could serve as a potential marker for adipocyte differentiation or maturation as it is highly expressed in adipocytes and not in preadipocytes (van Beek et al., unpublished results). Leptin, previously described as a gene that is differentially expressed between adipose depots [24, 50], was expressed at background levels, and was therefore, not included in the analysis. Furthermore, visfatin, also recently discovered as being differentially expressed between adipose depots [5], was not present on the microarray, and therefore was also

not included in the analysis. Finally, thrombospondin and carboxypeptidase E, differentially expressed between depots of very obese subjects [46], were also expressed at background levels and did not show differential expression between adipose depots in this study.

SPARC (osteonectin), ANXA5 and CD36 were previously identified with a higher expression in subcutaneous adipose tissue compared to omental adipose tissue [57]. The plasma level of SPARC, involved in extracellular matrix (ECM) maturation, correlates with BMI. Plasma levels of SPARC also correlate with coronary artery disease [52], and its expression was highly increased after myocardial infarction [34]. A correlation with cardiovascular disease also exists for Annexin A5, a structural cytosolic plasma membrane protein, which is an indirect inhibitor of thromboplastin specific complex [22] and for CD36, a fatty acid translocase with a role in fatty acid transport [37] which also functions as a thrombospondin receptor [3].

No functional relation between the genes with a higher expression level in the omental depot is apparent. RPL23a is a ribosomal protein but also contains a characteristic nuclear import signal [28] and is shown to bind to DNA in a histone H1 like manner [35]. Matrix Gla protein [matrix gamma-carboxyglutamic acid protein (MGP)] is a vitamin K dependent membrane bound signalling protein, involved in extracellular matrix calcification [41, 47].

Of the identified genes in this study, a large number of the identified genes can be linked to retinoic acid (RA). Apolipoprotein D, a member of the lipocalin family, is closely associated with lecithin:cholesterol acyltransferase (LCAT), and thus, involved in lipoprotein metabolism. It has a high homology to retinal binding protein, and could therefore, be involved in retinol/retinoic acid transport [51]. Strikingly, MGP [31], vimentin [29], FABP4 [19], CD36 [62], SPARC [27] and CRYAB [21] were shown to be directly regulated by RA. This suggests that RA may have a function as a local environmental regulator of the discriminating expression of the identified genes. Comparing promoter/enhancer sequences of the identified cDNAs and subsequent functional analysis could aid in the identification of RA or other factors responsible for the differences between the omental and subcutaneous depot. Furthermore, the differential expressed genes identified in this paper should be confirmed in larger number of normal weight subjects, as the amount of subjects used in this study is limited. Assessment of normal weight individuals is essential for the understanding of the physiological roles of the depots under normal weight conditions and to delineate specific alterations that occur during weight gain.

In conclusion, this study showed that expression patterns in the adipose tissue are highly homogeneous within one depot and even within persons. Using only non-obese subjects, distinct differences between depots could be identified, despite large interindividual variation. These differences may provide clues to local environmental modulators. This could reveal valuable targets in omental obesity related comorbidities such as insulin resistance. One of the four newly identified differentially expressed genes, FHL1, is a potential marker for adipocyte maturation, but this has to be confirmed in further studies.

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