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Up-regulation of CD36/FAT in preadipocytes in familial combined hyperlipidemia

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ABSTRACT

Familial combined hyperlipidemia (FCHL) shows many features of the metabolic syndrome. The strong genetic component makes it an excellent model to study the genetic background of metabolic syndrome and insulin resistance. Adipose tissue is believed to contribute to, or even underlie, the FCHL phenotype and is an interesting target tissue for gene expression studies. However, interpretation of adipose tissue gene expression experiments is complex since expression differences cannot only arise as a direct consequence of a genetic trait, but may also reflect an adaptation to metabolic influences at the cellular level. In the present study, we measured gene expression levels in cultured primary human preadipocytes from FCHL and control subjects. Since isolated preadipocytes were allowed to replicate for weeks under standardized conditions, the contribution of previous metabolic influences is rather small whereas genetic defects are preserved and expressed in vitro. The main finding was up-regulation of CD36/FAT in FCHL preadipocytes, confirmed in two independent groups of subjects, and a concomitant increase in CD36/FAT-mediated fatty acid uptake. CD36/FAT overexpression has previously been shown to be associated with other insulin-resistant states. The present data suggest that CD36/FAT overexpression in FCHL occurs very early in adipocyte differentiation and may be of genetic origin.

Key words: lipid metabolism • fatty acids • metabolic syndrome • gene expression

Familial combined hyperlipidemia (FCHL), a familial form of dyslipidemia with typical features of the metabolic syndrome, was delineated three decades ago (1–3) and has been associated with a fivefold increased risk of myocardial infarction before the age of 60 (4). Although substantial insight has been acquired in this common lipid disorder, the complex

genetics are not yet fully understood. Genome-wide scans revealed a number of chromosomal loci, among others 1q21, 1p31, 11p, and 16q, which are expected to be involved in the pathogenesis of FCHL (5–8). Recently, association was found in a Finnish FCHL study population with upstream stimulatory factor 1 (USF1) on 1q21 (9), a ubiquitous transcription factor directing a number of genes involved in lipid and glucose metabolism. Several organs, including liver, fat, muscle, and brain, are plausible targets in which genetic alterations may result in the typical FCHL phenotype of insulin resistance and dyslipidemia.

Arrays for gene expression studies are promising tools to identify the underlying genetic defects of complex disorders such as FCHL and diabetes mellitus type 2 (DM2) (10). A recent gene expression study from our laboratory in freshly isolated, subcutaneous adipocytes from matched FCHL and control subjects (11) revealed a number of consistently up- and down-regulated genes in FCHL, involved in lipid- and lipoprotein metabolism, cell growth, apoptosis, matrix remodeling, and fatty acid metabolism. However, an inherent limitation of gene expression analyses in tissue samples, obtained in vivo, is the difficulty to discriminate between primary genetic modifications that cause FCHL, and secondary changes resulting from cellular adaptations to the hyperlipidemia. In the present study, we used a novel approach in an attempt to identify primary genetic alterations, or the direct cellular consequences thereof, in FCHL. We prepared preadipocyte cell cultures obtained from subcutaneous fat biopsies from FCHL patients and matched healthy controls. Preadipocytes were repeatedly cultured under the same experimental conditions and studied for differential gene expression by arrays and quantitative RT-PCR (qRT-PCR). As the contribution of previous metabolic influences is rather small, this approach avoided overwhelming noise of adaptational gene expression, whereas genetic defects are preserved and expressed in vitro, thereby increasing—at least in theory—the chance of discovering the genetic defect.

MATERIALS AND METHODS

Subjects

FCHL probands and controls were recruited through the Lipid Clinic of the Maastricht University Hospital. FCHL families were ascertained as described previously (12). The affected FCHL subjects in this study had each been ascertained as an affected relative of a FCHL family that contained at least one other first-degree relative with a total cholesterol (TC) ≥6.5 mmol/l and/or triglyceride (TG) ≥2.3 mmol/l, combined with the presence of premature CAD within the family. Control participants were recruited from the spouse group or by an advertisement, and matched for body mass index (BMI) and sex with the FCHL patients. All controls had fasting TC <5.5 mmol/l, and fasting TG <1.7 mmol/l, and no family history of CAD. The clinical characteristics of the subjects at the time of the fat biopsy are listed in Table 1. The Human Investigation Review Committee of the Academic Hospital Maastricht approved the study protocol, and all subjects gave written informed consent.

Blood and adipose tissue sampling

Plasma TC and TG concentrations were measured after an overnight fast as described previously (12). Subjects had refrained from smoking and did not drink coffee or tea in the morning. To obtain unbiased lipid values, participants had abstained from alcohol for at least 72 h and any

lipid-lowering medication had been withdrawn 2 wk before blood sampling. Blood and liposuction samples were taken as described previously (11).

Sample preparation

Within minutes after the biopsy, the collagenase-treated cell suspension (11) was filtered through 500 μm nylon mesh and spun at 220 × *g* for 1 min to separate preadipocytes from mature adipocytes. Preadipocytes were suspended in 5 ml DMEM medium containing 10% fetal calf serum (FCS) and 1% glutamine/streptomycine/peniciline (GSP) and cultured. At passage 4, RNA was isolated using the TRIzol reagent (GibcoBRL, Grand Island, NY) according to the manufacturer's protocol. The Rneasy kit (Qiagen, Hilden, Germany) was used for total RNA clean up, and quality was assessed following the manufacturer's instructions.

Suppression subtractive hybridization

Human subcutaneous adipose tissue samples obtained from a single FCHL/control pair were used for suppression subtractive hybridization (SSH) (Fig. 1). The SMART PCR cDNA Synthesis Kit (Clontech, Palo Alto, CA) was used for preparation and amplification of double-stranded cDNA, and subtractive hybridization was carried out using the PCR-Select cDNA Subtraction Kit (Clontech), both according to the manufacturer's instructions. The amplified differentially expressed cDNAs were gel-purified using the QIAEX gel subtraction kit (Qiagen), and cloned into the pGEMT-easy vector before transformation into highly competent *Escherichia coli* JM109 cells (Promega, Leiden, The Netherlands).

Sequence identification

All SSH clones were sequenced using the T7 and SP6 primers and the Dye Terminator Sequencing Kit (Applied Biosystems, Nieuwekerk a/d IJssel, The Netherlands) on an ABI310 genetic analyzer (Applied Biosystems).The cleaned sequence reads, that is, SSH clone sequences without adaptor and vector sequences, were assembled into contigs, thus lowering the number of sequences to be evaluated and increasing the average length of the sequences. In this way, the original number of 547 sequences, representing 369 different clones, was reduced to 264 contigs. Megablast searches with these 264 contigs against the Unigene database showed that 167 contigs obtained a significant blast hit ($E<10^{-3}$; sequence identity >95%), which represented 73 unique genes and 65 unique ESTs. For 97 contigs, no significant blast hits were obtained.

As a quality control check, 32 of the 271 commercially obtained clones were sequenced, using M13 primers, to verify their cDNA content. In all, 15% of the clones did not contain the insert guaranteed by the supplier. Therefore, all commercial clones discussed in the current study were sequence verified.

Macroarray procedure

Clones derived by SSH, representing genes differentially expressed between FCHL and control adipocytes, and commercially obtained clones with conceptual candidate genes for FCHL, were used in macroarray analyses. Inserts were amplified by PCR using the T7/Sp6 primer set or the M13 primer set. PCR products were purified using a 96-well PCR fitration system (Millipore, Bedford, MA). Subsequently, the PCR products were spotted in duplicate on nylon filters using a

BioRobotics gridder (Eurogentec, Seraing, Belgium), and denatured in 0.66 M NaCl and 0.5 M NaOH, neutralized in 40 mM phosphate buffer pH 7.3, and subsequently UV cross-linked to the membrane. The macroarrays were hybridized with an α-^{32}P[dATP]-labeled cDNA probe generated from 10 μg of total RNA from either a FCHL or control subject using an oligo(dT)$_{12-18}$ primer according to standard procedures. Probe purification was done using ProbeQuant G-50 microcolumns (Amersham, Piscataway, NJ). The arrays were preincubated for 30 min in 5 ml ExpressHyb hybridization solution (Clontech) with denatured salmon sperm DNA (100 μg/ml, Invitrogen, Merelbeke, Belgium) at 55°C. Subsequently, denatured probes, and 25 μg of CoT-1 DNA (Invitrogen), were hybridized to the arrays for 16 h at 55°C. The arrays were washed once with 2× SSC/1% SDS and twice with 0.1× SSC/0.5% SDS and exposed to an imaging screen (Bio-Rad, Hercules, CA).

Macroarray data analysis

The array consisted of 369 clones from our cDNA library generated by SSH, 271 commercially acquired conceptual candidates for cardiovascular disease, 4 housekeeping genes in triplicate and 100 blanco spots. All were represented in duplicate on the array. The software package Imagene (Biodiscovery, El Segundo, CA) was used to construct and superimpose a series of grids over the blot. Radiation intensities of the spots were quantified, and the numerical output was exported to Microsoft Excel for further analyses. All spots were visually inspected, and only visible spots with a homogeneous round shape were taken into account. Expression in at least 4 of 5 pairs was required for a gene to be included for analyses. As a reference for background correction, the intensities of a number of empty spots in the blot and a complete row of empty spots at the top and the bottom of the blots were averaged. Intensities of the spots were corrected for background and mean intensities of 3 housekeeping genes (23 kD highly basic protein, hypoxanthine phosphoribosyltransferase 1, and β-actine) present in sixfold on each array. Akaike information criterion (AIC) (13, 14), the likeliness of a model based on the observed data penalized by the complexity of this model, was used to identify differentially expressed genes. The AIC complexity penalization was chosen to be more stringent than usual in order to match a two-sided test with a 95% significance level. A gene was therefore found to be differentially expressed when the AIC indicated the model, including a group effect, that is, being an FCHL patient or a control subject, was describing better the observed radiation intensities. Differences were expressed as a ratio of normalized spot intensities on the FCHL array and the control array.

qRT-PCR validation procedure

The accuracy and reproducibility of the macroarray data were verified in a three-stage validation procedure using qRT-PCR (Fig. 1). In the first stage of the validation procedure, the reliability of the array itself was tested. To this purpose, expression patterns of a selection of genes were determined in one FCHL/control pair by qRT-PCR, using the same RNA sample as used for the array. Fold expression differences were calculated and correlated with the array results of the same couple. In the second validation stage, the reproducibility of the results was tested. The same cell cultures as used for the macroarray were recultured and qRT-PCR was performed on a selection of genes, using newly extracted RNA. In the third validation phase, we tested whether observed differences in gene expression levels were also present in preadipocyte RNA of another group of five unrelated FCHL/control pairs.

qRT-PCR was carried out using the qPCR for SYBR Green core kit (Eurogentec, Seraing, Belgium) on an ABI PRISM 7700 sequence detection system (Applied Biosystems). The selected genes and primers used are listed in Table 2. Target gene mRNA levels were normalized to 18S rRNA and analyzed using a standard curve, or the $2^{-\Delta\Delta ct}$ method when appropriate. Absence of primer dimers and presence of a single amplification product were verified by a melting curve analysis at the end of each assay.

Fatty acid uptake and esterification

CD36/FAT-specific fatty acid uptake and subsequent esterification was studied using sulfo-N-succinimidyl esters (SSO). SSO specifically binds CD36 and arrests the LCFA transport function of this protein (15).

Preadipocytes were preincubated for 45 min at 37°C in the presence of SSO (concentration 0.2 mM) or DMSO (concentration 0.4%). Palmitate uptake was measured by addition of [14C] palmitate (0.3 μCi, final concentration, 0.6 mM) in a 2% BSA/MKR solution. The reaction was carried out at 37°C for 30 min. Cells were rinsed three times with 1 ml stop solution (MKR 0.2% BSA+0.2 mM phloretin) to terminate palmitate uptake. Cells were lysed in 1 ml isopropanol-heptane (2:3). Samples were applied to HP-TLC plates to separate triglycerides and phospholipids. Radioactivity was measured after exposure to an imaging screen with the Personal Molecular Imager FX (Bio-Rad).

RESULTS

Subjects

Initial macroarray experiments were done with 5 FCHL/control pairs, which were also used in the first and second stage of validation by qRT-PCR (Fig. 1). In the third stage of validation with qRT-PCR, 5 additional FCHL/control pairs were studied, all unrelated and independent subjects not used in earlier experiments. The clinical characteristics of all subjects are listed in Table 1. FCHL patients showed higher plasma levels of total cholesterol (TC), TG, FFA, glycerol, and apolipoprotein B, and lower levels of HDL cholesterol, as expected. Age was not different between patients and controls.

Differentially expressed genes

Subcutaneous fat from a female/female FCHL/control pair was used to create a differential mature adipocyte SSH library. The FCHL subject was 47 years old, BMI 26.8 kg/m^2, with a plasma TC of 7.77 mmol/l, TG 2.26 mmol/l, and HDL cholesterol 1.08 mmol/l. The control subject was 39 years old, BMI 25.0 kg/m^2, and a plasma TC of 5.59 mmol/l, TG 0.69 mmol/l, and HDL cholesterol 1.41 mmol/l. SSH procedures resulted in a forward and a reverse library containing up-regulated and down-regulated FCHL clones, respectively. In total, 369 clones were selected from both libraries and combined with 271 commercially obtained conceptual cardiovascular candidate gene clones on macroarrays (Fig. 1). Housekeeping genes were used to normalize macroarray expression data. Relative intensities of housekeeping genes were similar on the FCHL and control arrays (Table 3). This low variability in relative expression of

housekeeping genes in FCHL and control preadipocytes illustrated the suitability of these genes for normalization.

Macroarray analyses showed that 256 of 640 (40%) genes reported on the array were expressed in at least 4 of 5 FCHL/control pairs (Fig. 1). Twenty-seven (11%) of these genes exhibited statistically significant altered expression in FCHL. Five genes were up-regulated in FCHL and 22 were down-regulated. These genes, with their chromosomal location, are listed in Table 4 and divided into 4 main groups: receptors (3 genes), mitochondrial function (4 genes), matrix turnover (6 genes), and metabolism and others (8 genes). Six clones were ESTs.

Multistep validation procedure

Six genes (CD36/FAT, MMP 2, TIMP 3, TRA1, ST13 [mentioned in Table 4], and ADRP [not in Table 4]) were selected for the first stage of validation. Quantitative expression differences were measured by qRT-PCR in the same RNA samples as used for the array (Fig. 1). A highly significant correlation coefficient of 0.78 ($P<0.001$) was found between the logarithm of the fold differences obtained by macroarray and qRT-PCR (Fig. 2), which demonstrated the accuracy and validity of the array data obtained.

The second stage of the validation procedure tested whether the expression data of a selection of 8 genes was confirmed by qRT-PCR in newly isolated RNA from regrown cell cultures of the same 5 pairs of subjects (Fig. 1). The 8 genes were CD36/FAT, COL1A2, MMP2, ST13, SURF1, TIMP3, and TRA1 (mentioned in Table 4), as well as ADRP (not in Table 4). Very similar expression patterns to those observed on the arrays were found with CD36/FAT (5 of 5 pairs, shown in Fig. 3*A*) as well as TRA1 (4/5 pairs), COL1A2 (4/5 pairs), and to a lesser extent with MMP2, TIMP3, ST13, and ADRP (3 of 5 pairs). SURF1, which has low expression levels in preadipocytes, showed a similar expression difference as observed on arrays in only 2 of 5 couples. Combined, this second stage of the validation confirmed a true expression difference in CD36/FAT, TRA1, and COL1A2 genes in preadipocyte cell lines from the first 5 FCHL patients vs. paired controls.

In the third validation step, we evaluated which gene(s) remained differentially expressed in a new, independent set of 5 pairs of BMI- and sex-matched FCHL and control subjects (pairs 6–10 in Table 1 and Fig. 3*B*), aiming to answer the question of which genes can be regarded as consistently FCHL-specific. Up-regulation of CD36/FAT was confirmed in 4 of 5 new FCHL/control pairs (Fig. 3*B*). Down-regulation of TRA1 and COL1A2 could not be confirmed in the new pairs. All combined, CD36/FAT showed up-regulation in 8 out of 10 FCHL preadipocyte cell cultures compared with preadipocytes derived from sex- and BMI-matched controls.

CD36/FAT up-regulation is functional

To investigate whether CD36/FAT up-regulation was functional, we recultured FCHL and control preadipocytes, and measured total and CD36/FAT-specific LCFA uptake and incorporation in phospholipids and triglycerides. In the combined group (FCHL+control subjects) a significant correlation was found between CD36/FAT expression measured by

quantitative PCR and the fraction of fatty acids taken up via CD36/FAT and incorporated into phospholipids and triglycerides (Fig. 4).

DISCUSSION

In the present study, we investigated gene expression profiles in human preadipocyte cell cultures from human subjects with FCHL and pair-wise-matched normolipidemic controls. The main finding was up-regulation of CD36/FAT in FCHL preadipocytes, confirmed in two independent groups, and a concomitant increase in CD36/FAT-mediated incorporation of fatty acids into triglycerides and phospholipids. Since gene expression differences in cultured preadipocytes reflect the direct expression of a genetic trait, and adaptive expression differences are suppressed, these results indicate that CD36/FAT overexpression in FCHL impacts very early in adipocyte differentiation state and may be of genetic origin.

The application of the SSH library combined with a candidate gene approach offered some substantial advantages. Because a selection was made of genes that are differentially expressed, a limited number of genes needed statistical testing, thereby reducing the multiple-testing problem and the number of false-positive results. Given these relative advantages and its known limitations, in combination with a stringent statistical analysis and a rigorous validation procedure, the present results offer an intriguing insight into an FCHL preadipocyte and perhaps general fat tissue functioning in FCHL.

Adipose tissue is an interesting target tissue for gene expression studies in FCHL, since impaired function of adipose tissue is believed to contribute to, or even underlie, the expression of the FCHL phenotype. Previously, the expression profile of mature adipocytes, freshly isolated from fat tissue of FCHL adipose tissue, was investigated by our lab (11). However, an inherent drawback of gene expression studies on in vivo samples is the inability to distinguish between primary genetic defects, or direct cellular consequences thereof, and adaptive responses to, for example, hyperlipidemia. In the present study, we cultured preadipocytes of FCHL subjects and BMI- and sex-matched controls. This design is based on the assumption that gene expression differences due to previous metabolic influences are rather small, as isolated preadipocytes were allowed to replicate for weeks in a standardized environment, equal for FCHL and control preadipocytes. A similar approach was used by Gaster et al. (16), using cultured myotubes to study genetic effects of reduced lipid oxidation in muscle from type 2 diabetes subjects. This approach ruled out expression differences due to environmental factors, and allowed us to attribute the increased CD36/FAT expression to the underlying genetic background in FCHL (Fig. 5).

CD36/FAT is a multiligand class B scavenger receptor that has been implicated in multiple biological processes. Apart from its function as a scavenger receptor, CD36/FAT has been identified as a regulator of angiogenesis and as a fatty acid translocase (FAT) facilitating the transmembrane transport of long-chain fatty acids (LCFAs) (17). CD36/FAT has atherogenic and antiatherogenic aspects (18). Ligands for CD36/FAT are, among others, oxidized LDL, LCFAs, collagen, thrombospondin-1, and apoptotic cells. CD36/FAT expression is broad but especially abundant in tissues with high LCFA storage capacity, such as adipocytes, or oxidative capacity, such as heart and muscle (18).

The consistent up-regulation of CD36/FAT is remarkable given the metabolic (Fredrickson type IIa, Iib, or IV) and genetic heterogeneity of FCHL (1, 19). In preadipocytes, CD36/FAT was up-regulated in 8 of 10 FCHL patients, equally expressed in one pair, and down-regulated in another FCHL patient. Furthermore, in mature, freshly isolated adipocytes, CD36/FAT expression was also 50% increased in FCHL (unpublished data). Nevertheless, in contrast to the spontaneous hypertensive rat (SHR) in which CD36/FAT has been identified as the causal insulin-resistance gene (20), CD36/FAT is probably not the mutated gene in FCHL. CD36/FAT is located on chromosome 7q11.2, and to our knowledge, no genome-wide scan showed linkage in this region with FCHL or any related trait. Obviously, this may change when more data become available and phenotypes are refined. Furthermore, from a biological point of view, overexpression of CD36/FAT is expected to result in a more favorable plasma lipid spectrum than presently seen in FCHL, due to increased CD36/FAT-mediated FA uptake. This is supported by observations in transgenic mice overexpressing CD36/FAT in muscle. These animals typically exhibit reduced plasma triglycerides and fatty acids but increased plasma glucose and insulin (21). In line with this mechanism, CD36/FAT knockout mice have increased levels of fatty acids, triglycerides, and cholesterol (22). In our opinion, CD36/FAT up-regulation is therefore more likely a manifestation for a genetic alteration in FCHL, rather than the defect itself, in analogy to overexpression of the 3-HMG-CoA-reductase enzyme in fibroblasts from patients with an LDL receptor defect (23).

CD36/FAT overexpression may well reflect insulin resistance (24) or defective insulin signaling in FCHL. Previously, CD36/FAT was found to be increased in other insulin-resistant states. Cardiac myocytes from obese Zucker rats, a commonly used rodent model for obesity and insulin resistance, have increased sarcolemmal CD36/FAT and enhanced extracellular fatty acid uptake (25). Similarly, in skeletal muscle from human subjects with type 2 diabetes, sarcolemmal CD36/FAT was increased and associated with increased LCFA transport (26). CD36/FAT protein was also increased in diabetic macrophages as a response to defective insulin signaling (27). Finally, Griffin et al. (28) showed increased CD36/FAT translation efficiency in diabetic macrophages. These data and ours show that CD36/FAT is increased in insulin resistance.

In the present study, increased CD36 RNA and functional CD36/FAT protein in cultured preadipocytes suggests that CD36/FAT up-regulation is the direct consequence of a genetic defect, rather than a metabolic adaptation to, for example, hyperlipidemia, and that the actual defect in FCHL is already metabolically apparent in a very early adipocyte differentiation state. This defect may be increased genetic susceptibility to insulin resistance of FCHL adipose tissue.

In conclusion, CD36/FAT is the first gene found with consistent increased expression in preadipocytes of two independent groups of unrelated FCHL subjects. The use of cultured preadipocytes suppressed expression differences due to adaptive compensations at the cellular level and allowed us to specifically select for gene expression differences that are a direct consequence of a genetic trait. Increased CD36/FAT RNA and functional CD36/FAT protein in cultured preadipocytes implies that a genetic defect in FCHL affects fatty acid metabolism in a very early adipocyte differentiation stage and that FCHL adipocyte dysfunction may be of genetic origin. Future gene expression studies in other candidate tissues such as liver and muscle will further advance our understanding of insulin resistance and altered lipid metabolism in FCHL

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Table 1

Clinical characteristics of FCHL-control pairs for macroarray analyses and qRT-PCR[a]

	Familial combined hyperlipidemia						Control					
Pair	**Sex (M/F)**	**Age (years)**	**BMI (kg/m²)**	**TC (mmol/l)**	**TG (mmol/l)**	**FFA (mmol/l)**	**Sex (M/F)**	**Age (years)**	**BMI (kg/m²)**	**TC (mmol/l)**	**TG (mmol/l)**	**FFA (mmol/l)**
Array												
1	F	45	24.5	6.4	1.1	0.05	F	44	23.5	3.9	1.1	0.33
2	F	63	24.3	7.5	2.3	0.27	F	55	25.7	4.9	0.9	0.18
3	M	55	26.8	7.2	1.3	0.05	F	45	27.0	4.7	1.7	0.29
4	M	58	26.3	4.6	3.6	0.23	M	51	26.2	4.3	1.0	0.38
5	M	39	24.2	6.6	3.7	0.26	M	57	25.4	5.1	1.2	0.28
qRT-PCR												
6	M	51	27.0	5.9	7.1	0.59	M	48	26.9	5.5	1.4	0.05
7	F	46	22.0	7.7	1.8	0.40	F	48	22.2	3.3	0.7	0.43
8	M	53	23.5	7.2	1.8	0.49	M	61	23.4	4.3	0.5	0.50
9	F	48	25.3	7.4	2.8	0.58	F	42	24.5	4.7	0.8	0.19
10	F	54	26.4	6.7	3.9	0.47	F	41	25.7	3.9	0.8	0.41
Mean ± SD		51 ± 7	25.0 ± 1.6	6.7 ± 0.9	2.9 ± 1.8	0.32± 0.20		49 ± 7	25.1 ± 1.6	4.5 ± 0.7	1.0 ± 0.4	0.30 ± 0.13

[a]BMI, body mass index; TC, total cholesterol; TG, triglycerides; FFA, free fatty acids.

Table 2

Primers used for qRT-PCR validation

Gene name	Primers (5'-3')
Adipophilin (ADRP)	Forward: ACCAGTGCTCTGCCCATCA Reverse: CCCCTTACAGGCATAGGTATTGG
CD36 antigen (CD36/FAT)	Forward: AGTCACTGCGACATGATTAATGGT Reverse: CTGCAATACCTGGCTTTTCTCA
Surfeit1 (SURF1)	Forward: CCTATGTGGTCACTCCCTTCCA Reverse: TGGGAACGAACCCTCTATTTACC
HSP70 interacting protein (ST13)	Forward: AGAAGTTCAACCTAGGGCACAGA Reverse: TTGATCTCTCGCTCTTCACGTTT
Tumor rejection antigen gp96 (TRA1)	Forward: AAAAAGAGCGATTACATTAAGCTCTATG Reverse: CCACACCCTTGACAAAATTGAG
Collagen, type 1, alpha 2 (Col1a2)	Forward: TGAAAACATCCCAGCCAAGAA Reverse: CAAACTGGCTGCCAGCATT
Matrix metalloproteinase 2 (MMP2)	Forward: CATCTTTGCTGGAGACAAATTCTG Reverse: CTGCGATGAGCTTGGGAAA
Tissue inhibitor of metalloproteinase 3 (TIMP3)	Forward: CTGACAGGTCGCGTCTATGATG Reverse: TTACAACCCAGGTGATACCGATAGT
18S ribosomal RNA (18S rRNA)	Forward: TGCATGGCCGTTCTTTAGTTG Reverse: AGTTAGCATGCCAGAGTCTCGTT

Table 3

Relative housekeeping gene intensities (%) on FCHL and control macroarrays[a]

Housekeeping gene	FCHL (%)	Control (%)
23-kDa basic protein	5.1 ± 3.3	4.9 ± 2.0
HPRT	1.1 ± 1.1	0.9 ± 0.6
ACTB	93.8 ± 4.1	94.3 ± 2.4

[a]Data represent mean % ± SD

Table 4

FCHL-control ratios + 95% confidence intervals of 27 genes found statistically significant (*P*<0.05) differentially expressed in preadipocyte cellines

Gene Name	Chromosome	Ratio FCHL-control	95% Confidence interval
Receptors			
CD36 antigen (CD36/FAT)[a,b]	7q11.2	1.71	1.21–2.21
Platelet-derived growth factor receptor alpha polypeptide (PDGFRA)[b]	4q12	0.63	0.43–0.82
Adenosine A2a receptor (ADORA2A)[a]	22q11.2	0.47	0.18–0.76
Mitochondrion			
Surfeit 1 (SURF1)[a]	9q34	0.42	0.24–0.60
Succinate dehydrogenase complex, subunit C, integral membrane protein, 15kD (SDHC)[a]	1q21	0.58	0.31–0.85
ATP synthase, H+ transporting, mitochondrial F0 complex, subunit F6 (ATP5J)[a]	19p13.2	0.56	0.32–0.80
Translocase of inner mitochondrial membrane 17 homolog A yeast (TIMM17A)[b]	1q32	0.46	0.32–0.60
Matrix turnover			
Collagen, type VI, alpha 3 (COL6A3)[a]	2q37	1.73	1.02–2.45
Collagen, type XVIII, alpha 1 (COL18A1)[a]	21q22.3	0.60	0.43–0.76
Collagen, type I, alpha 2 (COL1A2)[a]	7q22.1	0.55	0.09–0.38
Matrix metalloproteinase 2 (MMP2)[a]	16q13	0.37	0.22–0.52
Matrix metalloproteinase 9 (MMP9)[a]	20q11.2q13.1	0.50	0.28–0.73
Tissue inhibitor of metalloproteinase 3 (TIMP3)[a]	22q12.1q13.2	0.41	0.10–0.73
Metabolism and others			
Methylene tetrahydrofolate dehydrogenase (NAD+ dependent) (MTHFD1)[a]	14q24	0.45	0.21–0.69
LIM domain-containing preferred translocation partner in lipoma (LPP)[a]	3q28	0.64	0.42–0.86
Suppression of tumorigenicity 13 colon carcinoma (ST13) (Hsp70 interacting protein)[a]	22q13	0.41	0.16–0.65
3-hydroxymethyl-3-methylglutaryl-coenzyme A lyase (HMGCL)[a]	1pter-p33	0.61	0.35–0.88
Glutamyl-prolyl-tRNA synthetase (EPRS)[b]	1q41q42	0.64	0.44–0.84
Vimentin (VIM)[b]	10p13	1.51	1.05–1.97
Tumor rejection antigen gp96 (TRA1)[b]	12q24.2q24.3	0.54	0.31–0.78
ATPase, H+ transporting, lysosomal 42kDa, V1 subunit C isoform 2 (ATP6V1C2)[b]	2p25.1	0.63	0.40–0.87
ESTs			
Est (BE621121)[b]		1.53	1.04–2.02
Est 4c.12 (AI570255)[b]		1.66	1.05–2.27
Est/hypothetical protein DC42 (AK025746)[b]		0.37	0.06–0.69
Est weakly similar to hypothetical protein FLJ20234 (AI090487)[b]		0.51	0.26–0.77
Est (NM_002085)[b]		0.32	0.14–0.49
Est/hypothetical protein My014 (NT_032962.5)[b]		0.66	0.44–0.88

[a]Genes from the candidate gene library. [b]Genes from the SSH library.

Fig. 1

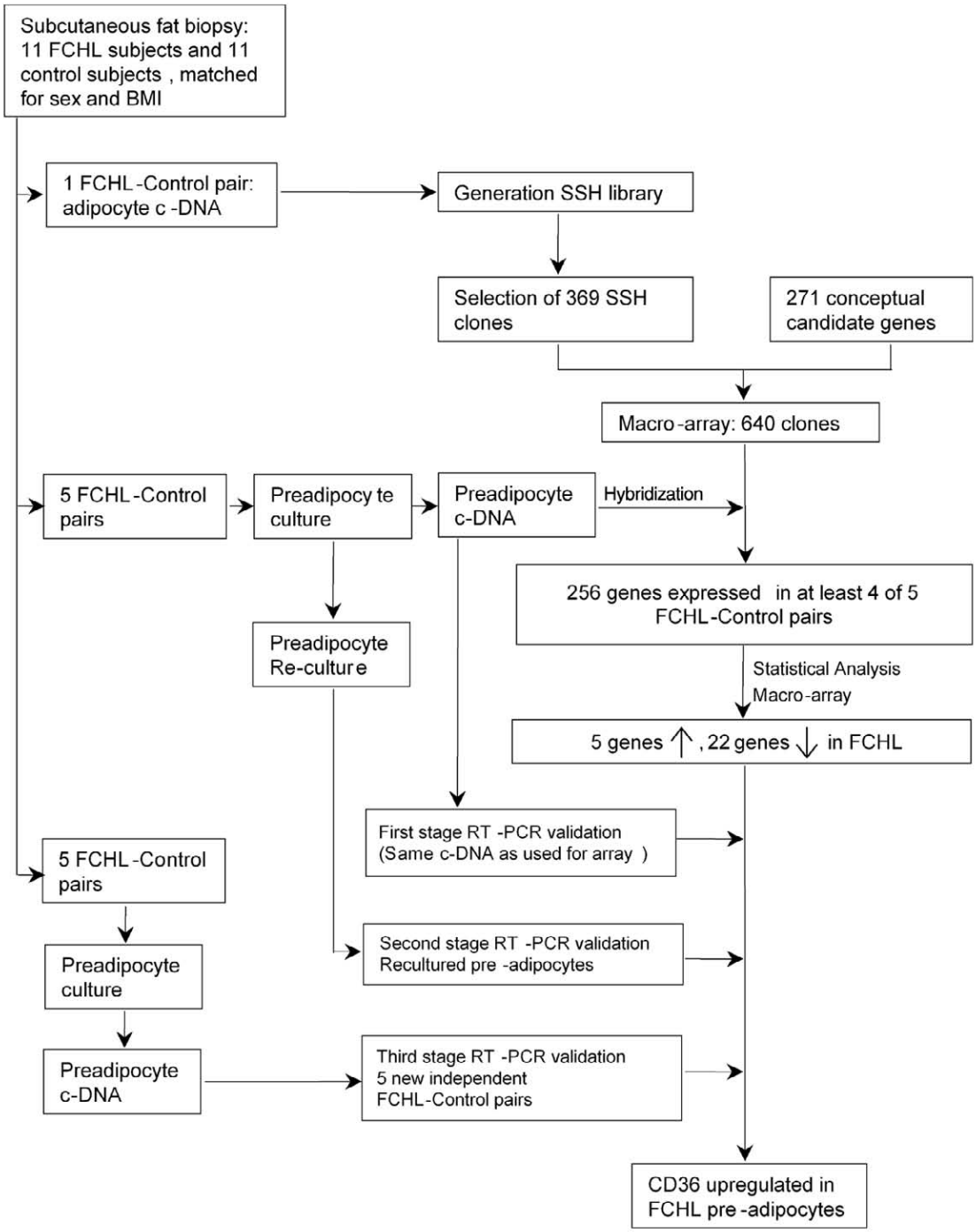

Figure 1. The design of the gene expression experiments is schematically depicted in this flow chart. Subcutaneous fat biopsies were taken from 11 familial combined hyperlipidemia (FCHL) subjects and 11 healthy controls, pair-matched for sex and body mass index (BMI). Suppression subtractive hybridization (SSH) was applied using one FCHL-control pair to generate a library of differentially expressed genes in adipose tissue, leaving 10 pairs for study. A selection of differentially expressed genes generated by SSH combined with conceptual candidate genes were spotted on macroarrays. Gene expression levels in preadipocytes from 5 FCHL-control pairs, cultured and harvested under the same conditions, were measured by macroarray. A three-step validation procedure was applied. First, reliability of the array was tested by measuring gene expression levels in a selection of genes by qRT-PCR with the same RNA as used for the macroarray. Second, a selection of differentially expressed genes was validated by qRT-PCR using RNA from regrown cell lines of the same subjects. Third, genes with consistent differential expression were validated in preadipocytes of 5 additional, genetically independent, FCHL-control pairs.

Fig. 2

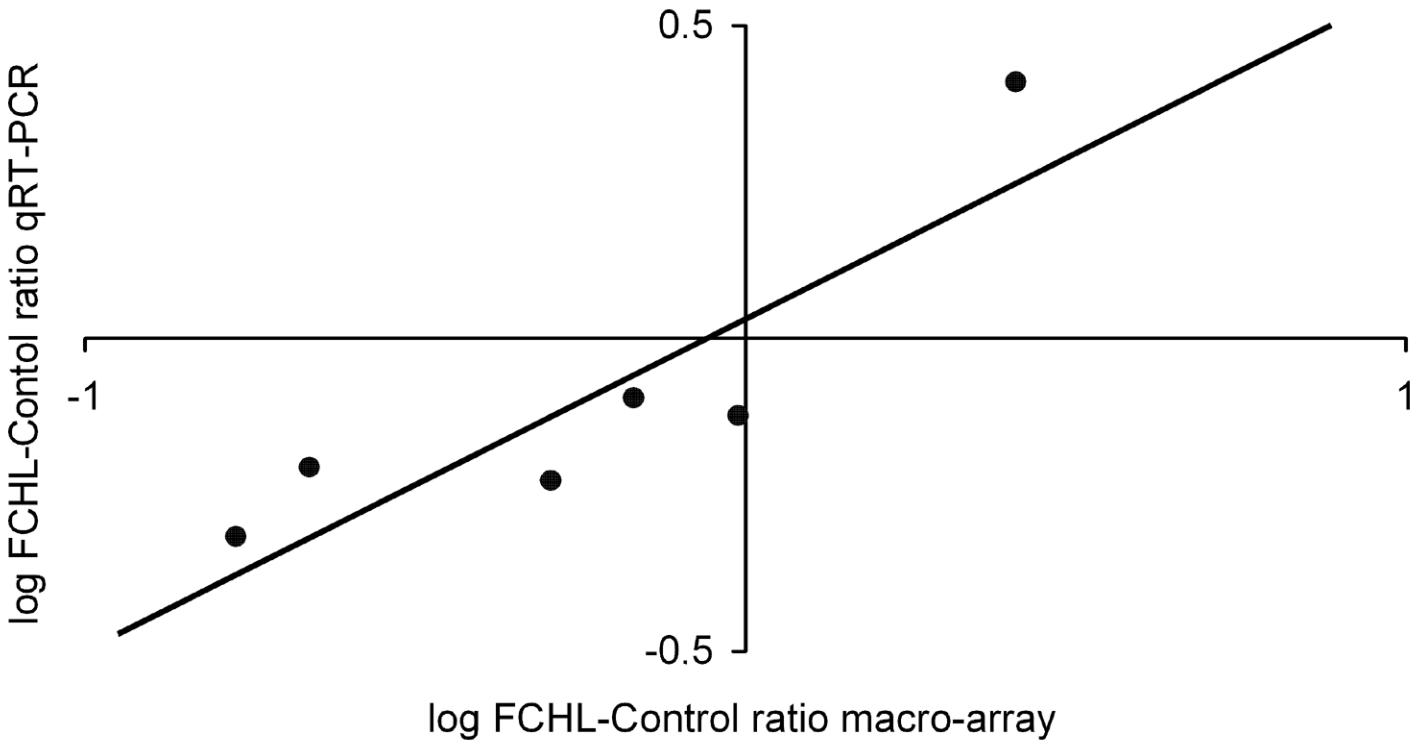

Figure 2. Fold changes of mRNA levels measured by macroarray and qRT-PCR (r^2=0.78, P<0.01).

Fig. 3

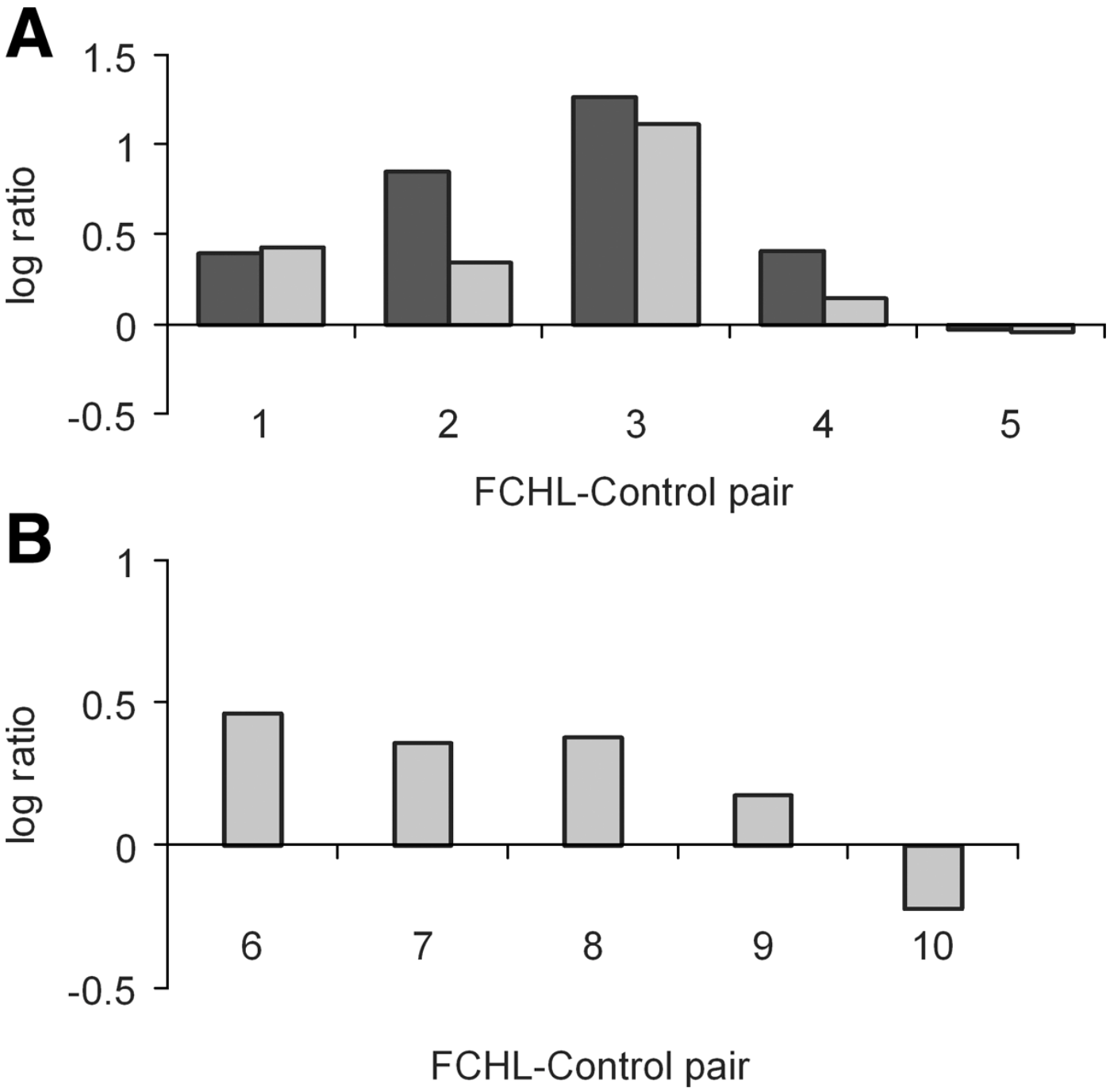

Figure 3. Black bars represent expression data of the macroarray. Gray bars represent qRT-PCR expression data. ***A***) CD36/FAT was up-regulated in 4 out of 5 FCHL patients on the macroarray. CD36/FAT expression levels measured by qRT-PCR in recultured preadipocytes closely followed expression levels of the macroarray, indicating the reproducibility of the data. ***B***) Up-regulation of CD36/FAT was confirmed by qRT-PCR in a second group of 5 independent FCHL-control pairs.

Fig. 4

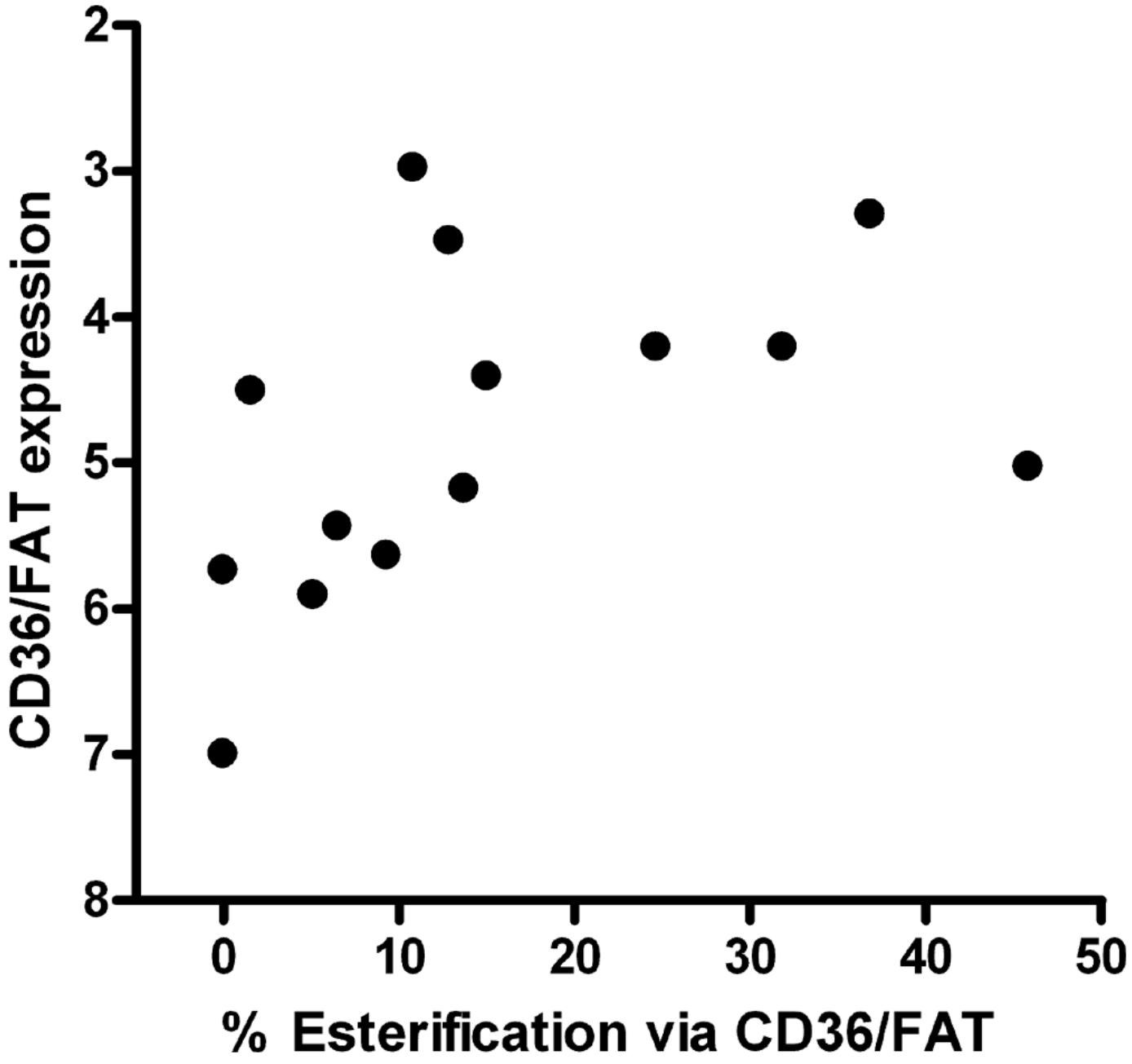

Figure 4. CD36/FAT RNA levels are positively correlated with the CD36/FAT mediated fatty acid esterification fraction (spearman's rho = 0.65, *P*=0.01). CD36/FAT expression is depicted as the CD36/FAT Ct value measured by qRT PCR, corrected for housekeeping gene (18srRNA) expression.

Fig. 5

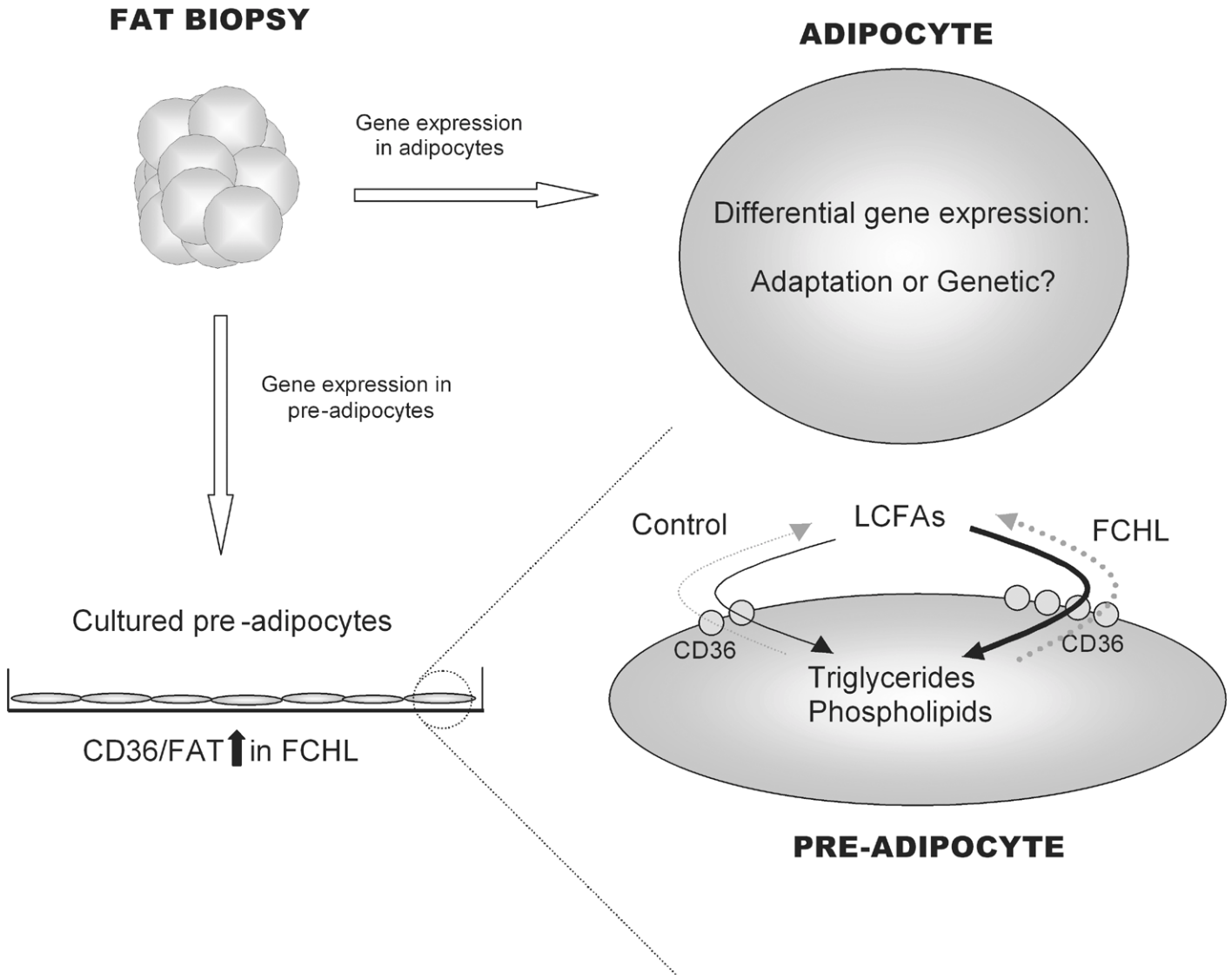

Figure 5. Gene expression experiments in mature freshly isolated adipocytes do not allow to distinguish between adaptations to metabolic influences or expression differences that are a direct result of a genetic trait. In cultured preadipocytes, the contribution of previous metabolic traits on gene expression is suppressed, whereas genetic defects, and direct consequences thereof, are preserved and expressed in vitro. CD36/FAT expression was increased in FCHL preadipocytes. Up-regulation was confirmed at the functional level, as FCHL preadipocytes demonstrated increased CD36/FAT-mediated uptake and esterification of fatty acids.