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ARTICLE

Downregulation of the acetyl-CoA metabolic network in adipose tissue of obese diabetic individuals and recovery after weight loss

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Abstract
Aims/hypothesis Not all obese individuals develop type 2 diabetes. Why some obese individuals retain normal glucose tolerance (NGT) is not well understood. We hypothesise that the biochemical mechanisms that underlie the function of adipose tissue can help explain the difference between obese individuals with NGT and those with type 2 diabetes.
Methods RNA sequencing was used to analyse the transcriptome of samples extracted from visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) of obese women with NGT or type 2 diabetes who were undergoing bariatric surgery. The gene expression data was analysed by bioinformatic visualisation and statistical analyses techniques.
Results A network-based approach to distinguish obese individuals with NGT from obese individuals with type 2 diabetes identified acetyl-CoA metabolic network downregulation as an important feature in the pathophysiology of type 2 diabetes in obese individuals. In general, genes within two reaction steps of acetyl-CoA were found to be downregulated in the VAT and SAT of individuals with type 2 diabetes. Upon weight loss and amelioration of metabolic abnormalities three months following bariatric surgery, the expression level of these genes recovered to levels seen in individuals with NGT. We report four novel genes associated with type 2 diabetes and recovery upon weight loss: *ACAT1* (encoding acetyl-CoA acetyltransferase 1), *ACACA* (encoding acetyl-CoA carboxylase α), *ALDH6A1* (encoding aldehyde dehydrogenase 6 family, member A1) and *MTHFD1* (encoding methylenetetrahydrofolate dehydrogenase).
Conclusions/interpretation Downregulation of the acetyl-CoA network in VAT and SAT is an important feature in the pathophysiology of type 2 diabetes in obese individuals. *ACAT1*, *ACACA*, *ALDH6A1* and *MTHFD1* represent novel

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biomarkers in adipose tissue associated with type 2 diabetes in obese individuals.

Keywords Acetyl-CoA · Adipocytes · RNA sequencing · Visceral adipose tissue

Abbreviations

IEM	Inborn errors of metabolism
KEGG	Kyoto Encyclopedia of Genes and Genomes
NGT	Normal glucose tolerance
SAT	Subcutaneous adipose tissue
SNP	Single-nucleotide polymorphism
VAT	Visceral adipose tissue

Introduction

Obesity is associated with increased risk of premature death and has reached epidemic proportions in modern societies [1]. Obesity results in decreased life expectancy due to associated metabolic and cardiovascular disorders, as well as several types of cancer [2, 3]. The majority of obese individuals develop insulin resistance and type 2 diabetes. However, approximately 10–25% of these individuals seem to remain insulin sensitive and metabolically 'healthy' [4]. Studies have shown that the expanded adipose tissue serves as an important pathogenic site in the development of type 2 diabetes [5]. Furthermore, the prevalence of metabolically healthy obesity has been attributed to a normal adipose tissue function [5]. A criterion for distinguishing between obese subtypes is of crucial importance to develop appropriate intervention and prevention strategies for these individuals [6]. Most studies have focussed on developing risk scores based on blood pressure, lipid levels, glucose homeostasis and inflammatory variables to distinguish the metabolically healthy from the metabolically abnormal [7, 8]. However, the biological mechanisms underlying the phenotypic differences observed among obese individuals are not fully understood. In view of the central role of adipose tissue in the manifestation of obesity pathology, we investigated gene expression and biochemical pathway profiles in visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) in a human cohort comprised of very obese individuals (BMI >40 kg/m^2) who had normal glucose tolerance (NGT) or who had type 2 diabetes.

Whole genome expression profiling of both SAT and VAT presents an opportunity to study the development of disease in the adipose tissue depots and to delineate biological processes that might explain the dysregulation of metabolism in these tissues. Earlier studies used microarray analyses to compare gene expression profiles in the SAT and VAT of obese individuals and found co-regulation of immune and metabolic genes with insulin resistance and metabolic syndrome [9–11]. We have employed next-generation RNA sequencing technology as it offers extensive coverage, precise quantification of transcripts and a large dynamic range [12–14].

The current study applied bioinformatic visualisation and statistical analyses techniques to the gene expression data and showed dysregulated acetyl-CoA metabolism as a distinguishing feature of obese individuals with type 2 diabetes. Multiple genes in the immediate vicinity of the acetyl-CoA reaction network were downregulated in diabetic obese individuals. To ascertain whether the downregulation of these genes was correlated to health status, we studied expression levels of these genes before and three months after bariatric surgery associated with significant weight loss and improvement of morbidity.

Methods

Participants The study group consisted of 17 obese women with NGT (with normal fasting glucose levels) and 15 obese women with type 2 diabetes (classified according to WHO standards). The groups were matched for age, weight and BMI (Table 1). All the women had been morbidly obese (BMI >40 kg/m^2) for at least 5 years. Participants who reported the use of weight loss medications during the 90 days before enrolment in the study were excluded. The body weight of all participants had been stable for at least 3 months prior to inclusion. The participants were investigated in the morning after an overnight fast. A venous blood sample was taken for the determination of plasma glucose (by the routine chemistry laboratory at the hospital) and insulin (by IRMA; Medgenix, Fleurus, Belgium). Thereafter, SAT was obtained from the paraumbilical region by needle aspiration under local anaesthesia using lidocaine. Around 4 weeks after the first examination all individuals underwent bariatric surgery (gastric bypass/banding). Within 1 h of opening the abdominal wall adipose tissue specimens were taken from the epigastric region of the abdominal wall (SAT) and from the major omentum (VAT). One piece of these adipose tissues was immediately put in RNA-later (Ambion; Life Technologies, Bleiswijk, the Netherlands) and subsequently stored at −80°C. Another piece of adipose tissue was used for the isolation of adipocytes using collagenase treatment, as described [15]. Three months after the operation, the participants were investigated again after an overnight fast. Plasma glucose and insulin was determined and another SAT needle biopsy was taken. The diets of the participants were not energy restricted in the period leading up to the bariatric surgery.

The study was approved by the Ethics Committee of Leiden University. All participants gave informed consent to participate in the study.

Table 1 Characteristics of participants with NGT and type 2 diabetes at baseline and 3 months post-bariatric surgery

Characteristic	NGT		T2DM		*p* value			
	Baseline	3 months post-surgery	Baseline	3 months post-surgery	T2DM vs NGT (baseline)	T2DM vs NGT (3 months)	NGT 3 months vs baseline	T2DM 3 months vs baseline
n	17	17	15	15				
Age (years)	49±6	49±6	53±5	53±5	NS	NS	NS	NS
BMI (kg/m^2)	42.9±3.2	36.9±3.3	43.4±4.4	35.9±4.0	NS	NS	1.38×10^{-15}	8.21×10^{-16}
Weight (kg)	122.2±3.1	105.0±2.8	118.9±4.5	98.9±3.7	NS	NS	1.23×10^{-14}	2.11×10^{-14}
HOMA-IR	2.79±2.05	1.72±1.62	4.25±3.26	1.68±0.91	0.06	NS	0.09	0.001
Fasting glucose (mmol/l)	5.08±0.54	5.08±0.76	9.28±2.61	5.87±1.21	2.03×10^{-10}	NS	NS	6.9×10^{-8}
Fasting insulin (pmol/l)	72.5±49.9	42.9±34.9	59.4±40.0	38.9±19.7	NS	NS	0.006	0.09
HbA_{1c}								
mmol/mol	37.6±2.3	34.1±0.9	55.0±4.3	40.2±1.8	8.2×10^{-6}	0.10	NS	0.0002
%	5.6	5.3	7.2	5.8	8.2×10^{-6}	0.10	NS	0.0002
Triacylglycerol (mmol/l)	1.49±0.17	1.30±0.13	2.02±0.19	1.32±0.14	0.03	NS	0.03	1.13×10^{-7}
NEFA (mmol/l)	0.99±0.07	1.16±0.08	1.18±0.11	1.14±0.09	NS	NS	0.06	NS
Total cholesterol (mmol/l)	4.84±0.25	4.20±0.18	4.34±0.22	3.49±0.20	NS	0.03	0.002	0.0006
HDL-cholesterol (mmol/l)	1.14±0.07	1.05±0.05	1.10±0.09	1.05±0.07	NS	NS	0.050	NS
LDL-cholesterol (mmol/l)	3.03±0.21	2.42±0.20	2.33±0.17	1.84±0.19	0.02	0.050	0.005	0.053
CRP (mg/l)	7.74±1.90	6.16±2.26	8.30±1.95	4.13±1.00	NS	NS	NS	0.004

Data are means±SD

Statistical differences between NGT and T2DM and pre- and post-intervention groups were determined with a mixed-effects model, where subject-specific deviances were modelled with random intercepts

CRP, C-reactive protein; T2DM, type 2 diabetes

Medication For obvious reasons we could not restrict the study to obese participants not using any type of medication. All participants were allowed to use cholesterol-lowering statins and antihypertensive medication. The use of drugs such as statins and antihypertensive drugs was slightly higher in the diabetic participants. At baseline, statins were used by 60% of patients with type 2 diabetes and 18% of patients with NGT. Of the diabetic patients 75% used antihypertensives against 40% in individuals with NGT. A substantial proportion of patients with type 2 diabetes received treatment with metformin (n=9; 60%) or sulfonylurea derivatives (n=4; 25%).

Isolation of RNA Total RNA was isolated using the Nucleospin RNA kit (Macherey-Nagel, Düren, Germany) according to the instructions of the manufacturer. The quality of each mRNA sample was examined using the Agilent 2100 Bioanalyzer (Santa Clara, CA, USA). All RNA samples had a RNA integrity number >7.

RNA deep sequencing RNA (50 μg) from the adipose tissue samples obtained during bariatric surgery was used for RNA deep sequencing, which was performed at the Beijing Genomics Institute using RNA-Seq (Transcriptome) sequencing on the HiSeq2000 (Illumina, San Diego, CA, USA) with 90-nucleotide-long Paired End reads, resulting in a minimum of 3 Gb clean data per sample. The reads were aligned to the Human reference genome build 19 (hg19) to obtain a histogram of coverage per exon and the associated count data (see electronic supplementary material [ESM] Methods 1). Differential expression analysis was performed on exon, gene and transcript levels as described in ESM Methods 1.

Bioinformatic analysis The bioinformatic analysis was performed as described in ESM Methods 2.

Quantitative real-time PCR for comparison of pre- and post-surgery gene expression data for select members of acetyl-CoA gene set The RNA of the needle biopsies obtained pre- and post-bariatric surgery, as well as the RNA obtained from the adipocytes during bariatric surgery, was used for quantitative real-time PCR (see ESM Methods 3 and ESM Table 1).

Results

Characteristics of participants at baseline and 3 months post-bariatric surgery Characteristics of the participants are shown in Table 1. At baseline fasting glucose, HbA_{1c} and triacylglycerol levels were significantly higher in individuals with type 2

diabetes than in those with NGT. Three months post-surgery, individuals with NGT and diabetes showed the same weight reduction. Fasting glucose, HbA_{1c} and triacylglycerol levels were significantly reduced in the diabetic individuals and were similar to levels in the individuals with NGT.

Gene expression analysis We used RNA sequencing to analyse the transcriptome of samples extracted from VAT and SAT of 32 (15 with type 2 diabetes, 17 with NGT) obese women undergoing bariatric surgery (Table 1). We first determined whether the overall gene expression profiles differed between obese women with type 2 diabetes and those with NGT and applied the global test [16] on all expressed genes. The global test on VAT and SAT yielded a p value of 3.7×10^{-3} and 9.4×10^{-4}, respectively, indicating a significant association of gene expression with health status. Gene-level analysis with the limma package in R identified 168 genes differentially expressed in VAT ($p<0.05$, after Benjamin–Hochberg FDR correction) between obese individuals with NGT and those with type 2 diabetes (Table 2 and ESM Table 2). Applying the same method on SAT yielded 121 genes that were significantly differentially expressed between obese individuals with NGT and those with type 2 diabetes (Table 3). There was an overlap of 24 of the differentially expressed genes between the two tissues.

Bioinformatic analysis to identify subnetworks in gene expression data We further investigated biological mechanisms underlying the differential health status among the participants. Statistically significant differential expression of genes ($p<0.05$ after FDR correction) in VAT and SAT was used as an input to a pathway-based over-representation analysis tool made available by ConsensusPathDB (http://cpdb.molgen.mpg.de/, accessed 14 January 2013). This analysis of genes from VAT identified pathways relevant to carbon, amino acid and fatty acid metabolism (ESM Table 3). A similar analysis strategy for SAT identified pathways relevant to several bacterial infections, regulation of actin cytoskeleton and Fc-γ R-mediated phagocytosis (ESM Table 4). The overlap between significant ($q<0.05$) pathways identified for the two tissues is limited to insulin signalling, branched-chain amino acid degradation and pyruvate metabolism. Furthermore, to determine whether significantly differentially expressed genes in each of the two tissues operate in close proximity in network space, we used 'Network-neighbourhood-based entity sets' (NEST), a software tool made available by ConsensusPathDB. ESM Table 5 shows the result for input of the top differentially expressed genes in VAT (168 genes, $p<0.05$ after multiple test correction). This analysis indicated that the differentially expressed genes in VAT operate in a network neighbourhood at the intersection of carbohydrate, amino acid and fatty acid metabolism. Importantly, the majority of the genes mapped onto these pathways were present in close proximity in network space to acetyl-CoA metabolism (Fig. 1). A similar approach, using NEST, with the significant hits from SAT did not yield any statistically significant sets.

Table 2 Top 25 genes up- or downregulated in VAT of diabetic individuals

Gene	Coefficient NGT vs T2DM	p value NGT vs T2DM	Adjusted p value NGT vs T2DM
ALDH6A1	−0.670	1.49×10^{-6}	0.005502
C14orf45	−0.462	1.59×10^{-6}	0.005502
ECHS1	−0.521	1.48×10^{-6}	0.005502
IRS1	−0.601	3.41×10^{-7}	0.005502
STBD1	−0.615	6.74×10^{-7}	0.005502
IARS2	−0.311	2.73×10^{-6}	0.006958
NAT8L	−0.745	2.81×10^{-6}	0.006958
AIFM2	−0.452	3.24×10^{-6}	0.007013
ATPAF1	−0.349	3.71×10^{-6}	0.007141
ACAD9	−0.311	8.28×10^{-6}	0.010501
GPI	−0.285	8.25×10^{-6}	0.010501
HADH	−0.575	8.49×10^{-6}	0.010501
HSPD1	−0.299	7.74×10^{-6}	0.010501
MTHFD1	−0.423	6.16×10^{-6}	0.010501
ACACA	−0.560	9.14×10^{-6}	0.010554
MAP3K15	−0.433	1.19×10^{-5}	0.012882
HK2	−0.712	1.32×10^{-5}	0.01298
PARVG	0.654	1.5×10^{-5}	0.01298
PDHA1	−0.375	1.48×10^{-5}	0.01298
PRKAR2B	−0.716	1.39×10^{-5}	0.01298
ACAT1	−0.406	1.81×10^{-5}	0.012994
ATP9A	−0.400	2.1×10^{-5}	0.012994
CEBPA	−0.566	1.97×10^{-5}	0.012994
DARS2	−0.379	1.64×10^{-5}	0.012994
NXPH4	−1.002	1.89×10^{-5}	0.012994

Coefficient NGT vs T2DM: log fold change of NGT vs T2DM; a negative value reflects downregulation whereas a positive value reflects upregulation of the gene in type 2 diabetic individuals

For the complete list of up- or downregulated genes in VAT of type 2 diabetic individuals see ESM Table 2

The adjusted p value NGT vs T2DM is the p value after Benjamini–Hochberg FDR correction

T2DM, type 2 diabetes

The acetyl-CoA metabolic network is downregulated in diabetic obese individuals The enriched network-neighbourhood-based sets described above hinted at the possibility of the acetyl-CoA metabolic network being a common feature of the statistically significant differentially expressed genes in VAT. To determine whether genes within two reaction steps of acetyl-CoA metabolism were significantly represented among the top hits in VAT, a gene set was generated using the Taverna workflow management system and the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway

Table 3 Top 25 genes up- or downregulated in SAT of diabetic individuals

Gene	Coefficient NGT vs T2DM	*p* value NGT vs T2DM	Adjusted *p* value NGT vs T2DM
DHTKD1	−0.39953	3.38×10^{-6}	0.027658
DPEP2	0.941324	3.63×10^{-6}	0.027658
S100A11	0.389024	4.79×10^{-6}	0.027658
IRS1	−0.64306	7.26×10^{-6}	0.027696
BIVM	−0.32809	8×10^{-6}	0.027696
CRABP2	0.889426	1.15×10^{-5}	0.033234
PXMP2	−0.46718	1.65×10^{-5}	0.03571
LSP1	0.826079	1.53×10^{-5}	0.03571
RNF14	−0.29276	2.01×10^{-5}	0.038745
FXYD5	0.508216	3×10^{-5}	0.041435
TYROBP	0.789776	2.74×10^{-5}	0.041435
CYBA	0.573909	2.8×10^{-5}	0.041435
THNSL1	−0.48462	3.11×10^{-5}	0.041435
ALDH6A1	−0.59541	5.12×10^{-5}	0.042281
C14orf45	−0.39723	0.000107	0.042281
HADH	−0.45138	0.000145	0.042281
MTHFD1	−0.3727	7.95×10^{-5}	0.042281
MAP3K15	−0.39465	9.36×10^{-5}	0.042281
SLC2A4	−0.73171	0.000105	0.042281
ME1	−0.45845	9.99×10^{-5}	0.042281
LDHD	−0.53027	9.59×10^{-5}	0.042281
FAN1	−0.26323	5.17×10^{-5}	0.042281
TMEM218	−0.39528	0.000128	0.042281
EEPD1	−0.45794	0.000156	0.042281
IL2RG	0.835802	0.000114	0.042281

Coefficient NGT vs T2DM: log fold change of NGT vs T2DM; a negative value reflects downregulation whereas a positive value reflects upregulation of the gene in type 2 diabetic individuals

The adjusted *p* value NGT vs T2DM is the *p* value after Benjamini–Hochberg FDR correction

T2DM, type 2 diabetes

database (ESM Methods 4). This approach involved finding all the genes that participate within a radius of two steps in the reaction space surrounding acetyl-CoA. This algorithm was implemented in Taverna and the pathway information present in the KEGG database was used to generate the gene set. The total number of genes in the acetyl-CoA set is 419.

We then performed statistical tests to determine whether members of the acetyl-CoA gene set were significantly represented among top hits in VAT. The number of genes among the 168 top hits in VAT that are also members of the acetyl-CoA gene set was 42 (ESM Table 2), ten times more than expected by chance ($p=1\times10^{-63}$, permutation test), indicating that the presence of the members of the acetyl-CoA gene set among the top hits due to chance alone was negligible. All these 42 genes were downregulated in the VAT of obese individuals with diabetes (ESM Table 2). Additionally, the global test to evaluate the acetyl-CoA gene set as a predictor of health status in VAT and SAT yielded a *p* value of 2.4×10^{-2} and 8.4×10^{-3}, respectively. The network-neighbourhood test did not yield a significant set for SAT, yet the acetyl-CoA gene set is more significant in SAT than in VAT because most of the genes in the acetyl-CoA gene set are borderline significant in SAT. These genes failed to make the cut-off necessary to be included for network-neighbourhood tests. However, the global test took into account the *p* value of all the entities in the gene set, and since most genes have modest *p* values in SAT, the overall *p* value generated for the acetyl-CoA gene set in that tissue type was lower than we would expect by examining the network neighbourhood of the most significant genes. In conclusion, genes in the acetyl-coA reaction network displayed a general downregulation in both VAT and SAT of individuals with type 2 diabetes.

Analysis at the transcript or exon level We investigated possible differential splicing events, comparing obese individuals with NGT and diabetes, for the 42 genes in the acetyl-CoA gene set. To do so, we analysed differences at the transcript level and at the level of expression of individual exons. Of the 42 genes, there were 16 genes with multiple annotated transcripts. All of the transcript variants were significantly downregulated in the individuals with type 2 diabetes as compared with the individuals with NGT (data not shown).

At the exon level, we did not identify any exon that deviated significantly from the overall gene expression pattern and did not obtain any evidence for alternative splicing between individuals with NGT and those with type 2 diabetes (data not shown).

Downregulation of genes in the acetyl-CoA reaction network recovers after weight loss Of the 24 genes that overlapped between the statistically significant top hits in VAT and SAT, nine genes were members of the acetyl-CoA gene set (*ACACA*, *ALDH6A1*, *MTHFD1*, *HADH*, *ME1*, *PC*, *LDHD*, *DHTKD1* and *GNPAT*). The gene expression profile of all the nine genes from the RNA-sequencing experiments showed a consistent downregulation among individuals with type 2 diabetes in both adipose tissues. The box plot depicting the expression levels in each of the tissues for both health types is shown, for some of the acetyl-CoA genes, in Fig. 2.

To ascertain whether the downregulation of the acetyl-CoA genes was correlated to type 2 diabetes, we compared the pre- and post-surgery (3 months after) expression levels of these genes in SAT by quantitative PCR. At this time the majority of diabetic obese women had a significantly improved metabolic health status as demonstrated by lower fasting glucose levels (Table 1). We observed a statistically significant upregulation of genes encoding acetyl-CoA carboxylase α (*ACACA*) ($p=9.3\times10^{-3}$), aldehyde dehydrogenase 6 family, member A1

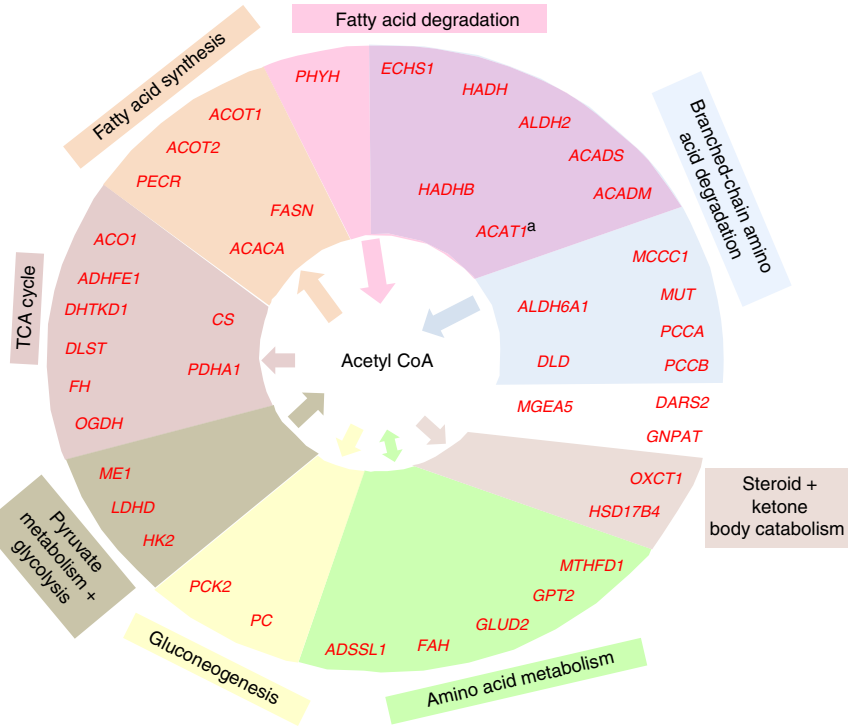

Fig. 1 Downregulation of the acetyl-CoA gene network in type 2 diabetes. Forty-two genes that are among the top differentially expressed genes in VAT are also members of the acetyl-CoA gene set. The genes within the inner circle act directly on acetyl-CoA while the genes in the outer circle participate one reaction step away from acetyl-CoA. All the genes were downregulated in VAT. [a]Also contributes to ketone body metabolism. TCA, tricarboxylic acid cycle

(*ALDH6A1*) ($p=4.1\times10^{-5}$) and methylenetetrahydrofolate dehydrogenase (*MTHFD1*) ($p=4.7\times10^{-2}$) post-surgery in individuals with type 2 diabetes, when compared with the changes in expression level observed in individuals with NGT (Fig. 3). Also acetyl-CoA acetyltransferase 1 (*ACAT1*), which is at the intersection of the acetyl-CoA network (Fig. 1), was upregulated post-surgery in type 2 diabetes ($p=2.3\times10^{-3}$). Three other genes, encoding dehydrogenase E1 and transketolase domain (*DHTKD1*), lactate dehydrogenase

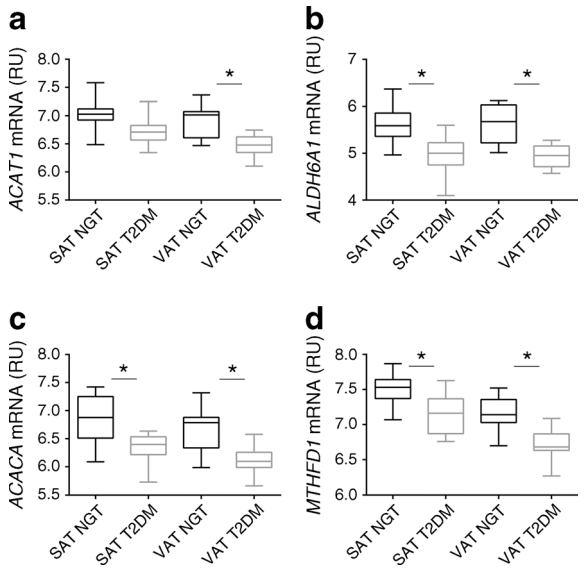

Fig. 2 Gene expression of acetyl-CoA network genes in VAT and SAT. Box plots of normalised gene expression profiles (relative units [RU]: $\log_2$-scale) of a few representative genes, *ACAT1* (**a**), *ALDH6A1* (**b**), *ACACA* (**c**), *MTHFD1* (**d**), in the acetyl-CoA reaction network that are downregulated (*adjusted p value <0.05 for indicated comparison) in both VAT and SAT of obese individuals with type 2 diabetes (grey bars) compared with NGT (black bars). The whiskers in the boxplots represent the upper and lower limits of the data. T2DM, type 2 diabetes

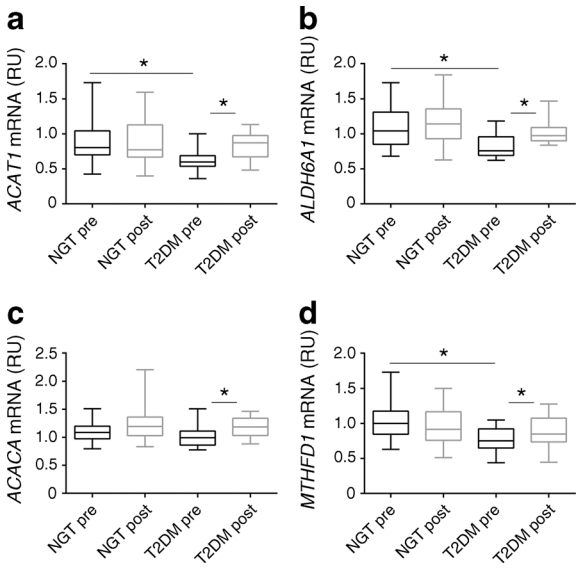

Fig. 3 Gene expression of acetyl-CoA network genes in obese individuals with type 2 diabetes are normalised after bariatric surgery. Box plots of expression levels of four representative genes, *ACAT1* (**a**), *ALDH6A1* (**b**), *ACACA* (**c**), *MTHFD1* (**d**) (as determined by quantitative PCR, corrected for housekeeping gene, linear scale: relative units [RU]), in type 2 diabetes and NGT before (black bars) and after bariatric surgery (grey bars). T2DM, type 2 diabetes. *$p<0.05$ (mixed-model-analysis). The whiskers in the boxplots represent the upper and lower limits of the data

(*LDHD*) and pyruvate carboxylase (*PC*), displayed a similar upregulation post-surgery in individuals with type 2 diabetes but did not reach the statistical *p* value threshold of 0.05. This indicates that the improved health status of diabetic individuals post-surgery is associated with a reversal of the disturbance in the acetyl-CoA metabolic network.

Gene expression of acetyl-CoA network in isolated adipocytes As adipose tissue not only consists of adipocytes but is a mixture of cells, including endothelial cells and leucocytes, we determined whether the downregulation of the acetyl-CoA network in diabetic individuals specifically takes place in the adipocytes. Indeed isolated adipocytes of diabetic individuals showed reduced gene expression levels for *ALDH6A1*, *ACAT1* and *MTHFD1* (Fig. 4).

Discussion

We have performed an in-depth comparison of gene expression in SAT and VAT of severely obese women with and without type 2 diabetes. Network analyses revealed that the acetyl-CoA network was dysregulated in type 2 diabetes and that specific genes directly associated with acetyl-CoA metabolism were downregulated in both VAT and SAT. Importantly, upon weight loss and amelioration of metabolic abnormalities, the expression of these genes in SAT recovered to a level corresponding to that seen in women with NGT.

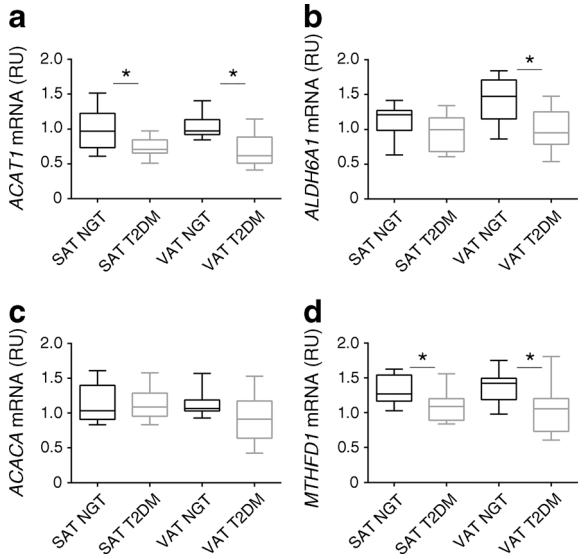

Fig. 4 Gene expression of acetyl-CoA network genes in adipocytes. Adipocytes were isolated from SAT and VAT of individuals with type 2 diabetes (grey bars) and NGT (black bars). Gene expression of four representative genes, *ACAT1* (**a**), *ALDH6A1* (**b**), *ACACA* (**c**), *MTHFD1* (**d**), was measured using quantitative PCR, corrected for housekeeping gene expression and plotted on a linear scale (RU). The whiskers in the boxplots represent the upper and lower limits of the data. T2DM, type 2 diabetes. *$p<0.05$ (*t* test) NGT vs T2DM

These results imply that downregulation of the acetyl-CoA network in VAT and SAT is a marker for the metabolic dysregulation characteristic of type 2 diabetes and, moreover, that it is reversible.

Network-based approaches have emerged as a powerful tool to unravel the mechanisms underlying complex traits [17–19]. Biological networks consist of molecular entities called nodes and functional interconnections between them called edges. An important property of these networks is that they are 'scale-free', in that some nodes called 'hubs' are connected to a substantially large number of other nodes and are therefore considered essential for maintaining the integrity of the cell [17]. In general, these systems are robust against random mutations but are vulnerable to attacks against the hub [17]. Acetyl-CoA is a key hub metabolite of the metabolic network and plays a critical role in maintaining cellular homeostasis [20]. Previous studies have implicated branched-chain amino acid degradation [21], fatty acid oxidation [22, 23] and citrate cycle [22, 23] dysregulation as a characteristic feature of type 2 diabetes and related traits. In this study, in addition to confirming the previous findings, we argue that the acetyl-CoA reaction network is a unifying principle and that its dysregulation distinguishes between obese women with type 2 diabetes and those with NGT.

Acetyl-CoA lies at the crossroads of glycolysis, citrate cycle, ketogenesis, lipid synthesis, amino acid and fatty acid metabolism, suggesting that the metabolite may play a key role as an energy sensor in the cell [20]. Carbon skeletons of sugars, amino acids and fatty acids are degraded to the acetyl group to form acetyl-CoA, which enters the citric acid cycle for energy generation. In addition, it is known to modulate gene expression through its role as a co-factor of histone acetyl-transferases, which enable the transcription of genes through histone acetylation at chromatin structures [24]. Cai et al argue that the primordial role of protein acetylation could have been to enable a cell to modulate gene expression/protein function in tune with the carbon source availability [25]. In other words, the acetyl-CoA is likely to serve as a fundamental and widely conserved gauge of metabolic state. A disturbance in this gauge may contribute to metabolic diseases, such as type 2 diabetes, as a consequence of altered cell metabolism and transcriptional regulation.

We report four genes associated with type 2 diabetes and recovery in the SAT of obese individuals: *ACAT1*, *ACACA*, *ALDH6A1* and *MTHFD1*. These genes all participate in the immediate vicinity of acetyl-CoA metabolism and are known hotspots of human metabolism, with *ACAT1*, *ALDH6A1* and *ACACA* recorded among inborn errors of metabolism (IEM) (Online Mendelian Inheritance in Man [OMIM]: 203750, 614105 and 613933, respectively). IEMs are congenital metabolic defects arising from single or multiple enzyme deficiencies [26]. Recently, IEMs have been mapped onto a mathematical reconstruction of human metabolism [27].

Analyses of IEMs in the context of network topology led to the observation that the IEMs are adjacent to each other, with acetyl-CoA acting as the central metabolite. This clearly suggests that the vicinity of acetyl-CoA in the network topology is a hub where abnormalities in individual genes potentially accumulate and, upon reaching a certain risk threshold, lead to the manifestation of disease.

The genes reported in this study function at critical decision points in cellular biochemical pathways, as illustrated by *ACAT1*. The enzyme encoded by this gene, acetyl-CoA acetyltransferase 1, mediates the reversible conversion of two molecules of acetyl-CoA to acetoacetyl-CoA [28]. This enzyme catalyses the final step in branched-chain amino acid and fatty acid degradation pathways and the acetyl-CoA produced here is used as an input for the citric acid cycle (www.genome.jp/dbget-bin/www_bget?hsa:38, accessed 18 September 2013). When energetics favours the production of acetoacetyl-CoA in this reaction step, the metabolite is used for ketone body synthesis [28]. Acetyl-CoA acetyltransferase 1 also mediates the first step in the mevalonate pathway, the end-product of which (Farnesyl-PP) is a precursor for cholesterol among other several important metabolites (www.genome.jp/dbget-bin/www_bget?hsa:38, accessed 18 September 2013). Therefore, acetyl-CoA acetyltransferase 1 is strategically placed at the intersection of important cellular pathways that respond to the energy status of the cell.

Intriguingly, additional genetic evidence for the role of *ACAT1* in type 2 diabetes mellitus is provided by a genome-wide association study (GWAS) in a UK prospective diabetes study that investigated the glycaemic response to metformin and reported a single-nucleotide polymorphism (SNP), rs11212617, associated with metformin's success [29]. Based on the proximity to the polymorphism, the study concluded that *ATM* was the causal gene that plays a role in metformin's success and that the variation at this gene alters the glycaemic response to metformin. However, re-analysing the polymorphism rs11212617, we found that the polymorphism is in fact an eQTL (http://eqtl.uchicago.edu/cgi-bin/gbrowse/eqtl/, accessed 30 November 2012) for the nearby *ACAT1* gene and not *ATM*. The confirmation for this eQTL is provided by two independent studies; Zeller et al who studied the monocyte transcriptome to determine eQTLs of relevance to human disease [30] and the data from the GEUVADIS consortium [31, 32], where the SNP was found to be an eQTL for *ACAT1* (nominal p value=1.1×10^{-6}). This means that the variation in the expression level of *ACAT1* alters the glycaemic response to metformin and therefore plays a role in the success of metformin's treatment. Furthermore, this clearly suggests that *ACAT1* plays a role in type 2 diabetes. Individuals with the polymorphism that alters its expression level may represent a subtype among individuals with type 2 diabetes, perhaps with different response to metformin.

There were differences in the usage of medication between the obese women with NGT and type 2 diabetes, especially in the usage of metformin, which was not used by any of the women with NGT and by 60% of the women with type 2 diabetes. As metformin acts on enzymes within the acetyl-coA network and affects lipid and glucose metabolism, the usage of metformin may have confounded our results. However, we have not found any evidence for this: (1) there was no difference in gene expression of *ACAT1*, *ALDH6A1*, *ACACA* and *MTHFD1* between metformin users and metformin non-users (ESM Fig. 1); (2) when metformin users were excluded from the comparison between individuals with NGT and those with type 2 diabetes, there was still a downregulation of *ACAT1*, *ALDH6A1*, *ACACA* and *MTHFD1* in the women with type 2 diabetes (ESM Fig. 2).

Our cohort consisted of severely obese women. We do not know whether the observed differences were a consequence of the metabolic defects that occur in type 2 diabetes (i.e. hyperglycaemia) or represented the underlying aetiology of type 2 diabetes. However, a previous study that used microarrays to analyse gene expression in adipose tissue showed that during the progression from the lean to the obese state and then further towards the metabolic syndrome, the genes involved in metabolic processing were gradually downregulated [10]. These data suggest that the downregulation of metabolic pathways underlies the pathology of type 2 diabetes.

Previous studies have postulated that low-grade inflammation of the adipose tissue plays an important role in the development of insulin resistance [33–36]. For example, a recent study in monozygotic twins discordant for obesity showed that the SAT transcript profile in the metabolically healthy obese is characterised by the maintenance of mitochondrial function and absence of inflammation [35]. This is in line with the results of our study, where we observe an inverse correlation pattern of differential expression of genes that are downregulated in metabolic and upregulated in inflammatory pathways in VAT and SAT of individuals with type 2 diabetes.

In summary, our results demonstrate that the acetyl-CoA network is dysregulated in VAT and SAT of obese women with type 2 diabetes. We report significant downregulation of several genes in the immediate vicinity of acetyl-CoA and a statistically significant recovery for four genes after amelioration of the metabolic abnormalities in SAT. Further research into the causal role of downregulation of the acetyl-CoA network in type 2 diabetes should indicate whether direct intervention in the acetyl-CoA network will provide novel therapeutic approaches.

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