ORIGINAL ARTICLE

Gene expression profile of subcutaneous adipose tissue in BMI-discordant monozygotic twin pairs unravels molecular and clinical changes associated with sub-types of obesity

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BACKGROUND: Subcutaneous adipose tissue (SAT) undergoes major changes in obesity, but little is known about the whole-genome scale patterns of these changes or about their variation between different obesity sub-groups. We sought to compare how transcriptomics profiles in SAT differ between monozygotic (MZ) co-twins who are discordant for body mass index (BMI), whether the profiles vary between twin pairs and whether the variation can be linked to clinical characteristics.
METHODS: We analysed the transcriptomics (Affymetrix U133 Plus 2.0) patterns of SAT in young MZ twin pairs ($n = 26$, intra-pair difference in BMI > 3 kg m^{-2}, aged 23–36), from 10 birth cohorts of adult Finnish twins. The clinical data included measurements of body composition, insulin resistance, lipids and adipokines.
RESULTS: We found 2108 genes differentially expressed (false discovery rate (FDR) < 0.05) in SAT of the BMI-discordant pairs. Pathway analyses of these genes revealed a significant downregulation of mitochondrial oxidative pathways ($P < 0.05$) and upregulation of inflammation pathways ($P < 0.05$). Hierarchical clustering of heavy/lean twin ratios, representing effects of acquired obesity in the transcriptomics data, revealed three sub-groups with different molecular profiles (FDR < 0.05). Analyses comparing these sub-groups showed that, in the heavy co-twins, downregulation of the mitochondrial pathways, especially that of branched chain amino acid degradation was more evident in two clusters while and upregulation of the inflammatory response was most evident in the last, presumably the unhealthiest cluster. High-fasting insulin levels and large adipocyte diameter were the predominant clinical characteristic of the heavy co-twins in this cluster (Bonferroni-adjusted $P < 0.05$).
CONCLUSIONS: This is the first study in BMI-discordant MZ twin pairs reporting sub-types of obesity based on both SAT gene expression profiles and clinical traits. We conclude that a decrease in mitochondrial BCAA degradation and an increase in inflammation in SAT co-occur and associate with hyperinsulinemia and large adipocyte size in unhealthy obesity.

INTRODUCTION

Subcutaneous adipose tissue (SAT) has an important metabolic function in storing and mobilising energy. During obesity development, SAT, containing adipocytes and stromal-vascular cells that include fibroblastic connective tissue cells as well as preadipocytes, macrophages and leucocytes,[1,2] undergoes major expansion and remodelling.[3–6] Changes include an increase in number and size of adipocytes, infiltration of adipose tissue (AT) by immune cells and relative rarefaction of blood vessels and neural structures.[3–6]

Whole-genome scale SAT transcriptomics studies have revealed various genes and pathways associated with upregulation of inflammation[7–11] and immune response,[7,8] and downregulation of mitochondrial pathways,[8,10,12] insulin-signalling[13] and lipid metabolism[8] in obesity. Further, targeted SAT transcriptomics studies comparing metabolically (MHO) healthy and unhealthy (MUO) obese individuals have found more downregulation of

branched chain amino acid (BCAA) degradation in MUO than in MHO group.[14] In a similar study, weight gain increased lipid metabolism and synthesis in MHO but not in MUO individuals.[15] These studies suggest that although obesity affects AT gene expression in general, the responses may vary depending on the obese person's metabolic health status.

Although there have been a few studies on SAT gene expression comparing obese and lean groups or groups with different clinical health parameters, little is known about whether a hypothesis-free transcriptomics analysis can identify distinct SAT gene expression profiles in obesity, which link to metabolic health. In ordinary studies comparing obese and lean individuals, part of the gene expression differences can be driven by differences in genetic background. To this end, we employ a study design of young adult MZ twin pairs highly discordant for body mass index (BMI). This study design allows us to analyse effects of acquired obesity independent of genetic background. We first examined

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whole-genome scale SAT gene expression differences between all BMI-discordant MZ co-twins, that is, an effect of overweight in general. We then clustered the twin pairs to determine whether the effects of acquired obesity show different molecular and clinical profiles manifesting as heterogeneity of obesity in general population. Lastly, we verified the results in an already published material of BMI-discordant MZ twins.[10]

MATERIALS AND METHODS

Subjects
The discovery phase of the study included 26 BMI-discordant (within-pair difference, ΔBMI $\geqslant$ 3 kg m^{-2}) MZ twin pairs (males $n = 9$, females $n = 17$) recruited from two longitudinal population-based studies of Finnish twins, FinnTwin16 (born between 1975–1979, $n = 2839$ pairs) and FinnTwin12 (born between 1983–1987, $n = 2578$ pairs).[16] The participants have been described previously as has the targeted analysis of genes regulating the mitochondria.[11,12] In brief, the mean BMI difference within the twin pairs was 6 kg m^{-2}, and mean age 30.2 years at the time of clinical study. The twins were healthy, with the exception of one obese twin with inactive ulcerative colitis (treated with mesalazine and azathioprine), and another obese twin with T2DM (treated with metformin and insulin). The replication set comprised 13 healthy BMI-discordant MZ twin pairs (males $n = 8$, females $n = 5$, mean BMI difference within-twin pairs = 5.25 kg m^{-2}, mean age = 25.6) from an already published data set,[10] obtained from (https://www.ebi.ac.uk/arrayexpress/). Four twin pairs exist in the discovery and replication data set but discovery data set samples were taken 6 years after the replication data set. We obtained written informed consent from all participants and designed the study protocol in accordance with the principles of the Helsinki Declaration with approval from the Ethics Committee of the Helsinki University Central Hospital.

Clinical measures were obtained as described previously (Table 1).[12,17] We used dual-energy X-ray absorptiometry (DEXA)[18] to measure body composition, magnetic resonance imaging to determine body fat distribution of abdominal subcutaneous and intra-abdominal fat, and proton magnetic resonance spectroscopy (^{1}H-MRS) to determine liver fat.[19] We measured fasting lipids, high-sensitivity C-reactive protein (hs-CRP), leptin, adiponectin, as well as glucose and insulin during a 2-h oral glucose tolerance test (OGTT). The plasma BCAA (valine, leucine and isoleucine) values were measured using a high-throughput NMR metabolomics platform.[17] We also recorded nutritional intake with a 3-day food diary, physical activity by Baecke indices.[12,20] Current daily smoking status for the twins was assessed by questionnaire.

AT morphology
We obtained SAT biopsies via a surgical technique under local anaesthesia. We photographed the SAT adipocytes with a light microscope and measured the diameters of 200 cells using ImageJ.[21,22]

Transcriptomics analyses
We used the RNeasy Lipid Tissue Mini Kit (Qiagen, Redwood City, CA, USA) to extract SAT RNA,[12] treated it with DNase I (Qiagen) and hybridised 250 ng to Affymetrix U133Plus2.0 microarray (Affymetrix, www.affymetrix.com) to detect gene expression.[11] We normalised the expression data using R-Bioconductor[23] package GCRMA,[24] and performed annotation with Brainarray cdf version 18.[25]

First, we analysed whether within-pair differences for heavy/lean status differed between males and females (by comparing the beta coefficients using linear models for microarray analysis [limma]).[26]

Second, we used limma to identify differentially expressed genes within the co-twins. P-values were corrected for multiple testing (Benjamini and Hochberg method).[27] We considered false discovery rate (FDR) < 0.05 as statistically significant. In this paired design model, as the twin pairs were perfectly matched for sex, sex did not provide any additional information to the model. We further examined the differential gene expression on pathway level using ingenuity pathway analysis tool (IPA, Qiagen).

Cluster analyses
Third, we explored whether the twin pairs cluster based on within-pair gene expression ratios (heavy/lean). We used weighted gene co-expression network analysis (R-WGCNA)[28] to perform hierarchical clustering. We then performed moderated two-sided t-tests (limma), using sex as a covariate, on the gene expression data to obtain differentially expressed genes within the pairs in each cluster. In this paired design model, although the twin pairs were matched for sex, sex distribution between the clusters differed and was adjusted for. Next, we tested whether the original top ten differentially expressed pathways within the 26 twin pairs were cluster-specific using IPA's comparison analysis. Lastly, we determined each cluster's pathways individually using the IPA's canonical pathway analysis. Fisher's exact test P-value < 0.05 was considered significant.

Replication data set
Clinical measures of the replication data set are as described previously[10] (Supplementary Table 1). However, we lacked data for insulin area under the curve (AUC) and glucose AUC during OGTT, adiponectin and BCAA, nutritional intake and physical activity that were measured in the discovery data set. Within-twin pair differences in gene expression for this data set have already been published,[10] and in this study, we used the data set to verify the cluster analysis results.

Basic statistics (discovery and replication data sets)
We used Wilcoxon's signed rank tests in R software[29] to measure means of the clinical measurements in the heavy and lean groups. In addition, we used one-way Anova with Tukey's posthoc tests in R software[29] to calculate mean differences within pairs in each cluster. FDR $\leqslant$ 0.05 was considered significant.

RESULTS

Twin pairs were highly discordant for clinical characteristics of obesity
As reported previously,[12] the lean and heavy co-twins were highly discordant ($P < 0.0001$) for all measures of adiposity (Table 1). In addition, the heavy co-twins were more insulin-resistant (higher fasting insulin and insulin AUC during OGTT), had higher levels of low-density lipoprotein, triacylglycerol, serum hs-CRP and leptin, and lower levels of high-density lipoprotein and adiponectin. The lean twins had significantly higher Baecke sports indices. Most twins were non-smokers, but three pairs were concordant and six pairs discordant for smoking status. The replication set[10] was similar to the current data set for all clinical measures (Supplementary Table 1). Four pairs were concordant and three pairs discordant for smoking status. Other pairs were not smokers.

2108 genes were differentially expressed in SAT within the BMI-discordant pairs
Altogether 2108 genes were differentially expressed (FDR < 0.05) between the lean and heavy co-twins in SAT, 945 (45%) of which were upregulated in the heavy co-twins. Between males and females, these genes had a correlation value of 0.899 (P-value < 0.0001) (Supplementary Figure 1). Although this correlation was high, we decided to altogether minimise the possible effects of sex by adjusting subsequent cluster models for sex. Among the 2108 genes differentially expressed, the top 10 up- and downregulated genes are shown in Table 2 (full list in Supplementary Table 2). The most upregulated gene in the heavy co-twins was EGFL6 (fold change 8.90), which has a role in the regulation of cell cycle, proliferation, and developmental processes. Genes involved in apoptosis and immune response were amongst the top 10 upregulated genes.

The most downregulated gene in the heavy co-twins, SLC27A2 (fold change 0.1848) is associated with lipid biosynthesis and fatty acid degradation. The remaining top nine downregulated genes are involved in lipid and fatty acid metabolism, and cell signalling processes.

Table 1. Characteristics of the 26 BMI-discordant monozygotic twin pairs

Variable	Lean co-twin	Heavy co-twin	P-value
BMI-discordant pairs (ΔBMI > 3 kg m^{-2}, n = 26 pairs)			
BMI (kg m^{-2})	25.3 ± 0.9	31.3 ± 1.0	< 0.0001
Body fat (%)	32.3 ± 1.8	41.1 ± 1.3	< 0.0001
Subcutaneous fat (cm^3)	3813.7 ± 416.8	6358.9 ± 540.4	< 0.0001
Intra-abdominal fat (cm^3)	790.2 ± 178.9	1643.7 ± 247.4	< 0.0001
Liver fat (%)	1.12 ± 0.32	4.52 ± 0.99	< 0.0001
Adipocyte diameter (μm) (n = 25 pairs)[a]	82.93 ± 9.01	97.37 ± 6.98	0.13
fP-glucose (mmol l^{-1}) (n = 25 pairs)[a]	5.1 ± 0.1	5.3 ± 0.1	0.17
AUC glucose in oral glucose tolerance test (mmol l^{-1} h^{-1}) (n = 25 pairs)[a]	14.16 ± 2.71	14.85 ± 2.79	0.35
fS-insulin (mU l^{-1}) (n = 25 pairs)[a]	4.9 ± 0.5	8.5 ± 1.2	0.0011
AUC Insulin (mU l^{-1} h^{-1}) (n = 25 pairs)[a]	87.6 ± 8.0	129.3 ± 24.6	0.031
Total cholesterol (mmol l^{-1})	4.4 ± 0.2	4.7 ± 0.2	0.14
low-density lipoprotein (mmol l^{-1})	2.6 ± 0.1	3.0 ± 0.2	0.14
high-density lipoprotein (mmol l^{-1})	1.6 ± 0.1	1.3 ± 0.1	0.00040
Triglycerides (mmol l^{-1})	0.94 ± 0.1	1.32 ± 0.2	0.014
fS-hs-CRP (mg dl^{-1}) (n = 25 pairs)[a]	2.6 ± 0.7	4.0 ± 1.1	0.065
fP-Adiponectin (μg ml^{-1})	2.8 ± 0.3	2.2 ± 0.2	0.0023
fP-Leptin (mg ml^{-1})	18.9 ± 4.1	34.6 ± 5.5	0.0015
Isoleucine (mmol l^{-1}) (n = 20 pairs)[a]	0.05 ± 0.01	0.06 ± 0.02	0.033
Leucine (mmol l^{-1}) (n = 20 pairs)[a]	0.08 ± 0.01	0.09 ± 0.02	0.0094
Valine (mmol l^{-1}) (n = 20 pairs)[a]	0.19 ± 0.04	0.21 ± 0.04	0.024
Total energy intake (KJs)	8532.36 ± 430.91	8822.3 ± 436.51	0.92
Protein intake (percent of total energy)	17.88 ± 0.96	16.71 ± 0.6	0.60
Fat intake (percent of total energy)	35.03 ± 1.3	36.06 ± 1.37	0.67
Carbohydrates intake (percent of total energy)	44.24 ± 1.46	42.93 ± 1.77	0.29
Alcohol intake (percent of total energy)	2.85 ± 1.03	4.3 ± 1.12	0.27
Baecke work index	2.87 ± 0.17	2.79 ± 0.17	0.88
Baecke sports index	2.88 ± 0.18	2.42 ± 0.17	0.049
Baecke leisure time index	2.88 ± 0.12	2.86 ± 0.1	0.73
Total Baecke index	8.63 ± 0.29	8.07 ± 0.28	0.12
One smoker[b]	3	3	
Non-smokers (pairs)		17	
Both smokers (pairs)		3	

Abbreviations: AUC, area under the curve; BMI, body mass index; fP, fasting plasma; fS, fasting serum; hs-CRP, high-sensitivity C-reactive protein. [a]Data not available for all pairs. [b]The number indicates which twin is the smoker. Wilcoxon's rank sum test was used to compare values of the lean versus the heavy co-twin. Data represent mean with s.e.

Top ten pathways mostly associated with mitochondrial function, lipid metabolism and inflammation

We then analysed the 2108 differentially expressed genes with IPA's pathway analysis to obtain their biological pathways. The ten most significant differentially expressed pathways are presented in Figure 1. Five of these are shown in detail in Supplementary Figures 2–6.

The top pathway was oxidative phosphorylation. Out of all the genes in this pathway, those that were also in our differentially expressed genes were downregulated in the heavy co-twins (Supplementary Figure 2). The mitochondrial dysfunction pathway included the same significantly downregulated genes as the oxidative phosphorylation pathway and in addition, upregulated genes GSR and CYB5R3, which are involved in mitochondrial oxidative stress reactions (Supplementary Figure 3). Majority of the genes in the BCAA pathways, valine degradation I (Supplementary Figure 4) and isoleucine degradation I also encode genes that function in the mitochondria and were downregulated in the heavy co-twins. The genes in the glutaryl-CoA degradation pathway (Supplementary Figure 5), all downregulated in the heavy co-twins, participate in tryptophan and lysine degradation and many of them are shared with the isoleucine degradation pathway.

In addition to the pathways discussed above, the differentially expressed pathways included triacylglycerol biosynthesis (Supplementary Figure 6), the transcripts of which were mostly downregulated in the heavy co-twins. Also ketogenesis and ketolysis pathways were differentially expressed in SAT between the co-twins. These two pathways share two genes (HADHA, HADHB), which are also involved in the fatty acid β-oxidation pathway[30] and two genes (ACAT1, ACAT2) also involved in isoleucine degradation (IPA). Nur77 Signalling in T-lymphocytes and unfolded protein response pathways had both up- and downregulated genes in our heavy co-twins. These two pathways have a role in inflammation and ER stress, respectively, suggesting an effect in these processes in obesity, although our data was unable to conclusively show upregulation or downregulation.

Gene expression profiling can be used in identifying obesity sub-types

Next, we explored whether the SAT transcriptomics profiles can characterise different forms of acquired obesity. Cluster analysis using gene expression ratios between heavy and lean co-twins revealed three clusters (Figure 2). The first cluster (Cluster 1) had two, the second cluster (Cluster 2) 19 and the third cluster (Cluster 3) five twin pairs. Because of unequal sex distribution in the clusters (Supplementary Tables 4 and 5), sex was included as a covariate in the cluster analysis. Cluster 1 had 413, Cluster 2 had 728 and Cluster 3 had 828 differentially expressed genes within the twin pairs (Supplementary Table 3) (FDR < 0.05). Most of the genes were specific for each cluster with four shared genes between Cluster 1 and 3 and 110 shared genes between Cluster 2 and 3 (Supplementary Figure 7). The clusters' clinical characteristics are presented in Supplementary Table 4.

We then performed IPA pathway analyses for the within-twin pair differentially expressed genes for each cluster separately. For

Table 2. Top 10 upregulated and downregulated genes in the heavy (as compared with lean co-twins) in the 26 BMI-discordant monozygotic twin pairs

Gene symbol	Gene name	Function[a]	Log-fold change[b]	Fold change[c]	Adjusted P-value[d]
Top 10 upregulated genes in the heavy co-twins				Fold-up	
EGFL6	EGF-like-domain, multiple 6	Cell cycle progression and angiogenesis	3.15	8.90	0.00040
UCHL1	Ubiquitin carboxyl-terminal esterase L1 (ubiquitin thiolesterase)	Cell proliferation and apoptosis	1.76	3.39	0.0015
SPP1	Secreted phosphoprotein 1	Cell proliferation and apoptosis	1.60	3.03	0.045
LINC00968	Long intergenic non-protein coding RNA 968	Unknown	1.58	2.98	0.0038
PHLDA2	Pleckstrin homoogy-like domain, family A, member 2	Regulation of glycogen metabolic process, regulation of cell migration, apoptotic process	1.53	2.88	0.00050
CHI3L1	Chitinase 3-like 1 (cartilage glycoprotein-39)	Apoptotic process, carbohydrate metabolic process	1.28	2.43	0.025
CCL18	Chemokine (C-C motif) ligand 18 (pulmonary and activation-regulated)	Immune response, inflammatory response	1.18	2.27	0.0099
TNMD	Tenomodulin	Endothelial cell orphogenesis, negative regulation of angiogenesis, negative regulation of endothelial cell proliferation	1.15	2.22	0.00090
TUBB2B	Tubulin, beta 2B class IIb	Cellular protein metabolic process	1.12	2.18	0.0013
IFI30	Interferon, gamma-inducible protein 30	Cytokine-mediated signalling pathway, immune system process	1.10	2.14	0.035
Top 10 downregulated genes in the heavy co-twins				Fold-down	
SLC27A2	Solute carrier family 27 (fatty acid transporter), member 2	Lipid metabolic process; fatty acid metabolic process; fatty acid transport; long-chain and very long-chain fatty acid metabolic process	−2.44	0.18	0.0016
COL6A6	Collagen, type VI, alpha 6	Cell adhesion; collagen catabolic process	−2.29	0.20	0.00040
C12orf39	Chromosome 12 open reading frame 39	Long-chain fatty acid import, negative regulation of appetite	−2.07	0.24	0.00040
CA3	Carbonic anhydrase III, muscle specific	Apoptosis, cell growth, response to oxidative stress	−2.05	0.24	0.00040
AZGP1	Alpha-2-glycoprotein 1, zinc-binding	Antigen processing and presentation, cell adhesion, immune response, negative regulation of cell proliferation	−1.47	0.36	0.0010
AGPAT9	1-acylglycerol-3-phosphate O-acyltransferase 9	Lipid metabolic process	−1.44	0.37	0.0026
TTC36	Tetratricopeptide repeat domain 36	Unknown	−1.41	0.38	0.0035
CISH	Cytokine inducible SH2-containing protein	Cytokine-mediated signalling pathway, intracellular signal transduction	−1.33	0.40	0.0026
NAALAD2	N-acetylated alpha-linked acidic dipeptidase 2	Metabolic process, proteolysis	−1.20	0.44	< 0.0001
FHOD3	Formin homology 2 domain containing 3	Protein binding	−1.16	0.45	0.0020

[a]Provided by IPA. [b]A positive value shows gene upregulation in the heavy co-twin, whereas a negative log-fold change indicates gene downregulation in the heavier co-twin. [c]A fold change > 1 means a fold change upwards and a fold change < 1 means a fold change downwards. [d]FDR (Benjamini and Hochberg's method). Values sorted by log-fold change.
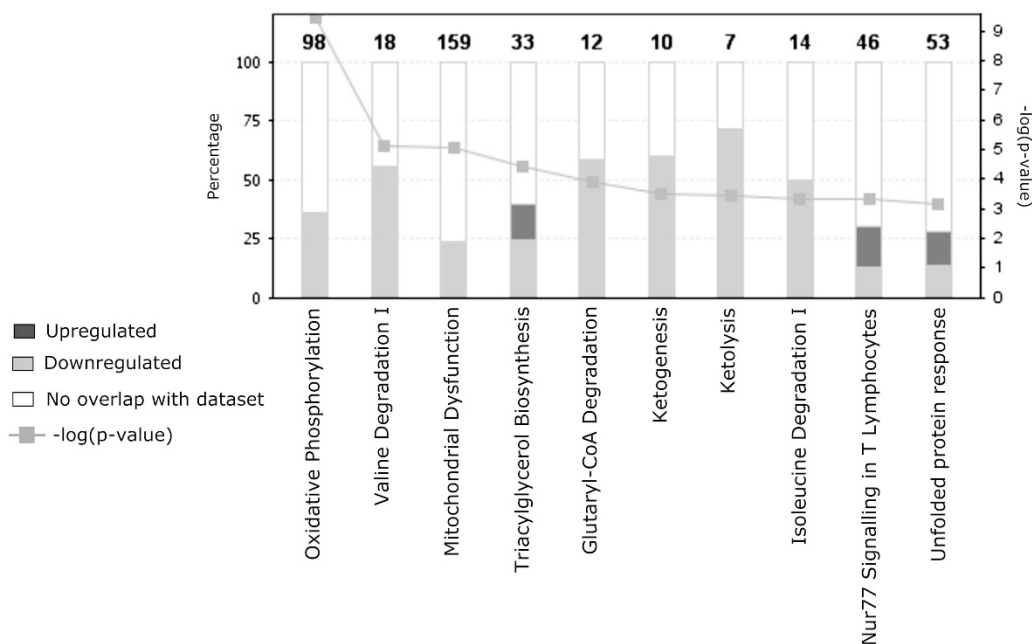
Figure 1. Top 10 biological pathways for the differentially expressed genes within 26 BMI-discordant monozygotic twin pairs. The numbers above the bars represent the genes belonging to the respective pathways (IPA). The left y axis represents the percentage of overlap between the study data set and the known genes belonging to the respective pathways. The y axis on the right displays the −log of P-value, calculated by Fisher's right-tailed exact test showing increased significance. A −log(P-value) of 1.3 is indicative of P-value of 0.05. The bars show the percentage of upregulated (dark grey) and downregulated (light grey) genes in the heavy co-twins in the study data set. The white blocks represent the genes belonging to the pathway but which did not reach significance.
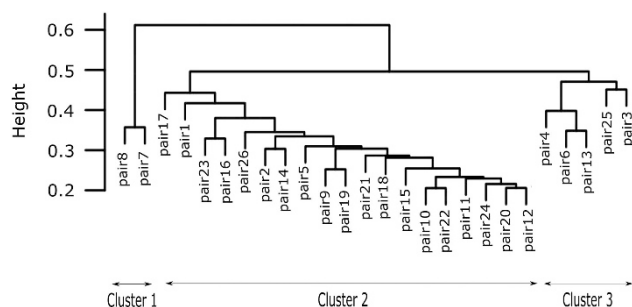
Figure 2. Cluster tree for the twin pair ratios of gene expression data ($n = 14\,665$ genes). Cluster 1 had a total of 274 differently expressed genes (139 upregulated, 135 downregulated), Cluster 2 had 728 differently expressed genes (290 upregulated, 438 downregulated) and Cluster 3 had 828 differently expressed genes (503 upregulated, 325 downregulated) within the twin pairs. Twin pair ratios ($n = 26$ BMI-discordant pairs) for the gene expression were used for the cluster analysis.

some pathways, IPA was able to (via z-scores) conclusively show upregulation/downregulation. Among the pathways that differed between the co-twins in Cluster 1 (Figure 3a), none reached significant z-scores. In Cluster 2 (Figure 3b), most of the top 10 pathways were involved in mitochondrial functions (two pathways were downregulated and one upregulated in the heavy co-twins). In Cluster 3, the significant pathways (Figure 3c) were strongly upregulated in the heavy co-twins and involved in immune response.

Then, we checked how the original top 10 pathways (Figure 1) obtained in the analysis on all 26 twin pairs behaved in each cluster. IPA's comparison analysis revealed that compared to Cluster 1, the other two clusters' pathways had more affected genes (Supplementary Figure 8). In Cluster 3, Nur77 signalling in T-lymphocytes (inflammation) and valine degradation were more

prominent than in Cluster 1 or 2. Compared to Cluster 1 and 3, Cluster 2 had more affected genes in the mitochondrial pathways.

Replication data set reveals similar patterns as the study data set for cluster 3

To validate the robustness of these clusters, we repeated the cluster analysis on previously published data on BMI-discordant MZ twin pairs. Owing to a very small replicate data set, we considered nominal P-values < 0.05 as significant. The differentially expressed genes on the replication data set revealed three clusters (Supplementary Figures 9 and 10) which we then analysed for the pathways (Supplementary Figure 11).

The pathways in Cluster 1 (Supplementary Figure 11a) and Cluster 2 (Supplementary Figure 11b) were not clearly similar to Cluster 1 and Cluster 2 of the study data set. However, like in the study data set, the replication data set showed upregulation of immune and inflammatory response for Cluster 3 (Supplementary Figure 11c). The results from the replication data set (Supplementary Figure 12) showed a general consistent pattern across the three clusters whereby, as we move from Cluster 1 to 2 towards Cluster 3, the representation of genes responsible for immune/inflammatory response increase.

Inflammatory response and BCAA degradation significantly different in cluster 3 in both data sets

We then selected a few pathways of interest to closely compare the clusters in both data sets. We selected two mitochondrial pathways (oxidative phosphorylation and BCAA degradation) and two inflammatory pathways (humoral immune response and inflammatory response) because of their top appearance either in the study or replication data set. We found that for both data sets (Figure 4 and Supplementary Figure 12) Cluster 2 and 3 showed higher enrichment for BCAA degradation and Cluster 3 for inflammatory response in the heavy co-twins compared to Cluster 1.
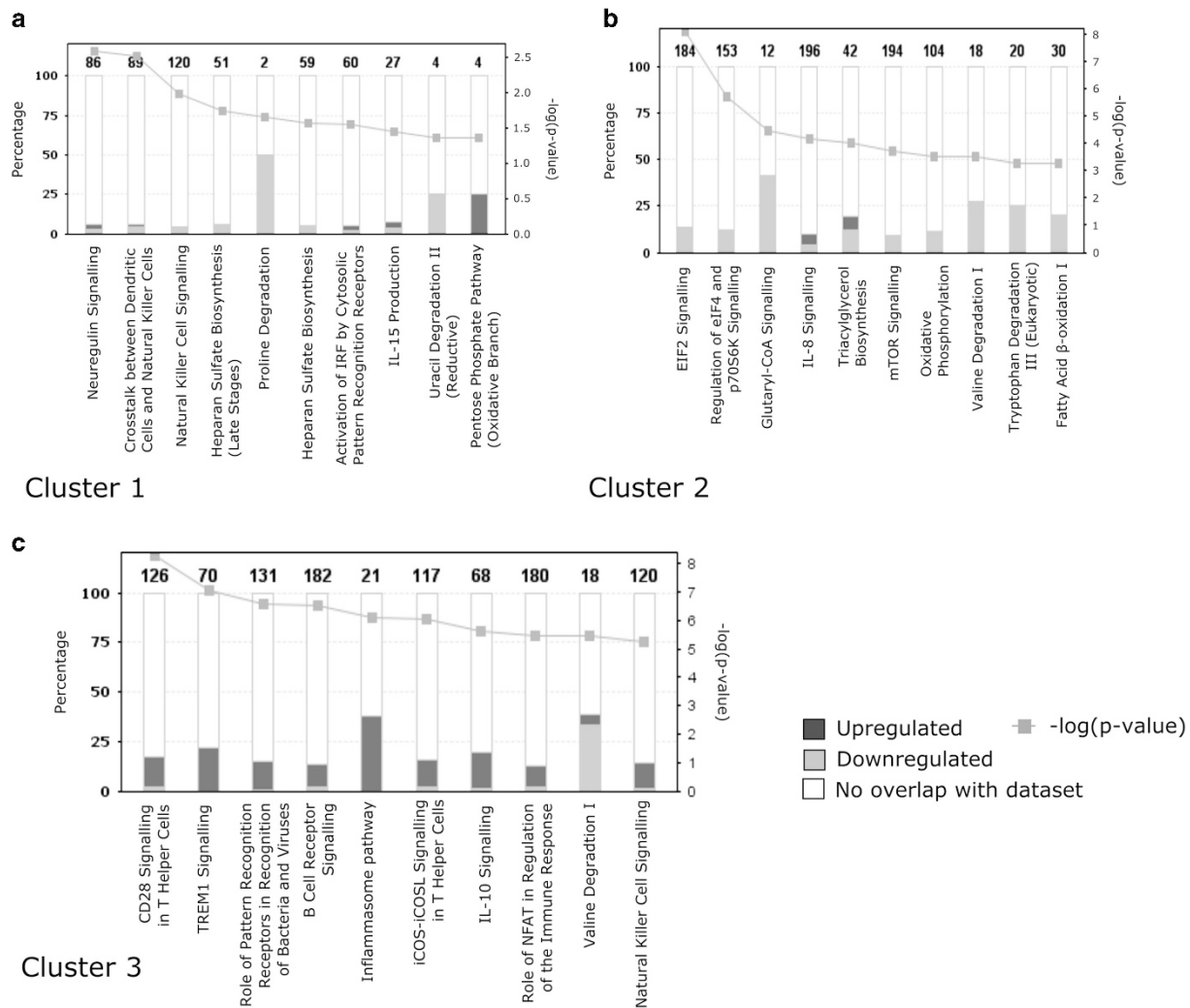
Figure 3. The top 10 pathways of the clusters based on the within-twin pair differences in gene expression. Differentially expressed genes for each cluster (FDR < 0.05, $n = 274$ for Cluster 1, $n = 728$ for Cluster 2, $n = 828$ for Cluster 3) were entered into IPA to produce the pathways. (**a**) Cluster 1, (**b**) Cluster 2 and (**c**) Cluster 3. The *y* axis displays the − log of *P*-value which is calculated by Fisher's exact right-tailed test. A-log (*P*-value) of 1.3 is indicative of *P*-value of 0.05. The percentage of upregulated (dark grey) and downregulated (light grey) genes in the heavy co-twins in the data set is represented. The white blocks represent the genes that belong to the pathway according to IPA analysis but did not reach significance. For some pathways, IPA was able to conclusively provide activation scores: (*z*-scores which show statistical significance of the observed number of 'activated' and 'inhibited' genes; < 0: inhibited, > 0: activated). *z*-scores > 2 or < − 2 are considered significant. For Cluster 2, IPA predicted the following pathways to be downregulated (EIF2 signalling ($z = -2.309$) and the following pathways to be upregulated: mTOR signalling ($z = 0.378$) and IL-8 signalling ($z = 1.414$). For Cluster 3, IPA predicted the following pathways in Cluster 3 to be upregulated: CD28 signalling in T-helper cells: ($z = 2.688$), TREM1 signalling ($z = 4.123$), B-cell receptor signalling ($z = 3.128$), role of pattern recognition receptors in recognition of bacteria and viruses ($z = 4.00$), role of NFAT in regulation of the immune response ($z = 3.578$), iCOS-iCOSL signalling in T-helper cells ($z = 2.309$).

Cluster 3 was associated with high-fasting insulin levels and adipocyte diameter

We then performed multiple group comparison analysis to ascertain clinical measurements differences in the three clusters. The results of the discovery data set (Supplementary Table 4) showed that differences between the heavy and leaner co-twins were larger for Cluster 3 than in Cluster 2 for fasting insulin levels (Bonferroni-adjusted $P = 0.041$) and adipocyte diameter (adjusted $P = 0.045$). The replication data set also showed that within-pair differences of fasting insulin were larger in Clusters 3 (adjusted $P = 0.02$) and 2 (adjusted $P = 0.005$) than in Cluster 1 (Supplementary Table 5). The within-pair differences in other obesity-associated clinical parameters did not differ between the three clusters.

DISCUSSION

This study reveals two fundamental changes in the SAT transcriptomics profiles of heavy versus lean co-twins of BMI-discordant MZ twin pairs: a downregulation of mitochondrial and upregulation of inflammatory pathways. The most pronounced differences between the co-twins were observed for oxidative phosphorylation and BCAA (especially valine and isoleucine) degradation pathways, suggesting an effect on mitochondrial function in obesity. Also, an effect on inflammation in the heavy co-twins was observed, although not nearly as prominent as the mitochondrial finding.

In addition, our extended hierarchical clustering analyses of gene expression of heavy/lean twin ratios revealed that the transcriptomics profiles defined three patterns of acquired obesity.
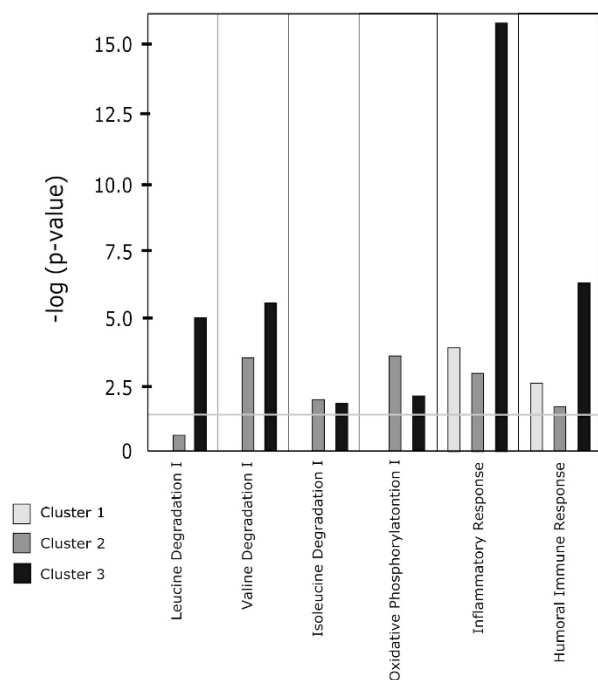
Figure 4. Pathways of Clusters 1–3 compared for selected functions. Differentially expressed genes for each cluster (FDR < 0.05, n = 274 for Cluster 1, n = 728 for Cluster 2, n = 828 for Cluster 3) were entered into IPA to compare the selected pathways. The y axis displays the − log of P-value which is calculated by Fisher's exact right-tailed test. A-log(P-value) of 1.3 is indicative of a P-value of 0.05.

Our results suggest that in Cluster 1, the transcriptional differences between the heavy and leaner co-twins were more benign, but that in Cluster 2 and 3, the intensification of mitochondrial downregulation was seen, and finally, in Cluster 3, a clear inflammatory pattern in the heavy co-twins. Many of these results, especially those of Cluster 3, were verified in a previously published replication data set of BMI-discordant MZ twin pairs.[10] Both the study and the replication data sets showed increased fasting insulin levels in the heavy twins of Cluster 3 than in the other clusters, perhaps suggesting that the defined AT transcriptomics profiles associate with development of the insulin-resistant phenotype in acquired obesity. In the discovery data set, the intra-pair difference in adipocyte size was also larger in Cluster 3.

In the first part (analysis of 26 twin pairs) of the study, we identified 2108 differentially expressed genes between the heavy and lean co-twins. These genes mapped to several mitochondria- and inflammation-related pathways. For the mitochondria-related pathways, genes from our data set were mostly downregulated in the heavy co-twins. For inflammation-related pathways, some genes from our data set were downregulated and some upregulated, in the heavy co-twins. Similar results were observed in the replication data set: mitochondrial oxidative pathways, especially BCAA catabolism, were downregulated together with upregulation of a number of inflammatory pathways in the heavy co-twins.[10] Mitochondrial downregulation (mitochondrial DNA copy number, mRNA and protein expression) in obese SAT was also observed in a series of targeted analyses performed for the same twins as in the current study.[21] A couple of other studies also suggest lower expression of genes encoding mitochondrial proteins in SAT in overweight subjects.[8,31] Studies also show that mitochondrial respiration in human fat cells is significantly reduced with increasing BMI.[32,33]

Although the triggering events for the mitochondrial down-regulation in obese fat are not known, it can be assumed that in energy overload, ATP production by cytosolic processes such as glycolysis are activated and mitochondria are needed less. Insufficiency of mitochondrial catabolism was noted for both fatty acids and BCAAs. It should also be noted that although ketogenesis and ketolysis appear as two significant pathways, these processes occur in the liver and thus showed up in our data set probably because of the shared genes with fatty acid oxidation and BCAA catabolism.

Altogether with overall oxidative phosphorylation, the most significantly enriched pathway in our heavy co-twins was BCAA degradation. The cluster analyses revealed that downregulation of mitochondrial and BCAA degradation-related pathways worsened towards the higher clusters 2 and 3, suggesting that these processes are related to a metabolically more disadvantaged obesity. We consider these as the most novel results of our study. BCAAs (valine, leucine and isoleucine) are essential amino acids catabolised by BCAT1 in the cytosol and thereafter by a number of genes in the mitochondria. We specifically show a significant downregulation of the mitochondrial genes, and interestingly, a twofold upregulation of the cytosolic BCAT1 (Supplementary Table 3). Similar findings have been reported in other studies.[31] Blood BCAA levels have been reported as increased,[34–36] and BCAA breakdown product alpha-ketoisocaproate as decreased along with decreased AT BCAA catabolism activity in obesity.[10] Unfortunately, our measured levels of valine, leucine and isoleucine did not show any significant differences between the Clusters, which could be because of lower statistical power owing to missing data for some of our twins. However, studies by other researchers suggest that the decrease in AT BCAA catabolism does contribute significantly to circulating BCAA levels[37,38] and that the gene expression of the BCAA catabolism enzymes in obesity is downregulated in AT, but not in liver nor muscle.[39] Increased BCAA levels have not only been correlated with insulin resistance[36,40–42] but also predict future risk of T2DM.[34,41] Here we show that, in what we assume as the more advanced stage of metabolic dysfunction in SAT, the decrease in BCAA catabolism pathways is more pronounced and associated with higher fasting insulin levels in the heavy co-twins. Previously, we showed that reduced BCAA catabolism in obesity correlates with a paradoxical reduction of adipogenesis pathways in SAT, and perhaps thereof, with accumulation of liver fat.[10] Interestingly, quite recently, isotope studies in adipocytes demonstrated that BCAA catabolism improves adipocyte differentiation.[43] With decreased BCAA catabolism in obesity, SAT may fail to enlarge adequately,[43] which in turn may favour deposition of ectopic fat and subsequent effects on insulin sensitivity.[44] Thus, our study provides a mechanistic link (reduction in mitochondrial catabolism of BCAAs) for the association between circulating BCAA and the risk of T2DM.

For reasons that are incompletely understood, downregulation of mitochondrial pathways and upregulation of inflammatory pathways in SAT often coexist.[10,11] In the present study, we noted the co-occurrence of these phenomena, but only in the higher clusters. Because mitochondrial 'dysfunction' only appeared in Clusters 2 and 3, and inflammation only in Cluster 3, these clusters possibly represent the metabolically more disadvantaged obesity. The order of causation remains unknown: it is possible that the mitochondria fail first as a reaction to overload of incoming energy (Cluster 2 and 3), and because the adipocytes are no longer viable, in a later stage (Cluster 3), inflammatory cells clear the dying adipocytes. Alternatively, inflammation in AT may hamper mitochondrial function.[45–48]

The inflammatory pathways enriched in Cluster 3 of the study data set were mainly involved in B and T-helper cell activation, indicating that infiltration of these cell types in the SAT is increased in the heavy co-twins of Cluster 3 but not in the other clusters. Why the inflammatory response seen in the present study is mainly of lymphocyte origin remains an open question. It is

possible that factors produced and secreted by adipocytes and/or macrophages enter the circulation and activate lymphocytes.[49] Alternatively, lymphocytes may precede macrophage infiltration in obese AT presenting an early inflammatory stage that may itself modify the number and the activation state of AT macrophages.[50–53] It is also possible that the true inflammatory response of AT involves a complex mixture of cell types and cellular events, as has been seen in other recent obesity studies.[54] Activation of the inflammasome pathway in Cluster 3 heavy co-twins in our study also points to a general inflammatory activation.

To see these molecular differences from a clinical perspective, we then analysed the clinical differences between twin pairs. The results (Supplementary Table 4) showed that Cluster 3 differed from Cluster 2 and 1 for fasting insulin levels and adipocyte size, suggesting that the heavy co-twins in Cluster 3 had a more marked insulin resistance along with larger adipocytes compared to their co-twins than the heavy twins in the other two clusters. The coexistence of mitochondrial pathway downregulation, large adipocyte size, inflammatory pathway upregulation in SAT and higher circulating levels of insulin in the heavy twins in Cluster 3 suggests that these phenomena may be biologically connected. Interestingly, large adipocytes have also previously been suspected to be linked to mitochondrial downregulation[21] and inflammation in AT,[55] and mitochondrial dysfunction and low-grade inflammation co-occur in insulin-resistant subjects' AT.[56] However, the mechanisms underpinning these connections require further studies.

The MZ co-twin study represents perhaps the best controlled study design available in humans because of the complete or close match for genes, age, gender, and intrauterine and childhood environment. As the genetic background is identical in each pair, acquired factors must account for the BMI discordance. With only six twin pairs discordant for smoking (both leaner and heavier co-twins being smokers), we cannot draw any conclusions about the effects of smoking on obesity. Alternatively, having leaner co-twins with higher Baecke sports index, hints at the possible positive effects of exercise. Food diaries, subject to significant underreporting especially by overweight people, did not reveal any differences between the co-twins.[57]

While comparing such matched groups of lean and heavy individuals has multiple advantages, there are also limitations. Despite extensive screening of nationwide cohorts, we only identified 26 BMI-discordant young adult twin pairs. Also, the independent contributions of the different SAT cells (stromavascular cells and adipocytes) to the results cannot be determined. In addition, measuring the transcripts levels does not necessarily translate to levels of functional proteins. This cross-sectional study also doesn't allow us to determine cause and effect. The twins may begin to exhibit different molecular and clinical symptoms with age or varying stages of obesity. The relatively low number of subjects per cluster also provided lower statistical power. Because Cluster 1 had only two twin pairs, no definitive conclusions may be drawn regarding this cluster. Also in the replication data set, the number of twin pairs is low. The clusters themselves may represent different genetic populations, a hypothesis worth studying in the future in larger data sets.

In conclusion, there are elements of mitochondrial downregulation and activation of inflammation and immune response in the SAT transcriptomics profiles in acquired obesity, and these changes may be specifically related to a certain subgroup or subgroups of obese persons. Our results highlight that not all obesities are the same. Accordingly, identifying obesity sub-types and profiling them using clinical traits and gene expression may facilitate improved diagnostics and personalised obesity treatment.

AUTHOR CONTRIBUTIONS

KHP designed the research, supervised the work, and participated in discussion and revision of the results. MM analysed all the data and wrote the full draft of the manuscript. KHP, SH, JK and AR collected the study data; and KHP, HYJ, JK and AR collected the replication data. AH, JL and NL participated in the imaging of the twins. JK was responsible for the base cohorts from which the discordant pairs were sampled. MO participated in discussions related to analysis and interpretation of the findings, helped in drafting the manuscript and critically commented on it. SH, HYJ, AH, JL, NL, JK, AR and KHP reviewed the manuscript draft. KHP is the guarantor of this work and as such had full access to the data and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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