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ARTICLE

Macrophage gene expression is related to obesity and the metabolic syndrome in human subcutaneous fat as well as in visceral fat

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Abstract
Aims/hypothesis Our goal was to identify a set of human adipose tissue macrophage (ATM)-specific markers and investigate whether their gene expression in subcutaneous adipose tissue (SAT) as well as in visceral adipose tissue (VAT) is related to obesity and to the occurrence of the metabolic syndrome.
Methods ATM-specific markers were identified by DNA microarray analysis of adipose tissue cell types isolated from SAT of lean and obese individuals. We then analysed gene expression of these markers by reverse transcription quantitative PCR in paired samples of SAT and VAT from 53 women stratified into four groups (lean, overweight, obese and obese with the metabolic syndrome). Anthropometric measurements, euglycaemic–hyperinsulinaemic clamp, blood analysis and computed tomography scans were performed.
Results A panel of 24 genes was selected as ATM-specific markers based on overexpression in ATM compared with other adipose tissue cell types. In SAT and VAT, gene expression of ATM markers was lowest in lean and highest in the metabolic syndrome group. mRNA levels in the two fat depots were negatively correlated with glucose disposal rate and positively associated with indices of adiposity and the metabolic syndrome.
Conclusions/interpretation In humans, expression of ATM-specific genes increases with the degree of adiposity and correlates with markers of insulin resistance and the metabolic syndrome to a similar degree in SAT and in VAT.

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Keywords Human adipose tissue · Inflammation · Macrophage · Metabolic syndrome · Obesity · Subcutaneous fat · Visceral fat

Abbreviations

ATM	Adipose tissue macrophage
SAT	Subcutaneous adipose tissue
SVF	Stromavascular fraction
VAT	Visceral adipose tissue

Introduction

In obesity, adipose tissue generates a local and systemic low-grade inflammatory environment. The first reports linking inflammation and carbohydrate metabolism date back to the 1800s and the clinical evidence of an association between inflammation and obesity to the 1950s [1]. Adipose tissue inflammation attracted increased attention after the key discoveries of a role for TNFα in obesity-linked insulin resistance in 1993 and of macrophage infiltration into adipose tissue in 2003 [2]. Since then, intense research on the factors initiating adipose tissue inflammation and promoting obesity-related metabolic abnormalities such as type 2 diabetes has been ongoing, but the problem remains imperfectly understood especially in humans. The term the metabolic syndrome was introduced to denote a cluster of metabolic disorders with an atherogenic, prothrombotic and inflammatory profile [3, 4]. The key components of the metabolic syndrome are insulin resistance and abdominal adiposity, with a predominance of intra-abdominal visceral fat accumulation [5].

The pathogenic potential of adipose tissue seems to be determined, besides total adiposity as such, by specific anatomic location [3, 6] or phenotypic and functional differences between subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). Indeed, although abdominal SAT and VAT are associated with metabolic risk profile [7], it has previously been reported [7–9] and recently confirmed in the Framingham Heart Study [10] that high VAT has a stronger correlation with metabolic risk factors and the metabolic syndrome than SAT. Several studies report depot-specific differences in inflammatory function based on higher number of macrophages [11–13], macrophage crown-like structures [14] and/or elevated levels of inflammatory molecules in VAT compared with SAT of obese individuals [11, 15–17].

The potential factors disturbing adipose tissue function in obesity are numerous. In expanding human adipose tissue, fat cell hypertrophy [18], altered production of adipokines, and increased number of macrophages [11–13] and lymphocytes [19] are considered to be major 'stressors'. Macrophages populate all tissues as quiescent but vital homeostatic cell populations with a primary role in clearing cellular debris [20, 21]. Macrophages differentiate from blood monocytes, which migrate into tissue in the steady state or in response to different stimuli that initialise macrophage activation. Depending on the type of stimuli, activated macrophages change phenotype and immune function in order to adapt to the local microenvironment. Interestingly, environmental signals do not necessarily increase macrophage immune function (secretion of pro- and anti-inflammatory cytokines), but can give rise to macrophages that are less equipped to produce cytokines [21]. Macrophage classification based on the two extreme states of polarisation ('classically activated' M1, 'alternatively activated' M2) has recently been discussed and new, more objective classification encompassing three basic macrophage populations has been proposed with anticipation that it may be further expanded [21]. Accordingly, recent data suggest that human adipose tissue macrophages (ATM) show a complex phenotype and are not strictly polarised into M1 and M2 [22, 23]. Several studies have examined expression levels of a few ATM genes in different groups of individuals [12, 13, 16, 24–26]. Given the interplay of biological systems, it is evident that reliance on a single or a few markers should be replaced by a spectrum of specific markers for study of ATM [1].

The present study was designed to investigate expression of specific human ATM genes in SAT and in VAT. We hypothesised that ATM gene expression may increase with adiposity and be related to the presence of the metabolic syndrome. We identified a panel of new human ATM-specific markers and determined mRNA levels in SAT and VAT of well-characterised groups of lean, overweight and obese individuals without and with the metabolic syndrome. The gene expression profile of ATM markers in both fat depots was correlated with indices of adiposity, variables of the metabolic syndrome, direct measurement of insulin sensitivity and computer tomography-derived measures of abdominal SAT and VAT compartments.

Methods

Identification of human adipose tissue cell type-specific genes Adipocytes and stromavascular fraction (SVF) were isolated from liposuction-obtained abdominal SAT of six women (BMI 24±1 kg/m^2, age 36±4 years [means ± SD]) by collagenase digestion. Macrophages, endothelial cells, progenitor cells and a negative fraction containing lymphocytes, mastocytes and other cells from SVF were obtained by

immunoselection and depletion [27, 28]. First, CD34-positive cells ($CD34^+$) and CD34-negative cells ($CD34^-$) were isolated from the SVF by use of CD34-coupled magnetic microbeads. Then macrophages ($CD34^-/CD14^+$) were sorted from $CD34^-$ population by immunoselection using CD14-coupled magnetic microbeads. The endothelial cells ($CD34^+/CD31^+$) were sorted from $CD34^+$ population by immunoselection using CD31-coupled magnetic microbeads. The progenitor cells were defined as $CD34^+/CD31^-$ population. The negative fraction ($CD34^-/CD14^-/CD31^-$) was sorted from $CD34^-/CD14^-$ population by depletion using CD31-coupled magnetic microbeads. Compared with the amount of cells from SVF, the mean yield was 3.0±0.4, 4.7±0.7, 8.7±1.2 and 4.6±0.7% for macrophages, endothelial cells, progenitor cells and negative fraction, respectively. DNA microarrays were used to measure mRNA levels in each cell fraction. Targets were generated from 500 ng total RNA (Low RNA Input Amplification kit; Agilent Technologies, Massy, France) and hybridised to whole genome 44 k oligonucleotide arrays (Agilent Technologies). Data acquisition and image processing were done with a scanner (GenePix 4000B; Axon Instruments, Molecular Devices, St Grégoire, France) and Feature Extraction 8.5 (Agilent Technologies). Raw data were normalised with a global Lowess procedure and filtered with R package LIMMA (Bioconductor; Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Genes specific for each adipose tissue cell type were identified with Significance Analysis of Microarray (Stanford University, Stanford, CA, USA) with an estimated false discovery rate of 5%. Principal component analysis was then used to optimise selection of ATM-specific genes with highly related SAT and VAT mRNA expression profiles in the 53 women enrolled in this study (see below). To validate the selection of ATM markers, a second series of DNA microarrays was performed on abdominal SAT cell fractions from six lean (BMI 24±1 kg/m^2, age 36±4 years [means±SD]) and six obese (BMI 36±6 kg/m^2, age 39±7 years [means±SD]) individuals. This series was scanned with Innoscan 700 (Innopsys, Carbonne, France) and analysed using Mapix (Innopsys). Gene expression of the markers was analysed by hierarchical clustering (uncentred Pearson's correlation, average linkage).

Participants Individuals scheduled to have abdominal surgery (laparoscopic or laparotomic cholecystectomy, hysterectomy and gastric banding) at the Departments of Surgery and Gynecology at Kralovske Vinohrady Faculty Hospital in Prague were monitored and 53 women (age 21–66 years, BMI 17.3–48.5 kg/m^2) were included. Exclusion criteria were: malignancy, current inflammatory conditions as diagnosed by clinical status and laboratory findings, known endocrinopathy, chronic liver or kidney disease, psychiatric disorders and body weight fluctuations >2% over the preceding 3 months. Each participant gave written informed consent and the study was approved by the Ethic Committee of the Third Faculty of Medicine, Charles University, Prague. According to BMI, and presence or absence of the metabolic syndrome evaluated according to the International Diabetes Federation criteria [29], the participants were stratified into four groups: lean (n=12), overweight (n=12), obese (n=15) and obese with the metabolic syndrome (n=14).

Study protocol A clinical investigation was performed 7–14 days prior to the surgery. Anthropometric measurements, blood sampling and euglycaemic–hyperinsulinaemic clamp were performed at rest after an overnight fast. Body composition was evaluated using bioelectrical impedance (QuadScan 4000; Bodystat, Douglas, Isle of Man). Visceral and subcutaneous fat areas were derived from computed tomography scans at the level L4 to L5 [30]. Blood samples were obtained before the clamp and plasma variables were measured using standard procedures. Insulin sensitivity was assessed using euglycaemic–hyperinsulinaemic clamp [31]. During the surgical procedure, paired samples of abdominal SAT and omental VAT were obtained and processed immediately. Adipose tissue was washed in physiological saline, homogenised in RLT lysis buffer (Qiagen, Courtaboeuf, France) and stored at −80°C until analysed.

RNA analysis Total RNA isolation was performed as previously described [32]. The mRNA expression of macrophage-specific genes was assessed by reverse transcription and real-time quantitative PCR using a sequence detection system (ABI PRISM 7900; Applied Biosystems, Foster City, CA, USA) and custom TaqMan low-density arrays and TaqMan gene expression assay pre-loaded targets (both from Applied Biosystems). Ribosomal 18S RNA was used as endogenous control.

Statistical analyses Clinical variables of the participants were first analysed by partial least square-discriminant analysis using SIMCA-P+ software (Umetrics/SIGMA PLUS, Siège social: 3, Toulouse, France). Partial least square-discriminant analysis is a principal component analysis-derived method used to optimise separations between groups of individuals [33]. Four groups of individuals were identified (lean, overweight, obese and the metabolic syndrome), thus confirming the initial stratification of participants according to BMI and the metabolic syndrome status. To compare clinical variables between the groups of individuals, log-transformed data were analysed by one-way ANOVA (four groups of individuals) with Bonferroni post hoc analysis using SPSS 17.0 statistical software (SPSS, Chicago, IL, USA). Gene expression analyses were done on log-transformed data using SPSS 17.0 software. Relative mRNA amounts were

expressed as $2^{\Delta C_t}$ values, where $\Delta C_t = C_t$ (endogenous control)–C_t (target gene). Two mean centroids ± SEM, one for SAT and one for VAT depot, were calculated from $2^{\Delta C_t}$ mRNA values after normalisation of gene expression levels to a mean of 0 and a variance of 1 across all individuals [34]. The mean centroid is a variable that represents weighted average of the 24 gene expression levels. The mean centroids were used to compare ATM marker gene expression between the groups and, also, to assess the correlations between the genes and physiological variables. Linear mixed-effect models for repeated measures were chosen as the most appropriate analysis for the comparison of macrophage marker expression represented as mean centroids between the groups of participants (between-group factor) and between adipose tissue depots (repeated measure). All models were assessed graphically for normality of the residuals and homogeneity of variances and covariances. As the homogeneity of variance and covariance was not respected, the heterogeneous Toeplitz covariance matrix was chosen for its low Akaike's information criterion compared with different covariance structures. Bonferroni adjustment was used for multiple comparisons. The results were considered statistically significant when the two-sided p value was $p<0.05$. Correlation analyses of gene expression between the two fat depots and vs clinical variables were performed using Pearson's test. To determine in which adipose tissue depot the genes were preferentially expressed, a comparison of $2^{\Delta C_t}$ values of each gene between SAT and VAT was realised by pair-wise analysis (Wilcoxon's signed rank test) for each group of participants.

Results

Human ATM-specific markers Comparison of DNA microarray gene expression data from different human adipose tissue cell types by Significance Analysis of Microarray biclass and pair-wise analyses revealed 332 ATM markers. To select highly specific ATM genes, the list was reduced according to the following criteria: (1) gene expression more than tenfold and twofold higher in macrophages than in adipocytes and other non-adipocyte cell types, respectively (DNA microarrays, data not shown); (2) more than 1.5-fold higher expression in macrophages than in SVF (DNA microarrays, data not shown); and (3) high inter-correlations of SAT and VAT gene expression in the 53 participants as tested by principal component analysis. A final list of 24 ATM-specific markers was obtained (Table 1). The selection of 24 ATM markers was validated by DNA microarray analyses on SAT of independent groups of lean and obese individuals. Hierarchical clustering clearly shows that the 24 genes discriminate ATM from other cell types in lean and obese individuals (Fig. 1).

Clinical variables of participants Anthropometric and metabolic characteristics of the four groups of participants are shown in Table 2. In the metabolic syndrome group, seven individuals had previously diagnosed type 2 diabetes. Body weight, fat mass, waist, WHR and insulin concentrations were lower in the lean than in the three other groups. The relative amount of visceral fat was significantly different between lean and the metabolic syndrome individuals, with higher amounts in the metabolic syndrome group. Glucose disposal rate corrected for body weight or for fat-free mass was lower in the metabolic syndrome than in lean and overweight patients. Glucose disposal rate related to body weight was decreased in obese when compared with the lean group.

Partial least square-discriminant analysis of clinical data shows distribution of 53 participants into four groups: lean, overweight, obese and the metabolic syndrome (Fig. 2). Most of the data variance was explained by two principal components. Component 1 (x-axis) discriminated individuals according to degree of obesity, i.e. lean, overweight, obese. Component 2 (y-axis) separated the two groups of obese individuals according to presence or absence of the metabolic syndrome. Five variables (WHR, insulin, triacylglycerol and computed tomography visceral/total fat) were the most important variables for discrimination of the metabolic syndrome group.

ATM marker mRNA expression in SAT and VAT of lean, overweight, obese and the metabolic syndrome individuals ATM marker mRNA levels, expressed as SAT and VAT mean centroids, were analysed in relation to the four groups of individuals. The expression of SAT and VAT mean centroids was lower in lean ($p<0.05$) and higher in the metabolic syndrome ($p<0.05$) than in the other three groups (Fig. 3). The rank order of gene expression was: lean<overweight=obese<the metabolic syndrome. We then determined the relation between ATM marker gene expression and clinical data in the entire group of 53 participants. Associations with clinical variables are shown in Table 3. In both adipose tissue depots, centroids of ATM genes were positively correlated with BMI, weight, fat mass, waist, WHR and computed tomography visceral/total fat, and negatively correlated with fat-free mass. Positive correlations between mean centroids of ATM marker mRNA levels and plasma levels of insulin, NEFA, triacylglycerol, and systolic and diastolic blood pressure levels were observed in the two fat depots. Next, the relation between ATM marker gene expression and insulin sensitivity was investigated. Pearson's correlation analysis revealed a

Table 1 List of 24 macrophage-specific markers derived from DNA microarray analysis of human adipose tissue cell types

Gene			Ratio of gene expression (macrophage : cell type listed)				
Official symbol	Other aliases	Official name	Adipocyte	EC	PC	NF	SVF
ACP5	*MGC117378, TRAP*	Acid phosphatase 5, tartrate resistant	88.9	4.0	5.1	2.8	1.7
CD14		CD14 molecule	17.8	2.5	3.6	17.6	1.8
CD163	*M130, MM130*	CD163 molecule	120.9	2.5	4.5	20.1	1.5
CD209	*CDSIGN, CLEC4L, DC-SIGN, DC-SIGN1, MGC129965*	CD209 molecule	25.3	2.1	2.8	9.7	1.6
CD33	*FLJ00391, SIGLEC-3, SIGLEC3, p67*	CD33 molecule	28.5	4.1	6.6	2.1	2.8
CD68	*DKFZp686M18236, GP110, SCARD1*	CD68 molecule	19.3	2.9	3.0	3.0	1.8
ADAP2	*CENTA2, HSA272195, CENT-B*	ArfGAP with dual PH domains 2	61.6	2.8	4.4	7.4	1.7
CLEC10A	*CD301, CLECSF13, CLECSF14, HML, HML2, MGL*	C-type lectin domain family 10, member A	224.4	6.8	11.6	8.0	3.2
FCGR2B	*RP11-474I16.2, CD32, CD32B, FCG2, FCGR2, IGFR2*	Fc fragment of IgG, low affinity IIb, receptor (CD32)	169.3	3.7	6.3	14.3	2.4
FCN1	*RP11-447M12.1, FCNM*	Ficolin (collagen/fibrinogen domain containing) 1	134.8	11.0	41.7	12.2	2.9
HLA-DMA	*DAAP-27A1.5, D6S222E, DMA, HLADM, RING6*	Major histocompatibility complex, class II, DM alpha	14.5	3.1	7.1	5.1	2.3
HLA-DRA	*DASS-397D15.1, FLJ51114, HLA-DRA1, MLRW*	Major histocompatibility complex, class II, DR alpha	45.6	2.8	6.6	4.0	2.0
IL10	*RP11-262N9.1, CSIF, IL-10, IL10A, MGC126450, MGC126451, TGIF*	Interleukin 10	27.0	4.3	9.0	9.9	2.5
IRF5	*SLEB10*	Interferon regulatory factor 5	32.5	4.2	6.6	3.0	2.2
KYNU		Kynureninase	50.5	6.6	10.6	6.3	4.1
LIPA	*RP11-341B24.1, CESD, LAL*	Lipase A, lysosomal acid, cholesterol esterase	17.9	4.2	4.7	2.7	1.7
MARCO	*AI323439, Ly112, Scara2*	Macrophage receptor with collagenous structure	36.3	2.5	4.1	15.3	1.6
MS4A4A	*HDCME31P, 4SPAN1, CD20-L1, CD20L1, MGC22311, MS4A4, MS4A7*	Membrane-spanning 4-domains, subfamily A, member 4	86.1	2.6	4.4	7.3	1.8
MS4A6A	*CDA01, 4SPAN3, 4SPAN3.2, CD20L3, MGC131944, MGC22650, MS4A6, MST090, MSTP090*	Membrane-spanning 4-domains, subfamily A, member 6A	76.4	4.2	7.9	15.5	2.7
MS4A7	*4SPAN2, CD20L4, CFFM4, MGC22368, MS4A8*	Membrane-spanning 4-domains, subfamily A, member 7	15.0	3.0	5.3	14.3	5.6
MSR1	*CD204, SCARA1, SR-A, phSR1, phSR2*	Macrophage scavenger receptor 1	68.1	3.5	4.6	6.2	1.9
SIGLEC1	*CD169, DKFZp667F058, FLJ00051, FLJ00055, FLJ00073, FLJ00411, FLJ32150, SIGLEC-1, SN, dJ1009E24.1*	Sialic acid binding Ig-like lectin 1, sialoadhesin	12.8	3.0	3.6	3.3	1.8
SLCO2B1	*DKFZp686E0517, KIAA0880, OATP-B, OATP2B1, OATPB, SLC21A9*	Solute carrier organic anion transporter family, member 2B1	45.0	2.5	5.7	3.5	1.5
TLR7	*UNQ248/PRO285*	Toll-like receptor 7	33.3	2.7	4.9	12.4	1.8

Gene symbols as listed in Entrez Gene (www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene)

EC, endothelial cells; PC, progenitor cells; NF, negative fraction

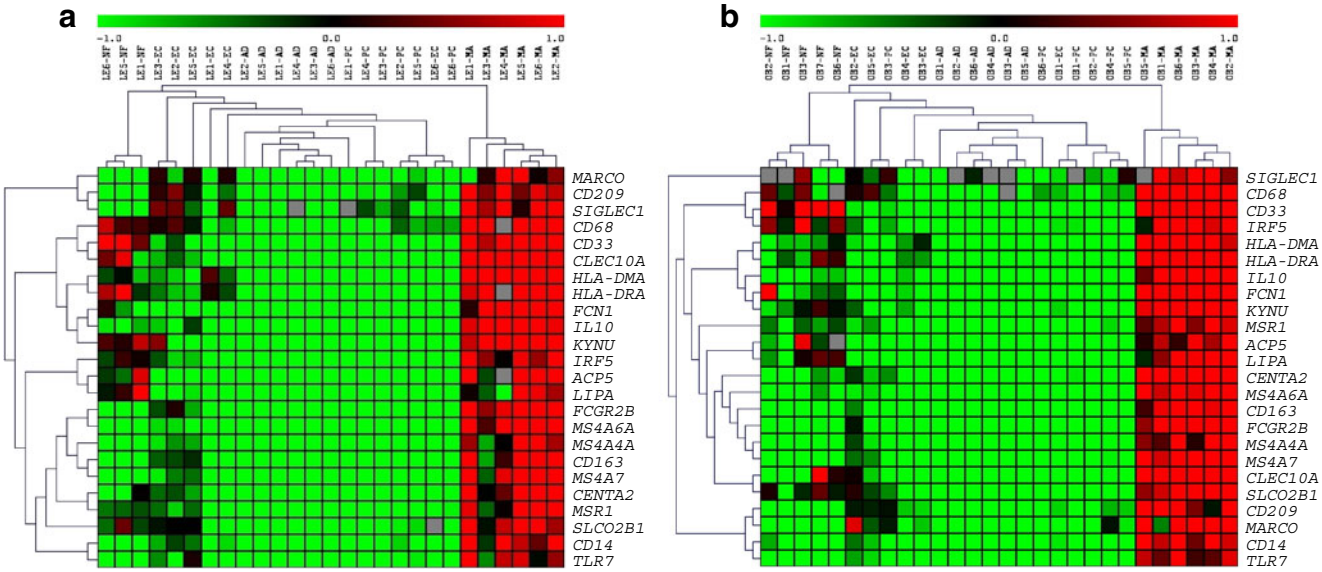

Fig. 1 Hierarchical clustering of 24 ATM marker genes expression in different human SAT cell types of lean (LE) (**a**) and obese (OB) (**b**) individuals. The ratio of the abundance of the transcripts of each gene in each cell type to a common reference pool is represented by the colour of the corresponding cell in the matrix file. Green, transcripts with lower expression than in reference pool; red, transcripts with higher expression than in reference pool. Horizontal dendograms, similarities in expression pattern between adipose tissue cell types in LE and OB individuals; vertical dendrograms, clustering of genes. AD, adipocytes; EC, endothelial cells; PC, progenitor cells; NF, negative fraction; MA, macrophages. For gene names, see Table 1

negative correlation when mean centroids of ATM markers in VAT and SAT were related to glucose disposal rate corrected for fat-free mass (Fig. 4a, b, Table 3) or glucose disposal rate related to body weight (Table 3).

Comparison of ATM marker mRNA expression in subcutaneous vs visceral fat depot Significant positive correlation was found between mean centroids of ATM-specific genes in SAT and VAT (Fig. 5). For each individual's group, a pair-wise analysis was realised to compare the expression of each macrophage-specific gene between VAT and SAT depot. The results are presented in Table 4. There was no significant difference between SAT and VAT in expression of *CD14*, *M130* (also known as *CD163*), *CD301* (also known as *CLEC10A*), *FCNM* (also known as *FCN1*) and *HLADM* (also known as *HLA-DMA*) in any of the individuals' groups. For the other genes, two noticeable patterns were observed: (1) in lean individuals, ten genes showed higher expression in VAT, while their expression was generally not different in other groups (except for three instances); (2) in overweight, obese or the metabolic syndrome individuals, four to ten genes showed higher expression in SAT, while their expression was generally not different in the lean group (except for two instances). *TRAP* (also known as *ACP5*) expression showed a clear difference between lean individuals and the other three groups, i.e. it was higher in VAT in lean individuals, while it was significantly higher in SAT in the overweight, obese and obese metabolic syndrome groups.

Discussion

In the present study, we used non-hypothesis-driven DNA microarray technology to identify 24 human ATM-specific markers by comparison of gene expression profiles in various adipose tissue cell types. We also investigated whether gene expression levels of these markers in abdominal SAT and VAT differ in obesity and in individuals with the metabolic syndrome. In 53 participants with different degrees of adiposity and presence or absence of the metabolic syndrome, analysis of expression of 24 ATM-specific marker genes in SAT and VAT showed the following rank order in both fat depots: lean<overweight = obese<the metabolic syndrome. The lack of difference in gene expression of ATM markers between the overweight and the obese groups may be related to comparable profiles in terms of variables associated with the metabolic syndrome. ATM gene expression in the two fat depots was clearly lower in lean individuals than in all other groups with higher adiposity; it was also higher in obese individuals with the metabolic syndrome than in the other three groups, including the group of equally obese individuals without the metabolic syndrome. mRNA levels of ATM markers in both adipose tissue depots positively correlated with BMI, weight, fat mass, waist, WHR and computed tomography visceral/total fat; they negatively correlated with fat-free mass. A positive correlation was also observed between ATM mRNA levels in the two fat depots and plasma levels of insulin, NEFA, triacylglycerol and diastolic blood pressure. Regarding insulin sensitivity, the analysis revealed a

Table 2 Clinical variables of four groups of individuals

Characteristic	Lean (n=12)	Overweight (n=12)	Obese (n=15)	Metabolic syndrome (n=14)
Age (years)	38±14	42±9	45±9	49±11
BMI (kg/m^2)	21.3±2.0	27.8±1.2[c]	37.0±6.0[c f]	34.0±3.1[c f]
Weight (kg)	61.0±7.6	79.6±4.4[c]	100.6±15.1[c f]	91.8±8.4[c d]
Fat mass (%)	25.8±5.8	34.7±4.5[c]	45.3±4.6[c f]	42.3±4.9[c e]
Fat-free mass (%)	74.3±5.8	65.3±4.5[b]	54.7±4.6[c f]	57.8±4.9[c f]
Waist (cm)	74.1±4.9	92.1±3.5[c]	109.2±11.2[c f]	107.3±6.1[c f]
WHR	0.76±0.03	0.84±0.04[c]	0.86±0.05[c]	0.92±0.05[c f h]
CT visceral/total fat	0.19±0.06	0.25±0.07	0.21±0.04	0.27±0.06[b]
Systolic BP (mmHg)	109.1±13.4	118.5±9.2	121.9±11.7[a]	129.4±10.5[c]
Diastolic BP (mmHg)	66.4±6.8	73.6±7.7	77.0±8.3[b]	79.0±6.7[c]
Glucose (mmol/l)	4.8±0.4	4.8±0.5	5.4±0.5	6.3±1.9[b e]
Insulin (pmol/l)	30.5±14.5	53.7±16.6[b]	64.1±27.9[c]	98.1±38.3[c e g]
NEFA (μmol/l)	510.8±201.6	668.6±246.4	652.1±218.0	762.8±260.5
Glycerol (μmol/l)	79.1±29.0	89.2±33.1	103.9±36.4	130.2±43.1[b d]
Triacylglycerol (mmol/l)	0.76±0.18	1.3±0.8	1.2±0.3	2.5±1.5[c d g]
Total cholesterol (mmol/l)	4.6±0.7	5.1±1.2	4.8±1.0	4.6±1.4
HDL-cholesterol (mmol/l)	1.6±0.3	1.4±0.4	1.4±0.3	1.2±0.2
Glucose disposal rate[i]	7.2±2.3	6.6±5.0	3.7±1.8[b]	2.8±1.2[c f]
Glucose disposal rate[j]	9.7±3.1	10.2±7.9	6.9±3.1	4.9±2.1[c e]

Values are means ± SD; participants (total) n=53

[a] p<0.05, [b] p<0.01, [c] p<0.001 compared with lean; [d] p<0.05, [e] p<0.01, [f] p<0.001 compared with overweight; [g] p<0.05, [h] p<0.01 compared with obese; [i] Corrected for body weight, mg kg^{-1} min^{-1}; [j] Corrected for fat-free mass, mg (kg fat-free mass)$^{-1}$ min^{-1}; CT, computed tomography

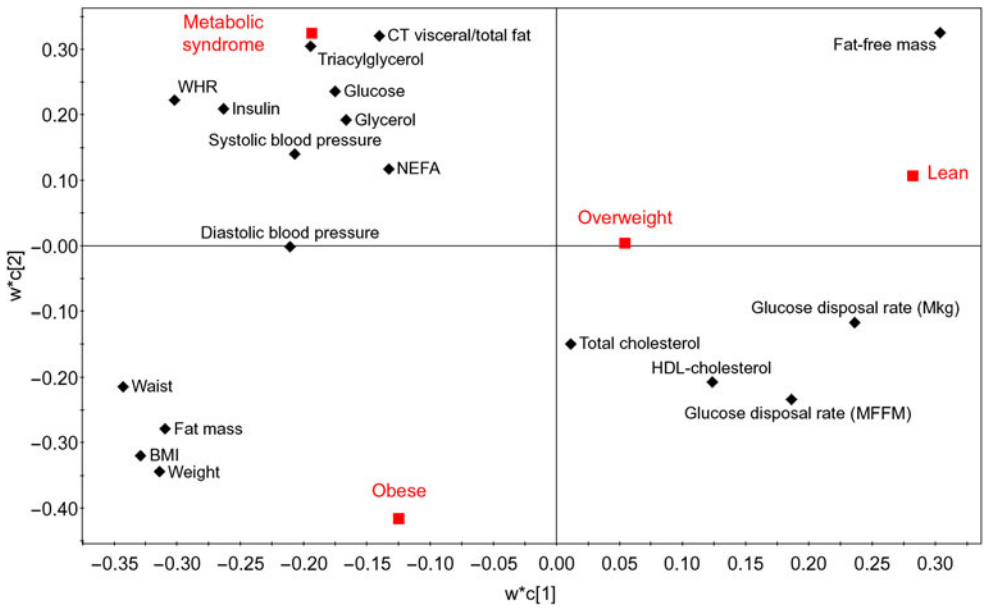

Fig. 2 Partial least square-discriminant analysis of clinical data was used as an exploratory analysis to represent the distribution of four groups of individuals (red font) on the basis of the values of their clinical variables. The w*c or loading plot gives a graphical summary of the correlation between clinical parameters (represented by vector w*) and groups of individuals (represented by vector c). Axes show w*c[component]. w*c[1] (x-axis, component 1, degree of obesity); w*c[2] (y-axis, component 2, presence or not of the metabolic syndrome). The two principal components explained most of the variance of data (by 36% and 27%, respectively). CT, computed tomography; MFFM, mg (kg fat-free mass)$^{-1}$ min^{-1}; Mkg, mg kg^{-1} min^{-1}

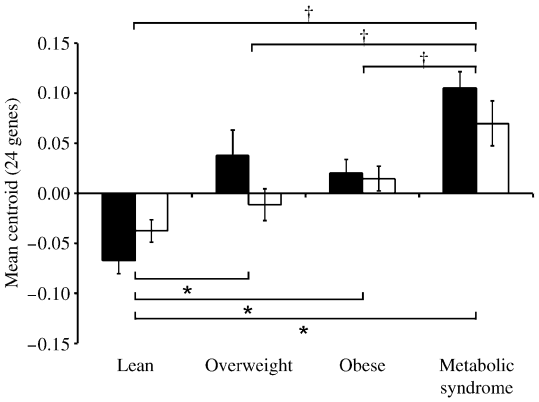

Fig. 3 ATM marker mRNA expression in SAT and VAT in participants as indicated. mRNA levels of 24 markers are expressed as mean centroids ± SEM in SAT (black bars) and VAT (white bars). *$p<0.05$ for comparison of gene expression in each fat depot between the lean and three other groups of individuals; †$p<0.05$ for as above, but between the metabolic syndrome and three other groups of individuals

negative correlation between ATM mRNA levels in each of the two fat depots and glucose disposal rate.

The limitations of this study include lack of information on macrophage quantity in adipose tissue, abundance of macrophage crown-like structures and adipocyte size. While these data could not be obtained due to the modest amount of adipose tissue material, it would have been interesting to correlate these variables with gene expression data of ATM-specific markers. In fact, it has been shown that an increased proportion of small adipose cells is associated with inflammation in SAT [25]. In addition, because various subpopulations of macrophages may exist and express distinct surface markers depending on the local microenvironment, different ATM markers could be identified in different macrophage subpopulations of adipose tissue. Several macrophage subpopulations have indeed been identified in human adipose tissue [23, 27, 35, 36]. Studies investigating mRNA variations of specific macrophage subpopulations in different adipose tissue locations and/or in different groups of individuals are currently not available due to technical limitations. Further work is therefore warranted.

We used a novel approach to investigate macrophage gene expression pattern in human SAT and VAT in different groups of individuals. First, we characterised and refined a selection of a set of ATM-specific markers by DNA microarray profiling and correlation analyses, confirming these by independent DNA microarray experiments in lean and obese individuals. Considering the plasticity of macrophages, using a spectrum of specific markers for identification of ATM is a more appropriate strategy than use of a single or a few markers [1, 21]. Our panel of 24 genes represents the most comprehensive list of human ATM-specific markers reported so far. Although the genes were selected by a 'no a priori hypothesis', it is interesting to note that some of the markers shared related characteristics. *CD204* (also known as *MSR1*), *M130*, *SCARA2* (also known as *MARCO*) and *GP110* (also known as *CD68*) encode macrophage scavenger receptors with wide roles in host defence and tissue homeostasis [37]. Other ATM marker genes (*CDSIGN* (also known as *CD209*), *SIGLEC3* (also known as *CD33*) and *CD169* (also known as *SIGLEC1*)) have been described as macrophage cell

Table 3 Correlations of centroids of macrophage marker gene expression in SAT and VAT with clinical variables of 53 individuals

Variable	SAT mean centroid	VAT mean centroid
BMI (kg/m^2)	0.473[c]	0.447[c]
Weight (kg)	0.480[c]	0.461[c]
Fat mass (%)	0.563[c]	0.417[b]
Fat-free mass (%)	−0.513[c]	−0.419[b]
Waist (cm)	0.592[c]	0.515[c]
WHR	0.653[c]	0.478[c]
CT visceral/total fat	0.480[c]	0.308[a]
Systolic BP (mmHg)	0.343[a]	0.279[a]
Diastolic BP (mmHg)	0.440[c]	0.351[b]
Glucose (mmol/l)	0.223	0.425[b]
Insulin (pmol/l)	0.654[c]	0.553[c]
NEFA (μmol/l)	0.318[a]	0.364[b]
Glycerol (μmol/l)	0.266	0.504[c]
Triacylglycerol (mmol/l)	0.401[b]	0.517[c]
Total cholesterol (mmol/l)	−0.14	0.028
HDL-cholesterol (mmol/l)	−0.363[b]	−0.221
Glucose disposal rate[d]	−0.631[c]	−0.587[c]
Glucose disposal rate[e]	−0.574[c]	−0.539[c]

Values are Pearson's *r* correlation coefficient

[a] $p<0.05$, [b] $p\leq0.01$, [c] $p\leq0.001$

[d] Corrected for body weight, mg kg^{-1} min^{-1}

[e] Corrected for fat-free mass, mg (kg fat-free mass)$^{-1}$ min^{-1}

CT, computed tomography

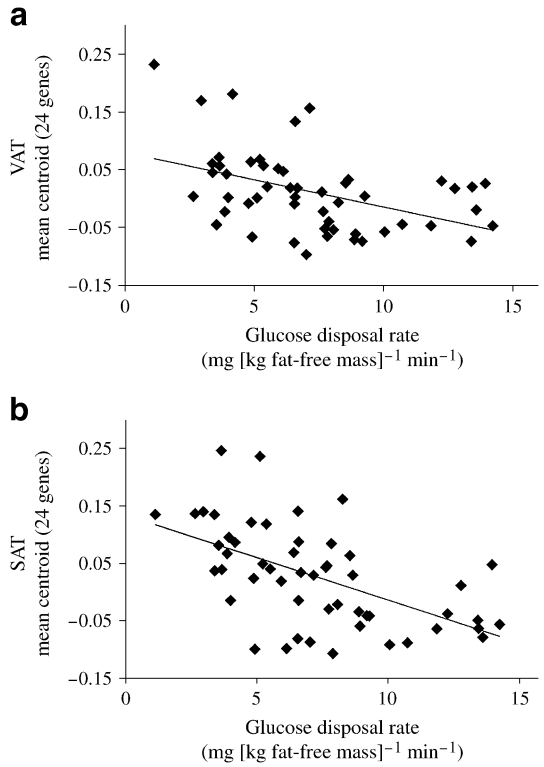

Fig. 4 Correlation analyses of macrophage-specific marker gene expression. Correlation between glucose disposal rate (mg [kg fat-free mass]$^{-1}$ min^{-1}) and (a) VAT and (**b**) SAT mean centroid for 24 genes; n=53 participants. (**a**) r=−0.539, p<0.001; (**b**) r=−0.574, p<0.001. Linear regression lines are shown

adhesion receptors binding carbohydrate ligands. *CD14*, *SLEB10* (also known as *IRF5*) and *UNQ248/PRO285* (also known as *TLR7*) belong to the Toll-like receptor pathways [38]. Our selection criteria picked up three marker genes (*CD20L1* (also known as *MS4A4A*), *CD20L3* (also known as *MS4A6A*), *CD20L4* (also known as *MS4A7*)) from a cluster of genes expressed in haemapoietic cells [39]. Two genes encoding class II major histocompatibility complex cell surface glycoproteins (*HLA-DRA1* (also known as *HLA-DRA*), *HLADM*) were represented. Moreover, *CD14* and *GP110* encode widely used cell-surface markers for identification of human ATM by immunohistochemistry or flow cytometry [11, 27, 40, 41]. Other genes of the list, such as *HLA-DR*, *M130*, *CDSIGN* and *IL-10* (also known as *IL10*), have been shown to be highly expressed in human ATMs [22, 23]. *TRAP* is highly expressed in adipose tissue of obese individuals where it is secreted from ATM [42]. Second, we investigated the association between ATM-specific gene expression levels and clinical variables in paired SAT and VAT samples from four thoroughly phenotyped groups of lean, overweight, obese and the metabolic syndrome individuals. Positive associations with obesity have been reported for a limited number of ATM transcripts, such as *GP110*, *M130* and *CD206* [12, 24, 26, 41, 43]. Accordingly, we found higher *GP110* and *M130* mRNA levels in obese than in lean individuals. Unlike previous studies, we used a large panel of 24 ATM-specific markers and showed positive correlation between ATM gene expression and fat mass. Data on ATM gene expression in the context of the metabolic complications of obesity are limited. Expression of five inflammatory genes in SAT was shown to be different between insulin-resistant and insulin-sensitive overweight patients [25]. In our study, we found significantly increased mRNA levels of 24 ATM-specific markers in the metabolic syndrome patients compared with all other groups in both fat depots.

An important predictive factor of the metabolic risk is the pattern of adipose tissue distribution. Generally, VAT is considered to be highly associated with the metabolic risk factors [44]. The existence of phenotypic and the metabolic differences between various adipose tissue depots implies differential gene expression profiles. Many studies with various experimental designs have searched for depot-specific differences of inflammatory molecules in humans [12, 13, 15–17, 24, 26, 45]. To date, only *M130* and *GP110* have been compared between SAT and VAT in different groups of individuals. For *M130*, our data showing no significant difference between SAT and VAT gene expression are consistent with the results of Shakeri-Manesch et al. for lean and morbidly obese individuals [26] and inconsistent with those of O'Rourke et al., who examined morbidly obese women and reported higher expression in VAT [13]. *GP110* has been reported to have an equal inter-depot expression in obese and lean individuals [16, 24, 26], which is in line with our findings. One team has reported contradicting results showing higher mRNA levels of *GP110* in omental adipose tissue of lean and obese patients [12]. However, these authors recruited women and men, while all our participants were all women. Our study revealed that there was no systematic difference between fat depots for expression of ATM-specific markers. For some genes, the degree of obesity influenced the relative expression in the two depots. Importantly, correlations between ATM gene expression and clinical variables related to obesity and the metabolic

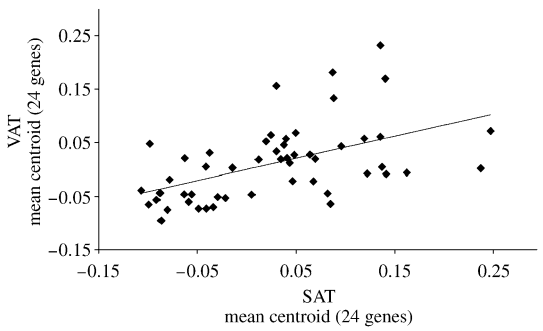

Fig. 5 Correlation between SAT and VAT mean centroids of 24 genes; r=0.510, p<0.001. The linear regression line is shown

Table 4 Comparison of macrophage markers mRNA expression between SAT and VAT depots in lean, overweight, obese and metabolic syndrome individuals

Gene symbol	Adipose tissue type per body weight group			
	Lean (n=12)	Overweight (n=12)	Obese (n=15)	Metabolic syndrome (n=14)
ACP5	VAT[a]	SAT[a]	SAT[b]	SAT[b]
CD14	NS	NS	NS	NS
CD163	NS	NS	NS	NS
CD209	NS	SAT[a]	NS	NS
CD33	NS	SAT[a]	NS	SAT[a]
CD68	NS	SAT[a]	NS	SAT[a]
ADAP2	VAT[b]	NS	NS	NS
CLEC10A	NS	NS	NS	NS
FCGR2B	NS	SAT[b]	NS	SAT[a]
FCN1	NS	NS	NS	NS
HLA-DMA	NS	NS	NS	NS
HLA-DRA	NS	SAT[b]	SAT[b]	SAT[b]
IL10	VAT[a]	NS	NS	SAT[a]
IRF5	NS	SAT[a]	NS	SAT[a]
KYNU	VAT[b]	NS	NS	NS
LIPA	NS	SAT[a]	SAT[a]	SAT[b]
MARCO	VAT[a]	NS	NS	NS
MS4A4A	VAT[a]	NS	NS	NS
MS4A6A	NS	SAT[a]	NS	NS
MS4A7	VAT[a]	NS	NS	NS
MSR1	NS	SAT[b]	SAT[b]	SAT[b]
SIGLEC1	VAT[a]	NS	NS	NS
SLCO2B1	VAT[b]	NS	VAT[a]	NS
TLR7	VAT[a]	NS	NS	NS

Gene symbols as listed in Entrez Gene (www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=gene)

VAT or SAT refers to higher mRNA levels in the indicated fat depot based on pair-wise analysis of $2^{\Delta C_t}$ mRNA values between SAT and VAT (Wilcoxon's signed rank test)

[a] $p<0.05$, [b] $p<0.01$

syndrome were as strong in SAT as in VAT. Hence, we found no evidence that the deleterious effect of VAT accumulation on the metabolic syndrome variables is related to higher expression of ATM markers in VAT.

Although this study was not designed to investigate the phenotypes of ATMs, comparison of the 24 ATM-specific markers with known human M1 and M2 macrophage markers revealed that six genes (*CD204*, *CDSIGN*, *CD301*, *CD20L1*, *CD20L3* and *CESD* (also known as *LIPA*)) are typical of human blood monocyte-derived macrophages polarised in vitro toward the M2 phenotype [20]. Other genes from our panel that could be assigned to M2 macrophages are *IL-10* and possibly *M130*. The haemoglobin scavenger receptor gene *M130* has been suggested to be rather M2-like [23], but has also been reported to be regulated by pro- and anti-inflammatory stimuli [46]. None of the 24 ATM markers were classified as M1. Importantly, it should be recognised that the current classification is imprecise and that there is a need for it to be expanded to encompass a spectrum of macrophage populations with different shades of activation [21]. However, according to the current nomenclature, our data are in agreement with a remodelling phenotype for human ATM expressing pro- and anti-inflammatory markers [22, 47]. These M2-like genes showed increased expression in SAT and VAT in the metabolic syndrome compared with lean individuals and were correlated with variables of adiposity and the metabolic syndrome. Therefore, we could not demonstrate a switch from M2 to M1 phenotype as reported in mice developing obesity [48, 49]. This is best exemplified by variations in the mRNA levels of the anti-inflammatory cytokine *IL-10*, which are higher in lean than in obese mouse ATM, but lower in lean than in obese and the metabolic syndrome SAT and VAT in humans [48]. As the link in mice of M1-activated ATM and adipose tissue inflammation to obesity and insulin resistance is not supported by data in humans, further work is now needed to determine the role of human ATM with remodelling phenotype in the development of the metabolic syndrome.

In conclusion, our data show that expression of ATM markers is increased with obesity, insulin resistance and occurrence of the metabolic syndrome in human SAT as well as in VAT. This suggests that the worsening of the metabolic status in obese individuals cannot be simply ascribed to ATM-mediated inflammation of visceral fat. Identification of specific ATM populations in different fat

depots in individuals with different the metabolic abnormalities is warranted to identify potiential pharmacological treatments of obesity and linked disturbances by targeted modulation of macrophage phenotype.

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