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ARTICLE

# Semaphorin 3C is a novel adipokine linked to extracellular matrix composition

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**Abstract**
*Aims/hypothesis* Alterations in white adipose tissue (WAT) function, including changes in protein (adipokine) secretion and extracellular matrix (ECM) composition, promote an insulin-resistant state. We set out to identify novel adipokines regulated by body fat mass in human subcutaneous WAT with potential roles in adipose function.
*Methods* Adipose transcriptome data and secretome profiles from conditions with increased/decreased WAT mass were combined. WAT donors were predominantly women. In vitro effects were assessed using recombinant protein. Results were confirmed by quantitative PCR/ELISA, metabolic assays and immunochemistry in human WAT and adipocytes.
*Results* We identified a hitherto uncharacterised adipokine, semaphorin 3C (SEMA3C), the expression of which correlated significantly with body weight, insulin resistance (HOMA of insulin resistance [$HOMA_{IR}$], and the rate constant for the insulin tolerance test [$K_{ITT}$]) and adipose tissue morphology (hypertrophy vs hyperplasia). SEMA3C was primarily found in mature adipocytes and had no direct effect on human adipocyte differentiation, lipolysis, glucose transport or the expression of β-oxidation genes. This could in part

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be explained by the significant downregulation of its cognate receptors during adipogenesis. In contrast, in pre-adipocytes, SEMA3C increased the production/secretion of several ECM components (fibronectin, elastin and collagen I) and matricellular factors (connective tissue growth factor, IL6 and transforming growth factor-β1). Furthermore, the expression of *SEMA3C* in human WAT correlated positively with the degree of fibrosis in WAT.

*Conclusions/interpretation* SEMA3C is a novel adipokine regulated by weight changes. The correlation with WAT hypertrophy and fibrosis in vivo, as well as its effects on ECM production in human pre-adipocytes in vitro, together suggest that SEMA3C constitutes an adipocyte-derived paracrine signal that influences ECM composition and may play a pathophysiological role in human WAT.

**Keywords** Bariatric surgery · Cancer cachexia · Fibrosis · Insulin sensitivity · Metabolism · Obesity · Type 2 diabetes

**Abbreviations**

| | |
|---|---|
| CTGF | Connective tissue growth factor |
| ECM | Extracellular matrix |
| ELN | Elastin |
| GO | Gene ontology |
| $HOMA_{IR}$ | HOMA of insulin resistance |
| $K_{ITT}$ | Rate constant for the insulin tolerance test |
| NRP | Neuropilin |
| PLXN | Plexin |
| SAM | Significance analysis of microarrays |
| scWAT | Subcutaneous WAT |
| SEMA3C | Semaphorin 3C |
| SVF | Stroma-vascular fraction |
| TGF-β1 | Transforming growth factor-β1 |
| WAT | White adipose tissue |

## Introduction

White adipose tissue (WAT) is a highly plastic organ that can change considerably in size within and between individuals. Excess fat mass is associated with insulin resistance, type 2 diabetes and dyslipidaemia, and correlates with distinct changes in fat-cell size and adipose function, including altered lipid metabolism, increased interstitial fibrosis (due to elevated extracellular matrix [ECM] protein production and deposition) and a chronic pro-inflammatory state [1]. Studies assessing the effects of weight loss have demonstrated that upon reduction of fat mass to a non-obese state, most aspects of WAT function are normalised [2, 3].

To maintain a healthy metabolic profile during fat mass alterations, adaptive mechanisms involving signals between cells present within the tissue (e.g. adipocytes, adipocyte progenitor cells, immune cells and endothelial cells) are necessary. Results in recent years have demonstrated that adipocytes secrete a number of polypeptides (collectively termed adipokines), which, in humans, predominantly act locally through auto- or paracrine mechanisms. However, in obesity the production of several adipokines can be maladaptive and can promote an insulin-resistant state [4]. Although a number of human WAT/adipocyte secretome studies have been performed, the overlap between different studies is rather poor (see for instance [5–9]). At present, the total human adipokinome is estimated to contain well over 600 members but the list is still growing.

In this study, we set out to identify novel adipokines regulated by weight alterations in human subcutaneous WAT (scWAT) that could have an impact on WAT function. To this end, transcriptomic data obtained from scWAT of individuals with different forms of fat mass alterations (obesity, weight reduction by bariatric surgery and cancer cachexia) were combined with a secretome analysis performed in human WAT. This unbiased approach enabled us to identify a hitherto uncharacterised adipokine, semaphorin 3C (SEMA3C), which belongs to a group of secreted factors including seven individual gene members (*SEMA3A–G*). Very little is known regarding the role of SEMA3 members in WAT. Only one publication has studied the expression of *Sema3a* in rat WAT [10]. SEMA3s signal through cognate receptors and co-receptors termed plexins (PLXNs) and neuropilins (NRPs), respectively, but the intracellular pathways are poorly characterised [11]. We demonstrate that SEMA3C is primarily secreted from fat cells and that the expression in scWAT correlates with insulin resistance, fat-cell morphology and other measures of the metabolic syndrome. Functional studies in human adipocytes did not reveal any significant effects of SEMA3C. However, incubation with recombinant SEMA3C in human pre-adipocytes increased the expression and secretion of several ECM components and matricellular factors. In addition, expression of the gene encoding SEMA3C in human WAT correlated with interstitial fibrosis.

## Methods

*Clinical cohorts* The participants included in the cohorts are described, including relevant references, in the electronic supplementary material (ESM) Table 1. Clinical assessments were performed as described in the corresponding references. Abdominal scWAT was obtained by needle/surgical biopsies or as a waste product from cosmetic surgical procedures, as described previously [12]. For tissues used for cell culture experiments, there was no selection on the basis of age, sex or BMI. None of the participants were on any regular medication that might be expected to affect adipocyte function. Cachexia was defined as described in [13]. BMI was classified according to the WHO definition. The metabolic syndrome

was defined according to recently described definitions [14] where waist circumference criteria from the International Diabetes Federation were used [15]. The project was conducted in accordance with the guidelines in The Declaration of Helsinki and the studies were approved by the Regional Ethics committee in Stockholm, Toulouse University Hospitals, the Third Faculty of Medicine in Prague and Hôtel-Dieu Hospital, Paris. Individual, written informed consent was obtained from all participants involved in the study.

*Adipocyte isolation, cell culture and tissue fractionation* Mature adipocytes and stroma-vascular fraction (SVF) cells were isolated as described previously [16, 17]. Adipocyte morphology (i.e. the relative fat-cell size in relation to total fat mass) has been shown to correlate with insulin sensitivity and was determined as described [18].

Distinct cell populations of the SVF were isolated using an immunoselection/depletion protocol and cultured as described [16, 17, 19–21]. $CD34^+/CD31^-$ cells were defined as progenitor cells, $CD34^+/CD31^+$ cells as endothelial cells, $CD34^-/CD14^+$ cells as macrophages and $CD34^-/CD14^-/CD3^+$ as lymphocytes. For time course analysis, cells were lysed to obtain RNA at day 4/5, 8 and 12 after the induction of differentiation. For the secretome analysis, mature adipocytes and the various cell populations from the SVF were maintained separately ex vivo at 37°C in endothelial basal culture medium (0.1% BSA) for 24 h and their conditioned media were collected.

*Transcriptome and secretome studies* For human transcriptome data, probesets identified to be differentially expressed, comparing obese with non-obese according to significance analysis of microarrays (SAM, false discovery rate 5%) [22], were used as a starting point. These probesets were extracted from the weight-loss studies and subsequently analysed with SAM. Thereafter, significantly regulated probesets corresponding to the same gene were averaged. This generated a set of genes that were regulated by obesity as well as by voluntary (energy restriction) and involuntary (cancer cachexia) weight loss. The list was filtered and genes with a low fold change (<15% comparing groups) were excluded, resulting in a list of 112 individual genes. Enrichment of predefined gene ontologies (GOs) were identified using Gene Ontology Tree Machine [23]. Messenger RNA levels of the class 3 SEMAs were separately extracted from human transcriptome profiling datasets and evaluated for differential expression in cohorts 1–3. Transcriptome analysis of different human subcutaneous WAT cell types was performed on cohort 4 as reported [24]. In the secretome analyses (cohort 5), media from each cell fraction were pooled. To optimise the detection of medium- and low-abundance proteins, depletion of highly abundant proteins from the media was performed using the ProteoMiner Protein Enrichment Kit (Bio-Rad, Hercules, CA, USA). Proteins were then separated on 1D SDS-PAGE gels (12% 12.5% constant concentration polyacrylamide slab gel; Bio-Rad). The resulting gel lanes were divided into slices (10/sample) before MS analysis. Proteins contained in gel slices were reduced, alkylated and digested with trypsin using standard in-gel digestion protocols. The digested peptides were then analysed using NanoLC/ESI LTQ-Orbitrap MS/MS (Thermo Fisher Scientific, Waltham, MA, USA), and the corresponding proteins were identified by automated database searching (Mascot Daemon, Matrix Science; http://mfpaq.sourceforge.net) against the Swiss-Prot/Trembl protein sequence database (http://www.uniprot.org). A comparative proteomic analysis of the proteins secreted by the different cell types contained in WAT (mature adipocytes, endothelial cells, macrophages, progenitor cells, endothelial cells and lymphocytes) led to the identification of 626 proteins (N. Viguerie, C.-I. Kolditz and D. Langin, unpublished data). To identify factors specifically produced by adipocytes, transcriptomic and proteomic data from the different cell types were compared. This list was cross-checked with the list of the 112 weight-regulated genes.

*RNA isolation, cDNA synthesis and quantitative PCR* RNA isolation, cDNA synthesis and real-time PCR were performed exactly as described [12]. For SYBR Green assays, 5 ng of cDNA was mixed with 2× iQ SYBR Green Supermix (Eurogentec, Ougrée, Belgium) and primers (Invitrogen, Carlsbad, CA, USA) in a final volume of 25 μl. For TaqMan assays, 10 ng of cDNA was mixed with 2× TaqMan Universal PCR Master Mix and TaqMan primers (Applied Biosystems, Foster City, CA, USA) in a final volume of 20 μl. Messenger RNA levels were normalised to an internal reference—18S rRNA (intact WAT, SVF and mature adipocytes) or *LRP10* mRNA (in vitro differentiated pre-adipocytes/adipocytes)—using a comparative $C_t$ method calculated by the formula $2^{\Delta C_t - \text{target gene}} / 2^{\Delta C_t - \text{reference gene}}$. TaqMan assays and SYBR Green primers are listed in ESM Table 2.

*ELISA* The level of SEMA3C secreted from intact scWAT (300 mg pieces in 3 ml medium) was assessed and incubated as described [25]. Medium was saved at −70°C for determination of SEMA3C levels according to the manufacturer's instructions (E80919Hu; USCN Life Science, Wuhan, Peoples Republic of China). The standard curve of the SEMA3C ELISA kit ranged between 78 and 5,000 pg/ml and the lowest detectable level was 27.7 pg/ml, according to the instruction manual provided by the manufacturer. The specificity of the ELISA kit was confirmed by western blot using two different antibodies (see ESM Fig. 1 and ESM Methods; Santa Cruz Biotechnology, Santa Cruz, CA, USA and R&D Systems, Minneapolis, MN, USA, respectively).

The secretion of SEMA3C protein from WAT explants of obese and non-obese participants was related to the number of fat cells in the incubated tissue sample. Connective tissue growth factor (CTGF, E90010Hu; USCN Life Science), ELN (E91337Hu; USCN Life Science), IL6 (D6050; R&D Systems) and transforming growth factor-β1 (TGF-β1) (DB100B; R&D Systems) protein secretion was measured in conditioned culture media from in vitro differentiated adipocytes according to the manufacturers' instructions.

*Immunofluorescence and confocal microscopy* Immunofluorescence analysis of collagen/fibronectin networks produced by human pre-adipocytes was performed as described previously [26] and as detailed in the ESM Methods.

*Immunohistochemical analyses* Subcutaneous WAT samples were prepared for immunohistochemical analyses as described in the ESM Methods and elsewhere [27, 28]. Representative microphotographs are presented in ESM Figs 2, 3.

*Hypoxia experiments* Conditioned media from mature adipocytes were obtained after 24 h culture in endothelial cell basal medium (1/3, vol./vol.) supplemented with 0.1% BSA, 100 U/ml penicillin and 100 g/ml streptomycin in CLINIcell culture cassettes (Mabio, Tourcoing, France) in normoxia (21% $O_2$) or hypoxia chambers (1% $O_2$; Sanyo, Avon, France).

*SEMA3C stimulation experiments* Cultured pre-adipocytes and adipocytes were stimulated, during the first 6 or last 2 days of differentiation, respectively, with 1–500 ng/ml recombinant human SEMA3C-Fc-fusion protein (5570-S3-050; R&D Systems). For pre-adipocytes, medium containing recombinant SEMA3C was changed on the third day of incubation. Following stimulation, conditioned culture medium was collected and cells were lysed for total RNA or effects on glucose transport [29] and lipolysis [30] were evaluated exactly as described. Experiments were performed in duplicate or triplicate with untreated cells as control. Incubations with an Fc:fusion-control protein (ALX-203-004-C050; Enzo Life Sciences, Farmingdale, NY, USA) were used as a negative control to rule out possible Fc-mediated effects on gene expression. No alterations in mRNA levels for SEMA3C-regulated genes were observed (ESM Fig. 4).

*Statistical analysis* Data shown in the figures are mean±SEM. For tables, results are presented as mean±SD, range, and/or fold change as detailed. For datasets that were not normally distributed, $\log_{10}$ transformed values were used. Results were analysed with appropriate parametric/non-parametric statistical tests, including Student's paired/unpaired *t* test, Wilcoxon signed-rank test, simple/multiple regression analysis and analysis of variance using standard software packages.

## Results

*Identification of novel candidate adipokines regulated by weight alterations* Our analysis of human scWAT transcriptional profiles included three cohorts with either excess (cohort 1, lean vs obese) or reduced fat mass due to voluntary (cohort 2, before and after energy restriction) or involuntary (cohort 3, cancer with or without cachexia) weight loss. The expression of a large number of genes was altered by either obesity or weight loss (data not shown). However, only 112 genes were regulated, predominantly in a reciprocal manner, in all three cohorts (ESM Table 3). Thus, the genes that were upregulated in obesity were mostly downregulated after weight loss and vice versa. GO analysis of these genes demonstrated that the pathways involved in ECM formation and development/organ morphogenesis were particularly enriched (both upregulated in obesity; results not shown). To determine which of these genes encoded proteins secreted by adipocytes, we cross-matched the list of weight-regulated genes with data from the combined transcriptome and secretome analyses of cell fractions present in human WAT (cohorts 4 and 5, respectively). Out of the 112 candidates, only six (*SEMA3C*, *NQO1*, *ABHD5*, *UCHL1*, *FMOD* and *GLUL*) were found to be selectively transcribed and secreted by adipocytes and to contain a predicted signal peptide sequence (ESM Table 3). Five of these six genes encoded proteins that had been identified in recently published human adipocyte secretome screens (*UCHL1*, *FMOD* and *GLUL*) and/or functionally studied in human adipocytes/WAT (*NQO1* and *ABHD5*). A single gene, *SEMA3C*, remained as a potentially novel adipokine not previously characterised in human WAT. Further studies were therefore focused on *SEMA3C*.

*Mapping of class 3 semaphorin expression in human adipose tissue* Since little is known about the class 3 SEMA family in human WAT, we mapped the expression of SEMA3s in cohorts 1–3. Although signals for all seven ligands were detected and two of the family members were regulated by either obesity (*SEMA3G*) or loss of fat mass (*SEMA3B*), only *SEMA3C* was regulated in all three cohorts (ESM Table 4). The results in obesity and weight loss were confirmed by quantitative RT-PCR, demonstrating that scWAT *SEMA3C* mRNA levels were increased in obesity and the metabolic syndrome (cohort 6, Fig. 1a) and were reduced upon voluntary weight loss (by bariatric surgery, cohort 7, Fig. 1b) and involuntary weight loss (cachexia, cohort 3, Fig. 1c).

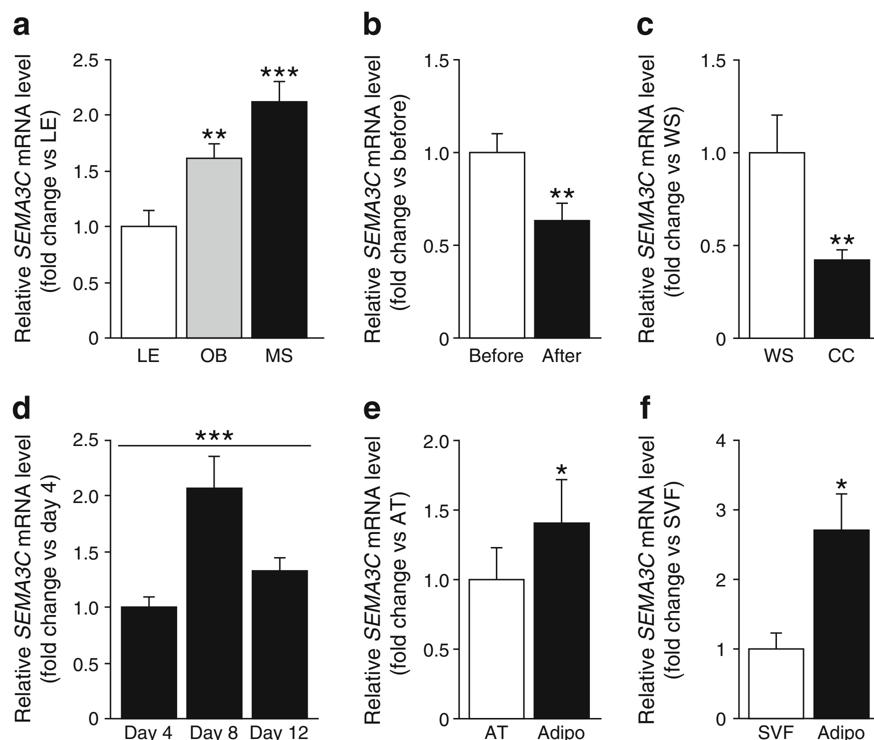


**Fig. 1** Human adipose *SEMA3C* mRNA expression. Expression of *SEMA3C* mRNA in subcutaneous WAT was compared between lean (LE) and obese (OB) participants and participants with the metabolic syndrome (MS) (cohort 6, $n$=24) (**a**), before and after bariatric surgery (cohort 7, $n$=13) (**b**) and between cancer patients with (CC) or without (weight stable [WS]) cancer cachexia (cohort 3, $n$=27) (**c**). *SEMA3C* mRNA expression was assessed during adipogenic differentiation of human pre-adipocytes in vitro (**d**), in intact subcutaneous adipose tissue (AT) and paired samples of isolated, mature adipocytes (Adipo) (**e**) and in adipocytes and corresponding SVF (**f**). Results are presented as fold change±SEM relative to control participants. Data are means±SEM. *$p$<0.05, **$p$<0.01, ***$p$<0.001

*SEMA3C is a novel adipokine predominantly expressed in fat cells* Adipose tissue is composed of several cell types including adipocytes, leucocytes and endothelial and progenitor cells. We therefore performed an extensive mapping of SEMA3C expression and secretion in human scWAT and distinct cell populations. *SEMA3C* mRNA expression increased during adipocyte differentiation in vitro (Fig. 1d) and was higher in isolated adipocytes than in paired samples of either intact WAT (cohort 8, Fig. 1e) or SVF (cohort 4, Fig. 1f). The presence of SEMA3C protein in WAT was further established by immunohistochemistry which detected the protein in both adipocytes and the stroma (ESM Fig. 3). Moreover, the secretion of SEMA3C from WAT explants was time dependent (cohort 9, Fig. 2a) and was significantly higher in samples from obese vs non-obese individuals (cohort 10, Fig. 2b). Since several SEMA3s, including SEMA3C, have been shown to be regulated by hypoxia in prostate cancer cells [31], we assessed whether SEMA3C secretion could be influenced by oxygen tension. However, following a 24 h incubation in either hypoxic (1%) or normoxic (21%) conditions, no differences in SEMA3C protein levels were observed in conditioned media from human adipocytes (cohort 11, data not shown). Additionally, in a recently published microarray study performed in human adipocytes [32], incubation under hypoxic conditions had no effect on *SEMA3C* expression, although *SEMA3A* expression was inhibited (2.9-fold reduction in mRNA level over 24 h).

*Adipose tissue SEMA3C levels are associated with insulin resistance and fat-cell morphology* While the results obtained so far demonstrate that SEMA3C is an adipokine that is regulated by weight changes, they do not yield any information on the role of SEMA3C in WAT function. We assessed whether *SEMA3C* mRNA levels were associated with any clinical variables in cohort 1. *SEMA3C* correlated positively with circulating glucose and insulin levels, measurements of whole-body insulin resistance (HOMA of insulin resistance [$HOMA_{IR}$] [33] and the rate constant for the insulin tolerance test [$K_{ITT}$]) and fat-cell size and morphology independent of BMI (Table 1). In addition, the levels of SEMA3C secreted from WAT explants correlated significantly with $HOMA_{IR}$ independent of BMI (Fig. 2c). These findings encouraged us to evaluate the function of SEMA3C in vitro.

*Functional characterisation of SEMA3C in primary human adipocytes* The effect of recombinant SEMA3C protein on primary cultures of human adipocytes was determined using concentrations in the range of 10–500 ng/ml. These concentrations were chosen based on ranges used in published studies in non-adipose cells [34]. Adipocytes were incubated with SEMA3C for 48 h and the effect on glucose transport, lipolysis and the expression of genes regulating lipid oxidation was determined. However, no significant effect on any of these variables was observed (ESM Fig. 5). The lack of response prompted us to determine whether SEMA3C

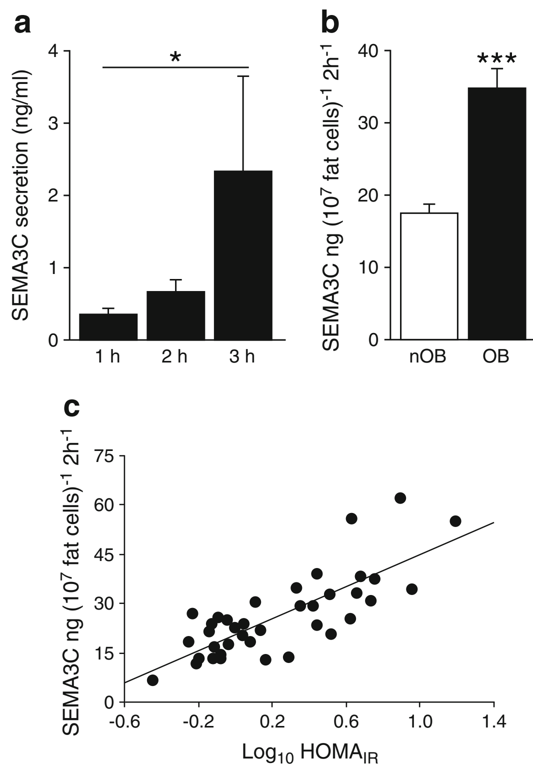


**Fig. 2** Human adipose SEMA3C secretion. SEMA3C protein secretion was determined by ELISA in intact adipose tissue incubates (n=6) at 1, 2 and 3 h (**a**) and intact tissue incubates from obese (OB) and non-obese (nOB) participants (**b**). All values were within the range of the standard curve. In part (**b**), values were corrected for fat-cell number and incubation time (to allow quantitative comparisons between groups). (**c**) The secreted levels of SEMA3C in part (**b**) were correlated to $HOMA_{IR}$ by multiple regression analysis using BMI as covariate ($p$ value, 0.0017; $r$ value, 0.441). The graph depicts a simple regression. Data are means±SEM. *$p<0.05$, ***$p<0.001$

receptors are expressed in differentiated human adipocytes. As detailed in Fig. 3a, the expression of all receptors, except for PLXNA2 (which was found at very low levels), was markedly downregulated during adipocyte differentiation, suggesting that SEMA3C may act on adipocyte progenitor cells (pre-adipocytes) present within WAT.

*SEMA3C regulates the production of ECM-related components in primary human pre-adipocytes* Pre-adipocytes are not only essential for adipogenesis. Recent studies demonstrate that human pre-adipocytes cultured in an inflammatory context (such as conditioned media from WAT macrophages) produce several factors that could promote the generation of WAT fibrosis [26]. Several structural components (e.g. collagen I/VI [encoded by *COL1A1*/*COL6A1*], fibronectin 1 [*FN1*] and elastin [*ELN*]), matricellular proteins (e.g. CTGF [encoded by *CTGF*] and TGF-β1 [*TGFB1*]) and inflammatory cyto-/chemokines (e.g. IL6 [encoded by *IL6*]) have been implicated in tissue remodelling and the development of fibrosis. We determined whether recombinant SEMA3C affected gene/protein expression of factors involved in differentiation, inflammation and ECM composition in human primary pre-adipocytes. Different concentrations of SEMA3C had no effect on adipogenesis determined by either lipid accumulation (assessed by microscopy, data not shown) or gene expression (of *FABP4*, *CEBPA* and *PPARG*, results not shown). Moreover, the expression of pro-inflammatory factors, including *TNF* and *CCL2*, *-3* or *-5*, was unaltered (Table 2). In contrast, SEMA3C increased mRNA levels of genes known to be associated with ECM remodelling and/or fibrosis (Table 2), including structural components (*COL1A1*, *ELN* and *FN1*), matricellular factors and cytokines (*CTGF*, *TGFB1* and *IL6*). These effects were confirmed at the protein level by ELISA (Fig. 3b), whereby levels of CTGF were found to be particularly induced (about fivefold). Moreover, immunofluorescence assays showed a more distinct extracellular network of fibronectin and collagen I induced by the addition of SEMA3C (Fig. 3c). The

**Table 1** Relationship between *SEMA3C* expression in human adipose tissue and clinical variables

| Measurement | Simple regression | | Multiple regression | |
|---|---|---|---|---|
| | $r$ value | $p$ value | Partial $r$ value | $p$ value |
| Waist circumference (cm) | 0.476 | 0.0002 | | |
| Waist–hip ratio | 0.427 | 0.0012 | | |
| BMI (kg/m$^2$) | 0.536 | <0.0001 | | |
| Body fat (%) | 0.591 | <0.0001 | | |
| Lean body mass (kg) | −0.03 | 0.8391 | | |
| Plasma glucose (mmol/l) | 0.496 | 0.0001 | 0.462 | 0.0019 |
| Plasma insulin (pmol/l) | 0.575 | <0.0001 | 0.328 | 0.0071 |
| $HOMA_{IR}$ | 0.645 | <0.0001 | 0.411 | 0.0004 |
| $K_{ITT}$ | −0.625 | 0.0001 | −0.415 | 0.0281 |
| Fat cell volume (pl) | 0.667 | <0.0001 | 0.359 | 0.0003 |
| Fat cell morphology | 0.426 | 0.001 | | |

Expression of *SEMA3C* was related to clinical variables in obese and non-obese participants (cohort 1, $n$=56). Results were evaluated with simple regression analysis and significant associations were further studied by multiple regression analysis using BMI as a covariate. Data are presented as $r$-, partial $r$- and $p$ value

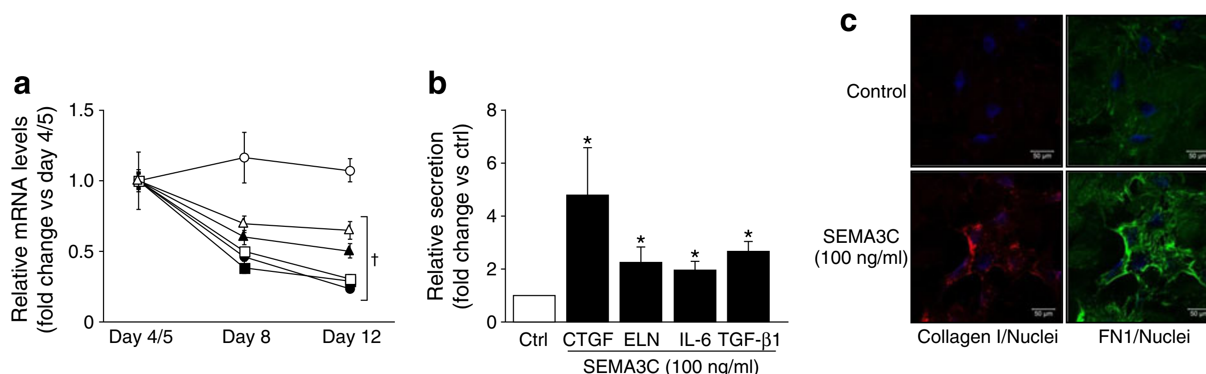


**Fig. 3** SEMA3C receptor levels and effects of SEMA3C in primary human pre-adipocytes. (**a**) mRNA expression of the SEMA3C receptor PLXNs (*PLXNA1-2*, *-4* and *-D1*) and co-receptors NRPs (*NRP1-2*) during adipogenesis ($n$=12). All receptors/co-receptors, except for *PLXNA2*, were significantly downregulated during differentiation ($^{\dagger}p<$ 0.0001for all, using repeated measures ANOVA). According to microarray results (data not shown), *PLXNA3* expression was extremely low in human adipose tissue and therefore not studied. Black circles, *NRP1*; black squares, *NRP2*; black triangles, *PLXNA1*; white circles, *PLXNA2*; white squares *PLXNA4* and white triangles, *PLXND1*. Data are means±SEM. (**b**) Five of the genes were significantly altered at the mRNA level (Table 2) and were evaluated at the protein level by determining the secretion in differentiating human adipocytes incubated with SEMA3C (100 ng/ml) for 6 days. The release of all but one (fibronectin) was increased by SEMA3C. Data are means±SEM ($n$=6); $^{*}p$<0.05 compared with control (Ctrl). (**c**) The effects of SEMA3C in promoting functional ECM matrix were determined by immunofluorescence in human pre-adipocytes after 6 days of incubation with SEMA3C (100 ng/ml). Cells incubated with SEMA3C displayed a significantly more pronounced staining for both fibronectin (FN1) and collagen compared with control cells

clinical relevance of these results was supported by the fact that *SEMA3C* mRNA levels correlated positively with interstitial fibrosis as evaluated by immunohistochemistry, at least in subcutaneous WAT of severely obese participants ($r$=0.546, $p$=0.0104, cohort 12).

**Table 2** Effect of recombinant SEMA3C on the expression of ECM and inflammatory genes in pre-adipocytes

| Gene | Control | Recombinant SEMA3C (ng/ml) | | | $p$ value |
|---|---|---|---|---|---|
| | | 1.0 | 10 | 100 | |
| *CCL2* | 1.0 | 0.85 | 0.86 | 1.12 | 0.363 |
| *CCL3* | 1.0 | 0.82 | 0.65 | 0.81 | 0.121 |
| *CCL5* | 1.0 | 0.84 | 0.78 | 0.88 | 0.334 |
| *COL1A1* | 1.0 | 0.91 | 1.54 | 1.61 | 0.003 |
| *COL6A1* | 1.0 | 0.93 | 0.89 | 1.08 | 0.581 |
| *CTGF* | 1.0 | 1.02 | 1.47 | 2.01 | 0.005 |
| *ELN* | 1.0 | 0.97 | 1.63 | 3.31 | 0.010 |
| *FN1* | 1.0 | 0.87 | 1.36 | 2.02 | 0.000 |
| *IL6* | 1.0 | 0.98 | 1.71 | 2.64 | 0.001 |
| *LOX* | 1.0 | 0.86 | 1.06 | 1.43 | 0.064 |
| *NOV* | 1.0 | 0.77 | 0.77 | 0.81 | 0.488 |
| *TGFB1* | 1.0 | 0.85 | 1.05 | 1.38 | 0.013 |
| *TNF* | 1.0 | 1.05 | 0.85 | 0.92 | 0.585 |

Human pre-adipocytes were incubated for 6 days with recombinant SEMA3C protein (1–100 ng/ml). Effects on the expression of ECM and inflammatory genes were measured and are summarised in the table. Six genes, *COL1A1*, *CTGF*, *ELN*, *FN1*, *IL6* and *TGFB1*, were significantly increased by SEMA3C in a concentration-dependent manner

Data are presented as fold change vs control treated samples (n=9)

## Discussion

By combining human adipose transcriptome and secretome data, we identified six potentialadipokines that were altered by changes in WAT mass. Out of these, five were described in previous secretome analyses and/or functionally studied in human adipocytes. Only SEMA3C was novel (i.e. previously not characterised in human WAT/adipocytes). We found that SEMA3C was predominantly expressed in fat cells and that its expression and secretion correlated with hypertrophic WAT (fewer but larger fat cells, a phenotype associated with in vivo insulin resistance [18, 35]) and independent measures of in vivo whole-body insulin resistance ($HOMA_{IR}$, $K_{ITT}$), regardless of BMI. Recombinant SEMA3C did not have any direct metabolic effects on human adipocytes in culture. This could be due either to the overall reduction in cognate receptor gene expression during differentiation or to a lack of effect on pathways controlling glucose/lipid handling. In contrast, in human pre-adipocytes, SEMA3C stimulated the mRNA/protein expression of structural and matricellular genes, resulting in ECM remodelling. Pre-adipocytes have recently been demonstrated to represent major cellular mediators promoting WAT fibrosis deposition in the pro-inflammatory microenvironment of obesity [26]. We observed that SEMA3C stimulates the production of several remodelling factors implicated in fibrosis deposition, including IL6 and TGF-β1 and CTGF, the latter two constituting important growth factors shown to be dysregulated in diseases with major alterations in tissue remodelling (such as systemic sclerosis) [36]. In the context of obesity, the finding that

*SEMA3C* expression correlated with human WAT fibrosis, at least in adipose samples from severely obese individuals, lends pathophysiological relevance to our data. Taken together, SEMA3C may influence insulin sensitivity via modified ECM deposition/interstitial fibrosis, a hypothesis which needs further experimental validation, preferably in an in vivo model.

Although human WAT displays a high adipocyte turnover, the total number of fat cells in adults remains constant over time and is unaltered by voluntary/involuntary weight reduction [37, 38]. The production of ECM from fibroblast-like adipose precursor cells is therefore essential in order to allow dynamic changes in fat-cell volume while retaining tissue stability. The relationship between fat-cell size and ECM remodelling was recently shown in WAT from healthy growing children [39]. However, ECM overproduction in obesity may have a negative impact on WAT plasticity and function through the development of interstitial fibrosis. The latter has been proposed to reduce oxygenation, leading to local hypoxia, which in turn attenuates adipogenesis and induces insulin resistance [40]. Hypoxia per se stimulates the production of ECM and this leads to a vicious circle further promoting a dysfunctional WAT [41]. These hypotheses are supported by findings in animal models demonstrating that knockout of ECM components (e.g. collagen type VI $\alpha 1$) protects against insulin resistance in diet-induced obesity [42] while overexpression of hypoxia-inducible factor-1$\alpha$ increases interstitial fibrosis and insulin resistance [41]. In line with these findings, fibrosis has been demonstrated in WAT of obese insulin-resistant individuals [43]. Altogether, this suggests that the amount and composition of the adipose interstitial compartment is important for tissue function/insulin sensitivity and that ECM production needs to be tightly regulated, most probably via dynamic crosstalks between adipocytes and adjacent cells. However, the signals that control ECM production in conditions of fat mass gain/loss have not been extensively studied [1, 40]. The mechanisms that regulate *SEMA3C* expression in human adipocytes are unclear. It is unlikely that increases in circulating glucose or NEFA levels stimulate *SEMA3C* expression in human WAT. This notion is supported by the finding that *SEMA3C* mRNA was reduced by weight loss following both bariatric surgery and cancer cachexia, the latter a condition characterised by increased NEFA and unaltered glucose levels [13]. Since SEMA3s, including *SEMA3C*, have been shown to be regulated by hypoxia in prostate cancer cells [31], it is tempting to speculate that hypoxia may induce ECM production via SEMA3C. In our experimental set-up, neither the expression nor secretion of SEMA3C by cultured human adipocytes was affected by reduced oxygen tension, suggesting that SEMA3C production by fat cells is regulated via hypoxia-independent mechanisms which need to be further investigated. The observation that *SEMA3C* expression correlated significantly with adipocyte morphology (a measure that is independent of body fat mass as discussed [18]) demonstrates that there is a link between relative fat-cell size and SEMA3C levels. Fat-cell morphology is determined by adipocyte [18] and lipid turnover [44]. However, the transcriptional networks that regulate these processes have not yet been determined and their potential role in regulating *SEMA3C* requires further investigation.

There are relatively few published studies on *SEMA3C* in general (<50 publications indexed in PubMed to date) and it has been studied predominantly in non-adipose tissue. Class 3 semaphorins have primarily been implicated in axon guidance and SEMA3C has been shown to act both as a neurochemoattractant and -repellent in vitro [45]. In addition, SEMA3C appears to play a role in angiogenesis, since it stimulates the proliferation of murine glomerular endothelial cells [34]. The physiological relevance of these findings is corroborated by the phenotype of *SEMA3C*$^{-/-}$ mice, which die shortly after birth due to congenital cardiovascular malformations [46]. We incubated human adipose-derived endothelial cells (CD34$^{+}$/CD31$^{+}$) with SEMA3C in vitro, but were unable to observe any pro-proliferative effects (not shown). However, this does not exclude the possibility that SEMA3C may have an impact on other aspects of angiogenesis/endothelial cell function in human WAT.

While SEMA3C displayed marked effects in vitro, the signalling pathways remain to be elucidated. Results in recent years suggest that SEMAs, including SEMA3s, signal via multimeric receptor complexes which activate intracellular signalling pathways that have only been partially unravelled [11]. It is at present unclear which receptor(s) mainly confer the effects of SEMA3C in human adipocytes; however, a comparison of relative mRNA levels shows that *NRP1* and *-2* and *PLXND1* are the most highly expressed isoforms in WAT (results not shown). A mapping in stroma-vascular-derived cells from human WAT (adipocyte progenitor cells, macrophages, lymphocytes and endothelial cells) demonstrated a similar expression pattern with clearly detectable levels of *NRP1* and *-2* and *PLXND1* mRNA in all fractions, while *PLXNA1* and *-A4* were expressed at considerably lower levels and the latter primarily in progenitor and endothelial cells (ESM Fig. 6). *PLXNA2* expression was below the detection limit ($C_t$ values >32, data not shown). This suggests that SEMA3C may also have effects in other cell types present in human WAT. However, future studies are needed to define the ligand–receptor stoichiometry, signalling pathways and SEMA3C-mediated effects in different cells of human WAT.

A caveat in our study is that the majority of the participants were women. However, in the men that were enrolled, there was no indication that sex influenced SEMA3C

expression. Taken together, our data suggest that SEMA3C is a novel adipokine, the expression of which correlates significantly with weight change, fat-cell hypertrophy, adipose fibrosis and insulin resistance in humans. While recombinant SEMA3C does not affect insulin sensitivity in adipocytes directly, it stimulates the production and release of structural and matricellular proteins in pre-adipocytes. We hypothesise that SEMA3C may be a factor that enables WAT remodelling, disturbances of which may contribute to the development of insulin resistance and type 2 diabetes.

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**Contribution statement** NM and MR designed the study, performed the in vitro experiments, analysed the data and wrote the manuscript. NM, ID and FW identified genes regulated by weight changes. C-IK, NV, VS and D. Langin collected adipose tissue and measured SEMA3C mRNA levels in cohorts 4 and 6. C-IK, NV, D. Langin and AB collected adipose tissue and performed secretome analysis in cohort 5. NM and MR measured secreted SEMA3C protein levels in cohorts 9 and 10. MR and EN collected adipose tissue and assessed *SEMA3C* mRNA levels in cohorts 1, 3, 7 and 8. PT, DE, JG and AB measured SEMA3C levels in cohort 11. KC and JT collected cohort 12, JT quantified fibrosis and VP and D. Lacasa performed the mRNA measurements of SEMA3C in the same cohort. VP, D. Lacasa and PL performed the immunocyto-/histochemistry experiments. All authors contributed to data interpretation, reviewed the manuscript and approved the final version.

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