Cancer-associated adipose tissue promotes breast cancer progression by paracrine Oncostatin M and Jak/STAT3 signaling.

Lapeire L1, Hendrix A2, Lambein K3, Van Bockstal M3, Braems G4, Van Den Broecke R4, Limame R5, Mestdagh P6, Vandesompele J6, Vanhove C7, Maynard D8, Lehuédé C9, Muller C9, Valet P10, Gespach C11, Bracke M2, Cocquyt V1, Denys H1, De Wever O2.

Authors’ Affiliations:
1 Department of Medical Oncology, 2 Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, 3 Department of Pathology, 4 Department of Gynaecology, 6 Center for Medical Genetics, 7 Institute Biomedical Technology; Ghent University Hospital, Ghent, Belgium
5 Center for Oncological Research (CORE), University of Antwerp, Antwerp, Belgium
8 Medical Genetics Branch, National Human Genome Research Institute, Bethesda, Maryland, USA
9 Institut de Pharmacologie et de Biologie Structurale, Université de Toulouse, UPS, Toulouse, France
10 Institut National de la Santé et de la Recherche Médicale, INSERM U1048, Université Paul Sabatier, Toulouse, France
11 Institut National de la Santé et de la Recherche Médicale, INSERM U938, Molecular and Clinical Oncology, Université Paris VI Pierre et Marie Curie, Paris, France
Financial support: this research was supported by Fund for Scientific Spearheads of Ghent University Hospital and Research Council of Ghent University, the National Cancer Plan (KPC_29_012), a grant from the ‘Bijzonder Onderzoeksfonds’ (BOF) of Ghent University (to HD), a postdoctoral grant (AH) and a travel grant (LL) from Fund for Scientific Research-Flanders. The study sponsors have no role in the design of the study; the collection, analysis and interpretation of the data; the writing of the manuscript; or the decision to submit the manuscript for publication.

Conflict of interest: the authors disclose no potential conflicts of interest.

Corresponding Author:

Olivier De Wever, Laboratory of Experimental Cancer Research, Department of Radiation Oncology and Experimental Cancer Research, Ghent University Hospital, De Pintelaan 185, 9000 Ghent, Belgium. Phone: +32 9 332 30 73; Fax: +32 9 332 49 91; Email: olivier.deweever@ugent.be
ABSTRACT

Increasing evidence supports the critical roles played by adipose tissue in breast cancer progression. Yet, the mediators and mechanisms are poorly understood. Here, we show that breast cancer-associated adipose tissue from freshly isolated tumors promotes F-actin remodelling, cellular scattering, invasiveness and spheroid reorganization of cultured breast cancer cells. A combination of techniques including transcriptomics, proteomics and kinomics enabled us to identify paracrine secretion of oncostatin M (OSM) by cancer-associated adipose tissue. Specifically, OSM, expressed by CD45+ leucocytes in the stromal vascular fraction, induced phosphorylation of STAT3 (pSTAT3-Y705 and S727 in breast cancer cells and transcription of several STAT3-dependent genes, including S100 family members S100A7, S100A8 and S100A9. Autocrine activation of STAT3 in MCF-7 cells ectopically expressing OSM induced cellular scattering and peritumoral neo-vascularization of orthotopic xenografts. Conversely, selective inhibition of OSM by neutralizing antibody and Jak family kinases by tofacitinib inhibited STAT3 signaling, peritumoral angiogenesis and cellular scattering. Importantly, nuclear staining of pSTAT3-Y705 identified at the tumor invasion front in ductal breast carcinomas correlates with increased lymphovascular invasion. Our work reveals the potential of novel therapeutic strategies targeting the OSM and STAT3 axis in breast cancer patients harboring nuclear pSTAT3-Y705.
INTRODUCTION

Cancer progression is the result of a complex interaction between the cancer cells and their microenvironment (1, 2). Breast tumors are surrounded by type I collagen-rich tissue including fibroblasts, blood and lymph vessels and adipose tissue. Insights in the function of adipose tissue have shifted from a static organ for energy storage to a dynamic one, excreting growth factors, cytokines and hormones, identifying the adipose tissue as an active player in the communication between the tumor and its microenvironment (3).

Human adipose tissue-derived adipocytes, stem cells, stromal cells, CD34+ progenitor cells and macrophages stimulate growth, migration and invasion of breast cancer cells by secretion of CCL5 (Rantes), interleukin (IL)-6, IL-8 and PAI-1 (4-7). A mouse mammary cancer model revealed tumor progression through secretion of adipocyte-derived type VI collagen (8). Dirat et al. showed that mature adipocytes promote the invasiveness of estrogen receptor α (ERα) positive and negative breast cancer cells (9). IL-6-mediated induction of epithelial-to-mesenchymal transition (EMT) was identified as key component of adipocyte-enhanced invasiveness of breast cancer cells (9). Zhang et al. reported stimulation of tumor growth and angiogenesis by recruitment of adipose stromal cells and endothelial cells in a breast cancer mouse model (10). All together, the interaction between breast cancer cells and associated adipose tissue is a multifactorial phenomenon driving breast cancer progression.

Signal transducers and activators of transcription (STATs) are important in cytokine receptor signaling. Several STATs contribute to normal mammary gland development and cellular response during pregnancy, lactation and involution. However, recent studies revealed their
seemingly contradictory participation in breast cancer progression (11). STAT3 is frequently activated in human breast cancer and correlates with poor prognosis (12).
MATERIALS AND METHODS

Cell Lines and Transfections

MCF-7, T47D, SKBR3 and MDA MB 231 cell lines were obtained from American Type Culture Collection (ATCC, Manassas, VA) (http://www.lgcstandards-atcc.org). Cells were maintained in Dulbecco’s minimal essential medium (DMEM) supplemented with 10% fetal calf serum, 100 U/mL penicillin, 100 μg/mL streptomycin (Invitrogen, Carlsbad, CA) and 2.5 μg/mL fungizone (Bristol-Meyers Squibb, Brussels, Belgium). All cell lines have been validated in-house by short tandem repeat profiling using the Cell ID™ System (Promega, USA) according to the manufacturer’s instructions. Upon receipt, cells were passaged and stored in liquid nitrogen. Every six months, a new aliquot of cells was resuscitated and used for experimentation. Every month cell cultures were tested for Mycoplasma contamination by using MycoAlert Plus kit (Lonza, Verviers, Belgium).

MCF-7 cells secreting turbo green fluorescent protein (tGFP)-OSM fusion protein (MCF-7-OSM) were generated by Fugene Transfection (Promega, Madison, WI). tGFP-OSM cDNA was purchased at Origene, Inc (Rockville, MD).

Antibodies and Reagents

Primary and secondary antibodies are described in Supplementary Materials and Methods. Recombinant human (rh)OSM, (rh)IL-6, (rh)IL-8, (rh)LIF, (rh)G-CSF and their respective neutralizing monoclonal antibodies were from R&D systems (Minneapolis, MN). Tofacitinib citrate was purchased at Bio-Connect Diagnostics (Huissen, The Netherlands). A neutralizing (n)OSM antibody, Akt-inhibitor (GSK2142795) and MEK-inhibitor (GSK1120212 or Trametinib) were kindly provided by GlaxoSmithKline (Brentford, UK) (13).
Conditioned Medium of Cancer Associated Adipose Tissue (CM\textsuperscript{CAAT}), isolation of Tumor Associated Adipocytes (TAA) and Stromal Vascular Fraction (SVF)

CAAT was obtained from breast cancer patients undergoing a mastectomy at Ghent University Hospital in accordance with the local ethics committee (Supplementary Table S1). Preparation of CM\textsuperscript{CAAT} and separation of adipose tissue in TAA and SVF and isolation of CD45+ and CD31+ fractions from SVF are described in Supplementary Materials and Methods. Supplementary Figure S1 demonstrates the use of CM\textsuperscript{CAAT} throughout the paper.

Functional assays

Experimental set-up for studying morphological changes induced by CAAT or CM\textsuperscript{CAAT} are described in Supplementary Materials and Methods. Factor shape was calculated as \((\text{perimeter}^2)/(4 \times \pi \times \text{area})\) for quantification.

MCF-7, T47D and MDA MB 231 type I collagen gel invasion assays are performed and quantified as described in Supplementary Materials and Methods and (14).

Matrigel invasion of MDA MB 231 and migration of MCF-7 and SKBR3 cells were performed using xCELLigence RTCA DP instrument (ACEA biosciences, San Diego, CA) (15).

Protein Analysis

Samples for western blot were prepared, runned and immunostained as described in (16).

Human phospho-kinase antibody array (R&D systems) was used to detect relative phosphorylation levels of 44 kinases. OSM concentrations in CM\textsuperscript{CAAT} were measured with a human OSM ELISA Kit (Sigma-Aldrich). Scanning densitometry was carried out with the Quantity One Program (Bio-Rad, Hercules, CA).
**Microarray Analysis**

Total RNA was isolated using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany) including DNAse I treatment. Quality control was performed using Agilent 2100 Bioanalyser (Agilent Technologies). Total RNA (0.5 μg) was processed and analysed on Human GE Agilent 4x44K microarrays. Four biological samples were studied. Data can be found on GEO (GEO accession number GSE58574).

**Quantitative Real-Time PCR (RT-qPCR)**

RNA was isolated using RNeasy Plus Universal Kit (Qiagen, Venlo, The Netherlands) including DNAse I treatment. cDNA synthesis and SYBR Green I RT-qPCR were carried out as described in (17). Prime PCR assays for S100A7, S100A12, OSM, NTN4, LRG1, LIFR, GP130 were purchased at Bio-Rad. Other primer sequences are described in Supplementary Table S2. RNA quality index (RQI >8) was assessed using Experion software (version 3.2, Bio-Rad).

**Mass Spectrometry**

CMCAAT samples were run on NuPAGE 4%-20% Bis-Tris gradient gels (Invitrogen) in denaturating SDS buffer, stained with 0.5% Coomassie Brilliant Blue (Bio-Rad) in 40% methanol and 10% acetic acid for 20 minutes, and destained. Gel bands were processed and analyzed by liquid chromatography-mass spectrometry/mass spectrometry as described in Supplementary Materials and Methods and (16).

**Animal Studies**

Animal studies were approved by the Local Ethics Committee of Ghent University Hospital (ECD 09/32). Immunodeficient mice were orthotopically injected with 1x10^6 cancer cells. After 4
weeks, the mice were sacrificed and tumors were resected for immunohistochemistry. Details can be found in Supplementary Materials and Methods.

**Patient Samples and IHC**

Clinical data and paraffin embedded primary breast carcinoma samples were collected at Ghent University Hospital (Supplementary Table S3). Written informed consent was obtained according to the recommendations of the local ethics committee. To evaluate nuclear pSTAT3-Y705, we considered an intensity score that was semi-quantitative scaled as score 0, weak or absent nuclear staining; score 1, between 5 and 30% nuclear staining; score 2, more than 30% nuclear staining. Three observers quantified independently.

**Statistics**

Statistical analyses were performed using IBM SPSS Statistics 21.0 software (Chicago, IL). Continuous data were analysed with Mann-Whitney test (mean ± standard deviation) or Student $t$ test (difference of means and 95% confidence interval) where appropriate. Spearman’s correlation was used to assess correlations. Categorical data were analyzed with Fisher’s Exact test. All data are representative of at least three independent experiments. Statistical tests were two-sided, $P$ values less than .05 were considered statistically significant.
RESULTS

The role of CAAT in morphological reorganization and invasion of breast cancer cells

CAAT was confronted with MCF-7 aggregates in type I collagen, the main structural component of the mammary gland. CAAT induced aggregate reorganization resulting in infiltration of CAAT by MCF-7, engulfing single adipocytes (Figure 1A). In contrast, MCF-7 aggregates not confronted with CAAT maintained a round shape up to 14 days of culture. The mean factor shape of CAAT-confronted MCF-7 aggregates was 4-fold that of controls, (control vs. CAAT; 1.290 ± 0.095 vs. 4.017, ± 0.603; \( P = .004 \); Figure 1A). We next questioned whether CMCAAT could mimick the effects induced by direct co-culture. Within 48h, 25 of 27 CMCAAT (93%) induced cellular extensions and reduced cell-cell contacts in MCF-7 (Figure 1B). Rescue experiments showed restoration of cobblestone shaped morphology and tight cell-cell contacts. F-actin staining of single cells revealed a rounded appearance for control cells and an elongated morphology with multiple protrusions upon CMCAAT treatment (Figure 1C). The mean factor shape of CMCAAT-treated MCF-7 cells was 2.5-fold higher than in controls (control vs. CMCAAT; 1.221, ± 0.123 vs. 4.070, ± 1.652; \( P = .002 \); Figure 1C). EMT-markers showed no major expression changes in control versus CMCAAT-treatment (Supplementary Figure S2). Confocal immunocytochemistry revealed a reorganized F-actin cytoskeleton and relocation of E-cadherin and \( \beta \)-catenin from the plasma membrane to the cytoplasm (Supplementary Figure S2). CMCAAT stimulated invasion of MCF-7 into type I collagen after 24h (control vs. CMCAAT; 9.750%, ± 2.790 vs. 46.750%, ± 2.408; \( P = .001 \), Figure 1D) and increased migration of MCF-7 with a 14-fold slope increment (\( P = .021 \), Figure 1E).
Similar morphological changes (Figure 1F) and stimulation of type I collagen invasion was seen upon CMCAAT treatment of T47D (control vs. CMCAAT; 9.340%, ± 3.872 vs. 33.880%, ± 5.065; \( P = .008 \), Figure 1G).

While induction of scattering is hard to assess in MDA MB 231 given their mesenchymal phenotype (Figure 1H), CMCAAT increased type I collagen invasion of MDA MB 231 in a 14 days assay (Figure 1I, upper panel). In addition, CMCAAT stimulated Matrigel invasion of MDA MB 231 with a 4.5-fold slope increment (\( P = .002 \), Figure 1I, lower panel).
**Paracrine activation of STAT3 by CAAT in breast cancer cells**

Phospho-kinase screening in CM\textsuperscript{CAAT}-treated MCF-7 cells revealed higher phosphorylation levels of STAT3 (Y705), ERK, JNK, AKT and CREB (Figure 2A).

Since pSTAT3-Y705 showed the highest relative increase, we explored CM\textsuperscript{CAAT}-stimulated pSTAT3-Y705 and -S727. Short-term exposure of MCF-7 to CM\textsuperscript{CAAT} increased pSTAT3-Y705 while long-term CM\textsuperscript{CAAT} exposure is necessary for pSTAT3-S727 (Figure 2B).

A rescue experiment decreased pSTAT3-Y705 and –S727 (Figure 2B). Increased pSTAT3-Y705 was confirmed in MCF-7 incubated with 9 out of 9 CM\textsuperscript{CAAT}. In agreement, confrontation of MCF-7 with CM\textsuperscript{CAAT} revealed strong nuclear pSTAT3-Y705 compared to control (Figure 2C).

Since activated STAT3 is a transcriptional regulator, we performed transcriptomics of MCF-7 cells under control conditions or with CM\textsuperscript{CAAT} prepared from two breast cancer patients. Using expression value cutoff of 5-fold, 67 up-regulated genes and 112 down-regulated genes were identified and assigned to clusters involving migration, inflammatory response, secretion and angiogenesis (Figure 2D). A proportion of these genes were associated with increased STAT3 transcriptional targets such as the most up-regulated genes S100A7 (70-fold), S100A8 (40-fold), and S100A9 (23-fold) (18). Down-regulated genes belonged to the connective tissue growth factor family (19) such as CTGF (40-fold), Nov (13-fold), CYR61 (6-fold), SERPINA1 (20), SERPINA3 and IGFBP5.

Differential expression of STAT3 transcriptional targets was validated through RT-qPCR (Con vs. CM\textsuperscript{CAAT}, \( P = .004 \) for all reported genes, except SERPINA1 \( P = .002 \), Figure 2E). Western blot confirmed increased expression of S100A7, S100A8 and S100A9 in CM\textsuperscript{CAAT}-treated MCF-7.
cells (Figure 2F). A rescue experiment showed restoration of S100A8 and S100A9 levels to basal conditions, while S100A7 levels were reduced by 32.5%.
Expression and secretion of Oncostatin M by CAAT

Using a biotin label-based assay, CM\textsuperscript{CAAT} from 2 patients revealed the presence of six proteins with a reported capacity to activate STAT3 signaling: OSM, IL-6, IL-8, G-CSF, LIF and leptin (Supplementary Figure S3). Addition of rhOSM dose-dependently increased Y705 and S727 pSTAT3 in MCF-7 cells. In contrast, only high concentrations of rhIL-6 and rhG-CSF phosphorylated STAT3 Y705 but not STAT3 S727 (Figure 3A), suggesting a reduced transcriptional activator capacity (as compared to rhOSM or CM\textsuperscript{CAAT}). Moreover, only rhOSM up-regulated S100A7 protein levels (Figure 3A). rhIL-6, rhIL-8 and rhLIF had no effect on morphology and addition of their neutralizing antibodies to CM\textsuperscript{CAAT} did not counter CM\textsuperscript{CAAT} induced morphological changes (Supplementary Figure S3). Only neutralizing (n)IL-6 antibody partially inhibited CM\textsuperscript{CAAT} induced phosphorylation of STAT3 Y705 but had no effect on S727 phosphorylation (Supplementary Figure S3). rhLeptin was not tested since it lacked the capacity to induce cellular scattering (21).

Mass spectrometry of CM\textsuperscript{CAAT} revealed 10 unique OSM peptides at the expected 25-30 kDa MW with 55% sequence coverage (Figure 3B). Western Blot identified OSM protein in CM\textsuperscript{CAAT} from 3 patients. ELISA of CM\textsuperscript{CAAT} from 16 patients revealed an OSM concentration between 3.7 and 15.7 pg/ml (Figure 3C). There was no correlation between the OSM concentration in CM\textsuperscript{CAAT} and BMI of these patients ($P = .948$, Spearman’s Rho = -0.115).

To determine the source of OSM production, tumor associated adipose tissue from 10 breast cancer patients was separated into tumor associated adipocytes (TAA) and the stromal vascular fraction (SVF). RT-qPCR revealed a 20-fold increased expression of OSM mRNA in the stromal
vascular fraction compared to adipocytes \((P < .001)\). OSM mRNA levels in TAA and SVF were not correlated with the BMI of the patients (OSM in TAA, \(P = .650\), Spearman’s Rho = 0.164; OSM in SVF, \(P = .235\), Spearman’s Rho = 0.413). OSM protein was found in the CD45+ leucocyte fraction and not in the CD31+ endothelial fraction or ‘Rest’ fraction containing CD34+/CD31- adipocyte progenitor cells (Figure 3D) (22).

Macrophages are CD45+ and an increased presence of tumor-associated macrophages has been associated with poor prognosis in human breast cancer. CD163 staining revealed an accumulation of tumor-associated macrophages in the adipose tissue at sites of cancer cells with high nuclear pSTAT3-Y705 infiltrating the adipose tissue (Figure 3E).

OSM engages heterodimeric receptors involving gp130 and either the OSM receptor (OSMR) or the leukemia inhibitory factor receptor (LIFR). Gp130/OSMR complex is specifically activated by OSM and is implicated in morphological changes (23). RT-qPCR revealed the relative expression of OSMR in MCF-7, T47D and MDA MB 231 while SKBR3 show a 60.000-fold lower OSMR expression (Figure 3F). rhOSM treatment of these cell lines induces morphological changes in all except SKBR3 (Figure 3G). Moreover, migration of MCF-7 and not SKBR3 is dose-dependently stimulated by rhOSM with a 5-fold (100 pg/ml, \(P = .071\)) and 9.9-fold (1 ng/ml, \(P = .001\)) slope increment in MCF-7 compared to 1.4-fold (100 pg/ml, \(P = .941\)) and 1.6-fold (1 ng/ml, \(P = .422\)) slope increment in SKBR3 (Figure 3H). rhOSM treatment of MCF-7 leads to an increase of OSMR (2.7-fold) and STAT3 (2.2-fold) expression (\(P = .029\) for both genes), suggesting positive feedback. The OSMR and STAT3 mRNA response is not observed in SKBR3. In both cell lines, LIFR and gp130 mRNA levels are not affected by rhOSM (Supplementary Figure S4).
Functional role of OSM and STAT3 signaling in CAAT-mediated morphological changes

To quantify CM<sup>CAAT</sup> potency on STAT3 phosphorylation, MCF-7 cells were treated with rhOSM. pSTAT3-Y705 was significant at 0.01ng/ml rhOSM, with a STAT3 phosphorylation capacity of CM<sup>CAAT</sup> equivalent to +/- 0.5ng/ml rhOSM (Figure 4A). rhOSM induced a gene signature (Con vs. rhOSM, \( P = .029 \) for all reported genes except IGFBP5 with \( P = .200 \), Figure 4B) and morphological changes (Figure 3G) similar to CM<sup>CAAT</sup> treatment. Preincubation of rhOSM with nOSM antibody or addition of the pan-Jak inhibitor tofacitinib abolished STAT3 phosphorylation (Figure 4C). Preincubation of CM<sup>CAAT</sup> with nOSM antibody reversed STAT3 activation (Figure 4C, lower left panel) and morphological responses (Figure 4D). Tofacitinib blocked CM<sup>CAAT</sup>-induced STAT3 activation and S100A7 expression (Figure 4C), morphological responses (Figure 4D) and nuclear localisation (Figure 4E). The AKT-pathway (inhibited by GSK2141795) but not the MEK/ERK-pathway (inhibited by trametinib) is necessary for pSTAT3-S727 and transcriptional activity (Supplementary Figure S5).

We established MCF-7 cells that ectopically secreted OSM (MCF-7-OSM) to examine the impact of constitutive OSM secretion and signalling in BC progression. MCF-7-OSM cells have a decreased proliferation rate, lost the ability to form aggregates and show an increased expression of OSMR compared to control MCF-7-GFP cells (Supplementary Figure S6). OSM in the secretome coincided with constitutive pSTAT3-Y705 and increased expression of S100A7 (Figure 5A). Tofacitinib reduced pSTAT3-Y705 and S100A7 expression (Figure 5A).
Histology revealed MCF-7-GFP cells organized into clusters with a compact pattern separated by Matrigel, whereas MCF-7-OSM xenografts displayed disorganized strands and single cells (Figure 5B). Quantification of cellular organization by calculating factor shape indicated a statistically significant deviation (GFP vs. OSM, 1.4 ± 0.09 vs. 4 ± 1.4, difference = 2.6, 95% CI = 1.24 to 3.95, \( P = .0016 \)). Pan-cytokeratin and vimentin showed no differential expression between MCF-7-GFP and MCF-7-OSM tumors. However, E-cadherin membrane expression and ER\(\alpha\) expression was reduced in OSM-secreting tumors compared to control (Supplementary Figure S2). Staining of pSTAT3-Y705 showed that 3.3% of MCF-7-GFP is positive compared with 69.8% of MCF-7-OSM cells (difference = 66.5%, 95% CI = 59% to 73%, \( P < .0001 \)) (Figure 5B).

All MCF-7-GFP xenografts had poor peritumoral vascularization (6 out of 6) while MCF-7-OSM tumors developed strong peritumoral vascularization (11 out of 12) as evidenced by macroscopic evaluation (Figure 5B) and contrast-enhanced micro-computed tomography (\( \mu \)CT) (Figure 5B). Microarray data revealed the respectively 11-fold and 7-fold upregulation of angiogenic factors NTN4 (24) and LRG1 (25) in CM\(^{CAAT}\)-treated MCF-7 cells. rhOSM and ectopic expression of OSM mimicked CM\(^{CAAT}\)-induced effects on NTN4 and LRG1 (CON vs CM\(^{CAAT}\), \( P < .0001 \); CON vs rhOSM and CON vs OSM, \( P = .004 \)) (Figure 5C).

Treatment with nOSM antibody alleviated OSM-induced peritumoral angiogenesis as demonstrated by reduced number of mice (1 out of 6) showing peritumoral blood vessels and blocks OSM-induced pSTAT3-Y705 (Figure 5B), with 21.1% of MCF-7-OSM cells showing a positive nuclear signal (difference compared to untreated = 47.9%, 95% CI = 38% to 57%, \( P < .0001 \)). Administration of tofacitinib prevented OSM-induced peritumoral angiogenesis in 4 out of...
of 6 mice and reduced pSTAT3-Y705 (Figure 5B) with 20% of MCF-7-OSM cells showing nuclear STAT3 (difference compared to untreated = 49%, 95% CI = 42% to 56%, \( P < .0001 \)). Both the nOSM antibody and tofacitinib restored MCF-7 cluster organization (Figure 5B). The mean factor shape of cell clusters from MCF-7-OSM tumors treated with nOSM antibody and tofacitinib was respectively 1.78 ± 0.44 and 1.62 ± 0.26 indicating compacted organization (difference nOSM antibody vs. untreated = 2.2, 95% CI = 0.8 to 3.63, \( P = .0058 \); difference tofacitinib vs. untreated = 2.3, 95% CI = 1.00 to 3.75, \( P = .0032 \)).
Nuclear expression of pSTAT3-Y705 in invasive ductal breast cancer

We studied the expression of nuclear pSTAT3-Y705 by immunohistochemistry in 50 ER-positive IDCA patients with histologically confirmed adipose tissue infiltration. Nuclear pSTAT3-Y705 staining was present at sites of adipose tissue infiltration in 18% of the samples (9/50). Interestingly, pSTAT3-Y705 positive samples showed a statistically significant association with lymphovascular invasion (LVI) ($P = .0032$, Figure 6). LVI is a poor prognostic factor in patients with lymph node-negative IDCA (26).
DISCUSSION

Adipose tissue is an endocrine organ producing effectors with local and systemic actions. Tumor infiltration into the adjacent fat is a risk factor for breast cancer progression (27). We investigated the impact of CAAT in breast cancer infiltration and progression. OSM is a CAAT-secreted factor activating STAT3 and inducing pro-inflammatory genes, cellular scattering and peritumoral angiogenesis (Figure 7).

While adipose tissue has been shown to secrete a number of cytokines potentially affecting breast cancer cells, our studies demonstrate that OSM is the most relevant factor stimulating cancer progression. First, in comparison with IL-6, IL-8 and G-CSF, OSM is the only inducer of cellular scattering and the most potent inducer of pSTAT3-Y705. Secondly, pSTAT3-S727, important for transcription, was only seen upon OSM stimulation. Thirdly, only OSM stimulates S100A7 expression described as poor prognosis marker in breast cancer (28). Fourthly, two distinct OSM-neutralizing antibodies (R&D systems and GSK) abolish CMCAAT-induced morphological changes and STAT3 activation. Fifthly, besides STAT3, CMCAAT activates downstream intermediates of OSM such as AKT, JNK, ERK and CREB (23, 29) and AKT activation is necessary for pSTAT3-S727. Sixthly, nIL-6, nIL-8, and nLIF antibodies do not affect CMCAAT-induced scattering. Seventhly, nIL-6 antibody partly inhibits CMCAAT-induced pSTAT3-Y705 but not pSTAT3-S727. nIL-8 and nLIF antibodies do not affect CMCAAT-induced pSTAT3-Y705. Finally, CMCAAT induces OSMR and STAT3 in a similar fashion as rhOSM. These findings are in agreement with Xiao et al. showing a positive feedback loop for further amplification of OSM-induced signaling (30).
OSM and CM\textsuperscript{CAAT} increase expression of S100 proteins. A rescue experiment showed a relative higher S100A7 protein level compared to S100A8/9 after rescue procedure. All studied S100 proteins have an intracellular and secreted pool, are associated with the actin cytoskeleton and contribute to increased scattering and migration. S100A8/9 proteins are uniquely reported as pro-migratory. However, S100A7 has a described pro- and anti-migratory function. This effect might be concentration dependent. Higher levels of S100A7 may stimulate migration; slightly lower levels support the inhibition of lamellipodia formation and restore the non-migratory state (31).

OSM and CM\textsuperscript{CAAT} shift E-cadherin membrane localization to the cytoplasm but have no impact on vimentin, fibronectin or cytokeratin expression. Reduction of cell surface E-cadherin expression is a prerequisite for cellular scattering (32) and a marker of EMT. In contrast to Guo et al. and West et al. (33, 34), we do not observe a full EMT in our set-up. Although all studies used breast cancer cells, both West et al. and Guo et al. used higher OSM concentrations (100ng/ml) and longer treatments that stimulate fibronectin, snail and vimentin expression (33). In accordance with West et al., withdrawal of OSM restores the epithelial phenotype, indicating that OSM does not induce permanent phenotypic changes. Concentrations of OSM provided at the adipose invasion front, a 1.000 to 10.000-fold lower compared to West et al. and Guo et al., were capable of activating STAT3 and increasing adipose tissue infiltration by reduced cell surface E-cadherin.

While OSM has been shown to inhibit cellular proliferation of MCF-7 and T47D (35, 36) and cell culture experiments with MCF-7 constitutively secreting OSM show a reduced growth compared to control transfected cells, our xenograft experiments revealed similar tumor sizes between control and OSM groups, most probably as a result of enhanced peritumoral angiogenesis. OSM may directly stimulate angiogenesis (37) and stimulates the secretion of VEGF by T47D cells.
(38). We discovered the stimulatory role of OSM on the expression of NTN4 and LRG1 genes encoding the pro-angiogenic factors netrin-4 and LRG1. OSM stimulates expression and secretion of S100 proteins which promote angiogenesis by stimulating endothelial cell proliferation (31). Whether in the xenograft model, the increased presence of blood vessels is caused directly by OSM or indirectly by OSM-induced NTN4, LRG1, S100 proteins or a combination of them, is not known.

OSM mRNA is high in the SVF and immune-isolation of CD45+ leucocytes revealed the presence of OSM protein compared to the endothelial and pre-adipocyte/fibroblast fraction. A study using subcutaneous adipose tissue supports our findings showing that mature adipocytes are not the main source of OSM but derives from cells in SVF including macrophages (39). CAAT is infiltrated by CD163+ macrophages and accumulate at sites of high pSTAT3-Y705 in cancer cells. CD163-positive macrophages are defined as tumor-associated macrophages correlated with poor prognosis in breast cancer (40) potentially by contribution of pro-inflammatory cytokines stimulating invasion (9). Mature adipocytes express the OSMR and OSM provided by infiltrated macrophages may contribute to dedifferentiation of mature adipocytes (41).

The underlying mechanisms linking obesity to breast cancer risk and progression are not yet fully understood. Obesity is involved in STAT3 activation by at least two mechanisms. First, high plasma levels of leptin, correlated with obesity, are mainly implicated in breast tumorigenesis and growth (42). Second, obesity is associated with a chronic state of low-grade inflammation, attracting macrophages and lymphocytes producing STAT3-activating cytokines (43, 44). Our data show no clear contribution of obesity mediated through CMCAAT. First, CMCAAT from 9
patients showed similar STAT3 activation, regardless of their BMI (5 normal, 3 overweight, 1 obese). Second, CM\textsuperscript{CAAT} from 25 patients with a BMI ranging from 19.1 to 41 (2 underweight, 10 normal, 9 overweight, 4 obese) induced cellular scattering. Third, from the 9 IDCA samples with high pSTAT3-Y705 staining only one was an obese patient (6 normal, 2 overweight, 1 obese). Fourth, OSM mRNA levels in SVF from 10 breast cancer patients were not correlated with BMI. Fifth, we found no correlation between OSM-concentration in CM\textsuperscript{CAAT} from 16 patients and their respective BMI. Admittedly, the number of patient samples in our study is low. Therefore, further studies using large series of breast CAAT samples from age-matched patients, including more detailed fat deposit information and metabolic biomarkers will yield more information whether OSM secretion by activated macrophages and pSTAT3-Y705 in infiltrating cancer cells is associated with obesity. Alternatively, recruitment of inflammatory cells is a consistent feature of the local tumor environment and an enabling characteristic of cancer progression (45). In agreement with Queen et al., local production of chemokines by cancer cells may recruit inflammatory cells at the adipose tissue invasion front leading to increased secretion of OSM (38).

Nuclear pSTAT3-Y705 at sites of adipose tissue infiltration revealed an association with lymphovascular invasion, a poor prognosis marker in breast cancer. Although all investigated CAAT and SVF samples expressed OSM, only one-fifth of ER-positive breast cancer patients showed pSTAT3-Y705 at invasion front. This apparent contradiction may be explained by differential expression of OSMR in breast cancer cells. Conflicting data exist about activated STAT3 as a marker for prognosis. One explanation is the use of tissue micro-array cores representing the bulk of the tumor, not allowing to discriminate cancer cells at the invasion front (46, 47). We used whole tissue slides allowing examination of cancer cell-CAAT interactions.
Our data are endorsed by recent findings underlining the link between pSTAT3 at the invasion front and tumor progression (48). Secondly, primary breast tumors displaying tyrosine phosphorylation of STAT3 and STAT5 are more differentiated and display more favourable prognostic characteristics than those with selective STAT3 activation. Indeed, both STAT’s mediate opposing effects on several key target genes, with STAT5 exerting a dominant role (49). In accordance, we observed a 2.3-fold higher STAT3 tyrosine phosphorylation compared to STAT5 tyrosine phosphorylation by CMCAAT (data not shown).

The OSM/STAT3 loop may have therapeutic potential in breast cancer. CMCAAT activates STAT3 through Jak family kinases as evidenced by tofacitinib. Tofacitinib has received FDA-approval as treatment of rheumatoid arthritis. Here, STAT3 plays a critical role in survival and proliferation of synoviocytes, the main component of the pannus that invades bone (50). Tofacitinib has been suggested for patients with natural killer T-cell lymphoma harboring constitutive Jak mutations (51). OSM neutralizing antibodies have been used in multiple xenograft models (52, 53). We used a monoclonal OSM neutralizing antibody that efficiently blocks OSM-induced peritumoral angiogenesis. Further studies should indicate the potential impact of OSM and Jak targeting in breast cancer.

This is the first report demonstrating a paracrine OSM/STAT3 activation loop at the level of adipose tissue versus epithelial interactions in ER-positive breast cancer.
AUTHOR CONTRIBUTIONS

LL and ODW: concept, design, acquisition of data, interpretation of data, drafting of the manuscript, statistics, approval of the final version; all others: acquisition of data, interpretation of data, statistics, revision of the manuscript, material and technical support, approval of the final version.

ACKNOWLEDGEMENTS

We thank G. De Bruyne, M. De Meulemeester, S. Decloedt, K. Van Wesemael, E. Wauters, S. De Geyter and S. Jeurissen for excellent technical assistance. M. Mareel is gratefully appreciated for critical revision of the manuscript.
REFERENCES:


FIGURE LEGENDS

**Figure 1:** CAAT stimulates invasivion. (A) H&E staining of MCF-7 spheroids cultured in CAAT or collagen and quantification by factor shape. (B) Phase-contrast images of MCF-7 cells treated with control medium or CM<br>CAAT for 48h. Afterwards, CM<br>CAAT was replaced by control medium for 48h (rescue). Images represent 1 of 25 experiments. (C) Confocal images of F-actin stained control or CM<br>CAAT-treated MCF-7 cells (left panel, scale bar is 15μm) and quantification of seven representative cells for each condition by factor shape (right panel). Data represent 1 of 3 experiments. (D) Phase-contrast images of MCF-7 cells on type I collagen gel, arrows indicate invasive extensions (left panel) and quantification (right panel). The data represent 1 of 4 experiments. (E) Graph representing migration of MCF-7 cells and quantification by calculating the slope increment of three replicates for each condition. Graph represents 1 of 3 experiments. Asterix indicates $P = .021$. (F) Phase-contrast images of control or CM<br>CAAT-treated T47D cells. Images represent 1 of 5 experiments. (G) T47D cells on type I collagen gel, arrows indicate invasive extensions (left panel) and quantification (right panel). The data represent 1 of 3 experiments. (H) Phase-contrast images of MDA MB 231 cells treated with control medium or CM<br>CAAT for 48h. Images represent 1 of 4 experiments. (I) H&E staining of MDA MB 231 transwell collagen invasion assay. Double head arrows indicate distance between front of infiltrating cells and bottom of collagen gel. Single head arrow depicts single cells reaching bottom of collagen gel (upper panel). Graph representing Matrigel invasion of MDA MB 231 cells and quantification by calculating the slope increment of three replicates for each condition. Asterix indicates $P = .002$ (lower panel). The data represent 1 of 3 experiments for each panel.
**Figure 2:** CAAT secreted factors activate STAT3 signaling. (A) Phospho kinase array demonstrating the relative phosphorylation levels of 44 kinases on CM\(^{CAAT}\)-treated MCF-7 cells (upper panel, only the upper part of the test with 28 kinases is shown) and measurement of the fold change (lower panel). (B) Western blot of pSTAT3-Y705 and –S727 in CM\(^{CAAT}\) treated MCF-7 cells. Duration of treatment is indicated: 5’ (five minutes), 10’ (ten minutes), 48h (two days). Rescue means 48h treatment with CM\(^{CAAT}\), followed by 48h with control medium. Total STAT3 and tubulin are loading controls. (C) pSTAT3-Y705 staining of paraffin-embedded MCF-7 pellets treated as indicated (scale bar is 50μm). (D) Pie chart for the distribution of at least 5-fold up- or downregulated genes in CM\(^{CAAT}\)-treated MCF-7 cells in comparison with control-treated MCF-7 cells according to their functional category (DAVID database). (E) Relative mRNA levels of indicated genes in MCF-7 cells treated for 48h with control medium or CM\(^{CAAT}\). (F) Western blot of S100 proteins in MCF-7 cells treated for 48h with control medium, CM\(^{CAAT}\) or after rescue.

**Figure 3:** CAAT-derived OSM phosphorylates Y705 and S727 STAT3, upregulates S100A7 and stimulates migration through OSM receptor. (A) Western blot of pSTAT3-Y705 and -S727 in MCF-7 cells treated with rhOSM, IL-6, IL-8 and G-CSF (upper panel). Western blot of S100A7 in MCF-7 cells treated with rhOSM, IL-6 or G-CSF (lower panel). (B) OSM peptides and peptide hits identified by CM\(^{CAAT}\) mass spectrometry. (C) Western blot of OSM in CM\(^{CAAT}\) of three patients (P26, P29 and P33). rhOSM (20ng) serves as positive control (upper panel). Scatter plot representing OSM concentration in CM\(^{CAAT}\) from 16 patients measured by ELISA (lower panel). (D) Scatter plot representing relative OSM mRNA levels in TAA and SVF of CAAT from 10 breast cancer patients (upper panel). Western blot of CD45 and OSM in total SVF and in isolated CD45+, CD31+ and ‘Rest’ fractions of SVF. (E) pSTAT3-Y705 staining of breast cancer cells...
and CD163 staining of activated macrophages (scale bar is 300μm for upper panel and 50μm for lower panel). (F) Relative mRNA levels of OSMR expression in 4 breast cancer cell lines. (G) Phase-contrast images of 4 different breast cancer cells treated with control medium or rhOSM (1ng/ml) for 48h. (H) Graphs representing migration of MCF-7 and SKBR3 cells treated with control medium or rhOSM at indicated concentrations using xCELLigence migration assay and quantified by calculating the slope increment of 5 replicates of each condition. Asterixes indicate p-value (MCF-7: one asterix is $P = .071$, two asterixes is $P = .001$; SKBR3: one asterix is $P = .941$, two asterixes is $P = .422$) After the experiment, membranes of control and rhOSM 1ng/ml were stained with crystal violet indicating migrated cells.

**Figure 4:** Tofacitinib and nOSM antibody inhibit rhOSM and CMCAAT induced scattering and STAT3 activation. (A) Western blot of pSTAT3-Y705 and -S727 in MCF-7 cells treated with a range of rhOSM or CMCAAT from three patients (P36, P40 and P44). pSTAT3-Y705 and -S727 by P36, P40 and P44 equals approximately 0.64, 0.86 and 0.90 ng/ml rhOSM respectively. (B) Relative mRNA levels of indicated genes in MCF-7 cells treated for 48h with rhOSM (2ng/ml). (C) Western blot of pSTAT3-Y705 and -S727 in MCF-7 cells treated for 48h with rhOSM (5ng/ml) (upper panels) or CMCAAT (lower panels), combined with a range of a nOSM antibody (left panels) or tofacitinib (right panels). (D) Phase-contrast images of MCF-7 cells treated for 48h with control medium, CMCAAT or CMCAAT combined with nOSM antibody (from R&D) or tofacitinib at the indicated concentrations. (E) pSTAT3-Y705 staining of paraffin-embedded MCF-7 cell pellet treated for 48h with CMCAAT or CMCAAT combined with tofacitinib (scale bar is 50μm).
**Figure 5:** Treatment with nOSM antibody or tofacitinib inhibits OSM-induced effects in MCF-7 xenografts. (A) Left panel: validation of MCF-7 cells stably transfected with OSM cDNA (MCF-7-OSM) compared to transfection with GFP control plasmid (MCF-7-GFP). Western blot of OSM, pSTAT3-Y705 and -S727, S100A7, S100A8 and S100A9 in cell lysates (LYS) and conditioned medium (CM) of MCF-7-GFP and MCF-7-OSM cells. Total STAT3 and tubulin serve as loading control. Right panel: western blot of pSTAT3-Y705 and -S727 and S100A7 in MCF-7-OSM cells treated for 48h with control medium or 500 nM tofacitinib. MCF-7-GFP serves as control cell line. (B) Swiss nu/nu mice injected with MCF-7-GFP or MCF-7-OSM cells in the right lower mammary fat pad and treated with neutralizing OSM antibody or tofacitinib. First row: macroscopic external view of tumors (indicated by arrows). Second row: macroscopic internal view of tumors. Third row: Contrast-enhanced μCT images of the blood vessels surrounding the tumor (asterix indicates the right hind leg bone, arrow depicts enhanced peritumoral angiogenesis). Fourth and fifth rows: H&E and pSTAT3-Y705 staining of resected tumors (scale bar is 50 μm). (C) Relative mRNA levels of NTN4 and LRG1 expression in MCF-7 cells treated with control medium, CM⁴⁴CAAT or rhOSM (2ng/ml) and in MCF-7-OSM cells.

**Figure 6:** pSTAT3-Y705 in breast cancer cells at the invasion front is correlated with lymphovascular invasion in breast cancer patients. (A) pSTAT3-Y705 staining of breast cancer cells invading surrounding adipose tissue in breast cancer patients (scale bar is 100 μm (upper panel) and 25 μm (lower panel)). (B) Contingency table showing association of pSTAT3-Y705 scores with lymphovascular invasion (LVI) for 50 ER+ IDC patients.

**Figure 7:** Schematic illustrating the role of CAAT-secreted OSM in the tumor microenvironment. Binding of OSM to its receptor (OSMR) on breast cancer cells (BCC) recruits
gp130 to form a heterodimer activating gp130-bound Jak. Activated Jaks lead to pSTAT3-Y705 which form homodimers translocating into the nucleus to regulate transcription. OSM phosphorylates STAT3 S727 through Akt signaling, potentiating transcriptional regulation. OSM induced Jak/STAT3 signaling stimulates invasion and peritumoral angiogenesis.
Figure 1:

A) Collagen spheroid

B) Con

C) F-actin + DAPI

D) Con

E) Invasion Index (%) vs. Time (Hours)

F) T47D

G) T47D

H) MDA MB 231

I) MDA MB 231
Figure 3:

A

<table>
<thead>
<tr>
<th>MCF-7</th>
<th>CON</th>
<th>OSM</th>
<th>IL-6</th>
<th>IL-8</th>
<th>G-CSF</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>5</td>
<td>1</td>
<td>10</td>
<td>100</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>10</td>
<td>100</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CON</td>
<td>OSM</td>
<td>IL-6</td>
<td>IL-8</td>
<td>G-CSF</td>
<td>CON</td>
</tr>
<tr>
<td>1</td>
<td>1</td>
<td>100</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

B

Mass spectrometry of CaaSY for OSM

<table>
<thead>
<tr>
<th>Unique peptides</th>
<th># hits</th>
</tr>
</thead>
<tbody>
<tr>
<td>EYRVLGGLQDK</td>
<td>2</td>
</tr>
<tr>
<td>QTDLMQDTSR</td>
<td>11</td>
</tr>
<tr>
<td>LLDDYR</td>
<td>2</td>
</tr>
<tr>
<td>IGGLDVPK</td>
<td>1</td>
</tr>
<tr>
<td>ERRGAPSEETLA</td>
<td>5</td>
</tr>
<tr>
<td>RGFLTNATLSCYLR</td>
<td>6</td>
</tr>
<tr>
<td>LADLEQQLPK</td>
<td>2</td>
</tr>
<tr>
<td>SGLNIDEKLMARPNIYLR</td>
<td>4</td>
</tr>
<tr>
<td>NNYYCMILQDNSTAEPTKAGR</td>
<td>5</td>
</tr>
<tr>
<td>GASDIPTPASDAEIR</td>
<td>12</td>
</tr>
</tbody>
</table>

C

D

E

F

G

H

Downloaded from cancerres.aacrjournals.org on April 13, 2017. © 2014 American Association for Cancer Research.
Figure 5:

A

<table>
<thead>
<tr>
<th></th>
<th>OSM (+ GFP)</th>
<th>OSM</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>OSM (+ GFP)</td>
<td>- 70 kDa</td>
<td>GFF</td>
<td>OSM</td>
</tr>
<tr>
<td>pSTAT3 (Y705)</td>
<td>- 70-86 kDa</td>
<td>GFF</td>
<td>OSM</td>
</tr>
<tr>
<td>pSTAT3 (S727)</td>
<td>- 70-86 kDa</td>
<td>GFF</td>
<td>OSM</td>
</tr>
<tr>
<td>STAT3</td>
<td>- 70-86 kDa</td>
<td>GFF</td>
<td>OSM</td>
</tr>
<tr>
<td>S100A7</td>
<td>- 11 kDa</td>
<td>GFF</td>
<td>OSM</td>
</tr>
<tr>
<td>S100A8</td>
<td>- 12 kDa</td>
<td>GFF</td>
<td>OSM</td>
</tr>
<tr>
<td>S100A9</td>
<td>- 13 kDa</td>
<td>GFF</td>
<td>OSM</td>
</tr>
<tr>
<td>Tubulin</td>
<td>- 55 kDa</td>
<td>GFF</td>
<td>OSM</td>
</tr>
</tbody>
</table>

B

C

Relative p-Signaling Level (relative to Con)
Figure 6:

(A) Immunohistochemical staining for pSTAT3 in Y705.

(B) Table showing the number of patients in different scores and LVI status.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total No. of patients</td>
<td>41</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>LVI status</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>32</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>positive</td>
<td>9</td>
<td>6</td>
<td>1</td>
</tr>
</tbody>
</table>

*P = 0.0032*
Figure 7:
Cancer Research

Cancer-associated adipose tissue promotes breast cancer progression by paracrine Oncostatin M and Jak/STAT3 signaling.

Lore Lapeire, An Hendrix, Kathleen Lambein, et al.

Cancer Res  Published OnlineFirst September 24, 2014.

Updated version
Access the most recent version of this article at:
doi:10.1158/0008-5472.CAN-14-0160

Supplementary Material
Access the most recent supplemental material at:
http://cancerres.aacrjournals.org/content/suppl/2014/09/23/0008-5472.CAN-14-0160.DC1

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.