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## Abstract

**Obesity is associated with a significantly increased risk for cancer suggesting that adipose tissue dysfunctions might play a crucial role therein. Macrophages play important roles in adipose tissue as well as in cancers. Here, we studied whether human adipose tissue macrophages (ATM) modulate cancer cell function.**

**Therefore, ATM were isolated and compared to monocyte-derived macrophages (MDM) from the same obese patients. ATM, but not MDM, were found to secrete factors inducing inflammation and lipid accumulation in human T47D and HT-29 cancer cells. Gene expression profile comparison of ATM and MDM revealed over-expression of functional clusters, such as cytokine-cytokine receptor interaction (especially CXC-chemokine) signalling as well as cancer-related pathways, in ATM. Comparison with gene expression profiles of human tumour-associated macrophages (TAM) showed that ATM, but not MDM resemble TAM. Indirect co-culture experiments demonstrated that factors secreted by pre-adipocytes, but not mature adipocytes, confer an ATM-like phenotype to MDM. Finally, the concentrations of ATM secreted factors related to cancer are elevated in serum of obese subjects. In conclusion, ATM may thus modulate the cancer cell phenotype.**

**MESH Keywords** Adipocytes ; cytology ; Adipose Tissue ; metabolism ; Azo Compounds ; pharmacology ; Cell Line, Tumor ; Chemokines ; metabolism ; Disease Progression ; Gene Expression Regulation, Neoplastic ; Humans ; Immunohistochemistry ; methods ; Inflammation ; Macrophages ; cytology ; metabolism ; Neoplasms ; metabolism ; Obesity ; metabolism ; Oligonucleotide Array Sequence Analysis ; Phenotype

**Author Keywords** macrophages ; obesity ; cancer ; adipose tissue ; chemokines

## INTRODUCTION

Obesity is a low-grade chronic inflammatory disease due to the infiltration of immune-inflammatory cells including monocytes which differentiate in macrophages and form aggregates in crown-like structures usually surrounding dead adipocytes. The number of adipose tissue macrophages (ATM) correlates with the body mass index (BMI) (1). Within adipose tissue (AT), ATM are a major source of chemokines and inflammatory cytokines, such as IL-6 and TNFα, which may on their turn promote the recruitment of additional immune-inflammatory cells. In animal models, AT macrophage infiltration and ensuing inflammation precedes the development of insulin resistance (2). Surface marker analysis revealed that human ATM present an anti-inflammatory phenotype (3), but are capable of producing pro-inflammatory mediators (4). ATM are surrounded by pre-adipocytes and adipocytes and it is likely that paracrine loops exist via the production of adipokines, free fatty acids and derived mediators, leading to inflammatory changes. In particular, pre-adipocytes produce higher levels of pro-inflammatory cytokines compared to adipocytes, suggesting that they play an important role in the induction and maintenance of inflammation (5,6), and may thus operate as ATM activators.

Interestingly, there are now epidemiological evidences establishing obesity as a risk factor for the development of cancer, such as colon, breast, oesophagus, kidney, liver and pancreas cancer (7). Whereas weight gain is accompanied by higher cancer incidence rates, recent longitudinal studies on metabolic surgery revealed that weight loss results in lower cancer rates (8). During obesity, the expanded AT could contribute to cancer development via a deregulated secretion of pro-inflammatory cytokines, chemokines and adipokines (9). Based on these observations we thus hypothesized that ATM could be involved in the development of cancer related to obesity. Interestingly, we found that conditioned medium from ATM, but not from autologous monocyte-derived macrophages (MDM) isolated from the same morbidly obese patients, induced phenotypic changes and activation of human breast cancer cells. To characterize the

phenotypical differences between human ATM and MDM, a global gene expression analysis was performed on ATM and MDM isolated from the same morbidly obese patients. Several genes over-represented in ATM, compared to MDM, were identified to belong to cytokine-cytokine receptor interaction pathways (including chemokine, hematopoietin, PDGF, interleukin, TNF, interferon and TGFβ pathways) identifying a specific ATM signature. Interestingly, ATM displayed a gene expression profile sharing similarities with human tumour-associated macrophages (TAM) (10,11). In line, ATM were found to produce a repertoire of growth factors, cytokines, chemokines and proteolytic enzymes involved in the regulation of tumour growth, angiogenesis, invasion and/or promotion of cancer metastasis, as observed in TAM (12). Finally, we show that the ATM phenotype is programmed by the cellular environment as demonstrated by indirect co-culture experiments applying the pre-adipocyte-conditioned media to MDM from lean subjects, resulting in an ATM-like phenotype.

These data indicate that the TAM-like human ATM phenotype is directed by surrounding cell types and suggest that factors released by ATM may contribute to cancer initiation and progression in obese patients. These observations suggest that ATM may be potential contributors to cancer development in obese humans.

## EXPERIMENTAL PROCEDURES

### Cell Culture

Visceral AT biopsies (n=11) were obtained from non-diabetic morbidly obese patients undergoing bariatric surgery at the Internal Surgery Department, Hospital of Lille, France (supplemental table 1 for patient characteristics). This study was approved by the ethics committee of the CHRU of Lille under the ABOS and OMENTOB frameworks. None of the patients had any clinical symptoms of systemic inflammation or cancer. All patients gave informed consent. To avoid contamination of ATM by blood monocytes, the vessels were carefully dissected and blood removed by extensive PBS washes before AT digestion. AT samples digested in Krebs buffer (pH 7.4) containing collagenase (1.5 mg/ml, Roche Diagnostic). After filtration through a 200μm filter (Spectra Mesh; Biovalley) and centrifugation, floating adipocytes were washed with PBS and released lipids collected. The stromal vascular fraction (SVF) was collected as described (13). ATM-$CD14^+$ cells were isolated using CD14-labelled magnetic beads (Miltenyi Biotec). ATM were cultured for 24h in Endothelial Cell Basal Medium (Promocell) supplemented with 0.1% BSA to dampen possible inflammation responses due to magnetic-bead separation and isolation procedure (13). Medium was then changed, cells cultured for another 24h with fresh medium and supernatants collected thereafter.

The $CD14^-$ fraction (containing endothelial cells, fibroblasts, lymphocytes, stem cells and pre-adipocytes) was cultured in Pre-adipocyte Basal Medium (Promocell) for 24h. Adherent pre-adipocytes were then cultured in selective Pre-adipocyte Growth Medium (PGM, Promocell) during 3 days before collecting the pre-adipocyte conditioned medium (PCM).

Human monocytes were isolated from fresh blood of the same obese or from lean patients, differentiated for 9 days in the presence of 10% pooled human serum (14) and treated for 72h with indicated PGM or PCM.

Human TAM were isolated as described (10,15) from solid tumours of untreated patients with histologically confirmed epithelial ovarian carcinoma (n=7) admitted to the San Gerardo Hospital in Monza (Italy). Human T47D breast ductal carcinoma and HT-29 colon adenocarcinoma cells were obtained from ATCC (Rockville, MD).

### Immunohistochemical analysis and laser capture microdissection (LCM) of human atherosclerotic plaques

Human atherosclerotic plaques (n=5) were removed from patients eligible for surgical carotid endarterectomy recruited at the Cardiovascular Surgery Department, Hospital of Lille, France. Informed consent was obtained from all patients. Pro-inflammatory CD68 positive and mannose receptor (MR) negative macrophages were isolated from lipid-rich areas within human atherosclerotic plaques by laser capture microdissection, as previously described (16).

### Oil Red O (ORO) staining

T47D cells were treated with ATM or MDM-conditioned media, fixed with 4% paraformaldehyde in PBS, stained using ORO and nuclei counterstained with hematoxylin (40X magnification).

### RNA extraction and real time quantitative polymerase chain reaction (Q-PCR) analysis

Cellular RNA was extracted using RNeasy kits (Qiagen). Total RNA was reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems, USA). cDNAs were quantified by Q-PCR on a MX4000 apparatus (Agilent Biotechnologies) using specific primers (supplemental table 2). Statistically differences were analysed by Student's t tests and considered significant when $p<0.05$.

### Sample preparation for gene expression and data analysis of Affymetrix oligonucleotide arrays

Total RNA was checked for quantity and quality using the Agilent 2100 Bioanalyzer (Agilent Biotechnologies). ATM, MDM and TAM RNA samples were processed using HG-U133 plus 2.0 (ATM and MDM) and HG-U133A (TAM) Affymetrix Genechips. The MatchProbes package (PMID: 14988118) was used to match the probe sequences between the GeneChips. Quality assurance of the hybridization was assessed by constructing a virtual image of the chip by plotting each residual obtained with each probe at the corresponding position using the affyPLM package. Raw gene expression data were processed and normalized with the Affymetrix GCOS 1.4 software and by multi-array analysis (RMA) (17).

**Statistical and informatic methods**

The raw microarray data were pre-processed using Bioconductor tools (http://www.bioconductor.org ) to generate a single expression value for each probe set in each sample. To identify expressed genes, a statistical analysis based on individual probe information was applied using the R2.6 software (http://www.r-project.org ). Statistical t-test was used to assess how likely changes in differential expression occurred by chance (threshold=0.05). Signal intensities ratios and p-values were calculated for each probe set using the linear model library (Limma) allowing identification of genes differentially expressed between ATM and MDM. Genes showing statistically significant differences of $p \leq 10^{-5}$ were selected for further analysis. Based on previous literature, the minimum cut off in intensities per gene was set at 1.5-fold change (FC) (18). Differential gene expression was verified by Q-PCR on ATM and MDM obtained from 7 additional donors.

**Functional classification, visualisation and pathway analysis**

Selected gene lists were uploaded in the DAVID (Database for Annotation, Visualization, and Integrated Discovery) website (19). Bioinformatics resource and the functional annotation and classification gene tools were used to generate clusters of related genes (20). Integrative data analysis, including multiple graphical visualizations was performed using GenespringGX10 (Agilent Biotechnologies) and pathway analysis by the Kyoto Encyclopedia of Genes and Genomes (KEGG). The profile of functional pathways was analysed using ProfCom software (21). This software allows comparing the proportion of genes related to specific gene ontology (GO) categories (biological process, cellular component and molecular function) among the ATM higher expressed genes to the proportion of genes related to the same category within the GO reference set of all genes from homo sapiens (21).

**Prediction of ATM protein secretion**

Secreted protein prediction was conducted by interrogating the list of the highly expressed ATM genes ($FC \geq 1.5$; $p \leq 10^{-5}$ ) using the SignalP3.0 (http://www.cbs.dtu.dk/services/SignalP/ ) (22,23) and SecretomeP2.0 (http://www.cbs.dtu.dk/services/SecretomeP/ ) (22) softwares to identify genes with the highest probability of encoding either signal peptides or protein sequences predictive of non-classic secretion, respectively, using a cut-off N-score of 0.7 (22). The corresponding genes were marked as predicted or not predicted. Gene encoding sequences that met the cut-off value for either classic or non-classic secretion were marked as classical or non-classical secretory genes.

**Protein extraction and western blot analysis**

MDM were harvested in ice-cold protein lysis buffer (RIPA). Cell homogenates were collected by centrifugation and proteins were separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane (Amersham). Membranes were then subjected to immunodetection using rabbit polyclonal antibodies against Stat3, Stat1, p44/p42 MAPK (ERK1/2), and their phosphorylated forms, Stat3 (Tyr705, Ser727), Stat1 (Tyr701, Ser727), p44/p42 MAPK (Thr202/Tyr204) (all from Cell Signaling) and β-actin (Santacruz Biotechnology). After incubation with appropriate secondary antibodies, immunoreactive bands were revealed using a chemiluminescence HRP Substrate detection kit (Immobilon™ Western, Millipore).

**Measurement of circulating CXCL proteins**

Sera were collected from obese and lean subjects (20 per group) and CXCL1, 2, 3 (Gro), CXCL6, CXCL7, CXCL9, CXCL10, CXCL11, sgp130 and TGFβ1 concentrations were measured by Luminex technology using commercially available kits (Millipore for all, except TGFβ1 (R&D Systems)). CXCL16 was measured by ELISA (R&D Systems). Statistical differences between groups were analyzed by Mann Whitney rank-sum test and were considered significant at $p < 0.05$.

## RESULTS

**ATM conditioned medium induces lipid accumulation and expression of inflammatory markers in human breast cancer cells**

Since several lines of evidences suggest a potential link between obesity and cancer and since macrophages play important roles in both pathologies, we determined whether secreted factors from ATM can impact on tumour cells. Therefore, indirect co-culture experiments were performed on human T47D breast cancer and HT-29 colon adenocarcinoma cells in the presence of conditioned media (CM) from ATM or MDM isolated from the same obese subjects. Interestingly, ATM-CM, but not MDM-CM, increased the expression of

CD36 and Fatty Acid Synthase (FAS) in T47D and HT-29 cells and enhanced lipid accumulation in T47D cells, as determined by Oil Red O staining (figure 1), a phenotype associated to a poor cancer prognosis (24). Moreover, ATM-CM and not MDM-CM also modulated the expression of several genes involved in angiogenesis and inflammation in HT-29 and T47D cells, such as VEGFα, TGFβ, ICAM-1, IL-6 and MCP-1 (figure 2).

Taken together, these results indicate that ATM-CM, but not MDM-CM can modulate the phenotype of human cancer cells.

**Identification of genes differentially expressed between ATM and MDM**

To characterize the differences in phenotype between ATM and MDM, a whole genome microarray analysis was performed on ATM and MDM from the same donors. To specifically evaluate the effect of obesity and not of other metabolic components such as diabetes, only obese non-diabetic subjects were included. Hierarchical clustering of the entire Affymetrix chip and after probe sets (PSN) filtration revealed a normal gene expression distribution and a strong similarity in the global gene expression profile between ATM and MDM (supplemental figure 1). Based on our criteria of selection stringency, more than 80% of PSN were similarly expressed and less than 20% of PSN showed differences. Normalized intensity values of gene expression in ATM were higher than the 75$^{th}$ percentile, whereas those of MDM were rather lower than the 25$^{th}$ percentile, indicating that gene expression is globally higher in ATM than in MDM (supplemental figure 2A). The numbers of transcript abundance differences (increased or decreased) between ATM and MDM are shown by a volcano plot (supplemental figure 2B). 1371 PSN were identified as higher expressed (Log2Ratio >0.58 corresponding to a FC>1.5; $p<0.01$) and 1771 PSN were lower expressed in ATM compared to MDM (Log2Ratio <0.58 corresponding to a FC<−1.5; $p<0.01$).

Functional pathway analysis revealed that the cytokine–cytokine receptor interaction (CCRI) pathway is the most significantly over-represented pathway in ATM (based on the p-value) (supplemental figure 2C). Moreover, a large number of other pathways, including the NOD-like receptor, chemokine, MAPK, Toll-like receptor, RIG-I-like receptor, complement and coagulation cascades, Jak-STAT, focal adhesion, ECM-receptor interaction and tight junction pathways, were over-expressed in ATM. Surprisingly, several cancer signalling pathways (acute myeloid leukaemia, small cell lung and bladder cancer) were also found to be higher expressed in ATM (supplemental figure 2C). Gene ontology (GO) analysis identified a number of genes associated with certain GO categories (biological process (BP), cellular components (CC), molecular function (MF)) to be higher expressed in ATM. ATM most prominently expressed genes related to transcription regulation, cell adhesion and signalling, inflammation and immune response. Genes implicated in the activity of transcription factors, signal transduction, growth factors and chemokines were also significantly enriched in ATM (supplemental figure 2D). By contrast, genes related to metabolism, ion binding, transferase and oxidoreductase activities were higher expressed in MDM, whereas genes related to general macrophage functions, such as phagocytosis and cell cytotoxicity, were expressed at the same level in ATM and MDM (data not shown).

**Genes belonging to the cytokine-cytokine receptor interaction (CCRI) pathways are highly expressed in ATM**

Since the cytokine-cytokine receptor interaction (CCRI) pathways are most significantly over-expressed in ATM, we focused our attention on these pathways, which include the chemokine (from the CC and C-X-C subfamilies), hematopoietin (Oncostatin M (OSM), gp130 (IL6ST)), PDGF, interleukin, TNF, interferon and TGFβ families. Interestingly, several members of the CC chemokine ligand family, such as CCL2, 3, 4, 5, 7, 8, 11, 18 and 20 (figure 3 and supplemental figure 3) and the majority of CXCL ligands (CXCL1, 2, 3, 5, 6, 7, 9, 10, 11, 12 and 14), but not their receptors (except for CCR6 and CCR7), were higher expressed in ATM than in MDM (supplemental figure 3), whereas only CXCL16 (SR-PSOX) was higher expressed in MDM (figure 3). Q-PCR analysis performed on ATM and MDM preparations from 7 additional obese individuals confirmed these results (figure 3).

Within the CCRI pathway, major differences were observed in the expression of genes of the hematopoietin family, such as IL-6, IL6-ST, IL-11, LIF, OSM and their receptors (supplemental figure 3C). Q-PCR analysis confirmed the different expression pattern of OSM, OSM-R and IL6-ST (supplemental figure 3D). Gene expression profiling and Q-PCR analysis also confirmed the differential expression of several members of the TGFβ signalling pathways which play an important role in the regulation of macrophage immune functions (supplemental figure 3E–G). To determine whether the expression profile of CXCL genes was a specific signature of ATM or a common characteristic of inflammatory macrophages, their expression levels were analyzed in pro-inflammatory CD68+ macrophages isolated from lipid-rich areas within human atherosclerotic plaques by laser capture microdissection and compared to levels in ATM.

Our results indicate that the expression level of CXCL1, CXCL10 and CXCL11 was much lower in CD68+ atherosclerotic plaque macrophages compared to ATM. Expression of CXCL9 and CXCL12 was highly variable between samples, also because of the relatively low expression levels of these two chemokines (supplemental figure 4). Moreover, CXCL5 mRNA expression was not detectable in CD68 + atherosclerotic plaque macrophages. Overall, these data indicate that the expression profile of CXCL members in ATM is different from those obtained in atherosclerotic plaque inflammatory macrophages.

**A large number of highly expressed genes in ATM encode predicted secreted proteins**

SignalIP 3.0 and SecretomeP 2.0 softwares were used to predict secreted proteins from ATM (supplemental table 3). 57 genes of the 1066 genes over-expressed in ATM were not identified by the software and are thus marked as unknown (supplemental figure 5). A total of 320 of the remainder 1009 genes (30%) were predicted to encode secreted proteins. Among these 30%, ~17% were classically secreted proteins and ~13% corresponded to non-classical secreted proteins. Proteins with anchor signals which represent approximately 4% of the gene list, were also considered since they can be processed by cleavage to soluble factors with biological functions. Thus 34% of ATM-enriched genes encode predicted secreted proteins, suggesting an over-representation of secreted proteins in ATM relative to their abundance in the human genome (15–20%). Grouping of genes in classes according to the FC (1 to 200; 201 to 400; 401 to 600; 601 to 800; 801 to 1066) (supplemental figure 5 and table 3) showed that the highest percentage of predicted secreted proteins was found in the gene class showing the highest FC, again suggestive of an enrichment of predicted secreted proteins in ATM, compared to MDM.

**Pre-adipocytes determine the ATM phenotype**

To address whether and how the AT environment (cells and lipids) can affect the ATM characteristics, indirect co-culture experiments were performed on MDM from lean subjects incubated with conditioned media from pre-adipocytes from obese subjects (PCM). A substantial increase of CXCL1, 5, 10, 11 and 12 as well as MCP-1, TNFα and IL-1β gene expression, resembling the ATM profile, was observed in lean MDM treated with PCM compared to control medium (PGM) (figure 4A). By contrast, no modification or a decreased expression of most CXCL genes was observed after incubation with released lipids from mature adipocytes directly isolated from AT (supplemental figure 6). These results indicate that pre-adipocytes secretion products determine the ATM phenotype.

Western blot analysis of total and phosphorylated forms of Stat3, Stat1, p44/p42 MAPK revealed that incubation of MDM with PCM very rapidly induced tyrosine phosphorylation of Stat3 and Stat1 as well as threonine/serine phosporylation of p44/p42 MAPK (figure 4B).

**The ATM phenotype resembles the phenotype of TAM**

Since ATM but not MDM were able to modify the cancer cell phenotype, we decided to determine whether a possible resemblance could exist between ATM and TAM by comparing the expression profile of ATM and MDM to that of TAM isolated from human tumours (10). Based on our stringency criteria, approximately 80% of PSN were similarly expressed between the three types of macrophages and approximately 20% of PSN showed differences (supplemental figure 7). Next, it was analysed which genes are similarly expressed between ATM and TAM and between MDM and TAM (−1.5>FC>+1.5). KEGG analysis revealed that among the 10 most significantly represented pathways shared between ATM and TAM (based on the p-value), the majority of them are cancer-related pathways (small lung cancer, chronic myeloid leukaemia, prostate cancer, pancreatic cancer, glioma). By contrast, the cancer-related pathways in MDM represented only a small number of the pathways shared with TAM (figure 5), indicating an enrichment of cancer-related pathways in ATM compared to MDM. Moreover, based on the literature (11), 29 genes highly expressed by TAM, including angiogenic factors, chemokines, cytokines, proteases and growth factors, were also higher in ATM compared to MDM (supplemental figure 7). Interestingly, the majority of these genes were higher or equally expressed in ATM compared to TAM (figure 6). By contrast, the expression of the same genes was generally lower in MDM compared to TAM (figure 6). These data demonstrate that human ATM display a TAM-like phenotype.

**Serum concentrations of CXCL1, 2, 3, 10 and 11 are elevated in obese subjects**

To determine whether circulating concentrations of CXCL chemokines and some factors belonging to the CCRI pathways, such as sgp130 and TGFβ1, which are over-represented in ATM, are altered *in vivo* in obese individuals, serum from obese and lean subjects was analyzed. The serum levels of total CXCL1, 2, 3 (Gro), CXCL 10, 11 and sgp130 were significantly elevated in obese compared to lean individuals, whereas the level of the anti-proliferative factor TGFβ1 was significantly decreased in obese individuals (figure 7). Altogether these results indicate that the expression of several factors highly expressed in ATM and related to cancer correlates with circulating levels in obese subjects without malignancies.

## DISCUSSION

Obesity is a low-grade chronic inflammatory state characterized by an increased number of infiltrated macrophages and altered adipocytokine production in AT (1). ATM are a major source of inflammatory cytokines and mediators involved in obesity induced-insulin resistance, such as IL-6 and TNFα. Interestingly, experimental and epidemiological studies have shown an association between excessive adiposity and increased cancer incidence and death, probably as a consequence of the obesity-associated metabolic and endocrine perturbations (7). Together, these observations raise the hypothesis that ATM can contribute to the link between obesity and cancer development.

Here we show that CM from ATM, but not from MDM isolated from the same obese donors, increase the expression of FAS and CD36 leading to lipid accumulation in cancer cells. Interestingly, tumours over-expressing FAS display a more aggressive behaviour

compared to those with normal FAS levels, since FAS plays a pivotal role in cancer cell survival and is directly involved in the maintenance or enhancement of the malignant phenotype (25). FAS is over-expressed in breast cancers with poor prognosis (24,26–28). In addition, several inflammatory molecules known to be dysregulated in cancer were enhanced in T47D and HT-29 cells by ATM-CM. Altogether these results indicate that factors released by ATM, but not by MDM, could affect tumour cell phenotype. Many tumours have increased levels of obesity-related factors, both adipokines and inflammatory components, in their microenvironment, rendering, in some cases, the tumours more aggressive (29). Thus, macrophages in peri-tumoural adipose tissue, which release obesity-related factors, could be locally involved in enhancing carcinogenesis.

Therefore, we compared global gene expression profiles of ATM to MDM isolated from the same obese patients. Functional cluster analysis revealed that genes involved in inflammatory pathways were found to be enriched in ATM compared to MDM, amongst which the CCRI pathway was the most significant. Surprisingly, several cancer-related signalling pathways were also higher expressed in ATM *vs* MDM. This phenotype was confirmed by comparing the MDM and ATM gene profiles to those of TAM isolated from solid human tumours (10).

Within the AT, ATM interact in a paracrine way with surrounding cells (T cells, pre-adipocytes, adipocytes, endothelial and stromal cells) via the production of hormone-like and angiogenic factors, which can promote survival of damaged cells and thus can actively participate in the promotion of tumours by endocrine and/or paracrine mechanisms (30). Within tumours, TAM participate in the progression/evolution of cancer and their infiltration correlates with cancer metastasis and poor prognosis in a variety of human carcinomas (31,32). TAM promote cancer metastasis in established advanced tumour stages, through mechanisms such as stimulation of angiogenesis (31,32), via a wide variety of factors which stimulate blood vessel growth and/or maturation. Our analysis revealed that the expression of angiogenic factors, chemokines, cytokines, proteases and growth factors (CCLs (MCP-1…), CXCLs (Gro, CXCL7…), IL-1α /β, IL-6, FGF2) which are also actively secreted by TAM (11), are higher expressed in ATM *vs* MDM. The majority of CC and CXC chemokine ligands (CCL, CXCL) are highly expressed in ATM. Chemokines mediate recruitment of immune cells (neutrophils, dendritic cells, lymphocytes, monocytes) at the site of inflammation, play a role in cell proliferation, inhibition of apoptosis as well as angiogenesis, both in AT and in the tumour micro-environment (33–35). CCL and CXCL are induced by the typical Th1 cytokine IFNγ alone or in combination with other inflammatory cytokines (36). Interestingly, different members of the IFNγ subfamily are also higher expressed in ATM (supplemental figure 4), suggesting a possible amplification of the Th1 immune response (12). It is worth noting that ATM over-expressed the ligands, but not the receptors of these chemokines, with an exception for CXCR6, CX3CR1 and CCR7, suggesting that the ligands produced by ATM may exert paracrine effects on surrounding cells possessing their cognate receptors, such as mature adipocytes (37). Surprisingly, the only CXCL lower expressed in ATM was CXCL16, also involved in leukocyte migration and adhesion (38). Importantly, high expression levels of CXCL16 in renal cancer tissue has been correlated with a better survival of patients (39,40). Our study also reveals that circulating levels of Gro (CXCL1, 2, 3), CXCL9, 10, 11 (CXCR3 ligands) and sgp130, key players in the maintenance and amplification of autoimmunity-related inflammatory processes (35), are elevated in obese subjects, while anti-proliferative TGFβ1 concentrations are lower (41). ATM over-expressed both OSM family members (IL-6, LIF, OSM, IL-11) and their receptors. Indirect co-culture experiments on MDM indicated that the phenotype of ATM is specifically related to factors released by the pre-adipocytes, whereas lipids released from mature adipocytes do not induce these effects. These data are in line with previous observations, suggesting the high pro-inflammatory potential of pre-adipocytes (5,6).

Collectively, our data indicate that ATM may contribute to the link between obesity and cancer and revealed for these cells a gene expression profile comparable to those of TAM.

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**Figure 1**

ATM conditioned medium induces lipid accumulation in human cancer cells

T47D (A, B, E) and HT-29 (C, D) cells were treated for 72h with ATM or MDM unconditioned (UC) or conditioned (CM) medium and mRNA levels of CD36 (A, C) and FAS (B, D) were analyzed by Q-PCR and normalized to cyclophilin mRNA. Results are expressed relative to the levels of cells in the presence of ATM or MDM unconditioned medium set at 1. Each bar is the mean value ± SD of triplicate determinations. Statistically significant differences are indicated (*p<0.05; **p<0.01; ***p<0.001). (E) ORO staining performed on T47D cells. Results represent a single experiment repeated 4 times with similar results.

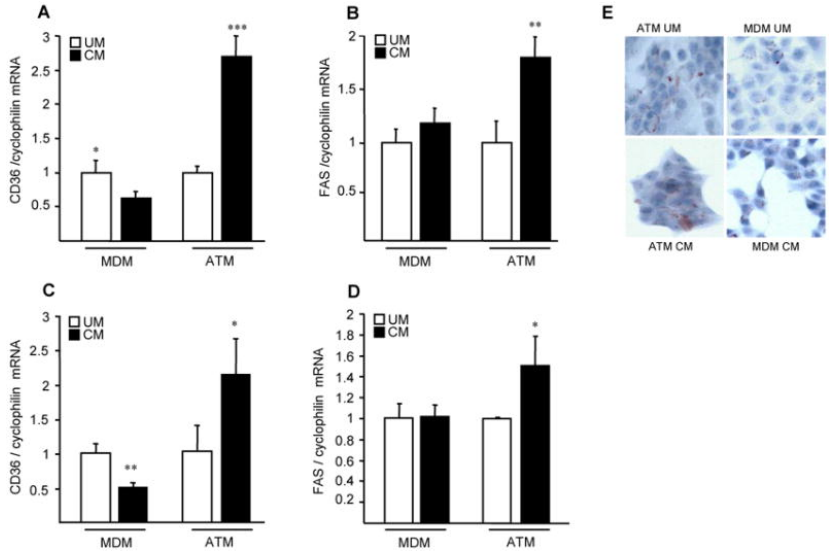

**Figure 2**

Different gene expression regulation by ATM vs MDM conditioned medium in human cancer cells

HT-29 and T47D cancer cells were treated for 72 hours with unconditioned (UM) or ATM or MDM conditioned medium (CM). mRNA levels of VEGFA, TGF-β1, ICAM-1, MCP-1 and IL-6, were subsequently analyzed by Q-PCR and normalized to cyclophilin mRNA. Results are expressed relative to the levels of cells in the presence of unconditioned medium set at 1. Each bar is the mean value ± SD of triplicate determinations. Statistically significant differences are indicated (*p<0.05; **p<0.01; ***p<0.001).

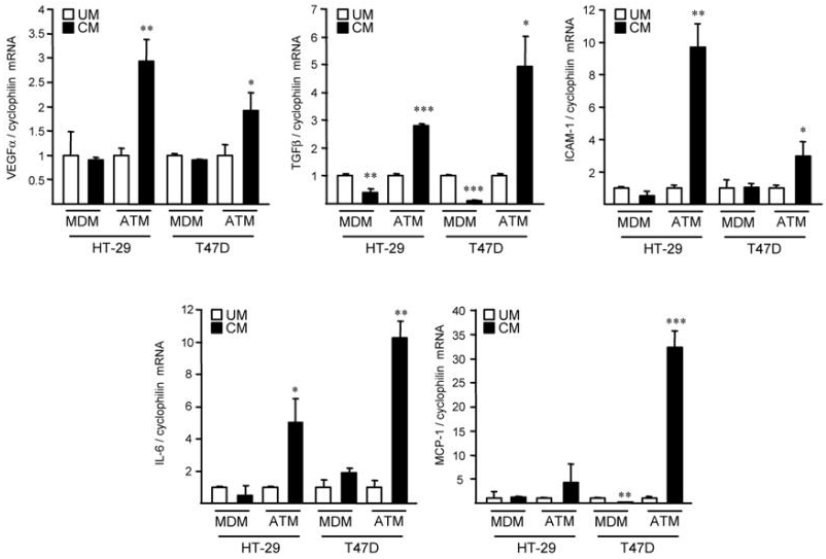


**Figure 3**

Genes belonging to the CC and CXC chemokine subfamily are highly expressed in ATM

Histogram of Affymetrix FC values of CXC chemokine ligand genes differently expressed between ATM and MDM and Q-PCR analysis of CXCL1, CXCL5, CXCL6, CXCL9, CXCL10, CXCL11, CXCL12, CXCL14 and CXCL16 in ATM and MDM from 7 donors. mRNA levels were normalized to cyclophilin mRNA and results expressed as mean ± SD of triplicate determinations relative to the levels in ATM set at 1. Statistically significant differences are indicated (***p<0.001). nd: non detectable

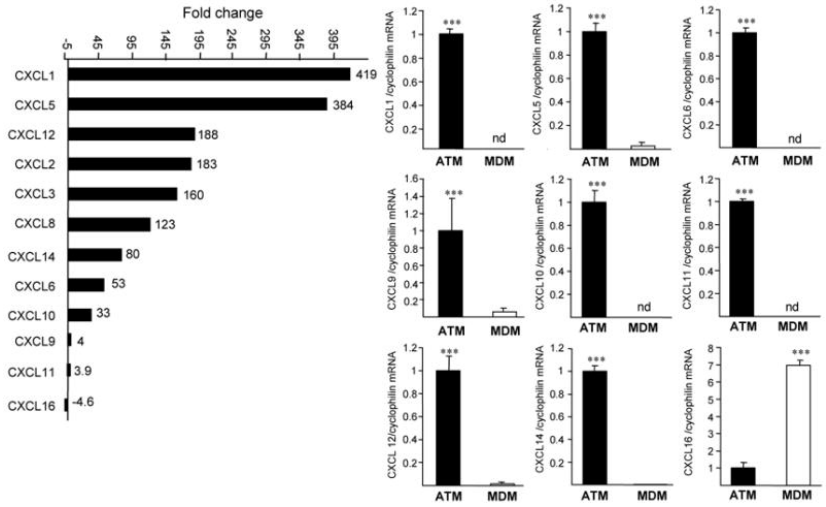

**Figure 4**

Pre-adipocyte conditioned medium (PCM) induces an ATM-like phenotype in MDM

MDM from lean subjects were treated with PCM or unconditioned pre-adipocyte growth medium (PGM) during 72h (A). mRNA levels of CXCL1, CXCL5, CXCL9, CXCL10, CXCL11, CXCL12, MCP-1, TNFα, IL-1b were analyzed by Q-PCR and normalized to cyclophilin mRNA. Results are representative of 3 experiments and are expressed relative to the levels of cells in the presence of PGM set at 1. Each bar is the mean value ± SD of triplicate determinations. Statistically significant differences are indicated (***p<0.001). MDM were treated with PCM for the indicated time periods (B). Western blot analysis was performed using antibodies against total or phosphorylated forms of Stat3, Stat1, p44/p42 MAPK (ERK1/2).

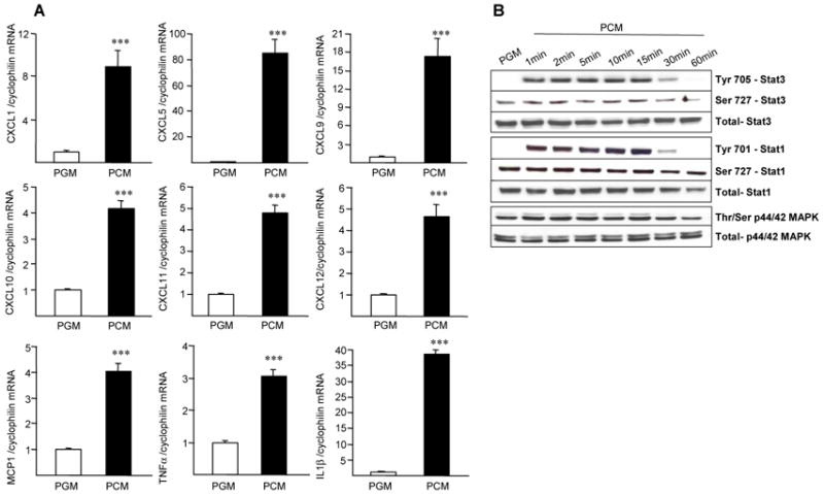


**Figure 5**

ATM exhibit a gene expression profile similar to TAM

(A) Top 10 of the most significant KEGG molecular pathways including genes similarly expressed between ATM/TAM and MDM/TAM, respectively. (B) Pie-chart representations of the top 10 KEGG pathways; cancer-related pathways have been considered together.

A

**ATM = TAM (-1.5 ≥ FC ≥ +1.5)**

| Pathway | P-value | Benjamini |
|---|---|---|
| Ribosome | 4.6E-07 | 9.1E-05 |
| Pathways in cancer | 4.5E-05 | 4.4E-03 |
| Small cell lung cancer | 2.4E-04 | 1.6E-02 |
| Homologous recombination | 7.6E-04 | 3.6E-02 |
| Chronic myeloid leukemia | 1.5E-03 | 5.6E-02 |
| Prostate cancer | 1.7E-03 | 5.5E-02 |
| Pancreatic cancer | 2.1E-03 | 5.6E-02 |
| Calcium signaling pathway | 3.4E-03 | 7.9E-02 |
| Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 8.5E-03 | 1.7E-01 |
| Glioma | 1.1E-02 | 2.0E-0.1 |

**MDM = TAM ( -1.5 ≥ FC ≥ +1.5)**

| Pathway | P-value | Benjamini |
|---|---|---|
| Tight junction | 4.6E-07 | 3.8E-03 |
| Ribosome | 4.5E-05 | 1.7E-02 |
| Purine metabolism | 2.4E-04 | 6.0E-02 |
| Homologous recombination | 7.6E-04 | 5.5E-02 |
| Calcium signaling pathway | 1.5E-03 | 1.3E-01 |
| Pathways in cancer | 1.7E-03 | 1.1E-01 |
| Maturity onset diabetes of the young | 2.1E-03 | 1.4E-01 |
| Huntington's disease | 3.4E-03 | 1.8E-01 |
| Melanoma | 8.5E-03 | 2.0E-01 |
| Regulation of actin cytoskeleton | 1.1E-02 | 2.2E-01 |

B

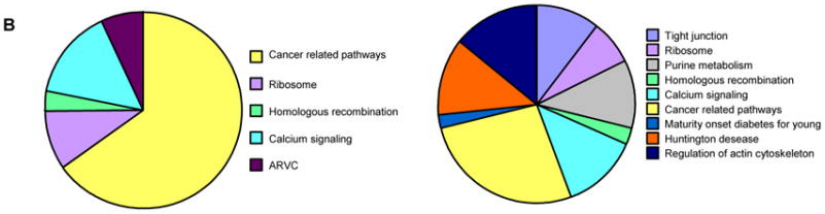

**Figure 6**

TAM-expressed genes are enriched in ATM compared to MDM

Histogram of Affymetrix FC values of selected TAM genes differently expressed between ATM/TAM and MDM/TAM, respectively.

| ATM vs TAM | Genes/total analysed (%) |
|---|---|
| Lower expressed | 6 / 29 (20.7 %) |
| Higher expressed | 18 / 29 (62.1 %) |
| Equally expressed | 5 / 29 (17.2 %) |

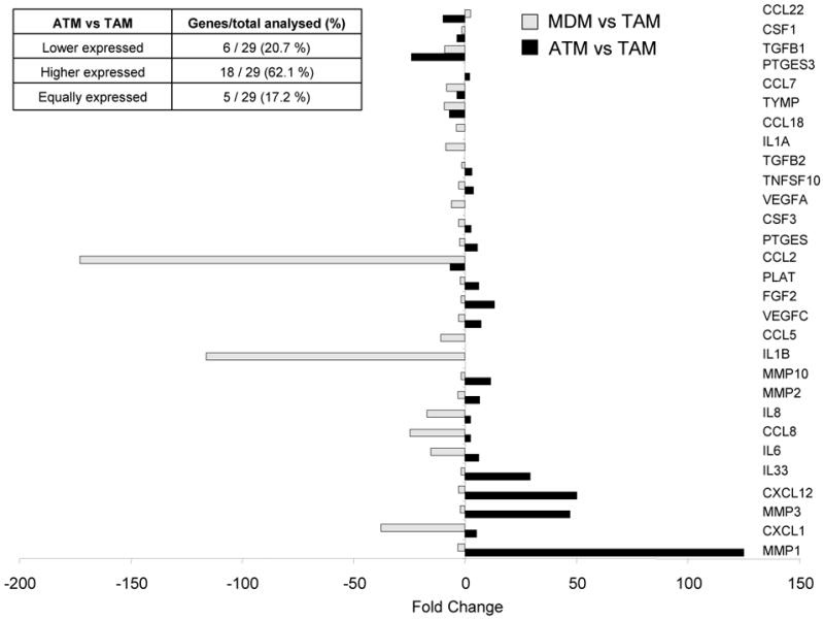


**Figure 7**

Obesity is associated with altered serum concentrations of ATM-expressed factors

Serum protein concentrations of CXCL1, 2, 3 (Gro), CXCL6 (GCP-2), CXCL7 (NAP-2), CXCL9 (MIG), CXCL10 (IP-10), CXCL11 (I-TAC), CXCL16 (S-PROX), TGFβ1, sgp130 were quantified in obese and lean subjects. Statistically significant differences are indicated (* $p<0.05$; ** $p<0.01$).