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Abstract

Background Adipose tissue is a major component of breast stroma. This study focused on delineating the effects of adipose stem cells (ASCs) derived from breast healthy women and cancer patients with normal or tumor breast cells.
Methods The ASCs were induced to differentiate into adipocytes, and the subsequent adipocyte conditioned media (ACM) were evaluated for their fatty acid profile, adipokine secretion and influence on proliferation, migration and invasion on tumoral (MCF7 and SUM159) and normal (HMEC) human breast cell lines.
Results An enrichment of arachidonic acid was observed in ACM from tumor tissues. Adipose tissues from tumor free secrete twice as much leptin than those from proximal or distal to the tumor. All ACMs display proliferative activity and favor invasiveness of SUM159 cells compared to MCF7 and HMEC. All ACMs induced lipid droplets accumulation in MCF7 cells and increased CD36 expression in tumor cells.
Conclusion We conclude that among secreted factors analyzed only arachidonic acid and leptin levels did discriminate ASCs from tumor-bearing and tumor-free breasts emphasizing the importance that others cell type could contributes to the adipose tissue secretome in a tumor context.

Keywords: Breast cancer; breast-associated adipocytes; adipose stem cells, microenvironment.

Introduction

Breast cancer (BC) remains the leading cause of cancer death in women worldwide. During the last decades, breast cancer has been stratified into different molecular subgroups that are associated with different prognosis. This molecular characterization defines luminal breast cancers (A and B), HER2/ErbB2-positive breast cancers and basal breast cancers [1]

During carcinogenesis, ductal or lobular epithelial cells proliferate abnormally. They may also acquire invasive characteristics and metastasize, forming secondary tumors in sites more or less distant from the primary tumor. Cancer cells are able to induce a remodeling of their microenvironment by activating or recruiting the healthy cells surrounding them, thus creating a niche favorable to tumor growth and dissemination [2, 3].

Research on the tumor microenvironment typically focuses on fibroblasts, immune cells, endothelial cells and the extracellular matrix (ECM). However, due to the distribution of adipose tissue all over the body, adipocytes are in close contact with invasive melanoma, prostate, colon and ovarian cancer cells [4, 5]. In breast cancer, adipose tissue is the main component of the tumor environment and there is a close proximity between cancer cells and adipocytes in the early stages of tumor development [6]. Moreover, although histological sections show that adipose tissue is excluded from the tumor center, adipocytes are present in a majority at the invasive front [6]. During their interaction with cancer cells, adipocytes are reprogrammed into cancer-associated adipocytes (CAA). These CAAs display specific phenotypes such as dedifferentiation, release of adipokines and proinflammatory cytokines and metabolic remodeling [7, 8].

The breast adipose tissue is a known endocrine organ that secretes many factors including adipokines, cytokines, chemokines and growth factors, therefore controlling various cellular process [9]. They can have a

paracrine or systemic effect and are involved in energy metabolism, angiogenesis and extracellular matrix remodeling. In response, breast cancer cells could induce signal lipolysis of adipose tissue, leading to a new secretory phenotype of adipose tissue [7, 10].

The secretory profile of adipocytes varies according to the location of the adipose tissue and is affected by a pathological context such as obesity and cancer [11, 12]. Many studies have revealed the importance of adipocyte-secreted factors such as leptin or interleukin 6 in controlling tumor progression [13]. We have recently shown that breast-associated adipocytes secretome induced invasiveness in breast cancer cells independently of body mass index, menopausal status and mammary density [14]. Considering the close localization between breast cancer and breast adipose tissue, the characteristics of peritumoral adipose tissue might have a significant association with the characteristics and clinical outcomes of breast cancer [15].

Here we investigated, the effect of adipocyte-conditioned media of human adipose tissue from healthy, or tumor breast on proliferation, migration and invasion on tumor and non-tumor breast cell lines.

Materials and Methods

Patients

Normal breast adipose tissues were collected from reduction mammoplasty from donors with no cancer history. Tumor proximal tissues were collected from donors undergoing mastectomy or tumorectomy. All tissues were collected in accordance with the ethical standards of the local ethical committee. All patients gave their informed consent to participate in the study, and investigations were conducted in accordance with the Declaration of Helsinki as revised in 2013. The study was approved by the French regulatory authorities (CPP Ile de France 1-2015 mars-DAP 18 and CPP Ile de France 10-2017 Janvier).

Table 1. Patients characteristics

	Cancer free group	Breast cancer group	p
n	14	17	
Age : range ; mean±sd	17-62 ; 43.8 ± 4.8	28-75 ; 54.6 ± 3,5	0,1532
BMI : range ; mean±sd	22.1-34.7 ; 30 ± 2.9	18.5-35.5 ; 26.9 ± 1.4	0,6192
Menopausal	9	10	
Pill or HRT	0	1	
Mammary density			
Low	7	9	
High	7	8	
Heterogeneous	0	1	
Cancer subtype	-		
Luminal A	-	7 (39%)	
Luminal B	-	10 (55,5%)	
HER2 overexpression	-	0	

Samples collection

For the cancer group, two samples of subcutaneous adipose tissue were collected, one at 1cm (Adjacent sample), another at a distance superior than 5cm (Distal sample) from the tumor. For the cancer free group, a unique adipose tissue sample (Normal sample) was harvested.

Cell lines and reagent

The human breast cancer cell lines MCF-7 obtained from American Type Culture Collection (ATCC, LGC Standards, France) was maintained in DMEM medium supplemented with 10% (v/v) FBS (fetal bovine serum) while the SUM159PT cell line was maintained in Ham's F12 medium supplemented with 5% heat inactivated FBS, 10 mM HEPES, 1 µg/ml hydrocortisone and 5 µg/ml of human insulin. The hTert-immortalized HMEC cell line was maintained in DMEM/F12 medium supplemented with 10% FBS, 10 ng/ml hEGF (human epidermal

growth factor), 0,5 µg/ml hydrocortisone, 10 µg/ml insulin, 0,5 µg/ml puromycin. All cell lines were maintained at 37 °C in a humidified atmosphere with 5% CO2.
All reagents were used at the following concentrations: Oil Red O: 0.6% (Sigma-Aldrich), SSO: 150µM (AbCam), BODIPY 493/503 (Thermofischer): 1 µM for microscopy imaging, DAPI (Euromedex): 1 µM.

Adipose-derived stem cell isolation, adipocyte differentiation and collection of conditioned media
ASCs were isolated by collagenase digestion and differentiated into adipocytes as previously described [14]. Lipid droplet analysis was performed on fixed, Oil Red O- and DAPI-stained cells with CellSens Dimension v1.16 (Shinjuku, Tokyo Japon). At least 90 cells were analyzed per condition [16, 17].

Western blot and ELISA
Samples for western blot were prepared, runned and immunostained as described in [14]. Antibodies directed against STAT3, P-STAT3, AKT, P-AKT, ERK, P-ERK and Vinculin were purchased from Cell Signaling Technology (Danvers, MA), were as the anti-actin-HRP antibody was obtained from Santa Cruz Biotechnology (Heidelberg, Germany). Leptin, adiponectin, IL6, MCP1 concentrations in the conditioned media were determined by using multi-analyte cartridge ELISA™ immunoassay according to the manufacturer's instructions (Bio-Techne, San Jose, CA, USA).

Cell proliferation
For the proliferation assay, cells were treated with control medium (DMEM) or adipocyte conditioned medium supplemented with 0.25% SVF. After Crystal Violet staining and solubilization in 1% SDS solution, OD were measured at 570nm using F200 Tecan device.

Cell migration, invasion and proliferation
This was performed using xCELLigence real-time cell analysis (RTCA) technology (Roche, Basel, Switzerland). For cell migration and invasion, cells in serum-free medium were added to the upper well of the CIM plate either uncoated (migration) or coated with a thin layer of Matrigel (Corning) basement membrane matrix. Control serum-free media or adipocyte conditioned media were used as chemoattractant in the lower chambers. The impedance value measured was expressed as the cell index (CI), divided by the background value.

Flow cytometry analysis
Cells cultured in control or adipose conditioned medium (ACM) supplemented with 2% serum for 48 hours were stained with antibodies against human CD36-APC (Beckman Coulter) at room temperature in the dark for 20 min. The labeled cells were analyzed on a FACS Gallios (Beckman Coulter) and data analysis was performed using Kaluza software.

Cytoplasmic lipid droplet staining
Cells were cultured in control or adipose conditioned medium supplemented with 2% serum and then were fixed with 4% paraformaldehyde for 10 min at room temperature. After three washes with PBS, the cells were incubated in the dark with 1 µM of Bodipy 493/503 (Thermofischer) and DAPI for 30 min at room temperature. The cells were subsequently visualized by a fluorescent microscope (Evos FL) with objective X40.

Adipokine array
Serum-free conditioned media was applied to an adipokine antibody array membrane (R&D systems) to detect relative levels of 58 adipokines and visualized on Chemidoc systems (Biorad). Protein expression was quantified by densitometric analysis of the immunoblots using Image Lab software developed by Bio-Rad.

Fatty Acid Methyl Ester Analysis
Fatty acid methyl esters (FAME) were prepared and assayed by GC-MS in the positive chemical ionization mode with ammonia as the reagent gas (GC6890-MS5975; Agilent Technologies, Les Ulis, France) as described previously [18]. The response factors of fatty acids were calibrated with a weighed mixture (FAME Mix Supelco® 37, Sigma-Aldrich Chimie, L'Isle d'Abeau Chesnes, Saint Quentin-Fallavier, France).

Statistical analysis
Data were expressed as mean ± SEM. Statistical analysis was performed using Mann-Whitney, Kruskal-Wallis test, two-way ANOVA or t test with GraphPad Prism 8 v8.0.2 (GraphPad Software, Inc).

Results

Participants and Samples Characteristics
Characteristics of the women who have participated in this study are detailed in Table 1. The cancer-free group included 14 women with a mean age of 43.8 (17-62) and a mean BMI of 30 (22.1-34.7). The case group included 17 women with a mean age of 54.6 (28-75) and a mean BMI of 26.9 (22.5-35.5). The intra-group heterogeneity

was important in terms of age, BMI, menopausal status in both cohorts. For mammary density, the two cohorts showed similar distribution between categories with almost half of women in low (B) or high mammary density (C and D) group. All patients from the case cohort had tumor with ER/PR expression a (Luminal A or B tumor). However, despite this heterogeneity, age and BMIs are not significantly different between the two groups.

Characterisation of ASC from healthy and breast cancer adipose tissue

ASCs from adipose tissue proximal and distal to the tumor and from cancer-free breast adipose tissue labelled as normal (N) were differentiated *in vitro* and the serum-free conditioned media were collected (ACM). The secreted adipokines were analyzed on human adipokine array. As shown in Fig.1 A, the levels of leptin were lower in ACM derived from proximal and distal adipose tissue compared to normal ACM. We did not detect any differences in adiponectin levels. No significant differences in adipokine secretion were observed between proximal and distal ACM. Consequently, we focused our work on the comparison between ASCs isolated from cancer-free women (N) and ASCs from proximal adipose tissue to the tumor (T). These results were confirmed by ELISA assays (Fig.1 B). We found an increase in Chitinase 3 like 1 level and a decrease in RBP4 levels in in ACM from tumor compared to normal ACM (Fig.1 C). These results were confirmed by Elisa and western blotting (Fig.1 D).

The differentiation capacity of the ASCs was assessed by Oil-Red-O staining. As shown in Fig.2 A, no significant difference in lipids accumulation were found after 14 days.

In addition, total free fatty acids (FFA) profiles were analyzed between the two groups (Fig.2 B). Interestingly, palmitic acid (C16:0) and arachidonic acid (C20:4) levels, were found elevated in the ACM from the tumor group oleic acid (C18:1 n-9) levels in the ACM from the tumor group were decreased compared to the normal group.

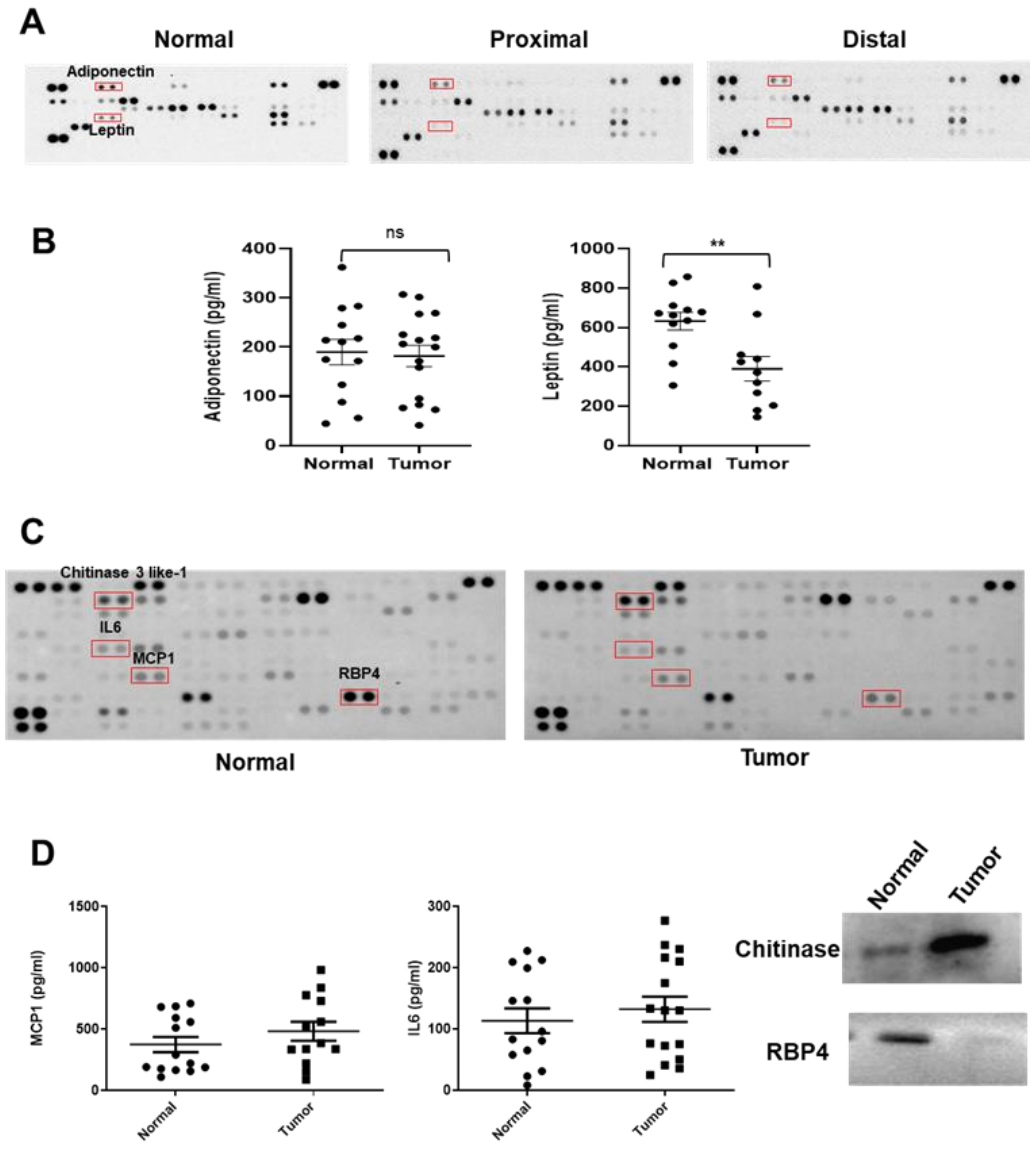


Fig.1 Analysis of adipokine and cytokine expression. Serum-free conditioned medium from the indicated adipocytes was applied onto human adipokine (A) or cytokine (C) array membranes. (B and D) Scatter plots of leptin, adiponectin, MCP1 and IL6 concentrations in conditioned medium are shown from 14 (normal) and 17 (tumor) differentiated breast adipocytes as measured by ELISA. **$p < 0.01$. Results are presented as means ± SEM. (D) Expression of secreted protein levels of RBP4 and Chitinase were measured by western blotting.

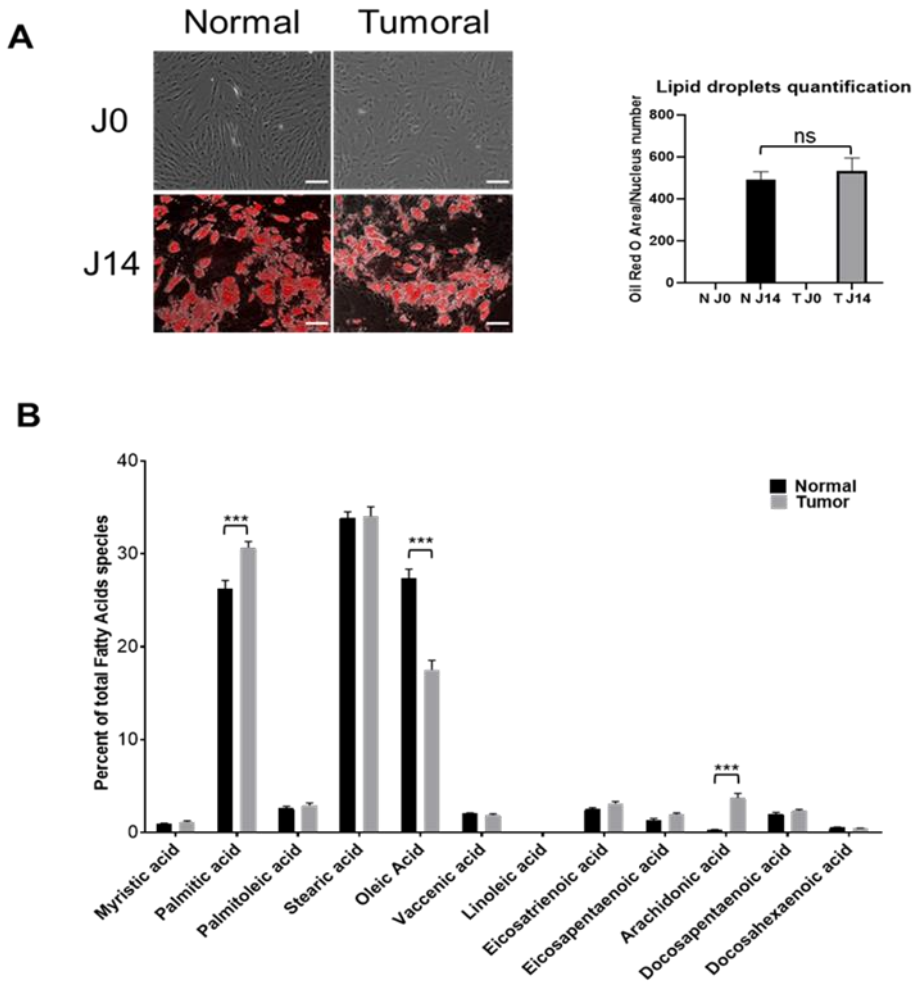


Fig.2 Adipocyte differentiation of ASCs populations. ASCs from normal and tumor proximal breast adipose tissue were differentiated into adipocytes for 14 days. (A) Monitoring of adipose differentiation was accomplished by microscopic imaging after Oil Red O-staining (left panel). Oil Red O-stained areas were then quantified by ImageJ analysis. Scale bars, 70 µm. Results are presented as means ± SEM (right panel). (B) Dosage of Fatty acids from total lipids. ***p<0.001. Results are presented as means ± SEM.

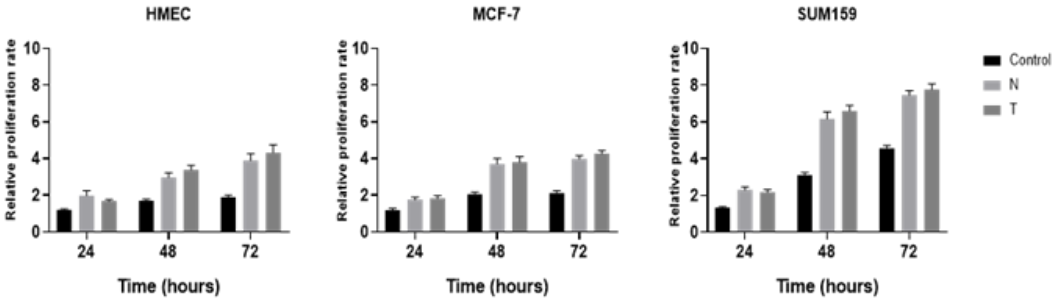
Breast ACM increase proliferation of mammary tumoral cells independently of ASCs origin

Then we evaluated the influence of the adipocyte secretome on the proliferation of normal mammary epithelial cells (HMEC) and breast tumoral cells corresponding to the luminal A (MCF-7) or the triple negative (SUM159) molecular subtypes (Fig.3 A).

ACM addition induced a strong proliferation increase in all cells, with the strongest induction found in SUM159 cells. Interestingly these findings were independent of the type of ACM.

To further understand the possible influence of microenvironment imprinting in tumor context we classified tumor bearing patients according to BMI, menopausal status and mammary density, critical factors known to be related to adipose inflammation of the breast [19-21]. As shown in Fig.3 B, we did not find any differences in the proliferative capacity of the three cell lines according to the type of ACM.

A


HMEC
MCF-7
SUM159
Relative proliferation rate
Time (hours)
24
48
72
Control
N
T

B

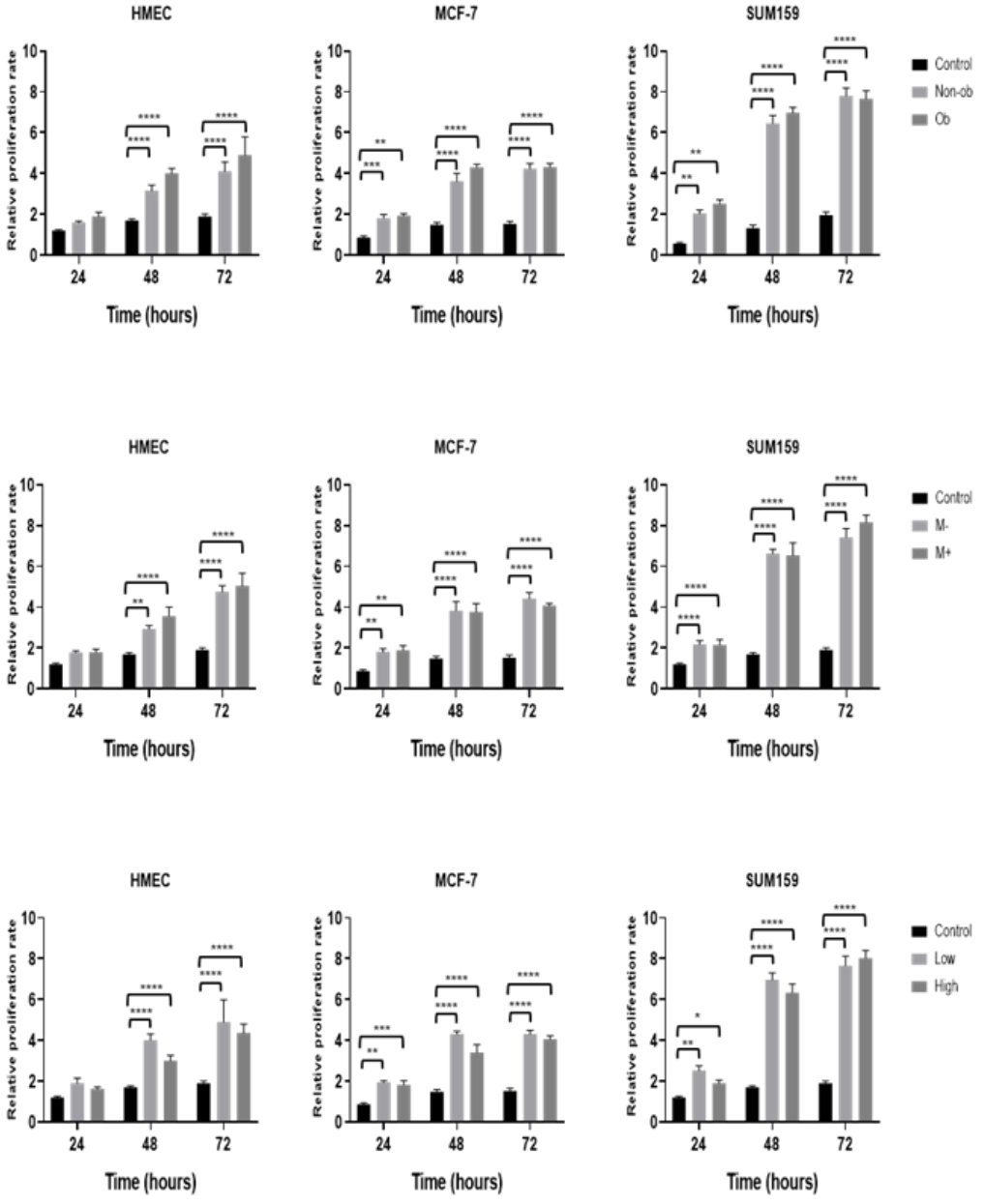

HMEC
MCF-7
SUM159
Relative proliferation rate
Time (hours)
Control
Non-ob
Ob
Control
M-
M+
Control
Low
High

Fig.3 Stimulation of tumor cells proliferation by adipocyte conditioned medium. (A) Histograms represent the proliferation of HMEC, MCF-7 and SUM159 cells treated with control medium (CTRL) or ACM from differentiated adipocytes from normal breast adipose tissue (N) or from tumor proximal adipose tissue (T). The proliferation was assessed using Crystal Violet assay. Data are representative of 3 individual samples from 3 independent experiments. (B) Histograms representing the proliferation of HMEC, MCF7 and SUM159 cells treated with control medium (CTRL) or conditioned medium from ASCs differentiated into adipocytes from tumor bearing breast adipose tissue with the indicated characteristics. (Non-ob; BMI<30) or obese women (Ob; BMI>30), non-menopaused (M-) or menopaused women (M+) and women with low (Low) or high mammary density (High). Results are presented as fold of time 0 (T0) which represent the basal level of untreated cells. ns: non-significant; *p < 0.05; **p < 0.01; ***p <0.001; ****p <0.0001. Results are presented as means ± SEM.

Next, we investigated which proliferative signaling pathways were activated in the different cell lines when treated with the different ACM. HMEC cells showed no detectable activation of any signaling pathways analyzed. On the contrary, MCF-7 and SUM159 cell line showed a clear activation of the AKT signaling pathway, however activation of both STAT3 and ERK1/2 signaling pathways was only observed in the SUM159 cell line (Fig.4). Taken together, these results indicate that adipocyte secretome increase both normal and tumoral mammary epithelial cells proliferation independently of ASCs origin with the activation of different proliferation and survival pathways depending on the mammary cell type.

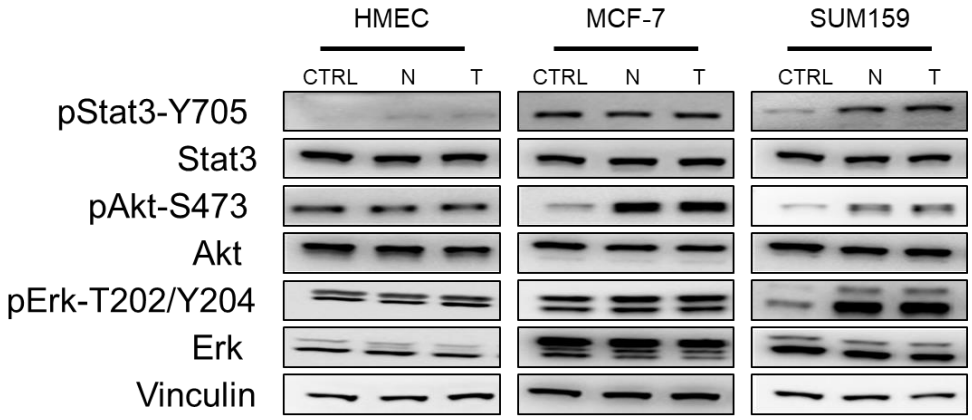


Fig.4 Proliferation and survival pathways activation analysis. Whole cell extracts were pre-pared from HMEC, MCF7 and SUM159 cells treated for 1 hour with either control medium or the indicated ACM and characterized by Western blot analysis.

Breast ACM increase migration and invasion of mammary tumoral cells independently of ASCs origin

Afterward, we analyzed the influence of ACM type on the migratory and invasive capacities of normal and tumor cells (Fig.5). No migration or invasion was detected for HMEC under any condition (data not shown). MCF-7 cells showed no significant effect on cell migration independently of the ACM type, in contrast the SUM159 cells showed a clear increase in migration with normal and tumor ACM (Fig.5 A). Similar results were obtained with invasion assay, with no effect on MCF-7 cells and an increase in SUM159 independently of the ACM types (Fig.5 B).

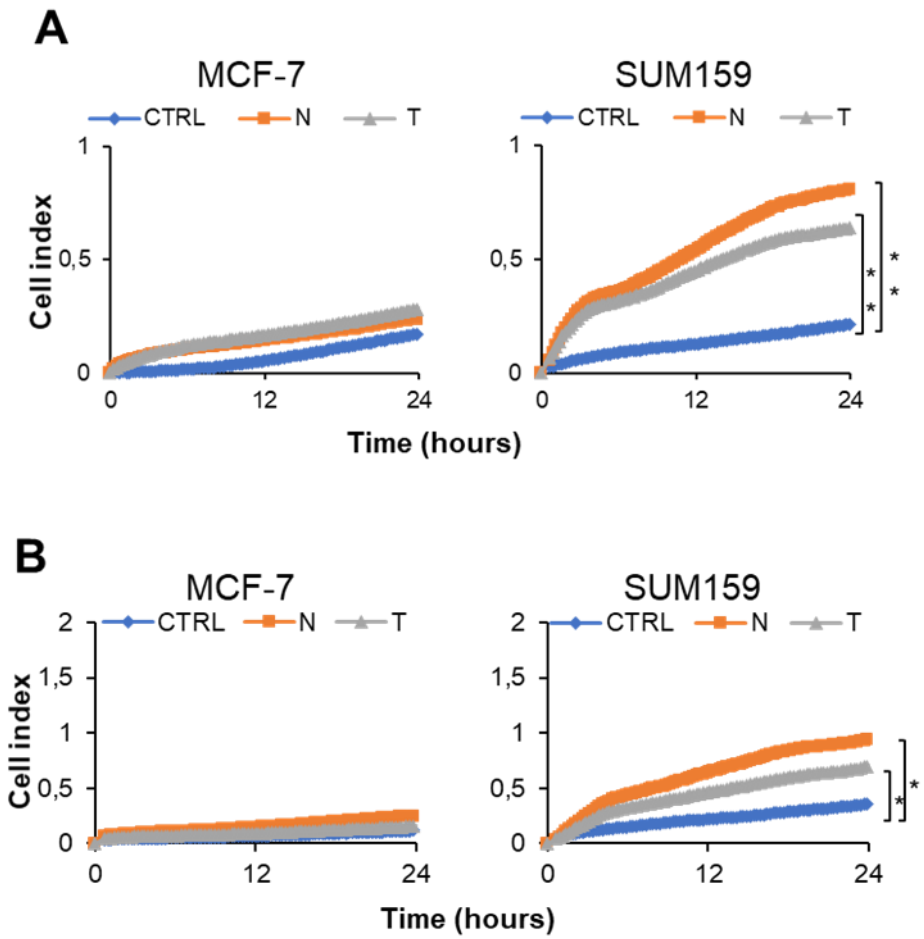


Fig.5 Migration and invasion of tumoral breast cells treated with adipocyte conditioned medium. Dynamic real time monitoring the migration (A) or invasion (B) of MCF-7 and SUM159 cells towards either control media or ACM from differentiated adipocytes from normal breast adipose tissue (N) or from tumor proximal adipose tissue (T). Results are expressed as mean +/- SEM for 3 individuals from 3 independent experiments. *p < 0.05; **p < 0.01

ACM increase the lipids droplets content of breast cancer cells

Next, we have compared the lipid droplets accumulation in the different cell lines treated with ACM from the two groups.

As shown in Fig.6, Bodipy staining revealed no lipid accumulation in HMEC cells. In clear contrast, MCF-7 cells showed a significant increase in lipid droplets while SUM159 cells showed no cytoplasmic lipids accumulation which is consistent with previous findings [14].

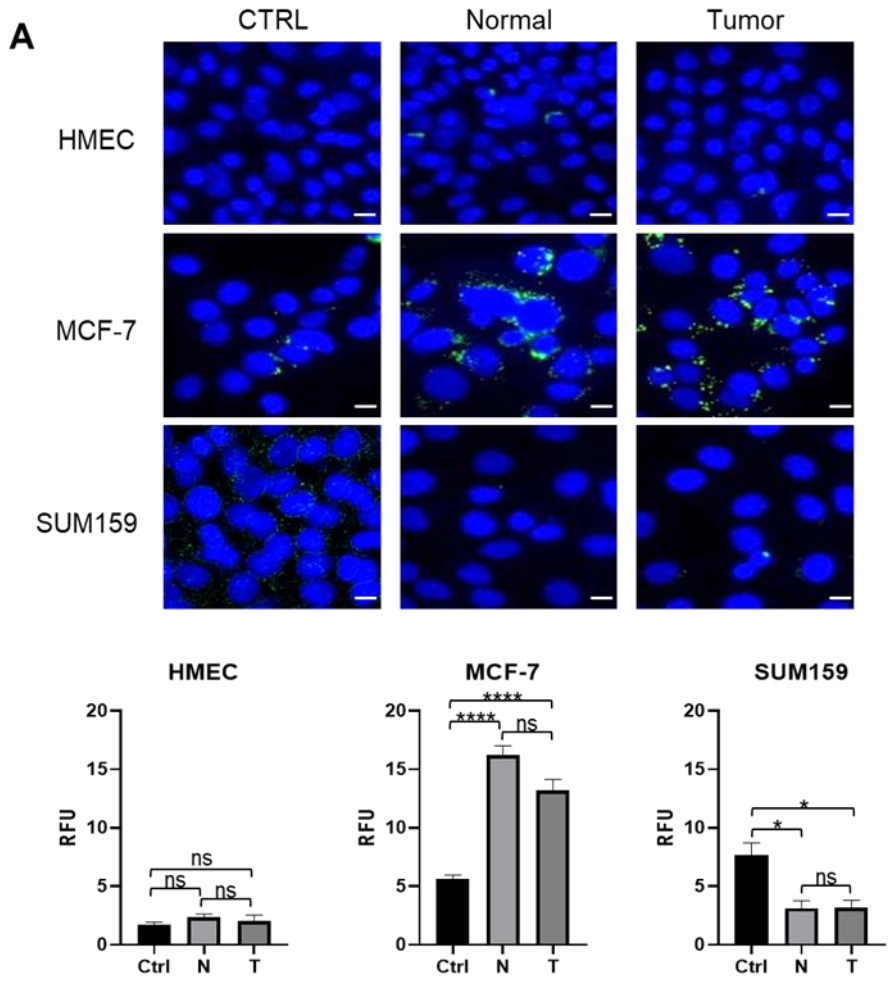


Fig.6 Influence of adipocyte conditioned medium on lipids droplets accumulation in breast tumor cells. Representative images of HMEC, MCF-7 and SUM159 cells cultured with control medium or ACM from differentiated adipocytes from normal breast adipose tissue (N) or from tumor proximal adipose tissue (T) for 24h followed by Bodipy staining. Scale bars, 10 µm (upper panels). The total lipid droplet area was calculated with ImageJ (lower panels). Results are presented as means ± SEM. *p < 0.05; ****p < 0.0001.

Adipocyte secretome up-regulates CD36 surface expression in tumor cells

To evaluate the CD36 surface expression in presence of ACM, flow cytometry analysis on cells incubated with ACM were performed. We did not detect any significant modulation of CD36 expression in HMEC cells but a clear although moderate increase in MCF-7 cells and a strong augmentation in SUM159 (Fig.7). The induction of surface CD36 expression was similar, independently of which ACM was used.

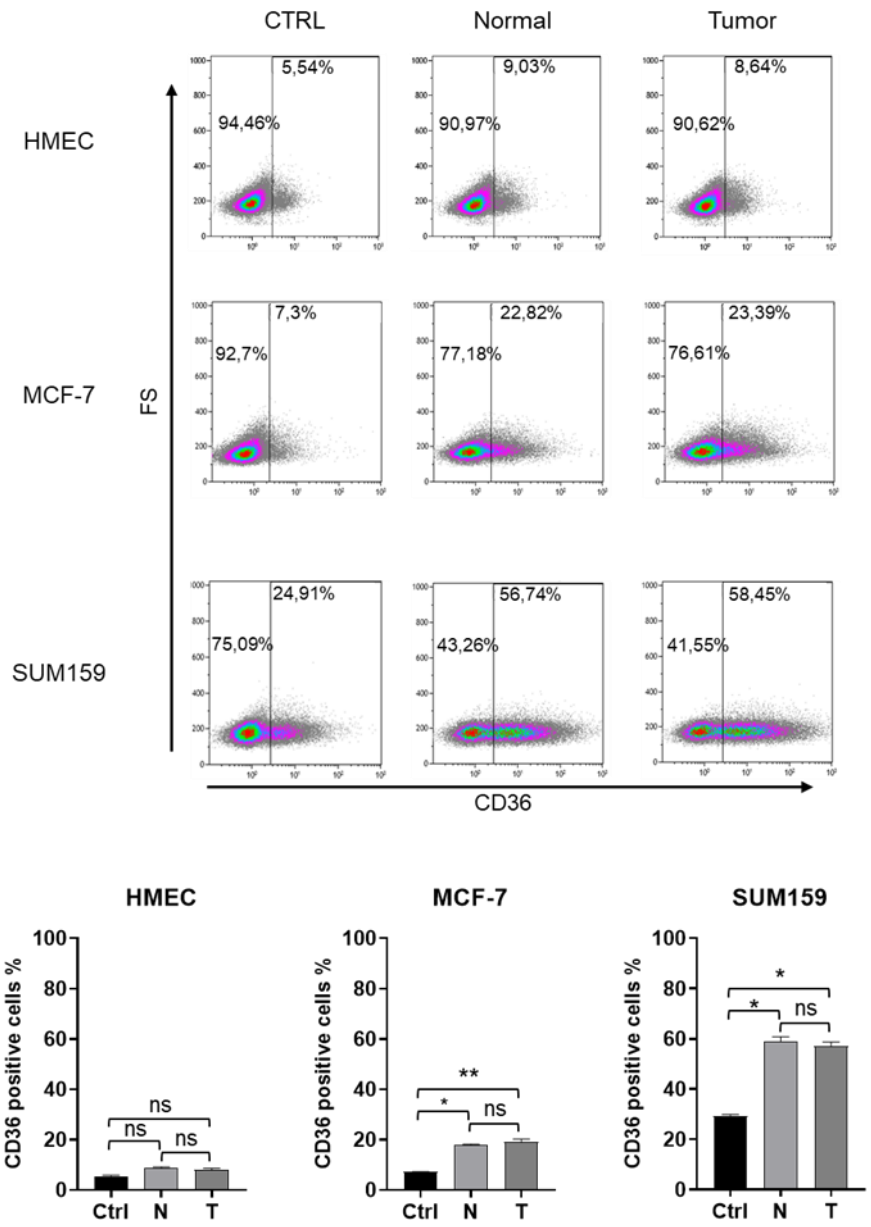


Fig.7 CD36 surface expression in normal and tumoral breast cells. HMEC, MCF-7 and SUM159 cells were cultured with indicated medium for 24h then labelled and subsequently cell surface CD36 expression was determined by flow cytometry for. Results are presented as means ± SEM. *p < 0.05; **p < 0.01.

Discussion

This study aimed to characterized the difference between differentiated adipocytes from ASCs isolated from tumor bearing from those of tumor-free breast adipose tissue. Our results show lower leptin levels in the tumor secretome and increased palmitate and arachidonic acids levels with lower oleate levels in the tumor group compared to normal group. Interestingly, polyunsaturated fatty acids (PUFA) n-6 such as arachidonic acid are associated with inflammation and cancer development, whereas PUFA n-3 such as docosahexaenoic acid (DHA) have anti-inflammatory and anti-cancer properties [22-24]. Our results showed that increased arachidonic acid content is not counterbalanced by DHA content in tumor adipose tissue. Furthermore, the secretion of adipokine is similar when the adipose samples were taken at a distance of 0.5-1 cm from the tumor or at a distance $\geq$ 5 cm. Interestingly we found lower levels of Retinol Binding Protein 4 (RBP4) and higher levels of Chitinase 3 like-1 (CHI3L1) in the tumor ACM groups. Numerous studies have shown that leptin have multiple roles on breast cancer as it can drive the breast cancer microenvironment, promote the breast cancer cells growth and invasion through activation of ERK1/2, PI3K-Akt and JAK2-STAT3 signaling pathways [25, 26]. RBP4 is an adipokine that belongs to the family of lipocalin. These lipophilic transporters specifically transport the retinol. We found higher RBP4 levels in normal adipocytes conditioned media which is confusing as higher RBP4 serum levels has been described as a potential breast cancer risk factor [27]. However, RBP4 serum levels cannot be directly correlated with RBP4 levels measured in adipocyte conditioned media because many cells types could contribute and regulate the RBP4 serum levels. Interestingly, a study shows that the level of RBP4 is lower in sera from patients with hormone receptor positive breast tumors levels compared to hormone receptor negative ones [27].

CHI3L1 is a non-enzymatic protein of the glycoside hydrolase family 18 that binds chitin. Many studies have shown a strong correlation between CHI3L1 and stages and outcomes of numerous cancers [28-30]. CHI3L1 has been reported to promote cancer cells growth, proliferation and metastasis but also angiogenesis, vasculogenic mimicry and tumor associated inflammation [31]. Increased adipose tissue gene expression levels of YKL-40 in obesity and T2D have been reported, being proposed as a new inflammatory marker related to insulin resistance [32, 33]. Moreover, circulating levels and adipose tissue expression of YKL-40 have been strongly associated with the inflammatory factors C-reactive protein (CRP), IL-6 and MCP-1 [32, 34]. Our findings show that an elevated CHI3L1 levels is also observed in breast adipose tissue particularly in a tumor environment, although we did not observe an increase in IL-6 and MCP-1 level.

Neither the proliferative, migratory or invasive abilities of normal or tumor cell lines distinguishes the effect of ACM from normal or tumor environment. To investigate further, we classified the ACM into subgroups according to BMI, menopausal status and mammary density to evaluate these parameters in an oncogenic context. This classification didn't allow to discriminate adipocyte origins.

Neither, STAT3, Akt and ERK pathways analysis nor lipids droplets and nor CD36 expression were consistent with ACM type.

These results are surprising, but it is noteworthy to consider the intragroup heterogeneity, particularly the differences in subjects 'median age and BMI and the limited size of the two groups that could occult some observation. In addition, we focused on ASCs derived adipocytes, yet adipose tissue is complex and multicellular and his modifications following his cross-talk with the tumor may not be fully represented with only differentiated adipocytes.

The use of differentiated ASCs as adipocyte models in our work can be questioned since some studies use mature adipocytes instead. The ASC used in our work come 32 different patients. These ASC were compared between them, and no significant difference was shown, either in their differentiation capacity or in their secretome. These ASCs are also exposed in the adipose tissue to the same environment as mature adipocytes and therefore marked with the same tissue imprint. Other studies use ASCs, and these also show similar results in stimulating tumor progression [35-37]. As such, it is more likely that the effect of adipocytes on tumor progression comes from the influence of components of the tumor microenvironment, rather than from an intrinsic phenotypic change in adipocytes. Furthermore, we have recently shown that ASCs from myeloma patients display similarly defective osteoblastic differentiation than MSC in bone marrow [38, 39].

Finally, the lack of adipose tissue samples from triple negative and HER2 overexpressed women, which is fortunate for the women of our cohort, could be an issue that must be addressed in our future work to have a complete understanding.

Conclusions

In conclusion, the data presented here show that conditioned media of adipocyte obtained after differentiation of ASC from breast adipose tissue containing a tumor is not fundamentally different from that of healthy breast tissue. Breast tumor imprinting persistence *in vitro* on the surrounding tissue should be further studied as it may be a critical factor for adipocytes/tumor cells studies. On the other hand, these findings would offer a positive and encouraging possibilities, as the loss or absence of tumor imprinting on breast adipose ASCs may be an opportunity of regenerative medicine for women undergoing mastectomy or lumpectomy as it could be a quick, available, compatible and safe source of graftable breast adipose tissue.

Conflicts of Interest: The authors declare no conflict of interest

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