TREBALL DE FI DE GRAU

BACHELOR'S DEGREE OF BIOCHEMISTRY AND MOLECULAR BIOLOGY

EXOSOMES, CROSSTALK MEDIATORS OF THE BREAST CANCER MICROENVIRONMENT

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This project is based on the results obtained over my curricular and extracurricular practices carried out in URLA's laboratory of IISPV, at Rovira I Virgili University, with the authorization of Sandra Guaita.

I would like to thank all the people of this group who have worked with me making possible this project. I would like to thank all the PhD students and technicians who have supported and encouraged me to not surrender these pasts months. Specially, I would like to thank Jose and Roser, for taking care of me whenever I needed to. Concluding, to my family and specially, to my supportive and beloved mother. Thank you for everything.

"We must have perseverance and above all confidence in ourselves. We must believe that we are gifted for something, and that this thing must be attained"

Marie Curie

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0. ABREVIATIONS

AKT. Protein kinase B.

BC. Breast cancer.

BCC. Breast cancer cells.

BRCA1/2. Breast cancer associated gene 1 and 2.

CAAs. Cancer associated adipocytes.

CAFs. Cancer associated fibroblasts.

CD36. Cluster of differentiation 36.

CM. Conditioned media.

DMEM. Dulbecco's modified Eagle media.

ECM. Extracellular matrix.

EGFR. Epidermal growth factor receptor.

ER. Oestrogen receptor.

FATP. Fatty acids transporter proteins

FBS. Foetal bovine serum.

HER2. Human epidermal growth factor 2.

MAPK. Mitogen-activated protein kinase.

PI3K. Phosphoinositide 3-kinase.

PR. Progesterone receptor.

TNBC. Triple negative breast cancer.

TME. Tumour microenvironment.

1. ABSTRACT

Adipocytes are the most abundant cell type in the breast tissue; hence, they play an important role in the tumour microenvironment crosstalk. Exosomes are being discussed as novel participants of the communication between cells in diverse biological processes, owing to the fact that they transfer cellular components to paracrine cells. This role opens up to a new issue of the participation of adipocyte-derived exosomes in the crosstalk of the tumour microenvironment. Previous research done in this laboratory has been focused on the study of the fatty acid-binding protein 4 (FABP4), due to the implication of this protein in the uptake, metabolism and homeostasis of fatty acids. Moreover, previously results done by this laboratory demonstrated that high levels of FABP4 detected in breast cancer patients lead to a less favourable prognosis. For this reason, this study aims to examine the role of this protein in adipocytes-derived exosomes.

For our different assays we worked with breast cancer cell lines MCF-7 and MDA-MB-231, as well as mature adipocytes. To examine the lipid droplet content, we stained mature adipocytes in these conditions by Oil Red assay. Moreover, we performed ELISA assay to analyse the levels of FABP4 and CD36 in the media.

On the other hand, in order to study the role of exosomes in tumour microenvironment, we isolated adipocyte-derived exosomes after their treatment with different conditions (Control, MCF-7 CM and MDA-MB 231 CM) and further we analyse the protein content of these exosomes by Western Blot.

We found that cancer-associated adipocytes release more fatty acids to the media, accompanied by the releasement of fatty acids transporter proteins and a higher number of exosomes than adipocytes. Moreover, the cargo of fatty acid transporters FABP4 and CD36 was increased by the influence of tumour cells.

To sum up, these results provide new insights into the role of FABP4 and CD36 within adipocyte-derived exosomes in the crosstalk of the tumour microenvironment.

2. OBJECTIVES

The aim of this project is to discuss the paper exosomes in the microenvironment of breast cancer. In order to do this, the following objectives need to be achieved:

a. To stablish a standardized method for exosome extraction and quantification.
b. To characterize the composition of FABP4 and CD36 within the exosome derived from mature adipocytes and CAAs.
c. To determine the effect of mature adipocytes and CAAs-derived exosomes in breast cancer cell lines MCF7 and MDA-MB 231.

3. INTRODUCTION

3.1. BREAST CANCER OVERVIEW

In 2018, there were 268,670 new breast cancer (BC) cases in the US and 41,400 deaths were BC-related. This data represents an increase of almost a 39% over that from 2009. It also represents the 30% of the cancer diagnosed in women during the last decade (1). In 2020, the diagnosis and treatment of all types of cancer were hampered by the COVID-19 pandemic, this means an estimation of 284,200 new cases and 44,130 deaths of BC in the US in 2021(2). Therefore, incidence rates of BC increases by about 0.5% per year (1,2).

Metastasis is responsible for approximately 90% of cancer-related deaths. Moreover, 25-50% of breast cancer patients will eventually develop deadly early metastasis, occurring even decades after the time of diagnosis and primary tumour removal (3).

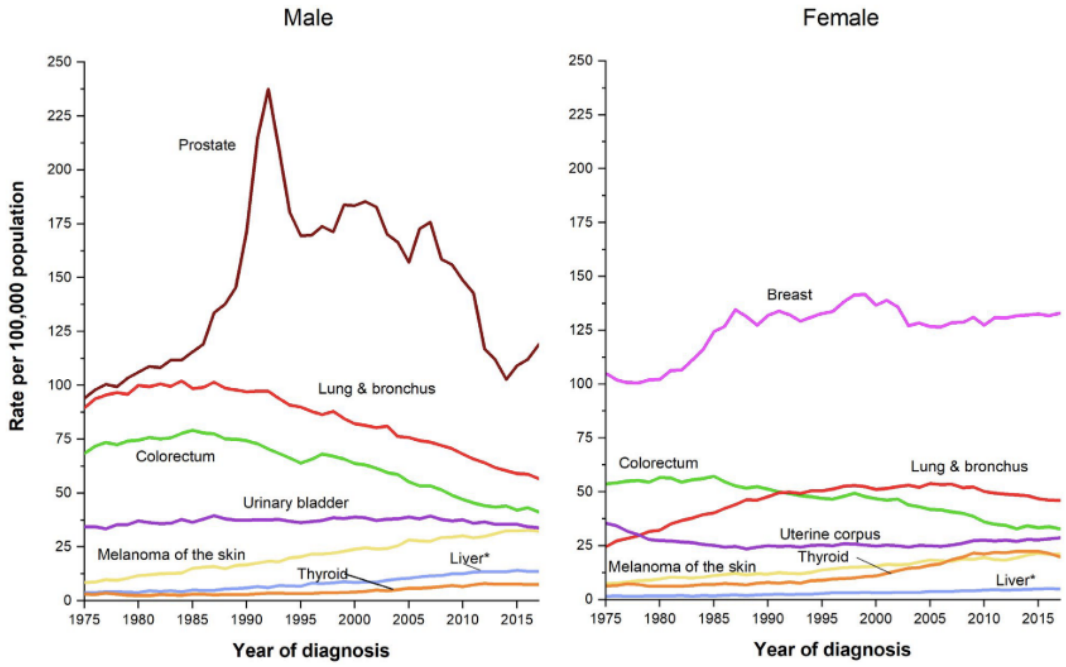

Figure 1. Incidence rates for different cancers by sex in the US population, since 1975 to 2017. Rates are adjusted to the US standard population in 2000. Breast cancer is shown as the cancer with the highest incidence in women, trending to an increase of 0.5% per year. Adapted from (2)

BC treatment is heavily influenced by histological and molecular characteristics of the tumour. According to tumour molecular characteristics, a stratification of BC into different subtypes has been stablished: receptor-positive, which express

oestrogen receptor (ER),progesterone receptor (PR) or human epidermal growth factor 2 receptor (HER2); meanwhile, triple-negative tumours are those which do not express ER, PR nor HER2 (4,5). ER and PR are nuclear sex steroid receptors that stimulate the growth of normal and neoplastic breast epithelium, which are expressed in approximately 75% of all BC. The expression of these receptors is related with a better prognosis indicator for the responsiveness to hormonal therapy (4). Approximately 10-15% of BC are triple-negative breast cancers, being usually associated with a poor prognosis due to the ineffectiveness of target therapies (6).

The initiation mechanism of BC is still unknown, although there are different risk factors have been stablished, both genetic and environmental, which can increase the possibility of developing BC (4,7).

Genetic-associated tumours represent only 10% of all tumours (4,8,9). BRCA1 and BRCA2, both genes which encode tumour suppressor proteins, are closely related with hereditary BC and mutations in these genes increase the risk of suffering BC. Actually, about 5-10% of all BC are caused by mutations in these genes (8). In addition, ERRB2 is a gene whose amplification causes an activation of the HER2 pathway in 13-15% of BC. The overactivation of HER2 pathways enhances the proliferation, cell survival, metastasis and adhesion through pathways such as PI3K, AKT and MAPK (4,8). Thus, monoclonal antibodies and immunotherapy against HER2 have shown to be effective (4,6,7). Furthermore, other associated genes have been described as BC initiating oncogenes, such as EGFR, c-Myc and Ras (8).

Nonetheless, environmental factors play an important role in the majority of BC cases, as about 90-95% of the cases don't follow a genetic profile mutation (4,8,10). The incidence of BC is highly related to the increasing age, besides nearly 25% of cases are related to family history. Moreover, reproductive factors related to menstruation (early menarche and later menopause) and procreation (nulliparity, late age at first birth and number of them) are highly related with the ER status and the BC progression. Furthermore, both endogenous (premenopausal state) and exogenous oestrogen (oral contraceptives and hormonal replacement therapy) are highly associated with BC risk. Alcohol

intake, high fat diets, weight and body fat distribution also are related to a major risk of BC (8). However, breastfeeding and physical activity have been indicated as protective factors (8,10). Obesity also arises the risk of developing breast cancer through chronic inflammation, being associated with poor prognosis and with an increase the risk of death in both premenopausal and postmenopausal BC patients (11,12).

3.2. TUMOR MICROENVIRONMENT

Nowadays, tumours have increasingly been recognized as organs whose complexity approaches and may even exceed that of normal healthy tissues (4,13). The tumour is surrounded by complex tissues that contain miscellaneous cell types (malignant and non-malignant) and their extracellular matrix (ECM), which interactions create the tumour microenvironment (TME) (14,15). Intercellular communication is driven by a complex and dynamic network of signalling molecules and enzymes against a background of major perturbations to the physical and chemical properties of the tissue (15). BC's microenvironment is characterized by a substantial inflammatory state and an activated phenotype of fibroblasts, which contribute to the nature of the neoplastic disease (16).

Hypoxia also has an important role in the TME, it has emerged as an essential factor in cancer because its promotion of tumour initiation, progression and resistance to therapy (17). This contribution to tumour progression has been described in several independent pathways that may be interconnected, therefore changes in metabolism, inflammation or other tumour hallmarks (18).

Cancer associated fibroblasts (CAFs) are crucial in TME. By the releasement of specific proteases and metalloproteinases into the extracellular space to alter the milieu, CAFs are able to enhance tumour progression (4,14).

The breast is compound by a vast majority of adipocyte cells, which are supplied by a network of nerves, connective tissue, blood and lymph vessels (19). Actually, BC TME is mainly formed by adipocytes (17). Adipocytes, as secretory cells, release diverse adipokines that actively aid tumour progression by the recruitment of malignant cells and immune cells that contribute to chronic

inflammation and promote the development of tumour cells by providing fatty acids as source of energy (14,15).

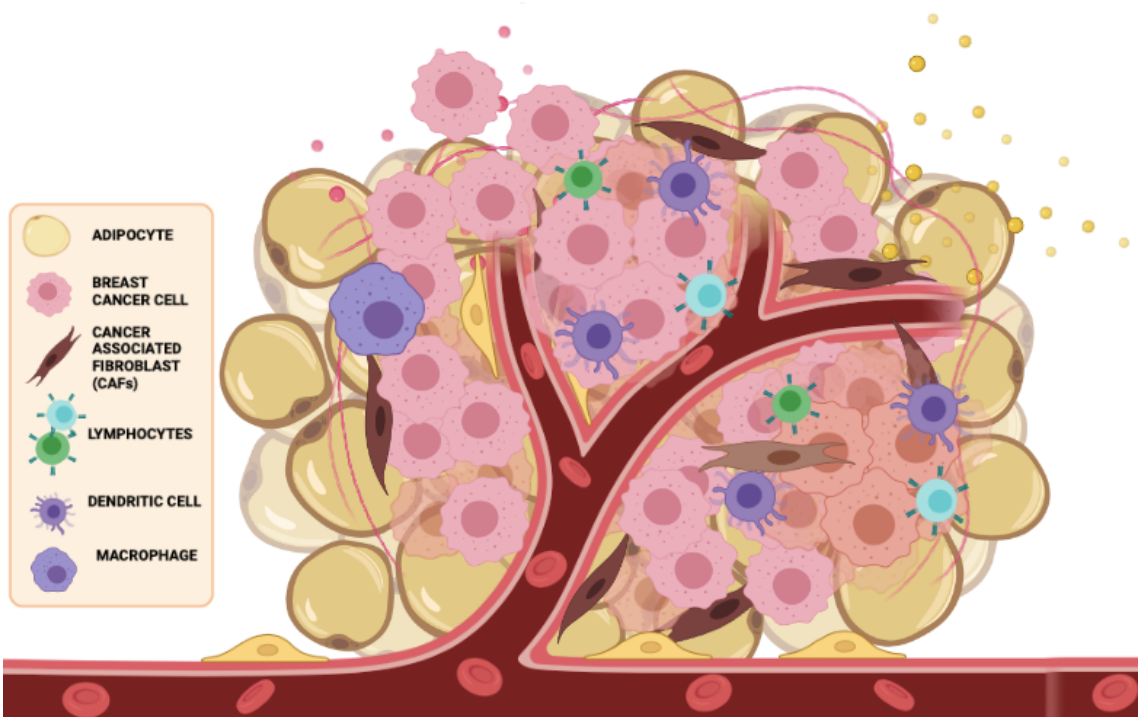

Figure 2. Breast cancer tumour microenvironment. The tumour lays in a vast majority of adipocytes, having a stretch crosstalk with this cell type. Moreover, other cells such as immune cells (including macrophages, dendritic cells and lymphocytes) and cancer associated fibroblasts (CAFs) compound the TME of BC. Adapted from Bio Render.

3.3. ADIPOSE TISSUE AND BREAST CANCER

Obesity, defined as a superior body mass index as 30 kg/m^2, is a major health problem which affects in different scale to the 38% of the world population (11,12). In women, a relation between obesity and different types of cancer has been stablished, including endometrial, renal, colon and breast cancer (17). It has long been recognized that there's an existing link between obesity and increased risk of receptor-positive post-menopausal BC but also with pre-menopausal TNBC in obese women (12).

The metabolic disturbances that characterize obesity result from disruption of energy balance, which causes stress and a dysfunctional state to the tissue (20). As said above, the tumour microenvironment is mainly formed by adipocytes (14,15).Adipocytes present in the tumour microenvironment are characterized by lipid droplet modification and a diminished size due to increased lipolysis,

overexpression of proinflammatory cytokines, distinct gene expression profile, higher browning activity and a decrease in adipocyte markers. These modified adipocytes are named cancer-associated adipocytes (CAAs) (1,11,17,21–23).

Therefore, the crosstalk between the tumour and the adipose tissue may be increased in obesity because of the alteration of normal adipokines pattern added to the fact that tumoral cells stimulate lipolysis and fatty acids mobilization (17) . Therefore, the interest in the study of adipose tissue in BC and the role of the adipocytes and their adipokines in cancer development and progression has increased as not only energy producers but also activators of different metabolic pathways (17,23).

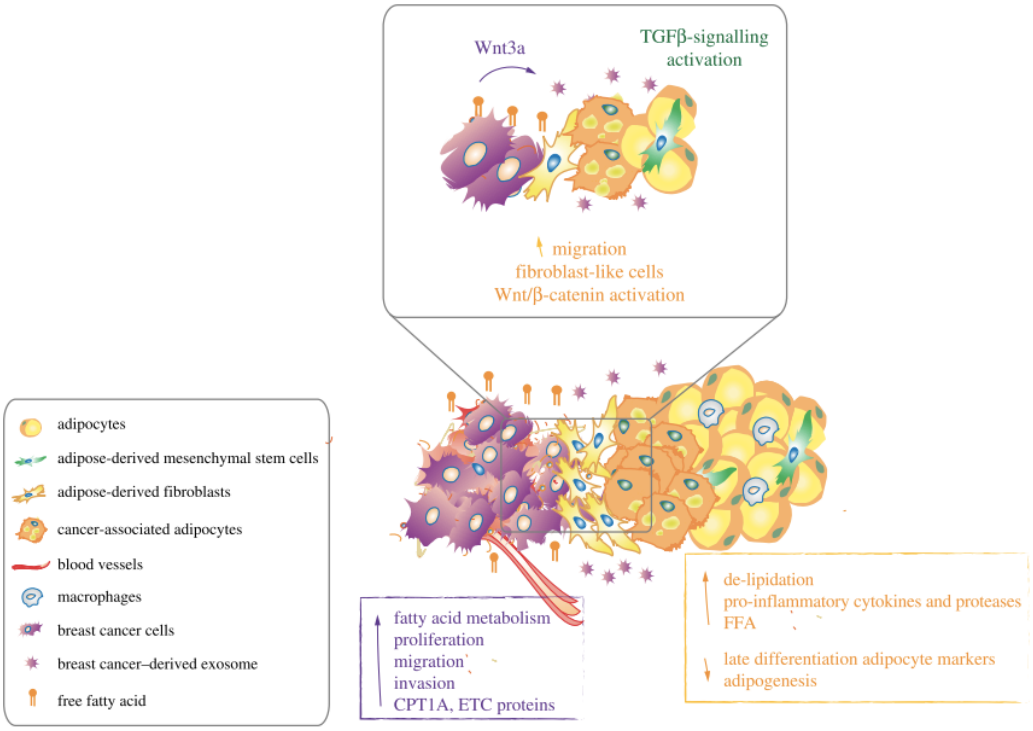

Figure 3. Adipocytes exposed to the tumour become into cancer associated adipocytes (CAAs). The peritumoral adipocytes change their phenotype, being characterized by a reduced cell volume, a gain of an irregular shape with smaller and dispersed lipid droplets and an increased delipidation. These phenotype modifications have an important role in tumour progression and metastasis. Adapted from (24).

As explained before, CAAs have a diminished size due to the increased lipolysis and the releasement of fatty acids to the peritumoral space. This transport is partially carried out by fatty acid-binding proteins (FABPs), a family of proteins that transport hydrophobic compounds (17,25). FABP4 is highly expressed by adipocytes of the white adipose tissue and brown adipose tissue, but also

expressed in macrophages, endothelial cells and some tumour cells (17). The production of FABP4 is regulated by fatty acids, insulin and peroxisome proliferator activated receptor-γ (PPAR- γ) agonists. FABP4 can also act as adipokine-like paracrine signalling molecule modifying homeostasis and cell function. The role of this protein in different pathologies such as obesity, type 2 diabetes, metabolic syndrome, atherosclerosis and breast cancer (23,26).

The important role of FABP4 in cancer has been described, enhancing the tumour initiation and progression by transporting the fatty acids from the CAAs to the cancer cells, inducing their oxidation to fuel tumour growth (23).

CD36 is a protein which can exhibit different signalling functions, such as scavenger receptor that binds to diverse ligands including fatty acids. This protein is expressed in tumour cells, immune cells and adipocytes and plays essential roles in lipid homeostasis (22). The expression of CD36 is upregulated in overweight, obesity and type 2 diabetes in the adipose tissue (27). Furthermore, this protein is increased during late stages of cancer, participating in angiogenesis, adhesion and metastasis of cancer (28). The inhibition of CD36 in tumour cells suppresses the epithelial-mesenchymal transition (EMT) and attenuated migration and invasion; this leads to the key role of CD36 not only as receptor of FA but also in the malignant phenotype (22,29). However, a new field is getting interest in cancer development in the TME context.

3.4. EXOSOMES

Exosomes are nano-sized vesicles released by all cell types with an endosomal origin. Not long ago, exosomes were regarded as a form of cellular garbage bags that carried excess or non-functional cellular contents. However, recent studies described exosomes as carriers of functional molecules, providers of shelter to the transported molecules and with an important role in intercellular communication. These vesicles present a wide heterogeneity in size, number and content as a result of the reflection of the phenotypic state of the cell which generates them (21,30–32).

The intercellular communication partially depends on the content of the exosome. Some proteins are consistently associated with biogenesis and membrane

markers of exosomes, such as ALIX, FLOTILIN or CD63, indirectly arguing for specificity in the exosomal protein components (21,30). In addition, different types of RNAs, including miRNA, in exosomes are dynamic mediators of intercellular communication. This miRNA have the ability of modulating cellular activities by modification the transcriptome of recipient via cell fusion with the exosome (30,31).

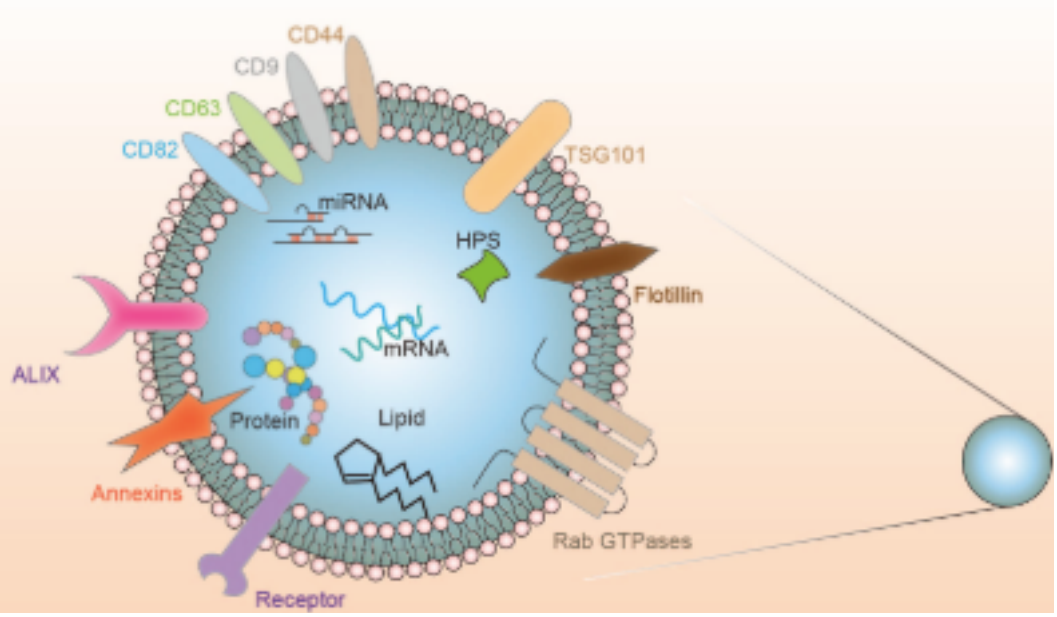

Figure 4. Exosome structure. Exosomes are small membrane bound vesicles which share a similar topology to the plasma membrane. Exosomes contain a variety of RNAs, cytosolic proteins, metabolites, hormones and bioactive lipids. The lipid bilayer membrane structures of exosomes contain typical transmembrane proteins which include receptors, proteins involved in exosome biogenesis (ALIX), membrane trafficking proteins, adhesion molecules, the lipid raft associated proteins as Flotillin-1, etc. The content from the exosomes is a reflection of the phenotype of the original cell, and it can be transferred to their target in the microenvironment. Adapted from (33).

Exosomes have emerged as a potential source of information to detect cancer and on potential regulatory drivers of tumour progression and metastasis (30). In BC, detachment of cells is an intense stimulation for the secretion of exosomes which transfer signalling molecules to each cell type within the tumour microenvironment enhancing tumour progression (31). Micro RNAs found in exosomes have been investigated as biomarkers in different cancers considering that exosomal miRNA profiles parallel those of the originating tumour cells, thus exosomes play a critical role in the development of tumours (21,32).

Adipocytes are able to enhance tumour metabolism towards the use of exosomal content, regulating BC cell proliferation, migration and apoptosis via intracellular transfer of oncoproteins (30,34). Exosomes secreted by tumour cells play a crucial role in the tumour microenvironment communication, being responsible for the transfer of proteins and genetic material to recipient cells, whom acquire a pro-tumorigenic phenotype (24). For instance, exosomes from cancer cells help CAFs to acquire protumorigenic properties while CAF-derived exosomes also promote cancer progression (30).

Furthermore, exosomes play a critical role in BC therapy. It has been demonstrated that exosomes provided by CAFs may confer chemotherapy and radiation therapy resistance to tumour-initiating cells via activation of STAT1-dependent antiviral signalling and NOTCH3 signalling (30,35). Otherwise, the utility of exosome as nano vehicles for therapeutic agents is being actively explored to deliver micro RNAs to specific targets. For instance, delivery of let-7 miR by modified exosomes targeting EGFR+ breast tumours reduced tumour size in mice (30).

4. MATERIALS AND METHODS

4.1. CELL CULTURE

3T3L1 were cultured in Dulbecco's modified Eagle's media (DMEM) High Glucose 10% foetal bovine serum (FBS) and incubated at 37ºC with 5% of CO_2 until they reached confluence. Afterwards, cells were differentiated for 10 days using DMEM High Glucose supplemented with 1 µm of dexamethasone (Sigma), 0.5 mM of 3-isobutyl-1-methylxanthine (IBMX) (Sigma) and 10 µg/mL of insulin (Sigma). After the differentiation, mature adipocytes were cultured with the necessary conditions for each experiment.

Luminal A cell line MCF-7 and TNBC cell line MDA-MB-231were provided by Dr. Eric Lam (Imperial College London). These cells were maintained in DMEM (Biowest-Labclinics) High Glucose supplemented with 10% FBS, 1% penicillin-streptomycin (Biowest-Labclinics) and 1 mM of non-essential amino acids (NEAAs) (Biowest-Labclinics) and incubated at 37ºC with 5% of CO_2.

4.1.1. CONDITIONED MEDIA OBTAINMENT

For CM obtainment, MCF-7 and MDA-MB-231 were cultured until they reached 80% of confluence. Then cells were deprived with DMEM High Glucose without FBS. After 24 hours of incubation, MCF-7 CM and MDA-MB-231 CM were collected.

Mature 3T3-L1 adipocytes were deprived for 60 hours with DMEM High Glucose without FBS. Then, the media was collected to be used as adipocyte CM. In order to obtain CAAs CM, mature adipocytes were cultured with MCF-7 CM or MDA-MB-231 CM for 60 hours, then CM was collected.

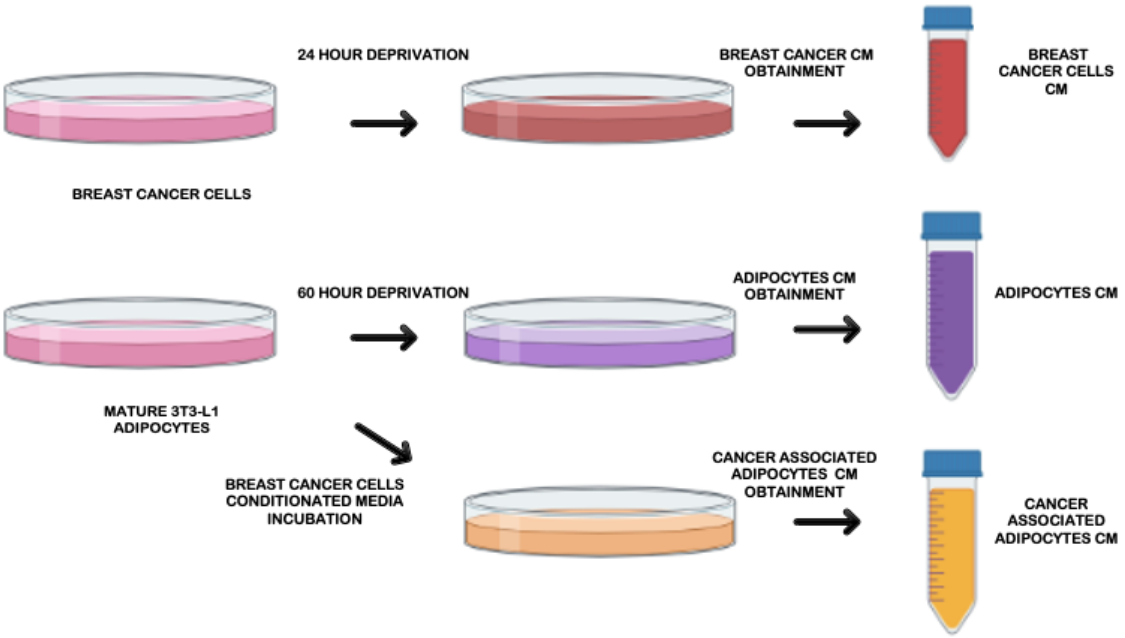

Figure 5. Outline of the methodologies followed to obtain conditioned media (CM). 3T3-L1, MCF-7 and MDA-MB-231 cell lines were incubated in different conditions (deprived DMEM High Glucose without FBS, MCF-7 CM or MDA-MB-231 CM) to obtain de conditioned media needed for further assays.

4.2. EXOSOME EXTRACTION

BC cell lines were cultured as monolayers in 10 cm culture plates. Once the cells reached 80% of confluency, the conditioned media was collected as explained above.

Then, CM was centrifuged at 300g for 10 minutes to remove cells. Supernatant was centrifuged at 15.000g for 20 minutes to discard cell-debris. Exosomes were harvested by ultracentrifugation at 120.000g for 70 minutes at 4 ºC using a TFT70.38 and the OPTIMA XPN-100 Ultracentrifuge. The exosome pellets were washed three times with PBS, ultra-centrifuged and finally resuspended with the appropriate buffer for each specific analysis (*Figure 6*).

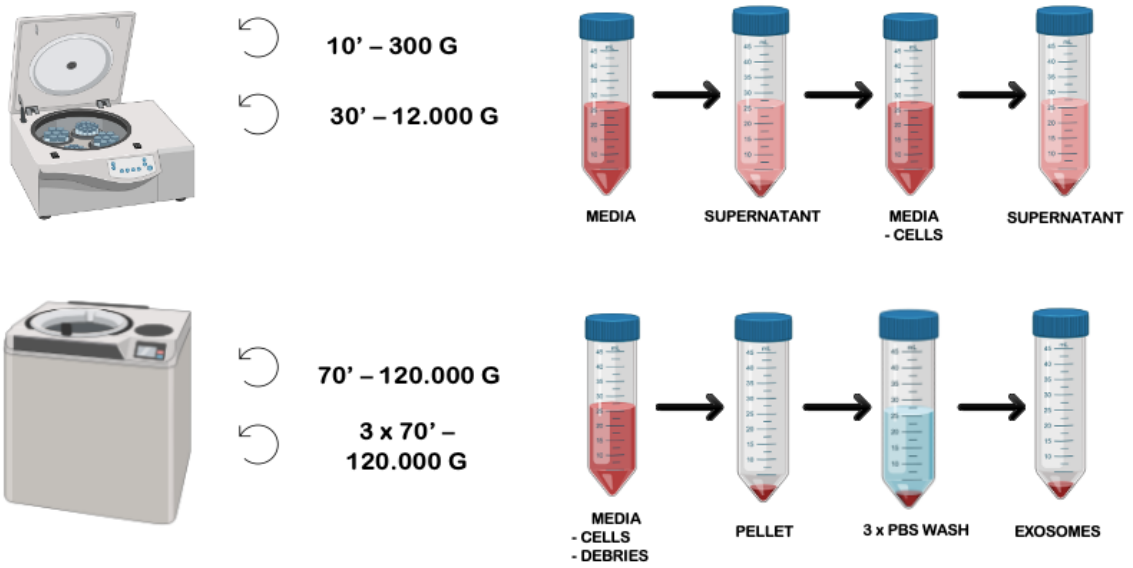

Figure 6. Ultracentrifugation process of exosome extraction. The media obtained from cells was differentially centrifuged in order to obtain the exosomes of each condition.

4.3. OIL RED O ASSAY

Oil Red O is a dye which strongly stains neutral lipids. This assay was performed in order to determinate the adipocyte lipid content. 3T3-L1 were cultured until they reached confluence. Then they were differentiated for 10 days into mature adipocytes. After adipocyte differentiation, mature adipocytes were cultured with different conditioned media (control CM, MCF7 CM and MDA-MB 231 CM).

After 3 days of incubation, treated mature adipocytes were stained with Oil Red dye following the protocol described (#K580-24) and pictures were taken with an inverted microscope (Olympus IX71). Then three washes with Isopropanol 60% were performed and for dye extraction, cells were incubated with Isopropanol 100% for 5 minutes. Afterwards absorbance at 490 nm was measured using SINERGY HT (Biotek).

4.4. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

In order to determine the protein levels of the media from adipocytes, ELISA assay was performed to detect the levels of FABP4 and CD36. The media from the adipocytes and the CAAs was obtained as explained above. The samples were analysed performing an ELISA following the company protocols (Antibodies Online).

4.5. PROTEIN EXTRACTION AND WESTERN BLOT

In order to analyse protein content, in each experiment cells or exosomes were lysed with RIPA buffer (0.5 mM Tris-HCl, 150 mM NaCl, 0.1 % SDS, 1 % Nonidet P40, 0.5 % Sodium Deoxycholic Acid) and inhibitors of both proteases and phosphatase (Roche). Then, protein concentration was quantified by the Bradford Assay and the plate reader Synergy HT (Biotek).

To analyse the protein extracted, 10-20 µg of protein were loaded and separated on electrophoresis gels (NuPage 10% Bis-Tris Gels) (Life Technologies). Proteins were transferred into nitrocellulose membranes using a semidry iBlot2 transfer device (Life Technologies). These membranes were blocked with 0,02 g/mL non fatty dry milk in TBS-Tween 0,1% for an hour at room temperature. Then, primary antibodies were added (Table 1) and incubated overnight at 4ºC while they were being shacked. Next, membranes were washed by six five-minute periods with TBS-Tween 0,1%. Secondary antibodies conjugated with peroxidase (HRP) (Dako) were added (Polyclonal Goat anti-Rabbit, Polyclonal Rabbit Anti-Goat or Polyclonal Goat Anti-Mouse at 1:10.000 dilution) to membranes for one-hour incubation.

Table 1. Primary antibodies and their corresponding secondary antibody used in Western Blot

Primary Antibody	Dilution	Company	Reference	Secondary Antibody
GAPDH	1:1000	Cell Signalling	#5174	Rabbit
CD36	1:10000	Abcam	AB133625	
FABP4	1:1000	R&D Systems	AF3150	Goat
FABP5	1:1000	R&D Systems	AF3077	
HSP90ß	1:10000	Abcam	AB32568	Rabbit
Flotilin-1	1:1000	BD Biosciences	610821	Mouse
Alix	1:1000	Cell Signalling	#2171	

Then, membranes were washed by six five-minute periods with TBS-Tween 0,1% and two ten-minute periods with TBS 1%. Afterwards, the chemiluminescent reagents (ECL Millipore reagent) in an Amersham Imager600 (GE Healthcare). Finally, images were quantified using ImageJ software.

4.6. BODIPY FLUORESCENT STAINING

In order to analyse the exosome uptake by BCC lines MCF-7 and MDA-MB 231 we differentiated 3T3-L1 fibroblasts for 10 days as previously described. During the differentiation process labelled palmitic-acid BODIPY (Sigma) every other day. Once it had obtained mature labelled adipocytes, they were deprived for 3 days, and media was collected. Labelled exosomes were isolated and as described above and approximately 50 µg of exosomes were added onto BCC lines MCF-7 and MDA-MB 231. After 3 days of treatment, BCC were washed up 3 times with PBS for 5 minutes and pictures were taken using fluorescent microscopy Olympus IX71.

4.7. PROLIFERATION ASSAY

Bromodeoxyuridine (BrdU) assay was carried out to measure cell proliferation according to the Cell Proliferation ELISA protocol (Roche, Barcelona, Spain). Briefly, $3x10^6$ tumour cells were seeded into 96 well microplates and different conditioned media were added for 24 h. After the addition of BrdU (100µM), the cells were fixed and incubated with anti-BrdU antibody for 90 min. Tetramethyl-benzidine (TMB) was then added and incubated for 15 min before the reaction was stopped by the addition of H_2SO_4 (1M). Absorbance of samples were measured in an ELISA reader at 370 nm (reference wavelength 492nm).

4.8. STATISTICAL ANALYSIS

The results were expressed as the mean ± standard error (SEM). The GraphPad Prism software was used for statistical analyses and a p-value <0.05 was considered to be statistically significant.

5. RESULTS

5.1. BCC CM INCREASES MATURE ADIPOCYTE DELIPIDATION

One of the characteristics of CAAs is the reduced cell volume due to the liberation of fatty acids to the peritumoral matrix to fuel the tumour (17). In order to determine the modification of the lipid content in adipocytes caused by tumour influence, mature adipocytes were cultured with BCC CM (both MCF-7 Luminal A cell type and MDA-MB-231 TNBC cell type).

Then, Oil Red staining was performed in order to measure the level of delipidation of these CAAs compared to control condition. Considering Figure 7, there's significant decrease in the amount of lipid droplets ($p<0.001$) in those adipocytes treated with tumoral media for three days. This may explain the reduced volume presented by cancer-associated adipocytes.

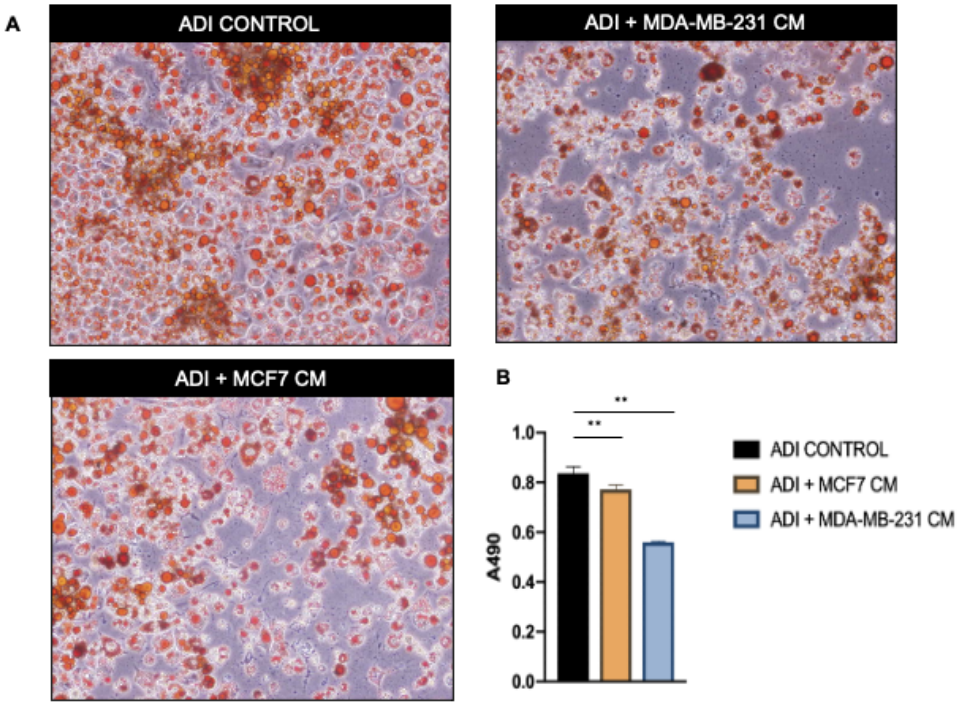

Figure 7. Oil red staining of mature adipocytes treated at different conditions. (A) Image show the lipid content in control adipocytes compared to those which have been cultured with MCF-7 and MDA-MB-231 CM for three days. The redder colour represents a higher amount of lipids in the adipocytes. (B) Graph represents the evolution of spectroscopic absorbance at 490 nm for six days. Significance is stablished at P value <0.05. **p<0.005

5.2. BC CM INCREASES FABP4 AND CD36 RELEASMENT FROM ADIPOCYTES

FABP4 and CD36 are two fatty acid transporter proteins (FATP) strongly related with BC and previous laboratory results have determined an increase in plasma levels of BC patients (23). Therefore, laboratory nonpublished results have stablished how FABP4 and CD36 have a crucial role in cancer progression and how mature adipocytes by the interaction with BC cell lines MCF7 and MDA-MB 231 are able to release these FATP to media in order to be taken up by tumour cells. For this reason, it is imperative to elucidate the role and behaviour of these proteins. As the communication is bidirectional, the first purpose is to understand how BC could affect the behaviour of the mature adipocytes and their effects on these proteins. For this reason, by an ELISA assay, FABP4 and CD36 levels (pg/ml) were analysed in media from mature adipocytes previously cultured with MCF-7 and MDA-MB-231 CM and it was compared to non-treated adipocytes media. The results show an increase in FABP4 and CD36 protein levels, suggesting a higher releasement of these proteins to the TME and as a FATP a correlation with the higher delipidation of the adipocytes mentioned above might be plausible (Figure 8).

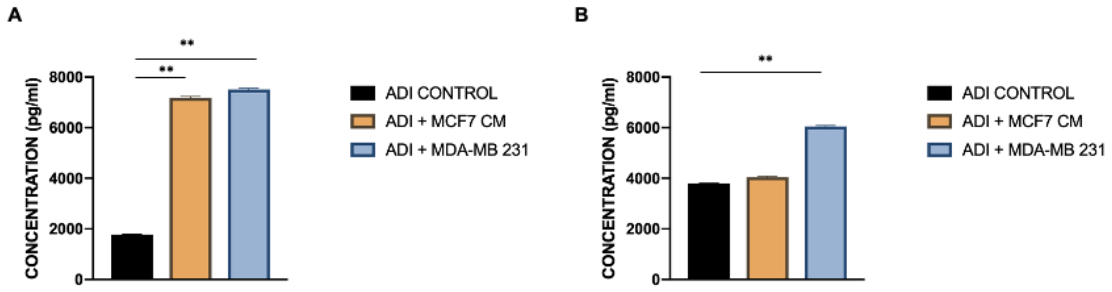

Figure 8. Breast cancer cell CM effect on FABP4 and CD36 levels in mature adipocytes' media. Adipocytes were cultured with BCC CM (both luminal A cell line MCF-7 and TNBC line MDA-MB-231) for 60 hours. (A) Comparison of FABP4 levels in the media of each condition analysed by ELISA. (B) Comparison of CD36 levels in the media of each condition analysed by ELISA. There's a statistical significance between the control media (non-treated adipocytes media) and those mediums from adipocytes treated with tumour cell CM. Significance is stablished at P value <0.05. **p<0.005. *(Modified from early research group results)*

5.3. OPTIMIZATION OF EXOSOME ISOLATION METHOLODY

At first sight, there were no precedents in this laboratory on the exosome isolation and characterization. In order to do further analysis with exosomes derived from mature adipocytes, different isolation tests were performed. Following the isolation methodology described by Théry et al. (36) the mature adipocytes-derived exosomes extraction and further characterization was performed. The exosome extraction process done in this study is slightly different to this one, due to the number of ultracentrifugations, the conditions of these and the limitations of our laboratory. The methodology used in further experiments is described above (*Material and Methods, 4.2*).

According to different exosomal databases, such as *Exocarta*, there are different exosomal markers that are used to verify the presence of exosomes on the samples. These exosomal markers are normally proteins involved in exosome biogenesis and releasement. Three of these proteins are ALIX, Flotillin-1 and HSP90ß. For this reason, in order to determine the presence of exosomes in our samples, the cargo of these markers was analysed by Western Blot.

In order to verify if the samples obtained were exosomes, exosomal protein markers ALIX, Flotillin-1 and HSP90ß were analysed by Western Blot (Figure 9).

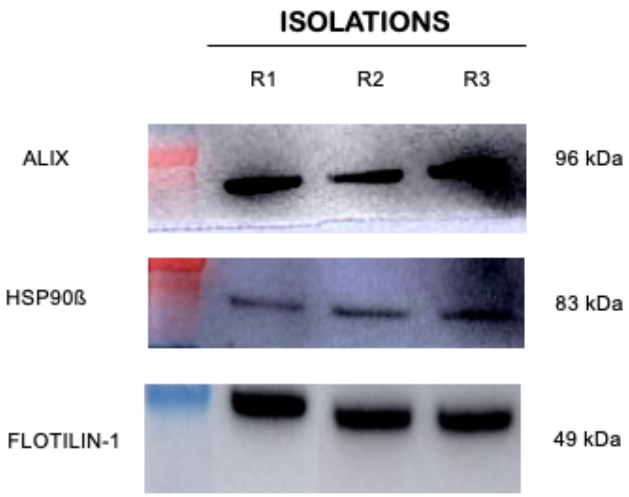

Figure 9. Immunoblots of exosomal markers. Immunodetection of ALIX, HSP90ß and FLOTILIN-1 within mature adipocytes-derived exosomes from three different isolations.

There are some proteins described as exosomes biomarkers, such as Alix, CD63 and Flotillin-1.Besides, based on previous studies whose protein endogen control

in Western Blot was an exosomal marker, we decided to use an exosomal marker as a loading control in our experiments to ensure that the concentration of the exosomes was the same in all the samples based on the different tests performed (37,38). In those tests, we demonstrated that the amount of Flotillin was not variating in the different conditions. For this reason, we decided to use Flotillin-1 as an endogen control in the following experiments.

5.4. ADIPOCYTE EXOSOME RELEASEMENT IS ENHANCED BY BREAST CANCER CM

Kalluri et al. suggests that cancer cells secrete significantly a higher number of exosomes than non-cancerous cells (30). According to previous results explained above, the higher delipidation, we wanted to assess how BCC CM might influence in the biogenesis or the releasement of exosomes in mature adipocytes to the TME. After 3T3-L1 differentiation into mature adipocytes, they were cultured with BCC CM and exosome isolation was performed as described before *(Material and Methods, 4.1).* As shown in Figure 10 we demonstrated that cancer associated adipocytes released a higher number of exosomes than adipocytes that were cultured in control conditions. We observed that mature adipocytes cultured with MCF7 CM produces a slightly higher number but not significant of exosomes. However, those mature adipocytes cultured with MDA-MB 231 CM increases in a significantly manner the number of released exosome to media.

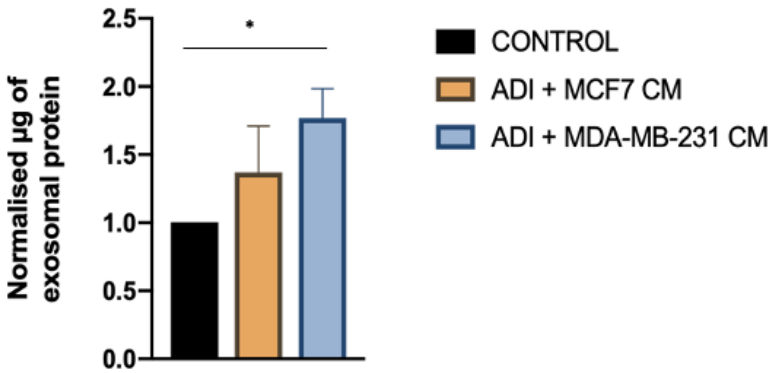

Figure 10. Exosome releasement in cancer associated adipocytes. The exosome production is compared within the protein concentration of each condition. Graph represents differences in the concentration of protein between the analysed conditions. There are statistical significance differences between the control group and those adipocytes treated with triple negative CM. Significance is stablished at p value <0.05.

5.5. FABP4 IS PRESENT WITHIN ADIPOCYTE-DERIVED EXOSOMES

FABP4 is involved in crosstalk between the adipocyte and the tumour, it is responsible for the transfer of lipids between cells, inducing the oxidation of fatty acids to fuel the tumour growth (17). It is known by previously results obtained in this laboratory that the cell culture of adipocytes with conditionate media from tumour cells imitating the microenvironmental surrounding of the tumour derivates in the release of FABP4 to extracellular media in order to transport fatty acids to the tumour. Also, it is described in exosomal database (*Exocarta*) that FABP4 is a protein which is included in adipocyte-derived exosomes. Different exosome isolations and protein extractions were performed in order to verify the presence of FABP4 within the mature adipocyte-derived exosomes. As shown in Figure 11, firstly, exosomes presence was demonstrated by the analysis of exosome marker Flotilin-1. Then, by Western Blot the presence of FABP4 within these exosomes was evaluated. We could observe that all different exosome isolations from mature adipocytes contained a high amount of FABP4, as the Exocarta bibliographic database describes.

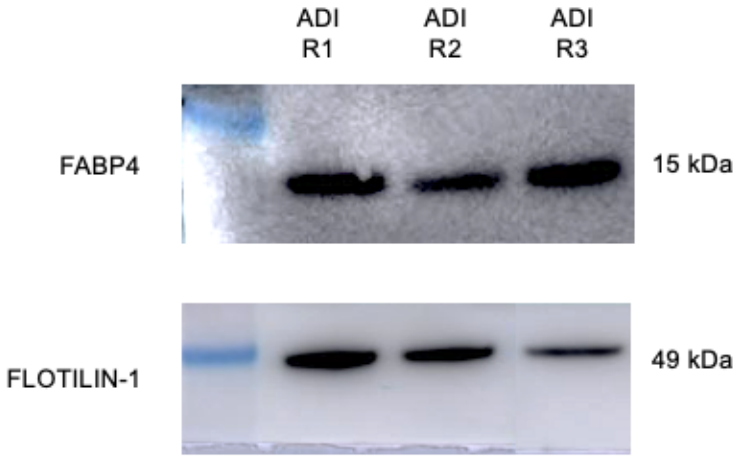

Figure 11. Exosomes isolated from adipocyte media contain a cargo of FABP4. Western blot of FABP4 and FLOTILIN-1 within exosomes isolation of three different replicates from mature adipocytes.

5.6. BC CM INCREASES FABP4 AND CD36 PROTEIN LEVELS IN ADIPOCYTE-DERIVED EXOSOMES

FABP4 and CD36, as explained above, are closely related with cancer progression. Next, we wanted to assess how CAAs treated with MCF-7 (Luminal A BC) and MDA-MB-231 (TNBC) CM could affect to the FABP4 exosomal cargo by adipocytes. The results obtained, shown in Figure 12, were that exosomal amount of FABP4 was significantly elevated in CAAs versus non-modified adipocytes (p=0.002). Moreover, the amount of exosomal FABP4 was higher in TNBC cell line than the Luminal A BC cell line (p=0.0016).

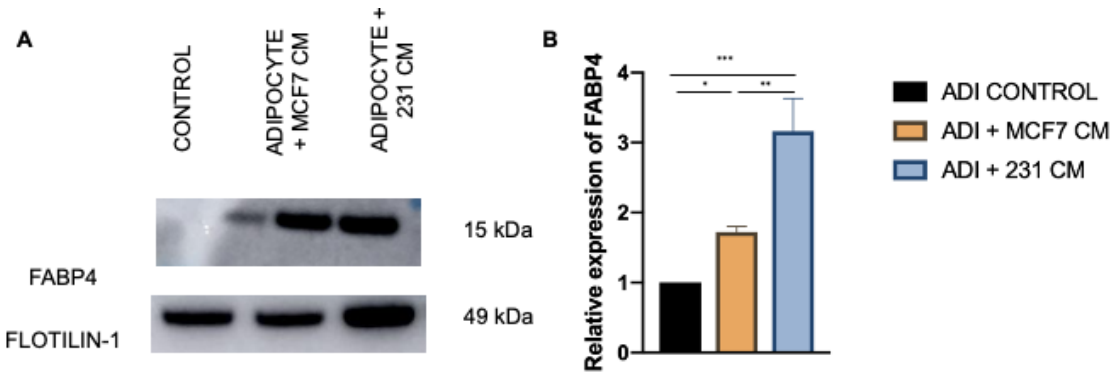

Figure 12. MCF7 and MDA-MB-231 conditionate media enhances the amount of FABP4 in mature adipocyte's exosomes. (A) Western Blot of FABP4 of exosomes derived from mature adipocytes previously cultured in different conditions (Control CM. MCF-7 CM and MDA-MB-231 CM). Flotilin-1 was used as the endogenous protein. **(B)** Graphs represent FABP4 protein differences between the analysed conditions There's a statistical significance between the control group (non-treated adipocytes) and those adipocytes treated with tumour cell CM (both luminal A cell type and triple negative cell type). Also, there's difference between the cargo of FABP4 between BC cell types. Significance is stablished at p value <0.05. **p<0.005. ***p<0.0005

In addition to those results, the amount of exosomal CD36 was also increased in CAAs treated with tumoral media versus the non-modified adipocytes (p=0.0015). Particularly, the relative expression of CD36 was significantly enhanced in the MDA-MB-231 cell line (p=0.0012) (Figure 13).

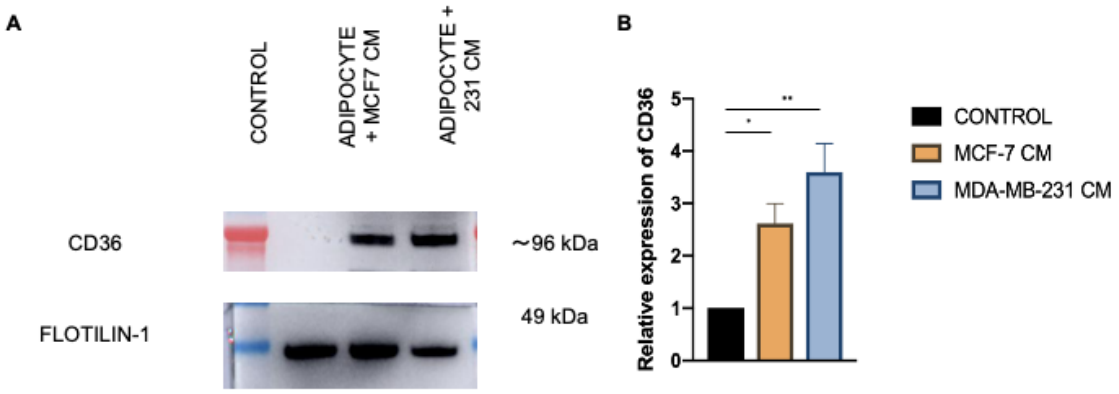

Figure 13. **MCF-7 and MDA-MB-231 conditionate media enhances the amount of CD36 in adipocyte's exosomes.** (**A)** Western Blot of CD36 of exosomes derived from mature adipocytes previously cultured in different conditions (Control CM, MCF-7 CM and MDA-MB-231 CM). Flotillin 1 was used as the endogenous protein. (**B)** Graphs represent CD36 protein differences between the analysed conditions. There are statistical significance differences between the control group (non-treated adipocytes) and those adipocytes treated with tumour cell media (Both luminal A cell type and Triple Negative cell type). Significance (*) is stablished at p value<0.05. **p<0.005.

These results suggest an increase of FATP via exosomes to tumoral cells by adipocytes, in order to fuel the fatty acid metabolism of the tumour.

5.7. MATURE ADIPOCYTES-DERIVED EXOSOMES ARE INTERNALISED BY BC CELL LINES MCF-7 AND MDA-MB-231

In order to study the effects of mature adipocytes-derived exosomes in BCC lines MCF-7 and MDA-MB-231, both cell lines were cultured for 3 days with labeled adipocyte-derived exosomes. For exosome labeling, previously, 3T3-L1 fibroblasts were differentiated into mature adipocytes using the fluorescent dye BODIPY. We could observe the increase of BODIPY fluorescent intensity during their differentiation (Figure 14). Then, mature adipocytes were serum starved for 60 hours and media was collected.

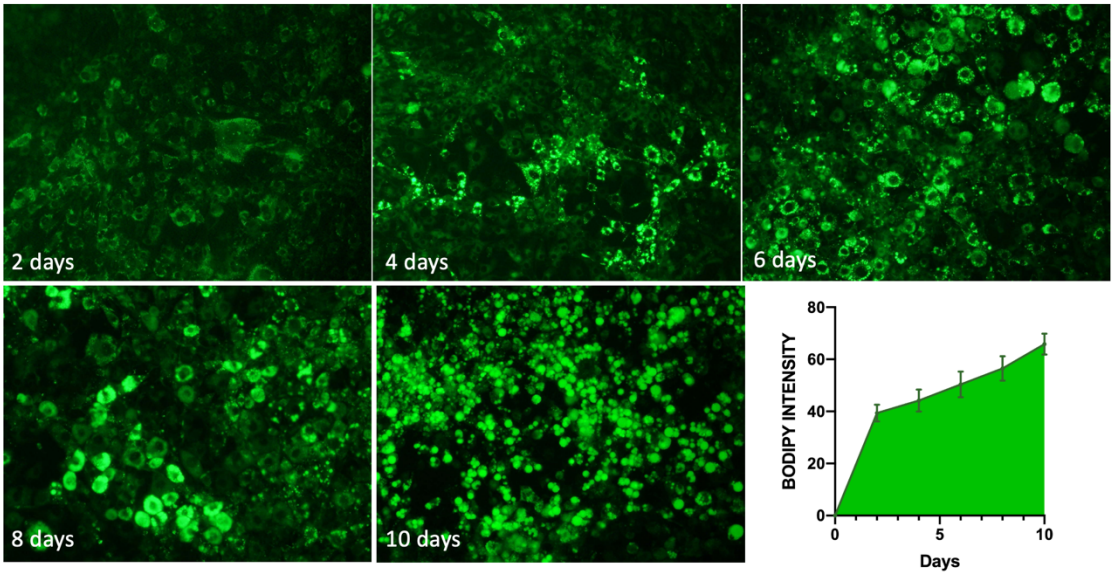

Figure 14. BODIPY uptake during 3T3-L1 fibroblasts differentiation. Fluorescent images at 10x magnification of 3T3-L1 differentiation after BODIPY addition. The bar chart shows the BODIPY intensity increases as the days go by until it reaches the maximum peak at 10 days of differentiation. *(Modified from early research group results).*

Afterwards, exosome isolation was performed and approximately 50µg of exosomes were added into BCC lines cultures. We could observe after 3 days of incubation that BCC lines acquired multiple fluorescent dots within the cytoplasm, suggesting that labeled exosomes were being internalized by both BCC lines.

In addition, we observed that the number of fluorescent dots tends to be higher in those BCC lines cultured with CAAs-derived exosomes compared to those tumor cells that had been cultured with control adipocytes-derived exosomes.

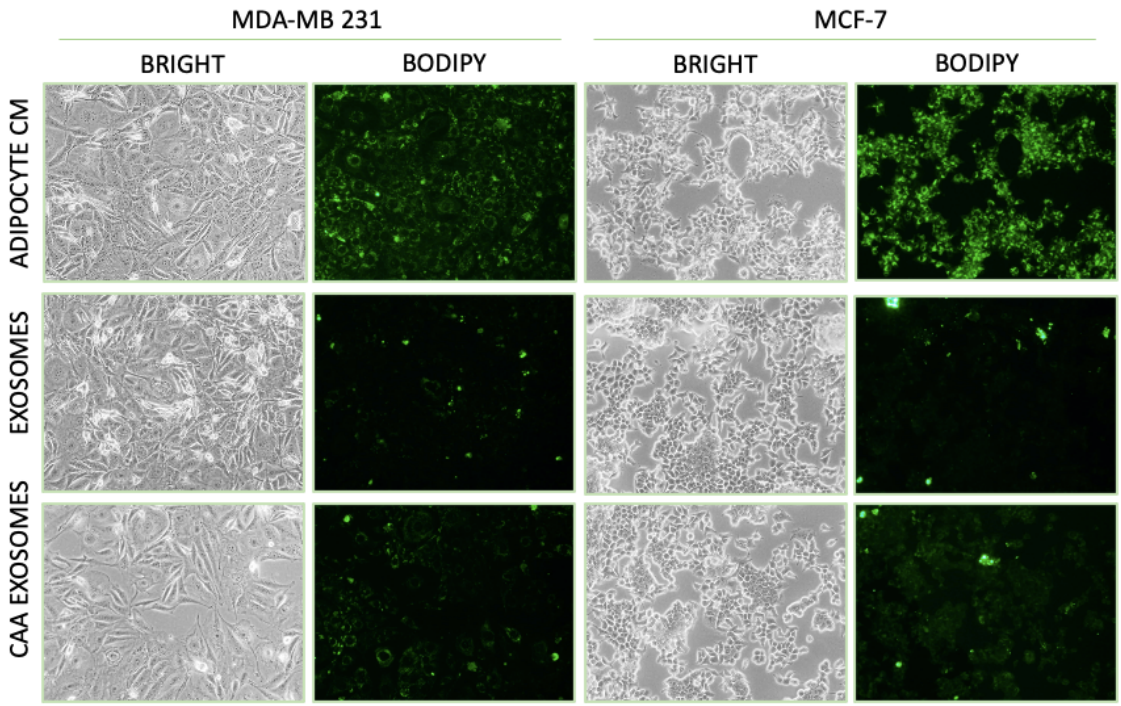

Figure 15. BODIPY uptake in BCC lines MCF-7 and MDA-MB-231. Bright and fluorescent images at 10x magnification of both cell lines at different conditions (Adipocyte CM, labelled adipocyte derived exosomes and labelled CAAs derived exosomes). We could observe a substantial uptake of palmitic acid after adipocyte CM. Therefore, after labelled exosome isolation and their addition into both BCC lines, we observe fluorescent dots within the cytoplasm of both tumour cell lines. We also observe a slight increase in the number of fluorescent dots after the addition of CAAs derived exosomes.

5.8. EXOSOMES DERIVED FROM BOTH ADIPOCYTES AND CAAs INCREASE BCC PROLIFERATION

In order to analyse how exosome communication could affect BC proliferation, one of the most important cancer hallmarks, we did a cell BrdU proliferation assay with BCC lines MCF-7 and MDA-MB-231. As shown in Figure16, there is increased proliferation once MCF-7 cells were treated with mature adipocytes-derived exosomes ($p<0.0001$). Moreover, the treatment of these cells with CAAs-derived exosomes shows also a significative differenced when compared to the non-treated cells ($p<0.0001$). Furthermore, the same results were obtained with the MDA-MB-231 cell line, showing a significative difference when these cells were treated with both mature adipocytes-derived exosomes ($p=0.0181$) and CAAs-derived exosomes ($p=0.0004$). In addition, we could observe there was a slight increase once both BCC lines were cultured with CAAs-derived exosomes. However, the number of replicates is too low to conclude that there is a positive

correlation between adipocytes nor CAAs- derived exosomes and a higher BC proliferation (n=4).

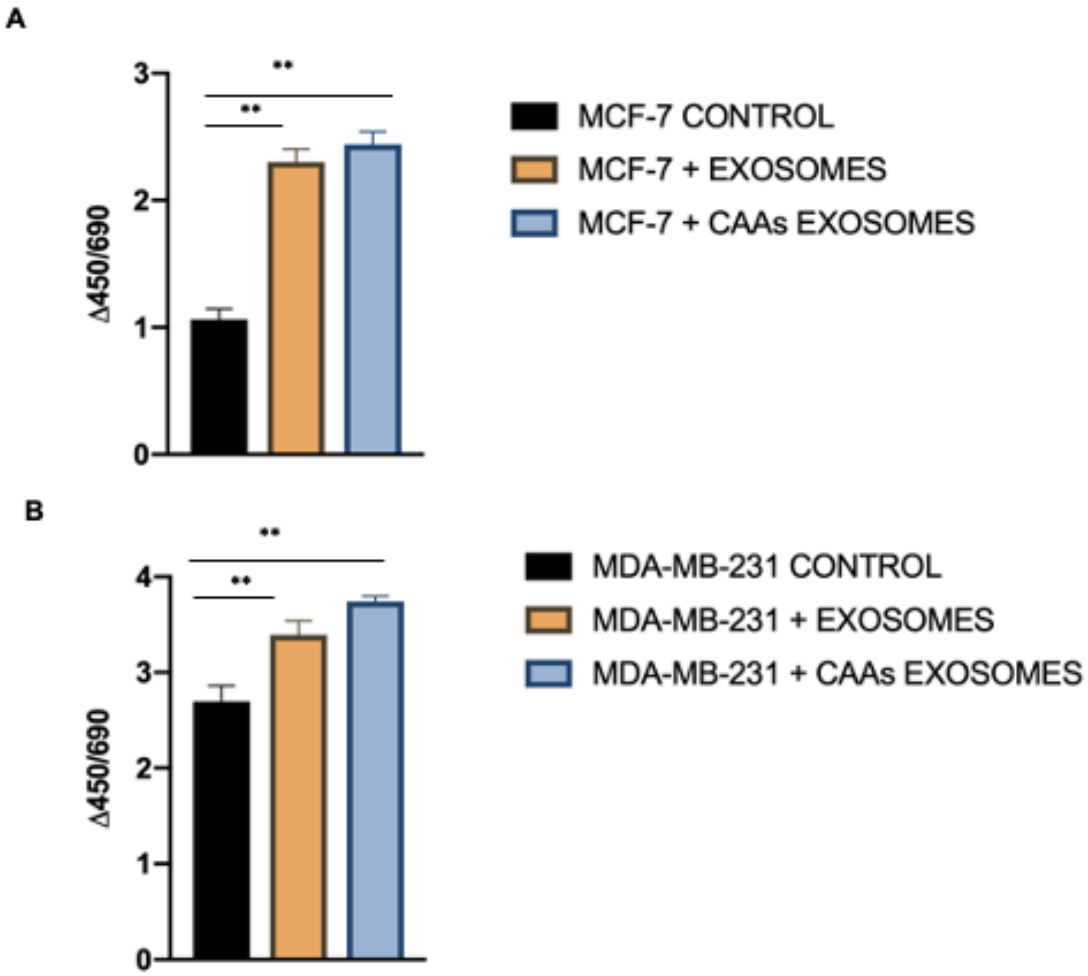

Figure 16. Exosomes derived from both adipocytes and CAAs increase BCC proliferation. Bar charts represent the BrdU rate of absorbance at 450/690 in MCF-7 cell line **(A)** and MDA-MB-231 cell line **(B)** at different conditions (control condition, adipocytes-derived exosomes and CAAs-derived exosomes). There is an increase in both BCC lines proliferation rates after exosome addition, as well as after CAAs-derived exosomes addition. Significance (*) is stablished at p value<0.05. **p<0.005.

These results suggest that exosomes have an important role in the tumour progression by increasing BCC proliferation, being principal actors in the crosstalk between the tumour and the adipose tissue.

6. DISCUSSION

In the last few years there has been a growing interest in the study of the tumour microenvironment, focused on the communication of the tumour and the surrounding tissue components. In breast cancer, the tumour is enveloped in a vast majority of adipose tissue. Thus, the dynamic and reciprocal communication of the adipose tissue and the tumour has been gaining importance.

The peritumoral adipocytes are known as CAAs and play an important role in cancer by promoting a chronic inflammation and the development of tumour cells (14–17). This CAAs have a diminished size of the lipid droplets due to increased lipolysis and the releasement of fatty acids into the peritumoral matrix. Not only these adipocytes have an increased secretion of fatty acids into the TME, but in this study it has been demonstrated that also have an increased secretion of FATP in order to transport these fatty acids to the tumour cells. The transport of these fatty acids is carried out by FATP such as FABP4 or CD36 (17,26,39,40). FABP4 is highly expressed in the adipose tissue but also in some tumour cells, and its role in breast cancer has been previously described, enhancing the tumour progression by collaboration in the transport, incorporation and oxidation of fatty acids to fuel tumour growth (23,26). CD36 is increased in late stages of cancer and participating in tumour progression and metastasis of cancer such as FABP4 (22,28,29).

Quite recently, considerable attention has been paid to exosomes as contributors to the crosstalk in the tumour microenvironment because they reflect the phenotype of the cell which generates them and transfer proteins, lipids and nucleic acids to the paracrine cells (21,30–32). Exosomes are nanosized particles that are released to the blood and lymphatic stream, as well as to the extracellular matrix. Thus, the profiling of exosomes of body fluids from breast cancer patients or even healthy patients serve as a non-invasive biomarker in diagnostic classification and prognostic (30,31,38).

As exosome isolation has not a well stablished methodology and different isolation protocols has been described according to each laboratory conditions and availabilities, it was imperative to stablish the correct isolation methodology in our laboratory. Therefore, we decided to stablish our method of exosome

extraction based on the existing protocol described by Théry et al. (36), taking in consideration the limitations of the laboratory and of the conditions of the experiment itself.

Much research has been done about exosomes as a potential source of information to detect cancer. However, one of the limitations of the exosome profile study is the imperative necessity of fresh samples. In this study we have been working with fresh and frozen exosomes for the protein quantification and analysis and we have demonstrated that freezing exosomes does not affect the protein composition of the exosomes leading us to analyse them by Immunoblotting techniques. We have been able to detect exosome markers in both conditions leading us the possibility of freeze samples for further protein analysis. However, we also have corroborated that the integrity of the exosome is disrupted after sample freeze.

All cell types are able to produce and release exosomes. Accordingly, their content varies depending on the cell type. Therefore, the amount of exosome production might vary also depending on the biological status of the cell. It has been described that tumour cells increase the production and releasement of exosomes to media in order to stablish a continuous communication with not only their close microenvironment but also distant host tissues (30–32). As communication mediators, the amount of released exosomes might be crucial in this process. According to our results, mature adipocytes treated with BCC CM suffered a higher lipolysis, therefore it may be accompanied by a higher production of exosomes. In this study we have demonstrated the importance of the bidirectional communication between BC and the adipose tissue in the production and release of exosomes to the matrix. We have demonstrated that the releasement of adipocyte-derived exosomes is significantly increased after the establishment of a communication with BCC lines MCF-7 and MDA-MB-231 and mature adipocytes, which might lead a crucial impact in the paracrine and distant pathological communication in the organism.

As mentioned above, in this study we have corroborated the mature adipocyte loss of lipid droplets after their culture with BCC CM. In addition, we have demonstrated that CAAs release a higher amount of the fatty acid transporter

protein FABP4 and CD36, both strongly related with BC tumorigenesis and progression. Exosomes, as reflect of their origin cell, might vary the protein cargo by the influence of the microenvironment. For this reason, we decided to test how BCC CM may modulate the mature adipocyte-derived exosomes cargo, focussing on the FATP CD36 and FABP4 as they were increased in media. We suggested that these proteins might be increased within the exosome. Indeed, after we culture mature adipocyte with BCC lines MCF-7 and MDA-MB-231 CM, we isolated the exosomes derived from control mature adipocytes as well as from both CAAs and analysed their cargo. As we expected, the protein FABP4 and CD36 amount within CAAs-derived exosomes was significant increased.

These proteins might increase the lipid transfer form mature adipocytes to the tumour cells. In addition, according to not yet published laboratory results, FABP4 is closely related with the activation of the survival pathway AKT-FOXO3A and the activation of different cancer hallmarks such as proliferation, migration and invasiveness. CD36, as Aznar describes in (28), is an important pro-tumorigenic protein and it enhances also different breast cancer hallmarks. For this reason, we suggest that the increase in these FATP cargo within CAAs might have a considerable influence in BC development as they may supply the altered fatty acid metabolism and enhance the tumour progression.

In fact, we could observe that BCC lines MCF-7 and MDA-MB-231 are able to internalize exosomes derived from mature adipocytes as well as from CAAs. Exosomes as cell communicators are released, transported by blood stream and finally internalized into a recipient cell type modifying its behaviour. In fact, some studies suggest that exosome cargo can modify the transcriptome, proteome, and metabolism of recipient cells (21,24,30–32,35).

Furthermore, as Wang et al. describe, exosomes derived from mature adipocytes modify the behaviour of MCF-7 and MFA-MB-231 cell lines, increasing their proliferation by the enhancement of the Hippo-WNT pathway (34). We have observed also that exosomes derived from mature adipocytes and CAAs increase the proliferation rate of MCF-7 and MDA-MB-231 cell lines.

Previous results obtained in this laboratory have demonstrated the role of both FABP4 and CD36 in the tumour progression. This project demonstrated that

these proteins are truly present in both adipocytes and CAAs-derived exosomes, thus implying their implication in the exosomal crosstalk between the tumour and the surrounding adipose tissue, which participates in the tumour progression. For this reason, our future research will be based on the plausible modulation of tumour progression by the adipocytes and CAAs-derived exosomes.

One of the main limitations of working with exosomes in this laboratory is that there's not a stablished protocol to work with, neither for the extraction nor working with exosomes and BCC cell lines. However, once the exosomes extraction method was stablished, we managed to do the exosomal content experiments.

Moreover, working with exosomes without having the resources to do a proper electron microscopy characterization means not knowing what you are working with until a repeated number of tests is done with other techniques like Western Blot. These techniques take longer to get results, so we were working also with the time against due to the COVID-19 pandemic, the deadlines and the limitations of the university.

7. CONCLUSIONS

In breast cancer, the dynamic and reciprocal communication of the adipose tissue and the tumour has been gaining importance in recent years. The peritumoral adipocytes suffer a phenotype switch, consisting in an increased secretion of fatty acids and FATP (such as FABP4 and CD36) to the TME in order to fuel the growth of tumour cells. Quite recently, considerable attention has been paid to exosomes as contributors to the crosstalk in the tumour microenvironment as potential regulatory drivers of the tumour progression and metastasis.

In this project we had stablished a standardized method for exosome extraction based on the characteristics and limitations of our laboratory. This protocol was necessary in order to analyse the adipocyte-derived exosomes. From our results we can conclude that:

- CAAs present a higher level of delipidation and FATP secretion in order to fuel the tumour growth.
- CAAs-derived exosome releasement is higher compared to those non-treated adipocytes.
- CAAs exosomes present higher levels of FABP4 and CD36 than control adipocytes.
- Exosomes are internalized by BCC cell lines, and this internalisation affects to the proliferation rate of this cells.
- Therefore, this suggests that CAAs-derived exosomes might contribute to the tumour progression.

However, one of the limitations of the study was that we hadn't enough time to do the proper assays on the BCC cells, so we couldn't analyse how the CAAs-derived exosomes affect to the tumour progression with enough replicated in order to stablish robust conclusions.

To sum up, in the present study we demonstrated how the CM from BC MCF-7 and MDA-MB-231 cell lines modify the amount and the cargo of adipocyte-derived exosomes, thus demonstrating that exosomes might play an important role in the crosstalk of the tumour microenvironment.

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