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ZACATENCO CAMPUS

CELL BIOLOGY DEPARTMENT

Effect of MDA-MB-231 breast cancer cell derived extracellular vesicles in the transformation of NIH 3T3 fibroblasts into cancer associated fibroblast-like cells.

THESIS

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Efecto de vesículas extracelulares derivadas de células de cáncer de mama MDA-MB-231 en la transformación de fibroblastos NIH 3T3 en células tipo "fibroblasto asociado al cáncer".

TESIS

Que presenta

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INDEX

I. ABBREVIATIONS

- **Arachidonic acid: AA**
- Bone marrow derived mesenchymal stem cells: BM-MSC
- **Cancer associated fibroblast: CAF**
- Dulbecco's modified Eagle medium: DMEM
- Epithelial to Mesenchymal transition: EMT
- Extracellular matrix: ECM
- **Extracellular Vesicle: EV**
- Fatty acid: FA
- Flotillin-2: Flot-2
- Foetal bovine serum: FBS
- Free fatty acid: FFA
- G protein couple receptor: GPCR
- Interferon: IFN
- Interleukin: IL
- Matrix metalloproteinase: MMP
- Mesenchymal stem cells: MSC
- Multivesicular body: MVB
- Phosphate buffered saline: PBS
- Stromal derived factor 1: SDF1
- Transforming growth factor β: TGF-β
- Tumour associated macrophages: TAM
- **Tumour microenvironment: TME**
- Tumour necrosis factor α: TNF-α
- Vascular endothelial growth factor: VEGF
- α smooth muscle actin: α-SMA
- Conditioned media: CM
- Receptor tyrosine kinase: RTK

II. ABSTRACT

Tumour and its microenvironment appear now as two sides of the same coin, denoting the tight relation that lays between them. Tumours need a supporting environment to grow and it is demonstrated that tumours themselves create it. Among the responsible agents for the formation of the tumour microenvironment, extracellular vesicles are in a predominant position. This membrane bound particles are able to induce deep changes both at signalling and genetic levels in recipient cells. Tumour cells secrete increased numbers of extracellular vesicles, and these markedly contribute to the creation of a reactive stroma that enhances tumour growth. One of the most important cell types in the tumour microenvironment is the so called cancer associated fibroblast (CAF). These cells exert many tumour promoting functions, and are essential in tumour development. In the same way, this cell type is very abundant in breast cancer, and considering the incidence and mortality rates in this malignancy, studying its milieu and communication mechanisms remains essential. We thus, examined the communication between tumour cells and fibroblasts, mediated by extracellular vesicles. We were able to evaluate the role of arachidonic acid (AA) in the process by stimulating breast cancer cells with it. We showed that breast cancer cell (MDA-MB-231) derived extracellular vesicles can induce the acquisition of a CAF-like phenotype in fibroblasts (NIH 3T3 cells). This is enhanced if EVs derive from AA-treated breast cancer cells. In turn, extracellular vesicles from CAF-like cells could induce a weak migration in MDA-MB-231 cells. We concluded that extracellular vesicles can solely carry out several important events in tumour progression, such as induction of the CAF phenotype, and the consequent tumour promoting functions such as migration. We also propose that AA can play an important role in the process. We suggest that this function enhancement in AA treated breast cancer cell derived extracellular vesicles is due to the AA induced cargo change in breast cancer EVs.

RESUMEN

El tumor y su microambiente se consideran a día de hoy como dos caras de la misma moneda, dada la estrecha relación que existe entre ambos. Los tumores necesitan un entorno que favorezca su crecimiento, y se ha demostrado que los propios tumores pueden crearlo. Entre los agentes responsables de la creación del microambiente tumoral, las vesículas extracelulares se encuentran en una posición predominante. Estas partículas rodeadas por doble membrana son capaces de inducir cambios tanto a nivel de señalización como a nivel genético en las células receptoras. Las células tumorales presentan una secreción aumentada de vesículas extracelulares, y éstas contribuyen marcadamente a la creación de un estroma reactivo que potencia el crecimiento tumoral. Uno de los tipos celulares más importantes en el microambiente tumoral son los "fibroblastos asociados al cáncer" (CAFs por sus siglas en inglés). Estas células llevan a cabo varias funciones que son esenciales en el desarrollo del tumor. Además, este tipo celular es muy abundante en los tumores mamarios, y considerando las tasas de mortalidad e incidencia de este cáncer, es esencial el estudio del entorno celular y los mecanismos de comunicación en el cáncer de mama. En consecuencia, examinamos la comunicación entre células tumorales y fibroblastos, mediada por vesículas extracelulares. Del mismo modo, evaluamos el rol del ácido araquidónico (AA) en el proceso, estimulando las células cancerosas con altas concentraciones de AA. Demostramos que las vesículas extracelulares derivadas de células de cáncer de mama (MDA-MB-231) inducen la adquisición de un fenotipo CAF-*like* en fibroblastos (NIH 3T3), y que este efecto esta potenciado cuando las vesículas extracelulares derivan de células de cáncer mamario tratadas con AA. Por otro lado, las vesículas extracelulares de células CAF-*like* pueden inducir una migración débil en células MDA-MB-231. Concluimos que las vesículas extracelulares pueden llevar a cabo por sí solas varios eventos importantes en la progresión tumoral, como la inducción del fenotipo CAF, y las funciones protumorales derivadas, como la inducción de la migración. También se muestra que el AA juega un papel importante en el experimento. Proponemos que éste potenciamiento en los efectos de las vesículas derivadas de las células de cáncer de mama, se debe a un cambio en el cargo ejercido por el AA.

III. INTRODUCTION

III.1. Breast cancer

III.1.1. Global epidemiology and breast cancer in Mexico

Breast cancer around the globe. Breast cancer has arisen as a global public health problem. According to the WHO, it is the most common cancer in women, both in developed and developing countries. In 2012 nearly 1.7 million new cases of breast cancer were diagnosed worldwide, making up to 25% of all cancers in women. Moreover, 15% of cancer deaths in females are attributed to breast cancer, with 522,000 deaths in 2012, (Torre *et al.*, 2015).

The incidence and mortality in breast cancer are not homogeneous around the globe, and developed/undeveloped regions show different trends (Torre *et al.*, 2015). Incidence rates are higher in developed regions (74.1 per 100,000), compared with developing regions (31.3 per 100,000). Mortality rates however, are much closer to each other, being 14.9 per 100,000 in developed regions and 11.5 per 100,000 in developing regions (Ferlay *et al.*, 2015).

Breast cancer in Mexico. Breast cancer is an important health issue in Mexico, being the second death cause in women older than 20 years old (INEGI, 2015). Both incidence and mortality rates in Mexico have been steadily growing in the last years. Incidence raised from 21.40 to 28.75 per 100,000 from 2007 to 2014, and mortality from 9 per 100,000 in the mid-1990s to 14 per 100,000 in 2013 (INEGI, 2015; Knaul *et al.*, 2009). One of the big problems in Mexico is the lack of a proper system for early detection of breast tumours, when the non-invasive tumour is localised in the breast. According to the data provided by the Secretariat of Health (Secretaría de Salud, 2002) only 5 to 10% of breast cancer cases are diagnosed at early stages, whereas in the US, early diagnosis reaches 61% of tumours (American Cancer Society, 2015). Interestingly, breast cancer incidence in Mexico is not homogenous and states with higher *per capita* income and more western lifestyle have higher incidence rates, including Durango, Sinaloa and Chihuahua (Figure 1).

III.1.2. Breast cancer progression

Breast Anatomy: The breast is a complex organ, responsible for milk production during lactation. It lays on the anterior thoracic wall from the second to the sixth rib. The breast is mainly made up by stroma, which is formed by adipose and connective tissues. The epithelial fraction of the breast is

constituted by mammary lobes, lobules and milk ducts, which are embedded in the stroma. There are 15 to 20 lobes in a normal adult breast, each one consisting in 20 – 40 lobules (Figure 2a). Lobules are tubuloalveolar glands responsible for milk secretion to the milk ducts, which in turn, converge in the nipple (Barshes *et al.*, 2004; Pandya and Moore, 2011).

The breast goes through many alterations in women's life. During early pregnancy, the epithelial compartment proliferates and replaces stromal elements; towards the end of pregnancy, the epithelia differentiate into milk secreting glandular cells. This happens under the control of several hormones, such as progesterone, oxytocin and oestrogen. A deep restructuration of the breast takes place during menopause where lobe and duct numbers are reduced, therefore the breast will consist predominantly of fat and stroma. Over time, stroma and fat are also reduced resulting in volume and contour loss (Pandya and Moore, 2011)

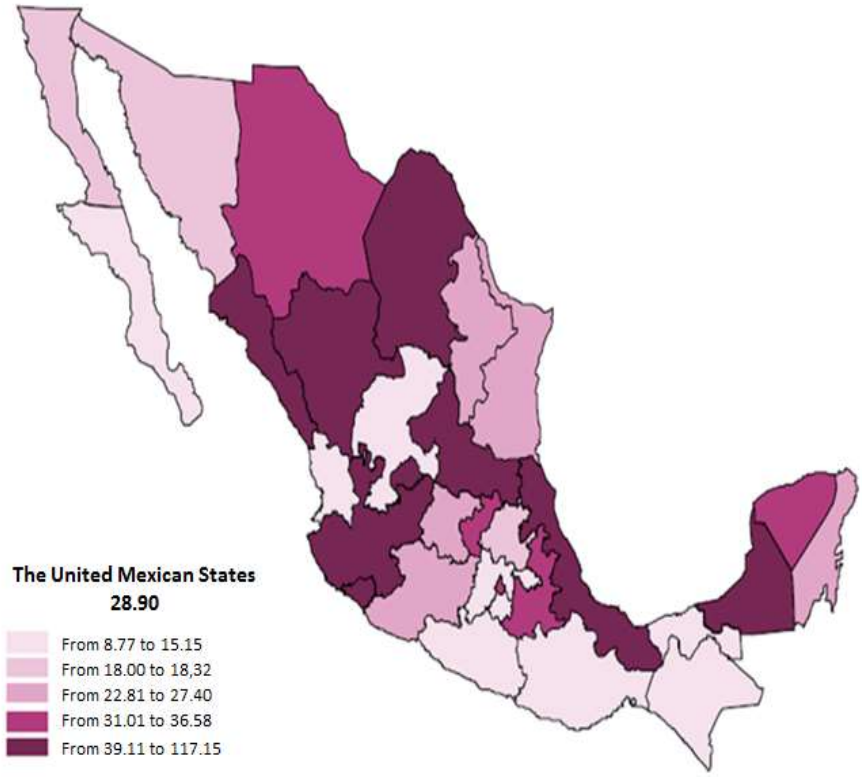

Figure 1. Incidence of breast cancer in Mexico by state for the year 2013. Higher incidence of breast cancer in Northern states can be seen. From "Estadísticas a propósito del día mundial de la lucha contra el cáncer de mama (19 de octubre)" (INEGI, 2015).

B*reast cancer progression.* About 80 - 85% of breast tumours are originated in the milk duct (Li and Daling, 2007). The duct is formed by a layer of luminal epithelial cells, surrounded by the

myoepithelial barrier that produces the components of the basement membrane, which is in contact with the stroma. The microenvironment of the milk duct is composed by extracellular matrix (ECM), fibroblast, adipocytes, immune cells and endothelial cells (Figure 2b). The progression of breast tumours starts with a benign proliferative lesion in the duct, which can develop into ductal carcinoma *in situ* (DCIS), this is, proliferation of ductal cells resulting in the clogging of the duct. If the tumour keeps progressing, it breaks the myoepithelial barrier and degrades the basement membrane, in consequence the duct losses its integrity, giving origin to an invasive ductal carcinoma (IDC). In this stage tumour cells are able to invade neighbouring and distant tissue, eventually leading to metastasis (Hu *et al.*, 2008; Place *et al.*, 2011).

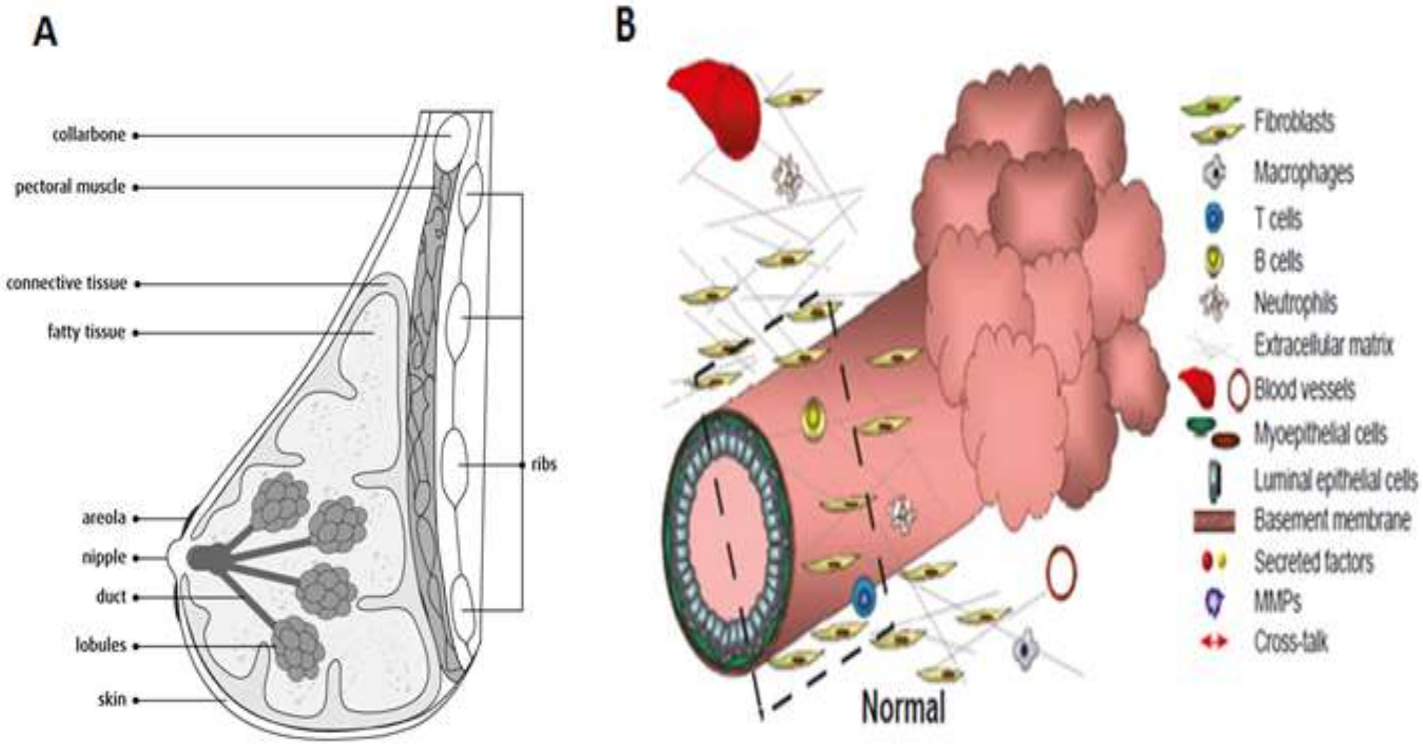

Figure 2. Anatomy of the breast and milk ducts. A) Anatomy of the breast showing the fatty stroma, lobes, lobules and ducts that form the mammary gland (Canadian Cancer Society). **B)** Structure of the milk duct, most common origin for breast cancer and its environment (Place *et al.*, 2011).

Metastasis. Most of breast cancer deaths are not due to the primary tumour in the breast, they are the result of metastases to distant organs (Weigelt *et al.*, 2005). Some steps in tumour progression must be fulfilled before metastasis occurs (Scully *et al.*, 2012). First, cells from the primary tumour must invade neighbouring tissue; this is mediated through the epithelial to mesenchymal transition (EMT)

(Kalluri and Weinberg, 2009). After EMT, tumour cells intravasate blood or lymph vessels and then extravasate in a given organ, where they tumour cells start proliferating, forming a new tumour far from the origin site (Scully *et al.*, 2012). Breast cancer shows a preference towards bone, brain, liver and lung metastases, whereas other sites are less common (Berman *et al.*, 2013).

III.1.3. Risk factors for breast cancer

Epidemiological studies have identified several risk factors for breast cancer. These risk factors can be classified in four groups.

A) Personal risk factors

Age: Breast cancer risk increases with age, twofold every 10 years, until menopause, when the risk increase slows down (McPherson *et al.*, 2000).

Sex: Only 1% of breast cancers are suffered by men worldwide (Fentiman *et al.*, 2006).

Geographical variation: Incidence is higher in developed countries. However, descendants of migrants from low-risk countries to high-risk countries eventually attain the incidence levels of the new country (Key *et al.*, 2001; Ziegler *et al.*, 1993).

B) Family history

Key genes: Mutations in BRCA1, BRCA2, p53 and PTEN are well described as risk increasing factors, although breast cancer caused by these mutations only account for 5% of all cases (Easton, 1999; McPherson *et al.*, 2000).

Benign proliferative lesions: A twofold increase in breast cancer risk has been reported in women bearing these lesions (Key *et al.*, 2001).

Family history: Women with first or second degree relatives bearing breast cancer present increased risk for developing breast cancer (McPherson *et al.*, 2000).

C) Reproductive and hormonal factors

Early menarche: Women that had an early menarche present a higher risk of developing breast cancer (Collaborative Group on Hormonal Factors in Breast, 2012).

Late menopause: For every year older at menopause, breast cancer risk is increased. In this way, premenstrual women have higher risk of breast cancer than postmenstrual women of the same age (Key *et al.*, 2001).

Childbearing: Childbearing is a protective factor in breast cancer and the risk diminishes with every birth. Age at firs child is essential, younger mothers have a lower risk to develop breast cancer compared to older mothers or childless women (McPherson *et al.*, 2000; Trentham-Dietz *et al.*, 2007).

Breastfeeding: The protective effect of breastfeeding and its duration has been reported, although the topic remains controversial (Lipworth *et al.*, 2000).

Oral contraceptives: Oral contraceptives increase breast cancer risk, but this risk decreases in time after use (Brinton *et al.*, 1995).

Menopausal hormone treatment: There is an increased risk in women using postmenstrual hormone treatment, and the risk is higher in combined hormone treatment than in oestrogen only treatment (Stefanick *et al.*, 2006).

D) Lifestyle

Alcohol consumption: A weak relation between alcohol consumption and breast cancer risk increase has been reported, especially in daily consumers (Trentham-Dietz *et al.*, 2000).

Obesity: It seems that obesity has a dual role in breast cancer risk. In premenstrual obese women there is a lower incidence, while obesity in postmenstrual women is an important risk factor (McPherson *et al.*, 2000; McTiernan, 2003).

High fat intake: It remains a controversial issue, but women with high fat intake present slightly increased risk for breast cancer (Cho *et al.*, 2003). The direct effect of dietary fatty acids has been widely discussed and results vary depending on the report (Holmes *et al.*, 1999; Howe *et al.*, 1990). However, it is clear that fatty acids are deeply involved in breast cancer development. In fact, as explained bellow, the specific composition of the fatty acid pool in the breast is related with breast cancer progression.

III.2. Fatty acids

Fatty acids (FAs). Fatty acids are carbon chains with a carboxyl group in the first carbon, widely found in the human body (Calder, 2015). Fatty acids are either saturated or unsaturated; the former are composed by a single bonded carbon chain, while the later contain double bounds, also called unsaturations (Rustan and Drevon, 2005).

Unsaturated fatty acids are classified as monounsaturated fatty acids (MUFAs), and polyunsaturated fatty acids (PUFAs). PUFAs in turn are classified according to the position of the first double bound after the carboxyl group. If it is located between the third and fourth carbon after the carboxyl it is an ω-3; if the unsaturation is between the sixth and seventh carbon, the fatty acid is an ω-6. PUFAs and their derivatives are essential in the human body, especially due to their involvement in signalling and inflammation (Rustan and Drevon, 2005).

Some fatty acids are indispensable for the correct functioning and homeostasis of the body. Although most fatty acids can be synthesised by the body from precursors, others must be obtained from diet. These fatty acids are called essential fatty acids, amongst which we can find linoleic acid (Das, 2006).

III.2.1 Functions of fatty acids

Functions of fatty acids. Fatty acids exert many function in the body and the cell. They are a component of membrane phospholipids, and the composition and relative abundance of specific unsaturated fatty acids in membranes regulates their fluidity. Fatty acids can also be used as energy source; triacylglycerol, made from fatty acids and glycerol, is the most common way to accumulate fat in the human body. In scarcity of glucose fatty acids from triacylglicerols are used as an energy source. Structural and energy reserve functions of fatty acids and related molecules are very well described (Calder, 2015).

Fatty acids are also important signalling molecules. Free fatty acids (FFA) in the extracellular milieu can be recognised by G protein coupled receptors (GPCR) called free fatty acid receptors (FFAR) (Hirasawa *et al.*, 2008). There are four FFARs, and each type recognises a different group of FFAs, with distinct specificity for individual fatty acids (Hirasawa *et al.*, 2008; Ichimura *et al.*, 2014). In the same way, FFAs can function as a transcription factors when stimulating certain intracellular receptors (Rustan and Drevon, 2005). This is the case of peroxisome proliferator activated receptor (PPAR), a transcription

factor that regulates lipid and glucose metabolism that can be weakly activated by certain fatty acids (Tyagi *et al.*, 2011). Some PUFA derivatives such as prostaglandins, have been proved to strongly activate PPARγ (Rustan and Drevon, 2005).

Fatty acids in cancer. The role of fatty acids in cancer has been extensively reported for PUFAs. The effects of ω-3 and ω-6 fatty acids are clearly different, and even opposite (Zhu *et al.*, 1995). The ω-3 PUFAs exert anti-apoptotic, anti-angiogenic, anti-proliferative and anti-inflammatory functions; in consequence, these fatty acids have anti-tumoural properties. The most potent ω-3 fatty acids are eicosapentanoic acid (EPA) and decosahexaenoic acid (DHA) (Spencer *et al.*, 2009). In contrast, ω-6 PUFAs are connected with cancer development; high ω-6 intake is a risk factor for colon, breast and prostate cancers. In the same way, a shift towards ω-6 in the ω-3:ω-6 ratio is considered to be a predictor for cancer progression (Xu and Qian, 2014). At least a part of these protumorigenic effects of ω-6 fatty acids is carried out by the metabolic pathways of arachidonic acid (Sakai *et al.*, 2012).

The predominant tissue in the breast is adipose tissue and fatty acids are used for milk production during lactation and as energy reserve (Jensen *et al.*, 1999). On the other hand, fatty acids act quite differently during tumorigenesis. The fatty acid composition of the breast changes in breast cancer patients, an increase of ω-6 fatty acid levels can be detected relative to ω-3 levels (Zhu *et al.*, 1995). In the same way, fatty acids can stimulate and signalise the tumour. Oleic and arachidonic acid, both ω-6 PUFAs, promote migration through FAK activation in a FFAR dependent manner in breast cancer cells (Navarro-Tito *et al.*, 2008; Navarro-Tito *et al.*, 2010).

Given the high obesity rates in Mexico (Secretaría de Salud, 2016), research on the role that fatty acids play in tumour progression is essential to conceive treatment methods and to tackle as effectively as possible this disease.

III.2.2. Arachidonic acid

Arachidonic acid (AA). It is a 20 carbon PUFA that plays a myriad of roles in the human body. AA is not an essential fatty acid and it can be synthesised from linoleic acid (Rett and Whelan, 2011) but it is mainly obtained from diet, with an average intake of about 200mg per day in western diets (Nelson *et al.*, 1997). Extracellular physiological concentration of AA has been reported to be around 13µM in skin, but in inflammatory contexts it can be increased up to 110µM (Hammarstrom *et al.*, 1975).

AA metabolism. AA is deeply involved in inflammation, mainly due to its metabolic products (eicosanoids). In consequence, intracellular concentration of AA is strictly controlled. It appears esterified as a phospholipid in the cell membrane and is only released by phospholipase A2 (Harizi *et al.*, 2008). Once AA is found free in the cytoplasm, it is rapidly metabolised by one of the three metabolic pathways of AA, lipoxygenase (LOX), cyclooxiygenase (COX) and P450 epoxygenase (CYP), whose products are prostaglandins, thromboxanes, epoxyds, leukotryenes and hydroxyeicosatetraenoic acids (HETEs) among others (Harizi *et al.*, 2008).

AA in cancer. The role of AA metabolites are well reported in cancer. Cyclooxygenase-2 (COX2), one of the enzymes responsible for prostaglandin production, is overexpressed in breast cancer (Hu *et al.*, 2009). In contrast, 15-PGDH, a prostaglandin oxidation enzyme, is downregulated (Wolf *et al.*, 2006). Overexpression of LOXL2, part of the lipoxygenase pathway, in colon cancer stroma is associated with poor survival (Torres *et al.*, 2015). In breast cancer, prostaglandin E_2 (PGE_2) has been shown to stimulate the production of aromatase in adipose tissue; this enzyme is necessary for the synthesis of oestrogen, which promotes tumour cell proliferation (Zhou *et al.*, 2005).

Eicosanoids are very important in the creation of the tumour microenvironment. PGE_2 is responsible for the immunological switch of the microenvironment, from an antitumoral T_H1 to an immunosuppressive T_H2 response. They mediate the downregulation of T_H1 cytokines such as interferon gamma (IFNγ), tumour necrosis factor alpha (TNFα) and interleukin 2 (IL-2) as well as the upregulation of T_H2 cytokines, such as IL-4, IL-6 and IL-10 (Wang and Dubois, 2010). It has been reported that PGE_2 can inhibit the antitumoural effects of cytotoxic T cells by preventing antigen presentation both directly by tumour cells and indirectly through dendritic cells (Ahmadi *et al.*, 2008).

Moreover, PGE_2 is linked with angiogenesis. In a mouse cancer model, the signalling of PGE_2 through its receptor Ep2, induced the secretion of VEGF in intestinal polyp stroma (Sonoshita *et al.*, 2001). In human colorectal cancer, PGE_2 is responsible for the secretion of CXCL1, a proangiogenic chemokine (Wang *et al.*, 2006).

III.3. Extracellular Vesicles

III.3.1. Classification

Extracellular vesicles (EVs) are nanosize membrane compartments that are secreted by cells to the extracellular medium. EVs are considered the third mechanism of intercellular communication together with cell-cell interactions and secreted factors mediated communication (Raposo and Stoorvogel, 2013). They are found in different body fluids such as blood (Caby *et al.*, 2005), saliva (Ogawa *et al.*, 2011), semen (Ronquist and Brody, 1985) and milk (Admyre *et al.*, 2007), both in physiological and pathological conditions. EVs have had several classification and nomenclature, based on size, function and cell origin among others (Gould and Raposo, 2013). However, a relative consensus has been attained with the classification based on biogenesis.

Exosomes. These EVs of endosomal origin range from 30nm to 100nm (Thery *et al.*, 1999). Exosomes are formed following a 4 step process: initiation, endocytosis, formation of the multivesicular body (MVB) and exosome secretion (Thery *et al.*, 2002). MVBs are endosomes that carry vesicles budded from the endosomal membrane into the lumen. This process can be carried both dependent and independently of ESCRT family proteins (Trajkovic *et al.*, 2008). Once these intraluminal vesicles are formed, there are two possible pathways: the lysosomal pathway, consisting in fusion with lysosomes and subsequent degradation, or the secretory pathway (Mobius *et al.*, 2002). Following the lated, the MVB fuses with the cell membrane and releases the intraluminal vesicles, exosomes, into the extracellular space (Raposo and Stoorvogel, 2013).

Microvesicles. Microvesicles bud directly from the cell membrane to the extracellular medium and have a size of 100nm to 1µm (Heijnen *et al.*, 1999). Processes such as cell activation and growth are accompanied with microvesicle shedding, and several stimuli such as cytokines, endotoxins, hypoxia and oxidative stress, have been reported as microvesicle secretion enhancers (Lynch and Ludlam, 2007).

Apoptotic bodies. Apoptotic bodies are in the range of 50nm to 2 µm, and they are secreted only by apoptotic cells during the late stages of apoptosis (Beyer and Pisetsky, 2010). They contain nuclear material, organelles and cytosolic fractions (Elmore, 2007). Apoptotic bodies have an irregular shape and possess high membrane permeability and expose phosphatydylserine, a trait shared with the apoptotic cell itself (Dignat-George and Boulanger, 2011; Thery *et al.*, 2001).

Due to the specific origins of apoptotic bodies – i.e. only apoptotic cells –, they are relatively left apart in some research areas. In other words, when EVs are addressed in papers about cancer for example, most of the studies encompass exosomes and microvesicles only. However, there are reports about apoptotic bodies in cancer (Bergsmedh *et al.*, 2001). Apoptotic bodies have also been left apart in this thesis.

III.3.2. Cargo, composition and uptake.

*Lipids***.** Most of the studies about lipid composition of EVs have been carried out in exosomes. The following sentences address to exosome lipid composition. EVs are delimited by a lipid bilayer, structurally similar to the cell membrane. The lipid cargo however, varies. There is an enrichment of sphingomyelin, gangliosides, and monounsaturated or saturated fatty acids, and decreased proportion of phosphatidylcholine and diacylglicerol (Laulagnier *et al.*, 2004). A significant portion of EVs expose phosphatidylserine in the outer membrane, which may facilitate fusion with target cells (Fitzner *et al.*, 2011). Cholesterol enriched exosomes have also been described (Llorente *et al.*, 2013). This particular lipid composition gives more rigidity to exosomes, which has been proposed to be pH dependant, the membrane losing its stiffness at lower pH. It is related with the acidic condition of the endosomal compartment, where the exosome membrane ought to be more fluid (Laulagnier *et al.*, 2004). The rigidity of exosome membranes in low pH resembles to that of cell membranes, consequently promoting membrane fusion and EV uptake in acidic conditions, such as the tumour microenvironment (Parolini *et al.*, 2009). Thus the specific composition of exosome membranes makes them stable and secure transporters for their cargo (Zaborowski *et al.*, 2015). It has also been reported that bioactive lipids can be carried by EVs, such as AA and PGE_2 as well as membrane lipid regulating enzymes, phospholipases (Subra *et al.*, 2010).

In respect of the lipid content of microvesicles, fewer reports have been published. Haraszti and colleagues carried out a high resolution proteomic and lipidomic analysis of microvesicles and exosomes from three different cell types: hepatocellular carcinoma, glioblastoma and human bone marrow derived mesenchymal stem cells (BM-MSC). They demonstrated that microvesicles are enriched in ceramides and sphingomyelins, whereas exosome lipids such as glycolipids, FFA and phosphatydylserine were absent or not enriched in microvesicles. These analyses show that although there is a strong EV type dependant lipid enrichment, the origin of EVs (i.e. cell type) is important (Haraszti *et al.*, 2016).

RNAs. An interesting feature of EV cargo is the abundance of RNAs. A varied collection of RNAs is found in EVs and its average size is shorter than RNAs found in the cell.

a) mRNAs. Full-length and fragmented mRNAs have been found in EVs. Interestingly, specific RNAs are enriched in EVs. A microarray analysis fulfilled in glioblastoma derived EVs showed that, out of 27,000 mRNAs, ~ 4,700 were exclusive from EVs and not detected in cells, and ~3,000 were preferentially included or excluded from EVs (Skog *et al.*, 2008). The authors also tested if EV-carried mRNAs could be translated in target cells. For that, they expressed luciferase in glioblastoma cells. Recipient cells were stimulated with glioblastoma derived EVs. Luciferase activity resulted in recipient cells, hence demonstrating that EV transported mRNAs can be translated (Skog *et al.*, 2008). Similar results were obtained in an analogous analysis carried out in a mast cell line (Valadi *et al.*, 2007).
b) miRNAs. They are found in body fluids, they appear associated to RNA binding proteins and lipoproteins or carried as cargo in EVs (Nishida-Aoki and Ochiya, 2015). miRNAs regulate gene expression by blocking the translation of specific mRNAs in the cytoplasm (Bartel, 2004). EV carried miRNAs are able to alter gene expression by inhibiting translation, but several other functions have also been described, mainly in the immune system. Mittelbrunn and colleagues reported that T cell derived exosomes were taken up by antigen presenting cells (APC) during immunological synapse. The authors carried out a luciferase reporter assay where the 3'-UTR of the luciferase gene was designed as a target for miR-335. Luciferase expression was strongly decreased when APCs were treated with miR-335 carrying exosomes (Mittelbrunn *et al.*, 2011). Among the functions not related to gene expression, it has been published that exosomal miRNAs can activate Toll like receptors (Chen *et al.*, 2013).

DNAs. It has recently been discovered that EVs can transport genomic double stranded DNA, in sizes bigger than 10 kilobase pairs (Kahlert *et al.*, 2014). Intact EVs were treated with DNases, and the resulting DNA fragments appeared shorter that those found in untreated EVs (Thakur *et al.*, 2014). This suggests that DNA is associated with EVs but not necessarily enclosed in them. DNA in EVs is very similar to nuclear DNA. EV-DNA is composed by DNA from all parts of the genome (Kahlert *et al.*, 2014). The methylation pattern also seems to be very similar to that found in nuclear DNA (Thakur *et al.*, 2014) and mutations in parental genome are also present in EV-DNA (Kahlert *et al.*, 2014).

*Proteins***.** A myriad of different proteins are found in EVs. Some of these are defined by the cell of origin, and others from the biogenesis and EV subtypes, (exosomes or microvesicles). However, there is a subset of proteins present in all EVs irrespective of cell origin and EV subtype. These protein are used as markers and purification control. This is the case for major histocompatibility complex (MHC) I and II, heat shock proteins (HSP), Alix1, tetraspanins and flotillin (Svensson *et al.*, 2013; Wubbolts *et al.*, 2003). A part from the proteins that organise and maintain EV integrity – those associated with lipids and responsible for biogenesis for example – several bioactive protein are selectively enriched as cargo. EVs derived from ovarian cancer cells showed high levels of effector proteins, such as PI3K and MAPK (Sinha *et al.*, 2014). These proteins are essential in many cellular responses, and control important processes like proliferation. Transcription factors, receptors, cytoskeletal proteins and enzymes are also common in EVs, as well as nucleic acid binding proteins. Thanks to the varied cargo of EVs, they can exert profound alteration in the behaviour of recipient cells, both in physiological and disease contexts. Proteomic analysis have been carried out to describe the protein cargo of EVs (Sinha *et al.*, 2014; Wubbolts *et al.*, 2003).

Uptake. Once EVs have been released they have to be taken up by target cells, both distant and adjacent. It is noteworthy that EVs are not captured at equal rates and randomly by any cell type. Keller and colleagues, showed that ovarian cancer cell derived EVs were profusely captured by natural killer cells but very little by T cells (Keller *et al.*, 2009). This target specificity of EVs is probably driven by membrane proteins both in EVs and target cells, such as immunoglobulins and integrins. As an example, manganese chloride, a strong inductor of a high affinity state of the lymphocyte function-associated antigen 1 (LFA-1), was added to resting T cells, which resulted in an increased uptake of EVs (Nolte-'t Hoen *et al.*, 2009).

Different uptake mechanisms have been described. EVs cannot be captured at 4ºC, which means the process is energy dependant (Escrevente *et al.*, 2011). EVs can stimulate target cells by internalisation and transfer of cargo, or by ligand-receptor interactions between the cell and EVs. The later has been proven in dendritic cell derived exosomes. These EVs harbour NKG2D ligands that can activate NK cells, through NKG2D receptors, exerting their effect in a receptor-ligand fashion (Viaud *et al.*, 2009). Although, cell membrane-EV fusion is possible (Parolini *et al.*, 2009), growing evidence suggests that the main way of EV uptake is endocytosis. Cytochalasin D, an actin polymerisation blocking agent and

endocytosis inhibitor, strongly decreases EV uptake (Svensson *et al.*, 2013). Knockout or inhibition of dynamin, an essential protein for the formation of endosomal vesicles, also reduces EV capture (Nanbo *et al.*, 2013). The role of clathrin dependant endocytosis remains under debate. A study using a clathrin dependent endocytosis inhibitor resulted in a decrease in EV uptake (Escrevente *et al.*, 2011), whereas a clathrin knockdown experiment showed no significant decrease (Svensson *et al.*, 2013). These contradicting data may respond to a differential implication of this pathway in EV capture, depending of parental and target cells, and specific conditions. To a lesser extent, EVs can also be internalised by phagocytosis and macropinocytosis in cell types that normally undergo these processes (Escrevente et al., 2011; Feng et al., 2010).

III.3.3 Role of extracellular vesicles

EVs in cancer. EVs are involved in many processes of tumour progression. In fact, a comprehensive review was published about the role that EVs play in the hallmarks of cancer proposed by Hanahan and Weinberg (Hanahan and Weinberg, 2011; Meehan and Vella, 2016). Some of these processes and roles are discussed below.

a) Tumour growth support. Tumours show altered proliferative pathways to enable continuous proliferation, such as PI3K/Akt and MAPK/ERK pathways (Hanahan and Weinberg, 2011). Bladder cancer derived EVs are able to provoke proliferation via the PI3K/Akt and ERK pathways (Yang *et al.*, 2013). In the same way, Qu and colleagues showed that gastric cancer derived exosomes were able to induce proliferation and ERK1/2 phosphorylation through the MAPK pathway (Qu *et al.*, 2009).
b) Evasion of cell death. EVs downregulate the expression of several propaoptotic or autophagic proteins. EVs released by BM-MSC inhibited the Jun-N terminal kinase (JNK) pathway, related with autophagic death, and downregulated Bcl-like protein 11 (Bim), a proapoptotic factor (Wang *et al.*, 2014) in myeloma cells. In the same way, bladder cancer derived exosomes have been shown to enhance survival and evade apoptosis by the upregulation of Bcl-2 and cyclin D1 and downregulation of Bax and caspase-3 (Yang *et al.*, 2013).
c) Angiogenesis. There are multiple means by which EVs induce or enhance angiogenesis. Nazarenko and colleagues reported that certain tetraspanins in EVs primed internalisation by endothelial cells, upregulating angiogenesis related genes (Nazarenko *et al.*, 2010). Moreover,

leukaemia derived EVs are capable of inducing endothelial cell migration and tubule formation, via the effects of miR-92a (Umezu *et al.*, 2013). It has also been reported, that melanoma derived exosomes were able to induce the secretion of proangiogenic factors in endothelial cells, such as Il-1α and TNF-α, among others (Hood *et al.*, 2009).

d) Invasion and metastasis. EMT confers a mesenchymal phenotype to epithelial cells in carcinomas. The acquisition of this phenotype enables tumour cells to migrate and invade tissue, permitting metastasis (Kalluri and Weinberg, 2009). Several EMT related factors have been found in EVs, Il-6, TGF-β and TNF-α for example (Ramteke *et al.*, 2015). Adipose tissue derived mesenchymal cell (AD-MSC) exosomes are able to induce migration and invasion in breast cancer via Wnt pathway (Lin *et al.*, 2013). Interestingly, there is a cargo change in EVs after tumour cells have undergone EMT: epithelial related protein contain decreases, and mesenchymal markers increase (Tauro *et al.*, 2013). Exosomes are also involved in the creation of the pre-metastasic niche. Peinado and colleagues reported that melanoma derived exosomes injected in naïve mice, tended to appear in specific sites such as lungs and spleen. After ortopic implantation of a melanoma cell line in mice, metastasis burden was increased in mice injected with EVs. This suggests that EVs induce changes in their "docking sites" – i.e. lungs and spleen – to create a pre-metastasic niche in melanoma (Peinado *et al.*, 2012).

EVs in BC. The role that EVs play in breast cancer is currently under active study. It is important to assess their function, because breast cancer patients show elevated concentrations of EVs (Galindo-Hernandez *et al.*, 2013). Proteins and miRNAs transported in EVs can promote neoplastic transformation and widely participate in different stages of breast cancer development (Jiang *et al.*, 2015; Zhang and Ma, 2012).

The most common sites for breast cancer metastasis are bone, brain, liver, and lung (Berman *et al.*, 2013). Several studies, using cell lines, preclinical *in vivo* studies, and clinical samples, have started to unravel the role of EVs in breast cancer invasion and metastasis. EVs secreted by breast cancer cells travel to normal lung tissue, the future metastasic niche, in an orthotopic nude mouse breast cancer models (Suetsugu *et al.*, 2013). In particular, miR-105 overexpression in local breast cancer cells enhances vascular permeability and induces metastasis. In fact, exosome-mediated transfer of miR- 105

can disrupt tight junctions and the integrity of endothelial monolayers, thereby promoting the metastatic progression of breast cancer (Zhou *et al.*, 2014).

Studies have shown that breast cancer cell derived exosomes can influence the immune system through interactions with T cells, dendritic cells, macrophages, and T regulatory cells. Exosomes can restrain immunological responses through downregulation of the NKG2D receptor in effector cells, which promotes breast cancer immune evasion (Clayton and Tabi, 2005).

EVs are important players in chemoresistance of breast cancer. HER-2 positive exosomes act as decoys to inhibit trastuzumab activity (Ciravolo *et al.*, 2012). Exosomal transport of P-glycoprotein (P-gp) has been described as another possible mechanism in exosome mediated drug resistance in breast cancer (Lv *et al.*, 2014). Exosomes from tamoxifen-resistant MCF-7 breast cancer epithelial cells were found to promote proliferation of MCF-7 wild-type cells. Functional assays (cell viability, apoptosis, and colony formation) assessed the involvement of miR-221 and miR-222 in the transfer of this tamoxifen resistance (Wei *et al.*, 2014).

III.4. Microenvironment and Cancer Associated Fibroblasts

III.4.1 Overview of tumour microenvironment

Tumour microenvironment. The tumour is not just a bulk of malignant cells growing uncontrolled. They are accompanied by a supportive microenvironment that promotes tumour growth. The complex crosstalk between non-malignant stromal cells and tumours results in a chronic inflammatory microenvironment with an altered signalling profile that enhances tumour progression (Mueller and Fusenig, 2004). The first notion of tumour microenvironment (TME) was proposed over 100 years ago by Stephen Paget (Paget, 1889). He noted that breast cancer metastases tended to spread to the same organs in different patients. Thus, he formulated the “Seed and soil” hypothesis, stating that there must be a fertile soil (i.e. the tissue of invaded organs) so the seeds (tumour cells) are able to grow there. The differences in the soil would explain the preference of certain tumours towards specific organs.

Harold F. Dvorak stated that “Tumours are wounds that do not heal”, due to the many events common both to wounds and tumours, such as, angiogenesis, cell infiltration and survival (Dvorak, 1986, 2015).

In fact, Dvorak proposes that tumours co-opt the wound healing response to create the stroma necessary for tumour progression (Dvorak, 2015). Tumour stroma is composed by various cell types, either recruited to the site or resident cells transformed by the tumour itself. These cells are not malignant *per se* but they acquire an abnormal phenotype that is associated to the tumour (Mbeunkui and Johann, 2009). Tumour microenvironment is also rich in ECM elements and soluble factors, such as cytokines and growth factors; these are secreted and produced both by tumour and stromal cells (Li *et al.*, 2007).

Cell types in TME. Many different cell types conform the tumour stroma, and the convergence of the signals and factors produced by these cells, organises the tumour supporting properties of the TME.

Immune cells are an essential part of tumour stroma. As mentioned above, a chronic inflammatory environment is necessary for tumour growth and immune cells are one of the main contributors to it (Coussens and Werb, 2002). In the first stages of tumour progression, an antitumoural immune response takes place, mainly driven by T_H1 cells (Xu, 2014). For the tumour to progress, the immune response must be evaded and the profile of T cells in the tumour site shifts towards a more tumour supporting population of T cells, consisting of T_H2, T_H17 and T regulatory cells (Coussens and Werb, 2002; Chang *et al.*, 2014; Pastille *et al.*, 2014). Macrophages are of great importance in the tumour site as well. Monocytes are recruited from the peripheral blood and differentiate into a specific subtype of macrophages in the tissue, the so called tumour associated macrophages, TAMs (Wynn *et al.*, 2013). These macrophages promote migration, invasion, ECM remodelling and angiogenesis, and they are considered a marker for poor prognosis (Qian and Pollard, 2010). Myeloid derived suppressor cells are dysfunctional precursor cells, abundant in many cancers (Gabrilovich and Nagaraj, 2009). They interfere with the normal antitumoural response of the immune system, thus contributing to the immune evasion of the tumour (Marigo *et al.*, 2008). Other immune cells, such as mastocytes, play a dual role in tumour progression, taking part both in antitumoural response and immune evasion of the tumour (de Souza *et al.*, 2012; Piconese *et al.*, 2009).

Angiogenesis is one of the most important hallmarks in tumour progression (Hanahan and Weinberg, 2011). Both tumour and tumour stroma induce angiogenesis through the secretion of angiogenic factors such as VEGF and SDF1/CXCL12 (Verheul and Pinedo, 2000; Weis and Cheresh, 2011). Endothelial and bone marrow derived stem cells migrate to the tumour site, where they will form the abnormal new

blood vessels. These abnormal blood vessels result in an insufficient O_2 diffusion which enhances tumour growth by various means (Hillen and Griffioen, 2007).

III.4.2 Cancer associated fibroblasts

Cancer associated fibroblasts (CAFs). CAFs are one of the most abundant cell types in TME. These cells are phenotypically very similar to myofibroblasts found in wounds, in accordance with Dvorak's hypothesis (Dvorak, 1986). CAFs are involved in all the stages of tumour progression, and they perform several functions through the secretion of soluble factors and ECM components, as well as extracellular vesicles (Madar *et al.*, 2013).

Origins of CAFs. Different cell types can give birth to the CAF population in TME. The rather obvious source is tissue resident normal fibroblasts (NFs), due to the fact that they are situated adjacent to the tumour. Some reports have shown that normal fibroblasts can acquire a CAF phenotype when put in contact with tumours or tumour derived factors. Normal mammary fibroblasts co-implanted with MCF-7 breast cancer cells in a mouse xenograft model, were transformed into CAFs (Kojima *et al.*, 2010). It is well documented that miRNAs are deregulated in human cancer cells. It was recently reported that this phenomenon takes place in CAFs as well. Mitra and colleagues mimicked the deregulated miRNA profile of CAFs in normal fibroblasts and it resulted in the acquisition of a CAF-like phenotype (Mitra *et al.*, 2012).

Another well-established source of CAFs is mesenchymal stem cells (MSC), derived from different sites. These cells can be recruited to the tumour site, and then they acquire a tumour supporting phenotype (Chang *et al.*, 2015). Human BM-MSC treated over a long period with tumour conditioned media resulted in the transformation of these cells into CAFs (Mishra *et al.*, 2008). The same results were obtained in a MSC – tumour xenograft model (Spaeth *et al.*, 2009). In a murine pancreatic cancer model that followed a BM-MSC transplant, 25% of CAFs in tumour were donor derived (Direkze *et al.*, 2004).

It has been reported that epithelial cells make up a significant fraction of myofibroblasts, analogue to CAFs, in kidney fibrosis (Iwano *et al.*, 2002). Carcinoma cells can undergo EMT and become CAFs in certain cases. Mink and colleagues reported that 24% of CAFs in the stroma of tumours treated with EGFR tyrosine kinase inhibitors were tumour derived (Mink *et al.*, 2010). The epithelial origin of CAFs in cancer has been thoroughly reviewed by Radisky and colleagues (Radisky *et al.*, 2007).

It has been demonstrated that endothelials cells can undergo an endothelial-to-mesenchymal transition (EndMT), an EMT counterpart. This process plays an important role in various pathogenic states, as reviewed by Potenta and colleagues (Potenta *et al.*, 2008). Therefore, endothelial cells can be a source of CAFs in some cancers.

Two conclusions can be drawn from the reports demonstrating the diverse origins of CAFs. First, CAFs are a very heterogeneous population, and second, tumours have the ability to induce and maintain the CAF phenotype in different cell types. Although the mechanisms for this transformation remains obscure, TGF-β is essential for the acquisition of the CAF phenotype (Casey *et al.*, 2008; Ronnov-Jessen and Petersen, 1993).

CAF markers. Several CAF markers have been defined by immunohistochemical assays (Garin-Chesa *et al.*, 1990; Strutz *et al.*, 1995). However, these markers are not exclusive or even shared by all CAFs. In accordance with the diverse origin of these cells, markers may vary among CAF subpopulations. A combination of several markers can shed light in the identification of CAFs in tissue (Shiga *et al.*, 2015).

The canonical CAF and myofibroblast marker is α smooth muscle actin (α-SMA), which is found significantly upregulated in comparison with normal fibroblasts (Ronnov-Jessen and Petersen, 1993). This protein enhances the contractile ability of fibroblasts, an important function during wound healing, related with stress fibre formation (Hinz *et al.*, 2001). Other cytoskeletal proteins such as the intermediate filament vimentin and desmin are also upregulated in CAFs (Togo *et al.*, 2013).

CAFs play an essential role in ECM reorganisation, permitting tumour growth and adjacent and distant tissue invasion (Kalluri and Zeisberg, 2006). Thus, some ECM related enzymes, both upregulated and downregulated, are considered CAF markers. Tenascin-c, neural glial antigen 2, periostin and MMP1, are found upregulated in CAFs and have been reviewed in several reports (Togo *et al.*, 2013). These proteins are related with ECM degradation, migration and invasion (Kalluri and Zeisberg, 2006).

Some membrane proteins have altered expressions in CAFs, compared to other stromal cells. Such is the case for fibroblast activation protein α (FAP α), platelet derived growth factor receptor (PDGFRβ) and caveolin 1 (Cav-1) (Augsten, 2014; Zhao *et al.*, 2013). FAP is a membrane serine protease whose main substrates are collagen I and gelatine, this is, collagen IV (Brokopp *et al.*, 2011). FAP can collaborate with MMPs, therefore the upregulation of FAP demonstrates the important function of CAFs in ECM

remodelling (Kelly *et al.*, 2012). PDGFR in turn, is a tyrosine kinase, involved in cell growth, proliferation and survival. In fibroblasts, PDGFRβ is essential for the activation and subsequent transformation into CAFs, moreover PDGF secreted by tumour cells stimulate CAFs and induce their proliferation (Anderberg *et al.*, 2009). CAFs also secrete PDGF that can act in a paracrine fashion, stimulating cancer cell invasion, migration, survival, and proliferation (Gialeli *et al.*, 2014). Caveolin 1, an essential protein for caveolae formation, is a prognosis marker in cancer, due to the many roles it plays in membrane trafficking, gene regulation and lipid transport. Overexpression of this protein correlates with good outcome (Shan-Wei *et al.*, 2012) . In CAFs however, this protein appears downregulated compared with other cells (Mercier *et al.*, 2008).

Finally, fibroblast specific protein (FSP), a cytosolic calcium binding protein, is a marker of activated fibroblast in different contexts, such as fibrosis or TME. CAFs have been reported to present increased expression of this protein (Sugimoto *et al.*, 2006).

Tumour supporting function of CAFs. CAFs are one of the most versatile cells in the TME. The functions of CAFs are not restrained to induce sheer tumour growth. CAFs take part in other events, such as the creation of a tumorigenic stroma, metastasis and angiogenesis.

In the primary site of the tumour, CAFs are involved in tumour initiation. CAFs can enter senescence the same way other cells do, and senescent CAFs can be found in tumour stroma (Hassona *et al.*, 2014). Krtolica and colleagues showed that senescent fibroblasts can enhance tumorogenicity of premalignant and malignant epithelial cancer cells, partly through the secretion of soluble factors (Krtolica *et al.*, 2001). CAFs promote proliferation of tumour cells. Treatment of endometrial and hepatic cancer cells with CAF conditioned media, induced proliferation in both cases (Jia *et al.*, 2013; Subramaniam *et al.*, 2013). In the later, hepatocyte growth factor (HGF) was demonstrated to be the main responsible for the effects on proliferation (Jia *et al.*, 2013). This shows that CAF secrete factors that have an impact in tumorogenicity and proliferation. In fact, CAFs produce several growth factors, essential for proliferation (Schmitt-Graff *et al.*, 1994).

CAFs can also contribute to the creation of a tumour promoting and supporting TME. Various reports show that CAFs play a role in angiogenesis. Some CAF secreted factors are able to indirectly induce angiogenesis. Quiescent endothelial cells are unresponsive for PDGR, but fibroblast growth factor (FGF)

stimulation, transcriptionally switches on PDGFR expression, thus activating endothelial cells (Cao *et al.*, 2008). Direct CAF-induced angiogenesis has also been reported. In a mouse gastric cancer model, VEGF expression was significantly upregulated in the tumour stroma. The stimulation of CAFs with tumour conditioned medium resulted VEGF overexpression and acceleration of tube formation in umbilical vein endothelial cells *in vitro* (Guo *et al.*, 2008). CAFs are involved in inflammation and modulation of the immune response and can directly provoke inflammation in the tumour site. Reports demonstrate that inflammation related genes are upregulated in CAFs from different cancers. Among these, cytokines IL-6 and TGFβ2, chemokines such as CXCL1 and the AA metabolising enzymes, LOXL2 and COX2 can be found, (Erez *et al.*, 2013; Nakagawa *et al.*, 2004; Torres *et al.*, 2015). This proinflammatory gene signature in CAFs enhances tumour growth and angiogenesis, mainly via NF-κB signalling pathway (Erez *et al.*, 2010). CAFs also contribute to the immunosuppressive characteristics of the tumour site. Liao and colleagues demonstrated that elimination of CAFs in a breast cancer murine model restored a normal antitumoural immune response in the tumour site, (Liao *et al.*, 2009). CAFs interact with other immune cells in the stroma. In fact, CAF-produced collagen and fibronectin hydrolytic products and SDF1, attract immune cells such as macrophages and T cells to the tumour site (Brundula *et al.*, 2002; Comito *et al.*, 2014). Finally, supernatant from pancreas stellate cells, a subpopulations of pancreas cancer CAFs, was able to promote differentiation of peripheral blood mononuclear cells into myeloid derived suppressor cells in a STAT3 dependent manner (Mace *et al.*, 2013).

CAFs further enhance cancer growth by altering tumour behaviour towards a more invasive phenotype. There is an interesting metabolic crosstalk between CAFs and tumour cells. The hypothesised Warburg effect in cancer cells – decreased oxidative phosphorylation and increased anaerobic glycolysis due to the lack of O_2 – is now discussed (Warburg, 1956). Another model has been proposed, the "Reverse Warburg effect". In contrast to the previous hypothesis, cancer cells induce Warburg effect in surrounding stromal cells (Pavlides *et al.*, 2009). Cancer cells are able to downregulate Cav1 expression in CAFs, thus enhancing NO production, oxidative stress and mitochondrial dysfunction (Gonzalez *et al.*, 2014; Martinez-Outschoorn *et al.*, 2010). With a disrupted mitochondrial metabolism, CAFs are unable to carry out the Tricarboxylic Acid (TCA) cycle, consequently energy is obtained from anaerobic glycolysis and fermentation. The high energy products from these processes (pyruvate and lactate) are secreted to the intercellular milieu where they are taken up by cancer cells and oxidised in the TCA cycle

(Martinez-Outschoorn *et al.*, 2010). Thus the reverse Warburg effect permits a better energy use in the tumour, enhancing its growth. CAFs also promote tumour cell migration and invasion, through different means. Secretion of certain soluble factors, remodelling of the ECM and EMT are important processes for invasion and metastasis. TGF-β is able to induce EMT in cancer cells, and CAFs secrete this cytokine (Yu *et al.*, 2014). CAF derived HGF and Tenascin C were able to induce invasion in colon cancer cells. Interestingly, neither of these was sufficient to promote invasion by itself, which illustrates the interdependent roles of ECM remodelling factors and secreted factors (De Wever *et al.*, 2004). CAF behaviour in ECM homeostasis and remodelling is disrupted when compared to normal fibroblasts, as an example MMPs are overexpressed in CAFs (Miles and Sikes, 2014). Gaggioli and colleagues showed that CAFs create tracks in collagen I matrixes, and lead the migration of cancer cells through those tracks, enabling invasion while cancer cells keep epithelial traits (Gaggioli *et al.*, 2007). CAFs can alter the ECM so it acquires an oriented alignment, permitting the directional migration and invasion of cancer cells (Lee *et al.*, 2011).

Finally, CAFs have also been involved in drug resistance. Cetuximab is a RTK (receptor tyrosine kinase) inhibitor that kills tumour cells in certain cancers (Li *et al.*, 2005), head and neck squamous cell carcinoma (HNSCC) among them. Different cell lines of HNSCC cocultured with CAFs and treated with Cetuximab, showed a decrease in cell death rate and in efficiency of Cetuximab. MMPs inhibitors abolished the protective effect against Cetuximab, suggesting that members of the MMP family might be responsible for cetuximab resistance (Johansson *et al.*, 2012). A similar experiment was carried out by Kharaziha and colleagues, were prostate cancer cells cocultured with CAFs showed resistance against Sorafenib treatment, another multi-RTK inhibitor, through the maintenance of Bcl-2 family member levels (antiapototic proteins) by CAFs (Kharaziha *et al.*, 2012).

*Tumour suppressive function of CAFs***.** It has been recently discovered that CAFs can also exert tumour suppressing functions. In a thorough paper, Özdemir and collaborators demonstrated in a pancreatic cancer mouse model that tumours were more aggresive when CAFs were depleted. Ganciclovir treatment in these mice, targeted proliferating α-SMA positive cells, CAFs, and eliminated them. CAF depleted mice showed markedly decreased survival rates, while tumours appeared smaller (weight loss) and more invasive and undifferentiated (Ozdemir *et al.*, 2014). Podoplanin (a membrane glycoprotein) overexpressing CAFs inhibited proliferation in small cell lung cancer cell lines in comparison

with control CAFs. The ablation of podoplanin expression restored the growth rates of cocultured cancer cells (Takahashi *et al.*, 2015). Finally, antitumoural behaviour of CAFs can be linked with the contradictory role of TGF-β, which inhibits tumour growth at early stages and enhances it in latter stages (Engle *et al.*, 1999; Ishii *et al.*, 2016). In conclusion, CAFs are able both to support and suppress tumour progression, emphasising the ambivalent role of this cell type, and its nature of "two blade spade".

CAFs in breast cancer. Breast tumours are desmoplastic, this is, there is an excessive production of connective tissue, and up to 90% of tumour mass can be composed by stroma (Dvorak, 1986). Sappino and colleagues showed that more than 80% of stromal cells in breast carcinomas are activated and positive for α-SMA (Sappino *et al.*, 1988).

Breast tumour CAFs have different origins. It has been demonstrated that human BM-MSCs acquire a CAF phenotype after 30 days exposure to tumour cell conditioned media (Katanov *et al.*, 2015). Normal fibroblasts co-cultured with breast cancer MDA-MB-468 cells resulted in hepatocyte growth factor secretion by fibroblasts and tumour growth enhancement, a typical CAF behaviour (Tyan *et al.*, 2011). As mentioned above, Mink and colleagues reported that 24% of CAFs in the stroma of tumours treated with EGFR tyrosine kinase inhibitors were tumour cells that underwent EMT and acquired a CAF phenotype (Mink *et al.*, 2010).

CAFs are able to induce growth and tumorigenesis in breast cancer (Huang *et al.*, 2010; Yamamura *et al.*, 2015). CAFs are partially responsible of the essential chronic inflammation in the tumour site, through the overexpression of inflammation related agents, such as COX2, IL-6 and CXCL-1 (Erez *et al.*, 2013). Angiogenesis is induced by breast CAFs through the elevated secretion of SDF1/CXCL12 which promotes endothelial cell recruitment to the tumour site, permitting the formation of new vessels and subsequently tumour growth (Orimo *et al.*, 2005). Soon and colleagues demonstrated that breast tumour CAFs increased mesenchymal marker expression in MCF7 breast cancer epithelial cell line, provoking EMT in these cells (Soon *et al.*, 2013). Other reports have shown that CAFs enhance tumour invasion through the overexpression of MMPs (Eck *et al.*, 2009; Wang *et al.*, 2002). In the same way, CAFs can confer chemoresistance in breast cancer as well. The secretion of IL-6 by CAFs results in the degradation of ER-α and resistance to Tamoxifen (Sun *et al.*, 2014). Breast CAFs can also induce the resistance to Doxorubicin in MDA-MB-231 breast cancer cells (Amornsupak *et al.*, 2014).

III.4.3. Relation between EVs and CAFs.

EVs and CAFs. EVs and CAFs are tightly related. EVs are one of the responsible for the acquisition of the CAF phenotype in some cells types. Cho and colleagues carried out an experiment, were stimulation of AD-MSC with breast cancer derived exosomes, resulted in expression of CAF marker α-SMA, as well as upregulation of several relevant cytokines such as VEGF and SDF1. Thus, tumour derived EVs modulate surrounding cells to create a supporting microenvironment (Cho *et al.*, 2012). In another report an increase of CAF markers was found in umbilical cord derived mesenchymal (UC-MSC) stem cells after stimulation with gastric cancer exosomes. The authors confirmed that TGF-β/SMAD pathway was responsible for the exosome exerted changes in UC-MSC recipient cells (Gu *et al.*, 2012).

As a conclusion, CAFs are also able to secrete EVs. Few reports have been published in recent years about the role of CAF-derived EVs in breast cancer. Luga and colleagues showed that CAF derived exosomes mobilise the autocrine-signalling pathway Wnt-PCP to enhance invasion in breast cancer cells (Luga *et al.*, 2012). A recent report demonstrated that murine fibroblasts deficient for the tissue inhibitors of metalloproteinases (TIMP) family genes, acquired a CAF phenotype with high expression of α-SMA. Exosomes secreted by these CAFs were able to induce migration and cancer stem cell marker expression in MDA-MB-231 breast cancer cells (Shimoda *et al.*, 2014). Reports about breast tumour CAF derived EVs are scarce, but several papers have been published about this topic in other cancers such as prostate, pancreas and stomach (Ji *et al.*, 2015; Richards *et al.*, 2017; Zhao *et al.*, 2016). The tight relationship between CAFs and tumours, is prominently driven by EVs. This represents the importance of EVs in tumour microenvironment and tumour-stroma communication. The essential functions played by CAFs, are at least partly dependant of EVs, due to the fact that EVs may transform parental cells into CAFs, and CAF derived EVs enhance tumour promoting processes. The CAF – EVs – tumour axis is thus, very relevant in tumour development and cancer aetiology.

IV. JUSTIFICATION

Given the importance of breast cancer burden, both globally and in Mexico, it is essential to understand the processes that drive tumour progression. The high incidence of obesity in Mexico has made the study of the role of fatty acids in breast cancer relevant. Tumour microenvironment has recently started to be

intensively studied also. The interaction between tumour and surrounding cells may define the tumour's path and tumours modulate their surroundings to create supportive milieu, with CAFs as one of the most prominent cell types. In the same way, EVs have been recognised as more and more important players in cancer, to the point of being connected with all Hallmarks of Cancer proposed by Hanahan and Weinberg. The complex communication driven by EVs between tumour cells and CAFs is a pivotal event in tumour progression. The combination of the aforementioned aspects makes up a passionate subject of study that is deeply involved in the field of cancer biology. This thesis tries to humbly shed some light into the matter.

V. HYPOTHESIS

MDA-MB-231 breast cancer cells stimulated with AA, release EVs that are able to induce a CAF phenotype in NIH 3T3 fibroblasts, characterised by the expression of CAF markers and secretion of MMPs, cytokines and EVs.

VI. AIMS

General aim: We try to elucidate the role that EVs derived from breast cancer cells (MBA-MB-231) play in the transformation of normal fibroblasts (NIH 3T3) into CAFs by the evaluation of protein markers expression and secretion of cytokines, metalloproteinases and EVs in these cells. All this is carried on under high AA concentration.

Specific aims. The general objective is driven by the integration of the next specific goals.

1. Determine the number of EVs secreted by MDA-MB-231 after stimulation with AA.
2. Examine the global phosphorylation state in NIH 3T3 treated with EVs.
3. Evaluate de expression of CAF markers in NIH 3T3 cells after treatment with EVs.
4. Determine the secretion of MMPs by NIH 3T3 after stimulation with EVs
5. Analyse the production of relevant cytokines in NIH 3T3 treated with EVs.
6. Determine the number of EVs secreted by NIH 3T3 after stimulation with EVs derived from MDA-MB231.

7. Evaluate whether NIH 3T3 stimulated with MDA-MB-231 EVs, release EVs that are able to induce migration in MDA-MB-231 cells.

VII. MATERIALS AND METHODS

VII.1. Cell lines and cell culture

Two cell lines were used in this project. Both cell lines shown in Table 1 were cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with 3.7g/L sodium bicarbonate, antibiotics and foetal bovine serum (FBS) at 5%. Cells were plated on 100mm, 60mm and 35mm dishes, depending on the experiment, and they were grown in incubators at 37ºC, 5% CO_2 and 95% O_2. MDA-MB-231 were cultured until reach confluence. In contrast, NIH 3T3 cells were passaged or stimulated at 80-90% confluence, as recommended by the provider and reported in literature (Yao and Rubin, 1992).

Table 1. Cell lines used for experiments and their main characteristics.

Cell line	Characteristics
MDA-MB-231	Metastasic human breast cancer cell line, epithelial origin, undergone EMT. (Jo *et al.*, 2009)
NIH 3T3	Embryonic murine fibroblast (*Mus musculus*). (Todaro and Green, 1963)

VII.2. Cell stimulation

MDA-MB-231 stimulation with AA. When reaching confluence, MDA-MB-231 cells were synchronized by serum deprivation for 24h. Next, 90µM of AA was added to fresh DMEM (without FBS) in a falcon tube, for adequate vortex mixing. Following that, the medium was added to the MDA-MB-231 plates, and incubated for 24, 36 and 48h.

Stimulation of MDA-MB-231 and NIH 3T3 with EVs. MDA-MB-231 cells were serum deprived for 24h before being stimulated with EVs. After starvation time, fresh DMEM (without FBS) and EVs were added to the plates. NIH 3T3 cells were cultured at low serum concentrations (0.5% FBS) for 12h as starvation. After that time elapsed, fresh DMEM 0.5% SFB was added together with EVs.

VII.3. Extracellular vesicle isolation.

The same procedure was followed for both MDA-MB-231 and NIH 3T3 cultures. Conditioned media were collected and subjected to *differential centrifugation*. First, they are centrifuged at 300g for 15 minutes. The pellet is discarded and the supernatant centrifuged at 600g for 15 minutes. This processes is repeated at 2,000g for 15 minutes and 10000g for 30 minutes, discarding the pellet and centrifuging the supernatant. At this point, is possible to storage the media (differentially centrifuged at 300, 600, 200 and 10,000g) at -20ºC for later use. After the 10,000g centrifugation, the last centrifugation is carried out, at 100,000g for 60 minutes. Finally, the supernatant is rejected and the pellet, enriched in EVs, resuspended in PBS, ready to use.

VII.4. Extracellular vesicle counting

BD Trucount™ tubes were used to quantify EVs secreted by both cell lines. The beads of Trucount™ tubes were dissolved in PBS and the resuspended EVs were added afterwards. The tubes with EVs were vortex mixed, and analysed in BF LSRFortessa™ flow cytometer.

VII.5. Zymography

NIH 3T3 conditioned media were collected and centrifuged at 2500 rpm using Centricon filters (pore ≥5000 Da). The concentrated media were mixed with red phenol metalloproteinase buffer (SDS 2.5%, sucrose 2% and phenol red 0.01%) and run in an 8% polyacrylamide gel, co-polymerised with gelatine (10mg/mL). The proteins were separated by electrophoresis at 75V during ~ 2.5h. Following electrophoresis, the gel was washed three times (30 minutes each time) with 2.5% Triton X-100 solution at room temperature. Next, the gel was incubated with MMP activation buffer (Tris 0.05M, CaCl2 0.01 M, NaN_3 0.02M and NaCl 0.150M, pH 7.4) for 24h. Finally, after incubation, the gel was dyed with Coomassie R-250 (Coomassie Brilliant Blue R-250, BioRad) for around 30 minutes. The activity of MMPs can be distinguished by the size of the clear areas of digested gelatine, in contrast with the blue background of undigested gelatine. Concentrated conditioned medium of MDA-MB-231 stimulated with 2µL/mL ethanol was used as control for MMP-2 and with PDB 0.2µL/mL for MMP-9.

VII.6. Protein extraction & Western Blot

NIH 3T3 cells plated in p60 dishes were stimulated with MDA-MB-231 EVs. After incubation time, cells were lysed with 200µL lysis buffer (RIPA and PMSF 1000:5) and scraped with a rubber policeman, the

p60 plates put on ice. The resulting extract is centrifuged at 12000 rpm for 12 minutes at 4ºC. After that, the supernatant is recovered and stored at -20ºC. For EV markers, the resuspended EV enriched pellet of the 100000g centrifugation is treated with a modified lysis buffer (RIPA, 1µL for 4µL of EVs, plus 2µL of PMSF).

The protein extract is separated in a polyacrylamide gel (10% for whole cell extract, 12% for EV markers) at 75V for 2h. Following electrophoresis, proteins were transferred to a nitrocellulose membrane (BioRad) by wet electrotransfer, either at 200mA for 2h or at 80mA overnight. To ensure that proteins were correctly transferred, the nitrocellulose membrane was dyed with Ponceau red, and then blocked with low fat milk (5% low fat milk powder dissolved in PBS-Tween-20 0.1%) for 2h to avoid unspecific interactions. The membrane was washed with PBS-Tween 0.1%, 3 times, 10 minutes each, and incubated with primary antibody overnight at 4ºC (Fot the list of antibodies used, see Table 2). The antibody was recovered and the membrane washed 3 times, 10 minutes each, with PBS-Tween 0.1%. Afterwards, the membrane was incubated with the secondary antibody, coupled to horseradish peroxidase, HRP, (dissolved in 5% low fat milk powder in PBS-Tween 1%) for 2h at room temperature (Table 2). The membranes were washed again, 3 times 10 minutes each, with PBS-Tween 0.1%. Specific proteins were detected by autoradiography (Kodak), employing a quimioluminiscent kit (Western Blot Luminol Reagent Santacruz sc-2046). The relative intensity of the bands were measured using Image J (version 1.42q). Actin was used as load control. The α-actin antibody was kindly provided by Dr. José Manuel Hernández Hernández. All the employed antibodies are listed in Table 2, together with their respective working dilutions.

VII.7. Electronic microscopy

After EV isolation, NIH 3T3 derived EVs were placed and absorbed (30µL) in cupper grids covered by a Formvar 0.3% layer and stabilised with carbon. The grids were dyed with a drop of uranyl 2% for 30 seconds, the excess of liquid removed, and the grids were let to dry at room temperature. The grids with EVs were analysed using a transmission electronic microscope (model JEM 1400) couple with a digital camera.

Table 2. Specificity, origin, working dilutions and providers of used antibodies.

Antibody	Specificity	Origin	Dilution	Provider
α-pY (phospho-tyrosine)	Monoclonal	Mouse	1:1000	Santa Cruz Biotechnology
α-Vimentin	Monoclonal	Mouse	1:1000	Santa Cruz Biotechnology
α-Caveolin-1	Polyclonal	Rabbit	1:1000	Santa Cruz Biotechnology
α-Flotillin-2	Monoclonal	Mouse	1:1000	BD Biosciences
α-Actin	Polyclonal	Mouse	1:300	Kindly provided by Dr. José Manuel Hernández Hernández
α – mouse Antibody coupled with HRP	Polyclonal	Goat	1:5000	Jackson ImmunoResearch
α – rabbit Antibody coupled with HRP	Polyclonal	Goat	1:5000	Jackson ImmunoResearch

VII.8. Scratch wound healing assay

MDA-MB-231 cells were grown until confluence in p35 plates. The cells were serum deprived for 24h, and 2h before the starvation time had elapsed, Mitomycin C was added for 2h to inhibit proliferation. After starvation and Mitomycin C (40µg/mL) treatment, the scratch was done with a 200µL pipette sterile tip. The plates were washed 3 times with PBS 1X to remove floating cells, and fresh DMEM with the stimuli, was added. The cells were incubated with fresh DMEM (negative control), 5% FBS DMEM (positive control) and DMEM + NIH 3T3 EVs (stimuli), for 48h. After incubation time, cells were washed twice with PBS 1X and fixed with paraformaldehyde 4% for 15 minutes. Afterwards, cells were dyed with crystal violet 0.5% and the migration measured with an inverted microscope couple to a camera.

VIII. RESULTS

VIII. 1. AA does not increase EV secretion in MDA-MB-231.

MDA-MB-231 were cultured to 100% confluence. After reaching this point, cells were serum deprived with DMEM for 24h. Following starvation, MDA-MB-231 were stimulated with AA. We tested four conditions: Stimulation with 90 µM AA for 24h, 36h and 48h, and a negative control (Ctrl EVs) incubated with fresh DMEM for 48h. Collected conditioned media were differentially centrifuged in order to isolate EVs.

To assure that the differential centrifugation method (explained in section VII.3) is adequate for EV isolation, we evaluated the presence of EV marker flotillin-2 (Flot-2) by Western Blotting in the EV enriched fraction (pellet of the 100,000g centrifugation) using a α-flotillin-2 antibody. To prove that the presence of flotillin-2 was specific for EVs, we designed two controls. The first one is "conditioned medium (CM)", this is, medium collected after stimulation time without going through any centrifugation (EVs are expected to be present, but in extremely low concentrations). The second control is "extracellular vesicle depleted fraction (EVDF)", supernatant of the 100,000g centrifugation, thus EVs are expected to be absent because they are concentrated in the pellet. Enriched EV fraction from unstimulated (serum deprived DMEM for 48h) and stimulated (90 µM AA for 48h) cells were used (Figure 3a).

Once the differential centrifugation method was shown to be effective for EV isolation, we wanted to test whether AA in inflammatory concentrations increases EV release in MDA-MB-231 cells. Isolated EVs from the four conditions of MDA-MB-231 (Ctrl EVs with fresh DMEM and AA stimulation for 24h, 36h and 48h) were quantified using BD Trucount™ tubes and analysing the samples by flow cytometry (Figure 3b). Interestingly, a stimulus as strong as AA in high concentrations, did not have an effect in EV production in MDA-MB-231 cells. The differences in EV concentration for the different conditions were not significant, although there is an increasing trend. To note, Ctrl EVs and 48h conditions were incubated the same amount of time, and although the difference is not significant, the average EV concentration is higher in conditioned medium derived from MDA-MB-231 stimulated with 90 µM AA for 48h than in Ctrl EVs.

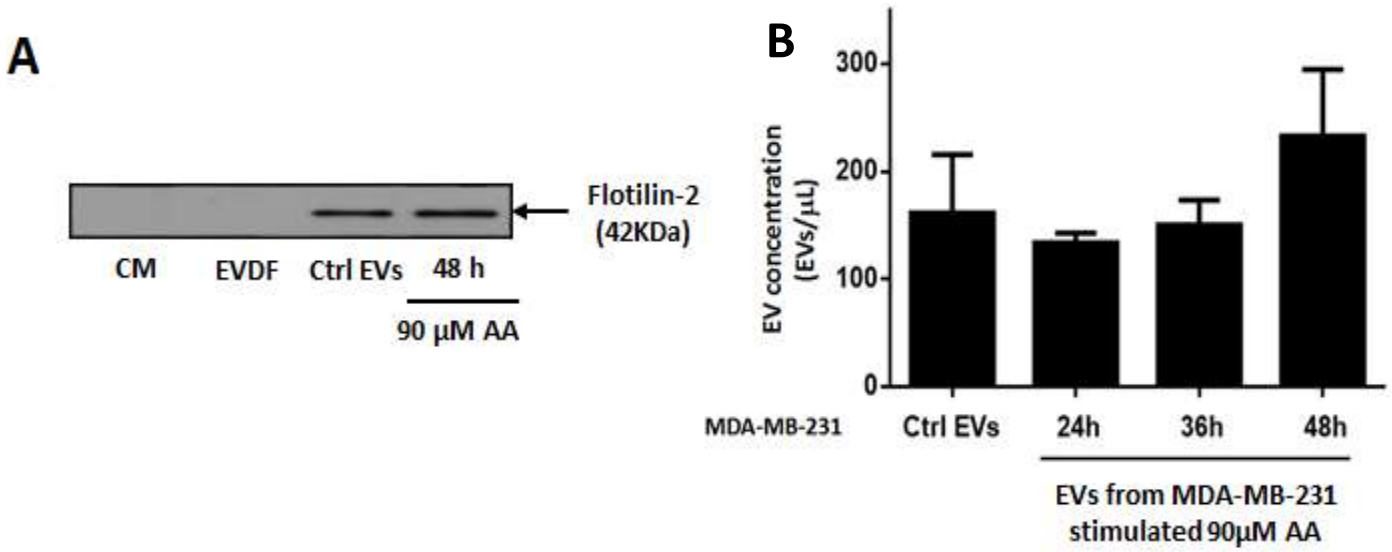

Figure 3. AA in high concentrations (90 µM) does not enhance EV secretion in MDA-MB-231 cells. A) Characterisation of EV isolation procedure. Flotillin-2 is enriched in Ctrl EVs and 48h conditions, both from the EV enriched pellet, but absent in unprocessed conditioned medium (CM) and EV depleted fraction (EVDF), showing the effectiveness of isolation. **B)** Quantification of MDA-MB-231 secreted EVs (n=3). The differences between conditions were not significant (p >0.05).

VIII.2. Extracellular vesicles derived from MDA-MB-231 cells stimulated with AA are able to change the phosphorylation profile in NIH 3T3 fibroblasts.

To assess the effect that MDA-MB-231derived EVs have in NIH 3T3, we first evaluated tyrosine phosphorylation profile in recipient cells. Tyrosine is one of the three aminoacids that can be phosphorylated. Determining the global phosphorylation state of tyrosine, we will get information about the activity of tyrosine kinases in the cell. Thus, higher levels of phospho-tyrosine (p-Tyr) is correlated with higher activity of tyrosine kinases, including RTKs that play an essential role in cancer progression. This experiment also serves as a control to test if MDA-MB-231 derived EVs (Human cells) can act on NIH 3T3 fibroblasts (Murine cells) initiating signalling pathways.

NIH 3T3 cells were cultured and serum deprived as mentioned in section VII.1. EVs from the 4 MDA-MB-231 conditions (Ctrl EVs and 90 µM AA for 24h, 36h and 48h) were used to stimulate p60 NIH 3T3 plates, one plate for each condition and a negative control consisting in NIH 3T3 incubated with fresh DMEM and 0.5% FBS. Cells were lysed after 24h incubation with the stimuli. p-Tyr profile was analysed by Western Blotting using a α-p-Tyr antibody (Figure 4a). Actin was used as load control. As seen in the quantification (Figure 4b), p-Tyr levels show a tendency to increase when NIH 3T3 cells are stimulated

with EVs. In particular, NIH 3T3 stimulated with EVs derived from AA treated MDA-MB231 cells showed the biggest increase trend in p-Tyr levels, although the enhanced tyrosine phosphorylation fades at 48h. Only one western blot could be fulfilled. Thus, our results suggest that these human cell derived EVs can affect murine recipient cells, showing that MDA-MB231 derived EVs can overcome the species barrier, although more experimentation is required.

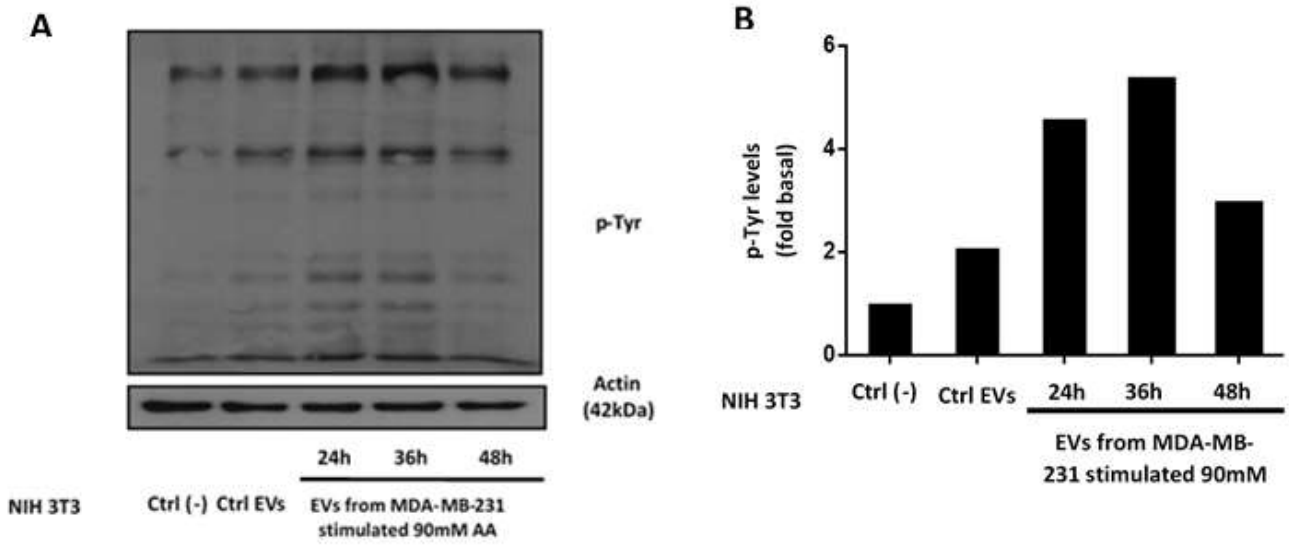

Figure 4. EVs derived from MDA-MB-231 increase tyrosine phosphorylation in NIH 3T3 cells, overcoming the species barrier. A) Western Blot showing protein bands with phosphorylated tyrosine of various molecular sizes. EV treated conditions present higher p-Tyr levels which in turn are particularly enhanced in conditions stimulated with EVs from MDA-MB-231 treated with 90 µM AA. **B)** Densitometric quantification of the Western Blot shown in panel (n=1). A strong increase can be seen in 24h and 36h conditions.

VIII.3. Extracellular vesicles derived from MDA-MB-231 cells stimulated with AA are able to alter the expression of vimentin and caveolin-1 in NIH 3T3 fibroblasts.

Following the same procedure described in the experiment above, we assessed the expression of two CAF markers, vimentin and caveolin-1. Vimentin has been reported to be upregulated in CAFs, while caveolin-1 is downregulated. We tried to elucidate whether stimulation of NIH 3T3 cell with MDA-MB-231 derived EVs was sufficient to induce a CAF phenotype in these cells. NIH 3T3 cells were stimulated for 24h with EVs (5 NIH 3T3 conditions: Ctrl (-), Ctrl EVs and EVs from MDA-MB-231 stimulated with 90 µM AA for 24, 36 and 48h) and were lysed to obtain the cell extract.

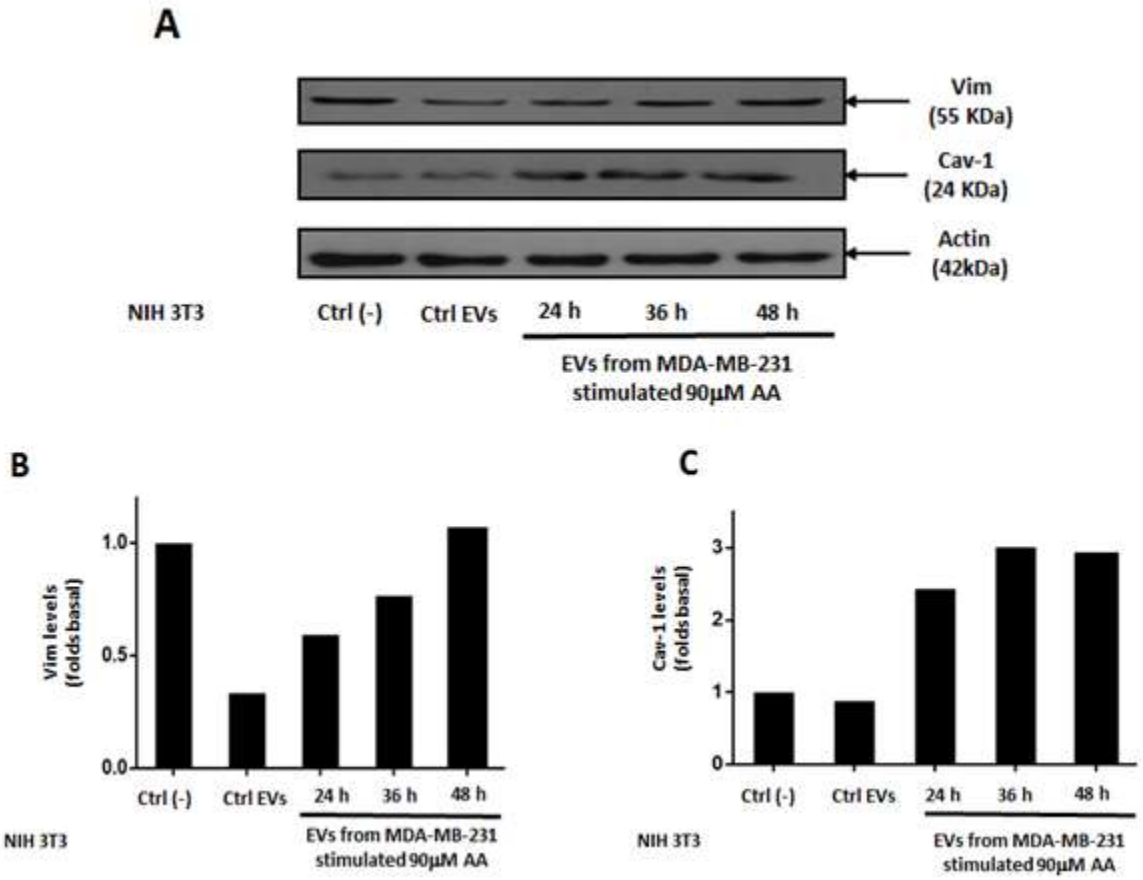

Figure 5. EVs derived from MDA-MB-231 seem to alter CAF marker vimentin and caveolin-1 expression in NIH 3T3. A) Western Blot showing vimentin and caveolin-1 expression in NIH 3T3 after 24h incubation with EVs. Actin was used as load control. **B)** Quantification of vimentin expression. Vimentin levels appear to decrease substantially with unstimulated MDA-231-MB EVs, and it is then recovered with AA treated MDA-MB-231 EVs (n=1). **C)** Quantification of caveolin-1 expression. An augmented expression of Cav-1 is reached when stimulation with EVs from AA treated MDA-MB-231 (n=1).

The expression changes of vimentin and caveolin-1 were evaluated by Western Blotting using α-vimentin and α-caveolin-1 antibodies (Fig 5a). Actin was used for load control. One independent experiment was carried out, but interesting information can be obtained. The results suggest that the expression of these two markers did not follow the expected trends. Even more, they followed the opposite tendency (Figure 5b,c). Vimentin expression appears strongly decreased when NIH 3T3 were stimulated with Ctrl EVs from MDA-MB-231, and the tendency gradually recovered to basal vimentin expression in the 48h condition (Figure 5b). Caveolin-1 expression seems to be upregulated in NIH 3T3 cells stimulated with EVs, but only

in those treated with EVs from AA stimulated MDA-MB-231. Therefore, stimulation of MDA-MB-231 cells with AA induces a cargo change in released EVs that seems to enhance caveolin-1 expression in NIH 3T3 (Figure 5c). Unfortunately, only one western blot could be fulfilled for each marker, thus further experimentation is needed to confirm the aforementioned tendencies.

VIII.4. Extracellular vesicles derived from MDA-MB-231 cells stimulated with AA enhance MMP secretion in NIH 3T3.

NIH 3T3 were cultured, serum deprived in 0.5% FBS and stimulated with EVs as in previous experiments. After 24h incubation, the conditioned media were collected and centrifuged with tubes containing Centricon filters (pore ≥5000 Da), at 2500 rpm for 2h. The concentrated media were run in a zimography gel (8% poly acrylamide, 1% gelatine). After incubation time and dying, relative levels of MMPs could be detected indirectly through gelatine hydrolysing activity of the enzymes. To assure that the number of cells was similar for every condition, cells were lysed and protein concentration of whole lysates was measured by microBradford technique.

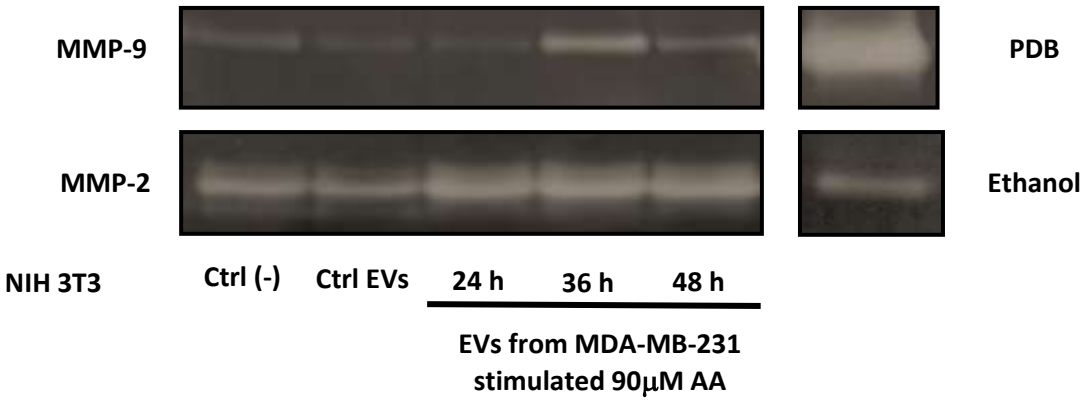

Figure 6. EVs derived from MDA-MB-231 increase MMP2 and MMP-9 secretion in NIH 3T3. Zymography showing the differential secretion of MMPs in NIH 3T3 cells after stimulation with EVs. MMP-9 secretion is enhanced in the 36h condition (this is, NIH 3T3 stimulated with EVs derived from MDA-MB-231 treated with AA for 36h). MMP-2 in turn, appears enhanced in all three conditions stimulated with EVs from AA treated MDA-MB-23. The zymography gel shown is representative for three different experiments (n=3).

It is worth mentioning that zymographies can only reflect the secretion of MMP-2 and MMP-9, due to the fact that both enzymes can hydrolyse gelatine. It is also important that zymographies give information about the secretion levels of MMPs, not the activity in the media. In fact, the hydrolysis of

gelatine by MMPs is artificially activated by a MMP activation buffer. The zymography gel shown in Figure 6, is representative for three independent experiments. As shown in the image, MMP-2 secretion is enhanced in all 3 conditions stimulated with EVs from MA-MB-231 cell treated with AA. In contrast, MMP-9 secretion is relatively constant, except for an increase in NIH 3T3 cells stimulated with EVs from MDA-MB-231 cells treated with AA for 36h and 48h conditions. In this manner, MDA-MB-231 cells secrete EVs that can increase MMP-9 and MMP-2 secretion in NIH 3T3 (Figure 6).

VIII.5. NIH 3T3 cells release extracellular vesicles.

To determine whether NIH 3T3 cells secrete extracellular vesicles, we carried out an electronic microscope analysis. We analysed NIH 3T3 derived EVs by transmission electronic microscopy. Employing a negative dying protocol (as explained in Materials and Method) we identified NIH 3T3 derived EVs under electronic microscope, as presented in Figure 7a,b. Typical features of EVs can be observed in the images. Double membrane appears clearly delineated in the microscopic image. In the same way, EVs of different sizes can be identified. Interestingly, most of the presented EVs are small sized, around 100nM considering scale bars. According to this, EVs are presumably exosomes, or small microvesicles.

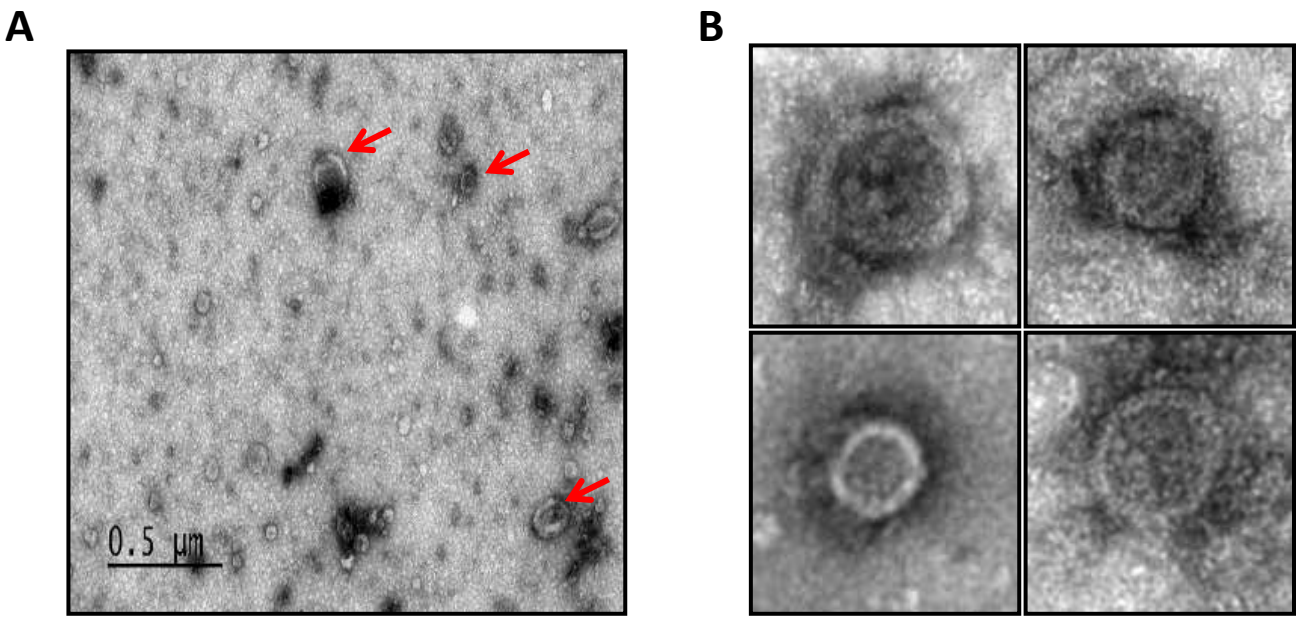

Figure 7. Transmission electronic microscopy image of NIH 3T3 derived EVs. A) Several EVs (red arrows) derived from NIH 3T3 in the transmission electronic microscopy image field. **B)** Selected NIH 3T3 EV images from different fields. The membrane can clearly be seen in all four images.

VIII.6. NIH 3T3 stimulated with MDA-MB-231 derived EVs, secrete augmented number of EVs.

After confirming that NIH 3T3 can release EVs by the previous experiment, we wanted to elucidate if stimulation of NIH 3T3 with MDA-MB-231 derived EVs, had any effect in NIH 3T3 released EV numbers. For that, NIH 3T3 cells were cultured and serum deprived as previously described and treated for 24h with MDA-MB-231 derived EVs unstimulated and stimulated with 90 µM AA for 36h. EVs derived from NIH 3T3 incubated with low serum (0.5%) for 24h were used as a negative control. Conditioned media were differentially centrifuged and EVs isolated. To assess if there was any change in NIH 3T3 derived EV numbers after stimulation, we quantified EVs with BD Trucount™ tubes and red the samples by flow cytometry. In the only experiment that could be read in the flow cytometer, EV numbers were strongly increased, when NIH 3T3 cells were stimulated with control EVs from MDA-MB-231. Surprisingly, this tendency was ablated and EV numbers returned to unstimulated levels when NIH 3T3 were treated with EVs from MDA-MB-231 incubated with AA. To note, it would seem that NIH 3T3 secrete more EVs than MDA-MB-231 cells, even without stimulation. To confirm this result, two other independent experiments have to be fulfilled.

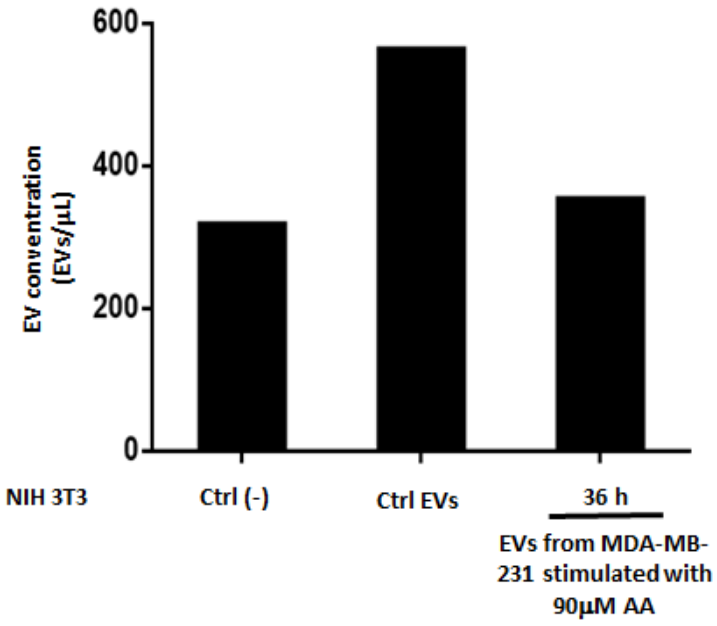

Figure 8. Quantification of NIH 3T3 released EVs. A strong increase in EV secretion appears when NIH 3T3 cells are stimulated with control EVs from MDA-MB-231. Basal level of EVs is recovered in the "36h" condition (n=1).

VIII.7. EVs derived from NIH 3T3 stimulated with MDA-MB-231 derived EVs, enhance migration in MDA-MB-231 cells.

We have demonstrated in section VIII.6 that NIH 3T3 fibroblasts secrete EVs and in section VII.7 we suggest that stimulation with MDA-MB-231 derived EVs has an effect in EV release of NIH 3T3, according to the tendency showed in Figure 8. Following this line of thought we decided to test whether NIH 3T3 EVs, both unstimulated and stimulated with MDA-MB-231 derived EVs, had any bioactive effect in MDA-MB-231. If NIH 3T3 cells had acquired a CAF-like phenotype, these cells ought to show tumour promoting behaviours. Thus, we designed a wound scratch assay for MDA-MB-231 stimulated with NIH 3T3 EVs, expecting to see enhanced migrations in stimulated conditions.

MDA-MB-231 cells were cultured with complete medium until they reached confluence. Then, cells were serum deprived for 24h and treated with Mytomicin C (40µg/mL) for 2h to inhibit proliferation. The scratch was performed as explained in Materials and Methods. MDA-MB-231 cells were stimulated with NIH 3T3 derived EVs for 48h (EV conditions: Ctrl (-) from unstimulated NIH 3T3, Ctrl EVs from NIH 3T3 stimulated with untreated MDA-MB-231 derived EVs, and 36h from NIH 3T3 stimulated with EVs from MDA-MB-231 treated with 90µM AA for 36h). The resulting migrations are shown in Figure 9a and quantified in Figure 9b. Although only one experiment could be carried out, a robust tendency is shown in the graphic. MDA-MB-231 cells stimulated with untreated NIH 3T3 derived EVs present basal like migration. Consequently, untreated NIH 3T3 EVs do no induce migration in MDA-MB-231 cells. Both conditions of stimulated NIH 3T3 on the contrary (Ctrl EVs and 36h), release EVs that are sufficient to induce a migratory behaviour in MDA-MB-231, both at a similar extent. We propose that treatment of NIH 3T3 fibroblasts with MDA-MB-231 derived EVs, confers these cells a tumour supporting behaviour induced by EVs.

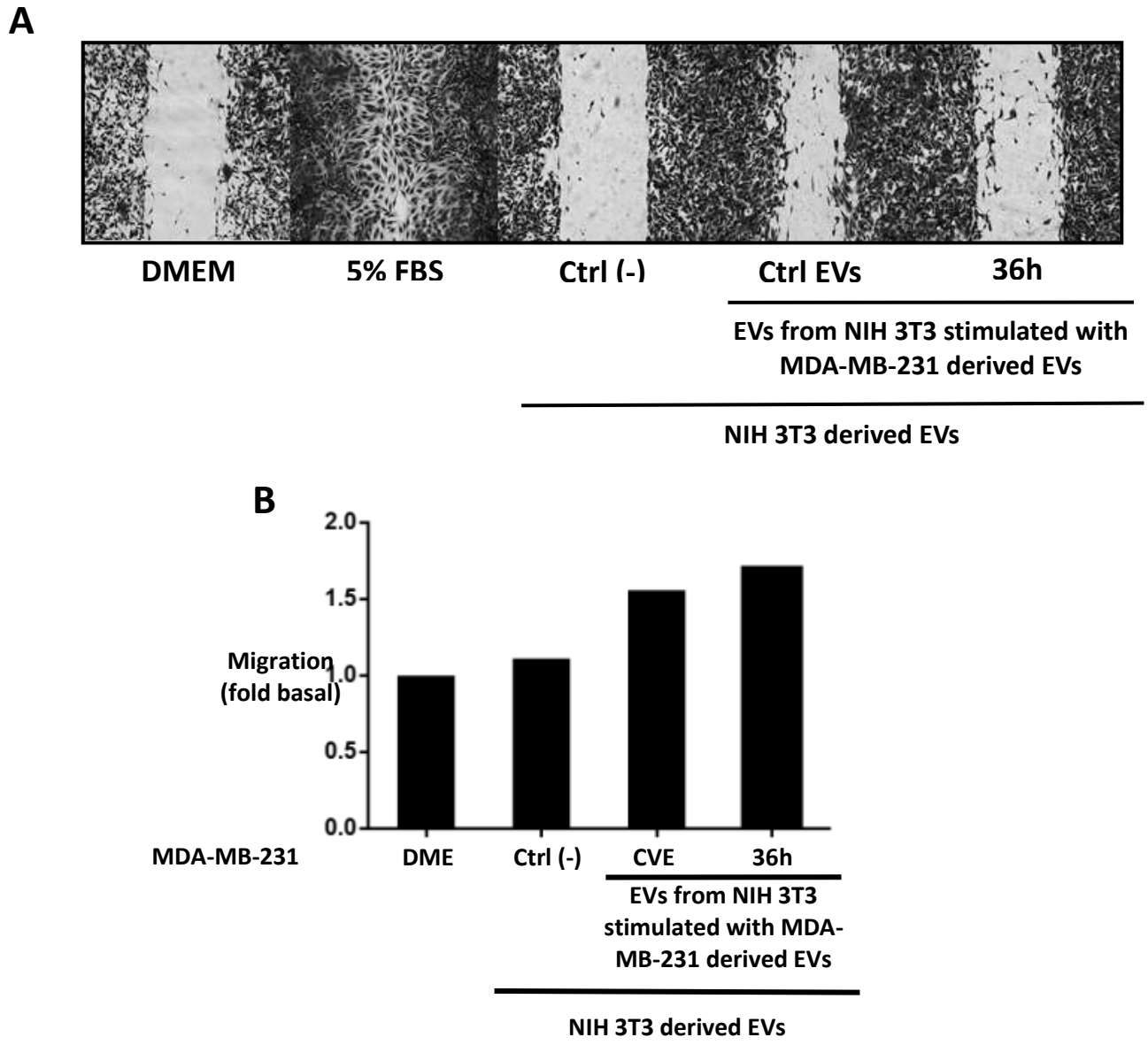

Figure 9. NIH 3T3 cells stimulated with MDA-MB-231 derived EVs, release EVs that induce migration in MDA-MB-231 cells. A) Images of the conditions used for this experiments. MDA-MB-231 cells incubated with serum free medium (DMEM) to assess basal migration, with DMEM at 5%FBS as positive control, and the three stimulated conditions Ctrl (-), Ctrl EVs and 36h. **B)** Quantification of the images above. Enhanced migration can be seen in CVE and 36h conditions, while Ctrl (-) migration is similar to that shown in DMEM negative control (n=1).

IX. DISCUSSION

Breast cancer is one of the deadliest cancers overall, and the deadliest cancer in women all around the world (Torre *et al.*, 2015). Among life style related risk factors, obesity and high fat intake seem to be tightly associated with breast cancer (Cho *et al.*, 2003; McTiernan, 2003). A part from that, it is demonstrated that fatty acids, especially ω-6 fatty acids, induce various processes that enhance tumour progression (Sakai *et al.*, 2012). This is why we studied the relation between arachidonic acid and breast cancer in MDA-MB-231 cells (Invasive cells, undergone EMT). In fact, our approach was to elucidate the role that AA has in MDA-MB-231 derived extracellular vesicles. EVs have become very popular recently, due to the versatility of these membrane bound compartments, and the myriad of roles they play, both in health and disease. EVs have been demonstrated to carry selectively enriched cargo of proteins, nucleic acids and lipids. Consequently, they play a predominant role in tumour progression and interestingly they are overproduced in cancers (Meehan and Vella, 2016).

One of the most important traits of cancer is the presence of a supporting surrounding. The existence of a friendly microenvironment permits the tumour to grow and develop to the point of metastasis and death of the patients unless adequate treatment is employed. Even more, tumour microenvironment does not only “permit” tumour growth, it also enhances the process and it is an essential player in tumour progression and resistance to anti-cancer treatment. Dvorak proposed that tumours co-opt the wound healing mechanisms to create a supporting microenvironment (Dvorak, 2015). Consequently, tumour cells are able to induce “supportive” phenotypes in different cell types, such as macrophages and the TAM phenotype (Tumour associated macrophage) and fibroblasts and the CAF phenotype (Cancer associated fibroblast) to generate a tumour-friendly stromal compartment (Dandekar *et al.*, 2011; Franco *et al.*, 2010). Considering the importance of tumour microenvironment in tumour biology, we focused on understanding one of the multiple processes that drive its creation: the acquisition of the CAF phenotype by fibroblasts.

Thus, we designed an experimental protocol where MDA-MB-231 were stimulated with high concentration of AA (90 µM) and secreted EVs were isolated to stimulate NIH 3T3 cells. The effect of EVs were evaluated in relation with the CAF-like traits of NIH 3T3.

We first showed that differential centrifugation employed to isolate EVs was adequate. The presence of EV marker flotillin-2 in the MDA-MB-231 EV enriched fraction but no in unprocessed conditioned medium and 100,000g centrifugation supernatant, showed that EVs were efficiently purified. We then quantified MDA-MB-231 secreted EVs with and without AA stimulus at different times (24, 36 and 48h). There was not a significant variation among conditions, even with a stimulus as strong as AA, which has been previously demonstrated by lab members (Navarro-Tito *et al.*, 2008). The small variations in the average number of EVs secreted, seem to be time-dependant (conditioned media from 24h incubation with AA showed smaller EV concentration comparing with 36h, and so on). However, the average EV concentration for CVE and 48h differs, despite the fact that both conditions have been incubated the same amount of time, although these changes are not significant. These results are not surprising though; previous reports by lab members showed that stimulation of MDA-MB-231 cells with linoleic acid, another ω-6 fatty acid, does not alter secreted EV numbers (Galindo-Hernandez *et al.*, 2014).

After incubation of MDA-MB-231 with AA, we purified EVs to assess whether these EVs were able to exert a biological response in NIH 3T3. To prove this, we chose to evaluate the phosphorylation of tyrosine in total lysates. Considering that EVs are carriers of rapid signalling molecules and membrane receptors such as PI3K/Akt and EGFR, one of the most probable effects is the initiation of signalling cascades, phosphorylation being an important trait of these (Yamashita *et al.*, 2013; Yang *et al.*, 2013). Plus, phosphorylation of tyrosine is related to activation of RTKs, very important signalling platforms in cancer (Regad, 2015). Although we only carried out one experiment, the results obtained suggest an interesting tendency and a very robust increase in phosphorylation of tyrosine was achieved in the obly western blot presented. All EV stimulated conditions showed a tendency of increase in p-Tyr levels compared with the control. In this manner, we propose that EVs from MDA-MB-231 are bioactive and can stimulate NIH 3T3 cells. It is also noteworthy that not all EVs induced the same increase in p-Tyr levels (from twofold for Ctrl EVs to fivefold for 36h condition, compared with the control). This would mean that the cargos from different conditions diverge. The three conditions stimulated with AA treated MDA-MB-231 EVs (24, 36 and 48h), show higher p-Tyr levels than Ctrl EVs, thus AA stimulation changed the cargo. But also differences in AA exposure times resulted in cargo alteration in EVs, based on the effect these had in NIH 3T3. Although more independent experiments are needed, this results shows exciting prospects. Finally, another relevant conclusion can be proposed from this experiment. Taking in

mind that MDA-MB-231 cells are human and NIH 3T3 are mouse cells, EVs derived from the former can stimulate murine cells. In this way, we suggest that MDA-MB-231 cells can overcome the species barrier, as previously shown by various reports (Costa-Silva *et al.*, 2015; Peinado *et al.*, 2012). This gives an idea of the powerful signalling capacities of EVs.

Next, we evaluated the acquisition of CAF phenotypic traits in NIH 3T3 stimulated with EVs from MDA-MB-231. First, we assessed whether there was a change in CAF marker expression after 24h incubation with EVs. We chose vimentin and caveolin-1, upregulated and downregulated in CAFs, respectively. Interestingly, the results obtained in one independent experiment were the opposite of what was expected. Vimentin expression appeared strongly downregulated in NIH 3T3 stimulated with Ctrl EVs and the expression gradually recovered in the next conditions until basal levels were reached for 48h condition. Caveolin-1 levels were in turn, upregulated when EVs from MDA-MB-231 treated with AA were used as stimulus. The trend showed in the graphic shows a fourfold upregulation in Cav-1 expression compared to the basal. Although further experimentation is needed to confirm these results, we can infer that EVs from MDA-MB-231 are able, not only to initiate quick signalling cascades as seen with p-Tyr levels, but also to change gene expression in NIH 3T3 cells. Regarding CAF markers and phenotype, our results do not match with what is reported in literature. However, the use of markers in CAFs has been recently put under debate. According to Sugimoto and colleagues, CAF markers such as α-SMA and FSP1, are not shared by all fibroblast in pancreatic cancer, and consequently do not always reflect CAF tumour supporting functions (Sugimoto *et al.*, 2006). In fact, due to the heterogeneity of CAF populations, markers are not necessarily shared by all CAFs, and specific subpopulation of CAFs may express specific CAF markers. In the other hand, the use of other markers could have given positive results for this experiment. α-SMA overexpression, the canonical CAF marker, can be achieved by stimulation of CAF parental cells, mesenchymal stem cells for example, with cancer derived EVs *in vitro* (Gu *et al.*, 2012). It has also been reported that NIH 3T3 cells express α-SMA (Negmadjanov *et al.*, 2015). So a change in the chosen protein could shed positive results for the acquisition of CAF phenotype by marker determination (we have showed that NIH 3T3 acquired a CAF-like phenotype in following experiments by other means).

We then evaluated the secretion of MMPs by NIH 3T3. ECM remodelling is an essential event in cancer progression, considering that is a previous step for migration, invasion and consequently metastasis.

CAFs play an essential role in ECM remodelling, mainly through the activity of certain upregulated proteins such as MMPs and FAP (Miles and Sikes, 2014; Togo *et al.*, 2013). Considering this, we determined the pattern of MMP secretion for CAFs unstimulated and stimulated with MDA-MB-231 EVs. According to our predictions, both MMP-2 and MMP-9 were upregulated in NIH 3T3 stimulated with EVs. In the former, the upregulation was caused after stimulation with all the three MDA-MB-231 AA treated conditions. In the later, EVs from the 36h condition were the most effective to induce MMP-9 secretion. Two conclusions can be attained with these results. First, we confirm the previous results showing that untreated MDA-MB-231 derived EVs and AA treated MDA-MB-231 EVs affect NIH 3T3 recipient cells differently. Considering that EV number for 36h condition, are lower than that of Ctrl EVs, we can affirm that the effect is not exerted through increased EV numbers. Thus, cargo alteration must be responsible for these effects, an event induced by AA treatment of MDA-MB-231 cells. Second, although EVs from MDA-MB-231 could not modulate the expression of CAF markers vimentin and caveolin-1 in accordance with the reported literature, an increase in MMP secretion was achieved. Upregulation of these crucial protein can be considered as another phenotypic marker of CAFs. In this way, EVs from AA treated MDA-MB-231 can confer a CAF-like phenotype in NIH 3T3.

After demonstrating that MDA-MB-231 EVs can induce the acquisition of a CAF-like phenotype in NIH 3T3, we wondered if these CAF-like cells were able to carry out tumour supporting functions. We proposed that CAF-like NIH 3T3 derived EVs could induce protumoural behaviours in MDA-MB-231 cells. Thus, we planned to close the circle: MDA-MB-231 derived EVs transform NIH 3T3 in CAF-like cells, and EVs derived from these CAF-like cells have protumoural effects in the former. In this way, we wanted to prove that an essential relationship between tumour and tumour microenvironment, the tumour-CAF crosstalk, can be carried out *in vitro* and, at least partially, by EVs. In order to elucidate this subject we first verified that NIH 3T3 secrete EVs. For this we analysed NIH 3T3 isolated EVs by transmission electronic microscopy. We identified EVs of different sizes but all ranging 100nm, and the double membrane could easily be seen.

We next quantified NIH 3T3 derived EVs by flow cytometry. We also analysed the effect that stimulation with MDA-MB-231 EVs has in NIH 3T3 EV secretion. Interestingly, EVs from unstimulated MDA-MB-231 induced the highest secretion of EVs in NIH 3T3, whereas EVs from MDA-MB-231 stimulated with AA for 36h, more bioactive *a priori* as demonstrated in previous experiments, induced basal secretion of EVs in

NIH 3T3. In consequence, stimulation with breast cancer cell EVs has a direct effect in EV secretion by NIH 3T3. Further experimentation is needed to confirm these results, considering that only independent experiment was carried out.

Our next and last, experiment was designed to test whether CAF-like NIH 3T3 derived EVs could in fact induce migration in MDA-MB-231 cells. Consequently, we would be testing if MDA-MB-231 derived EVs can induce protumoural CAF-like phenotype/behaviour in NIH 3T3 cells. Migration is a pivotal process in tumour development, related to intra- and extra-vasation and tumour spread. In a scratch-wound assay, we stimulated MDA-MB-231 cells with NIH 3T3 derived EVs from different conditions for 48h. In the one experiment carried out, EVs from stimulated NIH 3T3 cells (Ctrl EVs and 36h conditions) were able to induce migration to a similar extent in MDA-MB-231. Untreated NIH 3T3 EVs did not induce migration. This is in accordance with reported literature (Flaberg et al., 2011) that demonstrated that normal fibroblasts inhibit tumour growth. The fact that CAF-like NIH 3T3 derived EVs induce migration in MDA-MB-231 cells represents that a protumoural behaviour was provoked in NIH 3T3 cells at some extent, that would have to be proved in subsequent experiments.

Although further experimentation is needed to confirm our results, obtained data shows a strong trend supporting the induction of a CAF-like phenotype, both functionally and phenotypically, in NIH 3T3 fibroblasts stimulated with MDA-MB-231 derived EVs. It is also noteworthy, that the role of AA in this experiments is relevant, due mainly to the proposed cargo changes induced in EVs from MDA-MB-231 cells. Several biological effects by these EVs are enhanced when origin cells were incubated with AA.

X. CONCLUSION & PERSPECTIVES

As mentioned before, CAF phenotype induction and tumour-CAF crosstalk are very relevant events in tumour progression. We mimicked this processes *in vitro* and based on our results, we suggest that a significant part of it is carried out solely by EVs, both in CAF phenotype induction and in CAF promoted tumour development events, such as migration. Considering the immense variety of soluble factors that are secreted by tumour and CAFs, it is surprising that EVs can carry out, all by themselves, the above reported functions. This demonstrates the importance of EVs in TME crosstalk and communications as well as the effector power these agents possess.

Induction of migration in particular, shows the bidirectionallity of the tumour-CAF communication, the despite the statistical analysis and repetition are pending. Once tumours have transformed normal cells into tumour supporting cells, these enhance tumour development. It is impressive to realise how tumours can so efficiently create a “comfortable” niche to grow and spread. Fortunately, this field is starting to be deciphered by many research teams, as listed and cited in previous sections. Apart from suggesting and showinf the efficient transformation of fibroblasts in CAF-like cells, and what it would seem to be the induction of migration in breast cancer cells, we evaluated the effect of AA in these processes. We demonstrated that AA can potentiate the effect of MDA-MB-231 derived EVs, and in some experiments, AA treatment was indispensable to obtain positive results, in MMP-2 and MMP-9 activity detection by zymography for example. This highlights the importance of fatty acids, and AA particularly, in breast cancer progression and the profound changes they can trigger in the tumour niche.

In conclusion, we obtained fairly intriguing results that can serve as platform for further investigation. We here list some of the perspectives proposed.

1) Thoroughly study the role of AA stimulus in MDA-MB-231 cells, monitoring AA metabolising enzyme expression.
2) Determine the main effector pathway of EVs: ligand-receptor interaction, endocytosis or other.
3) Evaluate the EV-activated pathways in NIH 3T3 (PI3K/Akt, MAPK/ERK, …).
4) Analyse the expression of other CAF markers, such as α-SMA, in EV-treated NIH 3T3.
5) Matrigel invasion assay for MDA-MB-231 stimulated with NIH 3T3 derived EVs to test if CAF-like NIH 3T3 shed EVs able to induce invasion.
6) Evaluate the pathways that intervene in the invasive and migratory behaviour of MDA-MB-231 cells after stimulation with NIH 3T3 derived EVs.
7) Test whether CAF-like NIH 3T3 derived whole conditioned medium and EV-depleted conditioned medium are able to induce migration and invasion in MDA-MB-231 cells.

XI. BIBLIOGRAPHY

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