IMPACT OF FATTY ACID METABOLISM IN BREAST CANCER PERITUMORAL TISSUE. CLINICAL AND PATHOGENIC ASPECTS

Jose Adriá Cebrián

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IMPACT OF FATTY ACID METABOLISM IN BREAST CANCER PERITUMORAL TISSUE. CLINICAL AND PATHOGENIC ASPECTS

Jose Adriá Cebrián

DOCTORAL THESIS
2022

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DOCTORAL THESIS

Supervised by:
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I state that the present study, entitled **"Impact of fatty acid metabolism in breast cancer peritumoral tissue. Clinical and pathogenic aspects"** presented by Jose Adriá Cebrián, has been carried on under our supervision at the **Hospital Universitari Sant Joan de Reus** and the Department of **Medicine and Surgery** of the University.

And so that it is registered and has the appropriate effects, we signed this document in Reus, on February, 2022.

Doctoral Thesis Supervisors

Josep Gumà Padró

Sandra Guaita Esteruelas

No hay nada más bonito que agradecer a aquellos que están a tu lado la labor que han hecho en ti.

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PRESENTATION AND JUSTIFICATION

1. PRESENTATION AND JUSTIFICATION

This doctoral thesis has been carried on at Hospital Sant Joan de Reus, Institut d'Investigació Sanitària Pere Virgili and Research Unit on Lipids and Atherosclerosis (URLA), Department of Medicine. This doctoral thesis has been supervised by Dr Josep Gumà Padró and Dra Sandra Guaita Esteruelas.

Cancer is a complex disease with multiple risk variables that might accelerate its progression and development. Obesity is a disease rising the highest values of prevalence in the modern society. Strong association between cancer and obesity besides some cancers are embedded in adipose tissue, allows us to think that adipose tissue might have a role in cancer progression. Moreover, tumor microenvironment contains high number of infiltrated macrophages. Consequently, crosstalk between different elements of the tumor microenvironment might enhance tumor progression and development.

This thesis analyzes the crosstalk mechanisms between tumor cells and tumor microenvironment modification.

The disruption of normal cell metabolism, as a new cancer hallmark, leads to the acquisition of new competent mechanisms in the energy obtainment, thus enhancing the survival and proliferation of tumor cells.

Analyzing the metabolism behavior of the different BC molecular subtypes we try to understand their tumorigenicity, hence, to understand the possible effects of their altered metabolism in BC patient prognosis.

Furthermore, this thesis wants to assess new possible biomarkers for early breast cancer identification, hence improving the diagnosis and prognosis of this disease.

This thesis is based on 4 scientific publications, preceded by an introduction contextualizing the pathology and the important role of the tumor microenvironment; finally, being concluded with the discussion of our results and future perspectives in this field research.

INTRODUCTION

2. INTRODUCTION

2.1. Breast cancer overview

The World Health Organization (WHO) defines cancer as a disease originated by uncontrolled growth of abnormal cells. According to 2018 population data, 18.1 million new cancer cases and 9.5 million related-deaths were recorded [1]. However, the global number of cases is increasing continuously, and would rise to 24 million new cases per year in 2035 [2].

Breast cancer (BC) is the most common cancer among women in developed countries and the second leading cause of all cancer-related deaths [3].

2.2. Epidemiology

According to the American Cancer Society, one out of eight women in the United States will develop BC in their lifetime. In fact, the incidence of BC is rising up with an accumulative incidence of 3.1% per year [4]. Actually, BC incidence would reach approximately 3.2 million of new cases per year by 2050 [5].

However, incidence varies depending on different worldwide factors, including country development or ethnicity. In fact, in developed countries, the incidence of BC is higher than in underdeveloped countries. Actually, Europe and America's incidence and mortality were significantly higher than in Asia and Africa [1]. Accordingly, while 92 per 10.000 cases of BC are recorded in North America, only 27 per 10,000 cases are identified in middle-Africa and eastern Asia [6][7](Figure 1).

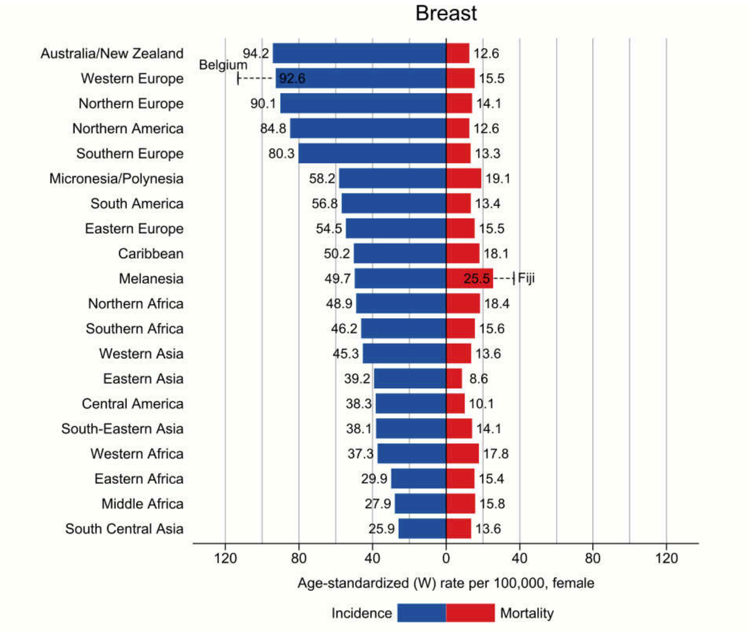

Figure 1. Epidemiology of Breast cancer*. Bar chart of region-specific incidence and mortality age-standardized for cancers of female breast in 2018 [1].*

However, the mammography accessibility is higher in high-income countries, thus might influence the number of BC diagnosticated cases in each region.

Analyzing the death rate in middle- and low-income regions, a higher death rate is observed compared to high income regions. One of the main factors in BC outcome is early diagnosis, being crucial for a good prognosis. In richest regions, an early diagnosis is frequently possible, while in low-income regions BC is often diagnosticated at late stages, being a decisive factor in the rate death in both regions [8]. Moreover, accessibility to a better treatment influences the death rate and in middle and low regions the availability of access to treatment is limited, and death rate remains high.

BC prevalence and mortality also vary depending on the ethnicity. According to cancer statistics from 2013 to 2017, BC prevalence is higher in white and black women compared to the Asian or Hispanic population [9]. However, developing Asian countries have a higher BC incidence in young women compared to western developed countries [10]. Actually, not only BC incidence is associated with ethnicity, but race. In fact, in non-Hispanic black women, the incidence of Triple Negative Breast Cancer (TNBC) is higher compared to white women or those of other races. In the same way, metastatic BCs and undifferentiated grade tumors have a higher prevalence in this ethnicity [11][12]. However, some variations in prevalence and incidence of BC could be explained by genetic predisposition or by the individual's lifestyle.

2.3. Breast anatomy

Breast is mainly composed of mammary glands surrounded by the connective stromal tissue (Figure 2). Mammary gland is a modified apocryphal gland made up of 15-20 mammary lobules. These lobules have a particular structure consisting of different cells (epithelial and myoepithelial).

Mammary glands are surrounded by a stromal connective tissue that forms the supporting structure. This tissue is composed of fibrous stroma and a fatty component. The main function of the fibrous stroma is to attach and secure the breast to the dermis and to separate the secretory lobules of the breast. On the other hand, fat component plays an important role in energy storage as well as breast development and the maturation.

Outside of the breast tissue is found the pectoral fascia, consisting of a thin sheet of connective tissue which acts as an attachment point for the suspensory ligaments.

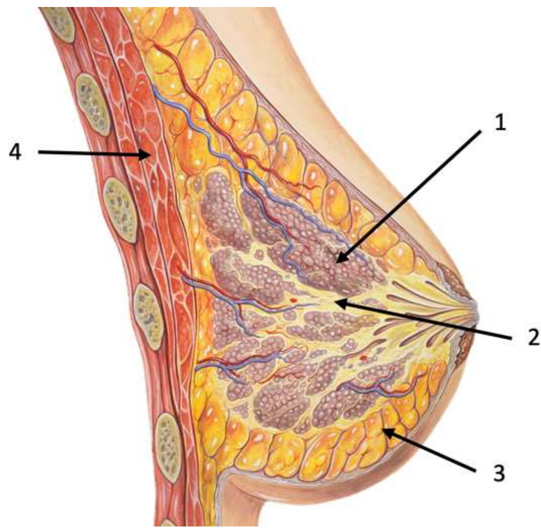

Figure 2. Breast anatomy*. An annotated illustration of a sagittal cross-section of breast anatomy. The tissues shown are 1. Mammary lobules 2. Fibroblast stroma 3. Fatty tissue. 4. Pectoral fascia. Source: Adapted from Patrick J. Lynch (Medical Illustrator)*

2.4. Breast cancer classification

Breast cancer is a heterogeneous disease, but traditionally was treated as a homogeneous disease. As a result, only a percentage of BC patients responded to treatments. For this reason, a better molecular stratification was studied for a correct diagnosis and treatment. Then, BCs were classified according to the presence or absence of hormone receptors (HR) and human epidermal growth factor receptor 2 (HER2). Accordingly, tumors that expressed estrogen receptor (ER) and/or progesterone receptor (PR) were classified as hormone receptor-positive, and those expressing high levels of HER2 as HER2 positive; meanwhile, tumors that do not express ER, PR or HER2 were catalogued as TNBC. After that, an improved method for BC molecular stratification was reported, involving four different subtypes: luminal A (estrogen and/or progesterone receptor positive), luminal B (estrogen and/or progesterone receptor positive and HER2 positive),

HER2 (human epidermal receptor 2 positive or enriched) and triple-negative (negative for these receptors) [11]. This stratification method was based on 50-gene expression signature (PAM50). Advances in gene characterization and molecular expression has led to the identification of a fifth molecular sub-type, the basal-like BC. However, in this thesis for simplicity, Perou classification will be referred, since it is the most widely used.

2.5. Inherited breast cancer

Although most BCs are not linked to an inherited factor (90-95% of BC are non-inherited), 5-10% of remaining cases can be explained by inherited genetic factors [13]. Actually, mutations in different genes are required for disease initiation and progression. There are specific predisposed genes that are mutated in different syndromes:

BRAC1/2: Two of the most important tumor suppressor genes are BC associated genes 1 and 2 (BRAC1/2). BRAC1 and BRAC2 are located on the chromosome 17q21 and 13q12, respectively, and they are two important anti-oncogenes that encode for two suppressor proteins involved in DNA repair. Once a harmful mutation in these genes occurs, there is an increased possibility to develop cancer, especially for breast and ovarian cancer. According to several studies, presence of a BRAC1/2 harmful allele increases drastically the risk to develop breast and/or ovarian cancer. In fact, while in a general population about 13% of women will develop BC during their lifespan, the incidence increases drastically with initial inherited mutation of BRAC1 or BRAC2, suggesting that 55-72% of women who inherit a mutation in BRAC1 and 45-60% of those women who inherit a harmful BRAC2 will develop BC in their lifetime [14][15][16]. Thus, BRAC1 and BRAC2 are two of the most commonly cancer mutated genes,

although other newly emerging genes are also appearing to explain BC hereditary [17].

TP53: Tumor suppressor gene TP53 encodes for an essential protein involved in DNA repair and in harmful stimuli response. TP53 is associated with a high spectrum of tumors, comprising sarcomas, adrenocortical carcinomas, brain cancers and very early onset BC [18][19][20]. Mutations in this this gene contribute to early onset BC compared to the general population [21]. As P53 protein is required for DNA repair, a chemotherapy approach is not considered for these patients due to the possibility of enhancing secondary tumor development [22].

PTEN: Phosphatase and Tensin Homologue (PTEN) gene is located in the chromosome 10. It encodes for a dual phosphatase with both protein and lipid phosphatase activities. PTEN acts as a tumor suppressor gene by negatively regulating a major cell growth and survival signaling pathway, the Akt cascade [23][24]. Germline mutation in PTEN leads to Cowden syndrome, characterized by a high risk of suffering benign and malignant tumors of thyroid, breast and endometrium [25][26]. BC research data confirm that individuals that suffer this syndrome have an increased BC risk and that over 90% of individuals with this syndrome will display some forms of clinical manifestations by their 20s [27].

STK11: The Serine/Threonine kinase gene (STK11/LKB1) is a tumor suppressor gene which mediates apoptosis and cell cycle regulation. Germline mutation that causes Peutz-Jeghers syndrome, characterized by mucocutaneous pigmentation and hamartomatous polyposis [28]. Mutations in this gene are also associated with higher BC risk, as well as other type of cancers such as pancreatic, ovarian, cervix, and lung cancer. In addition, mutations in

STK11/LKB1 increase drastically the possibility of suffering any other type of cancer by up to 85% [29].

CDH1: The E-cadherin gene (CDH1) encodes for an adhesion molecule that plays an important role in stablishing junctions between epithelial cells. Several studies have demonstrated a strong association between germinal mutations in CDH1 and hereditary diffuse gastric carcinoma [30]. Indeed, the cumulative risk of CDH1 mutations with gastric cancer is approximately 67% in males and 83% in females [31].Therefore, patients with CDH1 mutated also displayed a high predisposition to lobular BC and colorectal cancer [31][32][33]. Women with CDH1 mutations also face a 40-45% higher risk along their lifespan of developing lobular BC [30].

Nevertheless, hereditary BC accounts for only about 5-10% of all BCs. Most cases occur in a spontaneously. In fact, about 90-95% of BC cases do not occur because of an inherit profile mutation [13]. In fact, different risk factors can influence the incidence of BC.

2.6. Risk factors

Age is one of the most important risk factors in cancer. The probability of developing BC, as well as many other cancer types is higher at late ages. In fact, the probability of developing invasive BC increases from 2% in female before the 49 years old to 3.5% and 7% in women at the age of 60s and 70s, respectively [34]. Interestingly, during premenopausal stage, BC risk increases exponentially with the age, while it progresses slowly during post-menopausal period [35]. Breast is an estrogen and progesterone sensitive organ, and the dysregulation of these hormones can influence breast normal behavior. In fact, hormone

replacement therapy in post-menopausal women can also increase the BC risk [36][37].

Moreover, different reproductive characteristics can affect the BC incidence in women. Actually, low parity, late first menarche, or a delay in menopause, among others, are some risk factors that increase BC risk [13][38][39][40].

Albeit these factors can modulate the incidence of BC, one of the main risk factors that has an important role in BC development and progression is the population lifestyle. In fact, some studies suggest that a good lifestyle with a high percentage of physical activity and an equilibrated diet might reduce most cancer risk, including BC.

Nowadays, modern lifestyle includes high alcohol consumption and high fat diets as well as increased smoking habit. These factors are highly implicated in cancer incidence and progression. Alcohol consumption has been linked to higher estrogen related hormones levels and the activation of different estrogen receptor pathways [41][42]. Besides, strong associations between alcohol intake and BC cells invasiveness have been described by increasing the secretion of matrix metalloproteases (MMP) 2 and 9 [43], as well as by activating the canonical proliferative and invasive pathways in cancer stem cells [44].
Although there are controversial results about smoking habit and BC, accumulative studies suggest that smoking, especially at early ages, increases BC incidence in women [45]. Therefore, a positive correlation has linked BC to cigarettes and alcohol consumption [46]. These data, combined with studies correlating smoking habits and almost all type of cancers confirm these habits as risk factors.

Nevertheless, one of the most important modifiable risk factors is diet. Western countries lifestyle has generated diets based on a high percentage of fat, especially saturated fatty acids [47]. As a matter of fact, obesity is closely related to an increased risk of many type of cancers, including BC [48].

2.7. Obesity and breast cancer

Obesity is a chronic disease affecting high percentage of the population. This condition is characterized by weight gain and is associated with an increase in adipose cells in number and size.

Overweight and obesity are the fifth leading cause of death worldwide. Actually, approximately 3.4 million deaths are directly or indirectly associated with obesity [49][50]. However, obesity is also related to an increase of comorbidities. In fact, numerous studies have related obesity to different diseases, including diabetes mellitus, cardiovascular disease and cancer [51].

Essentially, in 2007, diverse cancer types have been associated with obesity. [52]. BC incidence is also highly influenced by a high fat diet, being obesity closely related to this kind of cancer. Controversially, premenopausal obese women BC risk is notably reduced [53], whereas postmenopausal obese women have higher BC risk [54]. In addition, obesity is associated with lower risk of ER-positive BC and higher risk of TNBC in premenopausal women. Though, in postmenopausal women, obesity is associated with a higher risk of ER-positive BC, but has not effect on the risk of ER-negative BC [55]. Besides these controversial data, it is well stablished that obesity increases the risk of death in both premenopausal and postmenopausal BC patients [56]. In order to understand how the obesity, hence the adipose tissue can influence tumor cells

development, we should focus on the behavior of these type of cells, as well as on the mechanisms that become crucial for their progression. These mechanisms are functional acquirable capabilities that allow cancer cells to survive, replicate and disseminate and are named as cancer hallmarks.

2.8. Cancer hallmarks

Six biological capabilities have been initially proposed as cancer hallmarks. These cancer hallmarks include sustained proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis [57](Figure 3). In addition, new studies have led to the consideration of further new cancer hallmarks such as avoiding immune destruction, tumor-promoting inflammation, genomic instability and deregulating cellular energetics [58][59][60](Figure 4).

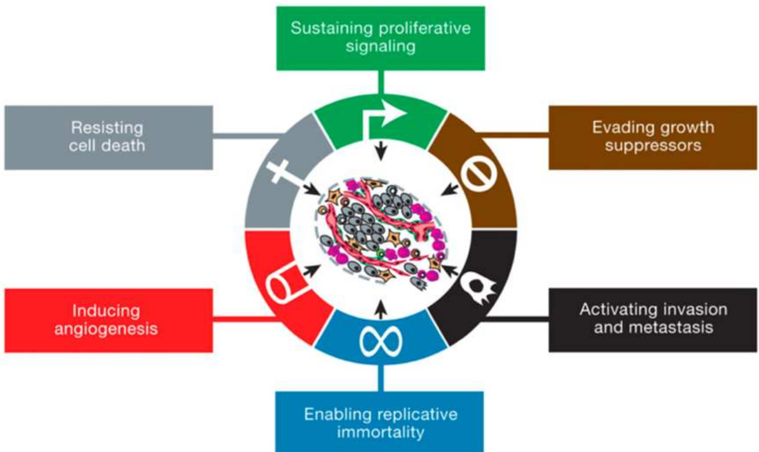

Figure 3. Classical cancer hallmarks. *Illustration of the six original cancer hallmarks: Sustaining proliferative signaling, Evading growth suppressors, Activating invasion and metastasis, Enabling replicative immortality, Inducing angiogenesis and Resisting cell death.*[61]

2.8.1. Sustained proliferative signaling

While normal tissue controls the cell number and homeostasis, tumor cells can chronically proliferate, modifying different mechanisms that control this homeostasis. In order to modify cell homeostasis, tumor cells uses mechanisms closely linked to growth signals and their receptors. This process is regulated by different growth factors that bind to surface receptors, whose tyrosine kinase domains amplify the signal cascade. Normal tissues control the production and releasement of growth-promoting signals in order to maintain tissue architecture and function. In addition, as a regulatory mechanism, cells control the production and functionality of their growth receptors. Tumor cells become masters of their fate, and by different mechanisms, they are able to proliferate uncontrollably.

Over-production of the receptor ligands can generate higher levels of signals from these receptors and sustain an autocrine-mediated proliferation. Moreover, tumor cells can also over-produce growth receptors, increasing the proliferative signals. Once growth factors have been recognized by the receptors, their stimulation leads to the initiation of downstream signal cascades that activate different kinases activating transcriptional mechanisms that increase cell proliferation [62][63][64][65].

Mutations in downstream proteins can also generate the sustained activation of the proliferative signals. Protein kinases are key molecules whose mutations are closely associated with overactivation of proliferative signaling pathways. Two of these signaling proteins are the phosphoinositide 3-kinase (PI3-kinase) and its downstream effector protein Akt (also called PKB/ Protein kinase B). In fact,

array analyses have detected mutations in the catalytic subunit of distinct Akt isoforms in different types of cancer [66][67].

Moreover, proteins, such as Ras, Raf or mitogen activated protein (MAP) kinase, components of the of the classical MAPK signaling pathway, are also mutated in many cancer types, especially in melanoma. In fact, RAS is mutated to an oncogenic active form in about 15% of human cancers [68].

A negative feedback loop controls the overactivation of these pathways and ensures homeostatic regulation of these proliferative signals in normal cells. Mutations in negative regulation mechanisms can sustain the proliferative signaling. Phosphatases, including PTEN, are key proteins that negatively regulate this pathway by the dephosphorylation of different molecules such as Inositol Phospholipid-3 (PIP3). Loss of function, mutations or gene promoter suppression of PTEN triggers to an amplification of PI3K signaling, thus promoting tumor progression [69].

In the same pathway, mTOR also plays an important role in the proliferation signals by the regulation of PI3K-Akt pathway [70]. Actually, pharmacological inhibition of mTOR by Rapamycin results in a reduction of cell proliferation, thus being an effective treatment in certain cancer cases [71].

2.8.2. Evading growth suppressors

Tumor cells have several powerful programs that negatively regulate cell proliferation via tumor suppressor genes. Two of the most studied tumor suppressor genes encode for the retinoblastoma-associated (RB) protein and TP53 protein which negatively regulate cell cycle progression. Tumors with

mutations in any of these key tumor suppressor genes will show a lack of the gatekeeper of cell-cycle progression genes , leading to uncontrolled proliferation [72][73].

In addition, a second mechanism that controls cell proliferation is cell-to-cell contact. NF2 gene product orchestrates the union of cell-surface adhesion proteins to transmembrane tyrosine kinase receptors. These adhesions block the activation of these receptors, thus limiting the efficiency of mitogenic signals [74][75].

2.8.3. Resisting cell death

Apoptosis is a mechanism that orchestrates programmed cell death, being a crucial barrier to cancer progression. This process is crucial for the correct functionality of an individual organism [76]. One of the main roles of cell death is involved in the eradication of cells with significant damages or out of control, avoiding the spread of aberrant information to descendant cells. In fact, TP53, as a DNA damage sensor, regulates the activation of cell apoptosis [77]. Pro and anti-apoptotic regulators control the apoptosis program in a balanced manner. The presence of a higher amount of pro-apoptotic regulators induces the releasement of cytochrome-c from the mitochondria that finally will activate a caspase complex, hence cell programmed death [78][79].

Moreover, FOXO family (FOXO3, FOXO1 and FOXO4) is a subclass of transcription factor involved in the homeostasis against oxidative stress, primarily regulated by Akt. The main role of FOXO proteins is to arrest cell cycle and proliferation before inducing cell death. FOXO inhibition represses the expression of Bim, a proapoptotic protein, enhancing cell survival [80][81][82].

Consequently, alterations in any of the different mechanism elements lead to an incompetent programmed cell death, hence to the enhancement of tumor cells resistance.

2.8.4. Enabling replicative immortality

This cancer hallmark is one of the most important and crucial for the generation of macroscopic tumors. Normal cells have different complex mechanisms that establish limits in cell proliferation and renewal, avoiding the situation of infinite replicability.

Telomers are composed by multiple tandems of hexanucleotide repeats, protecting both ends of the chromosome from telomerase action. Telomers length indicates how many successive cell duplications can be performed before telomers are completely shorted, becoming senescent cells [83]. By inhibition of telomerase as well as blockade of TP53 and RB pathways, tumor cells can avoid senescence, leading to the replicative immortality [84].

2.8.5. Inducing angiogenesis

As tumor cells are continuously proliferating, there is a high demand of nutrients and oxygen, being essential creation of new blood vessels for tumor irrigation. In normal conditions, the generation of new vasculature becomes quiescent in adulthood. Nevertheless, tumor can generate a pro-angiogenic switch as well as to maintain new vessels for continuous nutrient delivery [85].

2.8.6. Activating invasion and metastasis

Tumors progress continuously to higher pathological grade until tumor cells acquire the capacity to metastasize and invade new niches [86]. However, malignant cells are located in a complex tissue surrounded by different cells and extracellular matrix (ECM), hence developing different morphological modifications as well as ECM alterations are essential steps local and distal metastasis. In fact, dysregulation of ECM proteins, such as E-Cadherin, N-Cadherin or Vimentin, can lead to tumor cells attachment loss, increasing their mobility [87]. Moreover, changes in different enzymes expression, such as metalloproteases, can cause ECM degradation, increasing the possibility of invading the surrounding tissues and to arrive to the blood stream or the lymphatic nodes for dispersion [88].

Invading cancer cells can then enter in circulation by migrating directly through blood vessel walls (transmigration) [89]. If they survive the shear stress and the protective immune cells in the bloodstream, they can eventually attach to endothelial cells (ECs) that line the blood vessels. Circulating tumor cells should extravasate before proliferating in the stroma. Importantly, only a small proportion of these cells are able to accomplish all the steps and develop a new metastatic focus. To success, tumor cells require a high degree of “cell plasticity” referring to the ability of cells to reversibly change phenotypes. An example is seen in carcinomas, where epithelial cells acquire mesenchymal characteristics as well as the ability to migrate and invade adjacent tissues, a process termed Epithelial-Mesenchymal Transition (EMT) [90].

2.9. New cancer hallmarks

2.9.1. Genome instability and mutation

Acquisition of all different cancer hallmarks mentioned above requires a specific succession of alterations in cell genome, leading to an increase in random mutations that will finally orchestrate different cancer hallmarks. While normal cells have a very low rate of mutations, cancer cells acquire a high mutation rate, primarily by an increase in cells susceptibility to mutagenic agents and/or events [59]. This is due to an alteration in the machinery involved in the genomic surveillance and maintenance that will lead to accumulation of gene mutations. It will eventually result in the disruptions of many pathways involved in cell cycle checkpoints, DNA repair, and survival control, among others.

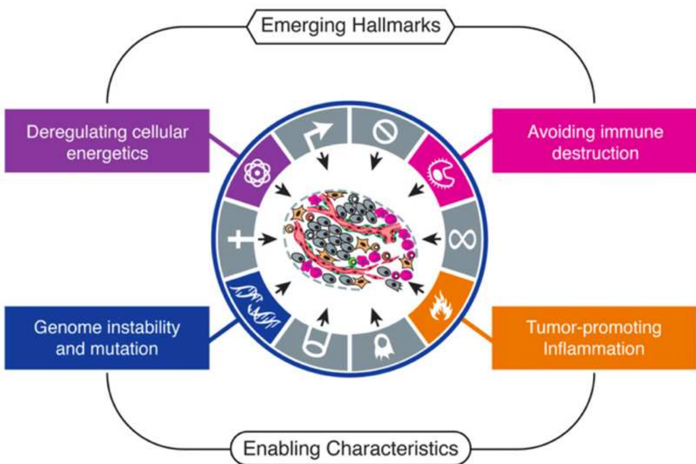

Figure 4. Emerging cancer hallmarks. *Illustration of the new hallmarks of cancer: Deregulating cellular energetics, Avoiding immune destruction, Tumor-promoting inflammation, and Genome instability and mutation* [61].

2.9.2. Tumor promoting inflammation

In addition, tumors are highly infiltrated by different immune cells. Usually, immune system produces a proinflammatory status once pathogens are recognized, increasing immune cell infiltration. [91] Tumors are recognized by the immune system, neutralizing these specific “pathogens”. However, recent studies have also demonstrated that chronic inflammation in specific areas might have important roles in cancer development and progression [92][93]. Inflammation can induce production and releasement of growth and survival factors, as well as extracellular enzymes, that are able to modify the ECM, facilitating tumor cell invasion or EMT [94][95].

Moreover, chronic inflammatory microenvironment elevates the number of macrophages and other leucocytes, generating high levels of reactive oxygen and nitrogen species. These molecules are potential mutagenic molecules and may ultimately produce DNA damage, thus increasing mutagenic ratio [96][16].

2.9.3. Avoiding immune response

Although proinflammatory status is crucial for cancer progression, inflammatory cell infiltration contributes either positively or negatively to tumor progression. As mentioned above, chronic inflammation might help in tumor progression. However, a lack of immune response against tumor cells will clearly allow malignant cells to proliferate and progress without any immune barrier [97]. In fact, an immunosuppressive state increases drastically the chance of developing cancer [98][99]. Experiments performed in immunodeficient mice have demonstrated that both innate and adaptative response contribute to tumor

elimination, thus, immune response deficiency significantly increases cancer development susceptibility [100][101].

However, a competitive and functional immune system does not guaranty the tumor eradication. In fact, tumor cells are able to evade immune system by disabling the different machinery that recognizes and destroys them. For example, tumor cells can avoid immune system recognition through cell surface modifications. In fact, loss of tumor antigens as well as the ability to modify MHC-1 antigen presentation are two of the main mechanisms used by tumor cells in the immune system evasion [102]. Therefore, “immunoediting” plays a decisive function in cancer progression. Tumor cells can modify the behavior of immune system for their own benefit. Actually, tumor cells can promote immunosuppressive status development by the production of immunosuppressor molecules that inhibit T cells function and viability [103][104]. Moreover, these molecules recruit immune cells whose function is to create an immunosuppressive status in the tissue [105][106].

2.9.4. Deregulated cellular energetics

Approximately ten decades ago, Otto Warburg observed an altered metabolism in tumor cells with enhanced glycolytic pathway compared to normal cells, which is now known as Warburg effect [107]. In fact, tumor cells are highly glucose dependent [108]. Anaerobic glycolysis provides cancer cells the energy to proliferate, as well as molecules for cellular building blocks synthesis. The reprogramming of metabolism in tumor cells will produce a considerable deregulation in certain metabolites classified as oncometabolites, which are crucial for cancer diagnosis [109]. Reprogramming energy metabolism improves cellular competence, giving tumor cells a selective advantage. On the other

hand, high energy demands also require an overactivated machinery to fuel tumor. However, as an energy production mechanism, glycolysis is less efficient in ATP production than mitochondrial oxidative phosphorylation. Tumor cells will produce glycolytic intermediates that will benefit in the assembly of the macromolecules and organelles that are required in cell division and proliferation [110]. Lipids are the principal cell membrane component. Thus, tumor cells produce high amount of lipids for cell membranes synthesis, as well as for metabolic reactions and signaling [111]. As tumor cells are embedded within a complex stroma, deregulation of cancer lipid, aminoacid and nucleotide metabolic programs might be mediated by the tumor microenvironment.

2.10. Tumor microenvironment

Tumors have been recently recognized as high complex organs, with numerous interactions between tumor cells and their surrounding microenvironment. Actually, tumor microenvironment can play a key role in cancer initiation and progression. There is a continuous exchange of information between different elements of the tumor microenvironment, through hormones, cytokines and growth factors, that are constantly released and uptaken by the cancer as well as stromal cells [112].

Breast is composed of diverse cell types, including endothelial cells, fibroblasts, immune cells and adipocytes. In normal physiological conditions, crosstalk between all these cells in the mammary microenvironment is responsible of the correct functionality and development of mammary gland (Figure 5).

The stroma maintains epithelial polarity and controls abnormal proliferation and neoplastic formation [113]. Myoepithelial cells are a clear example of how the normal stroma controls this process, acting as gatekeepers of tumor formation

by producing a basement membrane that acts as a barrier around luminal epithelial cells. In fact, their loss promotes conversion of ductal carcinoma *in situ* into an invasive carcinoma [114][115].

Each cell type that conforms the tumor microenvironment has a crucial role in tissue homeostasis, hence crosstalk between tumor cells and different tumor microenvironment components might induce tumor progression and development.

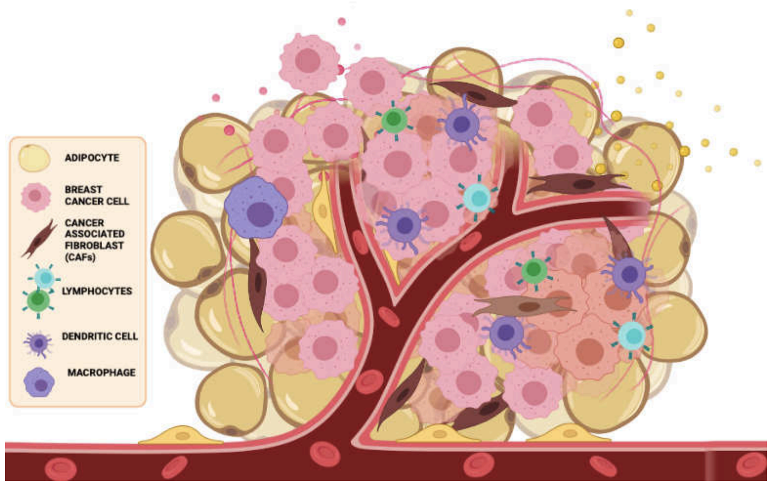

Figure 5. Tumor microenvironment*. Schematic representation of tumor microenvironment, where different cell types coexist with tumor cells. Tumor microenvironment is compound by a complex heterogenicity of adipocytes, fibroblasts, and immune system cells whose crosstalk prepare an excellent niche for cancer progression. Adapted from Bio Render.*

2.10.1. Fibroblasts

Fibroblasts are one of the most abundant cells in the organism and are located in the stroma of breast tissue. Bidirectional communication between these cell type and tumor cells has been described. In fact, tumor cells can modify fibroblast transcriptome, causing their transformation into cancer associated fibroblasts (CAFs) [116]. Moreover, progressive conversion of resident normal

fibroblasts into CAFs during tumor development has been observed *in vivo* [117], where an activation of transforming growth factor β (TGF-ß) and stromal cell derived factor 1 (SDF-1) pathways are notably present. These pathways activation is strongly associated with fibroblast differentiation to CAFs, and their inhibition significantly reduces this transformation.

Moreover, CAFs can modulate the tumor progression in different cancers, including BC. CAFs release a high number of factors and cytokines that contribute to tumor progression [118][119][120]. Besides, CAFs assist tumor cells in the acquisition of drug resistance. For example, CAFs protect MCF-7 cells against apoptosis induced by different anticancer treatments [121], as wells as CAFs can confer doxorubicin resistance to TNBC cell line MDA-MB-231[45]. Furthermore, EMT can be induced by the communication between BC cells and CAFs, leading to a high metastatic tumor behavior and a poor outcome in the organism [122].
Summarizing, high heterogenicity, plasticity and functionality of CAFs confer a pro-tumorigenic behavior to tumor cells, hence increasing cancer progression and development [123].

2.10.2. Immune system

Immune system acts as a natural barrier against foreign pathogens as well as aberrant cells from the organism. There are diverse immune cells in breast stroma, including macrophages, dendritic cells, and lymphocytes. Communication between the immune system and tumor cells is crucial for cancer development and progression. Through this crosstalk, tumor cells are able to modify immune system behavior for their own benefit [124], being

essential the analysis of the different immune system components that are susceptible to tumor modification.

The first layer of immune system is composed basically of macrophages and dendritic cells. Macrophages are the most abundant leucocyte population in breast stroma and are part of the innate immunity system, being the first defense barrier. Macrophages use to be categorized phenotypically as M1 and M2 macrophages. M1 phenotype is characterized by the activation of proinflammatory genes and releasement of a high amount of proinflammatory cytokines, whereas M2 phenotype is associated with the expression of anti-inflammatory genes and is involved in wound healing and tissue remodeling [125]. Various studies have determined that a M1 phenotype acts as an anti-tumor barrier by enhancing the inflammation in the affected region, that will lead to detection and elimination of tumor cells, while a M2 phenotype leads to progression of the tumor by inhibiting the proinflammatory status and immune response [126].

High levels of infiltrated macrophages are detected in solid tumors, described as tumor associated macrophages (TAMs) and they have crucial role in cancer progression. Indeed, TAMs have been closely associated with tumor growth, angiogenesis, tissue remodeling and suppression of adaptative immunity [127]. [128]. Furthermore, TAMs, can also facilitate tumor cells invasion and migration into blood stream; hence, TAMs are strongly involved in metastasis [16][129][130]. Higher number of infiltrated macrophages in tumor microenvironment have been described, correlating with worst prognosis in BC patients [127][131].
Moreover, TAMs are consistent of a major of M2 macrophage population, although they also contain elements of both polarized M1 and M2 subtypes

[132]. By releasing a diverse spectrum of cytokines, such as IL-10, the chemokine (C-C motif) ligand CCL2, CCL17, CCL22 and TGF-ß, TAMs help tumor cells survival and dissemination [129] [133] [134].
Although most of studies suggest that an anti-inflammatory status is essential for cancer development, a pro-inflammatory status has also been shown to be crucial in cancer development. Thus, inflammatory status might be beneficial but also harmful for cancer development and progression. Though M1 macrophages are known to be antitumoral, they can also enhance tissue damage and inflammation, which are associated with tumor progression during early stages of tumorigenesis [135].

Moreover, this inflammatory status can be controlled by a number of different secreted cytokines and chemokines, leading to infiltration of diverse immune cells, such as natural killers, dendritic cells, and lymphocytes [136] [137]. T-regulatory (T-reg) lymphocytes are one of the components of the inflammatory regulation system, that modulate the tumor behavior. These cells have a crucial role in host protection from autoimmune diseases by regulating inflammatory response. Tumor cells can hijack this function and enhance the role of T-regulatory (T-reg) lymphocytes. As a consequence, T-reg cells prepare an immune-suppressive microenvironment that allows tumor cells to avoid immune response favoring their escape and progression [138][38].

2.10.3. Adipose tissue

Cancer microenvironment, such as ovarian, colon, renal, gastric, and BC, among others, is commonly surrounded by adipose tissue. Traditionally, adipose tissue has been considered only as an energy storage tissue. However, numerous studies have now demonstrated that the adipose tissue is an active endocrine

organ with diverse functions in the organism. Adipose tissue is responsible of eating behavior modulation, steroid hormones, cytokines and adipokines production, and immune system control [139][140]. An imbalance in these physiological functions can lead to severe consequences in the organism. Accordingly, some studies have significantly correlated obesity to cancer progression [141][142].

As fat is the major component of breast tissue, the role played by adipocytes could be an important and decisive factor in BC progression. In fact, peritumoral adipose tissue, known as visceral tumor-surrounding adipose tissue, plays an important role in cancer development. Crosstalk between adipocytes and tumor cells benefit cancer progression. These interactions can alter the phenotype of peritumoral adipocytes, by modifying their transcriptome, proteome, and metabolome, culminating in the dedifferentiation of adipocytes into a fibroblast-phenotypic state, commonly termed as cancer associated adipocytes (CAAs) [143]. CAAs are characterized by a decrease in adipocyte markers, such as adiponectin and resistin, as well as by a reduction in transcriptional regulators, peroxisome proliferator activated receptor Y (PPARY) and co-activator CCAAT/enhancer binding protein α (C/EBP α), which ultimately produces a decrease in lipid droplet number and size due to increased lipolysis. Moreover, CAAs also display an overexpression of proinflammatory cytokines such as IL-1ß, IL-6 or tumor necrosis factor (TNF-α), MMPs such as MMP-11, and chemokines such as CCL2, CCL5, cytokines [141]. The increment of MMP-11 and stromelysin-3 can reduce the pre-adipocytes differentiation into mature adipocytes and revert mature adipocyte phenotype into a more fibroblast-like cells [93][92]. In addition, CAAs present alterations in the production and releasement of certain cytokines compared to normal adipocytes. In fact, high

levels of Interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP1) and CC-L5 are significantly upregulated in CAAs [144] (Figure 6).

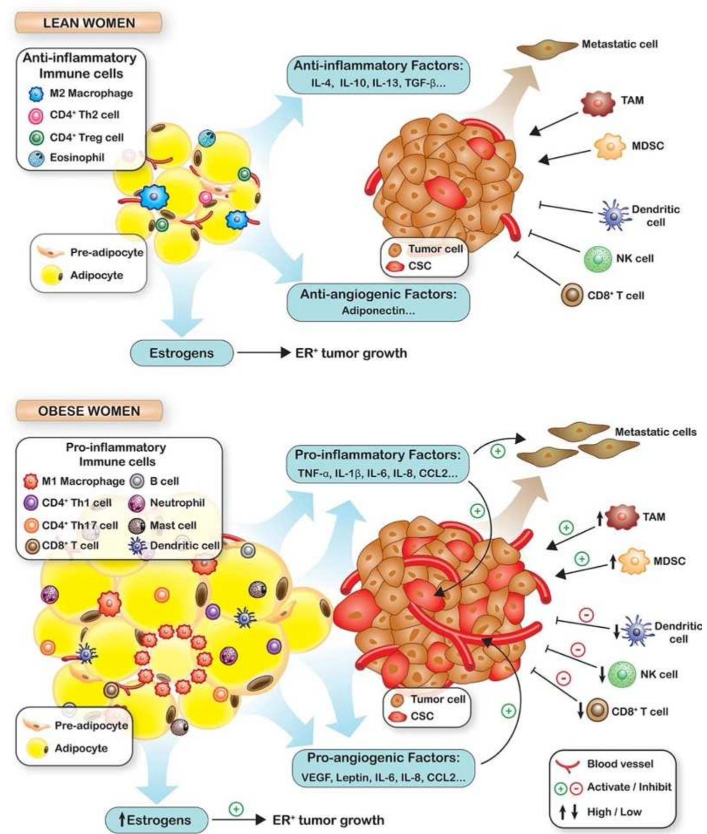

***Figure 6 Obese adipose tissue generates a pro-inflammatory and angiogenic environment**. Illustration where is described how healthy adipose tissue secretes anti-inflammatory factors such as IL-4, IL-10, IL-13 or TGF-ß as well as anti-angiogenic factors like adiponectin preventing cancer stem cells (CSCs) progression, whereas obese adipose tissue secretes pro-inflammatory molecules such as TNF-α, IL-1ß, IL-6, IL-8 or CCL2, as well as pro-angiogenic factors such as VEGF, Leptin, IL-6, IL-8, CCL2 leading to an increase in CSCs tumorigenicity. TNF, tumor necrosis factor; IL, interleukin; VEGF, vascular endothelial growth factor; CCL, chemokine (C-C motif) ligand* [55].

IL-6 is a proinflammatory cytokine involved in tumor progression by enhancing invasion and metastasis via the MAPK pathway [145]. MCP-1 and CCL-5 are chemoattractant cytokines which stimulate macrophage infiltration into

adipose tissue and promote a pro-inflammatory status that can lead to the development and progression of the tumor[144] [18]. Tumor crosstalk with peritumoral adipocytes can generate alterations in adipokines and cytokines production, thus enhancing cancer hallmarks [146] [140] (Figure 7).

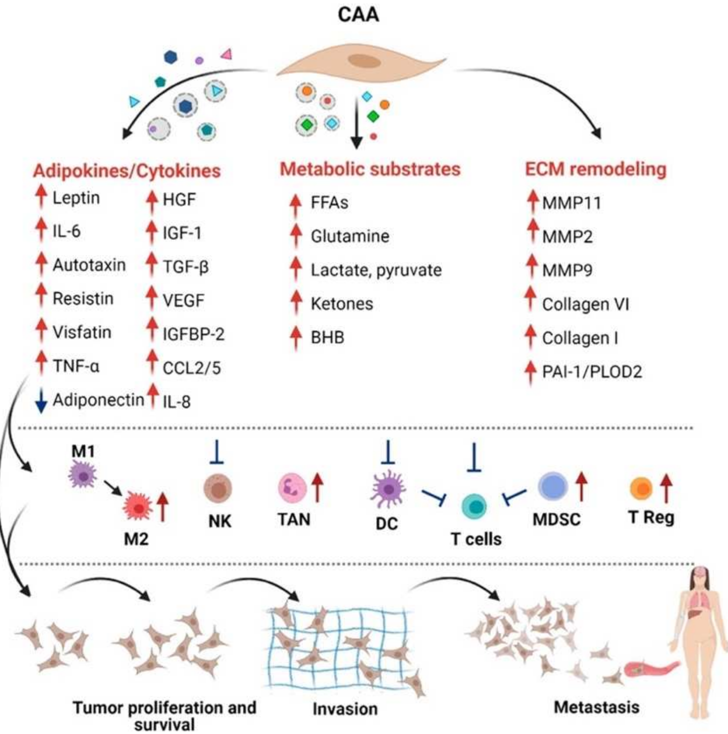

Figure 7. Pro-tumoral cancer associated adipocytes (CAAs) secretome. *CAAs release different molecules that, directly or indirectly, affect tumor cells progression. Red and blue rows represent the increment and decrease of molecules releasement from CAAs compared to normal mature adipocytes. Central panel represents a variety of immune system cells that are suppressed (blue lines) or activated (red arrows) by the releasement of certain CAAs molecules. IL, interleukin; TNF, tumor necrosis factor; HGF, hepatocyte growth factor; IGF, insulin growth factor; TGFβ, transforming growth factor β; VEGF, vascular endothelial growth factor; IGFBP2, IGF binding protein 2; CCL, chemokine (C-C motif) ligand; FFAs, free fatty acids; BHB, β-hydroxybutyrate; MMP, metalloprotease; PAI-1, plasminogen activator inhibitor 1; PLOD2, procollagen-lysine 2-oxoglutarate 5-dioxygenase 2; M, macrophages; NK, natural killer cells; TAN, tumor associated neutrophils; DC, dendritic cells; MDSC, myeloid derived suppressor cells; T Reg, regulatory T cells* [156].

CAAs exhibit several metabolic changes as well as an altered releasement pattern of metabolites such as lactate, pyruvate, free fatty acids, and ketone bodies to the ECM, fueling the malignant progression of adjacent tumor cells [147]. Tumor cells can also internalize these lipids into their metabolic pathway for oxidation and energy production and to activate different oncogenic signaling pathways [141].

Lipids are the main components of cellular membranes and organelles. These molecules are crucial for renewal and cell proliferation. As tumor cells are continuously proliferating, their demand for lipids increases drastically. In fact, several studies have demonstrated the role of lipid metabolism in cancer progression [45].

Lipids include an heterogenous variety of molecules. Depending on structure and composition, different biological roles are attributed, from membrane composition to energy storage or cell signal transduction [148][149]. Among huge spectrum of lipid families, fatty acids are the base of most of them. These molecules are used for the lipid biosynthesis, including diacylglycerides (DAG) and triglycerides (TAG). Interestingly, final products can be used also as alternative energy mechanism by the fatty acid oxidation (FAO) pathway. In fact, tumor cells show important changes in several enzymes promoting proliferation, metastasis, stemness and chemoresistance, partially by the increment in energy obtainment [150][151]. In addition, this pathway is significantly upregulated in TNBC overexpressing MYC oncogene [152][153]. Interestingly, FAO can also promote BC cell stemness and chemoresistance, whereas its inhibition sensitizes tumor cells to chemotherapy [154].

Within this heterogenicity of lipid families, phosphatidylcholines (PC), phosphatidyletilamides (PE), sphingomyelins (SM) or ceramides (Cer) constitute some different lipid families whose presence in plasma serum has been significantly altered in cancer [155].

Moreover, among different biologically active lipid molecules whose function is crucial in signaling regulation, steroidal hormones acquire a significant importance. They are hormones whose principal precursor is cholesterol and are involved in several mechanisms, such as the menstrual cycle, organism growth and development.

Interestingly, different studies have demonstrated a positive association between elevated cholesterol levels and cancer risk increment [157][158]. Synthesis and homeostasis deregulation, hence the deregulation of its biological functions might explain the mechanism that underlined cholesterol effects on cancer progression.

Cholesterol homeostasis is strongly regulated at different levels. Sterol regulatory element binding protein transcription factor 2 (SREBF2) and liver X receptors (LXRs) are the principal regulatory elements of cholesterol homeostasis [159][160]. According to Cancer Genome Atlas (TCGA) database, several cholesterol synthesis genes have been correlated to patient survival [161], thus suggesting a clear correlative link between cholesterol synthesis pathway and cancer outcome[162].

Moreover, CAAs also increase leptin production whiles adiponectin releasement. These two adipokines control several physiological processes related to feed behavior in the organism and their dysregulation has been

observed to be implicated in different metabolic disorders, such as cardiovascular disease, insulin resistance ,type 2 diabetes, and cancer [163]. *In vitro* studies have found that leptin deregulation tumor and stroma interaction, promoting the BC cells invasive growth [140]. Furthermore, high leptin and low serum adiponectin levels have been correlated to higher BC risk and aggressiveness, and worse patient prognosis [140] [164]. On the other hand, leptin is also implicated in angiogenesis enhancement, macrophage differentiation, inflammation, and anti-apoptotic factors releasement [144][165]. In addition, some adipokines upregulation, such as plasminogen activator inhibitor -1 (PAI-1), retinol binding, visfatin, resistin, TNF-alfa, retinol binding protein 4 (RBP4), or autotaxin increases BC pro-tumorigenicity [166][167][168][169][170][171].

Besides, fatty acid binding proteins or FABPs expression, is also dysregulated in obesity and can be influenced by the tumor cells [172].

Summarizing, link between an altered production of all these adipokines and BC opens new strategies in cancer diagnosis and treatment, especially regarding inflammation and tumor invasion.

2.11. <u>Fatty acid binding proteins (FABPs)</u>

FABPs are a family of small water soluble proteins (14-15 kDa) that can bind to long chain fatty acids (LCFA) or other ligands such as eicosanoids and thiazolidinediones, regulating lipid trafficking and cell signaling responses [173]. According to their presence in different tissues, they have been classified into specific FABP subtypes. However, some of these proteins are expressed in multiple tissues as well as they can be co-expressed in the same tissue [174].

Several studies have examined FAPBs role in fatty acids storage as lipid droplets, different gene expression modulation, as well as in cell proliferation and differentiation regulation. Changes in lipid metabolism, as a result of FABPs dysregulation are associated with different metabolic disorders, such as obesity, diabetes, hepatic steatosis and cancer [175][176][177][176].

2.11.1. FABP4

Fatty acid binding protein 4 (FABP4), also called adipocyte protein 2 (aP2) or adipocyte-fatty acid binding protein (A-FABP), is predominantly located in the adipose tissue, monocytes, and macrophages, although it can also be found in different cell types and tumors [176]. In consequence, FABP4 has also acquired special attention in the field of obesity and in many diseases including cancer. Interestingly, abdominal tumors, like ovarian cancer, have a high probability of metastasizing into omemtum [178]. Interestingly, whereas metastatic cells have significantly increased expression levels of FABP4, FABP4 expression is not usually detected in primary tumor. In addition, tumor cells in close contact with adipocytes also increase FABP4 expression [112].

Furthermore FABP4 transcript and protein levels are increased in obese mice compared to their lean counterparts [179]. FABP4 has been strongly associated with different metabolic diseases, including obesity, diabetes and atherosclerosis [180][168][181][40][182]. In agreement, high levels of FABP4 expression are linked to higher risk of developing BC [172], showing a possible association between cancer, obesity and FABP4. In fact, high levels of FABP4 are positively correlated to higher BC incidence [183]. In addition, Gharpure et al., have also reported significant reduction in progression-free survival of those ovarian cancer patients presenting higher expression levels of FABP4 [184].

Diverse signaling pathways have been described as downstream targets of FABP4. One of these pathways is PI3K/Akt signaling pathway. Uehara et al have described that exogenous FAPB4 promotes prostate cancer cell invasion *in vitro* through the enhancement of this pathway. In addition, they have demonstrated that FABP4 inhibition reduced lung cancer growth and metastasis [185].

Furthermore, previous results have reported that FABP4 addition enhanced of fatty acid binding proteins FABP5 and CD36 as well as FOXM1 expression. Moreover, these studies also demonstrated that exogenous FABP4 activated the MAPK and Akt pathways promoting the proliferation of BCC lines MDA-MB-231 and MCF-7 [187] (Figure 8).

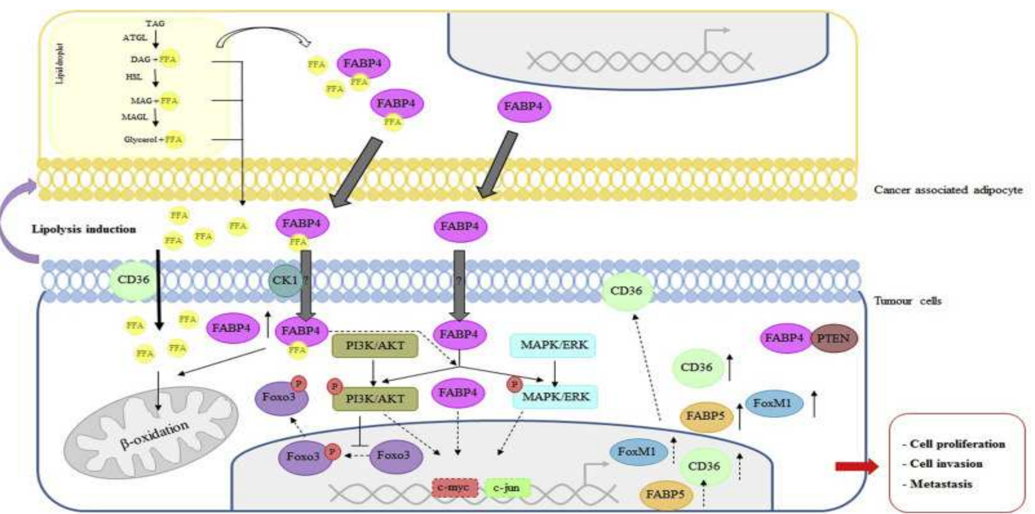

Figure 8. FABP4 role in cancer biology. *Illustration of the possible FABP4 functions in tumor cells. FABP4 plus FFA are released to the extracellular media and after their internalization within the tumor cell there is an activation of PI3K/Akt as well as MAPK/ERK signaling pathways, leading to the increase of FOXM1, FABP5 and CD36* [188].

Moreover, FABP4 is also implicated in prostate cancer development. *In vitro* studies with the prostate cancer cell line PC-3 have demonstrated that FABP4

enhanced the secretion of inflammatory cytokines, such as IL-6 and IL-8. In addition, FABP4 also increased the metalloproteases MMP-9 and MMP-2 expression, enhancing their metastatic behavior. Conversely, they also verified that FABP4 inhibition reduced these metastatic abilities [186].

FABP4 is also present in macrophages, and it has been detected a higher expression in a subpopulation of tumor associated macrophages predominantly present in late-stage BCs. These subpopulations presented an overactivation the nuclear factor κB (NFκB) pathway, leading to the enhancement of the pro-inflammatory and pro-tumorigenic cytokine IL-6. (Figure 9). FABP4 depletion in these macrophages resulted in a significant decrease of the tumor growth and metastasis [133].

The Nieman laboratory has shown that FABP4 binds to FFA, providing energy for ovarian tumor cell growth and metastasis [190]. *In vitro* and *in vivo* studies have also demonstrated that FABP4 is implicated in invasion, migration and epithelial-mesenchymal transition of cholangiocarcinoma cells. Furthermore, FABP4 inhibition by the specific small molecule inhibitor BMS309403 [2’-(5-Ethyl-3,4-diphenyl-pyrazol-1-yl)-biphenyl-3-yloxy] decreases its effects on metastasis and EMT [191].

Formation and assembly of new vessels is crucial for tumor cell dissemination and is mainly orchestrated by the protein vascular endothelial growth factor (VEGF). In glioblastoma, angiogenesis has been demonstrated to be mediated by FABP4-VEGF axis. Specifically, the overexpression of FABP4 in glioblastoma can potentiate the pro-angiogenic role of VEGF, leading to an enhancement of tumor cells metastasis [183][192]. In fact, FABP4 knockdown in endothelial cells can cause a significant decrease in angiogenesis. In addition, depletion of FAPB4

in ovarian tumor xenografts also decreases their growth and metastatic behavior [193].

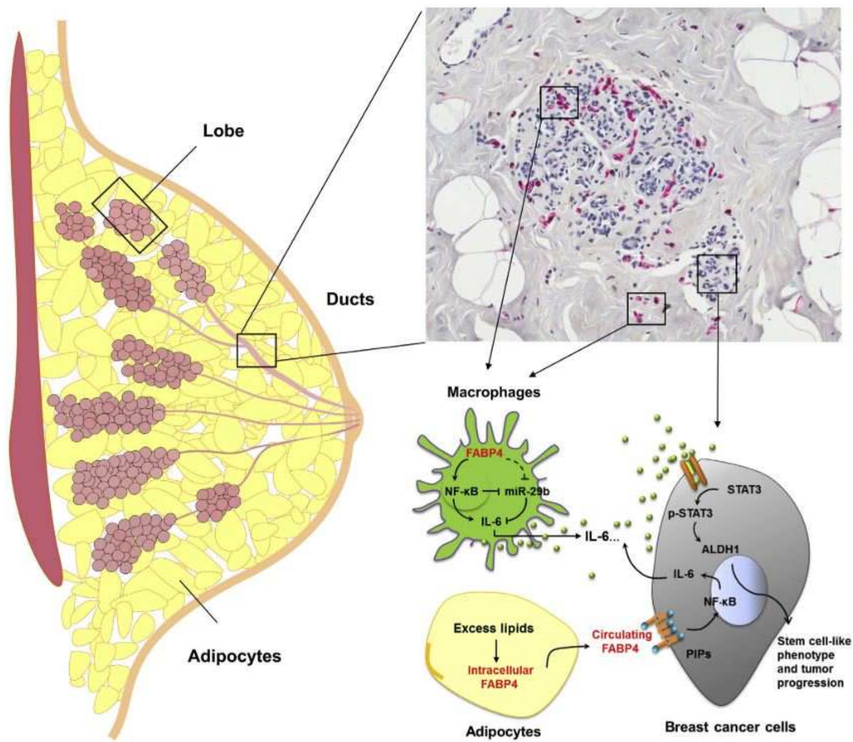

Figure 9. FABP4 inflammatory effects in breast cancer. *Schematic representation of the mammary gland, showing a transversal section of a mammary conduct affected by a tumor. Blue dots represent the tumor cells whereas pink dots represent the infiltrated macrophages. There is a communication between both elements where FABP4 expressed in macrophages enhances the pro-inflammatory cytokines production and releasement to the extracellular matrix. IL-6 activates the STAT3/ALDH1 pathway and stimulates tumor progression.IL, Interleukin; ALDH1 aldehyde dehydrogenase 1; STAT3, signal transducer and activator of transcription 3* [189].

In summary, FABP4 plays an important role in promoting malignant progression in different cancer types, including BC. However, FABP4 is not the only fatty acid transporter that contributes to BC development and dissemination. In fact, other fatty acid transporters, such as FABP5 and CD36, can also have an important role in BC progression.

2.11.2. FABP5

FABP5 is a 15kDa protein belonging to the FABP family that is expressed in many different cells and tissues, such as the skin, brain, kidney, mammary gland, lung, dendritic cells, adipocytes and macrophages [176]. Long chain fatty acids (LCFAs) transport, intracellular signaling, and gene regulation are some of the different functions that FABP5 interplay in the mammalian cell.

Interestingly, FAPB5 is expressed in two tissues where FABP4 is also expressed and released, suggesting a coordinate role of both fatty acid transport proteins in lipid metabolism, which may reflect a synergic effect of FABP4 and FABP5 in cell regulation. High FABP5 levels in the blood stream of mice has been strongly correlated to metabolic diseases, such as obesity and insulin resistance [194] [195], as a consequence of a high grade of inflammation [35].

Indeed, FABP5 has also been implicated in other diseases, including cancer. For example, Kazuyasu et al. have observed an upregulation of FABP5 in hepatocellular carcinoma (HCC) cell lines and cancer tissue compared to normal hepatocytes, thus promoting cell proliferation and invasiveness [196].

On the other hand, FABP5 inhibition by short hairpin siRNAs suppresses the pro-tumorigenic effects of FABP5 in HCC cells [197]. *In vitro* studies have also shown that FABP5 upregulation was associated with oral squamous cell carcinoma enhancement of proliferation and invasiveness. Besides, FABP5 upregulated the metastatic related enzymes MMP2 and MMP9 [198]

In cervical cancer, the association between FABP5 expression and the enhancement of cancer hallmarks has been also uncovered [199][200].

Furthermore, the regulation of cervical cancer cell metabolic reprogramming is also mediated by the upregulation of this fatty acid binding protein in this cell type [47].

Close relationship between BC development and the upregulation of FABP5 has been described too. In fact, Levi *et al*. have suggested a crucial link between HER2-induced tumorigenesis in BC and FABP5 expression [201]. Likewise, Liu *et al*. have discovered an association between the upregulation of FAPB5 in TNBC patients and poorer prognosis [202]. In concordance, FABP5 upregulation has also been shown to increase the metastatic behavior of TNBC cell lines [203].

Thus, in conclusion, FABP5 plays a crucial role in the progression of diverse cancers, including BC, by enhancing different cancer hallmarks such as proliferation, invasiveness, and metabolic reprogramming.

2.11.3. CD36

CD36 is a well-known scavenger receptor frequently expressed in a wide variety of tumor cells [204], as well as in different stromal cells [205]. The main function of CD36 in cellular biology consists on the absorption of LCFAs and the oxidated low density lipoproteins (ox-LDLs) [206].

CD36 is upregulated in different cancer types, including ovarian, gastric, glioblastoma, and oral squamous cell carcinoma (OSCC) [207] [208][209][204]. Several studies have correlated CD36 to cancer, promoting tumor growth. CD36 promotes tumor growth by activating the Src kinase and ERK1/ERK2 mediated signaling pathways, and its inhibition reduces tumor volume in cervical cancer xenograft models [210].

Pascual and colleagues have also described a relationship between higher CD36 expression and metastasis initiation in human OSCC [204]. Inhibition of CD36 also increased drastically the accumulation of lipid droplets in tumor cells, inducing cell lipo-toxicity and death and hence a significant decrease in tumor size. Moreover it has been described that fatty acids uptake mediated by CD36, activated Wnt and TGF-ß signaling, thus leading to EMT enhancement in hepatocellular carcinoma [211].

Together, evidence suggests that the fatty acid transporter CD36 plays an important role in cancer development and progression and can be a potential target for cancer treatment.

In conclusion, lipid transfer from the tumor microenvironment to cancer cells is crucial for cancer progression, and these three fatty acid transporters can impact on cancer development and malignant progression. Despite their role in fatty acid traffic, these fatty acid transporters are not the primary mediators of lipid transport. In fact, lipid traffic and transport through the blood stream and tissues is mediated by lipoproteins.

2.12. Lipoproteins

Lipoproteins are small spheroid vesicles that carry diverse lipid molecules, including cholesterol, triglycerides, and phospholipids [212]. Depending on the lipoprotein size, lipid composition and apolipoproteins (proteins that are contained within the lipoproteins), these lipoparticles can be classified in seven different subclasses (Table 1).

Lipid composition varies notably between these families. Actually, LDL has a higher proportion of triglycerides and cholesterol compared to HDL particles. Whereas LDL principal function is the cholesterol transport from liver to peripherical tissues, HDL is the principal responsible of the reverse transport of cholesterol, hence reducing cholesterol levels in peripherical tissues by its transport to the liver where this lipid will be eliminated from the organism.

Lipoprotein	Density (g/ml)	Size (nm)	Major Lipids	Major apoproteins
Chylomicrons	<0.930	75-1200	Triglycerides	Apo B-48, Apo C, Apo E
Remnants	0.930- 1.006	30-80	TG and Chol	Apo B-48, Apo E
VLDL	0.930- 1.006	30-80	Triglycerides	Apo B-100, Apo E, Apo C
IDL	1.006- 1.019	25-35	TG and Chol	Apo B-100, Apo E, Apo C
LDL	1.019- 1.063	18-25	Cholesterol	Apo B-100
HDL	1.063- 1.210	12-5	TG and Chol	Apo A-I,II, Apo C, Apo E
Lp (a)	1.055- 1.085	~30	Cholesterol	Apo B-100, Apo (a)

Table 1. Lipoprotein classification. *(VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; Lp (a), Lipoprotein a; TG, triglycerides, Chol, cholesterol, Apo, apolipoprotein* [212].

Consolidated evidence has described a causal role of LDL particles in atherosclerosis and cardiovascular disease where the HDL levels have been considered as cardio-protective [212][213][214]. Moreover, epidemiologic reports have shown that BC is correlated to higher levels of LDL in patient blood stream, whereas elevated HDL blood stream levels are considered as good prognostic factor in BC [215][216].

Although a number of studies have already associated these lipoproteins with cancer, these studies have used traditional correlation methods to establish a relationship between the number of these lipoproteins and cancer

development. New technologies can now measure the size and composition of these lipoparticles, including the molecules contained within, such as triglycerides and cholesterol, which can lead us to further understand the complex relationships between these lipoparticles and cancer in detail [217].

In fact, high levels of cholesterol and triglycerides have a positive impact in cancer progression. As tumor cells have a high demand of these lipids, it is plausible to think that the body cholesterol homeostasis, hence the circulating cholesterol levels might suffer a significant alteration. In agreement, numerous studies have established a correlation between high blood cholesterol levels and cancer prevalence, which also holds true for BC [157][158][218].

A recent metanalysis have demonstrated that total cholesterol, as well as cholesterol contained in LDL particles (LDL-C), were negatively associated with cancer survival [219]. Interestingly, it has been observed that the addition of statins, a common pharmacological inhibitor of the 3-hydroxy-3-methyl-glutaryl-coenzyme A reductase (HMGCR) had pleiotropic effects against cancer development [220], because of the reduction of liver cholesterol biogenesis, thus resulting in a reduction of total cholesterol and LDL-C [221]. Although statins use in cancer progression is yet controversial, research suggests plausible mechanisms that underline these anti-cancer effects [222].

In concordance, triglycerides importance in cancer progression has also been described above. These molecules are scaffold elements essential for the several molecules and organelle biosynthesis, hence they are important for cell biogenesis. An imbalance in triglycerides homeostasis might have an impact in cancer progression. Accordingly, high levels of plasma triglycerides and free fatty acids are positively correlated to an increased cancer risk [223][224].

Actually, triglycerides enrichment of the different lipoproteins has been positively correlated to an altered metabolism and inflammation status [225][226], hence to an increased cardiopathy [227]. Consistently, as obesity and inflammation are closely linked to cancer progression, the study of triglycerides composition of these lipoparticles in the development this disease might have an important role.

UNIVERSITAT ROVIRA I VIRGILI
IMPACT OF FATTY ACID METABOLISM IN BREAST CANCER PERITUMORAL TISSUE.
CLINICAL AND PATHOGENIC ASPECTS
Jose Adriá Cebrián

HYPOTHESIS

3. HYPOTHESIS

Breast cancer is the most common cancer in women and the second cancer with more related deaths. In last decades, the effect of obesity and adipose tissue in cancer progression, especially in BC, has been object of interest. Understanding the metabolic behavior of tumor cells in the microenvironment context might clarify the mechanisms underlined in adipose tissue, immune system, and tumor cells crosstalk. Consequently, these hypotheses have been stablished in this doctoral thesis:

Crosstalk between adipose tissue and BCC lines might enhance the different cancer hallmarks, partially mediated by FABP4. Thus, FABP4 may revert proliferation, chemo-resistance, migration, and invasion.

As a bidirectional communication, tumor cells are able to modify surrounding adipocytes, hence inducing their delipidation, fatty acid releasement, and lipid transfer.

Immune system, as one of the main components of tumor microenvironment plays an important role in cancer progression. Thus, crosstalk between different cell types of the tumor microenvironment might support a pro-inflammatory microenvironment status, hence in the chronic inflammation of BC patients, thus favoring cancer development.

Metabolism alteration might disrupt the metabolite signature in BC patients, hence supporting a new BC diagnosis methodology.

OBJECTIVES

4. OBJECTIVES

The main objective of this study is to understand the crosstalk in tumor microenvironment, generating a favorable niche for cancer development and progression. Understanding tumor microenvironment might open new therapeutic targets and lead us to the discover of new specific associated biomarkers.

Specific objectives:

1. To assess the effects of adipocyte CM on BCC lines hallmarks.

2. To identify the elements contained in adipocyte CM that supply BCC proliferation, survival, migration, and invasiveness.

3. To investigate the inhibition of proteins found through the second objective.

4. To study the bidirectional crosstalk between adipose tissue and tumor cells, as well as to assess the effect of metabolic behavior alterations in both cell types.

5. To track labeled palmitic acid incorporation and transformation in BCC lines.

6. To establish the role of the communication between adipose tissue, immune system, and tumor cells in the tumor microenvironment context.

7. To assess the pro-inflammatory status of tumor microenvironment, hence of BC patients.

8. To determine by 1H-NMR lipoprotein alterations in BC patients compared to control healthy women.

9. To determine new possible biomarkers for BC diagnosis.

RESULTS

5. RESULTS

For the different stablished objectives in this doctoral thesis, four scientific articles comprised the overall results.

Objectives:

1. **To assess the effects of adipocyte CM on BCC lines hallmarks.**

2. **To identify the elements contained in adipocyte CM that supply BCC proliferation, survival, migration, and invasiveness.**

3. **To investigate the inhibition of proteins found through the second objective.**

Article:

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Lipids and fatty acid transport proteins derived from adipose tissue increase BC lipid uptake and cancer hallmarks

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Keywords: Breast cancer, FAPB4, FABP5, CD36, lipids, cancer hallmarks.

FOR REVIEW

Abstract

Tumor microenvironment plays an important role in cancer progression. Adipose tissue is an endocrine tissue that produce and release a high amount of factors that are able to modify the transcriptome, proteome, metabolism, and the behavior of breast cancer cells. Fatty acid binding proteins and CD36 are implicated in cancer progression. In this study we have demonstrated that adipose tissue significantly enhances BCC lines MCF-7 and MDA-MB-231 proliferation, survival, migration, and invasiveness. Moreover, it is accompanied by an increase of lipid uptake from mature adipocytes as well as an increase in the transcript and protein levels of proteins FABP4, FABP5 and CD36. In addition, in this novel study, we have demonstrated by the inhibition of mature adipocytes FABP4 the important role of this adipocyte released protein in cancer progression, since the inhibition of FABP4 by BMS309403 significantly reduced the proliferation rate, the chemo resistance to Doxorubicin, the migration ability and the invasiveness behavior of both BCC lines. For this reason, it is plausible to think that FABP4 might be a novel BC target therapy.

1. Introduction

Breast cancer is the most common cancer among women in developed countries and the second leading cause of all cancer-related deaths (1). It is a highly heterogeneous disease with distinct morphological and phenotypic characteristics, as well as diverse clinical prognosis (2). One of the major risk factors for breast cancer is obesity (3). In fact, a myriad of studies have already established a strong correlation between body mass index (BMI) and many cancer types, including breast cancer (4)(5)(6). Obesity is characterized by impaired adipogenesis and deregulated adipose tissue (fat) function. Although the adipose tissue has historically been considered as an energy storage site (7), recent studies have revealed that the adipose tissue also functions as an endocrine organ responsible for the modulation of eating behaviour, the production of steroid hormones, cytokines and adipokines, and the regulation of the immune

system (8)'(9). For these reasons, the adipose tissue has gained importance in the pathogenesis of many metabolic diseases, particularly cancer. Adipose tissue is one of the principal components of the mammary gland as it accounts for over 50% of the non-lactating breast tissue. Being the predominant cell types of the adipose tissue (10), adipocytes could have an important influence in shaping the tumour microenvironment. The tumour microenvironment plays a key role in cancer initiation and progression. There is a persistent exchange of information between tumour cells and their microenvironment, through different hormones, cytokines and growth factors, being constantly released and taken up by the cancer cells as well as the stromal cells (11). Recent *in vitro* studies have demonstrated that the adipose tissue could be used by cancer cells as an energy reservoir and that adipocytes are able to transfer fatty acids to tumour cells for them to acquire energy and activate metabolic and signalling pathways (12)(13).

To understand how the adipose microenvironment can promote cancer progression, several studies have focused on its impact on different cancer traits. One of the primary cancer hallmarks is the unrestrained proliferative capability of the tumour cells. Some studies have demonstrated that close contact between tumour and stromal cells are able to enhance the proliferation of different breast cancer cell lines (14)(15). Many tumours have better ability to survive chemo- and radio-therapies due to their overactivation of distinct cellular survival pathways. The adipose tissue is a major contributor to the tumour microenvironment and can confer a higher survival rate to the tumour cells (16).

Although early diagnosed breast cancers usually have a good clinical outcome, some breast cancers also have a high probability for progression to metastasis, which is associated with poor prognosis and the main cause of breast cancer death. For this reason, a better understanding of the molecular mechanisms behind breast cancer metastatic progression is essential for breast cancer diagnosis and prognosis, development of novel therapeutic strategies, and ultimately improvement of patient outcomes. Some studies have demonstrated that breast cancer cell lines culturing with mature adipocytes show an increased capability for migration and invasiveness (14)(16)(17), but the exact mechanisms underlying these processes are still uncleared. Previous studies have described how adipose tissue increases the levels of circulating cytokines, and adipokines releasement, such as leptin, adiponectin, vascular endothelial growth factor (VEGF), tumour necrosis factor (TNF-α) and fatty acid binding proteins (FABPs) also have a physiological role in enhancing breast cancer progression and metastasis.

FABPs are a family of 14-15 kDa proteins that have an important role in fatty acid internalization and intracellular transport, as well as the regulation of lipid trafficking and cellular metabolic response (18).

FABP4, is present mainly in mature adipocytes and macrophages. It has been associated with pathological conditions, such as obesity, type 2 diabetes, and atherosclerosis. In addition, a positive correlation has recently been established between FABP4 and poor prognosis in breast cancer (19). A recent study has also demonstrated that FABP4 gene deletion decreases tumour growth in mice models of prostate cancer and that pharmacological inhibition of FABP4 by BMS309403 also restricts prostate cancer invasiveness in an *in vitro* model (16). Therefore, FABP4 might provide the link that facilitates the crosstalk between the adipose tissue and the breast cancer cells. Meanwhile, our group have demonstrated that exogenous FABP4 (eFABP4) can activate the AKT and MAPK pathways to enhance the proliferation rate of some breast cancer cell lines through the Forkhead box protein FOXO3-FOXM1 axis, which plays an important role in

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cancer progression (20). In fact, some studies have correlated FOX family expression with specific breast cancer subtypes (21).
In the present study, we examined the potential role of adipose tissue in the breast cancer microenvironment by exploring whether adipocytes can enhance different cancer hallmarks involved in breast cancer development and progression using the breast cancer cell lines MDA-MB-231 and MCF-7. We also sought to understand the underlying mechanisms by which adipocytes enhance these cancer phenotypes in breast cancer cells, focusing on the role of the fatty-acid transporters FABP4 and FABP5.

2. Materials and Methods

2.1. Cell culture

Human MCF-7 and MDA-MB-231 breast cancer cells were originally provided by Dr. Eric Lam from the American Type Culture Collection (ATCC). Cell lines were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% penicillin streptomycin and 1% non-essential amino acids (Biowest, *Barcelona, Spain)* at 37°C with 5% CO_2. 3T3-L1 cells were obtained from the American Type Culture Collection (ATCC). For 3T3-L1 cells differentiation, cells were seeded onto 10mm plates. Once the cells achieved confluency, they were cultured for 10 days in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% Non-essential Amino Acid Solution, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1µM dexamethasone and 10µg/ml insulin from bovine pancreas (Sigma, Barcelona, Spain).
For adipocyte conditioned medium (CM), 3T3-L1 cells were cultured and differentiated for 10 days. Mature adipocytes were then incubated with serum-starvation medium for 24 hours, and supernatant was then cleared by centrifugation at 1000 x *g*. Then, CM was collected.

2.2. Western Blot analysis

MCF-7 and MDA-MB-231 cells were lysed in RIPA buffer [0.5mM Tris-HCl (pH 7.4), 150mM NaCl, 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholic acid and protease and phosphatase inhibitors (Roche)]. Protein concentration was measured using the Bradford assay. Fifteen µg of protein were loaded and separated on electrophoresis gels (NuPage 10% Bis-Tris Gels) (Life Technologies, ThermoFisher, Barcelona, Spain). Then proteins were transfer to a Polyvinylidene Fluoride (PVDF) membrane (Immobilon-P) (Life Technologies) using a semidry iBlot2 transfer device (Life Technologies). PVDF membranes were blocked using Milk-TBS-Tween (5% non-fat dry milk in TBS 0.05% Tween) for 1 hour at RT.
Primary antibodies used were P-AKT (Ser473) (Cell Signaling, Leiden, The Netherlands, #4060), AKT (Cell Signaling, #4691), P-p44/42 MAPK (Thr402) (Cell Signaling, #4370), p44/42 MAPK (Cell Signaling, #4695), P-FOXO1/3 T24/32(Cell Signaling, #9464), FOXO3 (Cell Signalling, #2497), FOXM1 (C-20) (Santa Cruz Biotechnology, Heidelberg Germany, sc-502), P-p38 (T180/I182) D3F9 (Cell Signaling, #052016), p38 MAPK (D13E1) XP (R) (Cell Signalling #8690), FABP4 (R&D Systems, Bio-Techne R&D Systems, Madrid, Spain, AF3150), FABP5 (R&D Systems, AF3077), CD36 (Abcam, Cambridge, UK, AB133625), GAPDH (Cell Signaling, #5174), Actin (Santa Cruz Biotechnology, sc-

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1616). Primary antibodies were added to the PVDF membranes and incubated overnight at 4°C. The membranes were then washed 3x in TBST and incubated with secondary antibodies (Polyclonal Goat anti-rabbit, Polyclonal Rabbit anti-Goat or Polyclonal Goat anti-Mouse) (1:10.000) conjugated with peroxidase (HRP) (Dako, Sant Just Desvern, Spain). Signals were detected using chemiluminescent reagents (ECL Millipore reagent) by an Amersham Imager600 (GE Healthcare, Madrid, Spain) and analysed using the ImageQuanTL software (GE Healthcare).

2.3. Proliferation assay

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

2.4. Wound healing assay

MCF-7 and MDA-MB-231 cells were seeded in 12 well plates until they reached confluency. Cells were then serum-starved for 24 hours, before a single scratch was performed in the centre of the plate. Supernatant cells were removed, and wells were washed with PBS once. Different conditioned media were added, and images were taken every 2 hours until the wounds were closed using an Olympus IX71 microscope (Barcelona, Spain) and analysed using image software Image J.

2.5. Invasion assay

The invasiveness capacity of tumour cells was measured using a Transwell system (8 µm polycarbonate membrane; Corning New York, NY).
Firstly, fibroblasts cell line 3T3-L1 was cultured into the bottom chamber until confluence. Next, they were differentiated into mature adipocytes as described above. Then they were serum deprived for the invasion assay.
On the other hand, upper chambers were coated with Matrigel (Corning) in DMEM High Glucose 0,1% BSA for 2 hours at 37ºC.
Tumour cells ($8x10^4$) were seeded above the Matrigel and incubated for 24 hours at 37ºC. Then, inserts were washed out with PBS and cells were stained with Violet Crystal.
Then, images were taken of those cells that had invade the insert and they were counted using Image J software.

2.6. Lipid uptake assay

MCF-7 and MDA-MB-231 were seeded in 12 well plates. After serum-starvation, cells were incubated with the lipophilic fluorescent dye Nile Red (100ng/ml) (Sigma-Aldrich; Barcelona,

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Spain) diluted in PBS 1X for 5 min at room temperature in order to visualize the lipid droplets. Cell images were captured using an Olympus IX71 microscope and analysed using the Image J software obtaining the intensity of each image.

2.7. BMS309403 treatment

The FABP4 inhibitor BMS309403 was purchased from Tocris Bioscience (Bristol, UK) and solubilized in DMSO. Mature adipocytes generate high levels of FABP4, which are released into the extracellular matrix. To block FABP4 and its possible role in BC cells, once 3T3-L1 fibroblasts were differentiated into mature adipocytes, a concentration of 10µM of BMS309403 was added and after 24 hours of treatment conditioned medium was obtained as Adipocyte CM with 10µM BMS309403. BC cells were then cultured with this media, and different assays were performed.

2.7. RNA extraction and quantitative real time (qRT) PCR

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RNA extraction was performed according to the PureLink RNA Mini Kit (Invitrogen, ThermoFisher Scientific, Barcelona, Spain) protocol and it was quantified using Synergy HT (BioTek, Swindon, UK). Total RNA (1 µg) was reverse-transcribed using the PrimeScript RT Reagent Kit (Takara Bio, Saint-Germain-en-Laye, France). Levels of mRNA were assessed using LightCycler96 device (Roche) with the Taqman probes for respective genes acquired from Life technologies. The probes used were CD36 (Hs01090850_ml), FABP4 (Hs00609791_ml), FABP5 (Hs02339439_g1), GAPDH (Hs99999905_m1), MMP9 (Hs00234579_m1).

3. Results

3.1. ***Adipocyte-conditioned medium activates the Akt and MAPK pathways and promotes breast cancer cell proliferation and survival.***

The serine/threonine-specific protein kinase Akt has a crucial role in integrating the upstream phosphoinositide 3-kinase (PI3-K) survival signals with downstream transcription factors, such as FOXO3, to promote cell proliferation and viability. We first investigated whether adipocyte conditioned media can modify the canonical proliferative and survival pathway mediated through Akt. To this end, the MCF-7 and MDA-MB-231 cell lines were cultured with mature adipocyte CM or control CM. Immunoblots showed that adipocyte conditioned medium significantly increased Akt and FOXO3 phosphorylation in both MCF-7 and MDA-MB231 cell lines at 5 and 15 min after treatment with CM, but not with control medium. ($p<0,05$) (Fig 1A). Consistent with this data, we also detected increased ERK1/2-MAPK phosphorylation in both breast cancer cell lines ($p<0,005$) and p38-MAPK phosphorylation in MCF-7 cells ($p<0,005$). This indicated activation of ERK1/2- and p38-MAPK.

To examine the proliferative behaviour of these cell lines, we performed a BrdU cell proliferation assay and the results showed that the adipocyte CM significantly enhanced the proliferation rates of both the MCF-7 and MDA-MB-231 breast cancer cell lines ($p<0,05$) (Fig 1B). We next performed colony formation assay in the presence of Doxorubicin to test the drug resistance and survival of MCF-7 and MDA-MB-231cells. These assays demonstrated that the adipocyte CM significantly increased the clonogenicity of both MCF-7 and MDA-MB-231 BCC lines ($p<0,05$) (Fig 1D).

3.2. ***Adipocyte conditioned media increases migration and invasion of MCF-7 and MDA-MB-231 breast cancer cells***

Cell migration plays a key role in metastasis, a crucial multi-step process involved in cancer cell malignant progression. To assess if the adipocytes also modulate the ability of breast cancer cells to migrate, we performed wound healing assay on MCF-7 and MDA-MB-231 cells in the presence of control and adipocyte CM. The results showed that the gap closure rates were significantly faster in both the MCF-7 and MDA-MB-231 cells when they were cultured in the presence of adipocyte CM compared to the control CM (p<0,05) (Fig 2A).
Interestingly, the adipocyte CM also caused an increase of matrix metallopeptidase 9 (MMP-9) mRNA in MCF-7 and MDA-MB-231 breast cancer cell lines (p<0,05) (Fig 2B), which encodes for a metalloproteinase involved in the degradation of the extracellular matrix. Consistent with this finding, we performed an invasiveness assay. We counted the number of cells that had invaded the Matrigel and that had arrived at the bottom part of the insert. We observed that tumour cells were able to invade better once they were coculture with mature adipocytes than when they were culture only with control CM (p<0,05) (Fig 2C).

3.3. ***Adipocyte conditioned media increases lipid uptake and fatty acid transporter expression in MCF-7 and MDA-MB-231 cells***

Lipids released by adipocytes are taken up by cancer cells for their energy metabolism, membrane biogenesis as well as intracellular signalling. We next analysed if the adipocyte CM promotes lipid uptake in the breast cancer cells. To this end, we incubated the MCF-7 and MDA-MB-231 cells with the control or the adipocyte CM and studied their lipid uptake in forms of lipid droplets accumulation. The results showed that there was a significant increase in the amount of lipid droplets as revealed by Nile Red staining in both the MCF-7 and MDA-MB-231 cells following incubation with adipocyte CM when compared with control CM (p<0,05) (Fig 3A). This suggested that breast cancer cells take up the lipids released by the adipocytes.
The cellular uptake and transport of lipids are facilitated by fatty acid transporters, including FABP4, FABP5 and CD36. To investigate the mechanism by which adipocytes promote cell proliferation, survival, and migration, we also examined whether adipocyte CM modifies the expression of fatty acid transporters FABP4, FABP5 and CD36 to enhance lipid uptake and transport in the MDA-MB-231 and MCF-7 cell lines. RT-qPCR analysis showed that adipocyte CM significantly increased the expression of FABP5 and CD36 mRNA in both cell lines and FABP4 in MDA-MB-231 cell line when compared to control CM (p<0.05) (Fig 3B). MDA-MB-231 have very low expression of FABP4, whiles FABP4 has not expression of these transcript. Although,we observed high expression of these trancript in both cell lines after their culture with adipocyte CM, it was impossible to obtaain the fo9ld change in the Luminal A cell line MCF-7.
Consistent with this, Western blot analysis demonstrated that the FABP4 and FABP5 and CD36 protein levels were significantly induced in both breast cancer cell lines following incubation with adipocyte CM (p<0,05) (Fig 3C).

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3.4. ***FABP4 inhibition decreases proliferation mediated by adipocyte conditioned media in MCF-7 and MDA-M-231 breast cancer cell lines***

We have observed that adipocyte CM enhances breast cancer cell proliferation and migration, which are accompanied by the accumulation of lipid droplets and the induction of FABP4, FABP5 and CD36 expression. As FABP4 and FABP5 expression were the fatty acid transporters significantly induced both BCC lines following incubation with adipocyte CM, we decided to inhibit FABP4 with the well-established selective inhibitor BMS309403 to assess its role in breast cancer progression. When we incubated both cell lines with the adipocyte CM in the absence or presence of BMS309403, we observed that BMS309403 addition significantly decreased the phosphorylation status of AKT ($p<0,05$) and slightly the phosphorylation grade of FOXO3 (Fig 4A). Consequently, we also observed a significant reduction in cell proliferation in both the MCF-7 and MDA-MB-231 cell lines incubated with adipocyte-conditioned medium in the presence of BMS309403 compared to those without BMS309403 ($p<0,05$) (Fig 4B). This suggests that FABP4 has a key role in promoting breast cancer cell proliferation mediated by adipocyte conditional medium. In addition, we performed a survival assay using Doxorubicin as mentioned above and we observed that the inhibition of FABP4 in mature adipocytes and the subsequent culture of BCC lines decreases the clonogenicity of both cell lines, suggesting a plausible role of FABP4 in BCC lines MCF-7 and MDA-MB-231 chemo-resistance ($p<0,05$) (Fig 4C).

3.5. ***FABP4 inhibitor restricts cell migration and invasion mediated by adipocyte conditioned medium in MCF-7 and MDA-MB-231 breast cancer cell lines***

We next explored FABP4 role in breast cancer cell migration promoted by the adipocyte CM. To this end, we performed the wound healing scratch migration assays on both cell lines incubated with adipocyte CM in the absence of presence of BMS309403. Pertinently, the wound healing assays showed that this induction in cell migration by adipocyte CM was significantly limited by BMS309403 in both breast cancer cell lines ($p<0,05$) (Fig 5A), suggesting that FABP4 has a role in the induction of breast cancer cell migration mediated by adipocyte CM. In agreement, the induction of MMP9 expression by adipocyte CM was significantly reduced by BMS309403 in MDA-MB-231cell line ($p<0,05$) and there was a decrease tend in MCF-7 cell line MMP9 mRNA expression (Fig 5B).
Then, we tested the role of FABP4 in the invasiveness of these cell lines. We added the FABP4 inhibitor BMS309403 at a concentration of 10μM onto differentiated adipocytes. Then, we performed the invasion assay following the methodology previously described. Interestingly, we could observe that both cell lines, once they were cultured with adipocytes that previously had been treated with BMS309403, significantly reduced the invasiveness ability. In fact, the number of tumour cells that had transferred the insert was notably lower in this condition once we compared with the coculture condition ($p<0,05$) (Fig 5C).

3.6. ***FABP4 inhibition decreases lipid uptake mediated by adipocyte conditioned media in MCF-7 and MDA-MB-231 breast cancer cell lines***

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Lipid metabolism reprogramming has been shown to modulate breast cancer cell proliferation and migration. As the induction of cell proliferation and migration in both MCF-7 and MDA-MB-231 cells by adipocyte CM could be abrogated by BMS309403, we predicted that FABP4 might have a role in facilitating lipid uptake in the breast cancer cells. To test this idea, we incubated both cell lines with adipocyte CM in the presence or absence of BMS309403 and studied their lipid uptake by Nile red staining. Interestingly, when both breast cancer cell lines were incubated with the adipocyte CM from mature adipocytes previously incubated with BMS309403, we observed that the induction of lipid uptake was reduced ($p<0,05$) (Fig 6A), suggesting FABP4 plays a key role in lipid uptake by the breast cancer cells. To explore further the mechanism by which FABP4 inhibition affects lipid uptake by the breast cancer cells, we investigated the expression of fatty acid transporters FABP4, FAPB5 and CD36 in the BCC lines after incubation with CM from adipocytes previously treated with BMS309403. The results showed that there were significant changes in FABP4 at the RNA level in MDA-MB-231 cell line between incubation with CM from adipocytes with and without previous BMS309403 treatment ($p<0,05$). FABP5 mRNA levels were lower in MDA-MB-231 and MCF-7 cells incubated with adipocyte + BMS309403 CM when compared to untreated adipocyte CM, although these results were not significant. The expression levels of CD36 mRNA were significantly reduced in MDA-MB-231 cell line ($p<0,05$) and we could observe a slight but not significant reduction of CD36 expression in MCF-7 cells incubated with adipocyte + BMS309403 CM when compared to untreated adipocyte CM (Fig 6B). Consequently, we analysed the immunoblotting of these proteins, and we could observe that there was a slight reduction of the fatty acid transporters FABP4, FABP5 and CD36 in both cell lines incubated with CM from adipocytes previously treated with BMS309403 when compared to untreated adipocyte. However only FABP5 reduction in MCF-7 cell line and CD36 diminution in MDA-MB-231 cell lines were significant ($p<0,05$) (Fig 6C).

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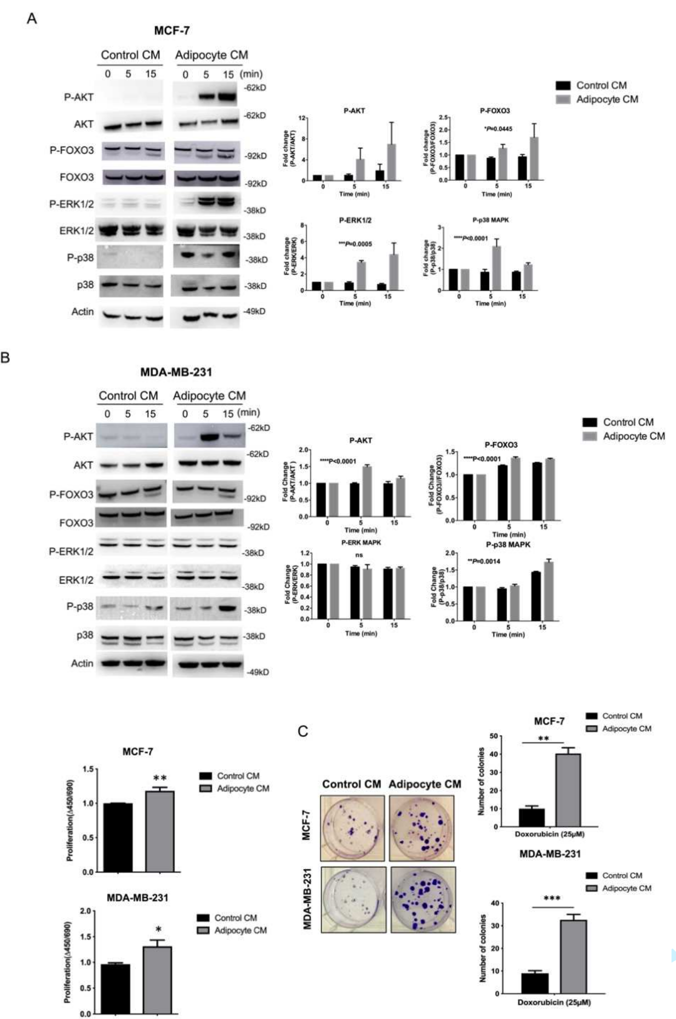

Fig 1. ***Adipocyte CM enhances proliferation and chemoresistance in MCF-7 and MDA-MB-231 BCC lines.*** *(A) Immunoblots and graph representation for, AKT, FOXO3, P38, ERK, and their phosphorylated forms of BCC lines MCF-7 and MDA-MB-231 at short times (0, 5 and 15 minutes) cultured with control CM or adipocyte CM. There is a significant increase of the phosphorylation forms of AKT and FOXO3 in both cell lines after the treatment with adipocyte CM. In addition, the activation of the P38 pathway is present in both cell lines whereas the phosphorylation of ERK is significantly increased in the luminal MCF-7 cell line. (B) Graph illustration of BCC lines MCF-7 and MDA-MB 231 proliferation rate, represented by the BrdU uptake (Δ450/690 nm) in different conditions (control CM and adipocyte CM) where a significant increase in BrdU uptake is observed in both cell lines after their culture with adipocyte CM. (C) Images and graph representation of survival assay where a concentration of 25 nM of Doxorubicin were used after the treatment with control CM and adipocyte CM. Cells cultured with adipocyte conditioned media were able to form a high number of colonies. One-way ANOVA where a p<0,05 was considered statistically significant.*

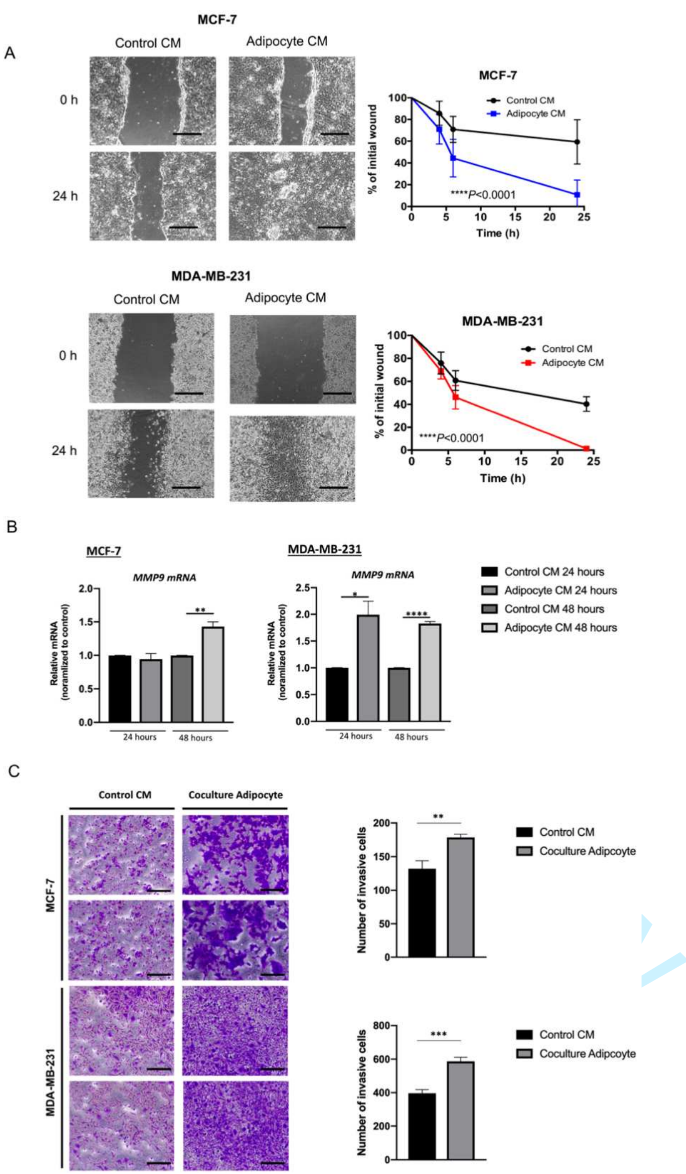

Fig 2. ***Adipocyte CM increases MCF-7 and MDA-MB-231 BCC lines migration and invasiveness.*** *(A) Images (4x magnification) and graph representation of a wound healing in different conditions (control CM and adipocyte CM) of BCC lines MCF-7 and MDA-MB-231 where wound was healed faster with adipocyte CM in both cell lines. (B) MMP9 mRNA levels of both BBC cell lines after 24 and 48 hours of treatment with control CM or adipocyte CM. It is found a significant increase in the transcript levels of this gene in both cell lines after adipocyte CM treatment. (C) Images (4x magnification) and graph representation of invasion assay after the culture of both BBC lines with control CM or mature adipocytes. Violet crystal stains those BCC that have invade and overpassed the Matrigel, therefore the invasive cells. It is observed a significant increase of BC invasive cells after the coculture with mature adipocytes. One-way ANOVA where a p<0,05 was considered statistically significant.*

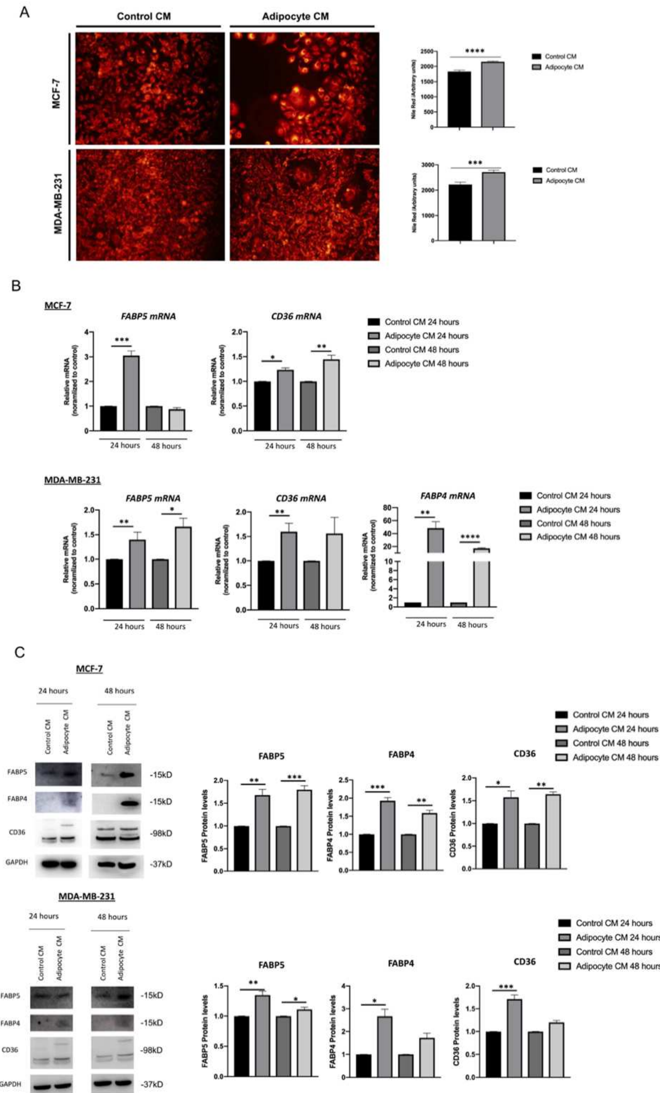

Fig 3. ***Adipocyte CM increases MCF-7 and MDA-MB-231 BCC lines lipid uptake and FABP4, FABP5 and CD36 levels***. *(A) Immunofluorescence images (10X magnification) of MCF-7 and MDA-MB-231 cell lines stained for fatty acids using Nile Red stain. Fatty acid uptake was increased once cells were cultured with adipocyte CM. Graphs represent Nile Red intensity, which could be translated to the amount of fatty acids taken up. (B) Graph representation of FABP4, FABP5 and CD36 transcripts in control CM and adipocyte CM at 24 and 48 hours, where it is observed a significant increase in all three transcripts after the treatment with adipocyte CM in the TNBC line MDA-MB-231 and an increase in FABP5 and CD36 transcripts in the luminal-A cell line MCF-7. (C) Immunoblots and graph representation of FABP5, FABP4 and CD36 protein levels in both BCC lines at 24 and 48 hours in the conditions described before where there is an increase in the cytoplasm levels of all three proteins in both cell lines after the addition of adipocyte CM, compared to control CM. One-way ANOVA where a p<0,05 was considered statistically significant.*

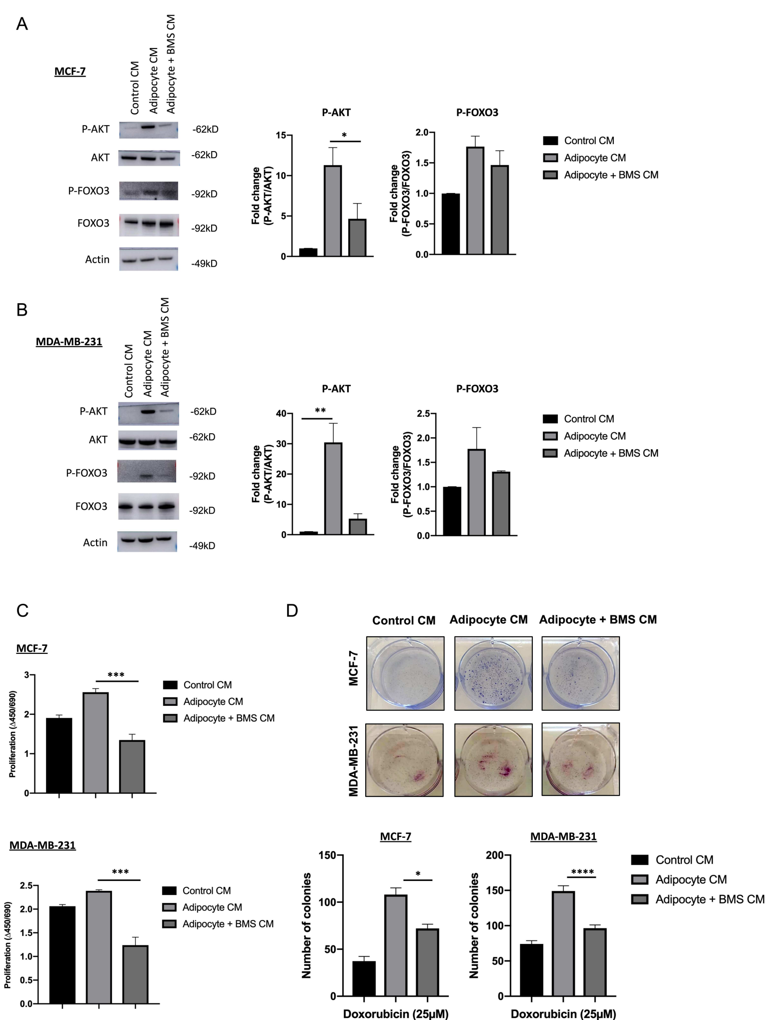

*Fig 4. **FABP4 inhibition reduces MCF-7 and MDA-MB-231 BCC lines proliferation and chemoresistance**. (A) Immunoblot and graph representation for, AKT, FOXO3, and their phosphorylated forms of BCC lines MCF-7 and MDA-MB-231 at 24 hours after their culture with control CM, Adipocyte CM and Adipocyte + BMS309403 CM. It is observed a significant decrease in the phosphorylation status of AKT protein in both cell lines and a slight decrease in the phosphorylation rate of FOXO3 although it is not significant. (B) Graph illustration of BCC lines MCF-7 and MDA-MB 231 proliferation rate, represented by the BrdU uptake (Δ450/690 nm) in the conditions mentioned before. There is a significant decrease in the proliferation rate of both cell lines after their incubation with the CM derived from mature adipocyte that previously had been treated with 10 µM of BMS309403. (C) Images and graph representation of survival assay where a concentration of 25 nM of Doxorubicin were used after the treatment with control CM, adipocyte CM and adipocyte + BMS309403 CM. There is a significant reduction in the clonogenicity of both BCC lines once they were cultured with adipocyte + BMS309403 CM compared to these cells cultured with adipocyte CM. One-way ANOVA where a p<0,05 was considered statistically significant.*

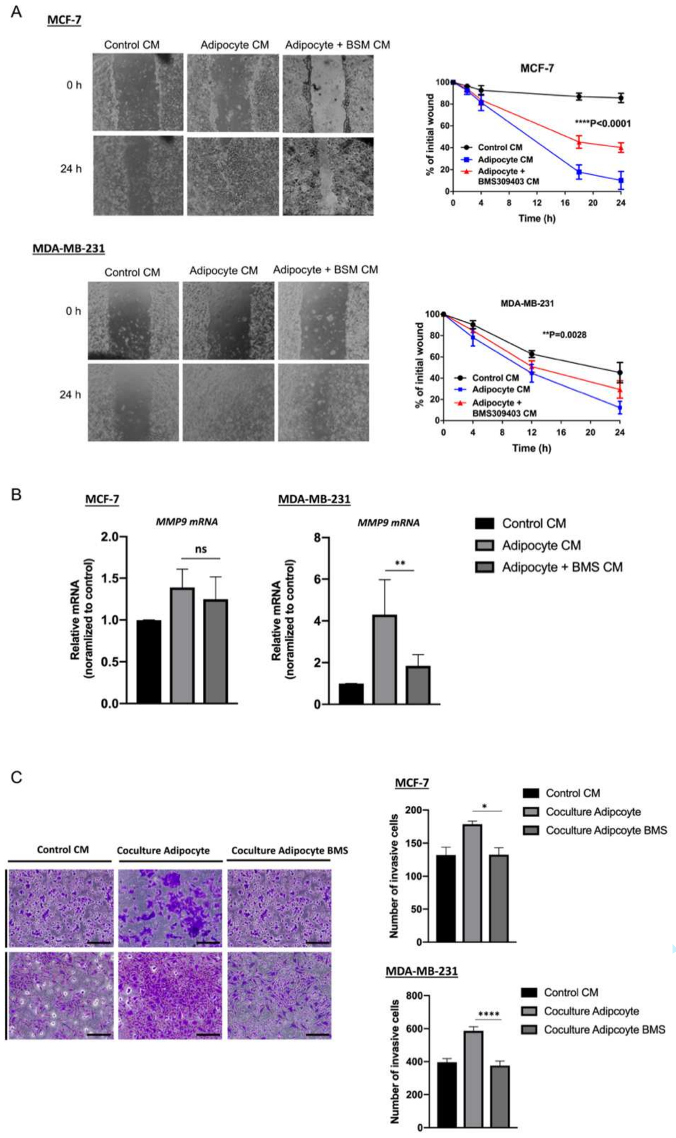

Fig 5. ***FABP4 inhibition decreases MCF-7 and MDA-MB-231 BCC lines migration and invasiveness.*** *(A) Images (4x magnification) and graph representation of a wound healing in different conditions (control CM, adipocyte CM and adipocyte + BMS309403 CM) where the addition of BMS309403 significantly decreases the wound healing of both BCC lines compared to their heling rate after the treatment with adipocyte CM. (B) MMP9 mRNA levels of both BBC lines in the conditions mentioned before at 24 hours of treatment. There is a significant decrease in this transcript after BMS309403 addition in the MDA-MB-231 cell line. (C) Images (4x magnification) and graph representation of invasion assay after the culture of both BBC lines with control CM, mature adipocytes or mature adipocytes previously treated with BMS309403. There is a decrease in the number of invasive stained cells in both cell lines after the BMS309403 addition compared to the number of invasive cells once BCCs were cultured with mature adipocytes without the FABP4 inhibitor. One-way ANOVA where a p<0,05 was considered statistically significant.*

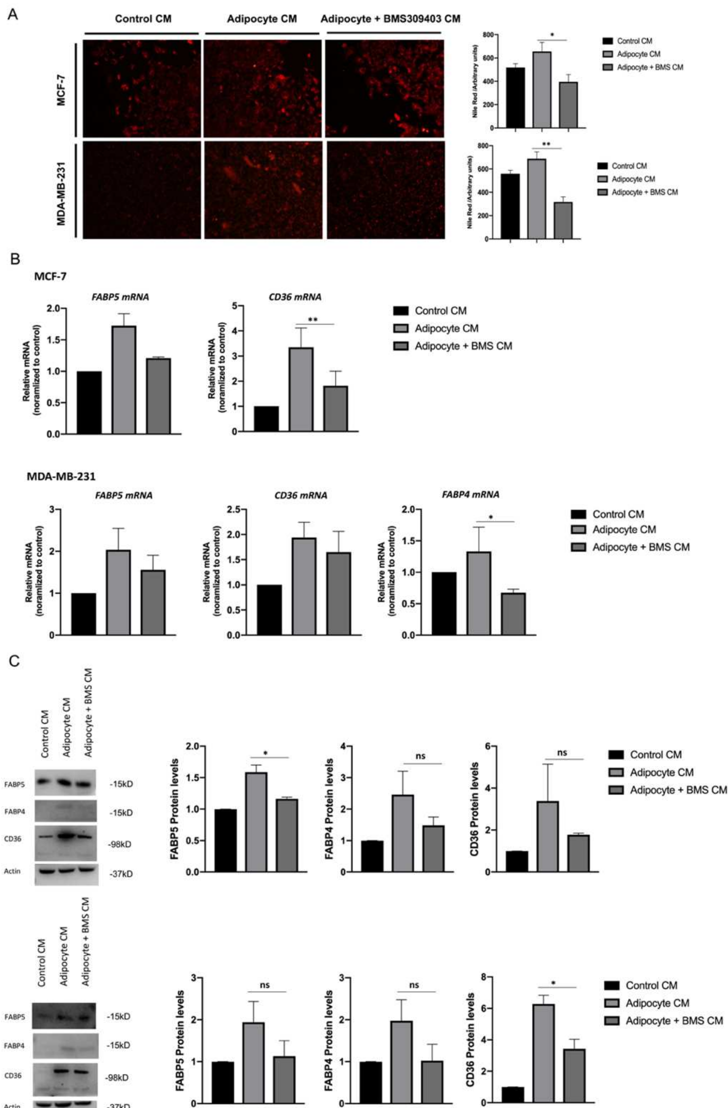

*Fig 6. . **FABP4 inhibition decreases MCF-7 and MDA-MB-231 BCC lines lipid uptake and FABP4, FABP5 and CD36 levels.** (A) Immunofluorescence images (10X magnification) of MCF-7 and MDA-MB-231 cell lines stained for fatty acids using Nile Red stain at 24 hours in different conditions (control CM, adipocyte CM and adipocyte + BMS309403 CM). It is observed a decrease in the lipid staining after the culture of both BCC lines with the adipocyte + BMS309403 CM compared to the adipocyte CM condition. (B) Graph representation of FABP4 FABP5 and CD36 transcripts in the conditions previously described at 24 hours, where it is observed a slight, although not significant decrease in all three transcripts after the treatment with adipocyte + BMS309403 CM. (C) Immunoblots and graph representation of FABP5, FABP4 and CD36 protein levels in both BCC lines at 24 in these conditions described. There is a slight decrease in all the three fatty acid transporters in both BCC lines although only FABP5 and CD36 were significant in MCF-7 and MDA-MB-231 respectively. One-way ANOVA where a p<0,05 was considered statistically significant.*

4. Discussion

The role of adipose tissue in the tumour microenvironment has recently been object of study, and different research stablish a close relationship between breast cancer progression and adipose tissue. Although many theories have been discussed, is not clear yet how adipose tissue could promote breast cancer.

In this study we demonstrated firstly how mature adipocytes can enhance several cancer hallmarks of the breast cancer cell lines MCF7 and MDA-MB 231.

The ability of tumour cells to survive in adverse situations is one of the most important aims in cancer progression. Drug resistance is a decisive factor in cancer treatment, and tumour cells use new tools to avoid apoptosis and cell death. Tumour microenvironment plays a crucial role in tumour survival, leading to activate different pathways to inhibit cell apoptosis and avoid drug effects (11). Akt signalling is the key protein in cell survival and its activation leads to the activation the intrinsic cellular mechanisms to subsist. FOX axis is in the second line of Akt activation and the phosphorylation of FOXO3 induces a signalling pathway that finally will promote cell survival and progression.

In this study we focus on this pathway, and we tested how adipocyte CM might activate it. Once we treated MCF-7 and MDA-MB -231 cell lines with adipocyte CM we observed that both cell lines suffer a significative phosphorylation at short times of both proteins, that was translated to an increase of their survival rates after doxorubicin treatment. Both cell lines were able to generate a higher number of colonies in the presence of doxorubicin when they were cultured with adipocyte CM. According to these results, adipocyte CM is able to activate the axis Akt-FOX pathway leading to a higher drug resistance and better survival in the studied BCC lines.

One of the main characteristics of tumours is the ability to grow without an exogenous stimulus. The overactivation of key regulator proteins in the proliferation pathway is crucial for tumour progression and proliferation enhancement.

Adipocytes have been stablished as tumour proliferation enhancers in different studies (22). In this study we analysed the effects in different proteins of the MAPK pathway. We have determined that adipocyte conditioned media is able to over-activate the phosphorylation of key proteins in the proliferation pathway, MAPK P44/42, and MAPK P38. Adipocyte CM, because of these proteins phosphorylation might impact on the proliferation rate of BC. Accordingly, once we cultured these cell lines with adipocyte CM, their proliferation rate increased significantly.

Tumour cell migration ability and invasiveness are two essential mechanisms for cancer to progress in the organism. Cancer must invade the surrounding tissues as well as attract the blood vessels, so that tumour cells can move on the blood stream and disseminate in the organism, invading and colonizing new organs as new metastatic niches (23).

Tumour migratory ability can be supplied for the stroma. In fact, some studies affirm that BC cells increase their migration rate when they are in close contact with other stroma cell types such as fibroblasts or adipocytes (24)(25)(26). We have observed that adipocyte CM enhance the migration rate of the BC cell lines MCF7 and MDA-MB 231, suggesting that certain molecule or a

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mixture of different factors included within the adipocyte CM improves their migratory capability.

Indeed, tumour invasiveness capability is essential for the metastasis formation. Tumour cells modifies the stroma within them, by certain enzymes releasement, such as metalloproteases and collagenases. One of the main stroma modulator enzymes is MMP9. This metalloprotease has been linked with an increase of metastasis formation *in vitro and in vivo* (27),(28). We have seen that adipocyte CM increased the transcription of this gene in both BCC, suggesting that adipose tissue may enhance BC metastasis, hence, being probably associated with poorest prognosis in BC. Moreover, once we performed the invasion assay using the coculture system we could observe that mature adipocytes were able to enhance the invasiveness ability of both BCC lines. These results confirm the idea that adipose tissue increases the ability of BC to invade the surrounding tissues and metastasize.

Different studies have stablished that tumour cells need a continuous supply of energy to proliferate and progress. Tumours are glucose-dependent (29),(30) and recent studies have suggested a special importance in the lipid metabolism for tumour progression (31) .

Several studies have demonstrated that tumour microenvironment acts as an energy supplier in the cancer progression (32),(33),(34). As adipose tissue is the most abundant tissue in breast, it is reasonable to think that adipocytes might be the major energy suppliers for breast tumour progression. In this study, as well as in other recent reviews, we have observed that the culture of MCF7 and MDA-MB-231 cell lines with adipocyte CM enhances the uptake of lipids from adipocytes. These lipids are taken by the tumour cells, and they are essential for their progression (13).

First, we observed that BC cell lines increased the lipid uptake when they were cultured with adipocyte CM. The amount of lipid droplets was increased once we tested by microscopy. Moreover, Nile Red analysis also confirmed that there was a higher amount of lipid droplet in those BCC cultured with adipocyte CM.

Then, we supposed that this accumulation of lipids from media might be accompanied with an increase of the classic fatty acid transporters.

FABP4, FABP5 and CD36 have been correlated with poor prognosis in BC patients (35),(36). We analysed by qPCR and by Western Blot how adipocyte CM might modify these fatty acid transporter mRNA and protein levels. Interestingly, we observed that there was an increase in all three fatty acid transporters at transcriptional and protein levels. According to all these data, these fatty acid transporters, specially FAPB4, which was the most upregulated in BC cell after adipocyte CM treatment, might play an important role in BC progression.

In consequence, the inhibition of this fatty acid transporter was one of the main objectives of this study to understand the role in the enhancement of different cancer hallmarks. BMS309403 is a highly selective inhibitor of FABP4, previously tested in different diseases such as type 2 diabetes or atherosclerosis (37). Several *in vitro* and *in vivo* studies have stablished that inhibition of FABP4improves the inflammatory status and significantly reduces atherosclerotic plaques (38).

Accordingly, the inhibition of FAPB4 in the tumour microenvironment might influence in the breast cancer cells behaviour, hence their cancer hallmarks.

Treatment with FAPB4 inhibitor BMS309403 in adipocytes was performed and the conditioned media was collected and used in different assays with both BC cell lines.

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Firstly, we could observe that conditioned media from adipocytes treated with BMS309403 reverts the proliferation rate of BC cell lines MCF7 and MDA-MB 231 that adipocyte CM by itself had enhanced. Hence, the inhibition of FABP4 in the tumour microenvironment is able to block partially the communication between BC cells and adipocytes, triggering in a significative reduction in BC cells proliferation.

Indeed, analysing the migratory effects of FABP4 inhibition, we also could observe that this cancer hallmark seems to be reduced. While MCF7 and MDA-MB 231 cell lines had suffered an enhancement in their migratory capabilities with adipocyte CM, they took longer to close the wound after FABP4 inhibition, hence, their migratory ability had been reduced partially.

The invasiveness competence of tumour cells depends on the ability to degrade the extracellular matrix where they are embedded. Metalloproteases are crucial for this objective and, as we mentioned before, *MMP9* transcript is enhanced by the culture of BC cell lines MCF7 and MDA-MB 231 with adipocyte CM. Inhibition of FABP4 in mature adipocyte reduces the transcript of this metalloprotease in MDA-MB-231 cell line. Furthermore, the inhibition of this fatty acid transporter also reduces notably the invasiveness ability of both BC cell lines suggesting FABP4 might play an important role in BC metastasis process.

We hypothesize that fatty acid transfer between adipocytes and BC cells are the main reason why tumour cells enhance their cancer hallmarks. As we have seen, there is an increase in the transcripts and protein levels of different fatty acid transporters, FABP4, FABP5 and CD36. Indeed, it is followed by an increase in the lipid content in both tumour cell lines. The inhibition of FABP4 in mature adipocytes, might reduce the transfer of fatty acids to the tumour cells. Actually, we observed that when we cultured BC cell lines with adipocyte CM previously treated with BMS309403 the transcripts and protein levels of FABP4, FABP5 and CD36 were slightly reduced in both cell lines. Theses fatty acid transporters have been linked strongly with poor prognosis in BC patients (39) and it is highly associated with different cancer hallmark activation (36)(40)(41)(19).

The reduction of these fatty acid transporters was also accompanied by a decrease of the lipid accumulation in both BC cell lines after treatment with CM derived from mature adipocytes previously cultured with BMS309403.

In conclusion, FABP4 inhibition in mature adipocytes blocked partially the communication between BC cells and the adipose tissue, leading to a significative reduction in the lipid uptake from media. This decrease is accompanied by a reduction in the proliferation rate of both BC cell lines as well as by a reduction in the migratory capability of tumour cell lines MCF7 and MDA-MB 231. Therefore, mature adipocytes FABP4 inhibition is followed by a reduction of certain metalloproteases such as MMP9 at the transcriptomic level in the tumour cells

According to these results, we have demonstrated that adipose tissue enhances BC hallmarks as well as in BC progression by the releasement of different soluble molecules to the media, including FABP4. In fact, by the inhibition of this protein we have observed its crucial role in MCF-7 and MDA-MB-231 cell lines hallmarks enhancement. Consequently, FABP4 inhibition in mature adipocytes by the addition of BMS3094 might be plausible strategy for the inhibition of the communication and the interaction between BC cells and adipose tissue, helping in the treatment of BC.

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1. Harbeck N, Penault-Llorca F, Cortes J, Gnant M, Houssami N, Poortmans P, et al. Breast

cancer. Nat Rev Dis Prim [Internet]. 2019 Dec 23 [cited 2021 Apr 13];5(1):66. Available from: http://www.nature.com/articles/s41572-019-0111-2

2. Kerr J, Anderson C, Lippman SM. Physical activity, sedentary behaviour, diet, and cancer: an update and emerging new evidence [Internet]. Vol. 18, The Lancet Oncology. Lancet Publishing Group; 2017 [cited 2021 Apr 13]. p. e457–71. Available from: https://pubmed.ncbi.nlm.nih.gov/28759385/
3. Hoyo C, Cook MB, Kamangar F, Freedman ND, Whiteman DC, Bernstein L, et al. Body mass index in relation to oesophageal and oesophagogastric junction adenocarcinomas: A pooled analysis from the international BEACON consortium. Int J Epidemiol [Internet]. 2012 [cited 2021 Apr 13];41(6):1706–18. Available from: /pmc/articles/PMC3535758/
4. Deng T, Lyon CJ, Bergin S, Caligiuri MA, Hsueh WA. Obesity, Inflammation, and Cancer. Annu Rev Pathol Mech Dis [Internet]. 2016 May 23 [cited 2021 Apr 13];11(1):421–49. Available from: http://www.annualreviews.org/doi/10.1146/annurev-pathol-012615-044359
5. Renehan AG, Tyson M, Egger M, Heller RF, Zwahlen M. Body-mass index and incidence of cancer: a systematic review and meta-analysis of prospective observational studies. Lancet [Internet]. 2008 [cited 2021 Apr 13];371(9612):569–78. Available from: https://pubmed.ncbi.nlm.nih.gov/18280327/
6. Booth A, Magnuson A, Fouts J, Foster M. Adipose tissue, obesity and adipokines: Role in cancer promotion. Horm Mol Biol Clin Investig [Internet]. 2015 Jan 1 [cited 2021 Apr 13];21(1):57–74. Available from: https://pubmed.ncbi.nlm.nih.gov/25781552/
7. Chan DSM, Vieira AR, Aune D, Bandera E V., Greenwood DC, McTiernan A, et al. Body mass index and survival in women with breast cancer—systematic literature review and meta-analysis of 82 follow-up studies [Internet]. Vol. 25, Annals of Oncology. Elsevier Ltd; 2014 [cited 2021 Apr 13]. p. 1901–14. Available from: https://pubmed.ncbi.nlm.nih.gov/24769692/
8. Hotamisligil GS. Inflammation, metaflammation and immunometabolic disorders [Internet]. Vol. 542, Nature. Nature Publishing Group; 2017 [cited 2021 Apr 13]. p. 177–85. Available from: https://pubmed.ncbi.nlm.nih.gov/28179656/
9. Coelho M, Oliveira T, Fernandes R. Biochemistry of adipose tissue: An endocrine organ [Internet]. Vol. 9, Archives of Medical Science. Arch Med Sci; 2013 [cited 2021 Apr 13]. p. 191–200. Available from: https://pubmed.ncbi.nlm.nih.gov/23671428/
10. Choi J, Cha YJ, Koo JS. Adipocyte biology in breast cancer: From silent bystander to active facilitator [Internet]. Vol. 69, Progress in Lipid Research. Elsevier Ltd; 2018 [cited 2021 Apr 13]. p. 11–20. Available from: https://pubmed.ncbi.nlm.nih.gov/29175445/
11. Krishna Muppalla JN, Muddana K, Reddy Dorankula SP, Rao Thokala M, Prakash Pasupula A. Microenvironment-A role in tumour progression and prognosis [Internet]. Vol. 7, Journal of Clinical and Diagnostic Research. J Clin Diagn Res; 2013 [cited 2021 Apr 13]. p. 2096–9. Available from: https://pubmed.ncbi.nlm.nih.gov/24179956/
12. Nieman KM, Kenny HA, Penicka C V., Ladanyi A, Buell-Gutbrod R, Zillhardt MR, et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. Nat Med [Internet]. 2011 Nov [cited 2021 Apr 13];17(11):1498–503. Available from: https://pubmed.ncbi.nlm.nih.gov/22037646/
13. Nieman KM, Romero IL, Van Houten B, Lengyel E. Adipose tissue and adipocytes support

FOR REVIEW

tumorigenesis and metastasis [Internet]. Vol. 1831, Biochimica et Biophysica Acta - Molecular and Cell Biology of Lipids. Elsevier B.V.; 2013 [cited 2021 Apr 13]. p. 1533–41. Available from: /pmc/articles/PMC3742583/

14. Lee YK, Jung WH, Koo JS. Adipocytes can induce epithelial-mesenchymal transition in breast cancer cells. Breast Cancer Res Treat [Internet]. 2015 Sep 7 [cited 2021 Apr 13];153(2):323–35. Available from: https://pubmed.ncbi.nlm.nih.gov/26285644/
15. Orecchioni M, Ghosheh Y, Pramod AB, Ley K. Macrophage polarization: Different gene signatures in M1(Lps+) vs. Classically and M2(LPS-) vs. Alternatively activated macrophages [Internet]. Vol. 10, Frontiers in Immunology. J Pathol; 2019 [cited 2021 Apr 13]. p. 221–8. Available from: https://pubmed.ncbi.nlm.nih.gov/14517839/
16. Herroon MK, Rajagurubandara E, Hardaway AL, Powell K, Turchick A, Feldmann D, et al. Bone marrow adipocytes promote tumor growth in bone via FABP4-dependent mechanisms. Oncotarget [Internet]. 2013 [cited 2021 Apr 13];4(11):2108–23. Available from: https://pubmed.ncbi.nlm.nih.gov/24240026/
17. Dirat B, Bochet L, Dabek M, Daviaud D, Dauvillier S, Majed B, et al. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. Cancer Res [Internet]. 2011 Apr 1 [cited 2021 Apr 13];71(7):2455–65. Available from: https://pubmed.ncbi.nlm.nih.gov/21459803/
18. Furuhashi M, Hotamisligil GS. Fatty acid-binding proteins: Role in metabolic diseases and potential as drug targets [Internet]. Vol. 7, Nature Reviews Drug Discovery. Nat Rev Drug Discov; 2008 [cited 2021 Apr 13]. p. 489–503. Available from: https://pubmed.ncbi.nlm.nih.gov/18511927/
19. Harjes U, Bridges E, McIntyre A, Fielding BA, Harris AL. Fatty acid-binding protein 4, a point of convergence for angiogenic and metabolic signaling pathways in endothelial cells. J Biol Chem [Internet]. 2014 Nov [cited 2021 Apr 13];289(33):23168–76. Available from: https://pubmed.ncbi.nlm.nih.gov/28701570/
20. Guaita-Esteruelas S, Bosquet A, Saavedra P, Gumà J, Girona J, Lam EWF, et al. Exogenous FABP4 increases breast cancer cell proliferation and activates the expression of fatty acid transport proteins. Mol Carcinog. 2017;56(1):208–17.
21. Lee JJ, Lee HJ, Son BH, Kim SB, Ahn JH, Ahn S Do, et al. Expression of FOXM1 and related proteins in breast cancer molecular subtypes. Int J Exp Pathol [Internet]. 2016 Apr 1 [cited 2021 Apr 13];97(2):170–7. Available from: https://pubmed.ncbi.nlm.nih.gov/27277416/
22. Manabe Y, Toda S, Miyazaki K, Sugihara H. Mature adipocytes, but not preadipocytes, promote the growth of breast carcinoma cells in collagen gel matrix culture through cancer-stromal cell interactions. J Pathol [Internet]. 2003 Oct 1 [cited 2021 Jun 30];201(2):221–8. Available from: https://pubmed.ncbi.nlm.nih.gov/14517839/
23. Seyfried TN, Huysentruyt LC. On the origin of cancer metastasis. Crit Rev Oncog [Internet]. 2013 [cited 2021 Jun 30];18(1–2):43–73. Available from: /pmc/articles/PMC3597235/
24. Dittmer A, Dittmer J. Carcinoma-associated fibroblasts promote growth of sox2-expressing breast cancer cells. Cancers (Basel) [Internet]. 2020 Nov 19 [cited 2021 Jun 3];12(11):1–22. Available from: http://www.ncbi.nlm.nih.gov/pubmed/33228022
25. Yu Z, Lou L, Zhao Y. Fibroblast growth factor 18 promotes the growth, migration and

FOR REVIEW

invasion of MDA-MB-231 cells. Oncol Rep [Internet]. 2018 Aug 1 [cited 2021 Jun 30];40(2):704–14. Available from: /pmc/articles/PMC6072296/

26. Bochet L, Lehuédé C, Dauvillier S, Wang YY, Dirat B, Laurent V, et al. Adipocyte-derived fibroblasts promote tumor progression and contribute to the desmoplastic reaction in breast cancer. Cancer Res [Internet]. 2013 Sep 15 [cited 2021 Jun 16];73(18):5657–68. Available from: https://pubmed.ncbi.nlm.nih.gov/23903958/
27. Mehner C, Hockla A, Miller E, Ran S, Radisky DC, Radisky ES. Tumor cell-produced matrix metalloproteinase 9 (MMP-9) drives malignant progression and metastasis of basal-like triple negative breast cancer. Oncotarget. 2014;5(9):2736–49.
28. Joseph C, Alsaleem M, Orah N, Narasimha PL, Miligy IM, Kurozumi S, et al. Elevated MMP9 expression in breast cancer is a predictor of shorter patient survival. Breast Cancer Res Treat [Internet]. 2020 Jul 1 [cited 2021 Jun 30];182(2):267–82. Available from: https://doi.org/10.1007/s10549-020-05670-x
29. Warburg O, Wind F, Negelein E. The metabolism of tumors in the body. J Gen Physiol [Internet]. 1927 Mar 7 [cited 2021 May 20];8(6):519–30. Available from: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC2140820/
30. Hay N. Reprogramming glucose metabolism in cancer: Can it be exploited for cancer therapy? [Internet]. Vol. 16, Nature Reviews Cancer. Nature Publishing Group; 2016 [cited 2021 May 25]. p. 635–49. Available from: /pmc/articles/PMC5516800/
31. Beloribi-Djefaflia S, Vasseur S, Guillaumond F. Lipid metabolic reprogramming in cancer cells. Oncogenesis [Internet]. 2016 Jan [cited 2021 Jun 30];5(1):e189–e189. Available from: /pmc/articles/PMC4728678/
32. Soysal SD, Tzankov A, Muenst SE. Role of the Tumor Microenvironment in Breast Cancer [Internet]. Vol. 82, Pathobiology. S. Karger AG; 2015 [cited 2021 Jun 30]. p. 142–52. Available from: https://pubmed.ncbi.nlm.nih.gov/26330355/
33. Giussani M, Merlino G, Cappelletti V, Tagliabue E, Daidone MG. Tumor-extracellular matrix interactions: Identification of tools associated with breast cancer progression. Vol. 35, Seminars in Cancer Biology. Academic Press; 2015. p. 3–10.
34. Yu T, Di G. Role of tumor microenvironment in triple-negative breast cancer and its prognostic significance. Vol. 29, Chinese Journal of Cancer Research. AME Publishing Company; 2017. p. 237–52.
35. Guaita-Esteruelas S, Saavedra-García P, Bosquet A, Borràs J, Girona J, Amiliano K, et al. Adipose-Derived Fatty Acid-Binding Proteins Plasma Concentrations Are Increased in Breast Cancer Patients. Oncologist. 2017;22(11):1309–15.
36. Ladanyi A, Mukherjee A, Kenny HA, Johnson A, Mitra AK, Sundaresan S, et al. Adipocyte-induced CD36 expression drives ovarian cancer progression and metastasis. Oncogene [Internet]. 2018 Apr 1 [cited 2021 Jun 30];37(17):2285–301. Available from: /pmc/articles/PMC5920730/
37. Furuhashi M, Tuncman G, Görgün CZ, Makowski L, Atsumi G, Vaillancourt E, et al. Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. Nature [Internet]. 2007 Jun 21 [cited 2021 Jun 30];447(7147):959–65. Available from: https://pubmed.ncbi.nlm.nih.gov/17554340/
38. Bosquet A, Girona J, Guaita-Esteruelas S, Heras M, Saavedra-García P, Martínez-Micaelo N, et al. FABP4 inhibitor BMS309403 decreases saturated-fatty-acid-induced endoplasmic

FOR REVIEW

reticulum stress-associated inflammation in skeletal muscle by reducing p38 MAPK activation. Biochim Biophys Acta - Mol Cell Biol Lipids [Internet]. 2018 Jun 1 [cited 2021 Jun 30];1863(6):604–13. Available from: https://pubmed.ncbi.nlm.nih.gov/29550588/

39. Enciu AM, Radu E, Popescu ID, Hinescu ME, Ceafalan LC. Targeting CD36 as Biomarker for Metastasis Prognostic: How Far from Translation into Clinical Practice? [Internet]. Vol. 2018, BioMed Research International. Hindawi Limited; 2018 [cited 2021 Jun 30]. Available from: https://pubmed.ncbi.nlm.nih.gov/30069479/
40. Hale JS, Otvos B, Sinyuk M, Alvarado AG, Hitomi M, Stoltz K, et al. Cancer stem cell-specific scavenger receptor 36 drives glioblastoma progression. Stem Cells [Internet]. 2014 [cited 2021 Jun 30];32(7):1746–58. Available from: /pmc/articles/PMC4063873/
41. Sakurai K, Tomihara K, Yamazaki M, Heshiki W, Moniruzzaman R, Sekido K, et al. CD36 expression on oral squamous cell carcinoma cells correlates with enhanced proliferation and migratory activity. Oral Dis [Internet]. 2020 May 1 [cited 2021 Jun 30];26(4):745–55. Available from: https://pubmed.ncbi.nlm.nih.gov/31603606/

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Objectives:

3. **To study the bidirectional crosstalk between adipose tissue and tumor cells, as well as to assess the effect of metabolic behavior alterations in both cell types.**

4 **To track labeled palmitic acid incorporation and transformation in BCC lines.**

Article:

Jose Adriá Cebrián, Sandra Guaita Esteruelas, Eric Lam, Marta Rodríguez Balada, Jordi Capellades, Josefa Girona, Ana Maria Jimenez Santamaria, Oscar Yanes, Luis Masana, Josep Gumà. MCF-7 Drug Resistant Cell Lines Switch Their Lipid Signature Metabolism to Triple Negative Breast Cancer Signature.

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Article

MCF-7 Drug Resistant Cell Lines Switch Their Lipid Metabolism to Triple Negative Breast Cancer Signature

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Simple Summary: Previously, we have demonstrated that lipid and lipoprotein profiles in breast cancer patients are altered compared to a control showing a new link between triglycerides enriched particles and breast cancer, hence suggesting an active role of lipids and their metabolism in this pathology. In this study, we have demonstrated the importance of tumor microenvironment crosstalk as well as the crucial role of lipid transfer between adipose tissue and cancerous cells. Moreover, we have demonstrated that each breast cancer subtype has their specific lipid signature. Interestingly, drug resistant luminal A cell lines switch this metabolic profile to the triple negative lipid signature. Knowledge of these signatures might help us to understand how these specific lipids are related with drug resistance and how it may clarify new treatments in these cancer patients.

Abstract: Obesity and adipose tissue have been closely related to a poor cancer prognosis, especially in prostate and breast cancer patients. The ability of transferring lipids from the adipose tissue to the tumor cells is actively linked to tumor progression. However, different types of breast tumor seem to use these lipids in different ways and metabolize them in different pathways. In this study we have tracked by mass spectrometry how palmitic acid from the adipocytes is released to media being later incorporated in different breast cancer cell lines (MDA-MB-231, SKBR3, BT474, MCF-7 and its resistant MCF-7 EPI^{R} and MCF-7 TAX^{R}). We have observed that different lines metabolize the palmitic acid in a different way and use their carbons in the synthesis of different new lipid families. Furthermore, we have observed that the lipid synthesis pattern varied according to the cell line. Surprisingly, the metabolic pattern of the resistant cells was more related to the TNBC cell line compared to their sensitive cell line MCF-7. These results allow us to determine a specific lipid pattern in different cell lines that later might be used in breast cancer diagnosis and to find a better treatment according to the cancer molecular type.

Keywords: breast; tumor microenvironment; adipocytes; FABP4; FABP5; CD36; lipids; mass spectrometry

1. Introduction

Breast cancer is the most common cancer in women and the second one overall [1]. Despite the advance in diagnosis techniques and treatment, nearly 12% of breast cancer patients eventually develop metastasis, and die from the disease [2]. A number of risk factors contribute to the poor outcomes, and the lifestyle is one of the most important. Obesity is one of the main known risk factors involved in the development and progression of breast cancer, as well as other cancer types, including pancreatic, hepatic and gallbladder cancer [3]. Although for many years adipose tissue has been consider as an energy reservoir, recent studies have demonstrated the importance of fat in different biological and physiological processes, including the production of steroid hormones, cytokines and adipokines, the control of immune system, and the regulation of eating behavior [4]. Due to the rapid proliferation rates of tumor cells, they require a substantial amount of lipid molecules for the biosynthesis of membranes, organelles, and other cellular factors. Adipose tissue, as a principal source of lipids, has an important influence in cancer progression through the transfer of lipids [5]. In fact, a number of studies have demonstrated that adipose tissue modulates cancer progression via the releasement of different factors and by the transfer of diverse lipid molecules [6]. Most of these factors are released as a consequence of the close communication between adipose tissue and the cancer cells [7]. Several studies have already demonstrated that there is an interaction between tumor cells and their microenvironment, which includes adipocytes [8,9]. This crosstalk of information and materials is mediated by the release of molecules, such as cytokines, hormones, metabolites and growth factors, that act on the closely located cells, as well as the more distant cells, modifying their behavior by the activation or inhibition of different metabolic pathways and/or by altering their proteomes and transcriptomes [7,10].

The communication between tumor cells and the adipocytes is bidirectional, and it has been described that tumor cells are able to modify the transcriptomes and metabolism of adjacent cells for their own benefit. In fact, adipocytes located within the tumor microenvironment are also subjected to dedifferentiation and a reprogramming of their metabolic behavior, transforming into cancer associated adipocytes (CAAs) [11]. CAAs release metabolites, such as lactate, pyruvate, free fatty acids, and ketone bodies, to the extracellular matrix, fuelling the proliferation of adjacent tumor cells [12]. Tumor cells internalized these lipids into their metabolic pathways for oxidation and energy production, and for activation of different signaling pathways [9,13].

Moreover, lipids are a high heterogenous variety of molecules. Depending on their structure and composition, lipids can play a plethora of roles in cell biology, such as membrane biosynthesis, energy storage, and cell signal transmission [14,15].

According to the Lipid Maps Alliance, lipids can be divided into eight different groups: fatty acids (FAs), glycerolipids (GL), glycerophospholipids (GP), sphingolipids (SP), sterol lipids (ST), prenol lipids (PR), saccharolipids (SL), and polyketides (PK) [16]. It is well established that tumor metabolism is subject to a number of modifications, and a switch to a lipidic program enhances tumor malignant transformation and progression [17].

These tumor lipid metabolic modifications also provide the possibilities for novel diagnostic strategies in cancer. As lipids can be found in urine, blood and other different accessible tissues, a lipid molecular screening by lipidomic technology can aide cancer diagnosis as well as in the understanding of the metabolic changes which cancer cells are subjected to within their microenvironment [18].

Some studies have found modifications in several lipid families in BC patients compared to the control population and correlated these alterations with a higher frequency of cancer occurrence [19]. Furthermore, it has been reported that there is an increase in levels of phospholipids, phosphocholines, cholines and glycerophosphocholines in tumors [20,21]. However, it is also important to note that plasma contains a high variety of lipids that can be altered according to dietary sources [22].

In order to understand the metabolic switch that tumor cells undergo within their microenvironment, we performed in-depth lipid profiling of four different BCC lines,

MDA-MB-231, BT474, SKBR3, and MCF-7 as well as the MCF-7 derived drug resistant MCF-7 TAX^R and MCF-7 Epi^R cells in close contact with mature adipocytes. Our main objective was to understand how the adipose tissue within the tumor microenvironment transfer lipids to adjacent cancer cells and how the tumor lipid metabolism is modified as a result, using lipidomics techniques and analysis.

Previous studies have detected a different lipidic and lipoprotein profile in breast cancer patients compared to healthy women [23]. Knowledge of a specific lipid signature and a particular lipid and lipoprotein profile might help in breast cancer diagnosis and in the improvement of new possible therapies.

2. Materials and Methods

2.1. Cell Culture

Human MDA-MB-231, BT474, SKBR3, MCF-7 and its resistant MCF-7 TAX^R and MCF-7 Epi^R were described previously and originally obtained from the American Type Culture Collection (ATCC). Cell lines were cultured Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS), 1% penicillin streptomycin and 1% non-essential amino acids (Biowest, Barcelona, Spain) at 37 °C with 5% CO_2.

Fibroblast cell line 3T3-L1 was obtained from the American Type Culture Collection (ATCC) and it was cultured until confluence. Then, they were cultured for 10 days with differentiation media, consisting in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% Non-essential Amino Acid Solution, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone and 10 μg/mL insulin from bovine pancreas (Sigma, Barcelona, Spain).

After differentiation, mature adipocytes were serum starved for 24 h. Then, they were cultured with the necessary conditions for each experiment.

2.2. Conditioned Media Obtainment

To obtain conditioned media (CM), MDA-MB-231, BT474, SKBR3, MCF-7 and its resistant MCF-7 TAX^R and MCF-7 Epi^R were cultured until they reached 80% of confluence. Then cells were serum starved. After 24 h of incubation with 0.1% FBS, different BCC lines CM were obtained for further assays. Moreover, mature adipocytes were cultured for 3 days with the different BCC lines CM. As a control condition, mature adipocytes were serum starved for 3 days.

2.3. Oil Red Staining

Oil Red O is a dye which strongly stains neutral lipids. To determinate the adipocyte lipid content, mature adipocytes were cultured with different BCC lines CM. For 3 days of incubation, treated and non-treated mature adipocytes were stained with Oil Red dye following the protocol described (#K580-24) and pictures were taken with an inverted microscope (Olympus IX71). Then, three washes with isopropanol 60% were performed and for dye extraction, cells were incubated with isopropanol 100% for 5 min. Afterwards absorbance at 490 nm was measured using SINERGY HT (BioTek, Swindon, UK).

2.4. RNA Extraction and Quantitative Real Time (qRT) PCR

RNA extraction was performed according to the PureLink RNA Mini Kit (Invitrogen, ThermoFisher Scientific, Barcelona, Spain) protocol and it was quantified using Synergy HT (BioTek, Swindon, UK). Total RNA (1 μg) was reverse-transcribed using the Prime-Script RT Reagent Kit (Takara Bio, Saint-Germain-en-Laye, France). Levels of mRNA were assessed using LightCycler96 device (Roche) with the Taqman probes for respective genes acquired from Life technologies CD36 (Mm00432403_m1), FABP4 (Mm00445878_m1), FABP5 (Mm00783731_s1), TBP (Mm01277042_m1). Then, transcript results were normalized with the gene TBP and the fold change was obtained.

2.5. Bodipy Fluorescent Staining

To observe the lipid transfer from mature adipocytes to BCC lines MDA-MB-231, BT474, SKBR3, MCF-7 and its resistant MCF-7 TAX^R and MCF-7 Epi^R, fibroblasts were differentiated for 10 days as previously described. During the differentiation process labeled palmitic-acid BODIPY (Sigma) every other day. In order to test the intake of labeled palmitic acid during the differentiation process, different images were taken using an inverted microscopy (Olympus IX71) and fluorescent intensity measure by ImageJ.

Alternatively, after maturation, the labeled adipocytes were serum-deprived for 3 days, and the medium was collected. Then, the BCC lines to be examined were seeded into 24 well plates until they reached 80% confluence and they were serum-starved with DMEM high glucose 0.1% FBS for 24 h. Next, the medium derived from labeled adipocytes were added to the BCC lines. After 72 h of treatment, cells were washed with PBS 1X, and images were taken using an inverted microscopy (Optimus IX71).

2.6. Enzyme-Linked Immunosorbent Assay (ELISA)

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

2.7. Lipid Uptake Assay: Nile Red Staining

Different BCC lines were seeded in 12 well plates. After serum-starvation, cells were incubated with adipocyte CM and they were stained after 24 h with the lipophilic fluorescent dye Nile Red (100 ng/mL) (Sigma-Aldrich; Barcelona, Spain) diluted in PBS 1× for 5 min at room temperature to visualize the lipid droplets. Cell images were captured using an Olympus IX71 microscope and analyzed using the Image J software obtaining the intensity of each image.

2.8. C^{13} Palmitic Assay

To assess the lipid transfer from mature adipocytes to the different BCC lines, a coculture system was performed. Firstly, 3T3-L1 fibroblasts were seeded and cultured in the upper chamber of a 12-well plate until they reached confluence. Then, they were differentiated as described above. Every other differentiation day, an isotope of palmitic acid (PA) with C^{13} was added. For PA formulation, C^{13}PA (200 μM) was mixed with free fatty acid BSA (33.32 μM) (Sigma). As a control condition, a parallel differentiation with non-isotope PA was performed.

In a 12 well plate, different BCC lines were seeded and cultured until they reached 80% of confluence. Then, the upper chamber with mature labeled and non-labeled adipocytes were put on above these BCCs. After 2 days of coculture, cells were harvested and collected until their analysis.

2.9. Lipidomics

Cells and culture media lipids were extracted using a biphasic extraction method. Cells were extracted in 220 μL methanol. After sample fragmentation by vortexing, immersion in liquid N2, and ultrasonication, 440 μL of dichloromethane (DCM) and 140 μL of water were added sequentially. A total of 200 μL of media was extracted via the same procedure as the cells without the immersion in liquid N2 and using methyl tert-butyl ether (MTBE) instead of DCM. Samples were incubated at 4 °C for 30 min and centrifuged (at 15,000 rpm for 15 min at 4 °C). 330 μL of the organic phase (lipidic) was collected for drying under a stream of nitrogen. Lipid pellets were resuspended in 150 μL of methanol/toluene (9:1) for liquid chromatography–mass spectrometry (LC–MS) analysis. Quality control (QC) samples consisting of pooled samples from each condition were injected at the beginning and periodically through the workflow.

Untargeted LC–MS analyses were performed using a UHPLC system (1200 series, Agilent Technologies) coupled with a 6550 ESI-QTOF MS (Agilent Technologies) operating in positive electrospray ionization (ESI+) mode. A total of 2 µL of cells extract and 3 µL of media extract were injected, and lipids were separated by reverse-phase chromatography with an Acquity UPLC C18-RP (ACQUITY UPLC BEH C18 1.7 µM, Waters). Mobile phase A was acetonitrile/water (60:40) (10 mM ammonium formate), and mobile phase B was isopropanol/acetonitrile (90:10) (10 mM ammonium formate). Solvent modifiers were used to enhance ionization and to improve the LC resolution in positive ionization mode. Separation was conducted under the following gradient: 0–2 min, 15–30% B; 2–2.5 min, 48% B; 2.5–11 min, 82% B; 11–11.5 min, 99% B; 11.5–12 min, 99% B; 12–12.1 min, 15% B; 12.1–15 min, 15% B. The ESI conditions were as follows: capillary voltage, 4000; gas temperature, 150 °C; drying gas, 12 L min^{-1}; nebulizer, 30 psig; fragmentor, 120 V; and skimmer, 65 V. The instrument was set to work over the m/z range from 50 to 1200 with an acquisition rate of 3 spectra/sec. For compound identification, MS/MS analyses were performed in targeted mode with an acquisition rate of 3 spectra/sec, applying three collision energies:10, 20, 30, and 40 V. Lipid structures were identified by matching tandem MS spectra against reference standards in LIPID MAPS [24] and/or LipidBlast [25] and/or Metlin [26].

LC–MS data were processed using the XCMS R package [2] for peak picking, retention time alignment and feature detection. Then, stable isotopic labelling was detected using geoRge R [27] package in order to find ^{13}C enriched lipid metabolites. This analysis provided a matrix containing the retention time, ion m/z value, number of ^{13}C enriched and the integrated the peak area of each feature for each sample. Features were then putatively associated to lipid identities found in above mentioned databases. Only those annotated features were statistically tested for significant changes across experimental groups using a one-way ANOVA, correcting for multiple comparisons using Tukey's honest significant differences and false discovery rate (FDR) method.

2.10. Statistical Analysis

All analyses were performed with the Statistical Package for Social Sciences (SPSS) software 27.0.1.0 (Madrid, Spain). Data were expressed as mean ± SEM. Statistical analysis was determined by one-way ANOVA and the differences between groups was analyzed using the Tukey post-hoc test. A p-value < 0.05 was considered to be statistically significant.

3. Results

3.1. Breast Cancer Cell Conditioned Media Enhances Delipidation of Mature Adipocytes

To assess if tumor cells can influence the amount of lipids liberated by the adipocytes, mature adipocytes were cultured with conditioned media from different BCC lines (MDA-MB-231, SKBR3, BT474, MCF-7 and the resistant MCF-7 EPI^R and MCF-7 TAX^R) and the amount of lipids accumulated in the mature adipocytes was then examined using Oil Red staining.

Interestingly, we observed that incubation with CMs from BCC lines caused the intracellular lipid droplet levels to decrease drastically when compared to the adipocytes incubated with the control CM (Figure 1). Accordingly, over 3 days of culture, the lipid levels of adipocytes incubated with BCC CMs declined significantly when compared with the control adipocytes (all CMs: $p < 0.001$; one-way ANOVA test).

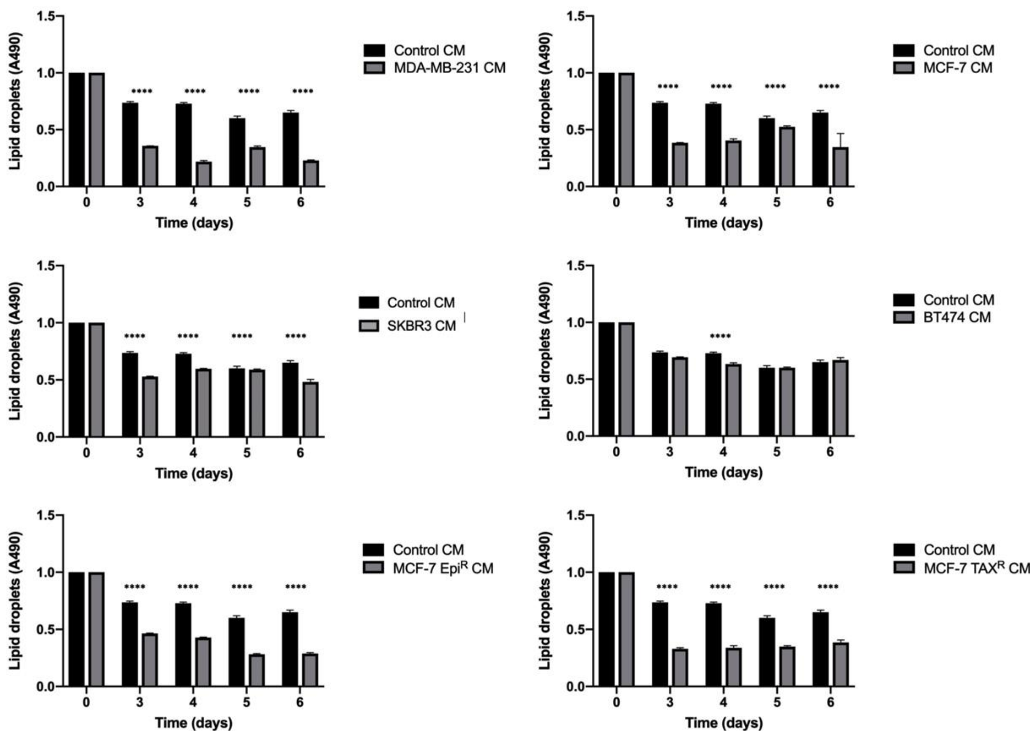

Figure 1. BCC CM increases mature adipocyte delipidation. Graph illustration of Oil Red dye in mature adipocytes at different conditions of culture for 6 days. The Oil Red tinction is represented according to colorimetric signal that is measured at an absorbance of 490 nm and symbolizes the amount and size of lipid droplets within mature adipocytes. In the graphic, black bars represent the control condition, while grey bars indicate those conditions where mature adipocytes have been cultured with a different BCC line CM. There is a significant decrease in Oil Red dye within those adipocytes that have been cultured with all the different BCC lines CM compared to those adipocytes cultured in control conditions. One-way ANOVA where a $p < 0.05$ was considered statistically significant (**** < 0.0001).

3.2. Breast Cancer Cell Conditioned Media Enhance FABP4, FABP5 and CD36 Release from Mature Adipocytes

Next, we explored the mechanism responsible for higher levels of fatty acid export and examined the expression levels of adipocyte-expressing fatty acid transporter proteins (i.e., FABP4, FABP5 and CD36) in the mature adipocytes upon culture with BCC CMs by RT-qPCR. The results showed that the mRNA levels of FABP4 and CD36 were significantly increased in the adipocytes when culture with BCC CMs (* $p < 0.05$) one-way ANOVA) (Figure 2A).

In addition, once we analyzed two of the most representative BCC lines (MDA-MB-231 and MCF-7) effects on adipocyte CM composition, we observed that there was an increase of all three fatty acid transporters within the media (Figure 2B). BCC lines MDA-MB-231 and MCF-7 CM produces an increase in fatty acid transporters mRNA in mature adipocytes, but also increases the releasement pattern of these fatty acid transporter proteins (* $p < 0.05$) one-way ANOVA.

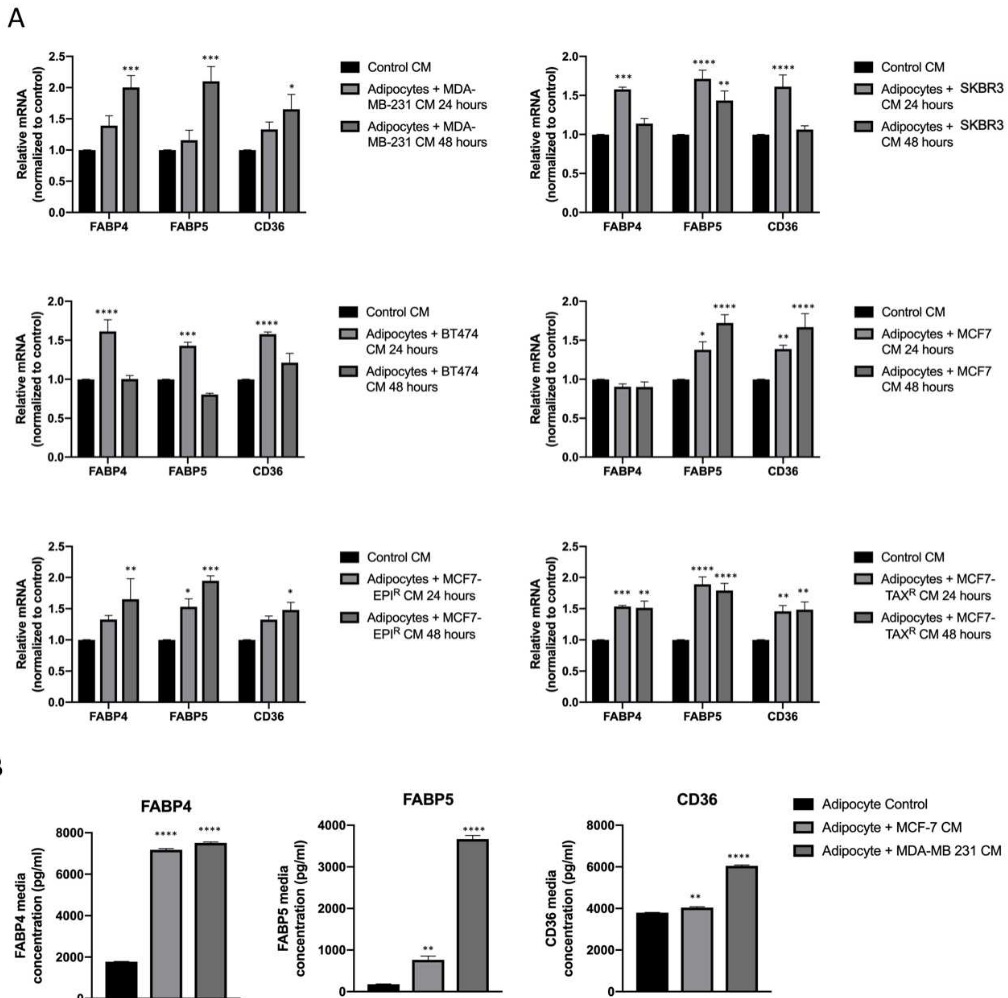

Figure 2. BCC CM enhances FABP4, FABP5 and CD36 mRNA levels and increases their releasement to media. (**A**) Graph representation of FABP4, FABP5 and CD36 transcripts in mature adipocytes at different conditions for 24 and 48 h of treatment. There is a clear and significant increase of all three transcripts in those mature adipocytes that have been culture with BCC line CM. Interestingly, FABP4 transcript in mature adipocytes culture with MCF-7 CM is not affected unlike the rest of conditions and transcripts. One-way ANOVA where a $p < 0.05$ was considered statistically significant. (**B**) Media from control adipocytes is compared with media from adipocytes cultured with MDA-MB-231 and MCF-7 cell lines conditioned media, for 24 h where a p-value < 0.05 is statistically significant (* < 0.05, ** < 0.005, *** < 0.0005, **** < 0.0001).

3.3. Mature Adipocytes Conditioned Media Increases Fatty Acid Uptake in Breast Cancer Cell Lines

Lipids are an important source of nutrients for the tumor cells, and they can be oxidized in order to generate energy and support growth. We have found a higher degree of delipidation and an increase in fatty acid transporter expression in mature adipocytes when cultured with BBC conditioned media. We hypothesized that those lipids secreted by the adipocytes are taken up by the BCCs. To test this conjecture, we cultured the BCC lines MDA-MB-231, SKBR3, BT474, MCF-7, MCF-7-EPI^R and MCF-7-TAX^R with the control or adipocyte CM and studied their lipid uptake by staining their intracellular accumulated lipid droplets using Nile Red. The results showed that there was a significant increase in

the amount of lipid droplets in all the BCC lines following incubation with adipocyte CM when compared with control CM ($p < 0.05$; one-way ANOVA) (Figure 3).

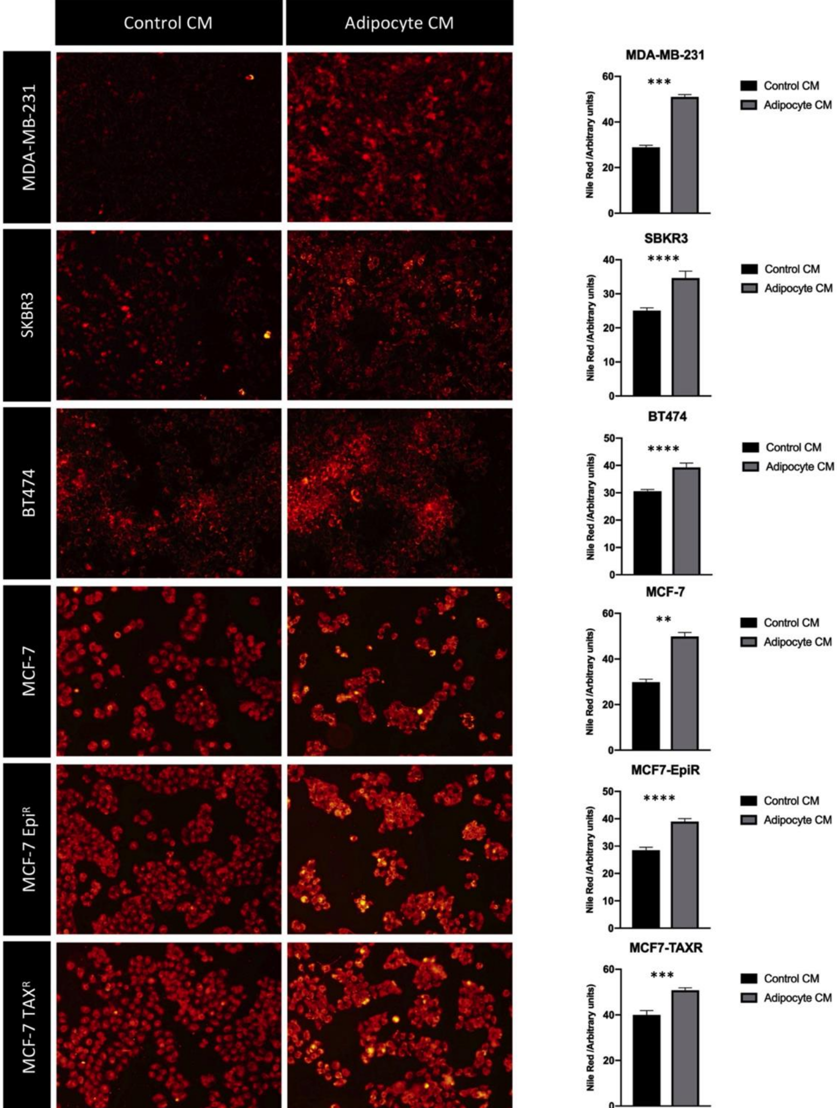

Figure 3. Mature adipocyte CM increases lipid uptake in BCC lines. Immunofluorescence images (10× magnification) of different BCC lines stained for fatty acids using Nile Red stain. There is an increase in Nile Red staining once cells were cultured with adipocyte CM compared to those cells cultured in control conditions. Graphs represent Nile Red staining intensity which could be translate to the amount of fatty acid taken up by the cancerous cells. One-way ANOVA where a $p < 0.05$ was considered statistically significant (** < 0.005, *** < 0.0005, **** < 0.0001).

3.4. Mature Adipocytes Internalize Palmitic Acid during Differentiation and Export it as Lipids to Breast Cancer Cells

Palmitic acid is a fatty acid closely linked to cancer progression [28]. We sought to confirm if lipid droplets accumulated in the BCCs are indeed derived from the uptake of

lipids released from the adipocytes. To this end, we first performed a negative and positive control to show a specific PA-BODIPY labelling (Figure S1). After that, we differentiated the murine fibroblast 3T3-L1 cells into adipocytes in the presence of the fluorescently labeled palmitic acid (BODIPY) and measured the fluorescent signal during the fibroblast differentiation into mature adipocytes. The result showed that the amount of lipid droplets increased as along with an increase in fluorescent BODIPY signal during adipocyte differentiation (Figure 4A).

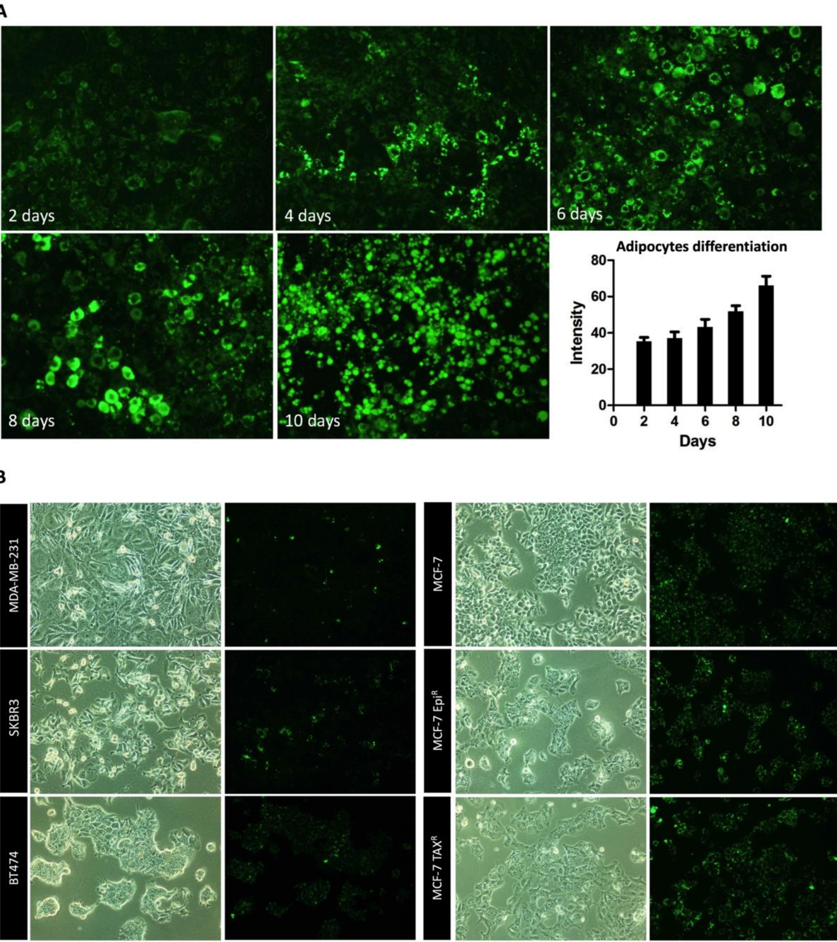

Figure 4. BCCs internalize palmitic acid from mature adipocytes. (**A**) Immunofluorescence images (10× magnification) of the fibroblasts 3T3-L1 cell line during their differentiation process for the labeled palmitic acid (BODIPY). Graph represents the BODIPY intensity within the preadipocytes during their differentiation until they reached the complete process. (**B**) Immunofluorescence images (10× magnification) of different BCC lines for BODIPY. Once it was obtained complete differentiated adipocytes with BODIPY incorporated, they were serum starved and adipocyte CM was obtained. Then, the different studied BCC lines were cultured with this adipocyte CM, and it was tested if there was BODIPY label within the cancerous cells. As it is represented in the pictures, all the BCC lines were able to uptake BODIPY from media within their cytoplasm.

Then, we assessed if the lipids accumulated in the mature adipocytes, are released by the adipocytes and uptaken to the different BCC lines. We analyzed the levels of intracellular BODIPY fluorescence in the BCCs after cultured them with PA-labeled adipocyte CM and found a substantial increase in labeled palmitic acid in the cytoplasm of BCCs, suggesting that the accumulation of lipids in the BCCs when co-cultured with adipocytes as a result of their uptake of lipids released by the adipocytes (Figure 4B).

3.5. Breast Cancer Cells have a Different and Specific Lipid Signature in the Palmitic Transformation and Resistant Cells Lipid Pattern Differs from Their Sensitive Cell Line Being Closely to TNBC Cell Line Pattern

Next, we sought to identify the specific changes in lipid compositions in the breast cancer cells in response to co-culture with adipocytes. ^{13}C labeled palmitic acid was added to differentiating adipocyte, which were then co-cultured with different BCCs, respectively. Further mass spectrometry analysis revealed that the ^{13}C labeled PA had transformed into different lipid species in the BCCs. The results showed that there were new lipid species, such as DAG, TG, ceramides, phosphatidylcholines, phosphoethanolamines and sphingomyelins, whose carbons were derived from the mature adipocytes derived ^{13}C-labeled PA. Once we analyzed the different BCC lines one by one, we observed that some BCCs had a similar but specific lipid pattern that were distinct from other BCCs. For example, we uncovered that SKBR3 and BT474 cells had a similar lipid signature. Through principal component analysis, we found that both SKBR3 and BT474 cells were closely related in terms of their lipid metabolism, particularly the way they convert the labeled fatty acids into DAGs and SMs. Moreover, we also detected that their lipid species, including diacylglycerols (DAGs), sphingomyelins (SMs), triglycerides (TGs) and phosphatidylcholines (PCs), are notably different from the other BCC lines studied. When we compared the luminal A MCF-7 cell line with its resistant derivatives, MCF-7 EPI^{R} and MCF-7 TAX^{R,} through principal component analysis, we observed that they were remarkably different in their lipid metabolic pathways by which the labeled fatty acids were transformed into intracellular lipids. Amongst the lipids which differed substantially between the parental and drug-resistant MCF-7 cells were ceramides (CMs), sphingomyelins (SMs), phosphatidylethanolamines (PEs) and phosphatidylcholines (PCs).

Surprisingly, the principal component analysis also showed that drug resistant MCF-7 TAX^{R} and MCF-7 EPI^{R} cells harbored a lipid signature more similar to the triple negative breast cancer (TNBC) cell line MDA-MB-231. (Figure 5).

Subsequently, the lipid families were also analyzed individually between different breast cancer cell lines. The principal component analysis also revealed a specific lipid metabolic pattern that is shared between the drug resistant MCF-7 cells and the TNBC MDA-MB-231 cell lines, which is distinct from the parental MCF-7 cells. In-depth individual lipid family analysis revealed that phosphatidylcholines (PCs), such as PC(O-33:3), PC(38:1) and PC(20:0/18:1) were increased in the drug-resistant MCF-7 and TNBC cells compared to the other drug-sensitive cell lines. Among the lipids, the phosphatidylethanolamines, including PE(P-34:2)///PE(P-16:1/18:1), PE(P-36:2)///PE(P-18:1/18:1), PE(O-34:2)///PE(O-15:1/19:1), PE(P-38:7)///PE(P-16:1/22:6), the ceramides (CMs), such as LacCer(18:1/16:0) and LacCer(18:1/24:0) and the sphingomyelins (SMs), such as SM(36:1) and SM(42:1), were also altered in both the drug resistant MCF-7 cells and MDA-MB-231 cells when compared with the rest of the drug-sensitive cell lines (Table 1).

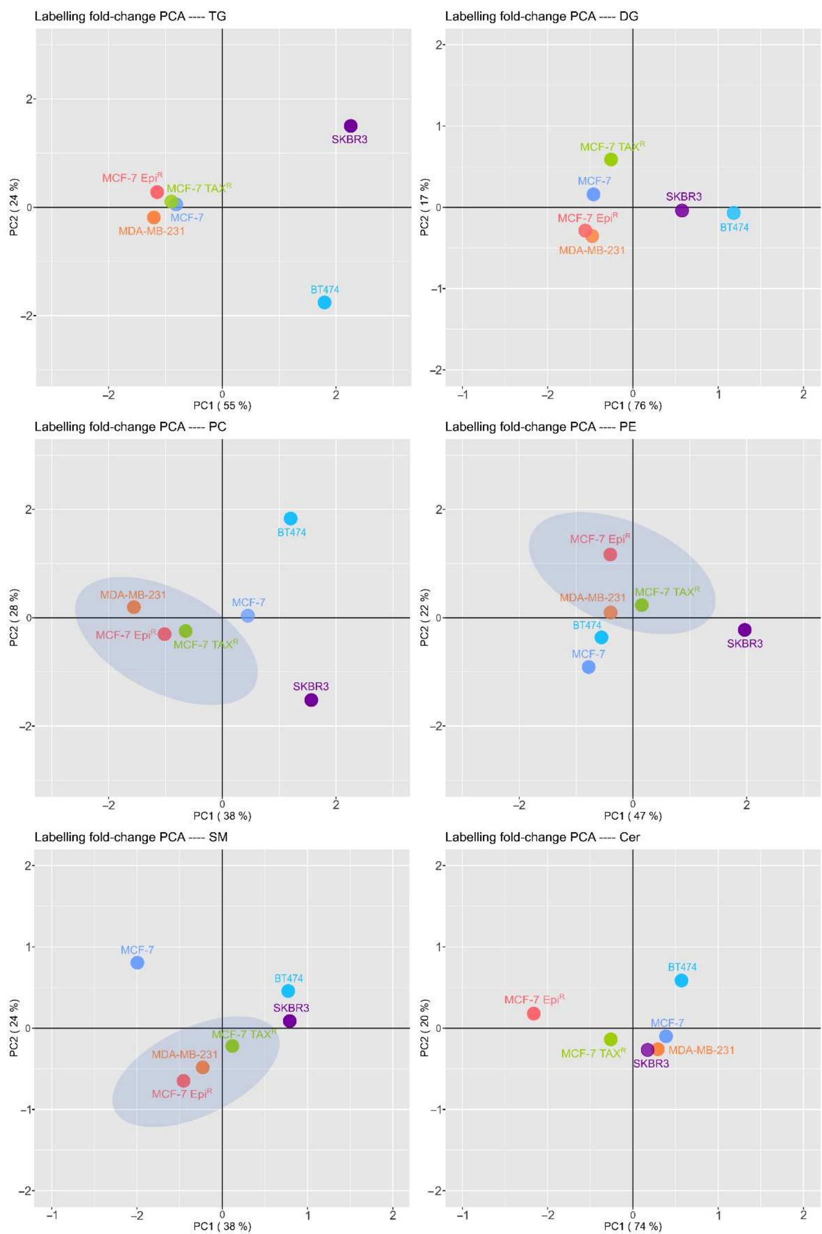

Figure 5. BCCs have a specific lipid signature that differs from each cell line. Principal component analysis (PCA) score plots of the different BCC lines analyzed by LC-MS. Scrutiny of the analyzed BCC lines indicated the similarities and differences between the different lipid species (DG, TG, PC, PE, SM and Cer). It is clear a differential lipid pattern between the studied BCC lines, where the luminal B cell line BT474 and the HER2+ cell line SKBR3 are notably differentiated in their lipid pattern from the rest of cell lines. Moreover, they are notably separated between themselves. Therefore, analyzing the lipid patterns of the BCC lines MCF-7 and its resistant cell lines, MCF-7 Epi^{R} and MCF-7 TAX^{R}, there were significant differences on them. In addition, resistant BCC lines lipid patterns were more similar to the lipid pattern of TNBC line MDA-MB-231 than the lipid profile of sensitive cell line MCF-7 (DG: diacylglycerol, TG: triacylglycerol, PC: phosphatidylcholine, PE: phosphatidylethanolamines, SM: sphingomyelin, Cer: ceramide).

Table 1. Resistant variants of MCF-7 have a lipid signature more similar to TNBC. Presence of different lipid families such as PC, PE, TG, SM and Ceramides in resistant cell lines MCF-7 Epi^R and MCF-7 TAX^R and the TNBC cell line MDA-MB-231 compared to the presence that is detected in the sensitive cell line MCF-7. It is described the fold change of each specific lipid within each family mentioned above that has been found within these four cell lines. There are differences in the presence of many PC, PE, SM and Cer, where an increased fold increase is present once their presence is compared between resistant and TNBC lines compared to sensitive cell line MCF-7 (green underlined).

	Lipid Species	MCF-7 Epi^R vs. MCF-7		MCF-7 TAX^R vs. MCF-7		MDA-MB-231 vs. MCF-7	
		Fold Change	*p* Value	Fold Change	*p* Value	Fold Change	*p* Value
PC	PC(28:0)///PC(14:0/14:0)	0.02990952	**0.001**	0.04615794	**0.001**	0.21447702	0.050
	PC(31:1)///PC(15:0/16:1)	0.0689704	**0.017**	0.09403492	**0.025**	0.20846594	0.108
	PC(30:1)///PC(16:1/14:0)	0.01112429	**0.002**	0.00700047	**0.000**	0.01721311	**0.002**
	PC(O-33:3)	1.27651749	**0.004**	3.99971727	**0.001**	2.80090791	**<0.001**
	PC(38:3)///PC(18:0/20:3)	0.27554774	**0.044**	0.26114733	**0.007**	0.65273596	0.738
	PC(44:1)///PC(26:0/18:1)	44.6634837	**0.092**	36.8772782	**0.032**	376.01122	**0.001**
	PC(38:1)///PC(20:0/18:1)	3.53236174	0.288	3.43871497	0.184	14.0520945	**0.004**
PE	PE(P-34:2)///PE(P-16:1/18:1)	28.2972788	**<0.001**	26.2359375	**<0.001**	22.5278479	**<0.001**
	PE(P-36:5)///PE(P-16:1/20:4)	8.81558854	**0.007**	10.4244025	**0.005**	14.2692477	**0.001**
	PE(P-36:2)///PE(P-18:1/18:1)	25.9952287	**<0.001**	19.896668	**<0.001**	114.690505	**<0.001**
	PE(34:1)///PE(16:0/18:1)	0.58971808	0.781	0.64063147	0.186	0.35193966	**0.009**
	PE(O-34:2)///PE(O-15:1/19:1)	29.4014166	**<0.001**	23.5110141	**<0.001**	22.0164115	**<0.001**
	PE(P-38:7)///PE(P-16:1/22:6)	14.7720082	**0.006**	12.4773801	**0.004**	9.72151443	**0.008**
TG	Mix TG(44:1)	0.03544991	0.003	0.04252924	**0.001**	0.04335925	**0.001**
	Mix TG(46:4)	0.05574601	**<0.001**	0.07728431	**<0.001**	0.07396131	**<0.001**
	Mix TG(46:1)	0.15810058	0.018	0.23540369	**0.004**	0.17216579	**0.002**
	Mix TG(48:4)	0.29777056	0.007	0.40895724	**0.008**	0.27500008	**0.001**
	Mix TG(50:5)	0.16080286	**<0.001**	0.17598899	**<0.001**	0.12880096	**<0.001**
	Mix TG(50:2)	0.48675871	0.399	0.53214893	**0.017**	0.39973327	**0.002**
	Mix TG(52:3)	0.24951721	0.092	0.30606399	**0.011**	0.34407207	**0.013**
	Mix TG(54:3)	0.47035591	0.322	0.38418791	**0.001**	0.40637479	**0.002**
SM	SM(36:1)	3.3947483	**0.001**	3.89343315	**0.002**	1.02089675	0.999
	SM(39:3)	0.22395786	**0.017**	0.21373755	**0.001**	0.21085366	**0.002**
	SM(42:2)	0.55261516	0.632	0.37982867	**0.013**	0.46507858	0.086
	SM(42:1)	2.07385942	0.331	2.05459388	0.309	5.87791467	**0.003**
Cer	LacCer(18:1/16:0)	57.8502342	**<0.001**	48.320848	**<0.001**	4.02257761	**0.003**
	DihydroCer(18:0(OH)/16:0)	0.20022379	0.073	0.18622892	**0.003**	0.10548975	**0.001**
	LacCer(18:1/24:0)	103.862204	**<0.001**	56.4934583	**<0.001**	10.0750913	**<0.001**

In summary, these alterations demonstrate a switch in lipid components and metabolism in the drug resistant MCF-7 Epi^R and MCF-7 TAX^R and the MDA-MB-231 cell lines when compared with the other relatively more drug-sensitive cell lines. Moreover, as MDA-MB-231 cells have previously been shown to be a more taxol and epirubicin resistant cell line compared to MCF-7 and some of the other breast cancer cell lines studied, our results also suggest that the more drug-resistant and malignant breast cancer cells have modified their lipid profiles and metabolism to enhance their drug-resistance and malignant progression.

4. Discussion

The interactions between different components of the tumor microenvironment and the cancer cells play a key role in cancer initiation and malignant progression [6]. In consequence, their cross-talks have been the objects of cancer research during last decades, and huge advances have been achieved in deciphering these interactions as well as the mechanistic details involved. One of the major components of the breast is adipose tissue; hence, the cross-communication between breast cancer cells and their nearby adipocytes

play a crucial part in breast cancer progression. Different mechanisms for interactions between the breast cancer cells and the adipocytes have been suggested, but the lipid transfer from adipocytes to cancerous cells have gained special attention. In fact, earlier studies have already established a close relationship between lipid transfer from adipocytes to breast cancer cells and the enhancement of several well-established cancer hallmarks in breast cancer. However, the specific mechanisms that underly these cross-communications between adipocytes and breast cancer cells are yet to be unveiled.

In this study, we also studied the interactions between adipocytes and six different breast cancer cell lines, including MDA-MB-231, SKBR3, BT474, MCF-7 and the MCF-7 derived drug-resistant cell lines MCF-7 Epi^R and MCF-7 TAX^R. The results have expanded our understanding of the effects of many types of breast cancer cells, including TNBC, HER2+, luminal A and luminal B as well as drug-resistant breast cancer cells on mature adipocytes and vice versa in the tumor microenvironment.

In this study, we firstly focused on how tumor cells modify the tumor microenvironment, focusing on the adipose tissue. We reviewed how different breast cancer cells modify the behavior of adipocytes within the tumor microenvironment and reconfirmed the previous finding that breast cancer cells promote lipid metabolism and increase its degree of delipidation [11]. We also found that breast cancer cells induce the transcription of different fatty acid transporters such as FABP4, FABP5 and CD36. We also obtained evidence that these fatty acid transporter proteins are also released with the lipids by the adipocytes into their surroundings, suggesting that their release might promote the transport and metabolism of fatty acids in the breast cancer cells. In agreement, these fatty acid transporter proteins have been intimately related to cancer development and malignant progression [29,30].

Moreover, it has also been suggested that tumor cells cocultured with mature adipocytes increases their lipid uptake, which is partially mediated by the fatty acid transporters [13,31,32].

Recently, palmitic acid has been shown to be strongly linked to cancer development and progression. In fact, it has been demonstrated tumor cells have a specific need for this lipid and it is capable of promoting a number of cancer hallmarks, particularly cancer survival and metastasis [33]. It has also been also demonstrated that there is a strong positive correlation between palmitic acid level and tumor progression [28].

As a result, we tracked the labeled palmitic acid, and confirmed that mature adipocytes first incorporated the labeled fatty acid, and then released it into the extracellular media, when they are treated with the conditional media from different breast cancer cell lines. We also obtained evidence that adipocyte-released fatty acids were taken up by the breast cancer cells and turned into lipids. Moreover, the internalized fatty acids will be used for the biosynthesis of new lipid compounds in the breast cancer cells, and the discovery of specific lipid signatures and metabolic pathways for distinct molecular breast cancer subtypes may help us to understand how different cancer cells internalize lipids from adjacent adipocytes and how they transform them into any lipid families in their own benefit. In fact, a previous study has demonstrated that cancer cells incorporate exogenous palmitic acid into their structural and oncogenic signaling lipid components [34]. It has been shown that fatty acid uptake and lipid metabolism promote cancer progression and metastasis.

To determine the lipid metabolic pathways involved in enhancing cancer progression and metastasis, we first cultured the fibroblast cell line 3T3-L1 in the presence of ^{13}C-palmitic acid and confirmed that this isotope-labeled fatty acid was internalized into mature adipocytes as lipid droplets. Then, the adipocytes carrying the isotope-labeled fatty acid were cocultured with our different breast cancer cell lines. We observed a significant increase of the isotopic labels in media, suggesting that there was a releasement of these lipids form mature adipocytes to their environment. Subsequently, we also observed that the isotope signals that had appeared in media disappeared slowly into the cancer cells, suggesting the labeled lipid internalization into the cancer cells.

Next, we analyzed the fate of the labeled palmitic acid carbon in all different breast cancer cell lines using a LC-MS analysis, and interestingly, we found that cancer cells were able to synthetize new lipid species.

As cancer cells need a huge amount of lipids in order to proliferate, to synthesize cellular structural components and to participate in intracellular signaling [5].

Phosphatidylcholines are one of the main lipid subtypes present in the mammalian cells and their metabolism has been widely studied in cancer. According to bibliography, it has been described that there is an increase in the presence of different PC in worst prognosis BC patients' blood stream [20]. Moreover, there is high correlation between lipid metabolism, including PC (14:0/16:0) for example, and the tumor grade [35].

Our results show significant differences between the PC pattern of each studied BCC line. Interestingly, the PC biosynthesis pattern was more similar between the resistant MCF-7 cell lines MCF-7 Epi^R and MCF-7 TAX^R and the TNBC cell line MDA-MB-231 compared to the sensitive cell lines MCF-7, the luminal B cell line BT474, and the HER2+ cell line SKBR3. Interestingly, PC saturations such as PC (36:1), PC (38:2) have been closely related with worst prognosis [36,37].As mentioned earlier, similar to MCF-7 Epi^R and MCF-7 TAX^R cells, MDA-MB-231 cells have previously been shown to be a more taxane (e.g., taxol) and anthracyclin (e.g., epirubicin) resistant cell line and more metastatic competence compared to MCF-7 and some of the other breast cancer cell lines used in the study [38]. Our results therefore suggest that the more drug-resistant and malignant breast cancer cells have modified their lipid profiles and metabolism to enhance their drug-resistance, metastatic competence and malignant progression.

We have found that the way that the drug resistant MCF-7 Epi^R and MCF-7 TAX^R cells and the TNBC MDA-MB-231 cells metabolize the palmitic acid derived from mature adipocytes in a different manner, in comparison to the parental drug-sensitive MCF-7 as well as to the luminal B and HER2+ cell lines, suggesting that more malignant and drug-resistant breast cancer cells acquire a specific PC biosynthesis pattern that differ from the drug-sensitive and less metastatic breast cancer cells. Furthermore, alterations in PE metabolism have been also linked with breast cancer progression. In fact, a number of studies have reported altered plasma and urine PE levels, specifically PE (15:0/19:1) to be linked to breast cancer development. In this study, we have also found this lipid family strongly altered in the drug-resistant cell lines (i.e., MCF-7 Epi^R, MCF-7 TAX^R and MDA-MB-231 cells) compared to the relatively more drug-sensitive cell lines.

Bioactive sphingolipids and ceramides are of special importance in promoting cancer progression due to their roles as signaling molecules and have been shown to regulate a number of critical cellular biological processes [10,39]. SM is one of the most abundant sphingolipids in normal cells and deregulated expression and function of this lipid family have also been correlated with breast cancer development and progression [36]. According to our results, we also detected an alteration in the signature of this family of lipids in the relatively more resistant cell lines. In our study, a similar signature of ceramide expression was also found in the drug-resistant breast cancer cells. Ceramides constitute the structural scaffold of sphingolipids and it has been described that metabolites derived from ceramides are involved in the signal transduction of a number of signaling pathways involved in apoptosis inhibition and cell growth promotion [40,41]. In addition, high levels of ceramide synthase and ceramide has been found in breast cancer compared to normal non-cancerous tissues [42].

5. Conclusions

In summary, our study shows that defining the lipid signatures in breast cancer might be a new approach to understanding the behavior of cancer cells within the tumor microenvironment, particularly when they are in the vicinity of the adipose tissue. Moreover, our data also suggest that altered lipid metabolism may play a critical role in the malignant transformation as well as drug-resistance in the breast cancer cells. The altered lipid signatures found in the drug-resistant and more metastatic breast cancer cells might

also be useful markers for breast cancer diagnosis and prognosis. Moreover, targeting the deregulated lipid uptake and metabolism in the drug resistant breast cancer cells may help to reverse the drug insensitivity of some breast cancer cells and lead to novel treatment strategies for breast cancer as well as drug resistance (Figure 6).

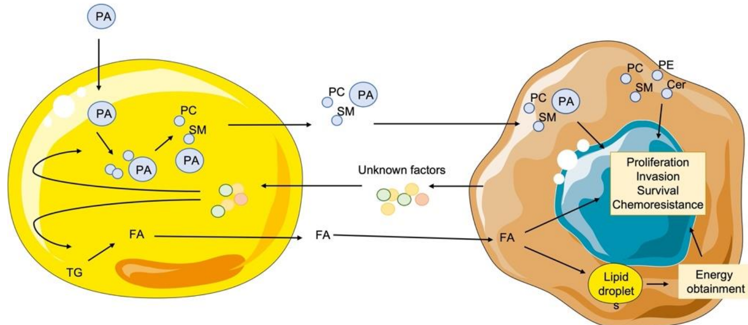

Figure 6. Schematic representation of the crosstalk between adipocytes and breast cancer cells. Palmitic acid (PA) is internalized into mature adipocytes during their differentiation. Then the coculture of both cell lines produces a releasement of unknown factors from cancerous cells that modifies the behavior of adjacent adipocytes increasing their catabolism, thus enhancing their delipidation. In addition, PA is metabolized, and new species are formed from this lipid, such as phosphatidylcholines (PC) or sphingomyelins (SM). Close contact between both cell types leads to the releasement of these lipid species, being internalized into the cancerous cells. Then, new lipidic species are produced form these lipids, including phosphatidylethanolamines (PE) and ceramides (Cer). The specific lipid pattern will confer an enhancement in the tumorigenic behavior on the breast cancer cells. In addition, free fatty acids also are released form adjacent adipocytes and are internalized into the cancerous cells. There, they are included into lipid droplets that will confer a new source of energy, although they also can induce the enhancement of the cancer cells hallmarks.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/cancers13235871/s1, Figure S1: Negative and positive control of PA-BODIPY labelling in BCC.

Author Contributions: Conceptualization, J.A.-C., S.G.-E., L.M. and J.G. (Josep Gumà); methodology, S.G.-E., J.G. (Josefa Girona) and J.A.-C.; validation, S.G.-E., J.G. (Josefa Girona), E.W.-F.L., L.M. and J.G. (Josep Gumà); formal analysis, S.G.-E., J.G. (Josefa Girona) and J.A.-C.; investigation, S.G.-E., E.W.-F.L. and J.A.-C.; resources, M.R.-B., A.M.J.-S. and J.C.; data curation, S.G.-E., M.R.-B., L.M., J.C., O.Y. and J.G. (Josep Gumà); writing—original draft preparation, S.G.-E., J.A.-C. and L.M.; writing—review and editing, J.A.-C., S.G.-E., L.M., E.W.-F.L., O.Y. and J.G. (Josep Gumà); visualization, S.G.-E., J.A.-C. and L.M.; supervision, S.G.-E., L.M. and E.W.-F.L.; project administration, S.G.-E. and J.G. (Josep Gumà). All authors have read and agreed to the published version of the manuscript.

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References

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J. Clin.* **2018**, *68*, 394–424. [CrossRef] [PubMed]
2. Smith, C.A.; Want, E.J.; O'Maille, G.; Abagyan, R.; Siuzdak, G. XCMS: Processing mass spectrometry data for metabolite profiling using nonlinear peak alignment, matching, and identification. *Anal. Chem.* **2006**, *78*, 779–787. [CrossRef] [PubMed]

3. Polednak, A.P. Estimating the number of U.S. incident cancers attributable to obesity and the impact on temporal trends in incidence rates for obesity-related cancers. *Cancer Detect. Prev.* **2008**, *32*, 190–199. [CrossRef] [PubMed]
4. Coelho, M.; Oliveira, T.; Fernandes, R. Biochemistry of adipose tissue: An endocrine organ. *Arch. Med. Sci.* **2013**, *9*, 191–200. [CrossRef]
5. Muller, C. Tumour-surrounding adipocytes are active players in breast cancer progression. *Ann. Endocrinol.* **2013**, *74*, 108–110. [CrossRef]
6. Bochet, L.; Lehuédé, C.; Dauvillier, S.; Wang, Y.Y.; Dirat, B.; Laurent, V.; Dray, C.; Guiet, R.; Maridonneau-Parini, I.; Le Gonidec, S.; et al. Adipocyte-derived fibroblasts promote tumor progression and contribute to the desmoplastic reaction in breast cancer. *Cancer Res.* **2013**, *73*, 5657–5668. [CrossRef]
7. Picon-Ruiz, M.; Pan, C.; Drews-Elger, K.; Jang, K.; Besser, A.H.; Zhao, D.; Morata-Tarifa, C.; Kim, M.; Ince, T.A.; Azzam, D.J.; et al. Interactions between adipocytes and breast cancer cells stimulate cytokine production and drive Src/Sox2/miR-302b-mediated malignant progression. *Cancer Res.* **2016**, *76*, 491–504. [CrossRef] [PubMed]
8. Krishna Muppalla, J.N.; Muddana, K.; Reddy Dorankula, S.P.; Rao Thokala, M.; Prakash Pasupula, A. Microenvironment-A role in tumour progression and prognosis. *J. Clin. Diagn. Res.* **2013**, *7*, 2096–2099.
9. Nieman, K.M.; Romero, I.L.; Van Houten, B.; Lengyel, E. Adipose tissue and adipocytes support tumorigenesis and metastasis. *Biochim. Biophys. Acta-Mol. Cell Biol. Lipids* **2013**, *1831*, 1533–1541. [CrossRef]
10. Ahmed, N.; Escalona, R.; Leung, D.; Chan, E.; Kannourakis, G. Tumour microenvironment and metabolic plasticity in cancer and cancer stem cells: Perspectives on metabolic and immune regulatory signatures in chemoresistant ovarian cancer stem cells. *Semin. Cancer Biol.* **2018**, *53*, 265–281. [CrossRef]
11. Rybinska, I.; Mangano, N.; Tagliabue, E.; Triulzi, T. Cancer-associated adipocytes in breast cancer: Causes and consequences. *Int. J. Mol. Sci.* **2021**, *22*, 3775. [CrossRef]
12. Attané, C.; Muller, C. Drilling for Oil: Tumor-Surrounding Adipocytes Fueling Cancer. *Trends Cancer* **2020**, *6*, 593–604. [CrossRef]
13. Yang, D.; Li, Y.; Xing, L.; Tan, Y.; Sun, J.; Zeng, B.; Xiang, T.; Tan, J.; Ren, G.; Wang, Y. Utilization of adipocyte-derived lipids and enhanced intracellular trafficking of fatty acids contribute to breast cancer progression. *Cell Commun. Signal.* **2018**, *16*, 32. [CrossRef] [PubMed]
14. Harayama, T.; Riezman, H. Understanding the diversity of membrane lipid composition. *Nat. Rev. Mol. Cell Biol.* **2018**, *19*, 281–296. [CrossRef] [PubMed]
15. Sunshine, H.; Iruela-Arispe, M.L. Membrane lipids and cell signaling. *Curr. Opin. Lipidol.* **2017**, *28*, 408–413. [CrossRef] [PubMed]
16. Fahy, E.; Subramaniam, S.; Murphy, R.C.; Nishijima, M.; Raetz, C.R.H.; Shimizu, T.; Spener, F.; Van Meer, G.; Wakelam, M.J.O.; Dennis, E.A. Update of the LIPID MAPS comprehensive classification system for lipids. *J. Lipid Res.* **2009**, *50*, S9. [CrossRef]
17. Beloribi-Djefaflia, S.; Vasseur, S.; Guillaumond, F. Lipid metabolic reprogramming in cancer cells. *Oncogenesis* **2016**, *5*, e189. [CrossRef]
18. Orešič, M.; Hänninen, V.A.; Vidal-Puig, A. Lipidomics: A new window to biomedical frontiers. *Trends Biotechnol.* **2008**, *26*, 647–652. [CrossRef]
19. Zeleznik, O.A.; Clish, C.B.; Kraft, P.; Avila-Pacheco, J.; Eliassen, A.H.; Tworoger, S.S. Circulating Lysophosphatidylcholines, Phosphatidylcholines, Ceramides, and Sphingomyelins and Ovarian Cancer Risk: A 23-Year Prospective Study. *J. Natl. Cancer Inst.* **2020**, *112*, 628–636. [CrossRef]
20. Chen, X.; Chen, H.; Dai, M.; Ai, J.; Li, Y.; Mahon, B.; Dai, S.; Deng, Y. Plasma lipidomics profiling identified lipid biomarkers in distinguishing early-stage breast cancer from benign lesions. *Oncotarget* **2016**, *7*, 36622–36631. [CrossRef]
21. Hammad, L.A.; Wu, G.; Saleh, M.M.; Klouckova, I.; Dobrolecki, L.E.; Hickey, R.J.; Schnaper, L.; Novotny, M.V.; Mechref, Y. Elevated levels of hydroxylated phosphocholine lipids in the blood serum of breast cancer patients. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 863–876. [CrossRef]
22. Li, X.; Liu, Z.L.; Wu, Y.T.; Wu, H.; Dai, W.; Arshad, B.; Xu, Z.; Li, H.; Wu, K.N.; Kong, L.Q. Status of lipid and lipoprotein in female breast cancer patients at initial diagnosis and during chemotherapy. *Lipids Health Dis.* **2018**, *17*, 1–6. [CrossRef]
23. Gumà, J.; Adriá-Cebrián, J.; Ruiz-Aguado, B.; Albacar, C.; Girona, J.; Rodríguez-Calvo, R.; Martínez-Micaelo, N.; Lam, E.W.F.; Masana, L.; Guaita-Esteruelas, S. Altered serum metabolic profile assessed by advanced 1h-nmr in breast cancer patients. *Cancers* **2021**, *13*, 4281. [CrossRef] [PubMed]
24. Fahy, E.; Sud, M.; Cotter, D.; Subramaniam, S. LIPID MAPS online tools for lipid research. *Nucleic Acids Res.* **2007**, *35* (Suppl. 2), W606–W612. [CrossRef]
25. Kind, T.; Liu, K.H.; Lee, D.Y.; Defelice, B.; Meissen, J.K.; Fiehn, O. LipidBlast in silico tandem mass spectrometry database for lipid identification. *Nat. Methods* **2013**, *10*, 755–758. [CrossRef] [PubMed]
26. Montenegro-Burke, J.R.; Guijas, C.; Siuzdak, G. METLIN: A Tandem Mass Spectral Library of Standards. *Methods Mol. Biol.* **2020**, *2104*, 149–163. [PubMed]
27. Capellades, J.; Navarro, M.; Samino, S.; Garcia-Ramirez, M.; Hernandez, C.; Simo, R.; Vinaixa, M.; Yanes, O. GeoRge: A Computational Tool to Detect the Presence of Stable Isotope Labeling in LC/MS-Based Untargeted Metabolomics. *Anal. Chem.* **2016**, *88*, 621–628. [CrossRef]
28. Aznar Benitah, S. Metastatic-initiating cells and lipid metabolism. *Cell Stress* **2017**, *1*, 110–114. [CrossRef]

29. Huang, M.; Narita, S.; Inoue, T.; Koizumi, A.; Saito, M.; Tsuruta, H.; Numakura, K.; Satoh, S.; Nanjo, H.; Sasaki, T.; et al. Fatty acid binding protein 4 enhances prostate cancer progression by upregulating matrix metalloproteinases and stromal cell cytokine production. *Oncotarget* **2017**, *8*, 111780–111794. [CrossRef]
30. Pan, J.; Fan, Z.; Wang, Z.; Dai, Q.; Xiang, Z.; Yuan, F.; Yan, M.; Zhu, Z.; Liu, B.; Li, C. CD36 mediates palmitate acid-induced metastasis of gastric cancer via AKT/GSK-3β/β-catenin pathway. *J. Exp. Clin. Cancer Res.* **2019**, *38*, 52. [CrossRef]
31. Wang, Y.Y.; Attané, C.; Milhas, D.; Dirat, B.; Dauvillier, S.; Guerard, A.; Gilhodes, J.; Lazar, I.; Alet, N.; Laurent, V.; et al. Mammary adipocytes stimulate breast cancer invasion through metabolic remodeling of tumor cells. *J. Clin. Investig.* **2017**, *2*, e87489. [CrossRef]
32. Nieman, K.M.; Kenny, H.A.; Penicka, C.V.; Ladanyi, A.; Buell-Gutbrod, R.; Zillhardt, M.R.; Romero, I.L.; Carey, M.S.; Mills, G.B.; Hotamisligil, G.S.; et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat. Med.* **2011**, *17*, 1498–1503. [CrossRef]
33. Fatima, S.; Hu, X.; Gong, R.-H.; Huang, C.; Chen, M.; Wong, H.L.X.; Bian, Z.; Kwan, H.Y. Palmitic acid is an intracellular signaling molecule involved in disease development. *Cell. Mol. Life Sci.* **2019**, *76*, 2547–2557. [CrossRef]
34. Louie, S.M.; Roberts, L.S.; Mulvihill, M.M.; Luo, K.; Nomura, D.K. Cancer cells incorporate and remodel exogenous palmitate into structural and oncogenic signaling lipids. *Biochim. Biophys. Acta-Mol. Cell Biol. Lipids* **2013**, *1831*, 1566–1572. [CrossRef]
35. Hilvo, M.; Denkert, C.; Lehtinen, L.; Müller, B.; Brockmöller, S.; Seppänen-Laakso, T.; Budczies, J.; Bucher, E.; Yetukuri, L.; Castillo, S.; et al. Novel theranostic opportunities offered by characterization of altered membrane lipid metabolism in breast cancer progression. *Cancer Res.* **2011**, *71*, 3236–3245. [CrossRef]
36. Nagahashi, M.; Tsuchida, J.; Moro, K.; Hasegawa, M.; Tatsuda, K.; Woelfel, I.A.; Takabe, K.; Wakai, T. High levels of sphingolipids in human breast cancer. *J. Surg. Res.* **2016**, *204*, 435–444. [CrossRef]
37. Qiu, Y.; Zhou, B.; Su, M.; Baxter, S.; Zheng, X.; Zhao, X.; Yen, Y.; Jia, W. Mass spectrometry-based quantitative metabolomics revealed a distinct lipid profile in breast cancer patients. *Int. J. Mol. Sci.* **2013**, *14*, 8047–8061. [CrossRef] [PubMed]
38. Huang, J.; Luo, Q.; Xiao, Y.; Li, H.; Kong, L.; Ren, G. The implication from RAS/RAF/ERK signaling pathway increased activation in epirubicin treated triple negative breast cancer. *Oncotarget* **2017**, *8*, 108249–108260. [CrossRef] [PubMed]
39. Ogretmen, B.; Hannun, Y.A. Biologically active sphingolipids in cancer pathogenesis and treatment. *Nat. Rev. Cancer* **2004**, *4*, 604–616. [CrossRef] [PubMed]
40. Gómez-Muñoz, A. Ceramide 1-phosphate/ceramide, a switch between life and death. *Biochim. Biophys. Acta-Biomembr.* **2006**, *1758*, 2049–2056. [CrossRef] [PubMed]
41. Regina Todeschini, A.; Hakomori, S. itiroh Functional role of glycosphingolipids and gangliosides in control of cell adhesion, motility, and growth, through glycosynaptic microdomains. *Biochim. Biophys. Acta-Gen. Subj.* **2008**, *1780*, 421–433. [CrossRef] [PubMed]
42. Schiffmann, S.; Sandner, J.; Birod, K.; Wobst, I.; Angioni, C.; Ruckhäberle, E.; Kaufmann, M.; Ackermann, H.; Lötsch, J.; Schmidt, H.; et al. Ceramide synthases and ceramide levels are increased in breast cancer tissue. *Carcinogenesis* **2009**, *30*, 745–752. [CrossRef] [PubMed]

Objectives:

6. To establish the role of the communication between adipose tissue, immune system, and tumor cells in the tumor microenvironment context.

7. To assess the pro-inflammatory status of tumor microenvironment, hence of BC patients.

Article:

Jose Adriá Cebrián, Sandra Guaita Esteruelas, Didac Llop, Juan Moreno Vedia, Kepa Amillano, Jana Repkova, Lola Delamo, Josefa Girona, Luis Masana, Josep Gumà. Tumor microenvironment crosstalk generates a pro-inflammatory status in breast cancer patients.

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Article

Tumor microenvironment crosstalk generates a pro-inflammatory status in breast cancer patients

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Abstract: Adipose tissue and immune system are two of the principal elements in tumor microenvironment. Intercommunication between these components and tumor cells is crucial in cancer progression. Adipose tissue as the principal energy reservoir might transfer their lipidic content to those breast cancer cells. Although immune system is the first defense barrier against tumor cells, alterations in its behavior could revert this protective effect. In this study, we have analyzed the influence of tumor cells on these two elements. Tumor cells can enhance the delipidation and the releasement of FABP4 protein to the extracellular matrix, thus facilitating the lipid transfer from mature adipocytes to adjacent tissue elements, such as THP-1 monocytes. Moreover, the intercommunication between tumor cells and adipose tissue generates a pro-inflammatory microenvironment in mature adipocytes by the enhancement of the cytokine transcript levels of IL-6 and MCP-1, as well as produces a notably activation of the inflammatory pathways and cytokine production in the monocytic cell line. In vivo analysis demonstrated also that breast cancer patients' plasma presents an alteration in the C-reactive protein, which is accompanied by an increment in the glycoprotein Glyc-A. Moreover, both pro-inflammatory parameters are positively correlated with the higher plasma levels of FABP4 that we found in breast cancer patients compared to control healthy women. In addition, the increment of total triglycerides and triglycerides enrich-lipoprotein are positively correlated with the inflammatory parameters Glyc-A and C-reactive protein, hence suggesting that breast cancer patients present a pro-inflammatory status that might be mediated by the intercommunication between the different components of the tumor microenvironment, thus opening new possible target therapies in the breast cancer treatment.

Keywords: Tumor microenvironment, immunology, inflammation, FABP4, glycoproteins.

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1. Introduction

Breast cancer is the most common cancer in women and the second leading cause of all cancer related deaths[1]. Tumor cells form part of a complex microenvironment with fibroblasts, vascular and lymphatic cells, pericytes, adipocytes and immune system cells[2]. It has been shown that tumor cells and their microenvironment stablish a close communication that mediates and promotes tumor progression by the enhancement of different cancer hallmarks [3].Nevertheless, the communication is bidirectional, hence tumor cells

are able to modify the transcriptome, proteome and metabolome of different adjacent cells in their own benefit [4][5]. Adipose tissue, as one of the principal components of the mammary gland, has an important role in breast cancer progression. Adipose tissue is not only an energy reservoir. In fact, the endocrine capacity of the adipose tissue has been highlighted since adipocytes produce hormones, growth factors and adipokines that are involved in most of breast cancer processes such as initiation, progression and metastasis[6].

Peritumoral adipocytes are characterized by lipid droplet modification, distinct gene expression profile, overexpression of proinflammatory cytokines, higher browning activity, a decrease in adipocyte markers and a diminished size due to an increased lipolysis. These modified adipocytes are named cancer-associated adipocytes (CAAs)[6][7][8]. The production of different adipose tissue cytokines and adipokines are dysregulated in these CAAs compared to adipocytes from normal breast tissue. In fact, high levels of Interleukin-6 or the chemoattractant protein-1(MCP-1), C-C motif chemokine ligand 5(CCL-5) or leptin have been detected in breast adipose tissue[9]. Some of these cytokines have an important impact in the attraction and infiltration of immune system cells within the tumoral microenvironment, thus disrupting the normal functionality of the tissue[10]. Moreover, the increment of leptin production has been associated with increased angiogenesis, macrophage differentiation, inflammation and anti-apoptotic factors releasement[7][9].

According to bibliography, fatty acid transport proteins FABP4, FABP5 and CD36 have an important role in cancer progression by the activation of some cancer hallmarks [11] and the active transfer of fatty acids from adipose tissue to breast cancer cells (BCC)[8]. The alteration of adipokines and cytokines production take a great interest as a new viewpoint in the tumor microenvironment regarding inflammation and tumor progression.

According to previous laboratory results, high FABP4 circulating levels has been associated with an increased risk of breast cancer[12]. Moreover, a direct role of FABP4 in breast cancer cells have been demonstrated in different studies [13][14]. In addition, FABP4 is highly expressed by adipocytes, macrophages, and endothelial cells [15] Thus, this cytokine could be implicated in breast cancer progression due to its role in these cell types from the tumor microenvironment. FABP4 increases mature adipocyte lipolysis, whereas in macrophages, FABP4 is involved in the inflammatory regulation. Both, the proinflammatory setting and the increased lipid transportation rate enhances tumor progression[16]. Inflammation has acquired importance as a new cancer hallmark, and different studies try to underly the mechanisms by which the pro-inflammatory status might influence cancer progression. The link between obesity and inflammation is under study. Thus, adipose tissue produces adipokines and cytokines that attract and stimulate macrophages, producing an histological structure referred to as crown like structure (CLS) with an increased production of proangiogenic factors and proinflammatory cytokines[17][18].

Moreover, whiles lean adipose tissue is predominantly infiltrated by alternative activated M2 macrophages, obese adipose tissue contains an upregulation in the number CLS that are basically formed by proinflammatory M1 macrophages with an increased production of cytokines such as Interleukin-1ß (IL-1ß),Interleukin-6 (IL-6), Tumor Necrosis Factor-α (TNF-α)[19].

In addition, the alteration of adipocytes and their transformation to CAAs also alter the production of these cytokines, hence the presence of the immune system within the tumor microenvironment. In fact, macrophages in healthy lean women constitute 5-10% of the total adipose tissue environment, whereas in peritumoral adipose tissue there is a significant increment of these immune cells[20]. According to the classical biology, the immune system, as the principal barrier against extrinsic and intrinsic pathogens, would benefit

the recognition and elimination of tumor cells. However, tumor can modify the behavior of surrounding immune system cells, including infiltrated macrophages, becoming into tumor associated macrophages (TAMs)[21]. These modified cells might have a dual role in cancer progression, thus, depending on the extracellular signals, they can either kill tumor cells or promote their growth and invasiveness. In fact, substantial bibliography has demonstrated that infiltrated macrophages promote different pro-tumorigenic features such as angiogenesis, metastasis and motility [22][23]. Moreover, higher macrophage recruitment and infiltration is partially mediated by the chemokine CCL-2[24]. According to different studies there is a correlation between a higher macrophage infiltration and poor patient prognosis and survival[25][26].

Clinically, even though C-reactive protein (CRP) has been traditionally used as an inflammatory marker, new biomarkers have been described in the analysis of the pro-inflammatory status of the patients. The ^{1}H-NMR-measured glycoproteins Glyc-A and Glyc-B belong to the family of acute-phase proteins, which are synthetized and released by the liver in response to an acute or chronic inflammation[27]. Glyc-A and Glyc-B parameters indicate the glycation status and levels of five of the most abundant acute-phase inflammatory proteins, reflecting the patient inflammatory status[28][29].

Summarizing, the tumor microenvironment is a highly heterogenous complex of tissues and cell types continuously in contact and communication. This communication can activate diverse transcriptional and metabolic changes in the tumor microenvironment to induce tumor cell growth and metastasis. Adipose tissue is able to overactive its lipolysis and fatty acid transport proteins releasement in order to supply energy demands of tumor cells. In addition, they can generate a pro-inflammatory status to attract a high number of new immune system, such as macrophages, lymphocytes or natural killers. These infiltrated cells might have a positive impact in cancer progression. Knowledge of the different processes that underlined this tumor microenvironment communication might lead us to open new therapeutic strategies targeted to block the information exchange between tumor microenvironment and tumor cells, allowing a better patient treatment and survival.

2. Experimental Design

2.1. Cell culture

Human MDA-MB-231, MCF-7 and its drug resistant variants MCF-7-TAX^{R} and MCF-7-Epi^{R} breast cancer cells were originally provided by Dr. Eric Lam from the American Type Culture Collection (ATCC). Cell lines were routinely maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% penicillin streptomycin and 1% non-essential amino acids (Biowest, *Barcelona, Spain)* at 37°C with 5% CO_2.

3T3-L1 cells were obtained from the American Type Culture Collection (ATCC) and maintained in DMEM High Glucose with 10% FBS, 1% penicillin streptomycin and 1% non-essential amino acids at 37°C with 5% CO_2. For 3T3-L1 cells differentiation, cells were seeded onto 10 mm plates. Once the cells achieved confluency, they were cultured for 10 days in DMEM supplemented with 10% FBS, 1% penicillin/streptomycin, 1% Non-essential Amino Acid Solution, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), 1 μM dexamethasone and 10 μg/ml insulin from bovine pancreas (Sigma, Barcelona, Spain).

THP-1 monocytic cell line was maintained in RPMI1640 (BioWest, Barcelona, Spain) supplemented with 10% FBS, 1% penicillin streptomycin and 1% non-essential amino acids at 37°C with 5% CO_2.

2.2. Conditioned media obtainment

To obtain BC conditioned media, methodology described in the previous paper was followed [8]. Briefly, BCC lines were cultured as described above and once they reached 80% of confluence, they were deprived for 24 hours with 0,1% FBS DMEM-High Glucose.
For adipocyte conditioned media obtainment, 3T3-L1 cells were cultured and differentiated for 10 days as described above. Then, mature adipocytes were incubated with serum-starvation media for 24 hours, and supernatant was then cleared by centrifugation at 900 rpm and collected. In order to obtain the CAAs conditioned media, mature adipocytes were treated with different Breast Cancer CM for 3 days and media was collected. Control CAAs were obtained using a deprivation media. Then, media was collected, centrifugated at 900 rpm and supernatant was collected.

2.3. Western Blot analysis

THP-1 monocytic cells were lysed in RIPA buffer [0.5mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.1% SDS, 1% Nonidet P40, 0.5% sodium deoxycholic acid and protease and phosphatase inhibitors (Roche)]. Protein concentration was measured using the Bradford assay. Fifteen μg of protein were loaded and separated on electrophoresis gels (NuPage 10% Bis-Tris Gels) (Life Technologies, ThermoFisher, Barcelona, Spain). Then proteins were transfer to a Polyvinylidene Fluoride (PVDF) membrane (Immobilon-P) (Life Technologies) using a semidry iBlot2 transfer device (Life Technologies). PVDF membranes were blocked using Milk-TBS-Tween (5% non-fat dry milk in TBS 0.05% Tween) for 1 hour at RT. Primary antibodies were added to the PVDF membranes and incubated overnight at 4°C. The membranes were then washed 3x in TBST and incubated with secondary antibodies (Polyclonal Goat anti-rabbit, Polyclonal Rabbit anti-Goat or Polyclonal Goat anti-Mouse) (1:10.000) conjugated with peroxidase (HRP) (Dako, Sant Just Desvern, Spain). Signals were detected using chemiluminescent reagents (ECL Millipore reagent) by an Amersham Imager600 (GE Healthcare, Madrid, Spain) and analyzed using ImageQuanTL software (GE Healthcare).

Primary antibody	Dilution	Company	Reference
p38 MAPK	1/1000	Cell Signaling	#8690
P-p38 MAPK	1/1000	Cell Signaling	#4511
p65 NF-kB	1/1000	Santa Cruz Biotechnology	sc-109
P-p65 NF-kB	1/1000	Cell Signaling	#3033
FAPB4	1/1000	R&D Systems	AF3150
Actin	1/1000	Santa Cruz Biotechnology	sc-1616

Table 1. Primary antibodies used for Western Blot analysis

2.3. RNA extraction and RT-qPCR

RNA extraction was performed according to the PureLink RNA Mini Kit (Invitrogen, ThermoFisher Scientific, Barcelona, Spain) protocol and it was quantified using Synergy HT (BioTek, Swindon, UK). Total RNA (1 μg) was reverse-transcribed using the PrimeScript RT Reagent Kit (Takara Bio, Saint-Germain-en-Laye, France). Levels of mRNA were assessed using LightCycler96 device (Roche) with the Taqman probes for respective genes acquired from Life technologies.

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Gene	Company	Reference
Il-6	Life Technologies	Mm00446190_m1
Mcp1	Life Technologies	Mm00441242_m1
Tbp	Life Technologies	Mm01277042_m1
TNF-α	Life Technologies	Hs00174128_m1
MCP1	Life Technologies	Hs00234140_m1
IL-1β	Life Technologies	Hs01555410_m1
IL-6	Life Technologies	Hs00174131_m1
GADPH	Life Technologies	Hs99999905_m1

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Table 2. RNA probes for RT-qPCR 189

2.4. Nile Red assay 190

To assess the lipid uptake capability of THP-1 in different conditions, 500.000 monocytic 191 cells were seeded in 12 well plates. After serum-starvation, cells were cultured in different 192 conditions for 24 hours and then they were incubated with the lipophilic fluorescent dye 193 Nile Red (100ng/ml) (Sigma-Aldrich; Barcelona, Spain) diluted in PBS 1X for 5 min at room 194 temperature to visualize the lipid droplets. Cell images were captured using an Olympus 195 IX71 microscope and analyzed using the Image J software obtaining the intensity of each 196 image. 197

2.5. Studied population 198

In this study, we used a population cohort of 240 individuals, 171 breast cancer patients 199 and 69 control healthy women, to assess a glycoprotein profile and different standard 200 clinical biochemistry analysis. Control patients were selected from the same family, simi- 201 lar Body Mass Index (BMI), age and geographical region. This project was approved by 202 the Hospital Ethical Committee (reference number: 99-05-20/04-5), the subjects signed 203 their written consent to participate in the study and accepted the publication of the results. 204

2.6. Blood sample collection 205

After subject overnight fasting, blood extraction was performed, and serum aliquots were 206 stored at -80ºC in the Biobank of the Institut d'Investigació Sanitària Pere Virgili (IISPV) 207 until their use. 208

2.7. Biochemical analysis 209

Standard biochemical parameters were analyzed previously[12]. In addition, Lipoprotein 210 and low molecular weight analysis was also performed[30]. 211

2.8. Glycoprotein analysis by H-NMR 212

The serum samples were sent to Biosfer Teslab in dry ice for the NMR analysis. Briefly, 213 200 μL of the sample were transferred into NMR tubes with phosphate buffer. High-res- 214 olution 1H-NMR spectroscopy data was acquired on a Bruker 600 MHz spectrometer, 215 while 1D nuclear Overhauser effect spectroscopy (NOESY, 4 scans) and Carr–Purcell– 216 Meiboom–Gill (CPMG, 64 scans) analysis was used to characterize small molecules such 217 as amino acids and sugars. LED diffusion (Diff) experiments (32 scans) were used to detect 218 larger molecules such as lipoproteins and glycoproteins compounds. The region of the ^{1}H- 219 NMR spectrum where the glycoproteins resonate (2.15–1.90 ppm) was analysed using 220 several functions according to the chemical shift: Glyc-A and Glyc-B. For each function, 221

we determined the total area and transformed it to concentration according to the number of sugar–protein bonds. The area, height, position, and bandwidth and their ratios were also calculated. The concentrations of Glyc-A and Glyc-B provided the amount of acetyl groups of protein bond N-acetylglucosamine, N-acetylgalactosamine (Glyc-A), and N-acetylneuraminic acid (Glyc-B), the predominant sialic acid found. 222 223 224 225 226

2.9. Statistical analysis 227

Regarding *in vitro* statistical analysis, at least 3 different assays were performed. On the other hand, in vivo results are expressed as mean ± standard deviation (SD) for the normally distributed variables, as the median and the interquartile range (IQR) for the non-normally distributed variables and as frequencies for categorical data. Shapiro test was used to ensure that the data were normally distributed. T-test was used to deter-mine significant differences between normal variables. Mann-Whitney test was used to detect significant differences between non-normally distributed variables. Chi square[2] was used to assess differences between categorical data. Correlations were evaluated using Pearson's test for normally distributed data and Spearman's test for data that were not normally distributed. P-values < 0.05 were considered statistically significant. SPSS software (IBM SPSS Statistics, version 20.0, North Castle, New York, http:// www.ibm.com) and R Studio version 4.0.5 (RStudio Team (2020). RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/) were used to perform the statistical analyses. 228 229 230 231 232 233 234 235 236 237 238 239 240 241

3. Results 242

3.1. Adipocyte and CAAs conditioned media increase THP-1 lipid and FABP4 uptake. 243

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Free fatty acids can play a crucial role in the inflammatory processes in the immune system. In fact, the accumulation of lipids within the immune system cells generates a proinflammatory cascade. THP-1 cell line undergo accumulation of lipid droplets once was cultured with adipocyte CM (Fig 1A) after one hour of treatment. Interestingly, the accumulation of lipid droplets was even higher once the monocyte cell line THP-1 was cultured with the CM derived from some CAAs (MCF-7-TAX[R], and MCF-7-Epi[R]) (Fig 1A). 245 246 247 248 249 250

Moreover, the amount of cytoplasmatic FABP4 in THP-1 cells cultured with adipocyte CM was increased compared to control conditions. Interestingly, intracellular FABP4 was even higher when THP-1 cell line was cultured with CAAs CM (Fig 1B). 251 252 253

In addition, previous laboratory results have demonstrated that CAAs contain higher amounts of FABP4 than control adipocytes. Moreover, the transcript of FABP4 as well as its releasement was enhanced in CAAs [8]. These results suggest that FABP4 protein might have a role in the transport of lipids from mature adipocytes to the monocyte cell line THP-1. 254 255 256 257 258

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3.2. MCF-7 and MDA-MB-231 conditioned media increases proinflammatory gene expression in mature adipocytes 260 261

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Recent studies have demonstrated that BC microenvironment presents a proinflammatory status within the tissue. For this reason, we wanted to assess if the tumor cells might induce this proinflammatory status in the surrounding adipose tissue. 263 264 265

For this purpose, mature adipocytes were cultured with MCF-7 and MDA-MB-231 CM and, after 24 hours, *Mcp-1*, and *Il-6* transcript levels were analyzed. Consequently, a significant increment of both transcripts was observed after treatment (Fig 2). 266 267 268

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3.3. Adipocyte and CAAs conditioned media promote a proinflammatory status in THP-1 monocytes. 270 271

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Monocytes are the precursor of infiltrated macrophages and tumor microenvironment generates a specific niche for they attraction and infiltration. Adipose tissue releases a high number of cytokines and adipokines to the extracellular matrix. Both processes are notably underlined, and their combination is essential for cancer progression. To assess how alteration of mature adipocytes and CAAs from tumor microenvironment might influence in the releasement of different factors, monocyte cell line THP-1 was grown with adipocyte or CAA CM and proinflammatory transcripts IL-6 and IL-1ß were measured. Monocytes cultured with adipocyte CM have a significant increment of both transcripts compare to control conditions. This increase was even higher once THP-1 cells were cultured with the different CAAs CM (Fig 3A).

Furthermore, two proteins closely related with the inflammation process, P65-NFKB and p38-MAPK, were analyzed. The phosphorylated forms of these proteins were increased under adipocyte CM condition. Furthermore, CAAs CM produces a significant increment of their phosphorylation compared those cultured only with adipocyte CM (Fig 3B).

3.4. Adipocyte and CAAs CM enhance monocyte activation and differentiation to M1 macrophages

Monocytes are the macrophages precursors, and its infiltration is essential for cancer progression. Adipose tissue as an endocrine organ produces high number of cytokines, including the chemoattractive cytokines IL-6 and MCP-1. The above results suggest that the peritumoral adipose tissue might influence the infiltration and posterior inflammation of monocytes. A methodologic approach to study the activation and the inflammatory status of monocytes is to identify their differentiation into macrophages by their attachment to the plate, hence their morphological alteration.

To analyze in vitro differentiation of THP-1 to M1 macrophages, the number of monocytes attached to the plate and with modified morphology were analyzed in the different treatment conditions. In control condition there were no attachment of monocytes to the plate, missing any type of differentiation. However, once monocytes were cultured with adipocyte CM an increment in the attachment and an alteration in the morphology was observed, appearing to become into macrophages. Interestingly, the treatment of THP-1 monocytes with CAA CM resulted in a significant increase in the monocytes attachment and in a substantial morphological modification, compared to those monocytes treated only with adipocyte CM (Fig 4).

3.5. Glycoproteins related to inflammation are increased in breast cancer patients and are correlated with an increment in plasma levels of C-reactive protein.

Traditionally CRP was a clinical marker widely used to describe inflammation in blood samples. Actually, the BC patients of this study showed an increased amount of CRP marker compared to control healthy women[12]. Nowadays, new metabolites and different compounds can be analyzed to discern a metabolic disruption, hence a specific disease. By ^{1}H-NMR, breast cancer patient serum was analyzed to describe new possible inflammatory biomarkers associated to breast cancer patients.

Glyc-A and Glyc-B, as a reflection of the levels and glycation of 5 of the most abundant acute phase proteins were used to understand the inflammatory status of these patients. Interestingly, breast cancer patients presented an altered glycoprotein pattern in plasma compared to control women. In fact, increased Gly-A as well as of Glyc-B were found in breast cancer patient serum compared to control healthy women (Fig 5A). Moreover, correlation analysis was also performed in both glycoproteins Glyc-A and Glyc-B with the CRP, and significant positive associations were observed (ϱ=0.37, $p<0.0001$ and ϱ=0.22, $p<0.01$ respectively) (Fig 5B).

3.6. Glyc-A is correlated with the increment of FABP4 plasma levels in breast cancer patients.

According to the biochemistry of these patients, an altered concentration plasma FABP4 was found in BC patients[12]. Interestingly, positive correlation was found between plasma levels of FABP4 and the ^{1}H-NMR-measured glycoprotein Glyc-A (ϱ=0.28, p<0.0001). Furthermore, this fatty acid transport protein presented also a positive correlation with the CRP (ϱ=0.36, p<0.0001) (Fig 6). However, we did not observe a significant correlation between FABP4 and Glyc-B (ϱ=0.025, p<0.74) (data not shown).

3.7. Free triglycerides and triglyceride-enriched lipoproteins were positively correlated with Glyc-A in breast cancer patients

Previous results showed a significant increase in plasma triglycerides levels in breast cancer patients compared to control healthy women. Moreover, BC patients of this cohort had an alteration in the composition of the plasmatic lipoproteins[30]. VLDL, IDL, LDL and HDL were notably enriched in triglycerides compared to control healthy women particles. Interestingly, BC patients' triglycerides plasma concentration correlated with the levels of ^{1}H-NMR glycoprotein Glyc-A (ϱ=0.53, p<0.0001) (Fig 7A). In addition, the triglycerides enriched particles, VLDL-TG, IDL-TG, LDL-TG and HDL-TG, had positive correlation with the plasmatic levels of Glyc-A in BC patients (ϱ=0.52, p<0.0001; ϱ=0.34, p<0.0001, ϱ=0.23, p=0.001, ϱ=0.21, p=0.004 respectively) (Fig 7B).

Fig1. **Adipocyte and CAAs conditioned media increase lipid uptake and FABP4 incorporation in the monocytic cell line THP-1.** (A) Immunofluorescent images (10X magnification) of THP-1 monocytes stained for fatty acid using Nile Red satin. Nile Red staining graphs compare the accumulation of lipid droplets on THP-1 cells after 1 hour of culture with control, adipocyte CM, and CAAs conditioned media, where we could observe an increment of the lipid uptake after the culture with adipocyte and CAAs CM. (B) Immunoblots representation for FABP4 of THP-1 after their culture with the different mentioned conditions. There is a significant increment in the amount of this protein within the cytoplasm of the monocytic cell line after their culture with adipocyte CM, being higher after their culture with the different CAAs CM. The results are expressed as the mean ± SEM of at least three independent experiments. A p- value < 0.05 is considered to be statistically significant. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

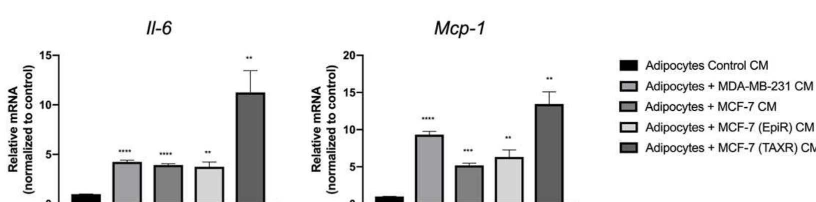

Fig2. Breast Cancer Cell Conditioned Media increases the transcript levels of *Il-6* and *Mcp-1* in mature adipocytes. Graph representation of *Il-6* and *Mcp-1* mRNA transcripts of mature adipocytes after their culture with different BCC CM (MDA-MB-231, MCF-7, MCF-7-Epi[R], and MCF-7-TAX[R]). Mature adipocytes suffered and increment in the transcript levels of both pro-inflammatory cytokines. The results are expressed as the mean ± SEM of three independent experiments. A p- value < 0.05 is considered to be statistically significant. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001.

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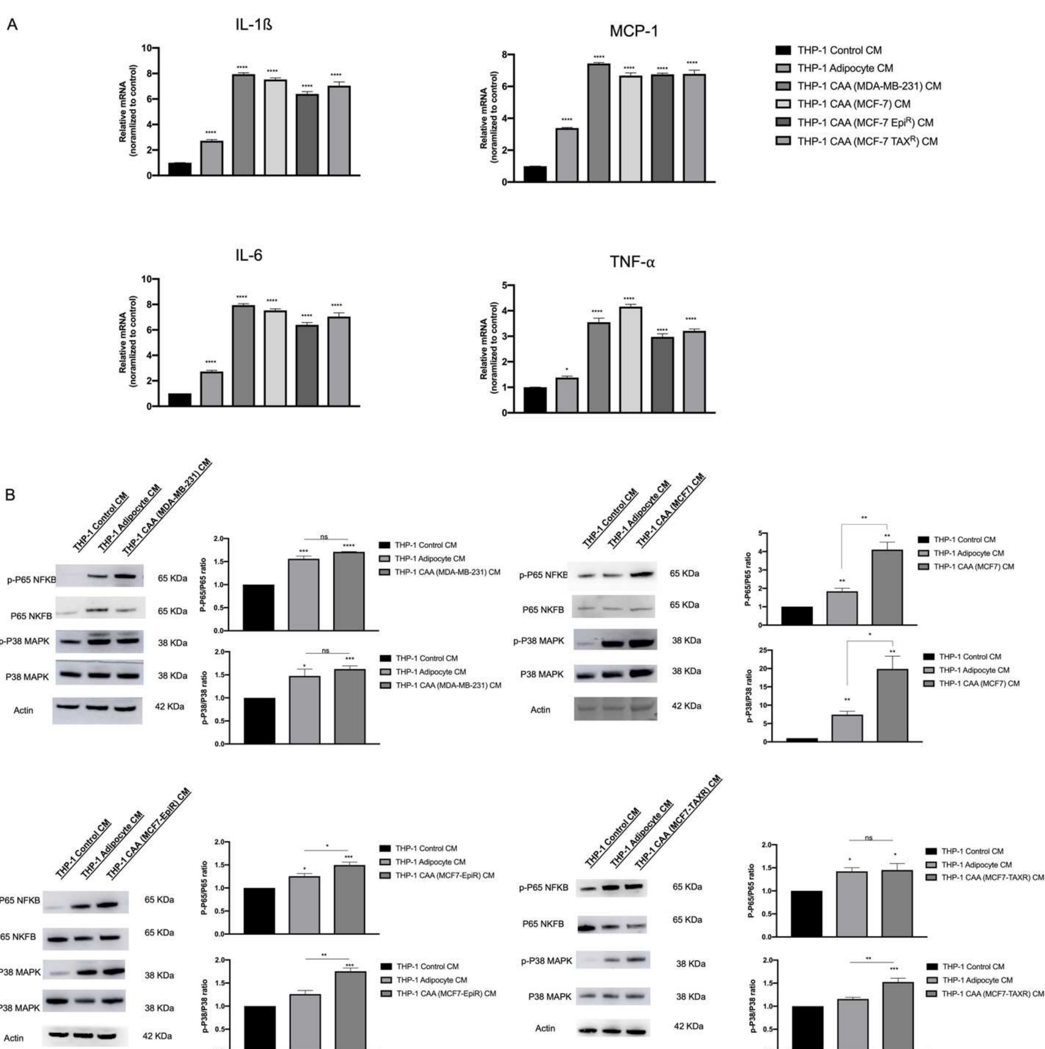

Fig3. Adipocyte and CAAs conditioned media promote a pro-inflammatory status in the monocytic cell line THP-1. (A) Graph representation of *IL-1ß*, *IL-6*, *TNF-α* and *MCP-1* mRNA transcripts of thp-1 cell line monocytes after their culture in different conditions (control conditioned media, adipocyte conditioned media or the different CAA conditioned media, derived from the culture of adipocytes with MDA-MB-231, MCF-7, MCF-7-Epi^R, or MCF-7-TAX^R conditioned media). The monocytic cell line THP-1 suffered and increment in the transcript levels of all the pro-inflammatory factors. (B) Immunoblots representation for NF-Kb, MAPK p38 and their phosphorylated forms in THP-1 monocytes after their culture with the different conditions previously described. There is an increment in the phosphorylation rate after their culture with adipocyte CM. However, this increment is significatively higher after their culture with all the different CAAs conditioned media The results are expressed as the mean ± SEM of three independent experiments. A p- value < 0.05 is considered to be statistically significant. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. 371 372 373 374 375 376 377 378

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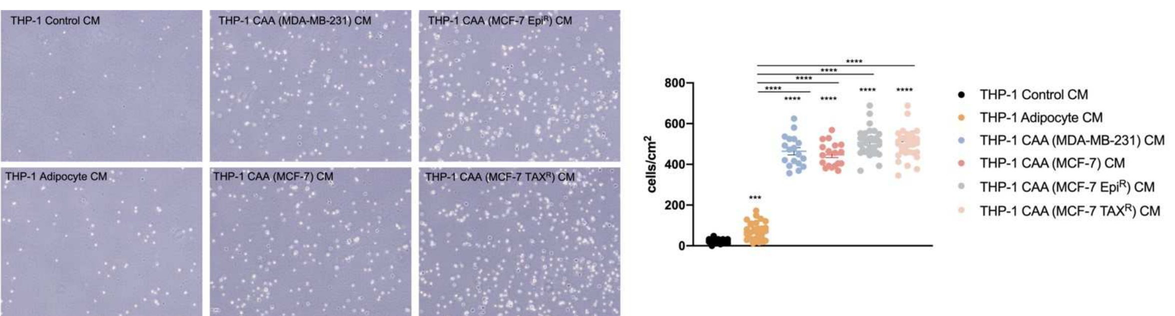

Fig4. Adipocyte and CAAs conditioned media activate monocyte THP-1 cells, increasing their plate attachment. Bright microscopic images (4x magnification) and graph representation of the amount of THP-1 monocytes that have attached to the plate after their culture with different conditions (control conditioned media, adipocyte conditioned media or the different CAA conditioned media, derived from the culture of adipocytes with MDA-MB-231, MCF-7, MCF-7-Epi[R], or MCF-7-TAX[R] conditioned media). Those monocytes that were cultured with adipocyte CM suffered an increment in the attachment to the plate after 24 hours. Moreover, it was enhanced after their culture with the different CAA CM mentioned before. The results are expressed as individual dots ± SEM of different independent experiments. A p- value < 0.05 is considered to be statistically significant. *p<0.05, **p<0.01, ***p<0.001, and ****p<0.0001. 380 381 382 383 384 385 386

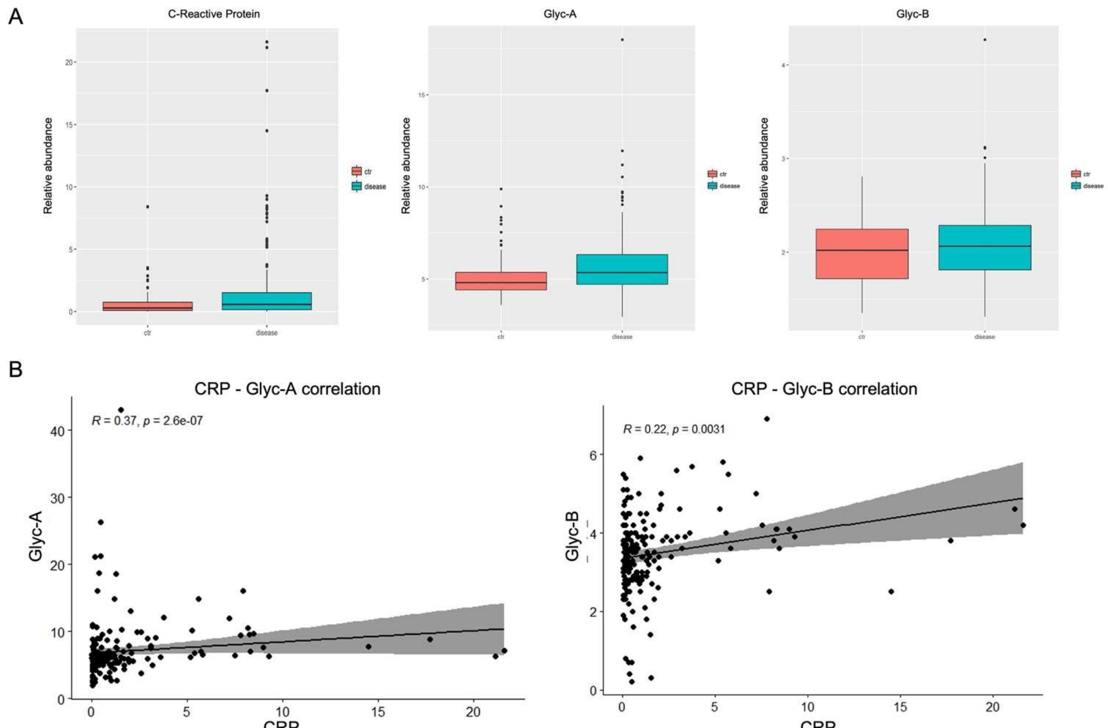

Fig5. Breast Cancer patients have an altered plasma pro-inflammatory biomarker pattern. (A) Blot representation of the acute pro-inflammatory biomarker C-Reactive Protein (CRP) and the glycoproteins Glyc-A and Glyc-B of control healthy women versus breast cancer patients, where an increment of all three parameters is presented in the plasma of the breast cancer patients compared to the control population. (B) Simple regression representation of the glycoproteins Glyc-A and Glyc-B versus the CRP parameter. There is a correlation of the 37% of the cases where an increment of Glyc-A could be explained with an increment of CRP, whiles a 22% of cases where an increment of Glyc-B could be explained with the increment of the CRP. 387 388 389 390 391 392

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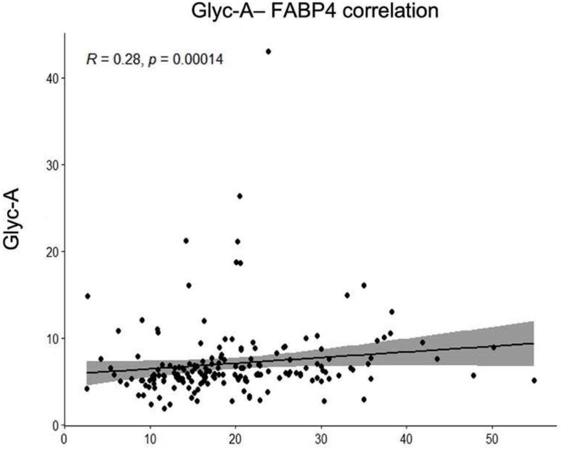

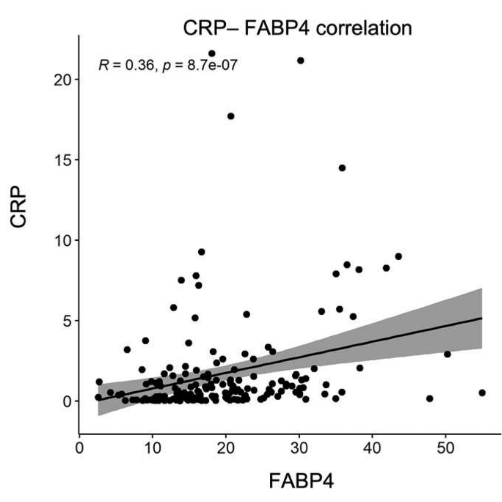

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Fig6. The pro-inflammatory biomarkers Glyc-A and CRP are positively correlated with FABP4 plasma levels in BC patients. Representation of a simple regression where is compared plasma levels of FABP4 versus CRP, as well as FABP4 versus Glyc-A. There is a positive correlation between breast cancer patients FABP4 and Glyc-A plasmatic levels (ϱ=0.28, p<0.0001), as well as between FABP4 and CRP (ϱ=0.36, p<0.0001). 396 397 398

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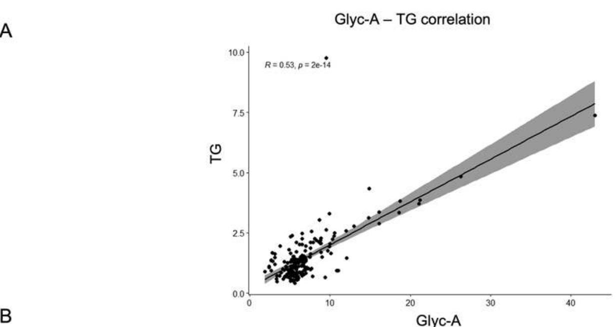

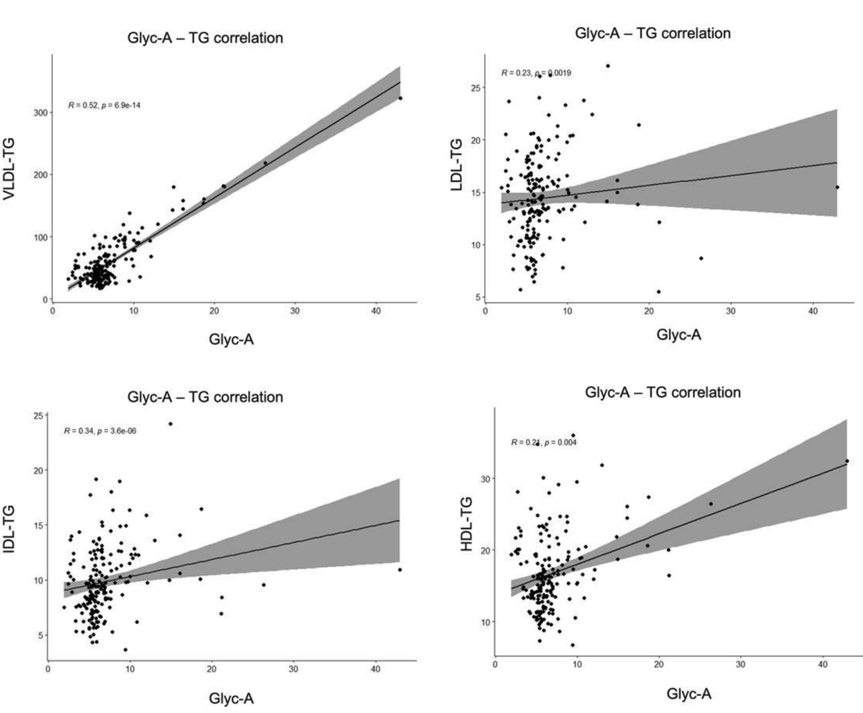

Fig7. There is a positive correlation between breast cancer patient Glyc-A plasmatic levels and the triglycerides composition of the different plasmatic lipoproteins. (A) Simple regression where the correlation between total amount of plasmatic triglycerides and Glyc-A is measured, where a positive correlation was found (ϱ=0.53, p<0.0001). (B) Simple regression where the correlation between Glyc-A plasmatic levels and the triglycerides composition of the different plasmatic lipoproteins (VLDL, IDL, LDL, and HDL) is measured. There is a positive correlation of this glycoprotein and the triglycerides enrichment of the lipoproteins (ϱ=0.52, p<0.0001; ϱ=0.34, p<0.0001, ϱ=0.23, p=0.001, ϱ=0.21, p=0.004 respectively). 406 407 408 409 410

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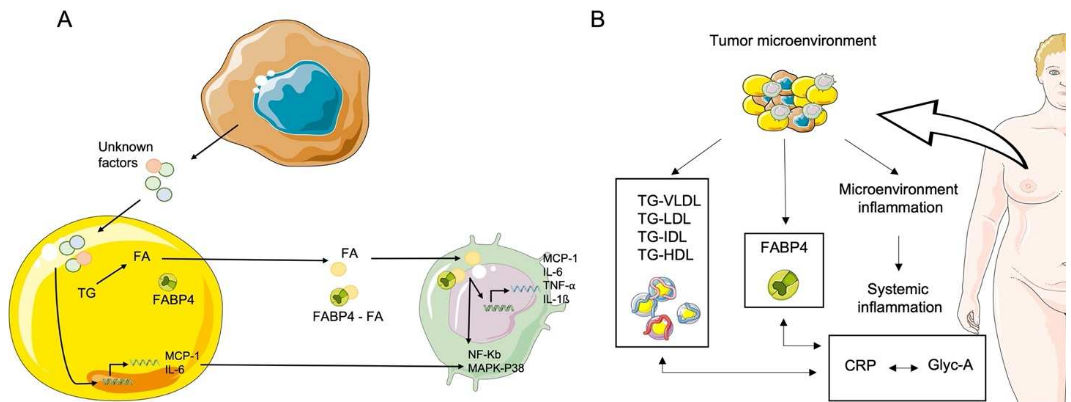

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Fig8. (A**) Schematic representation of in tumor microenvironment crosstalk between tumor cells, adipose tissue, and immune system monocytes.** Tumor cells are able to modify the behavior of mature adipocytes by the enhancement the pro-inflammatory transcripts MCP-1 and IL-6. Furthermore, they induce the lipid droplets delipidation, as well as an increasement of fatty acid releasement both in free form and attached to FABP4, a fatty acid transport protein that is also increased by the tumor cell influence. These molecules modify the behavior of the adjacent immune system cells, specially activating the inflammatory response in monocytes by the induction of pro-inflammatory transcripts such as MCP-1, IL-6, IL-1ß, and TNF-α, as well as by the phosphorylation of the pro-inflammatory proteins NK-kb and MAPK-p38. **(B) Schematic representation of the relationship between different plasma biomarkers in breast cancer patients.** Tumor microenvironment induce a pro-inflammatory tissue status, thus enhancing different pro-inflammatory cytokines and chemokines. This is reflected by an increasement of the C-Reactive Protein (CRP), which is positively associated with the increment of the acute phase glycoprotein-A (Glyc-A). Plasma levels of FABP4 are also significantly increased in BC patients and they are positively correlated with CRP and Glyc-A. Moreover, the lipoprotein profile and composition are altered in BC, thus enriched in triglycerides. This alteration is positively correlated with CRP and Glyc-A hence with the inflammatory status of the patient. 420 421 422 423 424 425 426 427 428 429 430

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4. Discussion 432

Adipose tissue is one of the main components of the mammary gland, thus the plausible effects of this endocrine organ in cancer progression have taken special attention in the tumor microenvironment field. Actually, there is a substantial exchange of information between tumor cells and each one of the different components of the tumor microenvironment, including the adipose tissue cells. In fact, by the releasement of certain unknown factors, tumor cells are able to modify surrounding adipocytes in their own benefit[4]. Previous laboratory results have demonstrated that BCCs enhance mature adipocyte delipidation, hence acquiring a more fibroblastic phenotype[8]. Different studies have demonstrated that these alterations are part of a modification process that leads to their transformation into CAAs and promotes cancer progression [31][32]. In addition, we have also demonstrated that BCCs increase the releasement of certain fatty acid transport proteins such as FABP4, FABP5 and CD36, hence mediating the lipid transfer from adipocytes to tumor cells[8]. Moreover, the addition of exogenous FABP4 had an impact in breast cancer cell lines MCF-7 and MDA-MB-231 progression, increasing their growth[11]. According to these results, it is plausible to think that this protein might have importance in the tumor microenvironment and cancer progression. Actually, FABP4 has a dual target in breast cancer progression. According to unpublished laboratory results the effects of this protein within the tumor cells include the enhancement of different cancer hallmarks of cancer such as proliferation, migration, or invasiveness. However, the role of this protein is not only limited to the tumor cells, but to the different components of the tumor microenvironment, including adipocytes and macrophages, where this protein is highly expressed. In this study we have focused on the intercommunication between adipocytes, tumor cells and infiltrated macrophages and the role of FABP4 in the process. 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455

Firstly, once the monocytic cell line THP-1 was cultured with adipocyte CM we observed a significant increment in the number of lipid droplets in THP-1 cytoplasm. Interestingly, 456 457

in those THP-1 cultured with the CM from CAAs, the accumulation of lipid droplets was even higher. According to these results, we also observed that those monocytes cultured with adipocyte CM had internalized a high amount of FABP4, but this incorporation was higher once they were cultured with CAAs conditioned media. The role of FABP4 on lipid accumulation in macrophages has been demonstrated previously in atherosclerosis[33], hence suggesting a plausible common role in this disease and cancer progression. This accumulation of lipids is closely related with chronic inflammation in obesity[34]. Moreover, some studies have demonstrated that adipose tissue suffered an alteration in the inflammatory status in obese individuals[35]. Furthermore, an increased chronic inflammatory status has been described in cancer progression [36], thus leading to a major infiltration of new immune system cells within the tumor microenvironment that conduct to worse prognosis. Consequently, the relationship between adipose tissue and the immune system seems to be crucial in the development of certain metabolic diseases and cancer by the generation of a chronic inflammation status.

Hence, in this study we wanted to assess the potential role of this inter-communication between adipocytes, tumor cells, and infiltrated macrophages in the generation of an inflammatory status. Firstly, we observed that those adipocytes that were cultured with BCC conditioned media increase the transcript levels of *Il-6* and *Mcp-1*. These cytokines are crucial in the formation of a pro-inflammatory status because of their implication in the extravasation and infiltration of different immune system cells as well as their role in immune system activation and cancer progression[37][38][39].

Once we have demonstrated that mature adipocytes acquired a more inflammatory profile, our next goal was to analyze the effects of these changes in the behavior of infiltrated macrophages. Accordingly, the transcript levels of MCP-1 and IL-6 were analyzed in those monocytes cultured with adipocyte and CAAs conditioned media. Moreover, two more cytokine transcripts were measured, TNF-α and IL-1ß. Monocytes cultured with adipocyte conditioned media suffered an increment in the level of all four transcripts compared to those monocytes cultured in control conditions. Interestingly, this increment in the pro-inflammatory cytokine transcripts was higher once these monocytes were cultured with different CAAs conditioned media compared to control conditions. Moreover, they were even higher compared to these transcript levels of THP-1 monocytes cultured with adipocyte conditioned media. In addition, by the analysis of two of the most important proteins related to inflammation in monocytes, MAPK p38 and NF-kb p65, we assessed the inflammation activation of these monocytes. Interestingly, both proteins suffered a higher phosphorylation, hence a higher activation once they were cultured with CAA conditioned media.

There is evidence that shows an increment in the number of infiltrated macrophages in obese animals and humans[40]. In addition, these infiltrated macrophages, by the activation of different cancer pathways, modulate cancer cell functions[41], thus suggesting that macrophages may mediate obesity-induced tumor progression. Actually, the density of tumor associated macrophages is closely associated with a poor prognosis in cancer[26]. The dual role of immune system in cancer progression might be controversial. In fact, according to bibliography, a ying-yang status exists in the inflammatory grade of tumor microenvironment, where both conditions coexist[42]. For many years, obesity was described to produce a polarization of infiltrated macrophages to a M1 phenotype. Nevertheless, a recent study has demonstrated by flow cytometry that both phenotypes coexist in infiltrated macrophages[43]. In this study we have detected that those monocytes that were cultured with adipocyte CM suffered a slight polarization to M1 macrophages, by attaching to the plate and modifying their morphology. However, once we culture these monocytes with CAA conditioned media where the amount of pro-inflammatory factors such as IL-6 and MCP-1 are notably increased, we observed that there was an increment

in the number of monocytes that have attached to the plate and have polarized to macrophages. 509 510

These results show that the communication between adipocytes and tumor cells increases the delipidation of mature adipocytes, hence the releasement of free fatty acids accoupled to different transport proteins such as FABP4 and that increases to the accumulation of fatty acids into the monocytic cell line THP-1. Thus, leads to monocyte activation to and the generation of a pro-inflammatory microenvironment status (Fig 8A). 511 512 513 514 515

Once *in vitro* analysis suggested a possible microenvironment inflammation, the next step was to analyze the chronic inflammatory status in breast cancer patients. Actually, an altered glycoprotein pattern was detected in breast cancer patients by ^{1}H-NMR. BC patients presented higher levels of C-reactive protein in serum compared to control healthy women. Interestingly, this protein was positively correlated to the plasmatic glycoproteins Glyc-A and Glyc-B. These plasma glycoproteins belong to the family of acute phase proteins, being released by the liver under a pro-inflammatory status [44][27]. Consequently, these findings suggest that these parameters might be a novel inflammatory markers. 516 517 518 519 520 521 522 523 524

Several studies has also deeply studied the relationship between inflammation and cancer[45]. In addition, a close relationship between different diseases, including cancer, and the presence of high levels of Gly-A in plasma have been described[46]. Accordingly, these results support the idea of the presence of an acute inflammatory status in breast cancer, orchestrated by the intercommunication between different microenvironment tissue cell types. Moreover, as we had previously described, THP-1 monocytes present a high amount of FABP4 in their cytoplasm. According to the important association of this fatty acid transport protein in different diseases, including atherosclerosis[47],[48] or even kidney disease[49], FABP4 might be correlated with the inflammatory status in breast cancer patients. Previous analysis of our BC patients revealed that they had incremented this protein in plasma compared to control healthy women[12]. After a simple linear correlation analysis, we detected that the 28% of the cases that presented an increment of Glyc-A could be explained with an increment in FABP4 plasma levels. These results suggested that this protein might have a crucial role in the generation of the inflammatory status, hence in the development and progression of cancer. 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539

The alteration of free fatty acids in cancer has been deeply studied. In fact, different studies have demonstrated that FFA levels were notably increased in patients with lung, gastric, thyroid, rectal, colon and ovarian cancer[50]. Furthermore, we had previously described that BC patients also present a higher plasma level of triglycerides, cholesterol, and non-esterified fatty acids. Obesity is another disease where the levels of triglycerides and different lipid species is altered. In addition, different studies have correlated this disease with an inflammation status in the studied population [51][52][53]. 540 541 542 543 544 545 546

Interestingly, our BC patients present an alteration in plasmatic levels of Gly-A compared to control healthy women. According to the relationship of this glycoprotein and inflammation and the correlation of the plasma levels of triglycerides and chronic inflammation mentioned above, there might exist a correlation of both diseases. Accordingly, after a simple correlation analysis of both parameters, we observed that there was a correlation of a 53% of cases where an increment of the Glyc-A plasma levels could be explained with the increment of total plasma triglycerides. Furthermore, previous laboratory results showed that breast cancer patients have an increment in plasmatic free fatty acids and triglycerides, both total and those contained within the lipoproteins IDL, VLDL, and HDL, thus suggesting that they might have a role in cancer progression[30]. Moreover, it has been demonstrated a correlation between obesity and chronic inflammation[54], 547 548 549 550 551 552 553 554 555 556 557

meanwhile it has been also described an increment in total triglycerides as well as of the triglycerides-enriched lipoproteins in obese individuals, hence our results have demonstrated that there is positive correlation between chronic inflammation and the presence of an enrichment of triglycerides in breast cancer patients (Fig 8B). 558 559 560 561

Summarizing, the communication within the tumor microenvironment is crucial in the development of cancer by the generation of an inflammatory status that might facilitate cancer progression, hence the understanding of the relationship and the communication mechanisms underlined between tumor cells, adipose tissue and immune system would allow us to investigate new possible therapies. 562 563 564 565 566

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References 586

1. Harbeck, N.; Penault-Llorca, F.; Cortes, J.; Gnant, M.; Houssami, N.; Poortmans, P.; Ruddy, K.; Tsang, J.; Cardoso, F. Breast cancer. *Nat. Rev. Dis. Prim.* **2019**, *5*, 66, doi:10.1038/s41572-019-0111-2. 587 588
2. Balkwill, F.R.; Capasso, M.; Hagemann, T. The tumor microenvironment at a glance. *J. Cell Sci.* **2012**, *125*, 5591–5596, doi:10.1242/jcs.116392. 589 590
3. Wang, M.; Zhao, J.; Zhang, L.; Wei, F.; Lian, Y.; Wu, Y.; Gong, Z.; Zhang, S.; Zhou, J.; Cao, K.; et al. Role of tumor microenvironment in tumorigenesis. *J. Cancer* 2017, *8*, 761–773. 591 592
4. Wu, Q.; Li, B.; Li, Z.; Li, J.; Sun, S.; Sun, S. Cancer-associated adipocytes: Key players in breast cancer progression. *J. Hematol. Oncol.* 2019, *12*, 85–97. 593 594
5. Houthuijzen, J.M.; Jonkers, J. Cancer-associated fibroblasts as key regulators of the breast cancer tumor microenvironment. *Cancer Metastasis Rev.* **2018**, *37*, 577–597, doi:10.1007/S10555-018-9768-3. 595 596
6. Dirat, B.; Bochet, L.; Dabek, M.; Daviaud, D.; Dauvillier, S.; Majed, B.; Wang, Y.Y.; Meulle, A.; Salles, B.; Le Gonidec, S.; et al. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer Res.* **2011**, *71*, 2455–2465, doi:10.1158/0008-5472.CAN-10-3323. 597 598 599
7. Wang, F.; Gao, S.; Chen, F.; Fu, Z.; Yin, H.; Lu, X.; Yu, J.; Lu, C. Mammary fat of breast cancer: Gene expression profiling and functional characterization. *PLoS One* **2014**, *9*, e109742, doi:10.1371/journal.pone.0109742. 600 601
8. Adriá-Cebrián, J.; Guaita-Esteruelas, S.; Lam, E.W.-F.; Rodríguez-Balada, M.; Capellades, J.; Girona, J.; Jimenez-Santamaria, A.M.; 602

Yanes, O.; Masana, L.; Gumà, J. Mcf-7 drug resistant cell lines switch their lipid metabolism to triple negative breast cancer signature. *Cancers (Basel).* **2021**, *13*, 5871, doi:10.3390/cancers13235871. 603 604
9. Picon-Ruiz, M.; Pan, C.; Drews-Elger, K.; Jang, K.; Besser, A.H.; Zhao, D.; Morata-Tarifa, C.; Kim, M.; Ince, T.A.; Azzam, D.J.; et al. Interactions between adipocytes and breast cancer cells stimulate cytokine production and drive Src/Sox2/miR-302b-mediated malignant progression. *Cancer Res.* **2016**, *76*, 491–504, doi:10.1158/0008-5472.CAN-15-0927. 605 606 607
10. An, G.; Wu, F.; Huang, S.; Feng, L.; Bai, J.; Gu, S.; Zhao, X. Effects of CCL5 on the biological behavior of breast cancer and the mechanisms of its interaction with tumor-associated macrophages. *Oncol. Rep.* **2019**, *42*, 2499–2511, doi:10.3892/or.2019.7344. 608 609
11. Guaita-Esteruelas, S.; Bosquet, A.; Saavedra, P.; Gumà, J.; Girona, J.; Lam, E.W.F.; Amillano, K.; Borràs, J.; Masana, L. Exogenous FABP4 increases breast cancer cell proliferation and activates the expression of fatty acid transport proteins. *Mol. Carcinog.* **2017**, *56*, 208–217, doi:10.1002/mc.22485. 610 611 612
12. Guaita-Esteruelas, S.; Saavedra-García, P.; Bosquet, A.; Borràs, J.; Girona, J.; Amiliano, K.; Rodríguez-Balada, M.; Heras, M.; Masana, L.; Gumà, J. Adipose-Derived Fatty Acid-Binding Proteins Plasma Concentrations Are Increased in Breast Cancer Patients. *Oncologist* **2017**, *22*, 1309–1315, doi:10.1634/theoncologist.2016-0483. 613 614 615
13. Herroon, M.K.; Rajagurubandara, E.; Hardaway, A.L.; Powell, K.; Turchick, A.; Feldmann, D.; Podgorski, I. Bone marrow adipocytes promote tumor growth in bone via FABP4-dependent mechanisms. *Oncotarget* **2013**, *4*, 2108–2123, doi:10.18632/oncotarget.1482. 616 617
14. McKillop, I.H.; Girardi, C.A.; Thompson, K.J. Role of fatty acid binding proteins (FABPs) in cancer development and progression. *Cell. Signal.* **2019**, *62*, doi:10.1016/J.CELLSIG.2019.06.001. 618 619
15. Furuhashi, M.; Hotamisligil, G.S. Fatty acid-binding proteins: Role in metabolic diseases and potential as drug targets. *Nat. Rev. Drug Discov.* 2008, *7*, 489–503. 620 621
16. Zeng, J.; Sauter, E.R.; Li, B. FABP4: A New Player in Obesity-Associated Breast Cancer. *Trends Mol. Med.* 2020, *26*, 437–440. 622
17. Kanda, H.; Tateya, S.; Tamori, Y.; Kotani, K.; Hiasa, K.I.; Kitazawa, R.; Kitazawa, S.; Miyachi, H.; Maeda, S.; Egashira, K.; et al. MCP-1 contributes to macrophage infiltration into adipose tissue, insulin resistance, and hepatic steatosis in obesity. *J. Clin. Invest.* **2006**, *116*, 1494–1505, doi:10.1172/JCI26498. 623 624 625
18. Fortner, R.T.; Katzke, V.; Kühn, T.; Kaaks, R. Obesity and breast cancer. In *Recent Results in Cancer Research*; Recent Results Cancer Res, 2016; Vol. 208, pp. 43–65. 626 627
19. Lumeng, C.N.; Bodzin, J.L.; Saltiel, A.R. Obesity induces a phenotypic switch in adipose tissue macrophage polarization. *J. Clin. Invest.* **2007**, *117*, 175–184, doi:10.1172/JCI29881. 628 629
20. Agurs-Collins, T.; Ross, S.A.; Dunn, B.K. The Many Faces of Obesity and Its Influence on Breast Cancer Risk. *Front. Oncol.* 2019, *9*. 630
21. Dehne, N.; Mora, J.; Namgaladze, D.; Weigert, A.; Brüne, B. Cancer cell and macrophage cross-talk in the tumor microenvironment. *Curr. Opin. Pharmacol.* 2017, *35*, 12–19. 631 632
22. Su, S.; Liu, Q.; Chen, J.; Chen, J.; Chen, F.; He, C.; Huang, D.; Wu, W.; Lin, L.; Huang, W.; et al. A Positive feedback loop between mesenchymal-like cancer cells and macrophages is essential to breast cancer metastasis. *Cancer Cell* **2014**, *25*, 605–620, doi:10.1016/j.ccr.2014.03.021. 633 634 635
23. Noy, R.; Pollard, J.W. Tumor-Associated Macrophages: From Mechanisms to Therapy. *Immunity* 2014, *41*, 49–61. 636
24. Qian, B.Z.; Li, J.; Zhang, H.; Kitamura, T.; Zhang, J.; Campion, L.R.; Kaiser, E.A.; Snyder, L.A.; Pollard, J.W. CCL2 recruits inflammatory monocytes to facilitate breast-tumour metastasis. *Nature* **2011**, *475*, 222–225, doi:10.1038/nature10138. 637 638
25. Chen, J.J.W.; Lin, Y.C.; Yao, P.L.; Yuan, A.; Chen, H.Y.; Shun, C.T.; Tsai, M.F.; Chen, C.H.; Yang, P.C. Tumor-associated macrophages: The double-edged sword in cancer progression. *J. Clin. Oncol.* **2005**, *23*, 953–964, doi:10.1200/JCO.2005.12.172. 639 640
26. Ryder, M.; Ghossein, R.A.; Ricarte-Filho, J.C.M.; Knauf, J.A.; Fagin, J.A. Increased density of tumor-associated macrophages is associated with decreased survival in advanced thyroid cancer. *Endocr. Relat. Cancer* **2008**, *15*, 1069–1074, doi:10.1677/ERC-08-0036. 641 642
27. Gabay, C.; Kushner, I. Acute-Phase Proteins and Other Systemic Responses to Inflammation. *N. Engl. J. Med.* **1999**, *340*, 448–454, doi:10.1056/nejm199902113400607. 643 644

28. Fuertes-Martín, R.; Correig, X.; Vallvé, J.C.; Amigó, N. Title: Human serum/plasma glycoprotein analysis by1H-NMR, an emerging method of inflammatory assessment. *J. Clin. Med.* 2020, *9*. 645 646
29. Connelly, M.A.; Otvos, J.D.; Shalaurova, I.; Playford, M.P.; Mehta, N.N. GlycA, a novel biomarker of systemic inflammation and cardiovascular disease risk. *J. Transl. Med.* 2017, *15*. 647 648
30. Gumà, J.; Adriá-Cebrián, J.; Ruiz-Aguado, B.; Albacar, C.; Girona, J.; Rodríguez-Calvo, R.; Martínez-Micaelo, N.; Lam, E.W.F.; Masana, L.; Guaita-Esteruelas, S. Altered serum metabolic profile assessed by advanced 1h-nmr in breast cancer patients. *Cancers (Basel).* **2021**, *13*, 4281, doi:10.3390/cancers13174281. 649 650 651
31. Nieman, K.M.; Romero, I.L.; Van Houten, B.; Lengyel, E. Adipose tissue and adipocytes support tumorigenesis and metastasis. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 2013, *1831*, 1533–1541. 652 653
32. Wu, Q.; Li, B.; Li, Z.; Li, J.; Sun, S.; Sun, S. Cancer-associated adipocytes: Key players in breast cancer progression. *J. Hematol. Oncol.* 2019, *12*. 654 655
33. Wang, X.Q.; Yang, K.; He, Y.S.; Lu, L.; Shen, W.F. Receptor mediated elevation in FABP4 levels by advanced glycation end products induces cholesterol and triacylglycerol accumulation in THP-1 macrophages. *Lipids* **2011**, *46*, 479–486, doi:10.1007/s11745-011-3542-4. 656 657 658
34. Weisberg, S.P.; McCann, D.; Desai, M.; Rosenbaum, M.; Leibel, R.L.; Ferrante, A.W. Obesity is associated with macrophage accumulation in adipose tissue. *J. Clin. Invest.* **2003**, *112*, 1796–1808, doi:10.1172/JCI19246. 659 660
35. Wellen, K.E.; Hotamisligil, G.S. Obesity-induced inflammatory changes in adipose tissue. *J. Clin. Invest.* 2003, *112*, 1785–1788. 661
36. Bednarczyk, R.B.; Tuli, N.Y.; Hanly, E.K.; Rahoma, G. Ben; Maniyar, R.; Mittelman, A.; Geliebter, J.; Tiwari, R.K. Macrophage inflammatory factors promote epithelial-mesenchymal transition in breast cancer. *Oncotarget* **2018**, *9*, 24272–24282, doi:10.18632/oncotarget.24917. 662 663 664
37. He, J.Y.; Wei, X.H.; Li, S.J.; Liu, Y.; Hu, H.L.; Li, Z.Z.; Kuang, X.H.; Wang, L.; Shi, X.; Yuan, S.T.; et al. Adipocyte-derived IL-6 and leptin promote breast Cancer metastasis via upregulation of Lysyl Hydroxylase-2 expression 11 Medical and Health Sciences 1112 Oncology and Carcinogenesis. *Cell Commun. Signal.* **2018**, *16*, doi:10.1186/s12964-018-0309-z. 665 666 667
38. Booth, A.; Magnuson, A.; Fouts, J.; Foster, M. Adipose tissue, obesity and adipokines: Role in cancer promotion. *Horm. Mol. Biol. Clin. Investig.* **2015**, *21*, 57–74, doi:10.1515/hmbci-2014-0037. 668 669
39. Wu, Q.; Li, B.; Li, J.; Sun, S.; Yuan, J.; Sun, S. Cancer-associated adipocytes as immunomodulators in cancer. *Biomark. Res.* 2021, *9*, 3423–3442. 670 671
40. Osborn, O.; Olefsky, J.M. The cellular and signaling networks linking the immune system and metabolism in disease. *Nat. Med.* 2012, *18*, 363–374. 672 673
41. Mayi, T.H.; Daoudi, M.; Derudas, B.; Gross, B.; Bories, G.; Wouters, K.; Brozek, J.; Caiazzo, R.; Raverdi, V.; Pigeyre, M.; et al. Human adipose tissue macrophages display activation of cancer-related pathways. *J. Biol. Chem.* **2012**, *287*, 21904–21913, doi:10.1074/jbc.M111.315200. 674 675 676
42. Allen, M.; Jones, J.L. Jekyll and Hyde: The role of the microenvironment on the progression of cancer. *J. Pathol.* 2011, *223*, 163–177. 677
43. Shaul, M.E.; Bennett, G.; Strissel, K.J.; Greenberg, A.S.; Obin, M.S. Dynamic, M2-like remodeling phenotypes of CD11c+ adipose tissue macrophages during high-fat diet - Induced obesity in mice. *Diabetes* **2010**, *59*, 1171–1181, doi:10.2337/db09-1402. 678 679
44. Otvos, J.D.; Shalaurova, I.; Wolak-Dinsmore, J.; Connelly, M.A.; Mackey, R.H.; Stein, J.H.; Tracy, R.P. GlycA: A composite nuclear magnetic resonance biomarker of systemic inflammation. *Clin. Chem.* **2015**, *61*, 714–723, doi:10.1373/clinchem.2014.232918. 680 681
45. Coussens, L.M.; Werb, Z. Inflammation and cancer. *Nature* 2002, *420*, 860–867. 682
46. Duprez, D.A.; Otvos, J.; Sanchez, O.A.; Mackey, R.H.; Tracy, R.; Jacobs, D.R. Comparison of the predictive value of GlycA and other biomarkers of inflammation for total death, incident cardiovascular events, noncardiovascular and noncancer inflammatory-related events, and total cancer events. *Clin. Chem.* **2016**, *62*, 1020–1031, doi:10.1373/clinchem.2016.255828. 683 684 685
47. Furuhashi, M.; Yuda, S.; Muranaka, A.; Kawamukai, M.; Matsumoto, M.; Tanaka, M.; Moniwa, N.; Ohnishi, H.; Saitoh, S.; 686

Shimamoto, K.; et al. Circulating fatty acid-binding protein 4 concentration predicts the progression of carotid atherosclerosis in a general population without medication. *Circ. J.* **2018**, *82*, 1121–1129, doi:10.1253/circj.CJ-17-1295. 687 688

48. Saito, N.; Furuhashi, M.; Koyama, M.; Higashiura, Y.; Akasaka, H.; Tanaka, M.; Moniwa, N.; Ohnishi, H.; Saitoh, S.; Ura, N.; et al. Elevated circulating FABP4 concentration predicts cardiovascular death in a general population: a 12-year prospective study. *Sci. Rep.* **2021**, *11*, doi:10.1038/S41598-021-83494-5. 689 690 691
49. Ebert, T.; Hopf, L.M.; Wurst, U.; Bachmann, A.; Kralisch, S.; Lössner, U.; Platz, M.; Kratzsch, J.; Stolzenburg, J.U.; Dietel, A.; et al. Circulating adipocyte fatty acid binding protein is increased in chronic and acute renal dysfunction. *Nutr. Metab. Cardiovasc. Dis.* **2014**, *24*, 1027–1034, doi:10.1016/j.numecd.2014.03.006. 692 693 694
50. Zhang, L.; Han, L.; He, J.; Lv, J.; Pan, R.; Lv, T. A high serum-free fatty acid level is associated with cancer. *J. Cancer Res. Clin. Oncol.* **2020**, *146*, 705–710, doi:10.1007/s00432-019-03095-8. 695 696
51. Balistreri, C.R.; Caruso, C.; Candore, G. The role of adipose tissue and adipokines in obesity-related inflammatory diseases. *Mediators Inflamm.* 2010, *2010*. 697 698
52. Rodríguez-Hernández, H.; Simental-Mendía, L.E.; Rodríguez-Ramírez, G.; Reyes-Romero, M.A. Obesity and inflammation: Epidemiology, risk factors, and markers of inflammation. *Int. J. Endocrinol.* 2013, *2013*. 699 700
53. Stępień, M.; Stępień, A.; Wlazeł, R.N.; Paradowski, M.; Banach, M.; Rysz, J. Obesity indices and inflammatory markers in obese non-diabetic normo- and hypertensive patients: A comparative pilot study. *Lipids Health Dis.* **2014**, *13*, 29, doi:10.1186/1476-511X-13-29. 701 702
54. Deng, T.; Lyon, C.J.; Bergin, S.; Caligiuri, M.A.; Hsueh, W.A. Obesity, Inflammation, and Cancer. *Annu. Rev. Pathol. Mech. Dis.* **2016**, *11*, 421–449, doi:10.1146/annurev-pathol-012615-044359. 703 704
55. Sproston, N.R.; Ashworth, J.J. Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front. Immunol.* **2018**, *9*, 754, doi:10.3389/FIMMU.2018.00754. 705 706

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Objectives:

8. **To determine by 1H-NMR lipoprotein alterations in BC patients compared to control healthy women.**

9. **To determine new possible biomarkers for BC diagnosis.**

Article:

Josep Gumà, **Jose Adriá Cebrián**, Belen Ruiz Aguado, Cinta Albacar, Josefa Girona, Ricardo Rodríguez Calvo, Neus Martínez Micaelo, Luis Masana, Sandra Guaita Esteruelas. Altered Serum Metabolic Profile Assessed by Advanced 1H-NMR in Breast Cancer Patients.

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Article

Altered Serum Metabolic Profile Assessed by Advanced 1H-NMR in Breast Cancer Patients

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Simple Summary: Previously, our group demonstrated high FABP4 circulating levels in breast cancer (BC) patients. Moreover, increased cholesterol and triglycerides (TG) were found. To deeply analyse the lipid metabolism in our BC cohort, lipid and low molecular weight metabolomics processes are performed in 240 women (171 BC and 69 control women). This paper provides original data related to a novel link between TG-enriched particles and BC. The main result of this study is that TG-enriched particles and some branched amino acids, as well as tyrosine and alanine, are positively associated with BC. This suggests that BC patients have a different metabolic signature that could be used for better stratification and treatment. To our knowledge, this is the first time that advanced NMR profiling has been used to identify relevant and specifically altered lipid and amino acid metabolites in BC serum samples, which could be used for early and reliable diagnosis and prognosis.

Abstract: Background: Altered lipid metabolism has been described in some types of cancer. To analyse in depth the metabolic modifications in breast cancer patients, advanced 1H-nuclear magnetic resonance was performed in these patients. The main objective of this paper was to define a specific lipidomic signature for these cancer patients. Materials and methods: Serum from 240 women (171 breast cancer patients and 69 control women) were studied and analysed by nuclear magnetic resonance. Results: Triglyceride-enriched particles, specifically very low-density lipoprotein triglycerides, intermediate-density lipoprotein triglycerides, low-density lipoprotein triglycerides, and high-density lipoprotein triglycerides, were positively associated with breast cancer. Moreover, alanine, tyrosine, and branched amino acids were also associated with increased risk of breast cancer. Conclusions: Breast cancer patients showed a modified metabolome, giving a very interesting tool to draw different radar charts between control women and breast cancer patients. To our knowledge, this is the first time that advanced nuclear magnetic resonance profiling has been used to identify relevant and specifically altered lipid or amino acid metabolites in BC serum samples. The altered metabolic signature could be analysed for early and reliable BC patient diagnosis and prognosis.

Keywords: lipoproteins; breast cancer; triglycerides

1. Introduction

Breast cancer is the most frequent tumour in women and the second leading cause of cancer-related deaths [1]. Recently, energy metabolism reprogramming has been described as an emerging hallmark in cancer [2,3]. Glucose and glutamine metabolism processes were identified as important metabolic changes in cancer cells [4]; however, recently alterations in the metabolism and regulation of lipids have gained increasing interest because of the definition of new roles for lipids in cancer progression [5]. These lipid modifications include lipid uptake, storage, lipogenesis, and lipolysis [6,7]; actually, cancer cells increase exogenous lipid uptake and endogenous synthesis in order to satisfy their needs for these molecules [3].

Lipids can promote cancer progression at the cellular level, although the epidemiological association is not clear. In fact, cholesterol has a crucial role in cell membrane regulation in mammalian cells, through modulating signal transduction [8]. Moreover, intracellular cholesterol levels are controlled by new biosynthesis, as well as extracellular and intracellular transport. Cholesterol levels are critical in some classical metabolic diseases, such as atherosclerosis, although can also be important for the pathogenesis of others, including cancer [9]. Moreover, cholesterol is a precursor for oestrogens and androgens, both of which are involved in the processes of tumour initiation and progression. Furthermore, oxysterols, molecules derived from cholesterol, are able to increase cancer cell growth and metastasis [8]. Indeed, lipid rafts are essential for cancer signalling and are enriched in cholesterol. Some modifications in their composition can lead to changes in signal transduction and cancer progression. For these reasons, cholesterol has increasing importance in the cancer development process [10].

In spite of the growing availability of these molecular data, there are still epidemiological discrepancies regarding lipid levels and cancer [10,11]. Particularly, opposing results have been found related to the association of lipids and breast cancer [12,13]. In concordance, despite the fact that there have been promising results regarding the use of statins for cancer treatment, the results are quite variable depending on the cancer type [8].

Metabolomic profiling has appeared as a good identification and quantification method for metabolic products such as diagnostic and prognostic biomarkers for certain disorders and diseases [14]. Traditional methods for lipid measurement only quantify circulating lipids by lipoparticle concentration, although their size, density, and triglyceride (TG) composition are usually not analysed during diagnostic analysis. Similarly, conventional measurements of circulating lipid particles only analyse the amounts of cholesterol in the lipid particles, although these methods are not focused on their composition (i.e., TG and phospholipids), particles size, or subclass concentration. Equally, circulating TG are usually measured in terms of total TG concentration and not based on their lipoparticle subclass [15]. Nevertheless, the relationship between lipids and cancer is affected by many factors other than lipid concentration alone; thus, the main goal of this study was to analyse lipoprotein particle subclasses, size, and composition characteristics in breast cancer patients in order to identify potential biomarkers for breast cancer diagnosis and prognosis.

2. Materials and Methods

2.1. Studied Population

In total, 240 individuals were enrolled into this metabolomics study, which included standard clinical biochemistry analysis. There were 171 breast cancer patients and 69 control subjects. Breast cancer patient samples were collected by the department of oncology from Hospital Universitari Sant Joan de Reus after breast cancer diagnosis. Common control subjects were selected from the same family to match age, body mass index (BMI), and geographical area characteristics where possible. Regular examinations were performed and recorded. The project was approved by the Hospital Ethical Committee (reference number: 99-05-20/04-5), and the subjects signed their written consent to participate in the study and accepted the publication of the results.

BMI was calculated as the body weight (kg) divided by the body height squared (m^2).

2.2. Blood Sample Collection and Storage

The blood samples were obtained after overnight fasting. Serum aliquots were stored at −80 °C in the Biobank of our centre in the Institut d'Investigació Sanitària Pere Virgili (IISPV) until their use.

2.3. Biochemical Analysis

Standard biochemical parameters were analysed previously [16]. Cluster of differentiation 36 (CD36) was analysed using a commercial ELISA kit (R&D Systems, Vitro, Madrid, Spain). CETP protein and activity were evaluated using commercial ELISA kits (Cusabio, Deltaclon, Madrid, Spain and Sigma-Aldrich, Merck, Madrid, Spain respectively).

2.4. Lipoprotein Analysis by NMR Spectroscopy (Advanced Lipoprotein Profile)

The serum samples were sent to Biosfer Teslab in dry ice for the NMR analysis. Here, 200 µL was transferred into NMR tubes with phosphate buffer. High-resolution 1H-NMR spectroscopy data were acquired on a Bruker 600 MHz spectrometer, while 1D nuclear Overhauser effect spectroscopy (NOESY, 4 scans) and Carr–Purcell–Meiboom–Gill (CPMG, 64 scans) analysis were used to characterise small molecules such as amino acids and sugars. LED diffusion (Diff) experiments (32 scans) were used to detect larger molecules such as lipoproteins and glycoproteins compounds. All of the sequences were run at 37 °C. The lipid concentrations, sizes, and particle numbers of the four main classes of lipoproteins and the particle numbers of nine subclasses were analysed as previously reported [17]. Briefly, particle concentrations and diffusion coefficients were obtained using the amplitudes and attenuation of their methyl group NMR signals using the 2D diffusion-ordered 1H NMR spectroscopy (DSTE) pulse. The methyl signal was surface-fitted with 9 lorentzian functions associated with each lipoprotein subclasses. The area was related to the lipid concentration of each lipoprotein and the size was calculated from their diffusion coefficient.

The coefficient between the lipid volume and the particle volume of a given class provided the subclass particle concentration. The common conversion factors used to transform concentration units into volume units gave the lipid volumes [18]. Finally, weighted average particle sizes were calculated by summing the known diameter of each subclass multiplied by its relative percentage of the subclass particle number.

2.5. Low Molecular Weight Metabolites Analysis

The CPMG spectra were phased, baseline-corrected, and referenced before performing the automatic metabolite profiling as previously reported using Dolphin software. The 14 low molecular weight metabolites (LMWMs) were identified and quantified. Identifications were analysed for all resonances along the spectra and quantification was performed using line–shape fitting methods on one of the signals.

2.6. Lipid Extraction

Lipophilic extracts were obtained from two 100 µL aliquots of freshly thawed plasma using the BUME method with slight modifications. BUME was optimised for batch extractions with diisopropyl ether (DIPE). This procedure was performed with a BRAVO liquid handling robot, involving drying of the upper lipophilic phase in a Speedvac until evaporation of organic solvents occurred and freezing at −80 °C for further NMR analysis. Lipid extracts were reconstituted in a solution of CDCl3–CD3OD–D2O (16:7:1, *v*/*v*/*v*) containing tetramethylsilane (TMS) at 1.18 mM and transferred into 5 mm NMR glass tubes. An Avance III-600 Bruker spectrometer was used to measure the 1H-NMR spectra at 600.20 MHz. A 90° pulse with a water presaturation sequence (zgpr) was used. Quantification of lipid signals was carried out with LipSpin6, an in-house software based on Matlab. Resonance assignments were performed based on literature values [19].

2.7. Statistical Analysis

The results are expressed as the means ± standard deviation (SD) for normally distributed data, the medians (interquartile range) for data that were not normally distributed, and frequencies for categorical data. The differences between groups were assessed using Student's t test, the Mann–Whitney U test, or chi-square tests. Binary logistic regression analysis was used to calculate the odds ratios (ORs) in serum parameters associated with the presence of breast cancer. In order to facilitate comparisons, the traits were standardised (metabolic marker divided by its standard deviation) (Holmes et al. 2018). Finally, we depicted the adjusted ORs and 95% confidence intervals (CIs) for each 1-SD higher metabolic measure.

SPSS software was used to perform the statistical analyses (IBM SPSS Statistics, version 20.0, North Castle, New York, http://www.ibm.com). Here, p values <0.05 were considered to be statistically significant.

3. Results

3.1. Initial Characteristics of the Study Population

Among the 240 participants with biochemical measurements, the mean age of breast cancer patients 44 (37–50) was similar to control women 43 (38–54) (p = n.s). The same trends were found regarding ages of menarche for control women (12; 11–13) and breast cancer patients (12; 12–14) (p = n.s), numbers of children for control women (2; 1–2) and breast cancer patients (2; 1–2) (p = n.s), and body mass index values for breast cancer patients (24.02; 22.20–28.13) and control patients (25.07; 22.72–28.36) (p = n.s). Finally, no significant differences were found in the percentages of menopausal women, with 30.3% in control women versus 20.2% in breast cancer patients (p = n.s)

For lipids measured using clinical chemistry, breast cancer patients had higher mean concentrations of total cholesterol (208.06 ± 31.40 in breast cancer patients compared to control women 194.18 ± 28.31; p = 0.002), Apo B100 101 (85–118 in breast cancer patients compared to 91 (78–104) in control women; p = 0.008), and triglycerides (86.73 (67.69–120.16) in breast cancer patients compared to 76.55 in control women (56.83–98.74); p = 0.004). In contrast, Apo A1, CETP, and CD36 protein concentrations were broadly similar between breast cancer and control women. Moreover, no significant differences were found in CETP activity (Table 1).

Table 1. Characteristics of the study group.

Study Group Data	Control (n = 69)	Breast Cancer (n = 171)	p Value
Clinical data			
Age	43 (38–54)	44 (37–50)	n.s
Children	2 (1–2)	2 (1–2)	n.s
Age of Menarche	12 (11–13)	12 (12–14)	n.s
Menopause (Yes, %)	30.3	20.2	n.s
BMI (Kg/m^2)	24.02 (22.20–28.13)	25,07 (22.72–28.36)	n.s
Biochemical data			
Cholesterol (mg/dl)	194.18 ± 28.31	208.06 ± 31.40	<0.005
Apo A1 (mg/dl)	147.22 ± 28.66	147.64 ± 27.34	n.s
Apo B100 (mg/dl)	91 (78–104)	101 (85–118)	<0.05
Triglycerides (mg/dl)	76.55 (56.83–98.74)	86.73 (67.69–120.16)	<0.005
FABP4 (ng/mL)	13.085 (8.73–18.05)	17.53 (13.15–23.33)	<0.001
FABP5 (ng/mL)	6.12 (5.44–7.91)	7.00 (5.26–9.04)	n.s
CETP activity (pmol/µL)	47.87 (26.95–69.54)	51.98 (31.71–76.43)	n.s
CETP protein (ng/µL)	596.51 ± 163.39	579.63 ± 155.76	n.s
CD36 (pg/mL)	908.67 (762.00–1037.56)	897.00 (763.37–997.56)	n.s

Data are expressed as medians (IQR) for non-normally distributed data, means ± SD for normally distributed data, or percentages for categorical variables. The statistical tests used were Student's t test (for data that were

normally distributed), Mann–Whitney U test (for data that were not normally distributed), or chi-square tests (for data gathered as categorical variables). Abbreviations: ApoAI, apolipoprotein AI; ApoB100, apolipoprotein B100; BMI, body mass index; CETP, cholesteryl ester transfer protein; CD36, cluster of differentiation 36; IQR, interquartile range; SD, standard deviation. Baseline measurements for the study group. Data are expressed as means ± SD for normally distributed data, medians (IQR) for non-normally distributed data, and percentages for categorical variables. Student's t test was used for data that were normally distributed, Mann–Whitney U test was performed for data that were not normally distributed, and chi-square tests were used for categorical variables. Abbreviations: ApoAI, apolipoprotein AI; ApoB100, apolipoprotein B100; BMI, body mass index; FABP4, fatty acid binging protein 4; FABP5, fatty acid binging protein 5; CETP, cholesteryl ester transfer protein; CD36, cluster of differentiation 36; IQR, interquartile range; SD, standard deviation.

3.2. Serum Metabolome Changes

Figure 1 depicts the OR for lipid particles and selected covariates in breast cancer compared with those in control women. Total cholesterol (OR: 1.702 (95% CI: 2.365–1.225)) ($p < 0.005$), IDL-C (OR: 1.659 (95% CI: 2.337–1.178)) ($p < 0.005$), LDL-C (OR: 1.440 (95% CI:1.965–1.055)) ($p < 0.05$), total-TG (OR: 1.745 (95% CI: 1.159–2.625)) ($p < 0.05$), VLDL-TG (OR: 2.196 (95% CI: 2.196–1.009)) ($p < 0.05$), IDL-TG (OR: 1.592 (95% CI: 1.131–2.243)) ($p < 0.05$), LDL-TG (OR: 1.812 (95% CI: 2.537–1.294)) ($p < 0.005$), HDL-TG (OR: 1.549 (95% CI: 2.192–1.095)) ($p < 0.05$), VLDL particles (OR: 1.460 (95% CI: 2.121–1.004)) ($p < 0.05$), small VLDL-*p* (OR: 1.462 (95% CI: 2.121–1.008)) ($p < 0.05$), LDL particles (OR: 1.503 (95% CI: 2.068–1.093))($p < 0.05$), large LDL-*p* (OR: 1.436 (95% CI: 1.951–1.057)) ($p < 0.05$), medium LDL-*p* (OR: 1.489 (95% CI:2.033–1.091)) ($p < 0.05$), large HDL-*p* (OR: 1.834 (95% CI: 2.641–1.274)) ($p < 0.005$), HDL-Z (OR: 1.533 (95% CI: 2.235–1.051)) ($p < 0.05$), and non-HDL-P (OR: 1.554 (95% CI: 2.152–1.122)) ($p < 0.05$) were associated with higher risks of breast cancer after adjusting for body mass index and age.

3.3. HDL Particles Are Altered and Transport a Higher Content of TG in BC Patients

HDL particles (HDL-P) have been studied in the reverse cholesterol transport context for many years, and they play an important role in the elimination of cholesterol in the body. Different metabolic alterations cause the disruption of HDL profiles in patients [20]. In this study, the number and composition of these particles were analysed in BC patients in order to understand their metabolic effects. HDL particle number profiles were compared between the healthy population and BC women and we observed that despite the fact that there were no differences in HDL-P in breast cancer patients compared to control women ($p = 0.159$), the large HDL-P ($p < 0.005$) and medium HDL-P ($p < 0.05$) were increased in breast cancer patients compare to control women (Figure 2A).

When we analysed these particles in more detail, we observed that their lipid profiles were also altered. In the BC population, the HDL particles transported a higher proportion of triglycerides in comparison with control women ($p < 0.005$) (Figure 2B).

3.4. LDL-P and Their Lipid Contents Are Increased in BC Patients

LDL particles have been established as particles that transport cholesterol from the liver to other parts of the body. Several studies have demonstrated a correlation between higher number of LDL particles and different metabolic diseases, particularly as a crucial factor in atherosclerosis [21]. In the cancer context, some studies have established LDL as a changeable factor, which is increased in patients with BC [10,22]. In agreement, we also detected that BC patients have a higher number of LDL particles compared to healthy individuals ($p < 0.005$) (Figure 3A). Moreover, when we analysed these particles in further detail, we also observed that the large ($p < 0.005$), medium ($p < 0.005$), and small LDL ($p < 0.05$) particles are increased in BC patients compared to control women. When we further analysed the LDL composition in our cohort, we again detected that the LDL particles in BC patients contain a higher amount of cholesterol compared to control women ($p < 0.005$). Moreover, when we studied the TG composition of LDL particles, we also observed a higher TG proportion in LDL particles in BC patients compared to control women ($p < 0.001$) (Figure 3B).

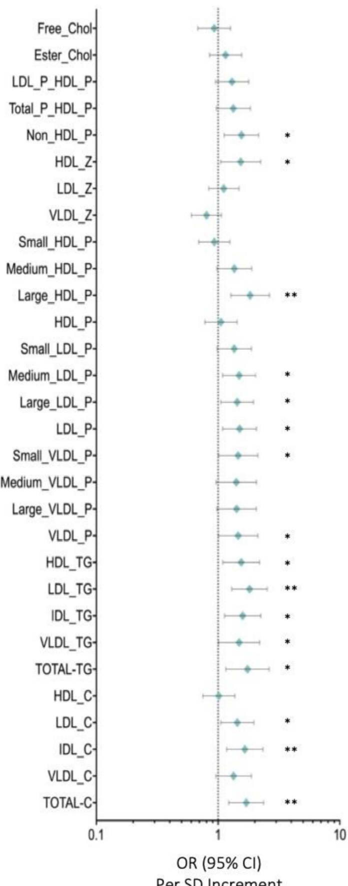

Figure 1. Serum metabolome. Data are presented as OR (95% CI) per 1-SD higher metabolic marker. The plot shows the OR of the effect of a 1-SD-deviation increase and the errors bars represent 95% of the effect estimate. The vertical line marks an OR of 1, i.e., no effect of the exposure on BC risk. Models are adjusted for body mass index and age. Abbreviations: Total-C, total cholesterol; VLDL-c, very low-density lipoprotein cholesterol; IDL-c, intermediate-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; Total-TG, total triglycerides; VLDL-TG, very low-density lipoprotein triglycerides; IDL-TG, intermediate-density lipoprotein triglycerides; LDL-TG, low-density lipoprotein triglycerides; HDL-TG, high-density lipoprotein triglycerides; VLDL-P, very low-density lipoprotein particle; large VLDL-P, large very low-density lipoprotein particle; medium VLDL-P, medium very low-density lipoprotein particle; small VLDL-P, small very low-density lipoprotein particle; LDL-P, low-density lipoprotein particle; large LDL-P, large low-density lipoprotein particle; medium LDL-P, medium low-density lipoprotein particle; Small LDL-P, Small low-density lipoprotein-particle; HDL-P, high-density lipoprotein-particle; Large HDL-P, large high-density lipoprotein particle; medium HDL-P, medium high-density lipoprotein particle; small HDL-P, small high-density lipoprotein particle; VLDL-z, very low-density lipoprotein size; LDL-z, low-density lipoprotein size, HDL-z, high-density lipoprotein size; non-HDL-p, non-high-density lipoprotein particle; T-P_HDL-P, total particles/high density lipoprotein particle; LDL-P_HDL-P, low-density lipoprotein particle/high-density lipoprotein particle; Esterified Chol, esterified cholesterol; Free chol, free cholesterol. Note: p values <0.05 were considered to be statistically significant (p <0.05 * and p < 0.005 **).

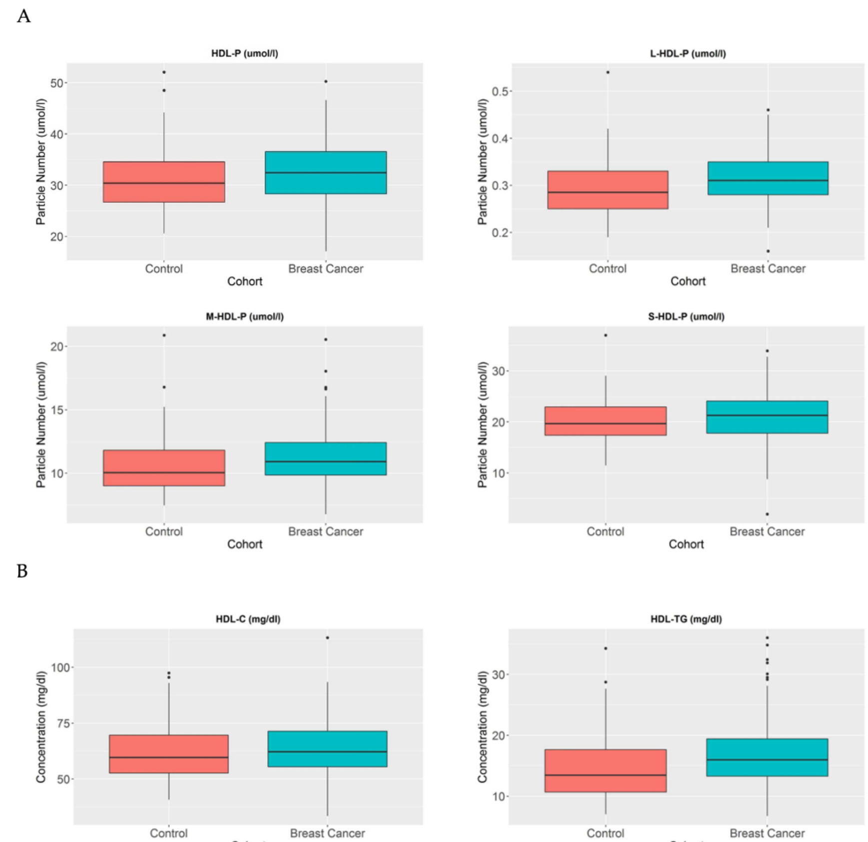

Figure 2. HDL-P, HDL-C, and HDL-TG serum concentrations in BC patients and control women. The blue blots represent BC patients and the red blots represent heathy women. The blots depict the mean and standard error of the mean: (**A**) HDL particles, large HDL particles, medium HDL particles, and small HDL particles; (**B**) HDL-C and HDL-TG. Note: p values < 0.05 were considered to be statistically significant. Abbreviations: BC, breast cancer; HDL, high-density lipoprotein.

To deeply analyse the lipidomics of breast cancer patients, lipid extraction and analysis by 1H-NMR were performed. No differences were found in lipidomics when breast cancer patient samples were analysed compared to control women.

3.5. Low Molecular Weight Metabolites Reveal an Imbalance in Branched Chain Amino Acids As Well As in Tyrosine and Alanine in BC Samples

The demand for glucose is often elevated in cancer patients. In advanced stages of cancer, an increase in proteolysis has been described to meet cancer cells' augmented energy demands. Moreover, in early stages, alterations in amino acid profiles have also been observed in some cancer types (Lieu et al. 2020).

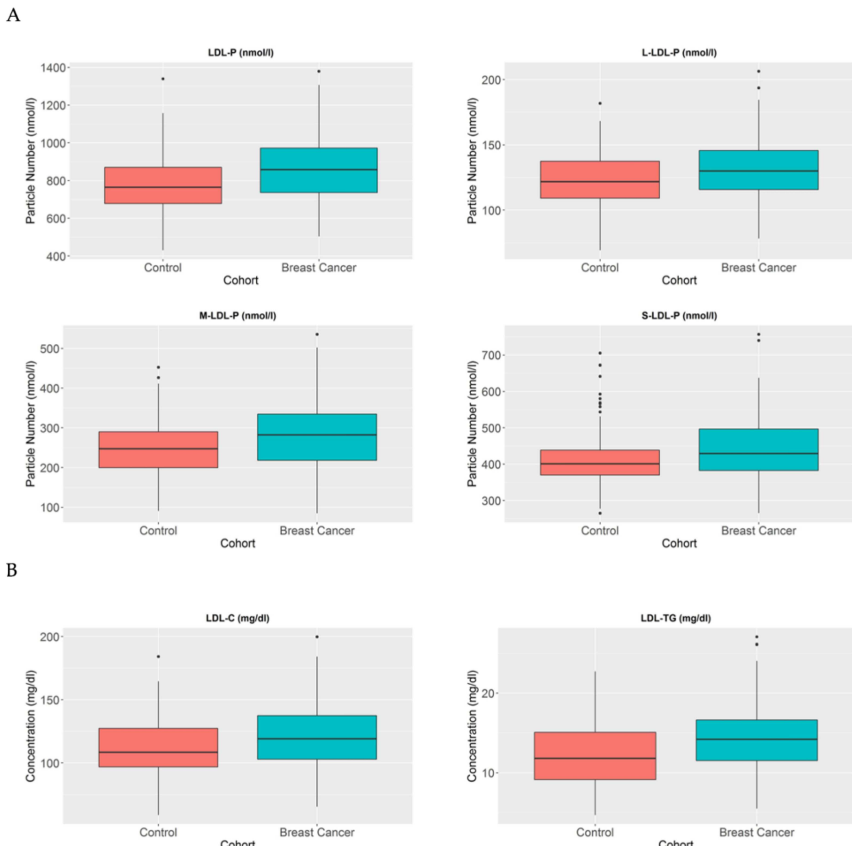

Figure 3. LDL-P, LDL-C, and LDL-TG serum concentrations in BC patients and control women. The blue blots represent BC patients, while the red blots represent heathy women. The blots symbolise the mean and standard error of the mean: (**A**) LDL particles, large LDL particles, medium LDL particles, and small LDL particles; (**B**) LDL-C and LDL-TG. Note: p values < 0.05 were considered to be statistically significant. Abbreviations: BC, breast cancer; LDL, low-density lipoprotein.

In this study, we analysed the profiles of low molecular weight metabolites (LMWMs) in BC patients using NMR in order to investigate the metabolic alterations in our cohort of breast cancer patients. Figure 4 depicts the OR for LMWMs and selected covariates in BC compared with those in control women. Correlation analysis showed that alanine (OR: 1.759 (95% CI: 2.466–1.255)) ($p < 0.005$), tyrosine (OR: 1.432 (95% CI: 1.979–1.036)) ($p < 0.05$), and isoleucine (OR: 1.382 (95% CI: 1.911–1.000)) ($p < 0.05$) were associated with higher risks of breast cancer after adjusting for body mass index and age (Figure 4). Conversely, lactate (OR: 0.689 (95% CI: 0.925-0.514)) ($p < 0.05$) and hydroxybutirate (OR: 0.696 (95% CI: 0.984-0.492)) ($p < 0.05$) were inversely associated with breast cancer risks after adjusting for body mass index and age (Figure 4).

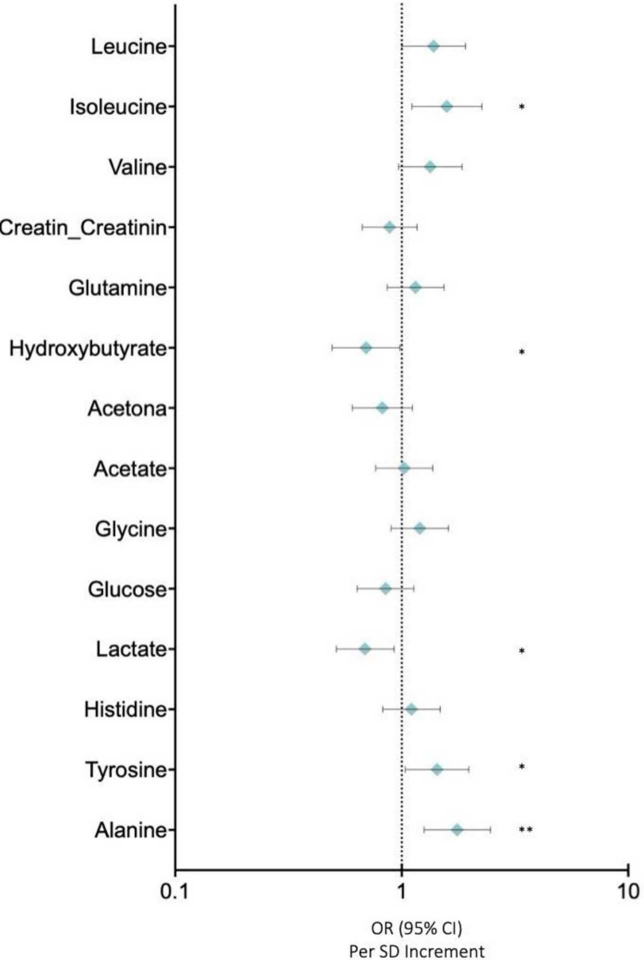

Figure 4. Low molecular weight metabolites. Data are presented as OR (95% CI) per 1-SD higher metabolic marker. Models are adjusted for body mass index and age. Note: p values <0.05 were considered to be statistically significant (p <0.05 * and p < 0.005 **).

Finally, presenting a metabolic chart that allows us to easily classify breast cancer patients from control women, Figure 5 compares quantitative lipid particles (Figure 5A) and LMWMs (Figure 5B) between control woman and breast cancer patients. Data were analysed as fold changes. The graphic representation reveals a modified trend in triglyceride-rich lipoproteins and LMWMs in breast cancer patients. The results show an altered spider chart in breast cancer patients compared to control women, giving an interesting tool for pathologic breast cancer metabolism characterization.

Figure 5. Radar charts presenting fold changes between control women and breast cancer patients, comparing quantitative variables: (**A**) lipidomic analysis showing an altered results for breast cancer patients compare to control women; (**B**) LMWMs showing a different spider chart for breast cancer patients compare to control women. Abbreviations: VLDL-c, very low-density lipoprotein cholesterol; IDL-c, intermediate-density lipoprotein cholesterol; LDL-c, low-density lipoprotein cholesterol; HDL-c, high-density lipoprotein cholesterol; Total-TG, total triglycerides; VLDL-TG, very low-density lipoprotein triglycerides; IDL-TG, intermediate-density lipoprotein triglycerides; LDL-TG, low-density lipoprotein triglycerides; HDL-TG, high-density lipoprotein triglycerides; VLDL-P, very low-density lipoprotein particle; large VLDL-P, large very low-density lipoprotein particle; medium VLDL-P, medium very low-density lipoprotein particle; small VLDL-P, small very low-density lipoprotein particle; LDL-P, low-density lipoprotein particle; large LDL-P, large low-density lipoprotein particle; medium LDL-P, medium low-density lipoprotein particle; Small LDL-P, Small low-density lipoprotein-particle; HDL-P, high-density lipoprotein-particle; Large HDL-P, large high-density lipoprotein particle; medium HDL-P, medium high-density lipoprotein particle; small HDL-P, small high-density lipoprotein particle; VLDL-z, very low-density lipoprotein size; LDL-z, low-density lipoprotein size, HDL-z, high-density lipoprotein size.

4. Discussion

In this study, we explored the use of advanced NMR profiling to identify relevant metabolic alterations in breast cancer patient serum samples. Advanced NMR profiling showed that lipid metabolites were modified in breast cancer patients when compared to control healthy women. Specifically, total cholesterol and some of the transport proteins, such as IDL and LDL, were increased in breast cancer patients compared to control women. Interestingly, the numbers of lipoprotein particles were also modified in the serum of breast cancer patients. In addition, these lipoproteins also showed increased amounts of TGs (i.e., LDL-TG and HDL-TG).

The present findings are not only interesting but also important because there have been many controversies related to the roles of lipoproteins in cancer development [23–25]. In these studies, the authors analysed the role of cholesterol lipoprotein, but not the number or composition of the lipoproteins. In particular, our results showed that high levels of not only LDL cholesterol particles but also of triglyceride-enriched LDL and HDL molecules are associated with an increased risk of breast cancer. Consistent with our findings, Girona et al. 2019 previously proposed that HDL-TG should be considered as a biomarker for metabolic and cardiovascular risk, as well as a marker of HDL dysfunction. In the present study, we also observed altered HDL-TG compositions in breast cancer patients. In concordance, LDL has been shown to be an important molecule for promoting breast cancer cell migration and proliferation. On the contrary, HDL has also been described as an antioxidative molecule having antiproliferative effects in prostate cancer cells. Specifically, synthetic HDL has been shown to be able to mediate its antiproliferative function through apoA-I and phosphatidylcholine [26]. The antioxidant activity of HDL has also been demonstrated to be able to limit cell proliferation induced by reactive oxygen species (ROS), and this ability is lost in TG-enriched HDL. Moreover, the apoA-I levels have also been reported to be inversely correlated with TG composition in HDL [27]. Structurally, HDLs are spherical molecules when they consist of a cholesteryl ester and a triglyceride rich core but are discoidal when they contain mainly apoA-I **(20)**. These alterations in HDL composition and structure could lead to errors in the quantification of HDL-C, which could explain some of the discrepancies related to HDL-C and breast cancer.

TG-enriched particles have been related to altered metabolism and inflammation [27]. In our population, breast cancer patients showed an altered lipid metabolism and increased levels of TG-enriched particles, which could also result in an inflamed phenotype. Nevertheless, our findings revealed that LDL and HDL particles enriched in TG may increase the risk of breast cancer. As a result, altered compositions and amounts of LDL and HDL can potentially directly contribute to breast cancer risk. Collectively, these previous findings and the present results would suggest proatherogenic profiling of patients for breast cancer diagnosis and prognosis.

In addition, we also analysed some of the low molecular weight metabolites in order to define the influence of these molecules on breast cancer risk. Amino acids are used to supply nutrients in cancer cells to sustain their high proliferative rates [28]. We found that branched amino acids (valine $p = 0.07$; leucine $p = 0.05$; isoleucine $p < 0.05$) are associated with increased breast cancer risk. In fact, branched amino acids have been described as essential nutrients for cancer growth and function and as sources of energy for cancer cells [29]. As a consequence, branched amino acids could also be used as metabolic markers for breast cancer. In agreement, we also observed an imbalance in other LMWMs, such as tyrosine and alanine, in the sera of BC patients compared with control women. Tyrosine could be used by BC cells as building blocks for proteins, as well as an alternative cellular energy source. As tyrosine metabolism is important for cancer progression, and according to our results, the tyrosine level could also be classified as a breast cancer risk factor [30]. The role of alanine in cancer is less well understood, but as an amino acid it could play a part in cancer cell proliferation and survival. Nevertheless, our data clearly demonstrate that alanine is increased in breast cancer patients and can be a potential breast cancer risk factor [31]. On the contrary, lactate and hydroxybutyrate

are inversely correlated with breast cancer risk. Lactate is inversely correlated with cancer risk, probably due to the fact that lactate can fuel tumour progression [32,33]. Moreover, hydroxybutyrate can potentially contribute to antiageing phenotypes, but its role in cancer remains controversial [34]. Several LMWMs were altered in the serum of BC patients and they can be used in conjunction with other lipid metabolites as reliable diagnostic and prognostic serum biomarkers for breast cancer.

This study provides clear correlations between triglyceride-enriched particles and breast cancer, although it has some limitations because the effects of hormone levels, post-menopausal state, and oral contraceptive use were not included due to the small size of this population. Further studies should be performed to increase the number of stratified breast cancer patients and to analyse the particles in these subpopulations.

5. Conclusions

In conclusion, our study has validated advanced NMR profiling as a valuable tool for detailed characterization of lipid and low molecular weight metabolites in cancer patient serum samples, and at the same time has contributed to a better understanding of the metabolic background of breast cancer. The use of advanced NMR profiling has allowed us to identify the relevant and specifically altered lipid and amino acid metabolites in breast cancer serum samples and to uncover specific metabolic signatures for early and reliable breast cancer patient diagnosis and prognosis.

Author Contributions: Conceptualization, S.G.-E., L.M. and J.G. (Josep Gumà); methodology, S.G.-E., J.G. (Josefa Girona), J.G. (Josep Gumà) and J.A.-C.; validation, S.G.-E., J.G (Josep Gumà), E.W.F.L., L.M. and J.G. (Josefa Girona); formal analysis, S.G.-E.; J.G. (Josefa Girona), J.G. (Josep Gumà), J.A.-C., N.M.-M. and R.R.-C.; investigation, S.G.-E., E.W.F.L., J.A.-C., N.M.-M. and R.R.-C.; resources, B.R.-A. and C.A.; data curation, S.G.-E., L.M. and J.G. (Josep Gumà); writing—original draft preparation, S.G.-E., J.A.-C. and L.M.; writing—review and editing, S.G.-E., L.M., E.W.F.L. and J.G. (Josep Gumà); visualization, S.G.-E., J.A.-C. and L.M.; supervision, S.G.-E., L.M. and E.W.F.L.; project administration, S.G.-E. and J.G. (Josep Gumà); funding acquisition, S.G.-E. All authors have read and agreed to the published version of the manuscript.

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Institutional Review Board Statement: This study was approved by Ethics Committee of Hospital Sant Joan de Reus. The hospital's ethical committee approved the project (reference number: 99-05-20/04-5).

Informed Consent Statement: All patients provided their written consent to participate in the study and agreed with the publication of the results.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

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Conflicts of Interest: The authors declare no conflict of interest.

References

1. Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics, 2018. *CA Cancer J. Clin.* **2018**, *68*, 7–30. [CrossRef]
2. Hanahan, D.; Weinberg, R.A. Hallmarks of Cancer: The Next Generation. *Cell* **2011**, *144*, 646–674. [CrossRef]
3. Beloribi-Djefaflia, S.; Vasseur, S.; Guillaumond, F. Lipid metabolic reprogramming in cancer cells. *Oncogenesis* **2016**, *5*, e189. [CrossRef] [PubMed]
4. Sun, L.; Suo, C.; Li, S.-T.; Zhang, H.; Gao, P. Metabolic reprogramming for cancer cells and their microenvironment: Beyond the Warburg Effect. *Biochim. Biophys. Acta. Rev. Cancer* **2018**, *1870*, 51–66. [CrossRef] [PubMed]
5. Baenke, F.; Peck, B.; Miess, H.; Schulze, A. Hooked on fat: The role of lipid synthesis in cancer metabolism and tumour development. *Dis. Model. Mech.* **2013**, *6*, 1353–1363. [CrossRef] [PubMed]
6. Cheng, C.; Geng, F.; Cheng, X.; Guo, D. Lipid metabolism reprogramming and its potential targets in cancer. *Cancer Commun.* **2018**, *38*, 1–14. [CrossRef] [PubMed]

7. Liu, Q.; Luo, Q.; Halim, A.; Song, G. Targeting lipid metabolism of cancer cells: A promising therapeutic strategy for cancer. *Cancer Lett.* **2017**, *401*, 39–45. [CrossRef]
8. Chimento, A.; Casaburi, I.; Avena, P.; Trotta, F.; De Luca, A.; Rago, V.; Pezzi, V.; Sirianni, R. Cholesterol and Its Metabolites in Tumor Growth: Therapeutic Potential of Statins in Cancer Treatment. *Front. Endocrinol.* **2019**, *9*, 807. [CrossRef]
9. Simons, K. How Cells Handle Cholesterol. *Science* **2000**, *290*, 1721–1726. [CrossRef]
10. Ding, X.; Zhang, W.; Li, S.; Yang, H. The role of cholesterol metabolism in cancer. *Am. J. Cancer. Res.* **2019**, *9*, 219–227.
11. Orho-Melander, M.; Hindy, G.; Borgquist, S.; Schulz, C.-A.; Manjer, J.; Melander, O.; Stocks, T. Blood lipid genetic scores, the HMGCR gene and cancer risk: A Mendelian randomization study. *Int. J. Epidemiol.* **2018**, *47*, 495–505. [CrossRef]
12. Abdelsalam, K.E.A.; Hassan, I.K.; Sadig, I.A. The role of developing breast cancer in alteration of serum lipid profile. *J. Res. Med. Sci.* **2012**, *17*, 562–565. [PubMed]
13. Hasija, K.; Bagga, H.K. Alterations of serum cholesterol and serum lipoprotein in breast cancer of women. *Indian. J. Clin. Biochem.* **2005**, *20*, 61–66. [CrossRef] [PubMed]
14. Ashrafian, H.; Sounderajah, V.; Glen, R.; Ebbels, T.; Blaise, B.J.; Kalra, D.; Kultima, K.; Spjuth, O.; Tenori, L.; Salek, R.; et al. Metabolomics—the stethoscope for the 21st century. *Med. Princ. Pr.* **2020**, *3*. [CrossRef]
15. Holmes, M.V.; Millwood, I.Y.; Kartsonaki, C.; Hill, M.R.; Bennett, D.A.; Boxall, R.; Guo, Y.; Xu, X.; Bian, Z.; Hu, R.; et al. Lipids, Lipoproteins, and Metabolites and Risk of Myocardial Infarction and Stroke. *J. Am. Coll. Cardiol.* **2018**, *71*, 620–632. [CrossRef]
16. Guaita-Esteruelas, S.; Saavedra-García, P.; Bosquet, A.; Borràs, J.; Girona, J.; Amiliano, K.; Rodríguez-Balada, M.; Heras, M.; Masana, L.; Gumà, J. Adipose-Derived Fatty Acid-Binding Proteins Plasma Concentrations Are Increased in Breast Cancer Patients. *Oncologist* **2017**, *22*, 1309–1315. [CrossRef]
17. Mallol, R.; Amigó, N.; Rodríguez-Gómez, M.; Ángel, H.M.; Vinaixa, M.; Plana, N.; Rock, E.; Ribalta, J.; Yanes, O.; Masana, L.; et al. Liposcale: A novel advanced lipoprotein test based on 2D diffusion-ordered 1H NMR spectroscopy. *J. Lipid. Res.* **2015**, *56*, 737–746. [CrossRef]
18. Jeyarajah, E.J.; Cromwell, W.; Otvos, J.D. Lipoprotein Particle Analysis by Nuclear Magnetic Resonance Spectroscopy. *Clin. Lab. Med.* **2006**, *26*, 847–870. [CrossRef]
19. Vinaixa, M.; Rodríguez, M.A.; Rull, A.; Beltrán, R.; Bladé, C.; Brezmes, J.; Cañellas, N.; Joven, J.; Correig, X. Metabolomic Assessment of the Effect of Dietary Cholesterol in the Progressive Development of Fatty Liver Disease. *J. Proteome. Res.* **2010**, *9*, 2527–2538. [CrossRef]
20. Ouimet, M.; Barrett, T.J.; Fisher, E.A. HDL and Reverse Cholesterol Transport. *Circ. Res.* **2019**, *124*, 1505–1518. [CrossRef]
21. Masana, L.; Girona, J.; Ibarretxe, D.; Rodríguez-Calvo, R.; Rosales, R.; Vallvé, J.-C.; Rodríguez-Borjabad, C.; Guardiola, M.; Rodríguez, M.; Guaita-Esteruelas, S.; et al. Clinical and pathophysiological evidence supporting the safety of extremely low LDL levels—The zero-LDL hypothesis. *J. Clin. Lipidol.* **2018**, *12*, 292–299. [CrossRef] [PubMed]
22. Cedó, L.; Reddy, S.T.; Mato, E.; Blanco-Vaca, F.; Escolà-Gil, J.C. HDL and LDL: Potential New Players in Breast Cancer Development. *J. Clin. Med.* **2019**, *8*, 853. [CrossRef] [PubMed]
23. Johnson, K.E.; Siewert, K.M.; Klarin, D.; Damrauer, S.M.; Chang, K.-M.; Tsao, P.S.; Assimes, T.L.; Maxwell, K.N.; Voight, B.F.; Program, T.V.M.V. The relationship between circulating lipids and breast cancer risk: A Mendelian randomization study. *PLoS Med.* **2020**, *17*, e1003302. [CrossRef]
24. Guan, X.; Liu, Z.; Zhao, Z.; Zhang, X.; Tao, S.; Yuan, B.; Zhang, J.; Wang, D.; Liu, Q.; Ding, Y. Emerging roles of low-density lipoprotein in the development and treatment of breast cancer. *Lipids. Health Dis.* **2019**, *18*, 1–9. [CrossRef] [PubMed]
25. Ganjali, S.; Ricciuti, B.; Pirro, M.; Butler, A.E.; Atkin, S.L.; Banach, M.; Sahebkar, A. High-Density Lipoprotein Components and Functionality in Cancer: State-of-the-Art. *Trends. Endocrinol. Metab.* **2019**, *30*, 12–24. [CrossRef] [PubMed]
26. Ruscica, M.; Macchi, C.; Pavanello, C.; Corsini, A.; Sahebkar, A.; Sirtori, C. Appropriateness of statin prescription in the elderly. *Eur. J. Intern. Med.* **2018**, *50*, 33–40. [CrossRef] [PubMed]
27. Girona, J.; Amigó, N.; Ibarretxe, D.; Plana, N.; Rodríguez-Borjabad, C.; Heras, M.; Ferré, R.; Gil, M.; Correig, X.; Masana, L. HDL Triglycerides: A New Marker of Metabolic and Cardiovascular Risk. *Int. J. Mol. Sci.* **2019**, *20*, 3151. [CrossRef]
28. Vettore, L.; Westbrook, R.; Tennant, D.A. New aspects of amino acid metabolism in cancer. *Br. J. Cancer.* **2020**, *122*, 150–156. [CrossRef]
29. Ananieva, E.A.; Wilkinson, A.C. Branched-chain amino acid metabolism in cancer. *Curr. Opin. Clin. Nutr. Metab. Care* **2018**, *21*, 64–70. [CrossRef]
30. Nguyen, T.N.; Nguyen, H.Q.; Le, D.-H. Unveiling prognostics biomarkers of tyrosine metabolism reprogramming in liver cancer by cross-platform gene expression analyses. *PLoS ONE* **2020**, *15*, e0229276. [CrossRef]
31. Choi, B.-H.; Coloff, J.L. The Diverse Functions of Non-Essential Amino Acids in Cancer. *Cancers* **2019**, *11*, 675. [CrossRef] [PubMed]
32. Hirschhaeuser, F.; Sattler, U.G.; Mueller-Klieser, W. Lactate: A Metabolic Key Player in Cancer. *Cancer Res.* **2011**, *71*, 6921–6925. [CrossRef] [PubMed]
33. de la Cruz-López, K.G.; Castro-Muñoz, L.J.; Reyes-Hernández, D.O.; García-Carrancá, A.; Manzo-Merino, J. Lactate in the Regulation of Tumor Microenvironment and Therapeutic Approaches. *Front. Oncol.* **2019**, *9*. [CrossRef] [PubMed]
34. Han, Y.-M.; Ramprasath, T.; Zou, M.-H. β-hydroxybutyrate and its metabolic effects on age-associated pathology. *Exp. Mol. Med.* **2020**, *52*, 548–555. [CrossRef] [PubMed]

SUMMARY OF RESULTS

6. SUMMARY OF RESULTS

The following results can be extracted from the articles presented in this doctoral thesis:

1) Adipocyte CM enhances MCF-7 and MDA-MB-231 BCC lines proliferation, survival, migration, and invasiveness. Moreover, an increment in lipid droplets accumulation and fatty acid binding proteins FABP4, FABP5 and CD36 expression and protein levels have been observed. BMS309403 treatment, reduces the effects on the different cancer cells, by significantly reducing the mature adipocytes FABP4 transcript levels.

2) BCC lines SKBR3, BT474, MDA-MB-231, MCF-7 and its Doxorubicin resistant cells MCF-7-Epi^{R} and Paclitaxel resistant cells MCF-7-TAX^{R} increase mature adipocyte delipidation as well as fatty acid binding proteins FABP4, FABP5 and CD36 expression and releasement. Moreover, crosstalk between both cell types increases the lipid transfer from mature adipocytes to tumor cell lines.

3) Palmitic acid derived from mature adipocytes is internalized in tumor cells and it is transformed into different lipid families such as PC, PE, DG, TG, Ceramides and SM. Interestingly, the lipid signature was distinctive between the different BCC lines.

4) Lipid signature of MCF-7 drug resistant cell lines underwent a lipid metabolism switch, becoming more similar to the lipid signature of the

TNBC cell line MDA-MB-231, increasing the palmitic acid transformation into PC, SM and SM compared to their derived sensitive cell line MCF-7.

5) Tumor microenvironment crosstalk increases the inflammatory behavior of mature adipocytes by the increment of transcript levels of IL-6 and MCP-1, hence producing a pro-inflammatory activation of the immune system THP-1 monocytic cell line, as well as an increment in the lipid droplets and FABP4 incorporation into this monocytic cells.

6) BC patients have an increment in the pro-inflammatory status as they presented higher levels of the CRP. This pro-inflammatory metabolite was notably correlated with an increment in serum levels of Glyc-A and Glyc-B, new pro-inflammatory biomarkers. In addition, CRP and Glyc-A were positively associated with the increment of serum FABP4 protein.

7) BC patients have incremented levels of HDL, LDL, and VLDL (small, medium, and large) particles compared to control healthy women.

8) BC patients’ lipoprotein profile is significantly as there is a triglycerides enrichment in all the studied lipoparticles. Moreover, the concentration of cholesterol in these particles was also higher compared to control healthy women.

9) Triglycerides enrich-lipoproteins are positively correlated with the pro-inflammatory metabolites CRP and Glyc-A, as well as with FABP4.

10) BC patients presented an altered low molecular weight metabolites profile (LMWM) compared with control healthy women, where the branched aminoacids valine, leucine, and isoleucine, as well as the aminoacids alanine and tyrosine were significantly increased in BC patients' serum compared to control healthy women.

GENERAL DISCUSSION

7. GENERAL DISCUSSION

Breast cancer is a disease that affects to a high number of woman population. In fact, according to the American Society of Cancer, one of eight women will develop breast cancer in their lifespan [4]. Despite the development of new techniques for diagnosis and new therapies against cancer progression and dissemination, BC mortality reaches 6,6% of all diagnosticated cases [1], becoming the leading cause of woman cancer related deaths in 103 countries, followed by cervix and lung cancers. Treatment of cancer has historically focused on cancer treatment itself. However, tumors are abnormal organs composed by multiple cell types and ECM, whose interactions support cancer progression and development [226][113]. This crosstalk confers new cancer abilities and skills that accelerate cancer cells development and progression. As breast is mainly composed by adipose tissue, the interaction between tumor cells and adipose tissue might have an important role in BC progression. For this reason, in this study, we have focused on BC and adipose tissue crosstalk. Traditionally, adipose tissue had been considered as an energy reservoir. However, adipose tissue has an important role in several endocrine biological processes such as the modulation of the eating behavior or production of different cytokines, adipokines and steroid hormones [140].

The role of adipose tissue in the tumor microenvironment has been recently object of study, and different reports have stablished a strong association between obesity, adipose tissue, BC development, and worse prognosis [227]. Accordingly, our research has focused on this relationship to clarify the intrinsic mechanisms that modulate BC progression within the tumor microenvironment, specifically with the adipose tissue.

Firstly, because tumor microenvironment, including adipose tissue, is able to enhance the cancer cells proliferation rates [126], we analyzed the effects of this dynamic crosstalk on the proliferation pathways activation. *In vitro*, we have demonstrated that mature adipocyte CM overactivated two crucial proliferative pathway proteins such as MAPK ERK1/ERK2 and MAPK p38 in two of the main studied cell lines, MDA-MB-231 and MCF-7. These proteins are crucial for tumor cells proliferation, survival and chemoresistance [228][81]. In addition, we have observed that the overactivation of these pathways was accompanied by an increment in the proliferation of both cell lines. As bibliography describes, tumor microenvironment, including the peritumoral adipose tissue, increases tumor cells proliferation [126].

Moreover, Akt is a central protein that regulates several processes, such as energetic balance, cell growth, cell cycle and apoptosis. In cancer, an overactivation of this protein by its phosphorylation enhances tumor cells growth [229]. FOX axis has also an important role in cancer progression, thus FOXO3A inhibition leads to the enhancement of tumor cells survival and proliferation. Our results showed that Akt was phosphorylated by the adipocyte CM, activating this pathway, thus, leading to the phosphorylation of FOXO3A.

Consequently, because of these pathways enhance the chemoresistance of cancer cells, we performed a clonogenicity assay to test how adipose CM might increase the BCC lines chemoresistance to Doxorubicin. Interestingly, we observed that after culturing both cell lines with adipocyte CM, the ability to form new colonies significantly improved

compared to those cells cultured with control CM, hence suggesting a relationship between adipocytes and tumor cells survival.

Cancer mortality is closely linked to tumor cells metastatic behavior [230]. Migration and invasiveness are essential for cancer progression, and they have been related to worse cancer patient prognosis. By a wound healing assay, we have demonstrated that both studied BCC lines were able to close the wound faster after the addition of adipocyte CM, compared to control CM. This suggests that adipocyte CM might enhance BC migration.

However, to arrive to lymphatic or blood stream, tumor cells must pass throw the ECM where they are embedded. ECM is a scaffold of interconnected macromolecules forming networks that encompass cells found in the different tissues and organs [231]. One of the most important steps in invasion is the degradation of this ECM. Certain enzymes are essential in this process, being metalloproteases (MMPs) one of the most important families [232]. In fact, MMPs have a crucial role in different biological processes, such as cell proliferation, cell survival, immune response, angiogenesis and invasion [233][234]. Moreover, these proteins are elevated in most cancer types, being strongly correlated to worse prognosis [235].

In this study, we have observed an increase of the MMP-9 transcript in both BCC lines after culturing with adipocyte CM. Certain studies have related this metalloprotease to an increase in metastasis formation *in vitro* and *in vivo* [236]. In addition, we have observed that the coculture of BCC with mature adipocytes increases the ability to invade the collagen matrix

in both cell lines, suggesting that peritumoral adipocytes might enhance BC invasiveness.

However, proliferation, migration, and invasion require a high demand of energy, hence, regulation of the energy metabolism, as a new cancer hallmark, is crucial for cancer progression [237]. The role of lipids, remarking the importance of lipid metabolism in cancer progression, has been recently studied [38][238]. In fact, tumors are able to activate *de novo* synthesis fatty acids and cholesterol [239]. BCCs are embedded mainly in adipose tissue. Consequently, this tissue might serve as an energy source for tumor cells. Actually, tumor microenvironment, specially adipose tissue, supplies different components for cancer progression [240][241][242].

Although diverse studies have stablished the influence of adipose tissue in cancer progression, the microenvironment crosstalk is bidirectional. Thus, tumor cells influence the behavior of surrounding cell types, including adipocytes, by metabolism alterations [141]. In fact, we have observed that mature adipocytes suffered a higher delipidation and lipid releasement to media when they were cultured with BCCs CM. Furthermore, by tracking labelled palmitic acid, we have demonstrated that mature adipocyte significantly decreased the number of cytoplasm lipid droplets once they were cultured with the different BCC lines CM. Moreover, labelled palmitic acid was incorporated into the different BCC lines, suggesting a transfer of lipids from mature adipocytes to tumor cells by an active crosstalk.

However, as lipids are hydrophobic, they require of specific transport proteins for their circulation. FABP4 and FABP5 are two of the most studied proteins for fatty acids transport. In fact, they have been strongly linked to cancer, including breast cancer [184][191]. Previous laboratory results have demonstrated that FABP4 and FABP5 levels were increased in breast cancer patients plasma compared to control women [172]. CD36 is another fatty acid transport protein whose relationship with cancer has gained notably importance. In fact, a recent study has demonstrated the importance of this protein in cancer metastasis [203]. Interestingly, our group has also observed higher levels of CD36 in BC patients plasma. Consequently, we decided to assess the effects of BCCs CM on mature adipocytes fatty acid transport proteins FABP4, FABP5, and CD36. In our study, we have observed that BCCs CM increased all three fatty acid transport proteins transcripts in mature adipocytes. Moreover, we have demonstrated that BCCs CM also increased mature adipocytes releasement of these fatty acid transporters to media, suggesting that they might have an impact in cancer fueling and progression.

Moreover, we decided to study the effects of adipose tissue in the transcript and protein levels of these fatty acid transport in our BCC lines. Interestingly, we observed a significant increment of FABP4, FABP5 and CD36 in both BCC lines, remarking the plausible importance of all three transport proteins.

According to correlation of FABP4 and cancer , we decided to inhibit this protein in order to assess its implication in BC development. BMS309403 is a well-known FABP4 inhibitor whose medical contribution in the atherosclerosis disease has been strongly proved [243]. In addition, some

studies have correlated this inhibitor to a significant decrease of tumor cells invasiveness and migration ability in cholangiocarcinoma and prostate cancers [190][244].

This study has demonstrated that BMS309403 reduced mature adipocytes lipolysis and triglycerides releasement. Actually, reduction of FABP4 has been associated with lower serum triglycerides and decreased atherosclerosis risk [245], partially because its implication in transcriptional metabolic program regulation [246].

Accordingly, we decided to assess the effects of BMS309403 on different cancer hallmarks previously studied. Firstly, after BCCs treatment with adipocyte CM plus BMS309403, we observed a reduction in the Akt and MAPK ERK1/ERK2 pathways activation, as well as a significant reduction in BCC lines proliferation, suggesting that FABP4 might have a key role in BCC lines proliferation. In fact, previous studies in our laboratory have demonstrated that exogenous FABP4 increases BCCs proliferation [188], hence the role of FABP4 might be crucial in this cancer hallmark. Moreover, FAPB4 deficiency impaired tumor growth in mouse models of ovarian cancer [189]. Therefore, knockdown of FABP4 results in a downregulation of ovarian cancer metastatic gene signature, as well as in the reduction of clonogenic tumor cell survival [247]. Our results have also demonstrated that in BCC lines MDA-MB-231 and MCF-7, FABP4 inhibition resulted in a reduction in the clonogenicity of both cell lines, hence in their survival capability once we administered Doxorubicin, suggesting that FAPB4 might influence survival and chemoresistance in tumor cells.

As we have previously observed, adipocyte CM enhanced migration and invasiveness of BCC lines MCF-7 and MDA-MB-231. We wondered if FABP4 might have a role in these processes. Interestingly, we have demonstrated that in mature adipocyte FABP4 inhibition significantly reduces the migration and invasion of both BCC lines, suggesting that FABP4 might influence the metastatic processes of BCCs. Actually, silencing FABP4 in ovarian cancer cells inhibited tumor progression in mouse models [184]. These findings suggest that FABP4 might regulate the invasive and aggressive behavior of tumor cells.

Previous laboratory results have demonstrated that the addition of exogenous FABP4 into the BCC lines activates the expression of fatty transport proteins FABP5 and CD36 [188]. In addition, our results have demonstrated also that adipocyte CM enhances the transcript and protein levels of these fatty acid transporters. By the inhibition of FABP4, we have demonstrated that this fatty acid protein significantly reduced the transcript levels of these fatty acid transport proteins and reduce the number of lipid droplets in BCCs cytoplasm, suggesting a diminution of lipid uptake in these cell lines. According to bibliography, FAPB4 inhibition reduces the triglycerides content in trophoblasts [248], hence the regulation of this fatty acid transport protein might regulate the lipid transfer from adipose tissue to the BCCs in the tumor microenvironment. These results suggest that the inhibition of FAPB4 in the tumor microenvironment has an important role in cancer progression by the partial inhibition of adipocyte CM effects on different cancer hallmarks, thus being useful as a possible new treatment.

As described above, tumor metabolism is a key factor in tumor cells progression. Lipid metabolism plays a remarkable function in the cancer metabolic switch, becoming an interesting point of study [237]. Actually, tumor cells are able to induce *de novo* synthesis of fatty acid and cholesterol [239], hence, enhancing their tumorigenicity [249]. In fact, fatty acid synthesis is required for breast cancer brain metastasis [250], remarking the importance of lipid metabolism in cancer progression. Moreover, peritumoral adipose tissue could influence the lipid metabolism of tumor cells by the transfer of molecules and lipid that would enhance the tumorigenicity of cancer cells [242][251]. Interestingly, this research has demonstrated that mature adipocytes released palmitic acid to media, then being incorporated in BCCs. This fatty acid has been strongly related to cancer progression and poor prognosis in cancer patients [252]. Moreover, palmitic acid diet promotes a pro-metastatic memory in breast cancer [253].

In this research, we have studied by ^{1}H-NMR lipid metabolism of six different BCC lines (MDA-MB-231, SBKR3, BT474, MCF-7 and its resistant cell lines MCF-7 Epi^{R} and MCF-7 TAX^{R}) once they internalize the labeled palmitic acid derived from mature adipocytes. We have observed that the isotopic carbon of labelled palmitic acid was present in new lipid species, suggesting a transformation of palmitic acid into new lipid families.

According to bibliography, PCs, PEs and SMs are three important lipid families whose dysregulation has been closely related to different metabolic diseases as well as to cancer worse prognosis [154],[254],[255]. We had detected that each BCC line had a lipid pattern that discerned notably from other cell lines, suggesting that depending on the

tumorigenicity of each BCC line, as well as its metabolic necessities, different lipid families might be required.

Novelty, we have worked with the drug resistant cell lines MCF-7 Epi^R and MCF-7 TAX^R derived from the sensitive Luminal A MCF-7 cell line. Interestingly, once we analyzed the lipid pattern of MCF-7 and its drug resistant derived cell lines, we observed that they notably differed in lipid synthesis of PCs, PEs, SMs, and Ceramides.

Surprisingly, once we analyzed lipid signature of the TNBC cell line MDA-MB-231 and both resistant MCF-7 cell lines, we observed a well-defined correlation in their lipid patterns. By a Principal Component Analysis (PCA) we demonstrated that resistant cell lines MCF-7 Epi^R and MCF-7 TAX^R lipid signatures were very similar to triple negative's lipid signature, especially the families of PC, PE and SM. According to bibliography, PCs are one of the most abundant lipid families within mammalian cells and alterations in BC patient's blood stream levels have been correlated to worse prognosis[154]. According to our results, PC saturations are not equally represented in our different BCC lines studied. In fact, we could observe that saturated PC were specifically more present in resistant MCF-7-Epi^R and MCF-7-TAX^R cell lines, as well as in the TNBC MDA-MB-231 compared to sensitive Luminal A MCF-7 cells. Some of the most statistically significant saturations overrepresented in the resistant and triple negative BCC lines compared to the sensitive MCF-7 were PC(44:1) /// PC(26:0/18:1) and PC(38:1) /// PC(20:0/18:1). We also found an increase in the PC form PC(O-33:3). Moreover, we also observed that there were other PC saturations highly altered, even though they did not reach statistical significance. PC(28:0) /// PC(14:0/14:0), PC(31:1) /// PC(15:0/16:1), PC(30:1) /// PC(16:1/14:0), PC(38:3) /// PC(18:0/20:3) were

notably increased in the resistant and triple negative BCC lines. According to bibliography, tumor grade is closely related to the metabolism of this specific lipidic family, including saturated form PC (14:0)[256]. Furthermore, evidence has also demonstrated that saturations such as PC(18:1), PC(36:1), or PC(38:2) are closely related to BC and worst prognosis [257][258][259]. PEs are also an important lipid family whose homeostasis disruption might influence in the cell functionality [260]. In our studies, we have found that the lipid pattern of PE also differs between resistant cells, TNBC and the sensitive cell line MCF-7. According to different studies where exhaustive plasma and urine analysis were preformed, specific saturations such as PE (15:0/19:1) are closely related to BC [261],[262].

In this study we have observed a specific PE saturations pattern in TNBC MDA-MB-231 that matches with the resistant cell lines MCF-7-Epi^{R} and MCF-7-TAX^{R} whereas it differs from the PE signature of the sensitive MCF-7. The most significant overrepresent PE saturations that we have found in the resistant cell and TNBC in comparison to the sensitive cell line were PE(P-34:2)///PE(P-16:1/18:1), PE(P-36:5)///PE(P-16:1/20:4), PE(P-36:2)///PE(P-18:1/18:1), PE(0-34:2)///PE(0-15:1/19:1) and PE(P-38:7)///PE(P-16:1/22:6). Therefore, this PE modifications might have a crucial role in the tumorgenicity of BCCs by the improvement of their metastatic abilities and resistance to a conventional chemotherapy.
However, not only modifications in these two lipid species have been identified. SMs, with different saturations, such as SM(36:1), SM(39:3), SM(42:2) or SM(42:1) are overrepresented in resistant and TNBC compared to the sensitive cell line MCF-7.

SMs have a central role in diverse biological processes such as death, proliferation, and migration [263], suggesting the importance of the homeostasis of this lipid specie.

Finally, ceramide family has similar alterations between the TNBC cells, and both resistant cell lines compared to the drug sensitive Luminal-A cells. This lipid family is closely related to inflammation, being a nexus to different metabolic disorders [264][265]. In addition, these lipids metabolism has also been associated with BC, being an important factor in critical steps of cancer development [266]. Consequently, these data suggest that resistance acquisition might be accompanied by an increase in malignancy, switching the lipid signature. Thus, knowledge of specific lipid signature from different BC molecular subtypes might lead to understand the metabolic behavior of each one, opening new ways of diagnosis and treatment in patients with unclear diagnosis. Moreover, understanding the intercommunication between adipose tissue, tumor cells and lipid metabolic pathways might let us to find new therapeutic targets.

However, as tumor microenvironment is not composed only by these adipocyte cells, but there are other cell types comprised, understanding the crosstalk of all these elements is crucial for a good treatment. In the tumor microenvironment, the immune system is a component that has gained a notably importance during last decades because of its implication in cancer progression. Infiltrated macrophages are one of the most studied immune cell types within the tumor microenvironment. The aim of this research was to understand the communication between all the different elements within the tumor microenvironment, including adipocytes,

tumor cells, and these immune system cells. As we have previously demonstrated, BCCs are able to increase the delipidation of mature adipocytes and the releasement of different lipid families to ECM in order to supply the energetic demand of tumor cells. Moreover, this is accompanied by an increment in the production and releasement of fatty acid binding proteins FABP4, FABP5 and CD36.

Our next purpose was to understand the possible effects of these proteins in the entire tumor microenvironment, including the immune system cells. For this reason, we cultured the monocytic THP-1 cell line with different conditioned media, including the adipocyte CM and the CCAs CM. Once we analyzed the lipid incorporation in the monocytic cell line, we observed a significant increment in the amount of lipid droplets in THP-1 cytoplasm once they were cultured with adipocyte CM. Interestingly, this increment was even higher once they were cultured with the CAAs CM. In addition, we also observed an increment of FABP4 in the cytoplasm of these monocytes. Bibliography have described an important role of FABP4 protein in lipid accumulation inside macrophage's cytoplasm of arteries in atherosclerosis disease [267]. Moreover, a novel study has demonstrated that the expression of FABP4 in TAMs promotes breast cancer progression[134].

One of the new cancer hallmarks is chronic inflammation of the tumor microenvironment. In fact, some studies have stablished a relationship between a chronic inflammation and cancer progression [268]. Accordingly, we have observed that mature adipocytes cultured with different BCC lines CM suffered a significant increment at transcript levels of *IL-6* and *MCP-1*. The importance of these two cytokines in the

inflammation process and cancer progression has been robustly described[269][166][270]. MCP-1 as a chemoattract cytokine might increase the infiltration of different immune cells, including circulating monocytes. The presence of a proinflammatory status would promote the differentiation of these monocytes into M1 macrophages increasing the inflammatory status in the area.

Firstly, we analyzed the effects of adipose tissue on the inflammatory status of THP-1 cell lines. After the transcriptome analysis of the monocytic cell line, previously treated with adipocyte CM, we observed that there was a significant increment at transcriptional level of IL-6, IL-1ß, TNF-α and MCP-1. Interestingly, after analyzing the effects of CAAs conditioned media, we could observe that the increment was significantly higher. Moreover, we also observed that monocytes cultured with matured adipocyte CM had an increment in phosphorylation forms of two of the most studied inflammation-related proteins, such as NF-κB p65 and MAPK-p38. Interestingly, the phosphorylation of both proteins was higher once these monocytes were cultured with the different CAAs conditioned media.

The extravasation and infiltration of monocytes in adipose tissue in obese animals and human individuals has been described[271]. In tumor microenvironment, infiltrated monocytes are able to activate different pathways in cancer cells, modulating their biological function, hence inducing cancer progression[272]. The activation of these extravasated monocytes into active inflammatory macrophages leads to the generation of a pro-inflammatory status in tumor microenvironment[273]. In this study we have observed that monocytes cultured with mature adipocyte

CM suffered a higher attachment to the plate than monocytes cultured in control conditions. Interestingly, the attachment and morphology modifications were even higher once the monocytic cell line was cultured with CAA conditioned medias derived from MDA-MB-231, MCF-7 and it drug resistant variants MCF-7-Epi^R and MCF-7-TAX^R,

Briefly, *in vitro* results, suggest that the intercommunication between adipocytes and tumor cells might induce the chemoattraction of new immune system cells by the increment of MCP-1 transcript and protein levels in mature adipocytes. Moreover, the activation of inflammatory proteins such as NF-κB and p38 MAPK as well as the induction of the transcription of inflammatory genes such as IL-6, IL-1ß, TNF-α and MCP-1 in infiltrated immune system cells might generate a pro-inflammatory status in tumor microenvironment.

Our next objective was to corroborate this evidence *in vivo*. We wanted to assess if breast cancer patients have an altered inflammatory status. ^{1}H-NMR analysis of different serum components is one of the most advanced techniques in the diagnosis field, including glycoprotein pattern detection. Glyc-A and Glyc-B, are two NMR-detectable plasma glycoproteins that belong to acute inflammation proteins family and they are released by the liver under a pro-inflammatory status in the organism [274],[275]. The presence of these glycoproteins in plasma of breast cancer patients might correlate to tumor microenvironment pro-inflammatory status. Interestingly, once we analyzed plasma levels of both glycoproteins, we observed that BC patients has a slight increment in Glyc-B plasma levels, meanwhile the levels Glyc-A were notably increased compared to control healthy women. According to bibliography, Glyc-A is a widely studied

inflammation-related protein and there is evidence that correlate its increment in plasma with different diseases, including cancer[276],[277]. Moreover, these BC patients also presented an increment of serum CRP levels compared to the control studied population. According to bibliography, CRP is an acute inflammatory protein, being significantly increased in plasma patients that suffer rheumatoid arthritis, some cardiovascular diseases, and infection [278]. Our analysis demonstrated that the increment of this well-known inflammatory protein was positively correlated to the increment of Glyc-A in our BC cohort, thus supporting previous *in vitro* results, where we suggested that cancer progression might be positively correlated to the generation of an inflammatory status.

Moreover, as we had previously described, THP-1 monocytes present a high amount of FABP4 in their cytoplasm. According to the important association of this fatty acid transport protein in different diseases, including atherosclerosis[279],[280] or even kidney disease[281], it might correlate to the inflammatory status in breast cancer patients. Interestingly, previous analysis of our BC patients cohort revealed an increment FABP4 in BC serum compared to control healthy women [172]. Consequently, by a simple linear correlation analysis, we detected that 27% of cases that presented an increment of Glyc-A could be explained by an increment in FABP4 plasma levels. These results suggested that this protein might have a crucial role in the generation of the inflammatory status, hence in the development and progression of the disease.

Interestingly, we also observed that the lipid profile of breast cancer patients was altered compared to control healthy women. Firstly, by standard clinical chemistry quantification, we detected that our BC

patients have higher plasma concentrations of total cholesterol, Apo B100 and triglycerides. Cholesterol, as central molecule of lipid metabolism, has an essential role in membrane biogenesis, hormone biosynthesis as well as in cell signaling transduction [282],[283]. This lipid is carried on lipoproteins, and it is incorporated into target cells. There are several studies that have associated breast cancer, as well as many other cancer with alterations in plasma lipoproteins [213][214]. However, the novelty of this study is that we have analyzed the composition of these lipoproteins in breast cancer patients and control healthy women, thus leading to improve the diagnosis potential.

According to bibliography, high levels of LDL are associated with higher risk of breast cancer [284]. In this study we have also demonstrated that higher proportion of triglycerides and cholesterol in these particles are related to BC. In concordance, LDL cholesterol, but not HDL cholesterol induces BCCs proliferation and invasion [285]. In addition, we have also demonstrated that alterations in LDL particles composition might also have a negative impact in BC. Actually, we have found that the composition of HDL lipoprotein in BC patients was notably altered, showing an increment in the triglycerides and cholesterol levels compared to control healthy women. Although many studies have associated high HDL plasma levels with better breast cancer prognosis [284][286], alterations in HDL composition might explain why the HDL protective effect is affected, becoming a breast cancer risk biomarker. In fact, TG enriched-HDL particles are positively correlated to an increment in metabolic and cardiovascular risk [223]. Moreover, there is a strong correlation between lipid alterations, higher serum triglycerides and chronic inflammation [287][288][289]. Consequently, we wanted to assess the possible

correlation of both parameters in our BC patients cohort. After a simple correlation analysis, we detected that 49% of cases where Glyc-A plasma levels are increased could be explained by the increment of total plasma triglycerides.

A recent study has demonstrated a strong association between triglycerides-enriched lipoproteins and Glyc-A [224]. Accordingly, as have we demonstrated that BC patients have an altered composition in lipoproteins, with increased levels of triglycerides, compared to control healthy women [290], the analysis of this triglycerides enriched particles has acquired a notable interest. The correlation between TG lipoprotein composition and Glyc-A levels was also positive, suggesting that there is a relationship between total triglycerides as well as TG enriched particles and pro-inflammatory protein Glyc-A, hence with the inflammatory status of the patient.

^{1}H-NMR technology allows us to study deeply BC patients metabolomics by the analysis of low molecular weight metabolites (LMWM). Aminoacids metabolism is altered in cancer, as they are able to facilitate the survival and proliferation of cancer cells under genotoxic, oxidative, and nutritional stress [291]. The metabolic reprogramming, as one of cancer hallmarks, includes amino acids metabolism. There are interconnections between amino acids metabolism and other metabolic and signaling pathways. Modifications in the aminoacids biosynthesis pathways would lead to modifications of the different final products, hence, producing alterations in multiple signaling cascades and metabolic signaling pathways [292][293].

In our BC patients cohort, we have found an increment of circulating branched aminoacids, particularly, valine, leucine, and isoleucine. These branched aminoacids have been described as a principal source of energy for cancer growth and progression[294]. Moreover, metabolism reprograming of these branched aminoacids can also induce cancer progression [295]. Interestingly, we also observed modifications in tyrosine and alanine in our BC patients cohort compared to control healthy women. Altered metabolism of tyrosine in liver cancer has been described, suggesting its importance in cancer development and cancer diagnosis [296]. Alanine is one of the principal components of different proteins and, although not published data suggest a clear correlation with cancer, it might have a crucial role in cancer progression [297].

Summarizing, the study of the tumor microenvironment effects on cancer development and progression, might open new strategies for BC cancer treatment and diagnosis.

CONCLUSIONS

8. CONCLUSIONS

The present thesis has the following conclusions:

1) Adipose tissue releases a high number of factors that are internalized in tumor cells, activating Akt and MAPK pathways, hence promoting tumor proliferation. Moreover, crosstalk between adipose tissue and tumor cells enhances chemoresistance, migration and invasiveness, thus promoting cancer progression and malignancy.

2) FABP4, FABP5 and CD36 are increased in BC cell lines after their culture with adipocyte CM. Concomitantly, an increment of cytoplasmatic lipid droplets is observed, thus suggesting that these fatty acid transport proteins might be partially responsible of lipid accumulation and cancer hallmark acquisition.

3) Inhibition of FABP4 significantly reduces lipid transfer between adipose tissue and tumor cells, thus reducing the different cancer hallmarks. These results suggests that this fatty acid binding protein might have a role in tumor microenvironment crosstalk, hence in cancer progression.

4) Tumor microenvironment crosstalk is bidirectional, hence BCCs modifies surrounding adipose tissue behavior by the increment of lipolysis and lipid uptake by the transport proteins FABP4, FABP5 and CD36.

5) Lipid synthesis profile of studied BCC lines significantly differs between them, thus explaining their different tumorigenicity grade.

6) MCF-7 drug resistant cells switch their lipid metabolism signature to TNBC cell line MDA-MB-231, hence explaining the augmented malignancy of these cell lines compared to their precursor sensitive cell line MCF-7.

7) BCCs generate a pro-inflammatory status in the surrounding adipose tissue by the increment of certain pro-inflammatory cytokines such as IL-6 and MCP-1. The chemoattraction of new immune system cells, lipid droplets increment and FABP4 uptake as well as the activation of the NF-κB and p38 MAPK pathways, partially mediated by the crosstalk between BCCs and adipose tissue, induce the generation of an inflamed microenvironment.

8) This pro-inflammatory status is notably reflected in BC patients, whose CRP serum values were significantly increased compared to control healthy women.

9) The biomolecule Glyc-A might be used as a new pro-inflammatory biomarker, as is positively correlated with CRP in our cohort of study.

10) CRP and Glyc-A serum levels are positively correlated with the protein levels of FABP4 in our BC patients' cohort. These results plus the association of FABP4 with the overactivation of the THP-1 monocytic cell line, suggest a possible association of FABP4 with the generation of a pro-inflammatory status in the tumor microenvironment.

11) BC patients undergo modifications in the lipoprotein profile composition, presenting a significant triglycerides and cholesterol enrichment, compared to control healthy women lipoproteins.

12) Total triglycerides as well as the triglycerides enriched-lipoproteins increment in BC patients are positively associated with CRP and Glyc-A serum levels increment, thus suggesting alterations in lipid metabolism and the generation of a pro-inflammatory status in BC.

13) Metabolism deregulation, as a new cancer hallmark, includes alterations in aminoacids metabolism. BC patients have elevated levels of LMWMs valine, leucine, isoleucine, tyrosine and alanine, important protein scaffold molecules as well as important signal mediators.

14) Advanced NMR profiling allows to identify relevant altered metabolites in breast cancer serum samples to uncover specific metabolic signatures for early and reliable BC patient diagnosis and prognosis.

FUTURE PRESPECTIVES

9. FUTURE PRESPECTIVES

Breast cancer incidence and mortality is notably increasing, and it is expected to rise to 24 million new cases per year in 2035. BC mortality raised to 9.5 million of deaths in 2018. Moreover, the incidence of obesity in modern society is notably elevated, as well as their related deaths. The relationship between obesity and cancer has been deeply studied, stablishing a close link of both diseases. Effects of adipose tissue in cancer progression has also been demonstrated and corroborated in this doctoral thesis. In fact, we have demonstrated that FABP4 might be a key protein in some cancer hallmarks. Knowledge of the action mechanisms of this protein in the tumor microenvironment crosstalk might open new therapeutic strategies. Accordingly, in this research we have demonstrated the effect of FABP4 inhibition by the blockade of the communication between adipose tissue and tumor cells.

However, although we have demonstrated that FABP4 can induce the proliferative and survival pathways of Akt and MAPK, as there is not a total reversion of adipocyte CM suppose that there are other action mechanisms that underlined the adipocyte and tumor crosstalk, and they must be investigated.

Moreover, as a limitation of this study, our research was performed mainly *in vitro* conditions. However, we are collecting tumoral and peritumoral BC samples for further studies, in order to analyze the effects

of crosstalk between the different tumor microenvironment elements in tumor progression.

Understanding that tumor microenvironment crosstalk is bidirectional opens a new action perspective. The alteration of the tumor microenvironment might become a new action strategy in the future. In this research we have demonstrated that mature adipocytes suffer a higher delipidation and an increment in the fatty acid transporters releasement, partially mediated by BCCs influence. Identification of those tumor released factors that alter the metabolism behavior of the peritumoral adipose tissue might be a plausible strategy in cancer progression attenuation.

In addition, the metabolic profile of the different cell lines, hence of the different molecular subtype of BC might be a reflection of their tumorigenicity. Understanding the upregulated lipid pathways in most malignant cell lines might open new therapeutic targets.

Besides, in this doctoral thesis, we have demonstrated that crosstalk between adipocytes, tumor cells and immune system macrophages generates a pro-inflammatory status in tumor microenvironment as well as in BC patients. As a new cancer hallmark, the enhancement of a pro-inflammatory behavior, hence its modulation, might be a potential treatment of this disease.

BIBLIOGRAPHY

10. BIBLIOGRAPHY

1. Bray, F.; Ferlay, J.; Soerjomataram, I.; Siegel, R.L.; Torre, L.A.; Jemal, A. Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA. Cancer J. Clin.* **2018**, *68*, 394–424, doi:10.3322/caac.21492.
2. Pilleron, S.; Sarfati, D.; Janssen-Heijnen, M.; Vignat, J.; Ferlay, J.; Bray, F.; Soerjomataram, I. Global cancer incidence in older adults, 2012 and 2035: A population-based study. *Int. J. Cancer* **2019**, *144*, 49–58, doi:10.1002/ijc.31664.
3. Harbeck, N.; Penault-Llorca, F.; Cortes, J.; Gnant, M.; Houssami, N.; Poortmans, P.; Ruddy, K.; Tsang, J.; Cardoso, F. Breast cancer. *Nat. Rev. Dis. Prim.* **2019**, *5*, 66, doi:10.1038/s41572-019-0111-2.
4. Bray, F.; Ferlay, J.; Laversanne, M.; Brewster, D.H.; Gombe Mbalawa, C.; Kohler, B.; Piñeros, M.; Steliarova-Foucher, E.; Swaminathan, R.; Antoni, S.; et al. Cancer Incidence in Five Continents: Inclusion criteria, highlights from Volume X and the global status of cancer registration. *Int. J. Cancer* **2015**, *137*, 2060–2071, doi:10.1002/ijc.29670.
5. Hortobagyi, G.N.; de la Garza Salazar, J.; Pritchard, K.; Amadori, D.; Haidinger, R.; Hudis, C.A.; Khaled, H.; Liu, M.C.; Martin, M.; Namer, M.; et al. The global breast cancer burden: Variations in epidemiology and survival. *Clin. Breast Cancer* 2005, *6*, 391–401.
6. Torre, L.A.; Siegel, R.L.; Ward, E.M.; Jemal, A. Global cancer incidence and mortality rates and trends - An update. *Cancer Epidemiol. Biomarkers Prev.* 2016, *25*, 16–27.
7. Torre, L.A.; Islami, F.; Siegel, R.L.; Ward, E.M.; Jemal, A. Global cancer in women: Burden and trends. *Cancer Epidemiol. Biomarkers Prev.* 2017,

26, 444–457.

8. Allemani, C.; Weir, H.K.; Carreira, H.; Harewood, R.; Spika, D.; Wang, X.S.; Bannon, F.; Ahn, J. V.; Johnson, C.J.; Bonaventure, A.; et al. Global surveillance of cancer survival 1995-2009: Analysis of individual data for 25 676 887 patients from 279 population-based registries in 67 countries (CONCORD-2). *Lancet* **2015**, *385*, 977–1010, doi:10.1016/S0140-6736(14)62038-9.
9. Howlader N, Noone AM, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, Cronin KAHowlader N, Noone AM, Krapcho M, Miller D, Brest A, Yu M, Ruhl J, Tatalovich Z, Mariotto A, Lewis DR, Chen HS, Feuer EJ, C.K. Cancer Statistics Review, 1975-2017 - SEER Statistics Available online: https://seer.cancer.gov/archive/csr/1975_2017/#contents (accessed on Apr 19, 2021).
10. Agarwal, G.; Ramakant, P.; Sánchez Forgach, E.R.; Rendón, J.C.; Chaparro, J.M.; Basurto, C.S.; Margaritoni, M. Breast cancer care in developing countries. *World J. Surg.* 2009, *33*, 2069–2076.
11. Kohler, B.A.; Sherman, R.L.; Howlader, N.; Jemal, A.; Ryerson, A.B.; Henry, K.A.; Boscoe, F.P.; Cronin, K.A.; Lake, A.; Noone, A.M.; et al. Annual report to the nation on the status of cancer, 1975-2011, featuring incidence of breast cancer subtypes by race/ethnicity, poverty, and state. *J. Natl. Cancer Inst.* **2015**, *107*, doi:10.1093/jnci/djv048.
12. Kong, X.; Liu, Z.; Cheng, R.; Sun, L.; Huang, S.; Fang, Y.; Wang, J. Variation in Breast Cancer Subtype Incidence and Distribution by Race/Ethnicity in the United States From 2010 to 2015. *JAMA Netw. open* **2020**, *3*, e2020303, doi:10.1001/jamanetworkopen.2020.20303.
13. Sun, Y.S.; Zhao, Z.; Yang, Z.N.; Xu, F.; Lu, H.J.; Zhu, Z.Y.; Shi, W.; Jiang, J.; Yao, P.P.; Zhu, H.P. Risk factors and preventions of breast cancer. *Int. J.*

Biol. Sci. 2017, *13*, 1387–1397.

14. Brose, M.S.; Rebbeck, T.R.; Calzone, K.A.; Stopfer, J.E.; Nathanson, K.L.; Weber, B.L. Cancer risk estimates for BCRA1 mutation carriers identified in a risk evaluation program. *J. Natl. Cancer Inst.* **2002**, *94*, 1365–1372, doi:10.1093/jnci/94.18.1365.
15. Antoniou, A.; Pharoah, P.D.P.; Narod, S.; Risch, H.A.; Eyfjord, J.E.; Hopper, J.L.; Loman, N.; Olsson, H.; Johannsson, O.; Borg, Å.; et al. Average risks of breast and ovarian cancer associated with BRCA1 or BRCA2 mutations detected in case series unselected for family history: A combined analysis of 22 studies. *Am. J. Hum. Genet.* **2003**, *72*, 1117–1130, doi:10.1086/375033.
16. Chen, S.; Parmigiani, G. Meta-analysis of BRCA1 and BRCA2 penetrance. *J. Clin. Oncol.* **2007**, *25*, 1329–1333, doi:10.1200/JCO.2006.09.1066.
17. Walsh, T.; Lee, M.K.; Casadei, S.; Thornton, A.M.; Stray, S.M.; Pennil, C.; Nord, A.S.; Mandell, J.B.; Swisher, E.M.; King, M.C. Detection of inherited mutations for breast and ovarian cancer using genomic capture and massively parallel sequencing. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 12629–12633, doi:10.1073/pnas.1007983107.
18. An, G.; Wu, F.; Huang, S.; Feng, L.; Bai, J.; Gu, S.; Zhao, X. Effects of CCL5 on the biological behavior of breast cancer and the mechanisms of its interaction with tumor-associated macrophages. *Oncol. Rep.* **2019**, *42*, 2499–2511, doi:10.3892/or.2019.7344.
19. Gonzalez, K.D.; Noltner, K.A.; Buzin, C.H.; Gu, D.; Wen-Fong, C.Y.; Nguyen, V.Q.; Han, J.H.; Lowstuter, K.; Longmate, J.; Sommer, S.S.; et al. Beyond li fraumeni syndrome: Clinical characteristics of families with p53 germline mutations. *J. Clin. Oncol.* **2009**, *27*, 1250–1256, doi:10.1200/JCO.2008.16.6959.
20. Merino, D.; Malkin, D. p53 and hereditary cancer. *Subcell. Biochem.* **2014**,

85, doi:10.1007/978-94-017-9211-0_1.

21. Garber, J.E.; Offit, K. Hereditary cancer predisposition syndromes. *J. Clin. Oncol.* 2005, *23*, 276–292.
22. Malkin, D. Li-fraumeni syndrome. *Genes and Cancer* 2011, *2*, 475–484.
23. Stiles, B.; Groszer, M.; Wang, S.; Jiao, J.; Wu, H. PTENless means more. *Dev. Biol.* 2004, *273*, 175–184.
24. Downes, C.P.; Ross, S.; Maccario, H.; Perera, N.; Davidson, L.; Leslie, N.R. Stimulation of PI 3-kinase signaling via inhibition of the tumor suppressor phosphatase, PTEN. *Adv. Enzyme Regul.* **2007**, *47*, 184–194, doi:10.1016/j.advenzreg.2006.12.018.
25. Eng, C. Role of PTEN, a lipid phosphatase upstream effector of protein kinase B, in epithelial thyroid carcinogenesis. *Ann. N. Y. Acad. Sci.* **2002**, *968*, 213–221, doi:10.1111/j.1749-6632.2002.tb04337.x.
26. Li, J.; Yen, C.; Liaw, D.; Podsypanina, K.; Bose, S.; Wang, S.I.; Puc, J.; Miliaresis, C.; Rodgers, L.; McCombie, R.; et al. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. *Science (80-.).* **1997**, *275*, 1943–1947, doi:10.1126/science.275.5308.1943.
27. Allain, D.C. Genetic counseling and testing for common hereditary breast cancer syndromes a Paper from the 2007 William Beaumont hospital symposium on molecular pathology. *J. Mol. Diagnostics* 2008, *10*, 383–395.
28. Tomlinson, I.P.M.; Houlston, R.S. Peutz-Jeghers syndrome. *J. Med. Genet.* 1997, *34*, 1007–1011.
29. Lim, W.; Olschwang, S.; Keller, J.J.; Westerman, A.M.; Menko, F.H.; Boardman, L.A.; Scott, R.J.; Trimbath, J.; Giardiello, F.M.; Gruber, S.B.; et al. Relative frequency and morphology of cancers in STK11 mutation carriers. *Gastroenterology* **2004**, *126*, 1788–1794,

doi:10.1053/j.gastro.2004.03.014.

30. Kangelaris, K.N.; Gruber, S.B. Clinical implications of founder and recurrent CDH1 mutations in hereditary diffuse gastric cancer. *J. Am. Med. Assoc.* 2007, *297*, 2410–2411.

31. Pharoah, P.D.P.; Guilford, P.; Caldas, C. Incidence of gastric cancer and breast cancer in CDH1 (E-cadherin) mutation carriers from hereditary diffuse gastric cancer families. *Gastroenterology* **2001**, *121*, 1348–1353, doi:10.1053/gast.2001.29611.

32. Berx, G.; Becker, K.F.; Höfler, H.; Van Roy, F. Mutations of the human E-cadherin (CDH1) gene. *Hum. Mutat.* 1998, *12*, 226–237.

33. Masciari, S.; Larsson, N.; Senz, J.; Boyd, N.; Kaurah, P.; Kandel, M.J.; Harris, L.N.; Pinheiro, H.C.; Troussard, A.; Miron, P.; et al. Germline E-cadherin mutations in familial lobular breast cancer. *J. Med. Genet.* **2007**, *44*, 726–731, doi:10.1136/jmg.2007.051268.

34. Siegel, R.L.; Miller, K.D.; Jemal, A. Cancer statistics, 2020. *CA. Cancer J. Clin.* **2020**, *70*, 7–30, doi:10.3322/caac.21590.

35. Yang, L.; Jacobsen, K.H. A systematic review of the association between breastfeeding and breast cancer. *J. Women's Heal.* **2008**, *17*, 1635–1645, doi:10.1089/jwh.2008.0917.

36. Gray, S. Breast cancer and hormone-replacement therapy: the Million Women Study. *Lancet* 2003, *362*, 1328–1329.

37. Beloribi-Djefaflia, S.; Vasseur, S.; Guillaumond, F. Lipid metabolic reprogramming in cancer cells. *Oncogenesis* **2016**, *5*, e189–e189, doi:10.1038/oncsis.2015.49.

38. Washbrook, E. Risk factors and epidemiology of breast cancer. *Women's Heal. Med.* **2006**, *3*, 8–14, doi:10.1383/wohm.2006.3.1.8.

39. Horn, J.; Åsvold, B.O.; Opdahl, S.; Tretli, S.; Vatten, L.J. Reproductive factors and the risk of breast cancer in old age: A Norwegian cohort

study. *Breast Cancer Res. Treat.* **2013**, *139*, 237–243, doi:10.1007/s10549-013-2531-0.

40. Rosato, V.; Bosetti, C.; Negri, E.; Talamini, R.; Dal Maso, L.; Malvezzi, M.; Falcini, F.; Montella, M.; La Vecchia, C. Reproductive and hormonal factors, family history, and breast cancer according to the hormonal receptor status. *Eur. J. Cancer Prev.* **2014**, *23*, 412–417, doi:10.1097/CEJ.0b013e3283639f7a.

41. Wang, Y.; Xu, M.; Ke, Z. ji; Luo, J. Cellular and molecular mechanisms underlying alcohol-induced aggressiveness of breast cancer. *Pharmacol. Res.* 2017, *115*, 299–308.

42. Fan, S.; Meng, Q.; Gao, B.; Grossman, J.; Yadegari, M.; Goldberg, I.D.; Rosen, E.M. Alcohol stimulates estrogen receptor signaling in human breast cancer cell lines. *Cancer Res.* **2000**, *60*, 5635–5639.

43. Etique, N.; Grillier-Vuissoz, I.; Flament, S. Ethanol stimulates the secretion of matrix metalloproteinases 2 and 9 in MCF-7 human breast cancer cells. *Oncol. Rep.* **2006**, *15*, 603–608, doi:10.3892/or.15.3.603.

44. Xu, M.; Ren, Z.; Wang, X.; Comer, A.; Frank, J.A.; Ke, Z.J.; Huang, Y.; Zhang, Z.; Shi, X.; Wang, S.; et al. ErbB2 and p38γ MAPK mediate alcohol-induced increase in breast cancer stem cells and metastasis. *Mol. Cancer* **2016**, *15*, doi:10.1186/s12943-016-0532-4.

45. Catsburg, C.; Miller, A.B.; Rohan, T.E. Active cigarette smoking and risk of breast cancer. *Int. J. Cancer* **2015**, *136*, 2204–2209, doi:10.1002/ijc.29266.

46. Knight, J.A.; Fan, J.; Malone, K.E.; John, E.M.; Lynch, C.F.; Langballe, R.; Bernstein, L.; Shore, R.E.; Brooks, J.D.; Reiner, A.S.; et al. Alcohol consumption and cigarette smoking in combination: A predictor of contralateral breast cancer risk in the WECARE study. *Int. J. Cancer* **2017**, *141*, 916–924, doi:10.1002/ijc.30791.

47. Zhang, C.; Liao, Y.; Liu, P.; Du, Q.; Liang, Y.; Ooi, S.; Qin, S.; He, S.; Yao, S.; Wang, W. FABP5 promotes lymph node metastasis in cervical cancer by reprogramming fatty acid metabolism. *Theranostics* **2020**, *10*, 6561–6580, doi:10.7150/thno.44868.
48. Hoyo, C.; Cook, M.B.; Kamangar, F.; Freedman, N.D.; Whiteman, D.C.; Bernstein, L.; Brown, L.M.; Risch, H.A.; Ye, W.; Sharp, L.; et al. Body mass index in relation to oesophageal and oesophagogastric junction adenocarcinomas: A pooled analysis from the international BEACON consortium. *Int. J. Epidemiol.* **2012**, *41*, 1706–1718, doi:10.1093/ije/dys176.
49. Flegal, K.M.; Graubard, B.I.; Williamson, D.F.; Gail, M.H. Excess deaths associated with underweight, overweight, and obesity. *J. Am. Med. Assoc.* **2005**, *293*, 1861–1867, doi:10.1001/jama.293.15.1861.
50. Smith, K.B.; Smith, M.S. Obesity Statistics. *Prim. Care - Clin. Off. Pract.* 2016, *43*, 121–135.
51. Abdelaal, M.; le Roux, C.W.; Docherty, N.G. Morbidity and mortality associated with obesity. *Ann. Transl. Med.* 2017, *5*.
52. Polednak, A.P. Estimating the number of U.S. incident cancers attributable to obesity and the impact on temporal trends in incidence rates for obesity-related cancers. *Cancer Detect. Prev.* **2008**, *32*, 190–199, doi:10.1016/j.cdp.2008.08.004.
53. Marmot, M.; Atinmo, T.; Byers, T.; Chen, J.; Hirohata, T.; Jackson, A.; James, W.; Kolonel, L.; Kumanyika, S.; Leitzmann, C.; et al. Food, nutrition, physical activity, and the prevention of cancer: a global perspective. *Choice Rev. Online* **2008**, *45*, 45-5024-45–5024, doi:10.5860/choice.45-5024.
54. Munsell, M.F.; Sprague, B.L.; Berry, D.A.; Chisholm, G.; Trentham-Dietz, A. Body mass index and breast cancer risk according to postmenopausal

estrogen-progestin use and hormone receptor status. *Epidemiol. Rev.* **2014**, *36*, 114–136, doi:10.1093/epirev/mxt010.

55. Picon-Ruiz, M.; Morata-Tarifa, C.; Valle-Goffin, J.J.; Friedman, E.R.; Slingerland, J.M. Obesity and adverse breast cancer risk and outcome: Mechanistic insights and strategies for intervention. *CA. Cancer J. Clin.* **2017**, *67*, 378–397, doi:10.3322/caac.21405.
56. Chan, D.S.M.; Vieira, A.R.; Aune, D.; Bandera, E. V.; Greenwood, D.C.; McTiernan, A.; Navarro Rosenblatt, D.; Thune, I.; Vieira, R.; Norat, T. Body mass index and survival in women with breast cancer—systematic literature review and meta-analysis of 82 follow-up studies. *Ann. Oncol.* 2014, *25*, 1901–1914.
57. Hanahan, D.; Weinberg, R.A. The hallmarks of cancer. *Cell* 2000, *100*, 57–70.
58. Colotta, F.; Allavena, P.; Sica, A.; Garlanda, C.; Mantovani, A. Cancer-related inflammation, the seventh hallmark of cancer: Links to genetic instability. *Carcinogenesis* 2009, *30*, 1073–1081.
59. Negrini, S.; Gorgoulis, V.G.; Halazonetis, T.D. Genomic instability an evolving hallmark of cancer. *Nat. Rev. Mol. Cell Biol.* 2010, *11*, 220–228.
60. Kroemer, G.; Pouyssegur, J. Tumor Cell Metabolism: Cancer’s Achilles’ Heel. *Cancer Cell* 2008, *13*, 472–482.
61. Hanahan, D.; Weinberg, R.A. Hallmarks of cancer: The next generation. *Cell* 2011, *144*, 646–674.
62. Witsch, E.; Sela, M.; Yarden, Y. Roles for Growth Factors in Cancer Progression. *Physiology* 2010, *25*, 85–101.
63. Hynes, N.E.; MacDonald, G. ErbB receptors and signaling pathways in cancer. *Curr. Opin. Cell Biol.* **2009**, *21*, 177–184, doi:10.1016/J.CEB.2008.12.010.
64. Perona, R. Cell signalling: Growth factors and tyrosine kinase receptors.

Clin. Transl. Oncol. **2006**, *8*, 77–82, doi:10.1007/s12094-006-0162-1.

65. Lemmon, M.A.; Schlessinger, J. Cell signaling by receptor tyrosine kinases. *Cell* 2010, *141*, 1117–1134.
66. Yuan, T.L.; Cantley, L.C. PI3K pathway alterations in cancer: Variations on a theme. *Oncogene* 2008, *27*, 5497–5510.
67. Noorolyai, S.; Shajari, N.; Baghbani, E.; Sadreddini, S.; Baradaran, B. The relation between PI3K/AKT signalling pathway and cancer. *Gene* 2019, *698*, 120–128.
68. Davies, H.; Bignell, G.R.; Cox, C.; Stephens, P.; Edkins, S.; Clegg, S.; Teague, J.; Woffendin, H.; Garnett, M.J.; Bottomley, W.; et al. Mutations of the BRAF gene in human cancer. *Nature* **2002**, *417*, 949–954, doi:10.1038/nature00766.
69. Jiang, B.H.; Liu, L.Z. PI3K/PTEN signaling in tumorigenesis and angiogenesis. *Biochim. Biophys. Acta - Proteins Proteomics* 2008, *1784*, 150–158.
70. Ji, J.; Zheng, P.S. Activation of mTOR signaling pathway contributes to survival of cervical cancer cells. *Gynecol. Oncol.* **2010**, *117*, 103–108, doi:10.1016/j.ygyno.2009.12.020.
71. Sudarsanam, S.; Johnson, D.E. Functional consequences of mTOR inhibition. *Curr. Opin. Drug Discov. Dev.* 2010, *13*, 31–40.
72. Burkhart, D.L.; Sage, J. Cellular mechanisms of tumour suppression by the retinoblastoma gene. *Nat. Rev. Cancer* **2008**, *8*, 671–682, doi:10.1038/NRC2399.
73. Muller, P.A.J.; Vousden, K.H. P53 mutations in cancer. *Nat. Cell Biol.* 2013, *15*, 2–8.
74. Curto, M.; Cole, B.K.; Lallemand, D.; Liu, C.H.; McClatchey, A.I. Contact-dependent inhibition of EGFR signaling by Nf2/Merlin. *J. Cell Biol.* **2007**, *177*, 893–903, doi:10.1083/jcb.200703010.

75. Okada, T.; Lopez-Lago, M.; Giancotti, F.G. Merlin/NF-2 mediates contact inhibition of growth by suppressing recruitment of Rac to the plasma membrane. *J. Cell Biol.* **2005**, *171*, 361–371, doi:10.1083/jcb.200503165.
76. Evan, G.; Littlewood, T. A matter of life and cell death. *Science (80-.).* 1998, *281*, 1317–1322.
77. Junttila, M.R.; Evan, G.I. p53--a Jack of all trades but master of none. *Nat. Rev. Cancer* **2009**, *9*, 821–829, doi:10.1038/NRC2728.
78. Adams, J.M.; Cory, S. The Bcl-2 apoptotic switch in cancer development and therapy. *Oncogene* 2007, *26*, 1324–1337.
79. Willis, S.N.; Adams, J.M. Life in the balance: How BH3-only proteins induce apoptosis. *Curr. Opin. Cell Biol.* 2005, *17*, 617–625.
80. Zhang, X.; Tang, N.; Hadden, T.J.; Rishi, A.K. Akt, FoxO and regulation of apoptosis. *Biochim. Biophys. Acta - Mol. Cell Res.* 2011, *1813*, 1978–1986.
81. Fu, Z.; Tindall, D.J. FOXOs, cancer and regulation of apoptosis. *Oncogene* 2008, *27*, 2312–2319.
82. Sunters, A.; Fernández De Mattos, S.; Stahl, M.; Brosens, J.J.; Zoumpoulidou, G.; Saunders, C.A.; Coffer, P.J.; Medema, R.H.; Coombes, R.C.; Lam, E.W.F. FoxO3a Transcriptional Regulation of Bim Controls Apoptosis in Paclitaxel-treated Breast Cancer Cell Lines. *J. Biol. Chem.* **2003**, *278*, 49795–49805, doi:10.1074/jbc.M309523200.
83. Blasco, M.A. Telomeres and human disease: Ageing, cancer and beyond. *Nat. Rev. Genet.* 2005, *6*, 611–622.
84. Artandi, S.E.; DePinho, R.A. Telomeres and telomerase in cancer. *Carcinogenesis* 2009, *31*, 9–18.
85. Hanahan, D.; Folkman, J. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell* 1996, *86*, 353–364.
86. Saha, T.; Solomon, J.; Samson, A.O.; Gil-Henn, H. Invasion and metastasis as a central hallmark of breast cancer. *J. Clin. Med.* 2021, *10*.

87. Berx, G.; van Roy, F. Involvement of members of the cadherin superfamily in cancer. *Cold Spring Harb. Perspect. Biol.* 2009, *1*.

88. Van Zijl, F.; Krupitza, G.; Mikulits, W. Initial steps of metastasis: Cell invasion and endothelial transmigration. *Mutat. Res. - Rev. Mutat. Res.* 2011, *728*, 23–34.

89. Zavyalova, M. V.; Denisov, E. V.; Tashireva, L.A.; Savelieva, O.E.; Kaigorodova, E. V.; Krakhmal, N. V.; Perelmuter, V.M. Intravasation as a Key Step in Cancer Metastasis. *Biochem.* 2019, *84*, 762–772.

90. Their, J.P. Epithelial-mesenchymal transitions in tumor progression. *Nat. Rev. Cancer* 2002, *2*, 442–454.

91. Medzhitov, R. Origin and physiological roles of inflammation. *Nature* 2008, *454*, 428–435.

92. Andarawewa, K.L.; Motrescu, E.R.; Chenard, M.P.; Gansmuller, A.; Stoll, I.; Tomasetto, C.; Rio, M.C. Stromelysin-3 is a potent negative regulator of adipogenesis participating to cancer cell-adipocyte interaction/crosstalk at the tumor invasive front. *Cancer Res.* **2005**, *65*, 10862–10871, doi:10.1158/0008-5472.CAN-05-1231.

93. Motrescu, E.R.; Rio, M.C. Cancer cells, adipocytes and matrix metalloproteinase 11: A vicious tumor progression cycle. In Proceedings of the Biological Chemistry; Biol Chem, 2008; Vol. 389, pp. 1037–1041.

94. DeNardo, D.G.; Andreu, P.; Coussens, L.M. Interactions between lymphocytes and myeloid cells regulate pro-versus anti-tumor immunity. *Cancer Metastasis Rev.* 2010, *29*, 309–316.

95. Mantovani, A.; Allavena, P.; Sica, A.; Balkwill, F. Cancer-related inflammation. *Nature* 2008, *454*, 436–444.

96. Murata, M. Inflammation and cancer. *Environ. Health Prev. Med.* 2018, *23*.

97. Vajdic, C.M.; Van Leeuwen, M.T. Cancer incidence and risk factors after

solid organ transplantation. *Int. J. Cancer* 2009, *125*, 1747–1754.

98. Penn, I.; Starzl, T.E. Immunosuppression and cancer. *Transplant. Proc.* **1973**, *5*, 943–947.

99. Mayor, P.C.; Eng, K.H.; Singel, K.L.; Abrams, S.I.; Odunsi, K.; Moysich, K.B.; Fuleihan, R.; Garabedian, E.; Lugar, P.; Ochs, H.D.; et al. Cancer in primary immunodeficiency diseases: Cancer incidence in the United States Immune Deficiency Network Registry. *J. Allergy Clin. Immunol.* **2018**, *141*, 1028–1035, doi:10.1016/j.jaci.2017.05.024.

100. Kim, R.; Emi, M.; Tanabe, K. Cancer immunoediting from immune surveillance to immune escape. *Immunology* 2007, *121*, 1–14.

101. Teng, M.W.L.; Swann, J.B.; Koebel, C.M.; Schreiber, R.D.; Smyth, M.J. Immune-mediated dormancy: an equilibrium with cancer. *J. Leukoc. Biol.* **2008**, *84*, 988–993, doi:10.1189/jlb.1107774.

102. Olson, B. m.; McNeel, D. g. Antigen loss and tumor-mediated immunosuppression facilitate tumor recurrence. *Expert Rev. Vaccines* **2012**, *11*, 1315–1317, doi:10.1586/erv.12.107.

103. Yang, L.; Pang, Y.; Moses, H.L. TGF-β and immune cells: an important regulatory axis in the tumor microenvironment and progression. *Trends Immunol.* 2010, *31*, 220–227.

104. Shields, J.D.; Kourtis, I.C.; Tomei, A.A.; Roberts, J.M.; Swartz, M.A. Induction of lymphoidlike stroma and immune escape by tumors that express the chemokine CCL21. *Science (80-.).* **2010**, *328*, 749–752, doi:10.1126/science.1185837.

105. Mougiakakos, D. Regulatory T cells in colorectal cancer: From biology to prognostic relevance. *Cancers (Basel).* 2011, *3*, 1708–1731.

106. Ostrand-Rosenberg, S.; Sinha, P. Myeloid-Derived Suppressor Cells: Linking Inflammation and Cancer. *J. Immunol.* **2009**, *182*, 4499–4506, doi:10.4049/jimmunol.0802740.

107. Warburg, O.; Wind, F.; Negelein, E. The metabolism of tumors in the body. *J. Gen. Physiol.* **1927**, *8*, 519–530, doi:10.1085/jgp.8.6.519.

108. Hay, N. Reprogramming glucose metabolism in cancer: Can it be exploited for cancer therapy? *Nat. Rev. Cancer* 2016, *16*, 635–649.

109. Khatami, F.; Aghamir, S.M.K.; Tavangar, S.M. Oncometabolites: A new insight for oncology. *Mol. Genet. Genomic Med.* 2019, *7*.

110. Abramson, H.N. The lipogenesis pathway as a cancer target. *J. Med. Chem.* 2011, *54*, 5615–5638.

111. Guo, D.; Bell, E.H. lavi.; Chakravarti, A. Lipid metabolism emerges as a promising target for malignant glioma therapy. *CNS Oncol.* 2013, *2*, 289–299.

112. Krishna Muppalla, J.N.; Muddana, K.; Reddy Dorankula, S.P.; Rao Thokala, M.; Prakash Pasupula, A. Microenvironment-A role in tumour progression and prognosis. *J. Clin. Diagnostic Res.* 2013, *7*, 2096–2099.

113. Folgueira, M.A.A.K.; Maistro, S.; Katayama, M.L.H.; Roela, R.A.; Mundim, F.G.L.; Nanogaki, S.; De Bock, G.H.; Brentani, M.M. Markers of breast cancer stromal fibroblasts in the primary tumour site associated with lymph node metastasis: A systematic review including our case series. *Biosci. Rep.* **2013**, *33*, 5635–5639, doi:10.1042/BSR20130060.

114. Hu, M.; Polyak, K. Microenvironmental regulation of cancer development. *Curr. Opin. Genet. Dev.* 2008, *18*, 27–34.

115. Hu, M.; Yao, J.; Carroll, D.K.; Weremowicz, S.; Chen, H.; Carrasco, D.; Richardson, A.; Violette, S.; Nikolskaya, T.; Nikolsky, Y.; et al. Regulation of In Situ to Invasive Breast Carcinoma Transition. *Cancer Cell* **2008**, *13*, 394–406, doi:10.1016/j.ccr.2008.03.007.

116. Kalluri, R.; Zeisberg, M. Fibroblasts in cancer. *Nat. Rev. Cancer* 2006, *6*, 392–401.

117. Kojima, Y.; Acar, A.; Eaton, E.N.; Mellody, K.T.; Scheel, C.; Ben-Porath, I.;

Onder, T.T.; Wang, Z.C.; Richardson, A.L.; Weinberg, R.A.; et al. Autocrine TGF-β and stromal cell-derived factor-1 (SDF-1) signaling drives the evolution of tumor-promoting mammary stromal myofibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 20009–20014, doi:10.1073/pnas.1013805107.

118. Krtolica, A.; Parrinello, S.; Lockett, S.; Desprez, P.Y.; Campisi, J. Senescent fibroblasts promote epithelial cell growth and tumorigenesis: A link between cancer and aging. *Proc. Natl. Acad. Sci. U. S. A.* **2001**, *98*, 12072–12077, doi:10.1073/pnas.211053698.

119. Dittmer, A.; Dittmer, J. Carcinoma-associated fibroblasts promote growth of sox2-expressing breast cancer cells. *Cancers (Basel).* **2020**, *12*, 1–22, doi:10.3390/cancers12113435.

120. Palmieri, C.; Roberts-Clark, D.; Assadi-Sabet, A.; Coope, R.C.; O'Hare, M.; Sunters, A.; Hanby, A.; Slade, M.J.; Gomm, J.J.; Lam, E.W.F.; et al. Fibrolast growth factor 7, secreted by breast fibroblasts, is an interleukin-1β-induced paracrine growth factor for human breast cells. *J. Endocrinol.* 2003, *177*, 65–81.

121. Martinez-Outschoorn, U.E.; Goldberg, A.; Lin, Z.; Ko, Y.H.; Flomenberg, N.; Wang, C.; Pavlides, S.; Pestell, R.G.; Howell, A.; Sotgia, F.; et al. Anti-estrogen resistance in breast cancer is induced by the tumor microenvironment and can be overcome by inhibiting mitochondrial function in epithelial cancer cells. *Cancer Biol. Ther.* **2011**, *12*, 924–938, doi:10.4161/cbt.12.10.17780.

122. Soon, P.S.H.; Kim, E.; Pon, C.K.; Gill, A.J.; Moore, K.; Spillane, A.J.; Benn, D.E.; Baxter, R.C. Breast cancer-associated fibroblasts induce epithelial-to-mesenchymal transition in breast cancer cells. *Endocr. Relat. Cancer* **2013**, *20*, 1–12, doi:10.1530/ERC-12-0227.

123. Biffi, G.; Tuveson, D.A. Diversity and biology of cancerassociated

fibroblasts. *Physiol. Rev.* **2021**, *101*, 147–176, doi:10.1152/physrev.00048.2019.

124. McAllister, S.S.; Weinberg, R.A. The tumour-induced systemic environment as a critical regulator of cancer progression and metastasis. *Nat. Cell Biol.* 2014, *16*, 717–727.
125. Orecchioni, M.; Ghosheh, Y.; Pramod, A.B.; Ley, K. Macrophage polarization: Different gene signatures in M1(Lps+) vs. Classically and M2(LPS-) vs. Alternatively activated macrophages. *Front. Immunol.* 2019, *10*.
126. Yang, M.; Ma, B.; Shao, H.; Clark, A.M.; Wells, A. Macrophage phenotypic subtypes diametrically regulate epithelial-mesenchymal plasticity in breast cancer cells. *BMC Cancer* **2016**, *16*, doi:10.1186/s12885-016-2411-1.
127. Komohara, Y.; Fujiwara, Y.; Ohnishi, K.; Takeya, M. Tumor-associated macrophages: Potential therapeutic targets for anti-cancer therapy. *Adv. Drug Deliv. Rev.* 2016, *99*, 180–185.
128. Allen, M.; Jones, J.L. Jekyll and Hyde: The role of the microenvironment on the progression of cancer. *J. Pathol.* 2011, *223*, 163–177.
129. Mantovani, A.; Schioppa, T.; Porta, C.; Allavena, P.; Sica, A. Role of tumor-associated macrophages in tumor progression and invasion. *Cancer Metastasis Rev.* 2006, *25*, 315–322.
130. Joyce, J.A.; Pollard, J.W. Microenvironmental regulation of metastasis. *Nat. Rev. Cancer* 2009, *9*, 239–252.
131. Cassetta, L.; Kitamura, T. Macrophage targeting: opening new possibilities for cancer immunotherapy. *Immunology* 2018, *155*, 285–293.
132. Sica, A.; Schioppa, T.; Mantovani, A.; Allavena, P. Tumour-associated macrophages are a distinct M2 polarised population promoting tumour

progression: Potential targets of anti-cancer therapy. *Eur. J. Cancer* **2006**, *42*, 717–727, doi:10.1016/j.ejca.2006.01.003.

133. Hao, J.; Yan, F.; Zhang, Y.; Triplett, A.; Zhang, Y.; Schultz, D.A.; Sun, Y.; Zeng, J.; Silverstein, K.A.T.; Zheng, Q.; et al. Expression of adipocyte/macrophage fatty acid–binding protein in tumor-associated macrophages promotes breast cancer progression. *Cancer Res.* **2018**, *78*, 2343–2355, doi:10.1158/0008-5472.CAN-17-2465.
134. Mantovani, A.; Locati, M. Tumor-associated macrophages as a paradigm of macrophage plasticity, diversity, and polarization lessons and open questions. *Arterioscler. Thromb. Vasc. Biol.* **2013**, *33*, 1478–1483, doi:10.1161/ATVBAHA.113.300168.
135. Bednarczyk, R.B.; Tuli, N.Y.; Hanly, E.K.; Rahoma, G. Ben; Maniyar, R.; Mittelman, A.; Geliebter, J.; Tiwari, R.K. Macrophage inflammatory factors promote epithelial-mesenchymal transition in breast cancer. *Oncotarget* **2018**, *9*, 24272–24282, doi:10.18632/oncotarget.24917.
136. Bremnes, R.M.; Al-Shibli, K.; Donnem, T.; Sirera, R.; Al-Saad, S.; Andersen, S.; Stenvold, H.; Camps, C.; Busund, L.T. The role of tumor-infiltrating immune cells and chronic inflammation at the tumor site on cancer development, progression, and prognosis: Emphasis on non-small cell lung cancer. *J. Thorac. Oncol.* 2011, *6*, 824–833.
137. Schreiber, R.D.; Old, L.J.; Smyth, M.J. Cancer immunoediting: Integrating immunity’s roles in cancer suppression and promotion. *Science (80-.).* 2011, *331*, 1565–1570.
138. Halvorsen, E.C.; Mahmoud, S.M.; Bennewith, K.L. Emerging roles of regulatory T cells in tumour progression and metastasis. *Cancer Metastasis Rev.* **2014**, *33*, 1025–1041, doi:10.1007/s10555-014-9529-x.
139. Coelho, M.; Oliveira, T.; Fernandes, R. Biochemistry of adipose tissue: An endocrine organ. *Arch. Med. Sci.* 2013, *9*, 191–200.

140. Wu, Q.; Li, B.; Li, Z.; Li, J.; Sun, S.; Sun, S. Cancer-associated adipocytes: Key players in breast cancer progression. *J. Hematol. Oncol.* 2019, *12*.

141. Nieman, K.M.; Romero, I.L.; Van Houten, B.; Lengyel, E. Adipose tissue and adipocytes support tumorigenesis and metastasis. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 2013, *1831*, 1533–1541.

142. Wang, Y.Y.; Attané, C.; Milhas, D.; Dirat, B.; Dauvillier, S.; Guerard, A.; Gilhodes, J.; Lazar, I.; Alet, N.; Laurent, V.; et al. Mammary adipocytes stimulate breast cancer invasion through metabolic remodeling of tumor cells. *J. Clin. Invest.* **2017**, *2*, doi:10.1172/jci.insight.87489.

143. Muller, C. Tumour-surrounding adipocytes are active players in breast cancer progression. *Ann. Endocrinol. (Paris).* **2013**, *74*, 108–110, doi:10.1016/j.ando.2013.02.007.

144. Picon-Ruiz, M.; Pan, C.; Drews-Elger, K.; Jang, K.; Besser, A.H.; Zhao, D.; Morata-Tarifa, C.; Kim, M.; Ince, T.A.; Azzam, D.J.; et al. Interactions between adipocytes and breast cancer cells stimulate cytokine production and drive Src/Sox2/miR-302b-mediated malignant progression. *Cancer Res.* **2016**, *76*, 491–504, doi:10.1158/0008-5472.CAN-15-0927.

145. Choi, J.; Cha, Y.J.; Koo, J.S. Adipocyte biology in breast cancer: From silent bystander to active facilitator. *Prog. Lipid Res.* 2018, *69*, 11–20.

146. Bochet, L.; Lehuédé, C.; Dauvillier, S.; Wang, Y.Y.; Dirat, B.; Laurent, V.; Dray, C.; Guiet, R.; Maridonneau-Parini, I.; Gonidec, S. Le; et al. Adipocyte-derived fibroblasts promote tumor progression and contribute to the desmoplastic reaction in breast cancer. *Cancer Res.* **2013**, *73*, 5657–5668, doi:10.1158/0008-5472.CAN-13-0530.

147. Attané, C.; Muller, C. Drilling for Oil: Tumor-Surrounding Adipocytes Fueling Cancer. *Trends in Cancer* 2020, *6*, 593–604.

148. Harayama, T.; Riezman, H. Understanding the diversity of membrane

lipid composition. *Nat. Rev. Mol. Cell Biol.* 2018, *19*, 281–296.

149. Sunshine, H.; Iruela-Arispe, M.L. Membrane lipids and cell signaling. *Curr. Opin. Lipidol.* 2017, *28*, 408–413.

150. Carracedo, A.; Cantley, L.C.; Pandolfi, P.P. Cancer metabolism: Fatty acid oxidation in the limelight. *Nat. Rev. Cancer* **2013**, *13*, 227–232, doi:10.1038/nrc3483.

151. Ma, Y.; Temkin, S.M.; Hawkridge, A.M.; Guo, C.; Wang, W.; Wang, X.Y.; Fang, X. Fatty acid oxidation: An emerging facet of metabolic transformation in cancer. *Cancer Lett.* 2018, *435*, 92–100.

152. Camarda, R.; Zhou, A.Y.; Kohnz, R.A.; Balakrishnan, S.; Mahieu, C.; Anderton, B.; Eyob, H.; Kajimura, S.; Tward, A.; Krings, G.; et al. Inhibition of fatty acid oxidation as a therapy for MYC-overexpressing triple-negative breast cancer. *Nat. Med.* **2016**, *22*, 427–432, doi:10.1038/NM.4055.

153. Casciano, J.C.; Perry, C.; Cohen-Nowak, A.J.; Miller, K.D.; Vande Voorde, J.; Zhang, Q.; Chalmers, S.; Sandison, M.E.; Liu, Q.; Hedley, A.; et al. MYC regulates fatty acid metabolism through a multigenic program in claudin-low triple negative breast cancer. *Br. J. Cancer* **2020**, *122*, 868–884, doi:10.1038/s41416-019-0711-3.

154. Wang, T.; Fahrmann, J.F.; Lee, H.; Li, Y.J.; Tripathi, S.C.; Yue, C.; Zhang, C.; Lifshitz, V.; Song, J.; Yuan, Y.; et al. JAK/STAT3-Regulated Fatty Acid β-Oxidation Is Critical for Breast Cancer Stem Cell Self-Renewal and Chemoresistance. *Cell Metab.* **2018**, *27*, 136-150.e5, doi:10.1016/j.cmet.2017.11.001.

155. Chen, X.; Chen, H.; Dai, M.; Ai, J.; Li, Y.; Mahon, B.; Dai, S.; Deng, Y. Plasma lipidomics profiling identified lipid biomarkers in distinguishing early-stage breast cancer from benign lesions. *Oncotarget* **2016**, *7*, 36622–36631, doi:10.18632/oncotarget.9124.

156. Rybinska, I.; Mangano, N.; Tagliabue, E.; Triulzi, T. Cancer-associated adipocytes in breast cancer: Causes and consequences. *Int. J. Mol. Sci.* 2021, *22*.

157. Pelton, K.; Freeman, M.R.; Solomon, K.R. Cholesterol and prostate cancer. *Curr. Opin. Pharmacol.* **2012**, *12*, 751–759, doi:10.1016/J.COPH.2012.07.006.

158. Shafique, K.; McLoone, P.; Qureshi, K.; Leung, H.; Hart, C.; Morrison, D.S. Cholesterol and the risk of grade-specific prostate cancer incidence: evidence from two large prospective cohort studies with up to 37 years' follow up. *BMC Cancer* **2012**, *12*, doi:10.1186/1471-2407-12-25.

159. Ikonen, E. Cellular cholesterol trafficking and compartmentalization. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 125–138, doi:10.1038/NRM2336.

160. Bengoechea-Alonso, M.T.; Ericsson, J. SREBP in signal transduction: cholesterol metabolism and beyond. *Curr. Opin. Cell Biol.* 2007, *19*, 215–222.

161. Weinstein, J.N.; Collisson, E.A.; Mills, G.B.; Shaw, K.R.M.; Ozenberger, B.A.; Ellrott, K.; Sander, C.; Stuart, J.M.; Chang, K.; Creighton, C.J.; et al. The cancer genome atlas pan-cancer analysis project. *Nat. Genet.* **2013**, *45*, 1113–1120, doi:10.1038/ng.2764.

162. Kuzu, O.F.; Noory, M.A.; Robertson, G.P. The role of cholesterol in cancer. *Cancer Res.* 2016, *76*, 2063–2070.

163. Grossmann, M.E.; Ray, A.; Nkhata, K.J.; Malakhov, D.A.; Rogozina, O.P.; Dogan, S.; Cleary, M.P. Obesity and breast cancer: Status of leptin and adiponectin in pathological processes. *Cancer Metastasis Rev.* 2010, *29*, 641–653.

164. Ye, J.; Jia, J.; Dong, S.; Zhang, C.; Yu, S.; Li, L.; Mao, C.; Wang, D.; Chen, J.; Yuan, G. Circulating adiponectin levels and the risk of breast cancer: A meta-analysis. *Eur. J. Cancer Prev.* **2014**, *23*, 158–165,

doi:10.1097/CEJ.0b013e328364f293.

165. Wang, F.; Gao, S.; Chen, F.; Fu, Z.; Yin, H.; Lu, X.; Yu, J.; Lu, C. Mammary fat of breast cancer: Gene expression profiling and functional characterization. *PLoS One* **2014**, *9*, e109742, doi:10.1371/journal.pone.0109742.

166. Booth, A.; Magnuson, A.; Fouts, J.; Foster, M. Adipose tissue, obesity and adipokines: Role in cancer promotion. *Horm. Mol. Biol. Clin. Investig.* **2015**, *21*, 57–74, doi:10.1515/hmbci-2014-0037.

167. Fazeli, M.S.; Dashti, H.; Akbarzadeh, S.; Assadi, M.; Aminian, A.; Keramati, M.R.; Nabipour, I. Circulating levels of novel adipocytokines in patients with colorectal cancer. *Cytokine* **2013**, *62*, 81–85, doi:10.1016/j.cyto.2013.02.012.

168. Nimptsch, K.; Pischon, T. Body fatness, related biomarkers and cancer risk: An epidemiological perspective. *Horm. Mol. Biol. Clin. Investig.* **2015**, *22*, 39–51, doi:10.1515/hmbci-2014-0043.

169. Schmidt, S.; Monk, J.M.; Robinson, L.E.; Mourtzakis, M. The integrative role of leptin, oestrogen and the insulin family in obesity-associated breast cancer: Potential effects of exercise. *Obes. Rev.* **2015**, *16*, 473–487, doi:10.1111/obr.12281.

170. Brindley, D.N.; Lin, F.T.; Tigyi, G.J. Role of the autotaxin-lysophosphatidate axis in cancer resistance to chemotherapy and radiotherapy. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **2013**, *1831*, 74–85, doi:10.1016/j.bbalip.2012.08.015.

171. Berry, D.C.; Levi, L.; Noy, N. Holo-retinol-binding protein and its receptor STRA6 drive oncogenic transformation. *Cancer Res.* **2014**, *74*, 6341–6351, doi:10.1158/0008-5472.CAN-14-1052.

172. Guaita-Esteruelas, S.; Saavedra-García, P.; Bosquet, A.; Borràs, J.; Girona, J.; Amiliano, K.; Rodríguez-Balada, M.; Heras, M.; Masana, L.; Gumà, J.

Adipose-Derived Fatty Acid-Binding Proteins Plasma Concentrations Are Increased in Breast Cancer Patients. *Oncologist* **2017**, *22*, 1309–1315, doi:10.1634/theoncologist.2016-0483.

173. Storch, J.; Corsico, B. The emerging functions and mechanisms of mammalian fatty acid-binding proteins. *Annu. Rev. Nutr.* 2008, *28*, 73–95.
174. Smathers, R.L.; Petersen, D.R. The human fatty acid-binding protein family: Evolutionary divergences and functions. *Hum. Genomics* 2011, *5*, 170–191.
175. Bernlohr, D.A.; Simpson, M.A.; Hertzel, A.V.; Banaszak, L.J. Intracellular lipid-binding proteins and their genes. *Annu. Rev. Nutr.* 1997, *17*, 277–303.
176. Furuhashi, M.; Hotamisligil, G.S. Fatty acid-binding proteins: Role in metabolic diseases and potential as drug targets. *Nat. Rev. Drug Discov.* 2008, *7*, 489–503.
177. Furuhashi, M.; Ishimura, S.; Ota, H.; Miura, T. Lipid Chaperones and Metabolic Inflammation. *Int. J. Inflam.* **2011**, *2011*, 1–12, doi:10.4061/2011/642612.
178. Cho, K.R.; Shih, I.M. Ovarian cancer. *Annu. Rev. Pathol. Mech. Dis.* 2009, *4*, 287–313.
179. Cao, H.; Sekiya, M.; Ertunc, M.E.; Burak, M.F.; Mayers, J.R.; White, A.; Inouye, K.; Rickey, L.M.; Ercal, B.C.; Furuhashi, M.; et al. Adipocyte lipid chaperone aP2 Is a secreted adipokine regulating hepatic glucose production. *Cell Metab.* **2013**, *17*, 768–778, doi:10.1016/j.cmet.2013.04.012.
180. Cabré, A.; Lázaro, I.; Girona, J.; Manzanares, J.M.; Marimón, F.; Plana, N.; Heras, M.; Masana, L. Fatty acid binding protein 4 is increased in metabolic syndrome and with thiazolidinedione treatment in diabetic

patients. *Atherosclerosis* **2007**, *195*, doi:10.1016/j.atherosclerosis.2007.04.045.

181. Cabré, A.; Babio, N.; Lázaro, I.; Bulló, M.; Garcia-Arellano, A.; Masana, L.; Salas-Salvadó, J. FABP4 predicts atherogenic dyslipidemia development. The PREDIMED study. *Atherosclerosis* **2012**, *222*, 229–234, doi:10.1016/j.atherosclerosis.2012.02.003.
182. Lázaro, I.; Ferré, R.; Plana, N.; Aragons, G.; Girona, J.; Merino, J.; Heras, M.; Cabré, A.; Masana, L. Cambios de estilo de vida disminuyen las concentraciones plasmáticas de FABP4 en pacientes con riesgo cardiovascular. *Rev. Esp. Cardiol.* **2012**, *65*, 152–157, doi:10.1016/j.recesp.2011.09.016.
183. Harjes, U.; Bridges, E.; McIntyre, A.; Fielding, B.A.; Harris, A.L. Fatty acid-binding protein 4, a point of convergence for angiogenic and metabolic signaling pathways in endothelial cells. *J. Biol. Chem.* **2014**, *289*, 23168–23176, doi:10.1074/jbc.M114.576512.
184. Gharpure, K.M.; Pradeep, S.; Sans, M.; Rupaimoole, R.; Ivan, C.; Wu, S.Y.; Bayraktar, E.; Nagaraja, A.S.; Mangala, L.S.; Zhang, X.; et al. FABP4 as a key determinant of metastatic potential of ovarian cancer. *Nat. Commun.* **2018**, *9*, doi:10.1038/s41467-018-04987-y.
185. Uehara, H.; Takahashi, T.; Oha, M.; Ogawa, H.; Izumi, K. Exogenous fatty acid binding protein 4 promotes human prostate cancer cell progression. *Int. J. Cancer* **2014**, *135*, 2558–2568, doi:10.1002/ijc.28903.
186. Huang, M.; Narita, S.; Inoue, T.; Koizumi, A.; Saito, M.; Tsuruta, H.; Numakura, K.; Satoh, S.; Nanjo, H.; Sasaki, T.; et al. Fatty acid binding protein 4 enhances prostate cancer progression by upregulating matrix metalloproteinases and stromal cell cytokine production. *Oncotarget* **2017**, *8*, 111780–111794, doi:10.18632/oncotarget.22908.
187. Guaita-Esteruelas, S.; Bosquet, A.; Saavedra, P.; Gumà, J.; Girona, J.; Lam,

E.W.F.; Amillano, K.; Borràs, J.; Masana, L. Exogenous FABP4 increases breast cancer cell proliferation and activates the expression of fatty acid transport proteins. *Mol. Carcinog.* **2017**, *56*, 208–217, doi:10.1002/mc.22485.

188. Guaita-Esteruelas, S.; Gumà, J.; Masana, L.; Borràs, J. The peritumoural adipose tissue microenvironment and cancer. The roles of fatty acid binding protein 4 and fatty acid binding protein 5. *Mol. Cell. Endocrinol.* **2018**, *462*, 107–118, doi:10.1016/j.mce.2017.02.002.

189. Zeng, J.; Sauter, E.R.; Li, B. FABP4: A New Player in Obesity-Associated Breast Cancer. *Trends Mol. Med.* 2020, *26*, 437–440.

190. Nieman, K.M.; Kenny, H.A.; Penicka, C. V.; Ladanyi, A.; Buell-Gutbrod, R.; Zillhardt, M.R.; Romero, I.L.; Carey, M.S.; Mills, G.B.; Hotamisligil, G.S.; et al. Adipocytes promote ovarian cancer metastasis and provide energy for rapid tumor growth. *Nat. Med.* **2011**, *17*, 1498–1503, doi:10.1038/nm.2492.

191. Nie, J.; Zhang, J.; Wang, L.; Lu, L.; Yuan, Q.; An, F.; Zhang, S.; Jiao, Y. Adipocytes promote cholangiocarcinoma metastasis through fatty acid binding protein 4. *J. Exp. Clin. Cancer Res.* **2017**, *36*, doi:10.1186/s13046-017-0641-y.

192. Cataltepe, O.; Arikan, M.C.; Ghelfi, E.; Karaaslan, C.; Ozsurekci, Y.; Dresser, K.; Li, Y.; Smith, T.W.; Cataltepe, S. Fatty acid binding protein 4 is expressed in distinct endothelial and non-endothelial cell populations in glioblastoma. *Neuropathol. Appl. Neurobiol.* **2012**, *38*, 400–410, doi:10.1111/j.1365-2990.2011.01237.x.

193. Harjes, U.; Bridges, E.; Gharpure, K.M.; Roxanis, I.; Sheldon, H.; Miranda, F.; Mangala, L.S.; Pradeep, S.; Lopez-Berestein, G.; Ahmed, A.; et al. Antiangiogenic and tumour inhibitory effects of downregulating tumour endothelial FABP4. *Oncogene* **2017**, *36*, 912–921,

doi:10.1038/onc.2016.256.

194. Furuhashi, M.; Sakuma, I.; Morimoto, T.; Higashiura, Y.; Sakai, A.; Matsumoto, M.; Sakuma, M.; Shimabukuro, M.; Nomiyama, T.; Arasaki, O.; et al. Independent and Distinct Associations of FABP4 and FABP5 With Metabolic Parameters in Type 2 Diabetes Mellitus. *Front. Endocrinol. (Lausanne).* **2020**, *11*, doi:10.3389/fendo.2020.575557.

195. Maeda, K.; Uysal, K.T.; Makowski, L.; Görgün, C.Z.; Atsumi, G.; Parker, R.A.; Brüning, J.; Hertzel, A.V.; Bernlohr, D.A.; Hotamisligil, G.S. Role of the fatty acid binding protein mal1 in obesity and insulin resistance. *Diabetes* **2003**, *52*, 300–307, doi:10.2337/diabetes.52.2.300.

196. Fujii, K.; Kondo, T.; Yokoo, H.; Yamada, T.; Iwatsuki, K.; Hirohashi, S. Proteomic study of human hepatocellular carcinoma using two-dimensional difference gel electrophoresis with saturation cysteine dye. *Proteomics* **2005**, *5*, 1411–1422, doi:10.1002/pmic.200401004.

197. Jeong, C.Y.; Hah, Y.S.; Cho, B.I.; Lee, S.M.; Joo, Y.T.; Jung, E.J.; Jeong, S.H.; Lee, Y.J.; Choi, S.K.; Ha, W.S.; et al. Fatty acid-binding protein 5 promotes cell proliferation and invasion in human intrahepatic cholangiocarcinoma. *Oncol. Rep.* **2012**, *28*, 1283–1292, doi:10.3892/or.2012.1922.

198. Fang, L.Y.; Wong, T.Y.; Chiang, W.F.; Chen, Y.L. Fatty-acid-binding protein 5 promotes cell proliferation and invasion in oral squamous cell carcinoma. *J. Oral Pathol. Med.* **2010**, *39*, 342–348, doi:10.1111/j.1600-0714.2009.00836.x.

199. Zhan, Y.Z.; Liu, F.; Zhang, Y.; Mo, X.Y.; Cheng, W.D.; Wang, W. FABP5 promotes cell growth, invasion and metastasis in cervical cancer. *Zhonghua Zhong Liu Za Zhi* **2019**, *41*, 200–207, doi:10.3760/cma.j.issn.0253-3766.2019.03.009.

200. Wang, W.; Chu, H. juan; Liang, Y. chun; Huang, J. ming; Shang, C. liang;

Tan, H.; Liu, D.; Zhao, Y. he; Liu, T. yu; Yao, S. zhong FABP5 correlates with poor prognosis and promotes tumor cell growth and metastasis in cervical cancer. *Tumor Biol.* **2016**, *37*, 14873–14883, doi:10.1007/s13277-016-5350-1.

201. Levi, L.; Lobo, G.; Doud, M.K.; Von Lintig, J.; Seachrist, D.; Tochtrop, G.P.; Noy, N. Genetic ablation of the fatty acid-binding protein FABP5 suppresses HER2-induced mammary tumorigenesis. *Cancer Res.* **2013**, *73*, 4770–4780, doi:10.1158/0008-5472.CAN-13-0384.
202. Liu, R.Z.; Graham, K.; Glubrecht, D.D.; Germain, D.R.; Mackey, J.R.; Godbout, R. Association of FABP5 expression with poor survival in triple-negative breast cancer: Implication for retinoic acid therapy. *Am. J. Pathol.* **2011**, *178*, 997–1008, doi:10.1016/j.ajpath.2010.11.075.
203. Powell, C.A.; Nasser, M.W.; Zhao, H.; Wochna, J.C.; Zhang, X.; Shapiro, C.; Shilo, K.; Ganju, R.K. Fatty acid binding protein 5 promotes metastatic potential of triple negative breast cancer cells through enhancing epidermal growth factor receptor stability. *Oncotarget* **2015**, *6*, 6373–6385, doi:10.18632/oncotarget.3442.
204. Pascual, G.; Avgustinova, A.; Mejetta, S.; Martín, M.; Castellanos, A.; Attolini, C.S.O.; Berenguer, A.; Prats, N.; Toll, A.; Hueto, J.A.; et al. Targeting metastasis-initiating cells through the fatty acid receptor CD36. *Nature* **2017**, *541*, 41–45, doi:10.1038/nature20791.
205. DeFilippis, R.A.; Chang, H.; Dumont, N.; Rabban, J.T.; Chen, Y.Y.; Fontenay, G. V.; Berman, H.K.; Gauthier, M.L.; Zhao, J.; Hu, D.; et al. CD36 repression activates a multicellular stromal program shared by high mammographic density and tumor tissues. *Cancer Discov.* **2012**, *2*, 826–839, doi:10.1158/2159-8290.CD-12-0107.
206. Glatz, J.F.C.; Luiken, J. From fat to FAT (CD36/SR-B2): Understanding the regulation of cellular fatty acid uptake. *Biochimie* 2017, *136*, 21–26.

207. Ladanyi, A.; Mukherjee, A.; Kenny, H.A.; Johnson, A.; Mitra, A.K.; Sundaresan, S.; Nieman, K.M.; Pascual, G.; Benitah, S.A.; Montag, A.; et al. Adipocyte-induced CD36 expression drives ovarian cancer progression and metastasis. *Oncogene* **2018**, *37*, 2285–2301, doi:10.1038/s41388-017-0093-z.

208. J, P.; Z, F.; Z, W.; Q, D.; Z, X.; F, Y.; M, Y.; Z, Z.; B, L.; C, L. CD36 mediates palmitate acid-induced metastasis of gastric cancer via AKT/GSK-3β/β-catenin pathway. *J. Exp. Clin. Cancer Res.* **2019**, *38*, doi:10.1186/S13046-019-1049-7.

209. Hale, J.S.; Otvos, B.; Sinyuk, M.; Alvarado, A.G.; Hitomi, M.; Stoltz, K.; Wu, Q.; Flavahan, W.; Levison, B.; Johansen, M.L.; et al. Cancer stem cell-specific scavenger receptor 36 drives glioblastoma progression. *Stem Cells* **2014**, *32*, 1746–1758, doi:10.1002/stem.1716.

210. Yang, P.; Su, C.; Luo, X.; Zeng, H.; Zhao, L.; Wei, L.; Zhang, X.; Varghese, Z.; Moorhead, J.F.; Chen, Y.; et al. Dietary oleic acid-induced CD36 promotes cervical cancer cell growth and metastasis via up-regulation Src/ERK pathway. *Cancer Lett.* **2018**, *438*, 76–85, doi:10.1016/j.canlet.2018.09.006.

211. Nath, A.; Li, I.; Roberts, L.R.; Chan, C. Elevated free fatty acid uptake via CD36 promotes epithelial-mesenchymal transition in hepatocellular carcinoma. *Sci. Rep.* **2015**, *5*, doi:10.1038/srep14752.

212. Feingold, K.R.; Grunfeld, C. *Introduction to Lipids and Lipoproteins*; MDText.com, Inc., 2000;

213. Borén, J.; John Chapman, M.; Krauss, R.M.; Packard, C.J.; Bentzon, J.F.; Binder, C.J.; Daemen, M.J.; Demer, L.L.; Hegele, R.A.; Nicholls, S.J.; et al. Low-density lipoproteins cause atherosclerotic cardiovascular disease: Pathophysiological, genetic, and therapeutic insights: A consensus statement from the European Atherosclerosis Society Consensus Panel.

Eur. Heart J. 2020, *41*, 2313–2330.

214. Khera, A. V.; Cuchel, M.; de la Llera-Moya, M.; Rodrigues, A.; Burke, M.F.; Jafri, K.; French, B.C.; Phillips, J.A.; Mucksavage, M.L.; Wilensky, R.L.; et al. Cholesterol Efflux Capacity, High-Density Lipoprotein Function, and Atherosclerosis. *N. Engl. J. Med.* **2011**, *364*, 127–135, doi:10.1056/nejmoa1001689.
215. Høyer, A.P.; Engholm, G. Serum lipids and breast cancer risk: a cohort study of 5,207 Danish women. *Cancer Causes Control* **1992**, *3*, 403–408, doi:10.1007/BF00051352.
216. Maran, L.; Hamid, A.; Hamid, S.B.S. Lipoproteins as Markers for Monitoring Cancer Progression. *J. Lipids* **2021**, *2021*, 8180424, doi:10.1155/2021/8180424.
217. Mallol, R.; Amigó, N.; Rodríguez, M.A.; Heras, M.; Vinaixa, M.; Plana, N.; Rock, E.; Ribalta, J.; Yanes, O.; Masana, L.; et al. Liposcale: A novel advanced lipoprotein test based on 2D diffusion-ordered 1H NMR spectroscopy. *J. Lipid Res.* **2015**, *56*, 737–746, doi:10.1194/jlr.D050120.
218. Hu, J.; La Vecchia, C.; de Groh, M.; Negri, E.; Morrison, H.; Mery, L.; Paulse, B.; Dewar, R.; Dryer, D.; Kreiger, N.; et al. Dietary cholesterol intake and cancer. *Ann. Oncol.* **2012**, *23*, 491–500, doi:10.1093/annonc/mdr155.
219. Zhou, P.; Li, B.; Liu, B.; Chen, T.; Xiao, J. Prognostic role of serum total cholesterol and high-density lipoprotein cholesterol in cancer survivors: A systematic review and meta-analysis. *Clin. Chim. Acta* 2018, *477*, 94–104.
220. Gazzerro, P.; Proto, M.C.; Gangemi, G.; Malfitano, A.M.; Ciaglia, E.; Pisanti, S.; Santoro, A.; Laezza, C.; Bifulco, M. Pharmacological actions of statins: A critical appraisal in the management of cancer. *Pharmacol. Rev.* 2012, *64*, 102–146.

221. Solomon, K.R.; Freeman, M.R. Do the cholesterol-lowering properties of statins affect cancer risk? *Trends Endocrinol. Metab.* **2008**, *19*, 113–121, doi:10.1016/J.TEM.2007.12.004.

222. Zaleska, M.; Mozenska, O.; Bil, J. Statins use and cancer: an update. *Future Oncol.* **2018**, *14*, 1497–1509, doi:10.2217/FON-2017-0543.

223. Chung, Y.W.; Han, D.S.; Park, Y.K.; Son, B.K.; Paik, C.H.; Lee, H.L.; Jeon, Y.C.; Sohn, J.H. Association of obesity, serum glucose and lipids with the risk of advanced colorectal adenoma and cancer: A case-control study in Korea. *Dig. Liver Dis.* **2006**, *38*, 668–672, doi:10.1016/j.dld.2006.05.014.

224. Tabuchi, M.; Kitayama, J.; Nagawa, H. Hypertriglyceridemia is positively correlated with the development of colorectal tubular adenoma in Japanese men. *World J. Gastroenterol.* **2006**, *12*, 1261–1264, doi:10.3748/WJG.V12.I8.1261.

225. Girona, J.; Amigó, N.; Ibarretxe, D.; Plana, N.; Rodríguez-Borjabad, C.; Heras, M.; Ferré, R.; Gil, M.; Correig, X.; Masana, L. HDL triglycerides: A new marker of metabolic and cardiovascular risk. *Int. J. Mol. Sci.* **2019**, *20*, doi:10.3390/ijms20133151.

226. Moreno-Vedia, J.; Rosales, R.; Ozcariz, E.; Llop, D.; Lahuerta, M.; Benavent, M.; Rodríguez-Calvo, R.; Plana, N.; Pedragosa, A.; Masana, L.; et al. Triglyceride-Rich Lipoproteins and Glycoprotein A and B Assessed by 1H-NMR in Metabolic-Associated Fatty Liver Disease. *Front. Endocrinol. (Lausanne).* **2022**, *0*, 1828, doi:10.3389/FENDO.2021.775677.

227. Holmes, M. V.; Millwood, I.Y.; Kartsonaki, C.; Hill, M.R.; Bennett, D.A.; Boxall, R.; Guo, Y.; Xu, X.; Bian, Z.; Hu, R.; et al. Lipids, Lipoproteins, and Metabolites and Risk of Myocardial Infarction and Stroke. *J. Am. Coll. Cardiol.* **2018**, *71*, 620–632, doi:10.1016/j.jacc.2017.12.006.

228. Egeblad, M.; Nakasone, E.S.; Werb, Z. Tumors as organs: Complex tissues that interface with the entire organism. *Dev. Cell* 2010, *18*, 884–901.

229. Protani, M.; Coory, M.; Martin, J.H. Effect of obesity on survival of women with breast cancer: Systematic review and meta-Analysis. *Breast Cancer Res. Treat.* 2010, *123*, 627–635.

230. McCubrey, J.A.; Steelman, L.S.; Chappell, W.H.; Abrams, S.L.; Wong, E.W.; Chang, F.; Lehmann, B.; Terrian, D.M.; Milella, M.; Tafuri, A.; et al. Roles of the Raf/MEK/ERK pathway in cell growth, malignant transformation and drug resistance. *Biochim. Biophys. Acta - Mol. Cell Res.* 2007, *1773*, 1263–1284.

231. Vivanco, I.; Sawyers, C.L. The phosphatidylinositol 3-kinase-AKT pathway in humancancer. *Nat. Rev. Cancer* **2002**, *2*, 489–501, doi:10.1038/nrc839.

232. Gupta, G.P.; Massagué, J. Cancer Metastasis: Building a Framework. *Cell* 2006, *127*, 679–695.

233. Karamanos, N.K.; Piperigkou, Z.; Theocharis, A.D.; Watanabe, H.; Franchi, M.; Baud, S.; Brézillon, S.; Götte, M.; Passi, A.; Vigetti, D.; et al. Proteoglycan chemical diversity drives multifunctional cell regulation and therapeutics. *Chem. Rev.* 2018, *118*, 9152–9232.

234. Conlon, G.A.; Murray, G.I. Recent advances in understanding the roles of matrix metalloproteinases in tumour invasion and metastasis. *J. Pathol.* 2019, *247*, 629–640.

235. Kessenbrock, K.; Plaks, V.; Werb, Z. Matrix Metalloproteinases: Regulators of the Tumor Microenvironment. *Cell* 2010, *141*, 52–67.

236. Shuman Moss, L.A.; Jensen-Taubman, S.; Stetler-Stevenson, W.G. Matrix metalloproteinases: Changing roles in tumor progression and metastasis. *Am. J. Pathol.* 2012, *181*, 1895–1899.

237. Hadler-Olsen, E.; Winberg, J.O.; Uhlin-Hansen, L. Matrix metalloproteinases in cancer: Their value as diagnostic and prognostic markers and therapeutic targets. *Tumor Biol.* 2013, *34*, 2041–2051.

238. Brown, G.T.; Murray, G.I. Current mechanistic insights into the roles of

matrix metalloproteinases in tumour invasion and metastasis. *J. Pathol.* **2015**, *237*, 273–281, doi:10.1002/path.4586.

239. Fernández, L.P.; Gómez de Cedrón, M.; Ramírez de Molina, A. Alterations of Lipid Metabolism in Cancer: Implications in Prognosis and Treatment. *Front. Oncol.* 2020, *10*, 577420.
240. Marino, N.; German, R.; Rao, X.; Simpson, E.; Liu, S.; Wan, J.; Liu, Y.; Sandusky, G.; Jacobsen, M.; Stoval, M.; et al. Upregulation of lipid metabolism genes in the breast prior to cancer diagnosis. *npj Breast Cancer* **2020**, *6*, doi:10.1038/s41523-020-00191-8.
241. Menendez, J.A.; Vellon, L.; Espinoza, I.; Lupu, R. The metastasis inducer CCN1 (CYR61) activates the fatty acid synthase (FASN)-driven lipogenic phenotype in breast cancer cells. *Oncoscience* **2016**, *3*, 242–257, doi:10.18632/ONCOSCIENCE.314.
242. D'Esposito, V.; Passaretti, F.; Hammarstedt, A.; Liguoro, D.; Terracciano, D.; Molea, G.; Canta, L.; Miele, C.; Smith, U.; Beguinot, F.; et al. Adipocyte-released insulin-like growth factor-1 is regulated by glucose and fatty acids and controls breast cancer cell growth in vitro. *Diabetologia* **2012**, *55*, 2811–2822, doi:10.1007/s00125-012-2629-7.
243. Wang, C.; Gao, C.; Meng, K.; Qiao, H.; Wang, Y. Human adipocytes stimulate invasion of breast cancer MCF-7 cells by secreting IGFBP-2. *PLoS One* **2015**, *10*, doi:10.1371/journal.pone.0119348.
244. Yang, D.; Li, Y.; Xing, L.; Tan, Y.; Sun, J.; Zeng, B.; Xiang, T.; Tan, J.; Ren, G.; Wang, Y. Utilization of adipocyte-derived lipids and enhanced intracellular trafficking of fatty acids contribute to breast cancer progression. *Cell Commun. Signal.* **2018**, *16*, doi:10.1186/s12964-018-0221-6.
245. Furuhashi, M.; Tuncman, G.; Görgün, C.Z.; Makowski, L.; Atsumi, G.; Vaillancourt, E.; Kono, K.; Babaev, V.R.; Fazio, S.; Linton, M.F.; et al.

Treatment of diabetes and atherosclerosis by inhibiting fatty-acid-binding protein aP2. *Nature* **2007**, *447*, 959–965, doi:10.1038/nature05844.

246. Herroon, M.K.; Rajagurubandara, E.; Hardaway, A.L.; Powell, K.; Turchick, A.; Feldmann, D.; Podgorski, I. Bone marrow adipocytes promote tumor growth in bone via FABP4-dependent mechanisms. *Oncotarget* **2013**, *4*, 2108–2123, doi:10.18632/oncotarget.1482.
247. Tuncman, G.; Erbay, E.; Hom, X.; De Vivo, I.; Campos, H.; Rimm, E.B.; Hotamisligil, G.S. A genetic variant at the fatty acid-binding protein aP2 locus reduces the risk for hypertriglyceridemia, type 2 diabetes, and cardiovascular disease. *Proc. Natl. Acad. Sci. U. S. A.* **2006**, *103*, 6970–6975, doi:10.1073/pnas.0602178103.
248. Garin-Shkolnik, T.; Rudich, A.; Hotamisligil, G.S.; Rubinstein, M. FABP4 attenuates PPARγ and adipogenesis and is inversely correlated with PPARγ in adipose tissues. *Diabetes* **2014**, *63*, 900–911, doi:10.2337/db13-0436.
249. Mukherjee, A.; Chiang, C.Y.; Daifotis, H.A.; Nieman, K.M.; Fahrmann, J.F.; Lastra, R.R.; Romero, I.L.; Fiehn, O.; Lengyel, E. Adipocyte-induced FABP4 expression in ovarian cancer cells promotes metastasis and mediates carboplatin resistance. *Cancer Res.* **2020**, *80*, 1748–1761, doi:10.1158/0008-5472.CAN-19-1999.
250. Scifres, C.M.; Chen, B.; Nelson, D.M.; Sadovsky, Y. Fatty acid binding protein 4 regulates intracellular lipid accumulation in human trophoblasts. *J. Clin. Endocrinol. Metab.* **2011**, *96*, doi:10.1210/jc.2010-2084.
251. Vazquez-Martin, A.; Colomer, R.; Brunet, J.; Lupu, R.; Menendez, J.A. Overexpression of fatty acid synthase gene activates HER1/HER2 tyrosine kinase receptors in human breast epithelial cells. *Cell Prolif.* **2008**, *41*, 59–

85, doi:10.1111/j.1365-2184.2007.00498.x.

252. Ferraro, G.B.; Ali, A.; Luengo, A.; Kodack, D.P.; Deik, A.; Abbott, K.L.; Bezwada, D.; Blanc, L.; Prideaux, B.; Jin, X.; et al. Fatty acid synthesis is required for breast cancer brain metastasis. *Nat. Cancer* **2021**, *2*, 414–428, doi:10.1038/s43018-021-00183-y.

253. Balaban, S.; Shearer, R.F.; Lee, L.S.; van Geldermalsen, M.; Schreuder, M.; Shtein, H.C.; Cairns, R.; Thomas, K.C.; Fazakerley, D.J.; Grewal, T.; et al. Adipocyte lipolysis links obesity to breast cancer growth: adipocyte-derived fatty acids drive breast cancer cell proliferation and migration. *Cancer Metab.* **2017**, *5*, doi:10.1186/S40170-016-0163-7.

254. Aznar Benitah, S. Metastatic-initiating cells and lipid metabolism. *Cell Stress* **2017**, *1*, 110–114, doi:10.15698/cst2017.12.113.

255. Pascual, G.; Domínguez, D.; Elosúa-Bayes, M.; Beckedorff, F.; Laudanna, C.; Bigas, C.; Douillet, D.; Greco, C.; Symeonidi, A.; Hernández, I.; et al. Dietary palmitic acid promotes a prometastatic memory via Schwann cells. *Nature* **2021**, *599*, 485–490, doi:10.1038/s41586-021-04075-0.

256. Yang, L.; Cui, X.; Zhang, N.; Li, M.; Bai, Y.; Han, X.; Shi, Y.; Liu, H. Comprehensive lipid profiling of plasma in patients with benign breast tumor and breast cancer reveals novel biomarkers. *Anal. Bioanal. Chem.* **2015**, *407*, doi:10.1007/s00216-015-8484-x.

257. Hammad, L.A.; Wu, G.; Saleh, M.M.; Klouckova, I.; Dobrolecki, L.E.; Hickey, R.J.; Schnaper, L.; Novotny, M. V.; Mechref, Y. Elevated levels of hydroxylated phosphocholine lipids in the blood serum of breast cancer patients. *Rapid Commun. Mass Spectrom.* **2009**, *23*, 863–876, doi:10.1002/rcm.3947.

258. Hilvo, M.; Denkert, C.; Lehtinen, L.; Müller, B.; Brockmöller, S.; Seppänen-Laakso, T.; Budczies, J.; Bucher, E.; Yetukuri, L.; Castillo, S.; et al. Novel theranostic opportunities offered by characterization of altered

membrane lipid metabolism in breast cancer progression. *Cancer Res.* **2011**, *71*, 3236–3245, doi:10.1158/0008-5472.CAN-10-3894.

259. Zhang, W.; Zhang, D.; Chen, Q.; Wu, J.; Ouyang, Z.; Xia, Y. Online photochemical derivatization enables comprehensive mass spectrometric analysis of unsaturated phospholipid isomers. *Nat. Commun.* **2019**, *10*, doi:10.1038/S41467-018-07963-8.

260. Nagahashi, M.; Tsuchida, J.; Moro, K.; Hasegawa, M.; Tatsuda, K.; Woelfel, I.A.; Takabe, K.; Wakai, T. High levels of sphingolipids in human breast cancer. *J. Surg. Res.* **2016**, *204*, 435–444, doi:10.1016/j.jss.2016.05.022.

261. Qiu, Y.; Zhou, B.; Su, M.; Baxter, S.; Zheng, X.; Zhao, X.; Yen, Y.; Jia, W. Mass spectrometry-based quantitative metabolomics revealed a distinct lipid profile in breast cancer patients. *Int. J. Mol. Sci.* **2013**, *14*, 8047–8061, doi:10.3390/ijms14048047.

262. Calzada, E.; Onguka, O.; Claypool, S.M. Phosphatidylethanolamine Metabolism in Health and Disease. In *International Review of Cell and Molecular Biology*; Academic Press, 2016; Vol. 321, pp. 29–88 ISBN 9780128047071.

263. Kim, H.; Min, H.K.; Kong, G.; Moon, M.H. Quantitative analysis of phosphatidylcholines and phosphatidylethanolamines in urine of patients with breast cancer by nanoflow liquid chromatography/tandem mass spectrometry. *Anal. Bioanal. Chem.* **2009**, *393*, 1649–1656, doi:10.1007/S00216-009-2621-3.

264. Jiang, N.; Zhang, G.; Pan, L.; Yan, C.; Zhang, L.; Weng, Y.; Wang, W.; Chen, X.; Yang, G. Potential plasma lipid biomarkers in early-stage breast cancer. *Biotechnol. Lett.* **2017**, *39*, 1657–1666, doi:10.1007/s10529-017-2417-z.

265. Taniguchi, M.; Okazaki, T. The role of sphingomyelin and sphingomyelin

synthases in cell death, proliferation and migration - From cell and animal models to human disorders. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* 2014, *1841*, 692–703.

266. Albeituni, S.; Stiban, J. Roles of Ceramides and Other Sphingolipids in Immune Cell Function and Inflammation. In *Advances in Experimental Medicine and Biology*; Adv Exp Med Biol, 2019; Vol. 1161, pp. 169–191.
267. Chaurasia, B.; Talbot, C.L.; Summers, S.A. Adipocyte Ceramides—The Nexus of Inflammation and Metabolic Disease. *Front. Immunol.* 2020, *11*.
268. García-González, V.; Díaz-Villanueva, J.F.; Galindo-Hernández, O.; Martínez-Navarro, I.; Hurtado-Ureta, G.; Pérez-Arias, A.A. Ceramide metabolism balance, a multifaceted factor in critical steps of breast cancer development. *Int. J. Mol. Sci.* 2018, *19*.
269. Wang, X.Q.; Yang, K.; He, Y.S.; Lu, L.; Shen, W.F. Receptor mediated elevation in FABP4 levels by advanced glycation end products induces cholesterol and triacylglycerol accumulation in THP-1 macrophages. *Lipids* **2011**, *46*, 479–486, doi:10.1007/s11745-011-3542-4.
270. Coussens, L.M.; Werb, Z. Inflammation and cancer. *Nature* 2002, *420*, 860–867.
271. He, J.Y.; Wei, X.H.; Li, S.J.; Liu, Y.; Hu, H.L.; Li, Z.Z.; Kuang, X.H.; Wang, L.; Shi, X.; Yuan, S.T.; et al. Adipocyte-derived IL-6 and leptin promote breast Cancer metastasis via upregulation of Lysyl Hydroxylase-2 expression 11 Medical and Health Sciences 1112 Oncology and Carcinogenesis. *Cell Commun. Signal.* **2018**, *16*, doi:10.1186/s12964-018-0309-z.
272. Wu, Q.; Li, B.; Li, J.; Sun, S.; Yuan, J.; Sun, S. Cancer-associated adipocytes as immunomodulators in cancer. *Biomark. Res.* 2021, *9*, 2.
273. Osborn, O.; Olefsky, J.M. The cellular and signaling networks linking the immune system and metabolism in disease. *Nat. Med.* 2012, *18*, 363–374.

274. Mayi, T.H.; Daoudi, M.; Derudas, B.; Gross, B.; Bories, G.; Wouters, K.; Brozek, J.; Caiazzo, R.; Raverdi, V.; Pigeyre, M.; et al. Human adipose tissue macrophages display activation of cancer-related pathways. *J. Biol. Chem.* **2012**, *287*, 21904–21913, doi:10.1074/jbc.M111.315200.

275. Corrêa, L.H.; Corrêa, R.; Farinasso, C.M.; de Sant'Ana Dourado, L.P.; Magalhães, K.G. Adipocytes and macrophages interplay in the orchestration of tumor microenvironment: New implications in cancer progression. *Front. Immunol.* 2017, *8*.

276. Gabay, C.; Kushner, I. Acute-Phase Proteins and Other Systemic Responses to Inflammation. *N. Engl. J. Med.* **1999**, *340*, 448–454, doi:10.1056/nejm199902113400607.

277. Connelly, M.A.; Otvos, J.D.; Shalaurova, I.; Playford, M.P.; Mehta, N.N. GlycA, a novel biomarker of systemic inflammation and cardiovascular disease risk. *J. Transl. Med.* 2017, *15*.

278. Sommer, G.; Ziegelmeier, M.; Bachmann, A.; Kralisch, S.; Lossner, U.; Kratzsch, J.; Blüher, M.; Stumvoll, M.; Fasshauer, M. Serum levels of adipocyte fatty acid-binding protein (AFABP) are increased in chronic haemodialysis (CD). *Clin. Endocrinol. (Oxf).* **2008**, *69*, 901–905, doi:10.1111/J.1365-2265.2008.03277.X.

279. Duprez, D.A.; Otvos, J.; Sanchez, O.A.; Mackey, R.H.; Tracy, R.; Jacobs, D.R. Comparison of the predictive value of GlycA and other biomarkers of inflammation for total death, incident cardiovascular events, noncardiovascular and noncancer inflammatory-related events, and total cancer events. *Clin. Chem.* **2016**, *62*, 1020–1031, doi:10.1373/clinchem.2016.255828.

280. Sproston, N.R.; Ashworth, J.J. Role of C-Reactive Protein at Sites of Inflammation and Infection. *Front. Immunol.* **2018**, *9*, 754, doi:10.3389/FIMMU.2018.00754.

281. Furuhashi, M.; Yuda, S.; Muranaka, A.; Kawamukai, M.; Matsumoto, M.; Tanaka, M.; Moniwa, N.; Ohnishi, H.; Saitoh, S.; Shimamoto, K.; et al. Circulating fatty acid-binding protein 4 concentration predicts the progression of carotid atherosclerosis in a general population without medication. *Circ. J.* **2018**, *82*, 1121–1129, doi:10.1253/circj.CJ-17-1295.

282. Saito, N.; Furuhashi, M.; Koyama, M.; Higashiura, Y.; Akasaka, H.; Tanaka, M.; Moniwa, N.; Ohnishi, H.; Saitoh, S.; Ura, N.; et al. Elevated circulating FABP4 concentration predicts cardiovascular death in a general population: a 12-year prospective study. *Sci. Rep.* **2021**, *11*, doi:10.1038/S41598-021-83494-5.

283. Ebert, T.; Hopf, L.M.; Wurst, U.; Bachmann, A.; Kralisch, S.; Lössner, U.; Platz, M.; Kratzsch, J.; Stolzenburg, J.U.; Dietel, A.; et al. Circulating adipocyte fatty acid binding protein is increased in chronic and acute renal dysfunction. *Nutr. Metab. Cardiovasc. Dis.* **2014**, *24*, 1027–1034, doi:10.1016/j.numecd.2014.03.006.

284. Maxfield, F.R.; van Meer, G. Cholesterol, the central lipid of mammalian cells. *Curr. Opin. Cell Biol.* 2010, *22*, 422–429.

285. Incardona, J.P.; Eaton, S. Cholesterol in signal transduction. *Curr. Opin. Cell Biol.* 2000, *12*, 193–203.

286. Shah, F.D.; Shukla, S.N.; Shah, P.M.; Patel, H.R.H.; Patel, P.S. Significance of alterations in plasma lipid profile levels in breast cancer. *Integr. Cancer Ther.* **2008**, *7*, 33–41, doi:10.1177/1534735407313883.

287. Rodrigues Dos Santos, C.; Domingues, G.; Matias, I.; Matos, J.; Fonseca, I.; De Almeida, J.M.; Dias, S. LDL-cholesterol signaling induces breast cancer proliferation and invasion. *Lipids Health Dis.* **2014**, *13*, 16, doi:10.1186/1476-511X-13-16.

288. Ahn, J.; Lim, U.; Weinstein, S.J.; Schatzkin, A.; Hayes, R.B.; Virtamo, J.; Albanes, D. Prediagnostic total and high-density lipoprotein cholesterol

and risk of cancer. *Cancer Epidemiol. Biomarkers Prev.* **2009**, *18*, 2814–2821, doi:10.1158/1055-9965.EPI-08-1248.

289. Balistreri, C.R.; Caruso, C.; Candore, G. The role of adipose tissue and adipokines in obesity-related inflammatory diseases. *Mediators Inflamm.* 2010, *2010*.

290. Rodríguez-Hernández, H.; Simental-Mendía, L.E.; Rodríguez-Ramírez, G.; Reyes-Romero, M.A. Obesity and inflammation: Epidemiology, risk factors, and markers of inflammation. *Int. J. Endocrinol.* 2013, *2013*.

291. Stępień, M.; Stępień, A.; Wlazeł, R.N.; Paradowski, M.; Banach, M.; Rysz, J. Obesity indices and inflammatory markers in obese non-diabetic normo- and hypertensive patients: A comparative pilot study. *Lipids Health Dis.* **2014**, *13*, 29, doi:10.1186/1476-511X-13-29.

292. Gumà, J.; Adriá-Cebrián, J.; Ruiz-Aguado, B.; Albacar, C.; Girona, J.; Rodríguez-Calvo, R.; Martínez-Micaelo, N.; Lam, E.W.F.; Masana, L.; Guaita-Esteruelas, S. Altered serum metabolic profile assessed by advanced 1H-NMR in breast cancer patients. *Cancers (Basel).* **2021**, *13*, 4281, doi:10.3390/cancers13174281.

293. Wei, Z.; Liu, X.; Cheng, C.; Yu, W.; Yi, P. Metabolism of Amino Acids in Cancer. *Front. Cell Dev. Biol.* 2021, *8*.

294. Vettore, L.; Westbrook, R.L.; Tennant, D.A. New aspects of amino acid metabolism in cancer. *Br. J. Cancer* **2020**, *122*, 150–156, doi:10.1038/S41416-019-0620-5.

295. Li, Z.; Zhang, H. Reprogramming of glucose, fatty acid and amino acid metabolism for cancer progression. *Cell. Mol. Life Sci.* 2016, *73*, 377–392.

296. Ananieva, E.A.; Wilkinson, A.C. Branched-chain amino acid metabolism in cancer. *Curr. Opin. Clin. Nutr. Metab. Care* **2018**, *21*, 64–70, doi:10.1097/MCO.0000000000000430.

297. Hattori, A.; Tsunoda, M.; Konuma, T.; Kobayashi, M.; Nagy, T.; Glushka,

J.; Tayyari, F.; McSkimming, D.; Kannan, N.; Tojo, A.; et al. Cancer progression by reprogrammed BCAA metabolism in myeloid leukaemia. *Nature* **2017**, *545*, 500–504, doi:10.1038/nature22314.

298. Nguyeni, T.N.; Nguyen, H.Q.; Duc-Haule Unveiling prognostics biomarkers of tyrosine metabolism reprogramming in liver cancer by cross-platform gene expression analyses. *PLoS One* **2020**, *15*, doi:10.1371/journal.pone.0229276.

299. Choi, B.H.; Coloff, J.L. The diverse functions of non-essential amino acids in cancer. *Cancers (Basel).* 2019, *11*.

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