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The impact of nutritional availability in the microenvironment on metabolic reprogramming and aggressiveness of breast cancer cells

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Abstract

Introduction: Metabolic reprogramming consists of adjusting cancer cells' metabolic profile to different environmental conditions. This enables them to adapt some stressful circumstances, namely shortage of nutrients or hypoxia. Hence, cancer cells, to survive and proliferate, rely on a variety of nutritional sources to support biomass and ATP production. Furthermore, metabolic reprogramming is considered a hallmark of cancer. It is known that, even in aerobiosis, cancer cells preferentially perform glycolysis, although it produces less ATP than oxidative phosphorylation (Warburg effect). Also, proliferating tumor cells increase their glutaminolysis rate. Lipidomic remodeling is another feature of cancer cells, with growing relevance, considering the fat-enriched western diet. Metabolic reprogramming enables cells to overcome stressful events, hence supporting metastatic cascade. Objectives: Our aims are to review the clinical importance of cancer metabolomic, while evaluating *in vitro* the metabolic reprograming of triple-negative breast cancer cell line MDA-MB-231 exposed to different concentration of Glucose, Glutamine and LDL-cholesterol. Also, compare the metabolic reprograming occurring in MDA-MB-231 *in vivo*, in breast cancer and lung metastasis, of NSG mice fed with normal diet, high cholesterol diet and high cholesterol diet after blockade of LDL receptor. Methods/Results: We used quantitative PCR to evaluate the expression of genes associated with oxidative phosphorylation (ND1), glycolysis (G6PD, PKM2 and LDHA), *de novo* fatty acids synthesis (FASN), lipid transporters (CD36 and LDLr) and glutaminolysis (GLS2 and IDH2). MDA-MB-231 cells *in vitro* are highly dependent upon glucose and glutamine, even when other carbon sources are present. *In vivo*, high cholesterol diet induces proliferation of primary breast cancer cells after blockade of LDLr. Lung metastases upregulate the expression of lipid transporters compared to primary tumors, both in normal diet and high cholesterol diet fed mice. Conclusion: MDA-MB-231 cells display metabolic plasticity *in vitro* and *in vivo*. High cholesterol diet benefits breast cancer cells, contributing to metastatic potential and consisting on a potential therapeutic target.

Keywords

Triple Negative Breast Cancer; Metastasis; Metabolic plasticity; High Cholesterol Diet

The present work expresses the opinions of the author.

Resumo

Introdução: A reprogramação metabólica consiste na adaptação do fenótipo metabólico das células tumorais às condições do microambiente. Para que possam sobreviver e proliferar, estas células utilizam uma grande variedade de substratos para produzir biomassa e ATP. A reprogramação metabólica é, portanto, um *hallmark* do cancro. As células tumorais utilizam preferencialmente a glicólise, mesmo em aerobiose, embora esta via lhes permita produzir substancialmente menos ATP (efeito de Warburg). Adicionalmente, nas células tumorais há aumento da taxa de glutaminólise, bem como reprogramação do metabolismo lipídico. Por permitir às células tumorais sobreviver a microambientes adversos, a reprogramação metabólica contribui para a mestastização. Objetivos: Procurámos rever a importância clínica da metabolómica tumoral, bem como avaliar a reprogramação metabólica que ocorre *in vitro* na linha celular de cancro da mama triplo negativo MDA-MB-231, quando expostas a diferentes concentrações de glicose, glutamina e LDL-colesterol; comparar a reprogramação metabólica que ocorre em células MDA-MB-231 *in vivo*, em tumores da mama primários e metástases pulmonares, em ratinhos NSG sujeitos a dietas normais, dietas enriquecidas em colesterol e em dietas enriquecidas com colesterol com bloqueio do recetor LDL. Métodos/Resultados: Por PCR quantitativo avaliou-se a expressão dos genes de enzimas chave da fosforilação oxidativa (ND1), glicólise (G6PD, PKM2, LDHA), síntese *de novo* de ácidos gordos (FASN), transportadores lipídicos (LDLr e CD36) e glutaminólise (GLS2 e IDH2*).* As células MDA-MB-231 *in vitro* são dependentes de glicose e glutamina, mesmo quando estão disponíveis outros substratos. *In vivo*, dietas enriquecidas em colesterol induzem proliferação dos tumores primários da mama após bloqueio do LDLr. As metástases pulmonares sobre expressam transportadores lipídicos comparadas com tumores primários em ambas as dietas. Conclusão: As células MDA-MB-231 apresentam plasticidade metabólica *in vivo* e *in vitro*. Dietas ricas em colesterol conferem vantagem às células de cancro da mama, contribuindo para o potencial metastático pulmonar e consistindo num eventual alvo terapêutico.

Palavras-chave

Cancro da mama triplo negativo; Metástases; Plasticidade metabólica; Dieta rica em colesterol

1. INTRODUCTION

Introduction

While quiescent cells have their metabolism designed to sustain baseline ATP production, highly proliferative cells are more engaged to biomass formation. Similarly, as highly proliferative cancer cells thrive, macromolecules have to be available for them to double cellular mass and accomplish cellular division (Palm & Thompson, 2017). Moreover, cell division is an energy consuming process, which means these cells have to sustain the production of many molecules of ATP (Koundouros & Poulogiannis, 2020).

Mammalian cells commonly acquire nutrients from extracellular sources, namely plasma and interstitial fluid through transmembrane transporters because of their absent ability to produce many macromolecules. This means that highly proliferative mammalian cells are dependent on the availability of nutrients in the microenvironment to proliferate (Vander Heiden & DeBerardinis, 2017) . Tumoral cells somehow resemble this dependency on the microenvironment and metabolic interactions established in the tumoral microenvironment are alike physiological ones observed in normal tissues. Nevertheless, there are abnormal metabolic features which contribute to shape the metabolic profile of tumoral cells repurposed to support tumor proliferation (Lyssiotis & Kimmelman, 2017).

In fact, the tumor microenvironment consists of the network of cancer cells, extracellular matrix, growth factors, stromal and immune cells that interact and evolve as the tumoral mass develops (**Figure 1**). The tumor microenvironment promotes and is involved in tumor progression, namely by providing micronutrients such as glucose, glutamine and fatty acids, used for ATP generation and biosynthetic purposes (Hui & Chen, 2015; Palm & Thompson, 2017; Pavlova & Thompson, 2016). However, the growth rate of most tumors exceeds the capacity of vascular beds to supply nutrients, which leads to establishment of hypoxic avascular areas within the tumor mass (Vander Heiden & DeBerardinis, 2017).

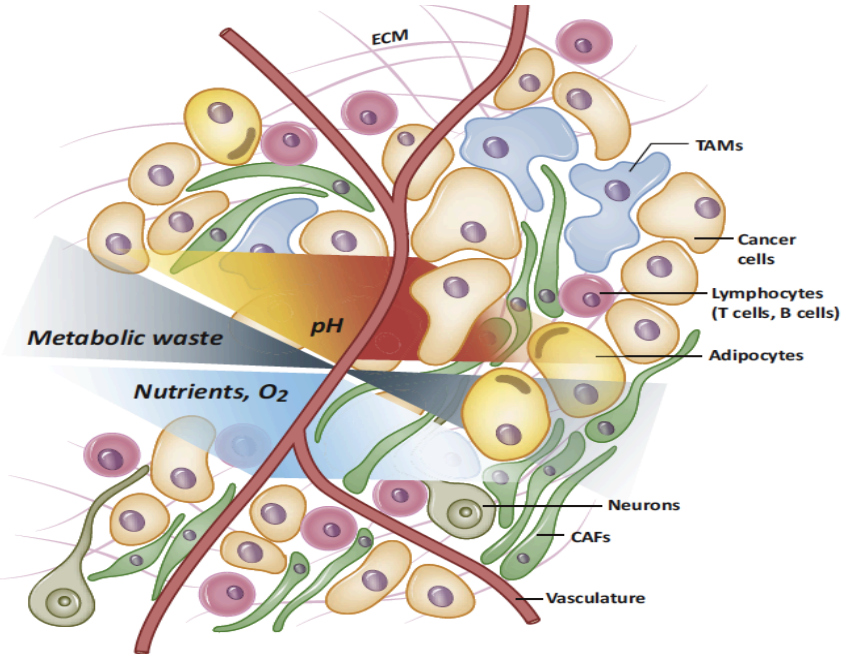

Figure 1- Features of the tumor microenvironment that contribute to metabolic heterogeneity (adapted from *Metabolic Interactions in the Tumor Microenvironment*, Lyssiotis & Kimmelman, 2017)

As a result, despite the increase uptake of nutrients from microenvironment, cancer cells must overcome nutrient shortfall that inevitably ensues, so that they can maintain proliferation. To survive this stress, cancer cells shape metabolic pathways that are preferably activated. This process is known as metabolic reprogramming and naturally confers cancer cells metabolic advantages by virtue of having access to specific nutrients that favor cellular proliferation and survival. This explains why metabolic reprogramming has been considered a new emerging hallmark of cancer (Pavlova & Thompson, 2016; Phelan, 2018). Subsequently, it is reasonable to assume that the disruption of cancer metabolism would be of clinical benefit and cancer metabolomic has emerged as a promising field of research (Cedó et al., 2019; Fendt et al., 2020; Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021; Vander Heiden & DeBerardinis, 2017).

Many solid tumors, such as breast and colon cancer, markedly increase their glucose consumptions, as descried by Otto Warburg in 1924. Warburg and colleagues observed that cancer cells preferentially adopt glycolysis independently of the oxygen availability, even though it produces substantially lower amounts of ATP. Despite being energetically inefficient, glycolysis appears to be profitable to cancer cells (**Figure 2**).

Actually, glycolysis is up to 100 times faster to produce ATP compared with oxidative phosphorylation, so that the amount of ATP produced per unit of time is comparable (Shestov et al., 2014). Moreover, glycolysis produces intermediate products that can be used in other anabolic pathways, allowing highly proliferative cells to produce themselves lipids, proteins and nucleic acids. Furthermore, these intermediate products can be used to modulating oxidative stress, chromatin state and cell signaling. By these mechanisms, Warburg effect appears to support biomass production and, consequently, proliferation. Apart from this intracellular effect, glycolysis modulates the tumoral microenvironment by increasing lactate secretion. Acidification of microenvironment can modulate tumoral-stroma interactions and promote polarization of macrophages to pro-tumoral (M2) phenotype (Estrella et al., 2013).

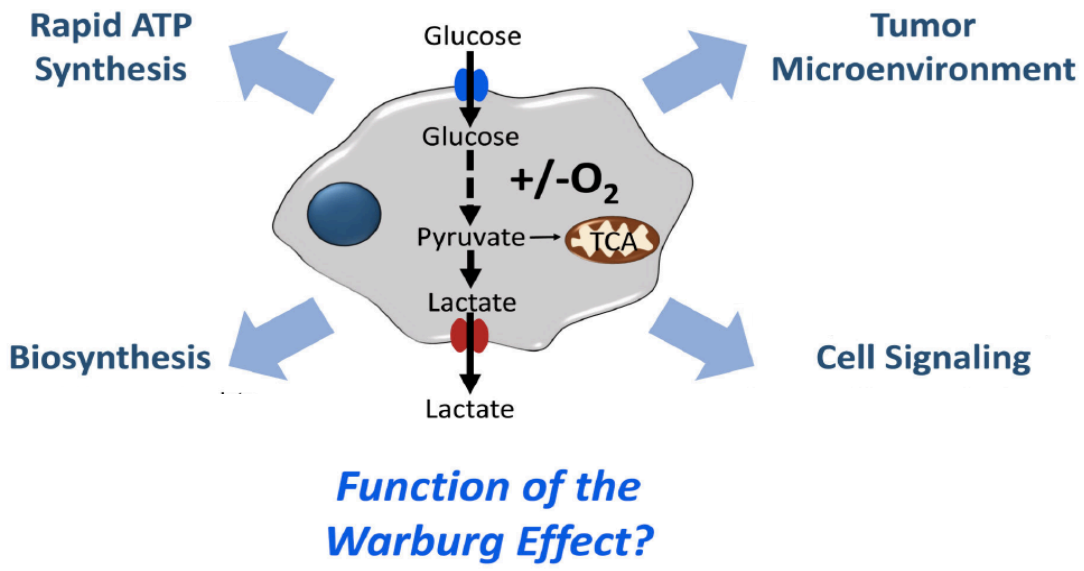

Figure 2- Summary of the proposed functions of the Warburg Effect (adapted from *The Warburg Effect: How does it Benefit Cancer Cells*, Liberti & Locasale, 2016)

Likewise, solid tumors such as brain, ovarian and pancreatic cancer, also accumulate glutamine, as was described by Harry Eagle in 1955. Glutamine is an amino acid used as energetic source, bearing in mind that glutaminolysis provides intermediate

molecules to tricarboxylic acid cycle. Also, glutamine can be used as a precursor of other amino acid synthesis or as a nitrogen source for synthesis of nucleic acids (de Oliveira et al., 2016). It has been showed that glutamate and lactate secreted by tumor cells are incorporated by tumor associated fibroblasts, which in turn produce and secrete glutamine. Additionally, blockage of glutamine synthesis in fibroblasts and glutamine catabolism in ovarian cancer cells slowed tumor growth (Yang et al., 2016).

Besides glucose and glutamine metabolic redefinition, lipidomic remodeling is another feature of cancer cells, although not so well studied. It is characterized by changes in fatty acid transport, *de novo* lipogenesis, storage and β oxidation (Koundouros & Poulogiannis, 2020). It has been described an higher intracellular lipid accumulation in different neoplastic processes, even though it has not been clearly established the causal role of these molecules in cancer origin (Cruz et al., 2020). As it may be, lipid metabolism reprograming is associated with cell growth, proliferation, differentiation and motility, leading to progression of cancer, metastasis and ultimately influencing biological aggressiveness (Santos & Schulze, 2012).

Lipids, mainly cholesterol, are essential components of biological membranes and are used as a fuel to energy production (**Figure 3**). Moreover, lipids control cell signaling (acting as second messengers and hormones) and modulate the crosstalk between cancer cells and microenvironment (Goossens et al., 2019; Santos & Schulze, 2012). Considering this, it is reasonable to believe that cancer cells have to accumulate higher levels of cholesterol in order to maintain an higher proliferative rate. Cancer cells can do so by upregulating lipid biosynthesis or increase cholesterol uptake from bloodstream (Goossens et al., 2019).

Uptake of cholesterol from bloodstream and microenvironment requires the expression of specialized transporters (Koundouros & Poulogiannis, 2020), the most studied ones being CD36 (fatty acid translocase- FAT), fatty acid transport protein family (FATP or SCLC27) and plasma membrane fatty acid-binding proteins (FABPpm). In prostate, gastric, ovarian and breast cancers, high CD36 expression correlates with poor outcomes, which suggests that fatty acid uptake may be associated with biological aggressiveness (Ladanyi et al., 2018). Lipid uptake leads to its accumulation in lipid droplets, which prevent toxicity and store lipids that can be oxidized in conditions of metabolic stress. In fact, β oxidation produces acetyl-CoA that can enter the Krebs cycle

and generate NADH and FADH2 to the electron transporting chain, producing huge quantities of ATP (Koundouros & Poulogiannis, 2020).

Even when lipids are available from exogenous sources, cancer cells can activate *de novo* lipogenesis, in which acetyl co-A is used to produce fatty acids. In breast and prostate cancer, it has been shown the overexpression of FASN, an enzyme essential to fatty acids synthesis. In breast cancer, FASN has been defined as a prognosis marker (OA-519)(Kuhajda et al., 1994).

Fatty acid oxidation provides large amounts of ATP and is carried out in high energy-demanding tissues. Moreover, cancer cells are highly dependent on this metabolic pathway during loss of attachment of extracellular matrix, because in this process cells require many molecules of ATP and NADPH, which are produced by this pathway (Carracedo et al., 2013).

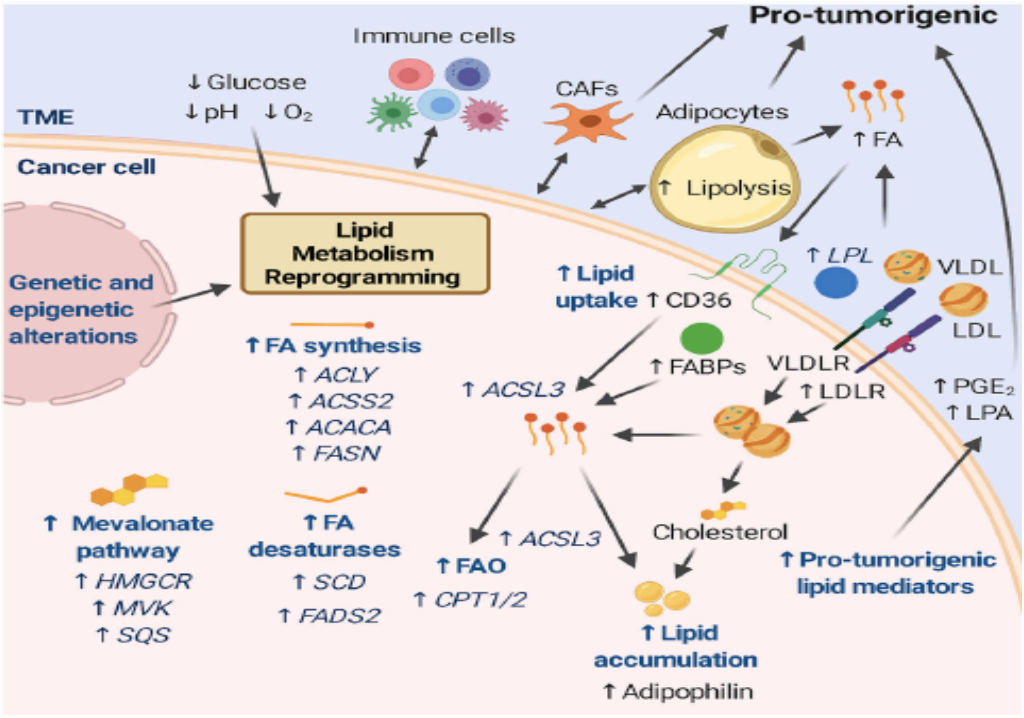

Figure 3- Cancer lipid metabolism reprogramming (adapted from *Lipid metabolism in cancer: New perspectives and emerging mechanism*, Schmidt, Patel, Kirsch, Lewis, Heiden, Locasale, et al., 2021)

Although lipid metabolism reprogramming is observed in almost all cancer cells, each cancer type has its specific metabolic alterations. Oncogenic pathways control lipid metabolism and shape expression of genes involved in lipid metabolism, which explains

why different cancers- which rely on different oncogenic pathways- express different lipid metabolic programs. On the other hand, lipid reprogramming itself is an early event in tumor development and can also modulate oncogenic pathways (Menendez & Lupu, 2007). These different tumoral lipidome may explain the biological behavior and aggressiveness of different tumors. As a result, defining tumoral lipidome would make it possible to predict tumoral phenotype and to define new therapeutic targets (Cedó et al., 2019; Koundouros & Poulogiannis, 2020; Santos & Schulze, 2012; Vander Heiden & DeBerardinis, 2017).

Breast cancer is the second most common in the world and the most common cancer among women. According to National Health Institute, in 2020 it is estimated that 276480 American women were diagnosed with breast cancer, which represents 15,3% of all new cancer cases. Also, 42170 American women died of this disease, which is 7% of all cancer deaths (Chopra & Davies, 2020; Momenimovahed & Salehiniya, 2019).

Breast cancer results from the interaction between genetic and environmental factors and this explains why the incidence rate of this cancer differs with race and ethnicity. In the last decade, prevalence of breast cancer increased, particularly in low income countries. This can be the result of investment in screening strategies, awareness of the importance of early diagnosis and accuracy of diagnosis methods. Alternatively, it can be due to changes in risk factors, with growing incorporation of western lifestyle (Cedó et al., 2019; Harbeck & Gnant, 2017; Momenimovahed & Salehiniya, 2019).

Actually, new insights on the epidemiological characteristics of breast cancer and its risk factors provide an opportunity to prevent, adjust screening strategies based on risk classification and to predict its biological behavior. Epidemiological studies have identified many risk factors for breast cancer, which are demographic, reproductive, hormonal, breast related, genetic and lifestyle factors. Lipid-enriched diet has been identified as one of the risk factors for breast cancer (Chopra & Davies, 2020; Harbeck & Gnant, 2017; Momenimovahed & Salehiniya, 2019). However, the impact of diet in breast cancer development is controversial (Cedó et al., 2019; Momenimovahed & Salehiniya, 2019). While some studies have failed to show association between lipoprotein levels and breast cancer, other large clinical studies have demonstrated a

link between LDL cholesterol levels and breast cancer risk (Momenimovahed & Salehiniya, 2019; Rodrigues Dos Santos et al., 2014).

It has also been showed that LDL cholesterol exposure leads to higher proliferation rate, loss of adhesion molecules and promotes migration, which suggests a relationship between LDL cholesterol and breast cancer biological aggressiveness (Rodrigues Dos Santos et al., 2014). In addition, a prospective trial has suggested that LDL levels at diagnosis is a prognostic factor of breast cancer (Rodrigues dos Santos et al., 2014). Mouse models of breast cancer have reinforced this relationship between hypercholesterolemia and breast cancer growth and metastasis (Llaverias et al., 2011).

Breast cancer is a heterogeneous disease and it is possible to define 4 main molecular subtypes of breast cancer based on its biological features, which are luminal A, luminal B, HER2 enriched and triple negative. Luminal A breast cancer is the most common one and overexpresses estrogen and progesterone receptors. Luminal B breast cancer also overexpresses these receptors but are more proliferative. HER2 enriched breast cancer does not over express either of these hormonal receptors but overexpresses HER2 receptor. Triple negative breast cancer does not overexpress hormonal receptors nor HER2 (Harbeck & Gnant, 2017; Momenimovahed & Salehiniya, 2019).

These molecular differences are associated with different biological behavior and, as a result, each type has its own profile of lipid metabolism. For instance, comparative mRNA expression analysis between receptor positive breast cancer and triple negative breast cancer shows that the former relies higher on the novo synthesis and fatty acid mobilization, whereas the later overexpresses specific transporters of lipids such as FABP5 and FABP7 (Monaco, 2017). Accordingly, LDL cholesterol mainly promotes proliferation and migration of ER-negative cells, but this is not evident in ER positive cell lines (Lu et al., 2017).

In fact, breast cancer is associated with 5 year relative survival of 90%, but it depends on the cancer stage at the diagnosis: cancers confined to the primary site have a 5 year relative survival rate of 98,9%, whereas only 6% of patients with metastasized breast cancer at diagnosis is alive after 5 years (Momenimovahed & Salehiniya, 2019). The most common organs affected by breast cancer metastasis are lung, bone, liver and brain. Lung metastasis are responsible for high burden of morbidity and mortality

(Chopra & Davies, 2020; Harbeck & Gnant, 2017). Metastatic disease consists of one of the major challenges in cancer treatment, as metastatic lesions are typically not sensitive to first line therapies. Disease prognosis becomes poorer once the metastatic disease ensues. Moreover, metastatic lesions are associated with increased morbidity associated not only with toxicity of therapies but also with specific organ compromise (Faubert et al., 2020; Gandhi & Das, 2019). Indeed, it is compelling to study the biology of metastasis in order to develop satisfactory treatment options that disrupt this process.

Metastasis consists in the development of secondary tumors at distance from the primary cancer mass. In this process, cancer cells have to detach, invade the blood vessels and form tumor emboli that would then adhere to blood vessel walls, establish in the distant organ and proliferate (Mehta et al., 1997). LDL appears to promote metastatic cascade by several mechanisms. LDL promote platelet adhesiveness and generation of tumor emboli. Also, LDL is an inflammatory stimulus in vessel walls, creating areas of disruption of endothelial cells in which tumor cells can escape to colonize the distant organ (Mehta et al., 1997; Rodrigues dos Santos et al., 2014). In general, it has been showed that LDL cholesterol exposure increases the proliferation rate, loss of adhesion molecules, promoting migration of the breast cancer cells which suggests that LDL cholesterol plays a role in breast cancer biological aggressiveness (Rodrigues dos Santos et al., 2014).

In recent years, it has become evident that metabolic phenotypes progress as the tumors progress, as these different metabolic phenotypes strengthens cancer cells to survive. Furthermore, selective pressures and clonal selection that takes place as the tumor evolves and eventually metastasizes creates metabolomic signatures which are specific of the steps of cancer progression (Faubert et al., 2020). Hence, metabolic reprogramming is now viewed as a dynamic process, which is essential to highly proliferative cells and can influence every step of metastatic cascade. For instance, some metabolic adaptations are associated with epithelial-mesenchymal-transitions (EMT), intravasation, circulation and colonization of secondary organs (X. Wang et al., 2019). In addition, organotropism, which is the propensity of an organ to accommodate metastatic lesions, is regulated by factors, including metabolic environment of the secondary organs (Faubert & Deberardinis, 2017). For this reason, it is important to

understand which metabolic pathways are so important to metastatic cancer cells that constitute vulnerabilities conceivably to be targeted in therapy (**Figure 4**).

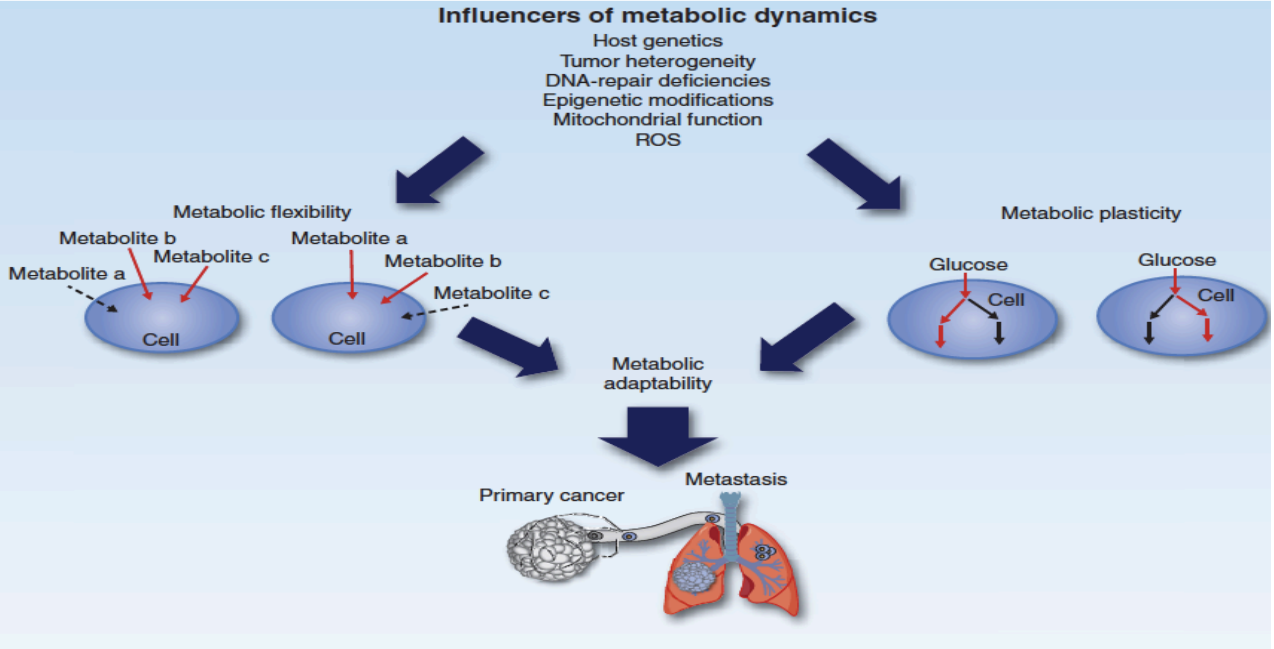

Figure 4- Metabolic flexibility and plasticity determine tumor metabolic adaptability and influences metastatic cascade (adapted from *Targeting Metabolic Plasticity and Flexibility Dynamics for Cancer Therapy,* Fendt et al., 2020)

Moreover, because metabolic reprogramming is so crucial to cancer initiation, progression and fitness, metabolomics in cancer is a promising area with huge clinical impact, specifically in prevention, diagnosis, staging and treatment of tumors (Fendt et al., 2020; Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021; Vander Heiden & DeBerardinis, 2017; Z. Wang et al., 2020)

2. OBJECTIVES

Objectives

The aim of this study consists of evaluating whether the nutrients availability in the microenvironment have a role on tumor aggressiveness, meaning metastasis, by remodeling the metabolism of breast cancer cells. To do that, we reviewed the clinical literature to evaluate the relevance of metabolomic approach in clinical practice and the clinical opportunity that emerge with the insight on the metabolic reprogramming. Then, we developed our practical work, which was divided in two objectives:

1) Evaluate in vitro the metabolic reprograming upon treated with high or low concentrations of Glucose, Glutamine and LDL in the triple-negative human breast cancer cell line MDA-MB-231.

2) Investigate and compare the possible metabolic reprograming occurring in the triple-negative human breast cancer cell line MDA-MB-231, in both primary and lung metastasis tumors, of NSG mice fed with normal, high cholesterol diet and high cholesterol diet and blockade of LDL receptor.

3. CLINICAL IMPACT OF CANCER METABOLISM

Clinical impact of cancer metabolism

There is evidence that support the concept of metabolic reprogramming as a dynamic process, which is essential to highly proliferative cells. Metabolic plasticity of cancer cells confers them benefits as allow them to proliferate and maximizes their fitness to an ever-changing microenvironment. Unsurprisingly, cancer metabolism is a hallmark of cancer. Hence, because metabolic reprogramming is so crucial to cancer initiation, progression and fitness, metabolomics in cancer is a promising area with huge clinical impact, specifically in prevention, diagnosis, staging and treatment of tumors (Fendt et al., 2020; Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021; Vander Heiden & DeBerardinis, 2017; Z. Wang et al., 2020).

Indeed, metabolism has been the center of attention in research aimed to define risk factors for cancer. It appears to be a relationship between some pathological metabolic states, such as obesity, diabetes and metabolic syndrome, as risk for some types of cancer (Karagozian et al., 2014; Lien & Vander Heiden, 2019). Moreover, some dietary factors and physical inactivity have also been implicated in cancer susceptibility (Fendt et al., 2020; Santos & Schulze, 2012; Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021). Beyond conferring risk to cancer initiation, the metabolic profile of patients have been correlated with disease progression, recurrence risk and even mortality (Cedó et al., 2019).

Metabolomic- based approaches of cancer risk stratification and prevention are important to virtual all types of cancer, but become critical to the most frequent ones to develop population prevention strategies. Breast cancer is in fact the second most common cancer in the world, the most frequent among women and it has become evident some metabolomic factors that are associated with increased risk of the disease, namely dietary factors. A prospective study has indeed demonstrated that LDL cholesterol levels at diagnosis correlates with tumor dimension, differentiation and proliferation, as well as with disease free survival at 25 months (Rodrigues dos Santos et al., 2014). However, it remains elusive how diet influences the prognosis and therapeutic response to when a cancer has already been diagnosed. Theoretically, food choices can impact the nutrients that circulate in bloodstream and that eventually become available to cancer cells (Lien & Vander Heiden, 2019) (**Figure 5**). Moreover, it is now known that metabolic syndrome is in fact an inflammatory disease which

promotes cancer progression. Nevertheless, it is very difficult to predict the impact of one specific dietary factor in tumoral microenvironment and current guidelines only suggest the adoption of a generic healthy diet and physical exercise (Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021).

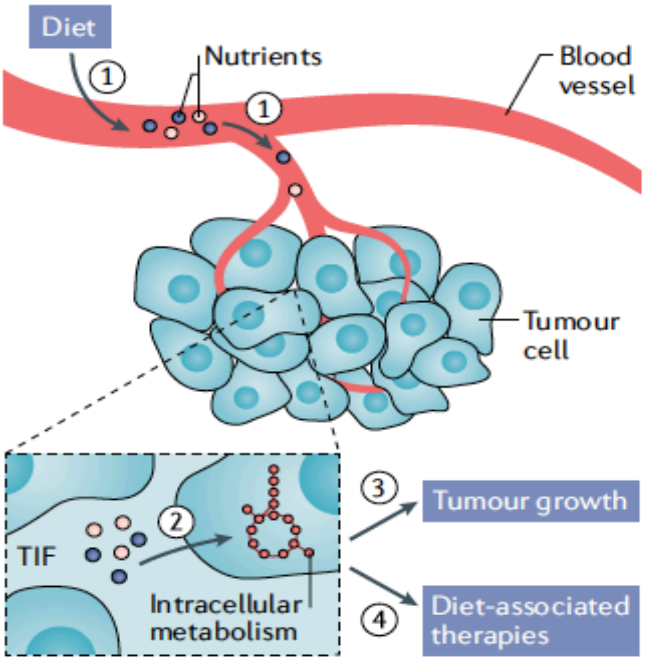

Figure 5- Framework for examining how diet impacts tumor metabolism, growth and progression (adapted from *A framework for examining how diet impacts tumor metabolism*, Lien & Vander Heiden, 2019*)*

Besides cancer risk and prognosis, cancer metabolic features have been applied in cancer screening, diagnosis and monitoring. Indeed, functional imaging scans such as 18F-FDG PET/TC are highly accurate tools to assess whole-body tumoral burden. These methods are based on the significantly higher uptake of glucose in cancer cells compared with normal tissues. In fact, this allows detection of small metastasis otherwise not recognized by other imaging modalities. This leads to a more accurate staging of the tumor and allows the choice of the therapy strategy that best fits that tumor. However, increased glucose uptake is also associated with some inflammatory states, which can limit the use of these scans in some settings. In addition, PET scans have other limitations, such as poor availability, short half-life of some radiotracers, image resolution and inability to differentiate tumors from benign hypermetabolic states. (Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021). To overcome these problems, recently novel radiolabeled molecules have been used in pre-clinical and

clinical studies, namely glutamine, fatty acids and carbohydrates-based tracers (Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021; Z. Wang et al., 2020).

Another potential value of metabolomics in cancer diagnosis is the identification of metabolic biomarkers of disease in tissue samples or biofluids. A wide variety of amino-acids, purines, pyrimidines and intermediates of metabolic pathways have been found to be differently present in cancer relative to normal tissues. Regarding breast cancer, there is evidence that different metabolic subtypes express distinct metabolic signatures. HER2 enriched tumors exhibit a glycolytic phenotype, as HER2 signaling promotes glucose uptake and glycolysis rate. Luminal breast cancer exhibits an intermediate metabolic phenotype, relying more in oxidative phosphorylation than and glycolysis Triple negative breast cancer display a highly glycolytic metabolism, based on high extracellular acidification rate (Gandhi & Das, 2019). Glutamate enrichment has also been identified in breast cancer tissue compared with normal mammary gland (Ghergurovich et al., 2021).

Nowadays, diagnosis is the major area in which metabolic reprogramming knowledge is used. PET scan is a noninvasive nuclear medicine tool that can help to assess the three-dimensional distribution of positron- emitting labelled radiotracers. The most used tracer is the 18F-labelled glucose analogue FDG, which accumulates in tissues that exhibit high glycolytic rate, namely cancer. The European Association of Nuclear Medicine (EANM) recommendations for PET scan include diagnosis purposes (differentiation of benign from malignant lesions; search for an unknown primary tumor when metastatic disease is discovered as the first manifestation of cancer or when the patient presents with a paraneoplastic syndrome); staging known malignancies and surveillance of disease (monitor the effect of therapy on known malignancies, determine whether residual abnormalities detected on physical examination or on other imaging studies following treatment represent tumor or posttreatment fibrosis or necrosis, detect tumor recurrence, especially in the presence of elevated tumor markers). Moreover, this tool allows the clinician to selection the region of tumor most likely to yield diagnostic information for biopsy and guide radiation therapy planning (Boellaard et al., 2015).

Apart from prevention and screening, cancer therapy is one of the most challenging fields of research. Not only does cancer metabolomics uncover novel drug

targets that can be exploited to cancer precision therapy, but it also helps to understand and overcome cancer resistance to previous effective therapies (Fendt et al., 2020; Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021). There is increasing evidence that metabolic reprogramming is one of the mechanisms by which cancer cells became resistance to therapy (Gandhi & Das, 2019; Vander Heiden & DeBerardinis, 2017), because of clonal selection of cells that have the metabolic phenotype suitable to overcome the stress induced by therapy. It has been observed that therapy-resistant tumors display distinctive metabolic phenotypes from treatment-naïve tumors (Vander Heiden & DeBerardinis, 2017). For instance, following cisplatin based chemotherapy, some glycolytic enzymes are overexpressed in cervical cancer (Fendt et al., 2020). In triple negative breast cancer, cytotoxic chemotherapy plays a central role in management of patients(Gandhi & Das, 2019). Accordingly, once patients became resistant to therapy, fewer options of treatment remain. In fact, taxane- resistant triple negative breast cancer display increased glucose uptake and overexpression of lactate dehydrogenase A (Sun et al., 2020). In these chemoresistant tumors, FASN is also overexpressed and FASN inhibition can enhance the effect of platinum-based treatment modalities (Sun et al., 2020).

In fact, several molecules have been developed to target the metabolic pathways in which cancer cells rely on the most.

Glycolysis is one of the most studied metabolic pathways and it is well established that it is essential to tumor cells, as observed by Otto Warburg (Liberti & Locasale, 2016). Hence, there have developed some drugs that inhibit some crucial enzymes of this pathway, namely the glycolysis transporter 1 (GLUT1), hexokinase, pyruvate kinase M2 (PKM2), lactate dehydrogenase A (LDHA) and monocarboxylate transporter 1 (MCT1) (Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021). However, glycolysis is also very important to normal tissues, which raises problems concerning safety and systemic effects of these drugs. It is reasonable to assume that such drugs would have a narrow therapeutic window in order to minimize the impact of shutting down glycolysis in normal cells. Nevertheless, different tissues express distinctive isoforms of the same glycolytic enzymes. For instance, most cancers express pyruvate kinase M2 (PKM2), which is different from the enzyme expressed in erythrocytes (PKR), liver (PKL), myocytes and brain (PKM1) (Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021).

Glutamine metabolism dependence is identified in many cancer cell lines (Altman et al., 2017; Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021; Sun et al., 2020), which makes glutaminolysis a targetable metabolic pathway in cancer therapy. There have been developed inhibitors of glutaminase 1 (GLS1) and glutamine transporter SLC1A5. Upregulation of glutamine metabolism is associated with immune evasion of cancer cells and this mechanism can impact the response to immunotherapy. In fact, the combination of a glutamine antagonist with immune-checkpoint blockade in an animal model was associated with potent antitumoral effect. In triple negative breast cancer, CB-839 (Telaglenastat), an inhibitor of GLS, had an antitumoral effect both in vitro and in vivo xenograft models (Gross et al., 2014; Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021; Sun et al., 2020). This molecule has been proved safe and exhibited a disease control rate of 55% in combination with paclitaxel in triple negative breast cancer patients who were refractory to taxane therapy. It has also been proved to be beneficial in renal cell carcinoma in combination with everolimus. However, the heterogeneity of metabolic plasticity within a specific malignant mass along with the rapid adaptability of TNBC threats metabolically targeted therapy in the clinical setting.

Considering amino acids' metabolism in cancer cells, other druggable targets include PHGDH and IDO1. PHGDH is the enzyme that initiates serine biosynthesis, which is amplified in many cancer types. Indoleamine-2,3-dioxygenase-1 is critical to tryptophan catabolism (Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021).

As cancer cells have increased needs to fatty acids, there is an upregulation of not only fatty acid transporters but also lipogenic enzymes. Hence, lipogenic enzymes such as ATP-citrate lyase, acetyl-CoA carboxylase and fatty acid synthase (Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021).

Nevertheless, metabolic intervention as a new strategy of cancer therapy faces some problems. On one hand, it is crucial to know which metabolic pathways are limiting to cancer cells to increase the effectiveness of treatment (Vander Heiden & DeBerardinis, 2017). However, theoretically these cells display metabolic plasticity, owing them to bypass any targeted pathway, hence it is unpredictable that these new agents will be useful as monotherapies. (Gandhi & Das, 2019; Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021).

On the other hand, it is possible that these therapies will lead prohibitive toxicities, because doses required to elicit a response are often high and can impact normal tissues that also rely on targeted metabolic pathways.

In order to surpass these questions, firstly it would be beneficial to stratify which patients would benefit the most from these approach by using biomarkers or evaluating the metabolomics of each tumor individually (Vander Heiden & DeBerardinis, 2017). Indeed, it has been observed that each primary tumor is characterized by a specific metabolic phenotype, considering that the tumoral microenvironment is distinct in each organ. Within the same tumor, cancer cells also have metabolic heterogeneity, resulting from the clonal selection that takes place as the tumor evolves (Faubert et al., 2020). On top of that, it is now clear that the same tumor can spread to many secondary organs, producing metastatic lesions whose metabolism is site-specific. Regarding breast cancer, it has been established that liver and lung metastasis are more dependent upon oxidative phosphorylation, while bone metastasis are more glycolytic (Gandhi & Das, 2019).

The patients selected for this therapeutic regimens would then be further categorized based on the specific metabolic features of their tumors, to choose which metabolic pathways are best to be inhibited in combination with other therapeutic modalities available (Fendt et al., 2020; Gandhi & Das, 2019; Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021).

Considering the Portuguese clinical practice, metabolic reprogramming- based guidelines for prevention of cancer and disease recurrence are currently not available. However, metabolomic of cancer cells, namely the increase uptake of glucose of cancer cells even in aerobiosis, is the principle for some diagnostic tools, such as 18F-FDG PET/TC scan.

4.
MATERIAL AND METHODS

Material and Methods

- Cell culture
  - In vitro assay:

The human breast cancer cell line MDA-MB-231 (92020424, ECACC, United Kingdom) was cultured in DMEM (Dulbecco's Modified Eagle Medium) supplemented with sodium pyruvate (0,11 g/L), sodium bicarbonate (1,8 g/L), 10 % fetal bovine serum (FBS), and 1% antibiotic penicillin-streptomycin solution (100x), with high levels of glucose (4,5 g/L) and glutamine (4mM). MDA-MB-231 cells are adherent tumorigenic basal breast cells that are ER negative, PR negative and HER2 negative.

Cells stored in liquid nitrogen were thawed and cultured into a T-25 flask. Later, at 80% of confluence, the cells were first detached by trypsinization, using 0,25% trypsin-EDTA (Gibco, California, USA), and then 1/4, about 20%, transferred to T-75 flask. Before exposing cells to experimental conditions (figure 1), the cells were starved, i.e. cultured for 24h with 1g/L glucose or for 24h with 1mM glutamine or for 6h with DMEM with Lipid free Serum. After, $1x10^5$ MDA-MB-231 cells, in log-phase growth, were exposed to the experimental conditions:

- High and Low Glucose Assays: Glucose culture medium DMEM supplemented with sodium pyruvate (0,11 g/L), sodium bicarbonate (1,8 g/L), 10 % fetal bovine serum (FBS), and 1% antibiotic penicillin-streptomycin solution (100x), with 4,5g/L (High) or 1g/L (Low) of glucose.
- High and Low Glutamine Assays: Cells were also cultured in a glutamine rich medium, composed by DMEM supplemented with L-glutamine (0,584 g/L or 4 mM, for high glutamine conditions, and 0,292 g/L or 2 mM, for low glutamine conditions), sodium pyruvate (0,11 g/L), sodium bicarbonate (1,8 g/L), 10% FBS and 1% antibiotic penicillin-streptomycin solution (100x).
- LDL Assay: Cells were cultured in different medium conditions (mentioned previously) and supplemented or not with 100 µg/mL of LDL.

A time course was performed for 6h, 24h and 72h. At each time point, cells were collected and processed to total RNA extraction (**Figure 6**). Each experiment was conducted 3 times (n=3).

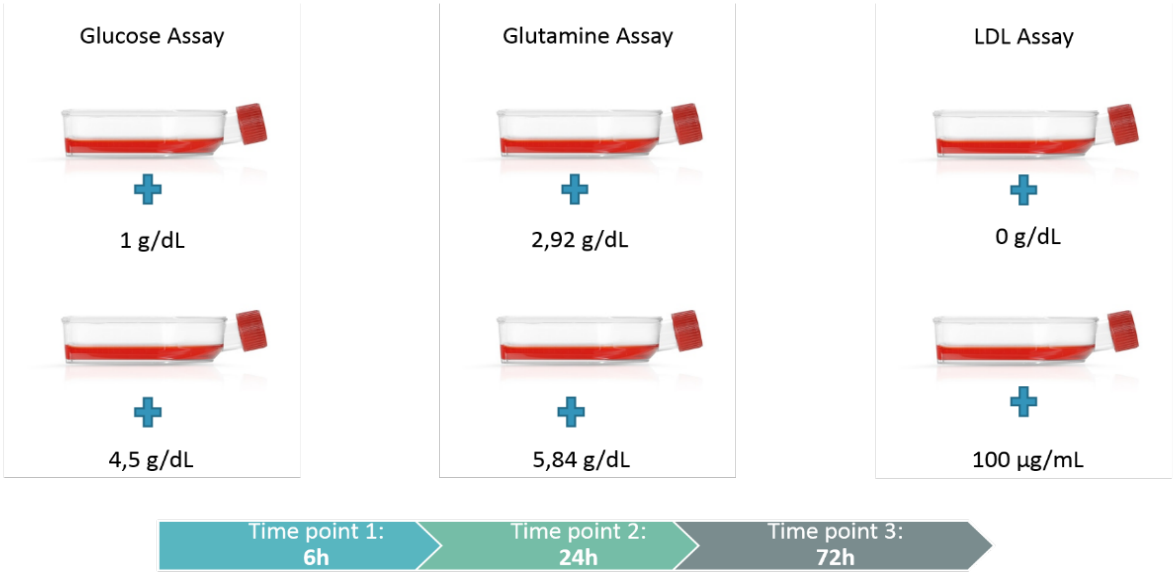

Figure 6- The experimental scheme of the *in vitro* assay. MDA-MB-231 cells were exposed to testing conditions (HGluc, LGluc, HGln, LGln, LDL or N/LDL) during different time-points (6h, 24h, and 72h). At the time 0h, triplicates were made for cells platted in each of the designed conditions.

- In vivo assay:

For the in vivo assay, 4 groups of NOD *scid* gamma mice (NSG) mice were used (**Figure 7**). Each group was composed of 3 mice, so that we had triplicates of each tissue samples. The control group (group 1) was not injected with MDA-MB-231 and was submitted to normal diet. The experimental groups (groups 2,3 and 4) were injected with approximately $1x10^6$ MDA-MB-231 GFP+ breast cancer cells directly in mammary gland. Then, the mice in group 2 were submitted to normal diet, whereas animals of group 3 and 4 were submitted to high cholesterol diet. Normal diet consisted of standard maintenance diet (3% of fat, without cholesterol). High Cholesterol diet consisted of 5.8 % Fat (coconut oil) +1.25 % Cholesterol, +0.5 % Na-Cholate, purchased from Ssniff®.

Besides the injection of MDA-MB-231, the experimental group was treated with human antibodies. Mice of group 2 and 3 were treated with human IgG control antibody and the animals of group 4 were injected with 0,3µg of an antibody anti-human LDLr (R&D AF2148). In fact, it was injected 0.1 µg of antibody in 50 µL of PBS

directly in mammary gland or in the tumor (depending if the tumor was already visible) and the remaining 0,2 µg was injected in 100µL of PBS into the peritoneal cavity. The antibodies were injected at the same time with the MDA-MB-231 cells and at day 3, 5,7,9,12 e 14. After 15 days, the animals were all euthanized, and mammary tumors and lungs were removed from mice exposed to normal and high cholesterol diet. These tissues were stored at -80ºC and then divided in pieces of approximately 30 mg each using sterile blade.

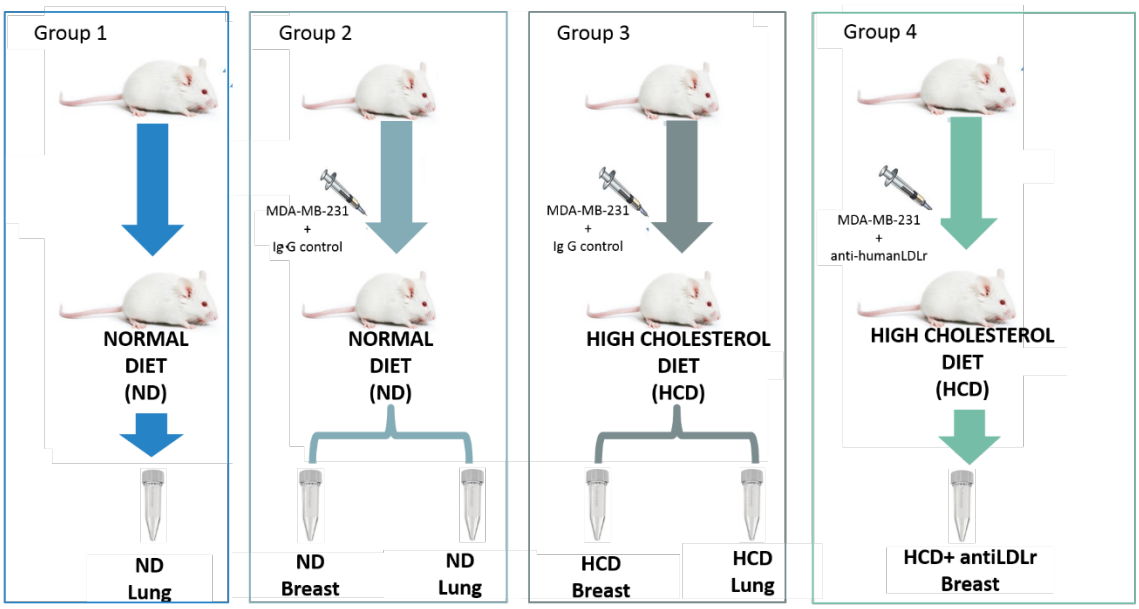

Figure 7- The experimental scheme of the *in vivo* assay. NSG mice were injected with 1x 10^6 MDA-MB-231 cells in mammary gland and treated with normal diet, high cholesterol diet. Also, the animals were injected with human antibodies, either control IgG or antibody anti-human LDLr. After 15days, the animals were euthanized and breast cancer and lung metastasis were sampled.

- RNA extraction

Total RNA extraction from MDA-MB-231 cells pellet was performed with RNeasy® Mini Kit (QIAGEN, Carnaxide, Portugal). Total RNA was extracted from MDA-MB-231 cells, which were resuspended in 1 volume of Buffer RLT. Then, it was used 1 volume of 90% ethanol, and 3 µl of DNase I. The final solution was then transferred to a RNeasy Mini spin column and centrifuged. Buffer RW1 and RPE were added to the column, centrifuged and discarded the flow-through between the addition of buffers. Finally, the membrane of the column was dried through a new centrifugation and the RNA collected was eluted in RNAse-free water for further cDNA production.

Regarding the total RNA extraction from NSG breast and lung samples, it was used the NZY Total RNA isolation kit. Briefly, 30 mg of tissue was cut into small pieces using a sterile blade and placed into a RNA free microcentrifuge tube with NI buffer and dithiothreitol. The lysate was then placed into a NZYSpin homogenization column and centrifugated. Ethanol 90% was added to flow-through and the solution was transferred to a NZYSpin binding column and centrifuged again. The flow-through was discarded, NI buffer was added and a new centrifugation was performed. For each isolation, it was prepared a digestion mix (digestion buffer and DNAse I), which was applied on the centre of the silica membrane and incubated for 15 minutes at room temperature. After this, it was added NWR1 buffer and NWR2 buffer, centrifugating the column and discarding the flow through between the two buffers. Finally, the column was again centrifugated to dry the membrane and the RNA collected was eluted in RNAse-free water to further cDNA production.

- cDNA production

To convert RNA extracts to cDNA, it was used the NZY First Strand cDNA Synthesis Kit, separate oligos (NZYTech). RNA was placed in a microcentrifuge tube and Oligo $(dT)_{18}$ primer mix and 10x Annealing Buffer were added. The solution was mixed and placed at 65ºC for 5 minutes and on ice for 1 minute. Later, NZYTech 2x Master Mix and NZYTech Enzyme Mix were added. After gently mixing the samples, they were submitted to a defined program in the BioRad T100™ Thermal Cycler: 10 min on 25ºC, 50 min on 37ºC, and 5 min at 85ºC. Finally, NZY RNase H (*E.coli*) was added and the solution was placed at 37ºC for 20 minutes. The final cDNA was quantified using NanoDrop™ spectrophotometer and DNAse-free water was added as needed to a final concentration of 800 ng/µl.

- RT-PCR

For RT-PCR analysis a MicroAmp™ Optical 384-Well Reaction Plate (Applied Biosystems™) was used. In each well 3,4 µl of cDNA (with a concentration previously corrected to 800 ng/µl) was used, 5 µl of NZYSpeedy qPCR Green Master Mix, 0,8 µl of forward primer (400nM), and 0,8 µl of reverse primer (400nM). For each sample replicates were performed, meaning 2 identical wells were prepared. The 12k Flex & ViiA™ 7 Real-Time PCR System was programmed for: 1 cycle at 95ºC for 2 min and 40

cycles at 95ºC for 5s, followed by 15s at 65ºC. The curves obtained were evaluated based on the mean of Ct value for each gene target quantification.

Also, we used the Livak method (Ct of gene of interest-Ct of housekeeping gene followed by 2^(-ΔΔCt)) to verify the relative expression of each gene to the control condition and housekeeping gene (RNaseP). Using the Livak method, it was considered that a fold expression higher than 1 represented an upregulation of the considered gene, while a fold expression less than 1 represented a downregulation of the considered gene. The following primers were used:

Table 1—Human' primers list for quantitative real-time PCR: mitochondrial complex I, Lipid Uptake, *De novo* Lipid synthesis, Glycolysis, Oxidative Phosphorylation, Glutamine pathways and housekeeping gene.

Metabolic Pathway Key-players/molecules	**Gene Name**	**Primer**	**Sequence**
Housekeeping	RNASE P	hRNASEP-F hRNASEP-R	CCCCGTTCTCTGGGAACTC TGTATGAGACCACTCTTTCCCATA
Mitochondrial Complex I	ND1	hND1-F hND1-R	ACGCCATAAAACTCTTCACCAAAG TAGTAGAAGAGCGATGGTGAGAGCTA
Lipid uptake	LDLr	hLDL-F hLDL-R	GCTTGTCTGTCACCTGCAAA AACTGCCGAGAGATGCACTT
	CD36	hCD36-F hCD36-R	GGTGTGGTGATGTTTGTTGC CAGGGCCTAGGATTTGTTGA
***De novo* lipogenesis**	FASN	hFASN-F hFASN-R	CGACAGCACCAGCTTCGCCA CACGCTGGCCTGCAGCTTCT
Glycolysis	PKM-2	hPKM-2-F hPKM-2-R	CCACTTGCAATTATTTGAGGAA GTGAGCAGACCTGCCAGACT
	LDHA	hLDHA-F hLDHA-R	ACCCAGTTTCCACCATGATT CCCAAAATGCAAGGAACACT
	G6PD	hG6PD-F hG6PD-R	GGAGGGCGACGACGACGAAG TCGGGCAGAAGGCCATCCCG
Oxidative Phosphorylation	IDH2	hIDH2-F hIDH2-R	GGAGCCCGAGGTCAAAATAC TGGCAGTTCATCAAGGAGAA
Glutamine pathway	GLS2	hGLS2-F hGLS2-R	GCCTGGGTGATTTGCTCTTTT CCTTTAGTGCAGTGGTGAACTT

- Quality control assay: Agarose gel

To prepare 1% agarose gel, TAE stock solution and agarose were mixed. The solution was boiled, GreenSafe (NZYTECH) added and the gel was poured in the cast. The samples product from RT-PCR were then mixture with DNA loading dye. The samples were loaded in the gel and ran for 40 minutes at 100V. The gel was transferred to Chemidoc XRS+ to evaluate the samples quality.

- Statistical analysis

A Two-Way ANOVA (Turkey Test) was performed using GraphPad Prism 9.0.2 program to compare the gene expression:

1) The *in vitro* assay:
    a) High versus Low Glucose;
    b) With versus without Glutamine;
    c) With versus without LDL-treatment;
    d) Multi-comparations between time-points within each group;
2) The *in vivo* assay:
    a) Normal diet versus high cholesterol diet fed NSG mice breast cancer without blockade of LDL receptor;
    b) Normal diet versus high cholesterol diet fed NSG mice breast cancer with blockade of LDL receptor;
    c) High cholesterol diet versus high cholesterol diet fed NSG mice after blocking LDL receptor;
    d) Normal diet versus high cholesterol diet NSG mice lung metastasis;
    e) Normal diet fed mice breast cancer versus normal diet fed mice lung metastasis;
    f) High cholesterol diet fed breast cancer versus high cholesterol diet lung metastasis.

In the statistical analysis, it is considered that * (P<0,05) is significant, ** (P<0,01) is highly significant, *** (P<0,001) is very highly significant and ****(P<0,0001) is extremely highly significant.

5.
RESULTS

Results

The metabolic phenotype of triple negative breast cancer cells has already been characterized. In fact, these cells have glucose and glutamine as the two preferential sources of carbon substrates, being glycolysis and glutaminolysis highly active in these cells. Moreover, fatty acid oxidation and uptake are both used simultaneously by triple negative breast cancer (Sun et al., 2020; Z. Wang et al., 2020). Mitochondrial oxidative phosphorylation also plays a role in metabolic phenotype of these cells, even though it appears that TNBC can acquire an hybrid metabolic state, switching from glycolysis to mitochondrial metabolism depending on environmental circumstances (Jia et al., 2019). In spite of these metabolic clues already characterized, cancer cells are highly heterogenous and evolve as the microenvironment changes (Lyssiotis & Kimmelman, 2017) .

Therefore, glycolysis, glutaminolysis, oxidative phosphorylation, fatty acid synthesis and lipid uptake were the metabolic pathways considered the most relevant to analyze to characterize the metabolic reprogramming of triple negative breast cancer cells that takes place when nutrients' availability changes.

The premise here was that higher metabolic pathway activity would compel higher levels of enzymes operating in that specific pathway. Then, it was assumed that upregulation and downregulation of genes of those enzymes indicated that these metabolic pathways were respectively more or less required by these cells. With this assumption, a total of 9 enzyme genes' expression was analyzed by quantitative PCR (**Table 1**), namely 1 gene of oxidative phosphorylation (mitochondrial complex I- ND1), 3 genes from glycolysis (glycose-6-phosphate dehydrogenase- G6PD; pyruvate kinase M2- PKM2 and lactate dehydrogenase A- LDHA), 1 gene from fatty acids synthesis (fatty acid synthase- FASN), 2 genes from lipid transporters (LDL receptor- LDLr and fatty acid translocase- CD36) and 2 genes form glutaminolysis (glutaminase 2- GLS2 and isocitrate dehydrogenase- IDH2). The expression of these genes was normalized to a housekeeping gene- RNAse P- whose expression does not change in cancer cells compared to normal ones. The normalization of expression to human RNAse P made us certain that only human cells' genes- MDA-MB-231 cell line- were being amplified, which was particularly relevant in our *in vivo* assay.

♦ **High glucose exposure stimulates overall metabolism of MDA-MB-231 cells**

Considering that glucose oxidation is a crucial feature of the metabolic phenotype of triple negative breast cancer cells, we wanted to study the metabolic rewire that took place by exposing these cells to high concentration of glucose.

In fact, quantitative PCR analysis indicates that MDA-MB-231 cells upon exposure to high glucose medium for 6 hours (h) appear to upregulate LDHA, CD36, LDLr, GLS2 and IDH2 genes and none of the remaining genes appeared to be downregulated at this time point (**Figure 8**). Nevertheless, none of these genes had an expression statistically different from controls (low glucose exposure).

In contrast, at 24h time point, these cells seemed to upregulate the expression of G6PD, LDHA, LDLr and IDH2 and downregulated the expression of PKM2. Only the downregulation of PKM2 was statistically significant (**Figure 8**). At 24h time point, the expression of G6PD, LDLr and IDH2 was higher than at 6h, while the expression of PKM2, FASN and CD36 was lower than at 6h. Again, none of these differences were statistically significant using two-way ANOVA.

Considering the 72h time point, MDA-MB-231 upregulated the expression of ND1 and PKM2 genes and downregulated CD36 and IDH2 in high glucose medium, even though there was none statistically significance difference in expression compared to controls (low glucose) (**Figure 8)**. At 72h time point, the ND1 gene was more expressed than at 6 h, but G6PD, PKM2, LDHA, FASN, CD36, LDLr, GLS2 and IDH2 were less expressed than at 6h. Moreover, at 72h time point, the expression of ND1 and PKM2 was higher than at 24h time point, while the expression of G6PD, LDHA, CD36 LDLr, GLS2 was decreased at 72h compared to 24h time point.

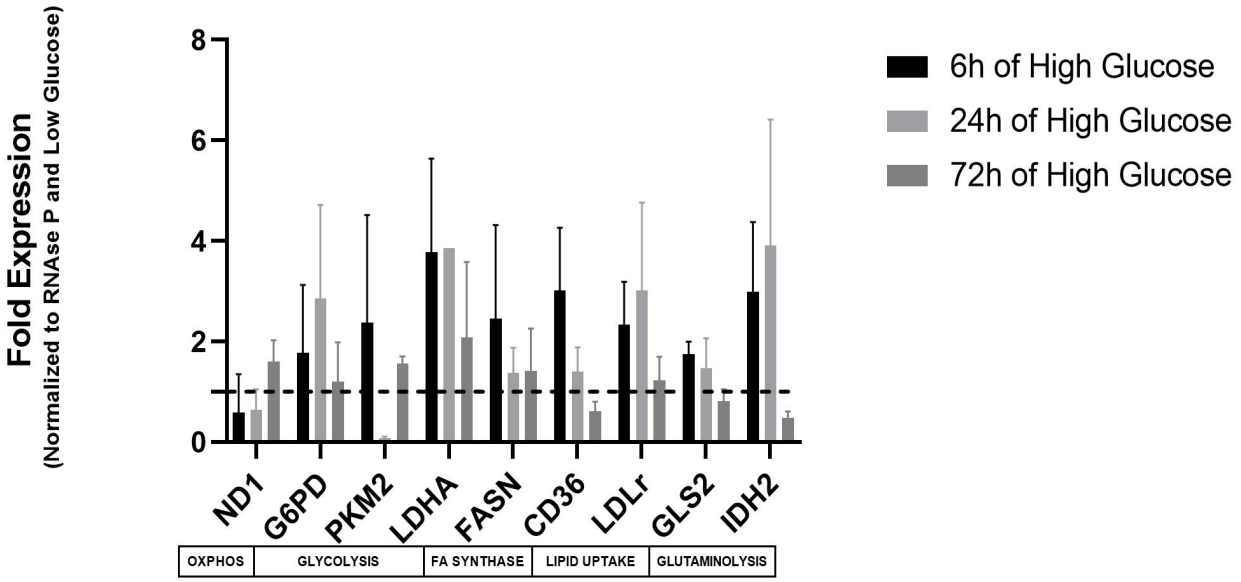

Figure 8- Gene expression signature induced by high glucose exposure in MDA-MB-231 cells. qPCR analysis of the relative expression of the indicated target genes in MDA-MB-231 cells exposed to high glucose for 6 hours (black bar), 24 hours (light gray bar) or 72 hours (dark gray bar). There were evaluated the expression of genes of the mitochondrial complex I (ND1), glycolysis (G6PD, PKM2 and LDHA), de novo lipid synthesis (FASN), lipid transporters (CD36 and LDLr) and glutaminolysis (GLS2 and IDH2). Data are presented as mean ±SD after a two-way ANOVA analysis.

- **High glutamine exposure leads to upregulation of glycolysis enzymes' genes, lipid transporters and *de novo* lipid in triple negative breast cancer cells**

Besides glucose, triple negative breast cancer cells rely on glutamine metabolism. For this reason, we also cultured MDA-MB-231 cells in high glutamine medium to evaluate the metabolic rewire that would take place.

In fact, quantitative PCR analysis demonstrated that after 6h of exposure to high glutamine, MDA-MB-231 cells upregulate G6PD, LDHA and FASN genes, even though the expression of these genes was not statistically significant compared to controls (low glutamine) (**Figure 9**). In addition, at 6h time point, these cells overexpressed PKM2, LDLr and IDH2. These 3 genes had an expression statistically increased compared to controls (low glutamine exposure). Besides, ND1, CD36 and GLS2 genes appeared to be downregulated at 6h, compared to controls (low glutamine exposure), but these differences were not statistically significant.

At 24h time point, ND1 was significantly downregulated compared to controls (low glutamine exposure). Also, CD36 and GLS2 seemed to be downregulated compared to controls (low glutamine exposure), regardless of not being statistically significant (**Figure**

9). None of the evaluated genes appeared to be upregulated compared to controls (low glutamine exposure). Moreover, G6PD, PKM2, LDHA, FASN, CD36, LDLr and IDH2 genes are less expressed at 24h than at 6h.

Interestingly, at 72 h time point, all the genes appeared to be significantly downregulated compared to controls (low glutamine), while mitochondrial complex I ND1) was approximately 30 times more expressed than controls (low glutamine exposure) (**Figure 9**). In fact, these cells are very dependent upon glutamine and, as a result, the deprivation of glutamine for such a long period (72 hours) had a profound impact on cells exposed to low glutamine (control), therefore justifying these intriguing results. Moreover, the profound downregulation of almost all genes observed at 72h justifies the statistically significant differences in the comparisons of the 3 time points.

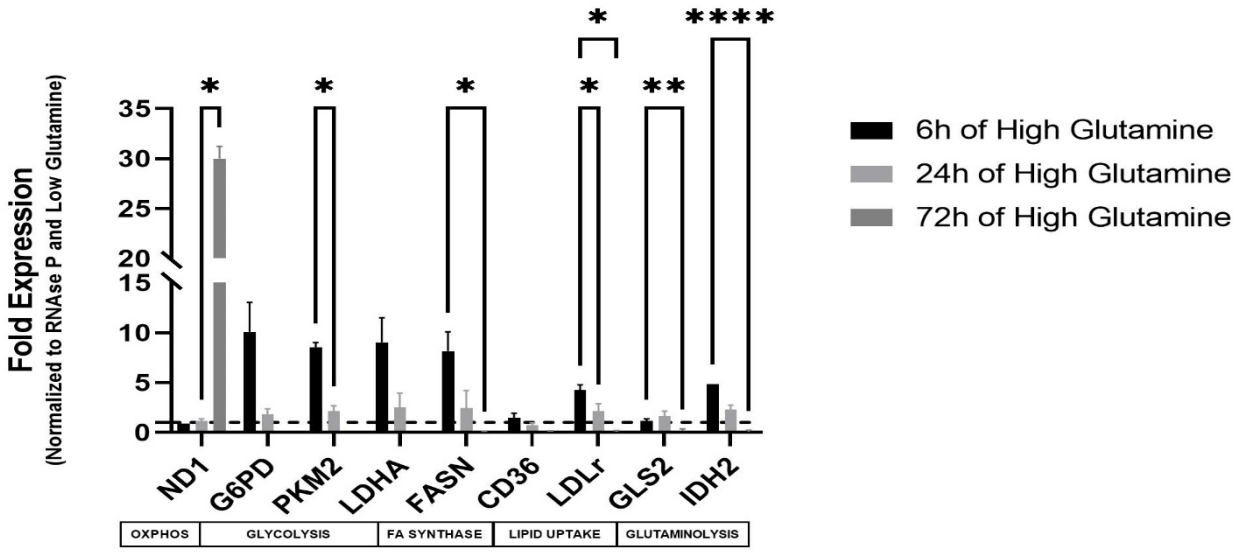

Figure 9- Gene expression signature induced by high glutamine exposure in MDA-MB-231 cells. qPCR analysis of the relative expression of the indicated target genes in MDA-MB-231 cells exposed to high glutamine for 6 hours (black bar), 24 hours (light gray bar) or 72 hours (dark gray bar). There were evaluated the expression of genes of the mitochondrial complex I (ND1), glycolysis (G6PD, PKM2 and LDHA), de novo lipid synthesis (FASN), lipid transporters (CD36 and LDLr) and glutaminolysis (GLS2 and IDH2). Data are presented as mean ±SD after a two-way ANOVA analysis (p*<0,05, p**<0,001 and p****<0,0001).

- **LDL exposure leads to metabolic reprogramming of MDA-MB-231 cells in a glucose and glutamine dependent fashion**

Aside from glucose and glutamine, triple negative breast cancer cells lean on lipid metabolism, either lipid synthesis and fatty acid oxidation to support proliferation, invasion and metastasis (Sun et al., 2020). These cells can obtain fatty acids by uptaking dietary lipids from bloodstream, mainly in form of LDL particles. Therefore, we hypothesized that LDL exposure would lead to metabolic reprogramming of MDA-MB-231 cells.

Upon high glucose and high glutamine, LDL exposed MDA-MB-231 cells significantly reprogrammed their metabolism (**Figure 10.1**). In fact, at 6 h time point, LDHA and FASN appeared to be upregulated compared controls (not exposed to LDL), but this difference in expression was not statistically significant. CD36 and IDH2 were significantly overexpressed compared to controls, while GLS2 had an expression significantly lower than controls (not exposed to LDL).

After 24h of LDL exposure, none of the studied genes were upregulated. Indeed, PKM2, LDLr and isocitrate dehydrogenase IDH2 were all significantly downregulated compared to controls (not exposed to LDL). LDHA and FASN were downregulated compared to controls, but the difference in expression was not statistically significant. At this time point, ND1, PKM2, LDHA, FASN, CD36, LDLr and IDH2 were less expressed compared to 6h time point.

Finally, at 72 h time point, PKM2, LDHA and IDH2 appeared to be upregulated compared to controls (not LDL exposed). FASN gene upregulation compared to controls (not LDL exposed) was statistically significant. ND1 and CD36 and LDLr were downregulated at this time point, but only CD36 downregulation was statistically significant compared to controls (not LDL exposed). At 72h there were an important reduction in expression of CD36 compared to 6h time point. Moreover, the expression of PKM2 and FASN appreciably increased at 72h, comparing to 24h time point.

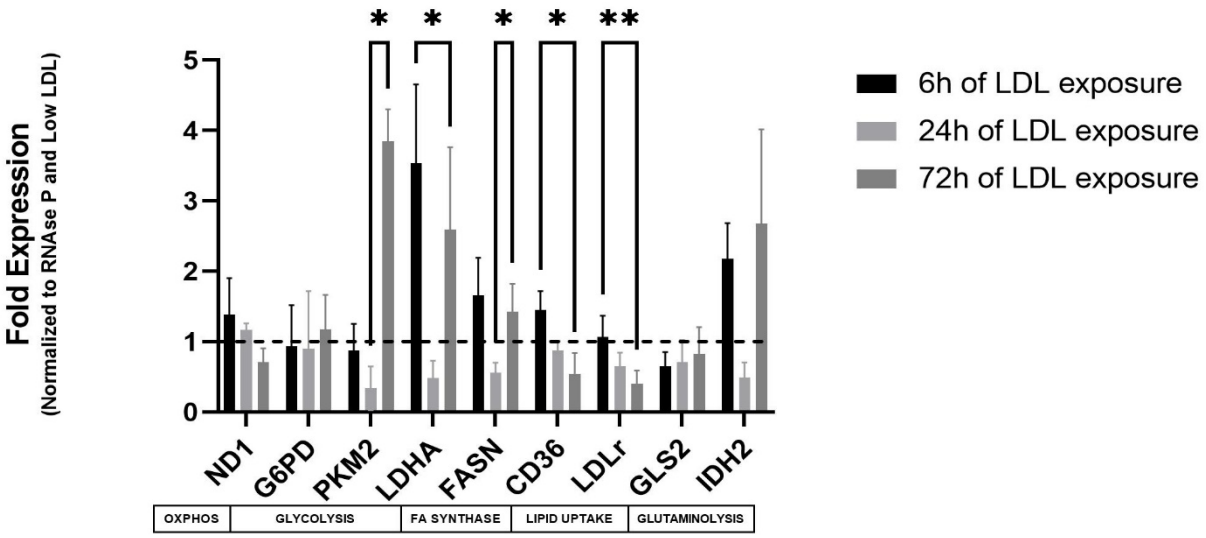

Figure 10.1- Gene expression signature induced by LDL in high glucose and high glutamine medium in MDA-MB-231 cells' metabolism- qPCR analysis of the relative expression of the indicated target genes in MDA-MB-231 cells exposed to high glucose, high glutamine and LDL for 6 hours (black bar), 24 hours (light gray bar) or 72 hours (dark gray bar). There were evaluated the expression of genes of the mitochondrial complex I (ND1), glycolysis (G6PD, PKM2 and LDHA), de novo lipid synthesis (FASN), lipid transporters (CD36 and LDLr) and glutaminolysis (GLS2 and IDH2). Data are presented as mean ±SD after a two-way ANOVA analysis (p*<0,05 and p**<0,01).

However, these metabolic reprogramming observed following LDL exposure appeared to be dependent from glutamine exposure, (**Figure 10.2**) considering that LDL exposure in a glutamine deprived medium lead to downregulation of almost all target genes. Actually, at 6h of LDL exposure in glutamine deprived medium, G6PD, PKM2, LDHA, FASN, LDLr, GLS2 and IDH2 were downregulated compared to controls (low glutamine exposed without LDL), of which only the downregulation of G6PD and LDHA was statistically significant compared to controls (low glutamine exposed without LDL).

Considering 24h time point, none of the target genes were upregulated. G6PD, PKM2, LDHA, FASN, LDLr and IDH2 were significantly downregulated compared to controls (low glutamine without LDL). Also, GLS2 was downregulated compared to controls, even though this downregulation was not statistically significant (**Figure 10.2**).

At 72h time point, ND1 gene was significantly overexpressed, while G6PD, PKM2, FASN, GLS2 and IDH2 are downregulated compared to controls (low glutamine without LDL), being this difference in expression statistically significant. LDHA and LDLr also appeared to be downregulated compared to controls, but this regulation was not statistically significant (**Figure 10.2**). Apart from ND1 expression, that increases at 72h

compared to 6h, none of the remaining genes appear to have an expression that is different comparing the 3 time points.

Moreover, MDA-MB-231 cells exposed to LDL in a glucose deprived medium (**Figure 10.3**) at 6h time point, did not upregulate the expression of any of the studied genes compared to controls (low glucose exposed without LDL). CD36 appeared to be downregulated at this time point compared to controls (low glucose exposed without LDL), but this difference was not statistically significant.

After 24 hours of LDL in a low glucose medium, ND1 and CD36 appeared to be upregulated compared to controls (low glucose exposed without LDL), although this difference was not statistically significant (**Figure 10.3**). In addition, G6PD, PKM2, LDHA, FASN, LDLr, GLS2 and IDH2 were downregulated compared to controls, but only G6PD, LDHA and IDH2 had a difference in expression, that was statistically significant. At 24h, ND1 and CD36 are more expressed than at 6h, while G6PD, PKM2, LDHA, FASN, LDLr and IDH2 are less expressed than at 6h. None of these differences in expression at these time points were statistically significant.

Lastly, 72h time point was associated with a statistically significant downregulation of G6PD, PKM2, LDHA, FASN, LDLr and IDH2 compared to controls (**Figure 10.3**). GLS2 was also downregulated at this time point compared to controls, but the difference in expression was not statistically significant. At 72h, CD36 was more expressed than at 6h, while G6PD, PKM2, LDHA, FASN, LDLr, GLs2 and IDH2 were less expressed than at 6h. This differences in expression comparing 6h and 72h time points were not statistically significant. At 72h, IDH2 was more expressed than at 24h, while ND1, FASN, LDLr and GLS2 were all less expressed than at 24h. This differences in expression were not statistically significant.

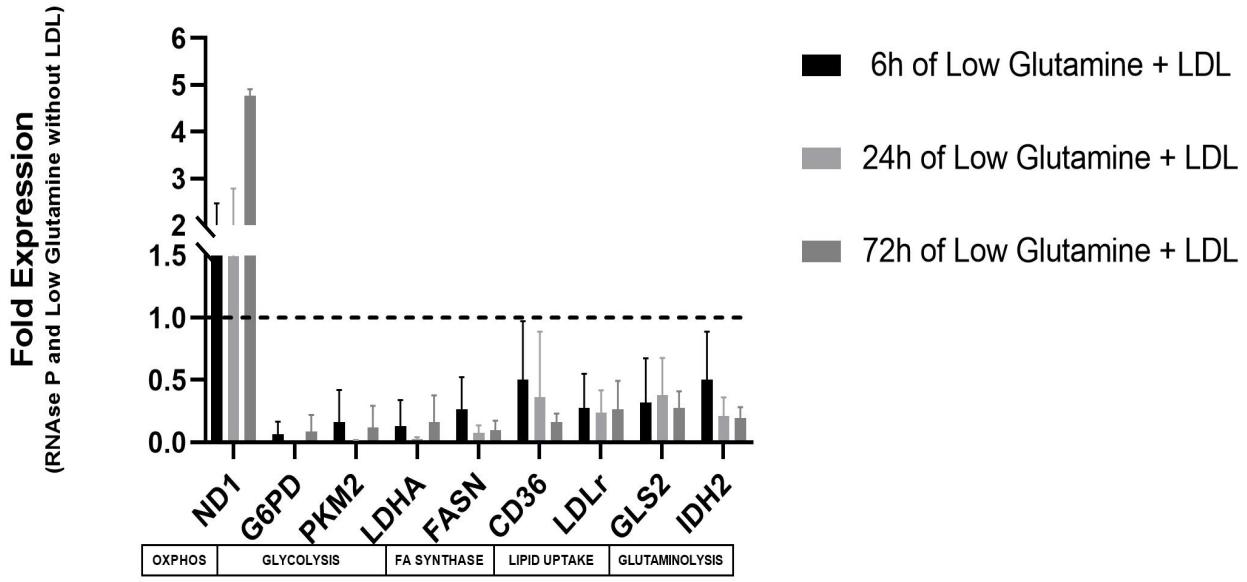

Figure 10.2- Gene expression signature induced by LDL in glutamine deprived medium in MDA-MB-231 cells' metabolism- qPCR analysis of the relative expression of the indicated target genes in MDA-MB-231 cells exposed to low glutamine and LDL for 6 hours (black bar), 24 hours (light gray bar) or 72 hours (dark gray bar). There were evaluated the expression of genes of the mitochondrial complex I (ND1), glycolysis (G6PD, PKM2 and LDHA), de novo lipid synthesis (FASN), lipid transporters (CD36 and LDLr) and glutaminolysis (GLS2 and IDH2). Data are presented as mean ±SD after a two-way ANOVA analysis.

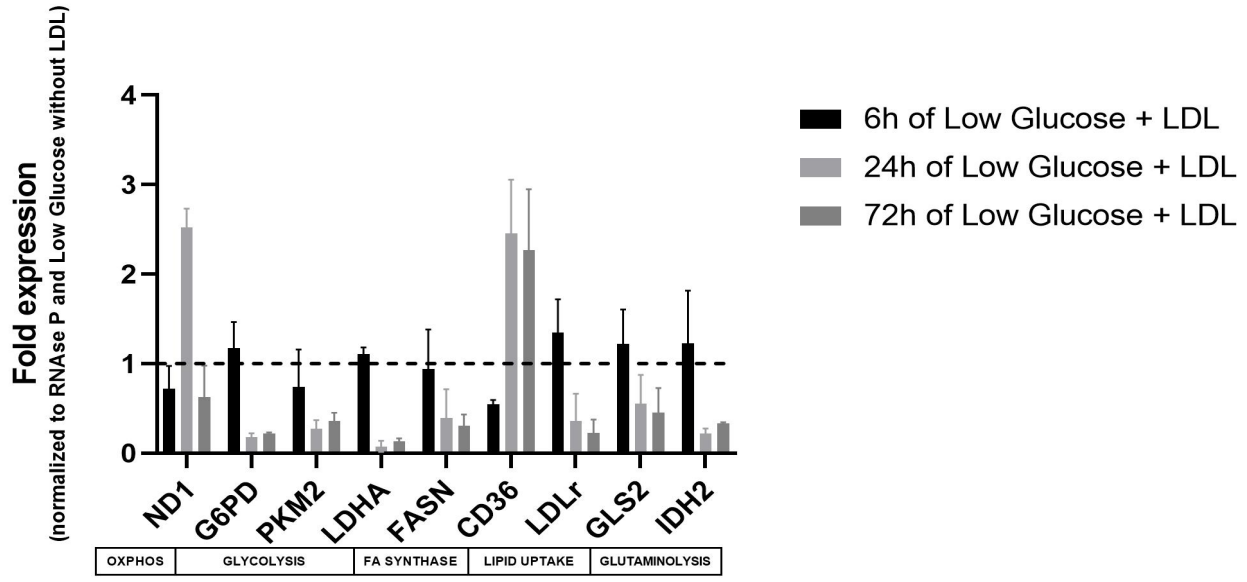

Figure 10.3- Gene expression signature induced by LDL in glucose deprived medium in MDA-MB-231 cells' metabolism- qPCR analysis of the relative expression of the indicated target genes in MDA-MB-231 cells exposed to low glucose and LDL for 6 hours (black bar), 24 hours (light gray bar) or 72 hours (dark gray bar). There were evaluated the expression of genes of the mitochondrial complex I (ND1), glycolysis (G6PD, PKM2 and LDHA), de novo lipid synthesis (FASN), lipid transporters (CD36 and LDLr) and glutaminolysis (GLS2 and IDH2). Data are presented as mean ±SD after a two-way ANOVA analysis.

- ***In vivo*, MDA-MB-231 breast cancer cells in high cholesterol diet fed mice rewire lipidomic metabolism after blockade of LDLr**

In addition to metabolic reprogramming that takes place in triple negative breast cancer cells with change in nutritional availability, these cells rewire their metabolism in response to the interactions that occur in tumoral microenvironment. Besides, metastatic triple negative breast cancer cells also rewire their metabolism as they establish at secondary site (Sun et al., 2020). Therefore, we studied the metabolic reprogramming that takes place in MDA-MB-231 cells *in vivo* in response to high cholesterol diet with and without access to the main lipidic particle: LDL.

Quantitative PCR analysis demonstrates that high cholesterol diet fed mice had triple negative breast cancer cells that upregulated ND1 and CD36 compared to housekeeping gene (RNAse P) in normal diet fed mice's tumor cells. These differences in expression were not statistically significant (**Figure 11**). Additionally, the blockade of LDL receptor conducted to upregulation of PKM2, LDHA, CD36, LDLr and GLS2 compared to normal diet fed mice's tumors. Of these differences in expression, only the overexpression of CD36 was statistically significant.

High cholesterol diet fed mice with blockade of LDLr had an higher expression of PKM2, FASN, CD36, LDLr and GS2 compared to high cholesterol diet fed mice without blockade of LDLr (**Figure 11**).

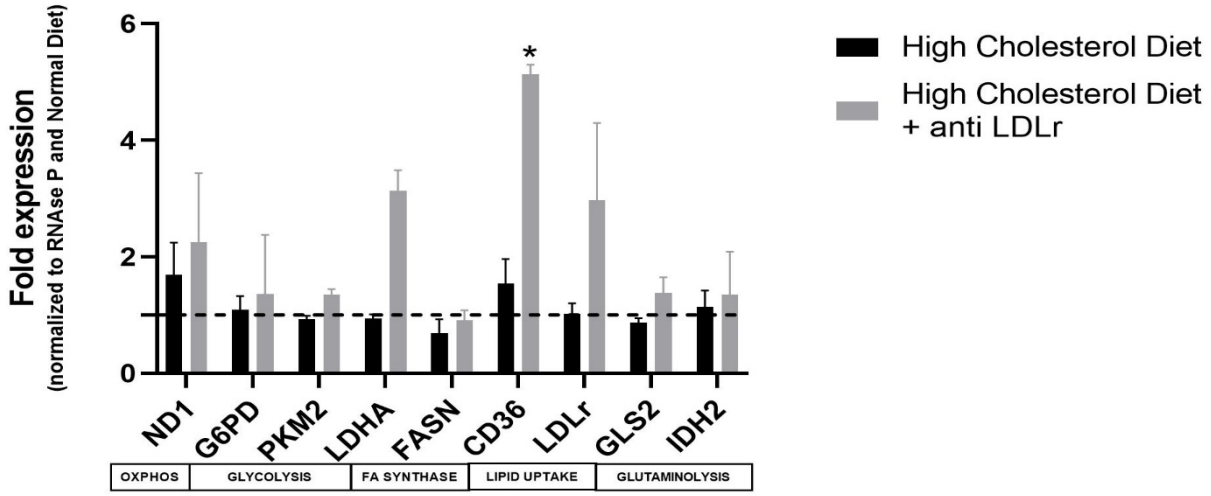

Figure 11- Gene expression signature induced by High cholesterol diet and by blockade of LDLr in triple negative breast cancer. qPCR analysis of the relative expression of the indicated target genes in breast cancer exposed to high cholesterol diet and high cholesterol diet (black bar) and high cholesterol diet after blockade of LDLr, (gray bar). There were evaluated the expression of genes of the mitochondrial complex I (ND1), glycolysis (G6PD, PKM2 and LDHA), de novo lipid synthesis (FASN), lipid transporters (CD36 and LDLr) and glutaminolysis (GLS2 and IDH2). Data are presented as mean ±SD after a two-way ANOVA analysis (*p<0,05).

- **<u>Inhibition of LDL receptor in high cholesterol diet fed mice increased overall metabolism of MDA-MB-231 cells</u>**

Quantitative PCR analysis demonstrates that in high cholesterol diet fed mice, blockage of LDL receptor significantly upregulates all the glycolysis genes, namely G6PD, PKM2, LDHA, as well as FASN, LDLr, and IDH2 compared to high cholesterol diet fed mice without blockade of LDLr. ND1 and GLS2 were also upregulated, but the difference in expression was not statistically significant (**Figure 12**).

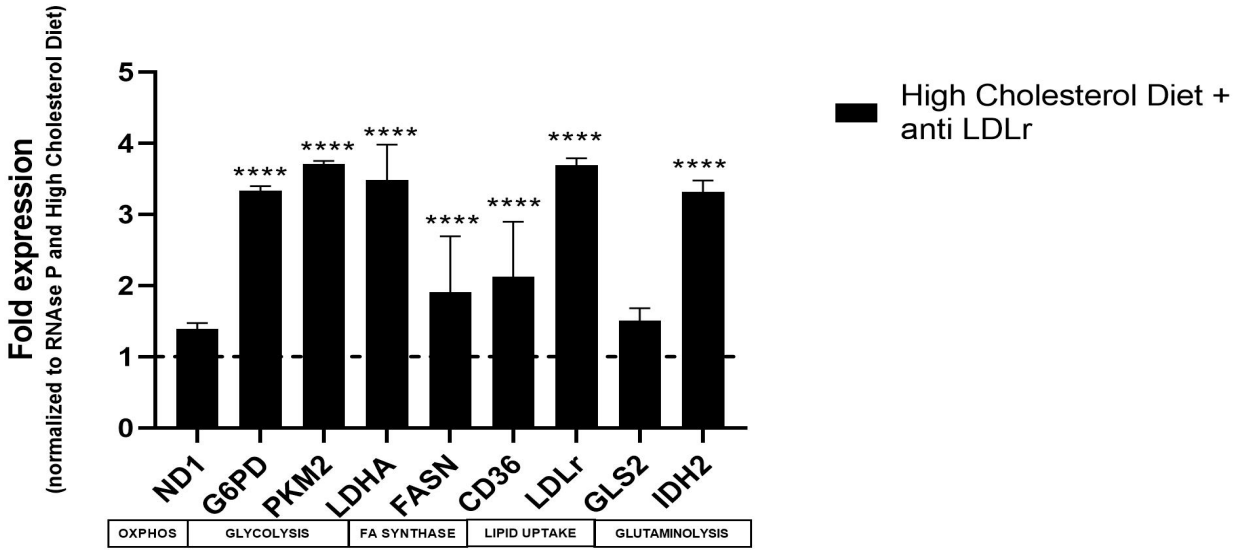

Figure 12- Gene expression signature induced in triple negative breast cancer by blockade of LDL receptor in High cholesterol diet exposed mice. qPCR analysis of the relative expression of the indicated target genes in breast cancer exposed to high cholesterol diet after blockade of LDLr(black bar). There were evaluated the expression of genes of the mitochondrial complex I (ND1), glycolysis (G6PD, PKM2 and LDHA), de novo lipid synthesis (FASN), lipid transporters (CD36 and LDLr) and glutaminolysis (GLS2 and IDH2). Data are presented as mean ±SD after a two-way ANOVA analysis (****p<0,0001).

- **<u>High cholesterol diet promotes glycolysis, oxidative phosphorylation and lipid uptake in MDA-MB-231 lung metastasis</u>**

Besides, metastatic triple negative breast cancer cells also rewire their metabolism as they establish at secondary site (Sun et al., 2020). Bearing this in mind, we wanted to study the impact of high cholesterol diet in the metabolism of lung metastasis of MDA-MB-231 cells, particularly what metabolic pathways these cells would upregulate and downregulate in this environment.

Quantitative PCR analysis demonstrates that high cholesterol diet drove the upregulation of ND1, PKM2, LDHA and LDLr genes in lung metastasis of triple negative breast cancer compared to lung metastasis of normal diet fed mice. G6PD, FASN and GLS2 genes seemed to be upregulated, compared to lung metastasis of normal diet fed mice, but the differences in expression of these genes were not statistically relevant. IDH2 appeared to be downregulated in high cholesterol diet fed mice lung metastasis compared to normal diet fed mice lung metastasis, but this difference in expression was not statistically important (**Figure 13)**.

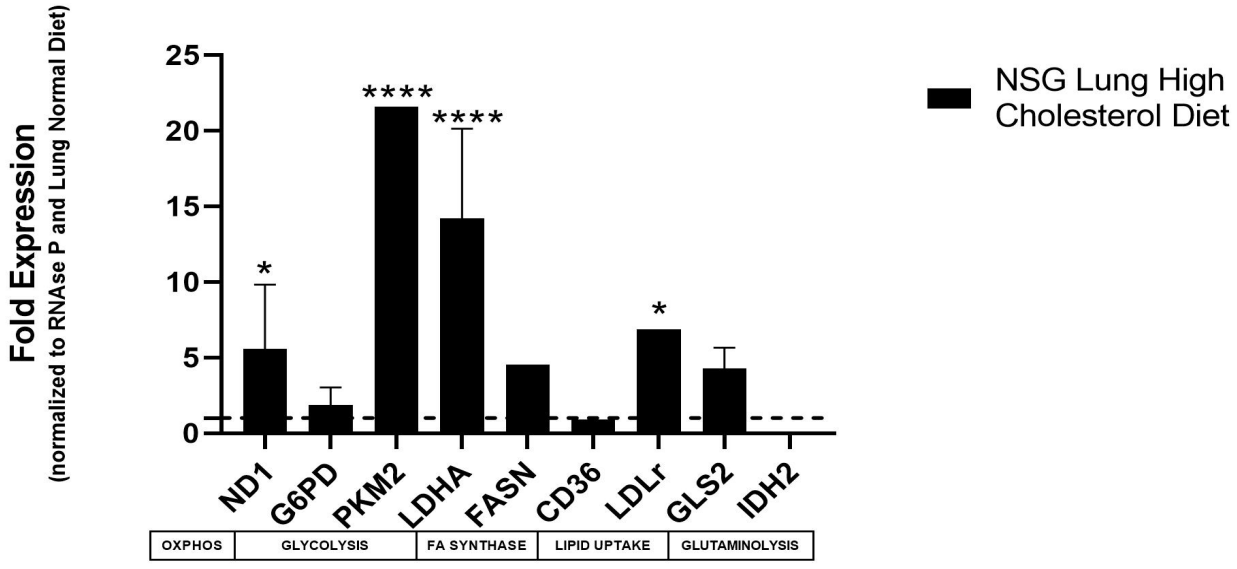

Figure 13- Gene expression signature induced by high cholesterol diet in lung metastasis of triple negative breast cancer. qPCR analysis of the relative expression of the indicated target genes in lung metastasis exposed to high cholesterol diet (black bar). There were evaluated the expression of genes of the mitochondrial complex I (ND1), glycolysis (G6PD, PKM2 and LDHA), de novo lipid synthesis (FASN), lipid transporters (CD36 and LDLr) and glutaminolysis (GLS2 and IDH2). Data are presented as mean ±SD after a two-way ANOVA analysis (*p<0,05 and ****p<0,0001).

- **In high cholesterol diet fed mice, lung metastasis of MDA-MB-231 cells become more reliant upon lipid uptake compared to primary breast cancer**

Considering that the metabolic rewiring of metastatic cells is specific to the organ of metastatic lesion (Faubert et al., 2020; Gandhi & Das, 2019), we wanted to compare the metabolic profile of primary breast cancers and respective lung metastases.

In fact, comparing the gene expression of these enzymes in primary tumors and lung metastasis of high cholesterol diet fed mice lung metastasis, only CD36 was significantly overexpressed in lung metastasis compared to primary tumors (**Figure 14**). ND1, G6PD, PKM2, LDHA and GLS2 were downregulated in lung metastasis compared to breast cancer in high cholesterol diet fed mice but had a difference in expression that was not statistically significant (**Figure 14**).

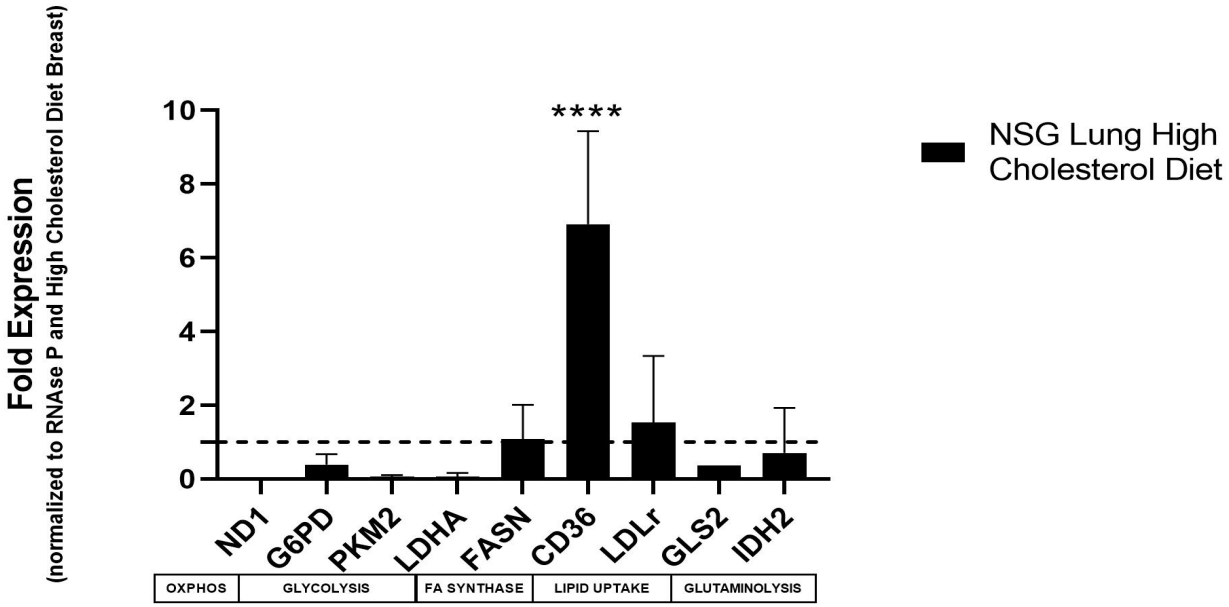

Figure 14- Gene expression signature induced by high cholesterol diet in lung metastasis of triple negative breast cancer compared to primary tumors. qPCR analysis of the relative expression of the indicated target genes in lung metastasis exposed to high cholesterol diet (black bar). There were evaluated the expression of genes of the mitochondrial complex I (ND1), glycolysis (G6PD, PKM2 and LDHA), de novo lipid synthesis (FASN), lipid transporters (CD36 and LDLr) and glutaminolysis (GLS2 and IDH2). Data are presented as mean ±SD after a two-way ANOVA analysis (****p<0,0001).

- **Lung metastasis and primary tumors of normal diet fed mice also appear to rewire their lipid metabolism**

These differences in CD36 expression in primary breast cancer and lung metastasis of MDA-MB-231 cells in high cholesterol diet fed mice raised the question of whether the type of diet was the reason for the upregulation of CD36 or was the different conditions in primary tumor and lung metastasis that led this upregulation (**Figure 15**). Hence, we compared the metabolic reprogramming that occurred in lung metastasis and primary tumors in normal diet fed mice. In fact, we observed that in mice fed with normal diet, there was an upregulation of CD36 in lung metastasis compared to primary breast tumor. However, this difference was not statistically significant, probably because of hide standard deviation. Actually, in normal diet fed mice, only in 2 of the 3 lung samples occurred amplification of human RNAse P, pointing that only in these 2 samples were present MDA-MB-231 cells (lung metastasis). In one of the metastases CD36 was approximately 12 times more expressed than in normal diet fed breast cancers, while in the other CD36 was not overexpressed.

Additionally, in lung metastasis, LDL receptor seemed to be upregulated, but the difference in expression was not statistically significant.

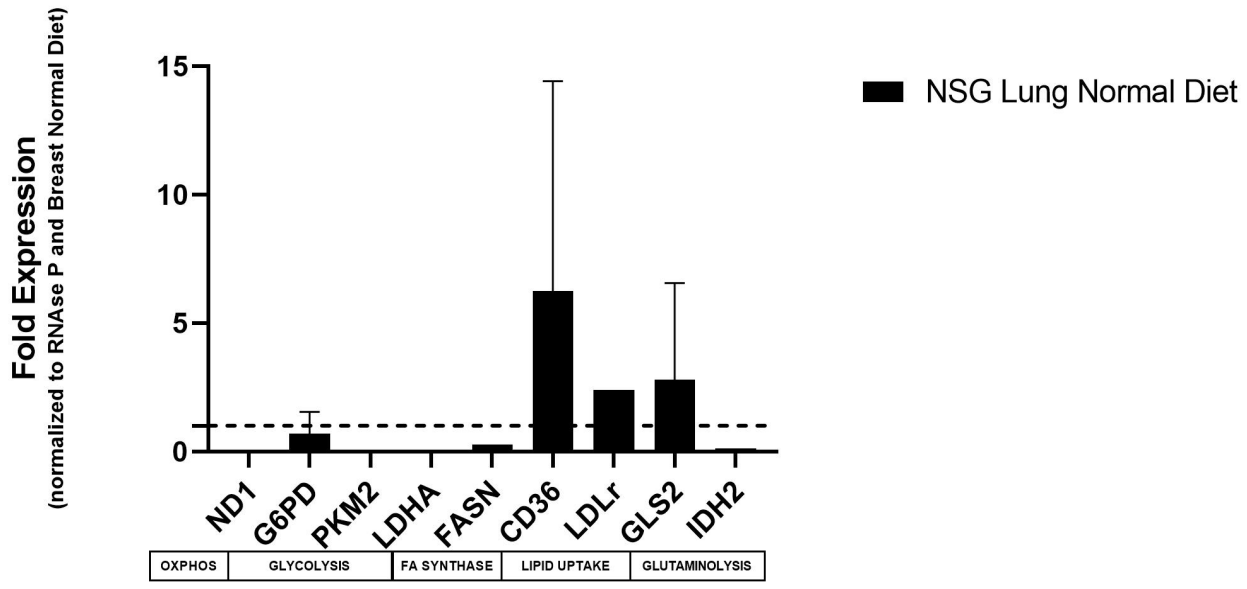

Figure 15- Gene expression signature induced by normal diet in lung metastasis of triple negative breast cancer compared to primary tumors of normal diet fed mice. qPCR analysis of the relative expression of the indicated target genes in lung metastasis exposed to high cholesterol diet (black bar). There were evaluated the expression of genes of the mitochondrial complex I (ND1), glycolysis (G6PD, PKM2 and LDHA), de novo lipid synthesis (FASN), lipid transporters (CD36 and LDLr) and glutaminolysis (GLS2 and IDH2). Data are presented as mean ±SD after a two-way ANOVA analysis.

6. DISCUSSION

Discussion

Triple negative breast cancer cells, like other highly proliferative cancer subtypes, display metabolic adaptability, which is the capacity to use different metabolic substrates to fulfill their macromolecules needs (Fendt et al., 2020). Compared with other molecular subtypes, triple negative breast cancer cells appear to be the most dependent upon glycolysis, showing elevated lactate production as well as upregulating glycolytic enzymes and glucose transporters (Gandhi & Das, 2019).

In our *in vitro* assay, MDA-MB-231 cells exposed to high glucose rewired their metabolism, upregulating the expression of genes involved in oxidative phosphorylation, glycolysis, lipid synthesis, lipid uptake and glutaminolysis (**Figure 8**), despite none of the enzymes of these metabolic pathways appeared to be more upregulated than the others. Moreover, none of these genes had an increase in expression statistically different from controls. Furthermore, it is possible that glucose exposure leads to a metabolic activation, displaying an intermediate metabolic phenotype. It has been extensively described the importance of glucose to proliferation of MDA-MB-231 cells (Sun et al., 2020; Z. Wang et al., 2020), considering that it can feed ATP production and biosynthesis of amino acid, lipid and nucleic acids synthesis, which are required for cell growth (Gandhi & Das, 2019) . Our results are concordant with other observations, in which high glucose exposure increased the proliferation rate of MDA-MB-231 cells and was associated with higher oxygen consumption rate (higher oxidative phosphorylation rate), extracellular acidification rates (higher glycolytic activity) and uptake of fatty acids, namely palmitate (Ocaña et al., 2020). Moreover, stable-isotope-tracing studies in patients with TNBC shown that these tumor cells uptake huge quantities of glucose from the bloodstream (indicated by high isotopic enrichment glucose at tumor bulk) and that glucose is metabolized to produce lactate, amino-acids synthesis including serine, aspartate, glutamate, proline and glutamine, as these molecules display isotopic carbon atoms (Ghergurovich et al., 2021).

Indeed, it has been observed that knocking down glucose transporter GLUT4 and limiting glucose uptake leads to reallocation of glycolytic flux to oxidative phosphorylation, compromising cell proliferation and viability under hypoxia (Sun et al., 2020; Z. Wang et al., 2020).

Glutamine is the most abundant circulating amino acid and many cancers, including TNBC rewire glutaminolysis to support biomass, energy and glutathione production. Thus, many cancer cells display a glutamine-addiction phenotype (Gwangwa et al., 2019).

Exposure MDA-MB-231 cells to high glutamine for 6 hours was associated with upregulation of the glycolysis enzymes G6PD, PKM2 and LDHA, as well as LDLr and FASN (**Figure 9**). The relationship between glutamine availability and glycolysis is in concordance with previous described data, in which supplementation of MDA-MB-231 cells with glutamine was associated with an increase in the extracellular acidification rate (Ocaña et al., 2020). This indicates that MDA-MB-231 cells uptake glutamine for enhances glutaminolysis and glycolysis (Gwangwa et al., 2019). In fact, glycolysis is essential to metabolomic phenotype of TNBC and these cells upregulate glucose transporters (GLUT) and other glycolytic enzymes such as lactate dehydrogenase (LDHA) and hexokinase 2 (Jia et al., 2019; Sun et al., 2020; Z. Wang et al., 2020). Metabolite abundance assays comparing breast cancer cells and normal breast tissue show that lactate (end product of glycolysis) is importantly more abundant in breast cancer samples compared to normal tissues, which suggests an increase in glycolysis' rate in cancer cells (Jia et al., 2019). This is consistent with the advantages that the activation of this metabolic pathway provides to highly proliferative cells (Vander Heiden & DeBerardinis, 2017). Considering the relation of glutamine exposure and lipid metabolism, previous studies had demonstrated that glutamine alone did not affect fatty acids uptake in MDA-MB-231 cells, unless it was added glucose (Ocaña et al., 2020). In our assay, cells were exposed to high glutamine and high glucose medium, which can explain why glutamine exposure increased LDL receptor expression. It is important to note that glutamine allows cells to grow and proliferate. Glutamine enrichment benefits triple negative breast cancer cells, providing intermediates to biomass production, fueling the TCA cycle and producing the precursors for glutathione, which is part of the antioxidant cellular system (Sun et al., 2020). As a result, it is reasonable that exposure to high concentration of glutamine leads to proliferation, hence upregulation of lipid uptake and, at the same time, fatty acids biosynthesis.

Considering the reliance of MDA-MB-231 upon glutamine, we hypothesized that following 72 hours of high glutamine exposure, these cells would display an increased

metabolic activity with upregulation of the enzymes 'genes of all the studied metabolic pathways. In contrast, our results indicate that at 72 hours time point, apart from ND1, which was upregulated, all the remaining enzymes' genes were profoundly downregulated (**Figure 9**). In fact, our results are a comparison between cells exposed to high glutamine and a control group deprived from glutamine for the same period. As a result, we propose that the cells in control group were extremely affected by glutamine deprivation. Hence, these puzzling results illustrate the glutamine-addicted phenotype of these cells. Some other studies have established that MDA-MB-231 cell line can survive 48 hours without glucose, but not in deprivation of glutamine (Ocaña et al., 2020). It has been demonstrated that glutamine deprivation decreases cell growth because of high generation or reactive oxygen species, aberrant mitochondrial potential and disrupted cell cycle progression in a time-dependent fashion in MDA-MB-231 cell line (Gwangwa et al., 2019). Moreover, in TNBC, GLS expression is significantly correlated with low levels of tumor infiltrating lymphocytes, which can be due to a metabolic competition between cancer and other cells of the microenvironment (Z. Wang et al., 2020). These findings point glutaminolysis as a reasonable target to cancer therapy.

Besides glycolysis and glutaminolysis, cancer cells have fatty acids (from extracellular uptake or *de novo* synthesis) as an alternative source of energy production, allowing them to resist hypoxia and glucose deprivation. In fact, TNBC display enhanced lipid uptake through upregulation of CD36 (fatty acid translocase). Fatty acid binding proteins such as FABP5 and FABP7 have been identified as biomarkers of poor clinical outcome, probably due to their role as promotors of fatty acids uptake and proliferation (Z. Wang et al., 2020). In addition to fatty acids, these cells have increased requirements of cholesterol. *In vivo* studies had revealed that MDA-MB-231 cells upregulate LDL receptor gene and increase protein expression of these receptor, in contrast to other ER positive breast cancer cell lines (Cedó et al., 2019). Accordingly, LDL-cholesterol promotes proliferation and migration in MDA-MB-231 cells.

Our results suggest that in high glucose and high glutamine presence, LDL exposure promotes metabolic reprogramming of MDA-MB-231 cells. 6 hours of LDL exposure upregulated glycolytic enzyme LDHA, as well as CD36 and IDH2, whereas GLS2 was downregulated (**Figure 10.1**). This points to an activation of glycolysis, fatty acid

uptake and glutaminolysis. In other words, following 6 hours of LDL exposure, MDA-MB-231 cells globally activate all the metabolic pathways, which is a hint to a proliferation engagement. However, at 24 hours, there was a downregulation of glycolysis genes (PKM2, LDHA), FASN, LDLr and IDH2 (**Figure 10.1**). In fact, lipid uptake drives to cellular stress, because increase in lipid content can lead to lipid peroxidation and toxicity (Santos & Schulze, 2012; Schmidt, Patel, Kirsch, Lewis, Heiden, & Locasale, 2021). As a result, these cells must adapt to avoid the pernicious effects of lipid accumulation. Thus, at 24h time point, there was probably a reset in metabolism. Actually, at 72h, there is again an upregulation of glycolytic enzymes (PKM2 and LDHA), FASN and IDH2 and downregulation of both lipid transporters (LDLr and CD36), hinting that these cells are again committed to proliferation (**Figure 10.1**). The downregulation of LDL receptor and CD36 is in line with previous evidence that LDL receptor regulation is mediated by LDL available to cells in a negative feedback loop (Mehta et al., 1997; Zhang et al., 2012). Previously, it has been shown that LDL rewires the metabolism of MDA-MB-231 cell line and significantly changes the expression of glycolysis and lipid transporters' genes. LDL exposure for 48 hours lead to upregulation of CD36, LDHA and PKM2 and downregulation of LDLr (Monteiro, 2017).

Nevertheless, it seems that this effect of LDL in metabolism of MDA-MB-231 cells depends on the availability of other subtracts, namely glutamine and glucose. When MDA-MB-231 cells are exposed to low glutamine and LDL (**Figure 10.2**), we observed a metabolic phenotype that reminds the glutamine addiction of these cells. Apart from ND1, all enzyme genes' were downregulated when exposed to low glutamine and LDL, compared to controls (low glutamine without LDL) (**Figure 10.2**). For this reason, we assume that the established effect of LDL cholesterol in proliferation and aggressiveness of MDA-MB-231 cells (Mehta et al., 1997; Schmidt, Patel, Kirsch, Lewis, Heiden, Locasale, et al., 2021) is conditional to the presence of glutamine. Moreover, high cholesterol exposure did not appear to mitigate the glutamine-addiction of MDA-MB-231 cells. This observation is concordant with the functions of glutamine in TNBC cells, particularly maintaining redox balance and providing nitrogen to nucleic acids and other amino-acids synthesis. Furthermore, we speculated that the metabolic rewire associated with LDL exposure was also dependent from glucose. As a result, we exposed MDA-MB-231 cells to low glucose and LDL. At 6 hours, cells did not upregulate any of

enzymes' genes (**Figure 10.3**), which is not in favor with metabolic activity needed to proliferate. This contrasts with upregulation of enzymes' genes and consequently metabolic activity observed at 6 hours of LDL exposure in the presence of high glucose and glutamine (**Figure 10.1**). At 24 and 72 hours, glycolysis, lipid synthesis and glutaminolysis genes and LDL receptor are downregulated compared to controls (low glucose without LDL) which is in favor with a low metabolic rate. Our results are concordant with the observation that fatty acids uptake in MDA-MB-231 cells increase in the presence of glucose and decreases in the presence of glucose analog 2-deoxyglucose (2-DG), which cannot be metabolized through glycolysis (Ocaña et al., 2020). Thus, we can also speculate that LDL impact on metabolism of MDA-MB-231 is somehow dependent upon glycose availability and, consonant with glutamine, LDL did not mitigate the dependence of glucose of MDA-MB-231 cells. Nonetheless, 72 hours of glucose deprivation did not appear to affect the viability of MDA-MB-231 cells as profoundly as glutamine. We hypothesize that following the LDL exposure lipotoxicity ensues and high glycolytic rate produces intermediates to cellular redox system, hence helping cells to overcome this stress. Indeed, it has been shown that palmitate exposed hepatocytes for 1 hour increase glycolytic rate (Kakimoto et al., 2021). To clarify the impact of glucose and glutamine deprivation in MDA-MB-231 cells, further studies are needed, for instance cell viability and proliferation assays.

Our *in vitro* assay suggested that MDA-MB-231 cells display metabolic flexibility, despite being hugely dependent upon glutamine and glucose. Nevertheless, these metabolic changes do not consider the effect of microenvironment interactions, immune regulation and other stressful events that cancer cells must face in vivo. As a result, to further study the metabolic reprogramming of these cells we analyzed how MDA-MB-231 cells reshape their metabolism in vivo, when facing distinct nutrient availability from different diets.

For this reason, we studied MDA-MB-231 cells *in vivo* using NSG, which are non-obese diabetic mice (Pearson et al., 2008), which allowed us to assume an environment of high glucose exposure both in primary tumors and lung metastasis.

In fact, the metabolic profile of primary breast cancer in high cholesterol-diet fed mice is not significantly different from primary breast cancer in normal diet fed mice (**Figure 11**). On the other hand, breast cancer MDA-MB-231 in high cholesterol diet fed

mice after blockade of LDL receptor upregulated of all enzymes' genes, compared to cells of breast cancer in high cholesterol diet fed mice without blockade of LDL receptor (**Figure 12**).

Previously, it has been established that high fat diets increase the incidence and growth of some tumors, including breast cancer. Thus, we expected that breast cancer in high cholesterol diet fed mice would have a metabolic phenotype different from those breast cancers in normal diet, namely an upregulation of glycolysis, glutaminolysis and oxidative phosphorylation, indicating more proliferation. However, we did not observe these differences. In truth, it is known that diet-induced changes in blood metabolites (namely high fat diet and hypercholesterolemia) may not be perfectly reflected in changes in the availability of such nutrients in tumor microenvironment, because within tumor mass there are areas with different degrees of access to blood vessels, which results in areas more deprived in nutrients (Lien & Vander Heiden, 2019). It is possible that we sampled primary breast cancer of an hypovascular area. Moreover, cancer cells are heterogeneous within the same tumor mass, which means different degrees of engagement in proliferation. Thus, in future, it would be interesting to couple metabolomic studies with single-cell transcriptome profiling, to associate individual metabolomic features of cancer cells with other genes' expression, namely genes associated with proliferation or metastatic cascade.

Blockade of LDL receptor in MDA-MB-231 in high cholesterol diet fed mice lead to a significant upregulation of CD36 and a less pronounced upregulation of glycolysis, oxidative phosphorylation and LDL receptor genes. LDL receptor regulation, comparing to normal diet fed mice. In fact, cells can have access to cholesterol by LDL receptor mediated endocytosis, de novo synthesis from acetyl co-A or by receptor-independent transport. This third mechanism is not regulated and nonsaturable but is not relevant in normal conditions. LDL receptor expression is increases when cells are cholesterol depleted and decrease in cholesterol- enrichment circumstances (Mehta et al., 1997). As a result, when MDA-MB-231 cells have LDL receptors blocked, a cholesterol-depleted condition ensues and LDL receptor is overexpressed, which is in tune with our observations. Moreover, it is reasonable to assume that cholesterol depletion leads to upregulation of other lipid transporters, namely CD36 (Schmidt, Patel, Kirsch, Lewis, Heiden, Locasale, et al., 2021). Having in mind the mechanisms of having access to

cholesterol, we hypothesize that MDA-MB-231 cells upregulate the genes of the enzymes involved in biosynthesis of cholesterol, namely HMGCoA reductase. Cholesterol biosynthesis requires acetyl-coA, which can be diverted from oxidative phosphorylation. This may be the reason why these cells upregulate oxidative phosphorylation gene. On the other hand, blocking LDL receptor in MDA-MB-231 cells and preventing endocytosis of this particle promotes a tumoral microenvironment enriched in LDL cholesterol. Cholesterol enrichment of stromal cells modulate some paracrine or autocrine signaling pathways, which can lead to proliferation (Koundouros & Poulogiannis, 2020), hence the increased glycolysis and oxidative phosphorylation enzymes' genes. Moreover, LDL accumulation in tumoral microenvironment can affect immune system cells. It has been demonstrated that immune-suppressive regulatory T cells are more dependent on the lipid metabolism than effector T lymphocytes, which rely on glycolysis. Furthermore, while M1 macrophages (pro inflammatory and anti-tumoral) rely on aerobic glycolysis, M2 macrophages are more engaged to lipid uptake and fatty acid oxidation (Schmidt, Patel, Kirsch, Lewis, Heiden, Locasale, et al., 2021). We suppose that the accumulation of LDL in microenvironment drives the immune evasion and tumoral progression. However, one should not forget that NSG mice are highly immunodeficient. As a result, in this particular model, the immune surveillance may not significantly affect MDA-MB-231 cells.

Besides the metabolomics of primary breast cancer in normal diet and high cholesterol diet fed mice, we wanted to understand whether these different diets had an impact in development of metastatic disease in lungs.

Metastasis formation is the leading cause of death and morbidity of cancer patients. The pathophysiology of each step of the metastatic cascade is not yet revealed, but metabolic reprogramming seems to allow metastasis outgrowth. As a result, in vivo models of metastasis are essential to understand metastasis origin and development, in order to prevent and treat them (Altea-Manzano et al., 2020). In fact, the in vivo model using human breast cancer xenografts in NOD *scid* gamma mice (NSG) is useful to study the metastatic process, as human breast cancer cells reliably metastasize to distant organs from primary tumors within the mammary fat pads (Iorns et al., 2012; Puchalapalli et al., 2016). It has been established that these model develops macro-metastases frequently and consistently, particularly in axillary lymph nodes and lungs

(Iorns et al., 2012). We used this model to compare gene expression of enzyme genes involved in key metabolic pathways of MDA-MB-231 cells.

We considered lung samples metastatic positive in cases in which human RNAse P primer amplified in quantitative PCR. In fact, all of lungs of high cholesterol diet fed mice had microscopic metastatic disease, while 2 of 3 of lungs of normal diet fed mice had metastatic disease.

Lung metastasis of high cholesterol diet fed mice significantly upregulate the oxidative phosphorylation, glycolysis enzymes' genes, as well as LDL receptor gene compared to normal diet fed mice (**Figure 13**). In fact, some studies using ^{13}C-glucose had revealed that lung cancer samples display enrichment of both glycolysis and oxidative phosphorylation intermediates, which can indicate that even normal lung cells are reliant upon these 2 pathways (Hensley et al., 2016). Thus, it is possible that metastatic cells established in lung tissue have to adapt their metabolism to new microenvironment, becoming more dependent upon both glycolysis and oxidative phosphorylation. When these cells are additionally exposed to high cholesterol diet, the proliferation induced by lipids will increase even more the activity of both glycolysis and oxidative phosphorylation, as we observed (**Figure 13**).

After observing the metabolic rewire associated with exposure to different diets, we wanted to understand what changed in metabolomics of lung metastasis in comparison to primary tumors. For this purpose, we compared the metabolomic profile of lung metastasis and primary tumors of high cholesterol fed mice (**Figure 14**). Lung metastasis of high cholesterol diet fed mice importantly upregulate CD36 comparing to primary tumors of high cholesterol fed mice, which resembles the metabolic reprogramming of in vitro MDA-MB-231 cells exposed to low glucose and LDL (**Figure 10.3**). This resemblance seems to indicate that breast tissue performs like a low glucose without LDL environment while lung tissue is a low glucose with LDL environment. Considering that our mouse model was non-obese diabetic and hence hyperglycemic, we suppose that both breast and lung tissues are hypovascular. This is in line with our previous assumption. In addition, in other mouse models of gastric cancer and squamous cell carcinoma in high fat diet fed mice, CD36 had been implicated in lung metastasis development (Schmidt, Patel, Kirsch, Lewis, Heiden, Locasale, et al., 2021).

Assuming that CD36 would promote lung metastasis of MDA-MB-231 breast cancer, we then compared the metabolomics of lung metastasis and primary breast cancer of normal diet fed mice. We wanted to establish whether the upregulation of CD36 was due to high cholesterol exposure or due to the metastasis development itself. We observed that, in one sample, lung metastasis of normal diet fed mice, CD36 was upregulated, comparing to primary tumors of normal diet fed mice. However, in the other sample, CD36 was not overexpressed compared to controls, producing a hide standard deviation of fold difference of CD36 expression (**Figure 15**). This seems to indicate that in high cholesterol exposed cells, CD36 is essential to lung metastasis formation. However, in normal diet, CD36 overexpression was not observed in all samples, which means that this lipid transporter may facilitate lung metastasizing but is not compulsory to the establishment of secondary lung lesions.

CD36 is a fatty acid transporter that allows cells to increase their lipid content. Consequently, CD36 upregulation allows cells to increase their lipid droplets, which not only is beneficial as energy storage, but also mediates other functions such as ROS production and signaling (Schmidt, Patel, Kirsch, Lewis, Heiden, Locasale, et al., 2021).

Interestingly, the metabolic profiles of primary breast cancer lung metastasis are similar, excepting the upregulation of CD36 in lung metastasis. In spite of colonizing a new organ with specific environmental features, these cells manage to maintain their original metabolic phenotype, apart from increasing lipid uptake. Considering the metabolic dependence of MDA-MB-231 cells upon glutamine, we can speculate that lung tissue is in fact enriched in this amino-acid.

Nevertheless, our work has some limitations. In fact, we assumed that an enzyme's gene upregulation or downregulation reflected activity of the associated metabolic pathway. However, there are some post-transduction changes in proteins that can affect their activity without modifying gene expression (Jia et al., 2019; van Gorsel et al., 2019). As a result, metabolomic studies not only rely on gene expression but also on proteomic, metabolite quantification and enzymatic activity assays. Further work is hence needed to confirm our findings. Moreover, we only studied oxidative phosphorylation, glycolysis, fatty acid synthesis, lipid uptake and glutaminolysis, but there is evidence that another metabolic pathways are also important to TNBC, namely cholesterol synthesis, other amino acids synthesis and uptake, namely cystine and

serine, tryptophane, arginine (Sun et al., 2020; Z. Wang et al., 2020). In the future, these metabolic pathways should also be studied to understand their contribution to the metabolic phenotype. In our in vivo assay, we used a NSG mice model, lacking T and B lymphocytes as well as NK cells (Iorns et al., 2012). For this reason, it was not possible to assess eventual immune interactions established between cancer and immune cells in the microenvironment which would contribute to modulation of metabolomics of both primary breast cancer and lung metastasis.

7. CONCLUSION

Conclusion

MDA-MB-231 cells *in vitro* display metabolic plasticity and adaptability, having the capacity to use different substrates depending on the availability of nutrients in microenvironment. However, this metabolic reprogramming is not enough to decrease the dependence of these cells from glucose and glutamine. *In vivo,* high fat diet induces proliferation of primary breast cancer cells after blockade of LDL receptor. Also, this diet induces proliferation of lung metastasis cells. Lung metastases upregulate the expression of lipid transporters compared to primary tumors, both in normal diet and high cholesterol diet fed mice, which can disclosure the roles of lipid accumulation in tumor cells.

These observations suggest low cholesterol diets may be adequate to prevent lung metastatic disease. Moreover, diagnosis of lung metastatic lesions based on new isotope labeled fatty acids would enable better accuracy. Once metastatic lung disease ensues, lipid lowering drugs, coupled with a specific low cholesterol diet, would be an adjuvant to current therapeutic approaches.

Understanding cancer metabolism and specific features of metabolism in the different steps of tumor development opens a new opportunity in management of oncologic patient, namely in prevention, diagnosis, treatment and surveillance.

8. REFERENCES

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