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Characterizing the effect of arginine deprivation on breast cancer cell adhesion and cytoskeletal remodeling

By

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Characterizing the effect of arginine deprivation on breast cancer cell adhesion and cytoskeletal remodeling

Chantal Issam Fayad

ABSTRACT

Arginine is a non-essential amino acid that becomes required in several diseases, including cancer, whereby its production via endogenous biosynthesis becomes insufficient. Targeted arginine deprivation can thus be an attractive therapeutic strategy. Little is known about the cellular and molecular effects of targeted arginine deprivation in breast cancer. We set out of test how human recombinant arginase HuArgI(Co)-PEG5000, an emerging arginine deprivation therapeutic, impacts adhesion, autophagy, EMT and cytoskeletal organization in three cell line models for luminal (MCF-7) and triple-negative (UACC-2087 and MDA-MB-231) breast cancers. Immunofluorescence staining for the canonical autophagy marker LC3 revealed no nuclear-to-cytoplasmic shift in LC3 in any HuArgI(Co)-PEG5000-treated cell line, suggesting the absence of canonical autophagy induction during arginine deprivation. HuArgI(Co)-PEG5000-treatment induced a decrease in E-cadherin expression without inducing a clear EMT, and inhibited cell-cell adhesion in all tested cell lines. Furthermore, drug-mediated arginine deprivation inhibited focal adhesion and stress fiber formation in UACC-2087 cells. Altogether, our *in vitro* findings suggest that arginine deprivation could regulate ECM adhesion, cell-cell junctions, and cell motility in breast cancer.

Keywords: Arginine, Arginase, LC3, Focal adhesions, EMT, Adhesion, Breast cancer.

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LIST OF ABBREVIATIONS

ADI: Arginine deiminase

ADI-PEG: Pegylated arginine deiminase

AML: Acute myeloid leukemia

Arg1: Arginase 1

ASL: Arginosuccinate lyase

ASS: Arginosuccinate synthetase

CAF: Cancer associated fibroblasts

CAT: Cationic amino acid trasnporter

CPS: Carbamoyl phosphate synthetase

DMEM: Dulbecoo's modified eagle media

ECM: Extracellular matrix

EMT: Epthilial to mesenchymal transition

FA: Focal adhesions

FAK: Focal adhesion kinases

FBS: Fetal bovine serum

HCC: hepatocellular carcinoma

Her2: Human epidermal receptor 2

HuArg1: human pegylated arginase 1

HuArgI(Co)-PEG5000: Human recombinant arginase I cobalt PEG 5000

LAT: L-type amino acid transporter

OTC: Ornithine transcarbamoylase

PBS: Phosphate-buffered saline

Peg-rhArg1: Pegylated recombinant human arginase 1

SLC7: Solute carrier 7

TNBC: Triple negative breast cancer

Chapter 1

Literature review

1.1 Breast cancer

1.1.1 Definition

Breast cancer is a malignant tumor that has developed from the uncontrolled growth of the epithelial cells of glandular milk ducts or lobules of the breast and it is the most common cancer in women and the second most common cancer in the world (Ferlay et al., 2013; Benson et al. 2009).

Breast tumors typically begin as ductal hyper proliferation and progress to benign tumors or even metastatic carcinomas as a result of continuous stimulation by various carcinogenic factors. Breast cancer initiation and development are influenced by tumor microenvironments such as stromal factors and macrophages. The sporadic cases are around 70 to 80%, the familial cases between 15 and 20% and the hereditary around 5 to 10% (Sun et al., 2017).

1.1.2 Classification

Breast cancers are heterogeneous, with a wide range of morphologic and biological characteristics, as well as clinical behavior and treatment response (Tsang & Tse 2020). They can be classified based on histological and molecular characteristics.

Histological classification of breast cancers relies on the pathologic growth pattern. It will be generally classified into in situ carcinoma and invasive (infiltrating) carcinoma. Breast carcinoma in situ is also sub-classified as either ductal or lobular. Invasive carcinomas are also a diverse category of tumors divided into histological subtypes.

While this classification scheme has been useful for decades, it is based solely on histology and does not incorporate newer molecular markers with proven significant clinical outcome and thus limit the ability to develop targeted therapeutics against specific

types of breast cancer (Li et al., 2005). Research identified many intrinsic breast cancer molecular subtypes, which were later confirmed and categorized as luminal subtype A, luminal subtype B, Her2+, triple negative, normal breast-like, based on the expression of three receptors: estrogen, progesterone and human epidermal receptors (Perou et al., 2000; Sørlie et al., 2001; Sorlie et al., 2003).

a. Luminal A

It is the most prevalent form of breast cancer; it accounts for 50–60% of all cases. This subtype is characterized by the expression of ER, PGR as well as absence of HER2 expression and by a low proliferation rate discovered by measuring the expression of ki67. It also has a low histological grade, a good prognosis and a low relapse time compared to other subtypes (Eroles et al., 2012).

b. Luminal B

Tumors having these molecular profile constitute about 10 to 20% of all breast cancer subtypes. It differs from the luminal A subtype by being more aggressive, having a worse prognosis, higher histological grade, and index of proliferation. A main difference between A and B is the expression of HER2 as well as the presence of proliferation genes in the luminal B (Eroles et al., 2012).

c. $Her2^{+}$

These tumors accounts to 15 to 20% of all breast cancer subtypes. This subtype is characterized by the high expression of Her2 and genes involved in cell proliferation and by being highly proliferative. It is linked with a poor prognosis despite the survival improvement that was seen upon treatment (Eroles et al., 2012).

d. Triple negative (basal-like)

10 to 20% of breast cancer subtypes belongs to this group. They do not express any receptor and they are highly aggressive. They have worse prognosis that luminal subtypes and a higher relapse rate although they are chemosensitive (Eroles et al., 2012).

e. Normal-like

They constitute about 5 to 10% of all the breast cancer subtypes. They also do not express any of the receptors, but express genes that are seen in adipose tissues, thus they have intermediate prognosis between luminal and triple negative breast cancer subtypes (Eroles et al., 2012).

1.2 Epithelial to mesenchymal transition

1.2.1 Definition

Epithelial to mesenchymal transition (EMT) is a biological process that allows a polarized epithelial cell, which normally interacts with the basement membrane via its basal surface, to undergo a series of biochemical changes that allow it to adopt a mesenchymal cell phenotype, which includes increased migratory capacity, invasiveness, resistance to apoptosis, and significantly increased production of ECM components (Kalluri & Weinberg, 2009).

1.2.2 EMT and metastasis

Metastasis is the process by which cells from the primary tumor break off and enter the bloodstream and/or lymph vessels, eventually settling at a secondary site. EMT is crucial for this phenomenon, as it is regarded as a promoter of metastasis due to the transformations that allow cells to acquire mobility, a mesenchymal feature. The first step in metastasis is detachment, in which the cells of the primary tumor lose contact with each other and become mobile as a result of EMT-induced mesenchymal acquisition. Following that, the cells can invade neighboring tissues and the basal membrane (invasion phase), eventually reaching the blood and lymphatic vessels, defining the stage of intravasion where no more epithelial characteristics are observed (Ramos et al., 2017).

1.2.3 EMT in cancers

EMT is characterized by the loss of epithelial markers such as E-cadherin, cytokeratins and gain of mesenchymal markers such as vimentin, N-cadherin and fibroblast specific protein (FSP1). In fact, several types of cancers including breast, prostate, melanoma and lung cancers had an increase in vimentin's expression and this indicates an increase in tumor growth and invasiveness and decrease in its prognosis. For instance, in MCF7, a breast cancer subtype, overexpressing vimentin lead to an increase in cell stiffness, cell motility and directional migration. It also contributed to the reorientation of microtubule polarity, and increased EMT phenotypes (Ribatti et al., 2020).

1.3 Amino acid metabolism

1.3.1 Synthesis

Amino acids constitute the basic units of proteins and they are made of carboxyl, amino groups and a side chain that determines the uniqueness of each amino acid. As intermediate metabolites, amino acids are essential to several pathological and physiological pathways and to cellular processes such as gene expression, cell signaling, and development (Hoffer, 2016).

Amino acids are classified into 3 groups: essential amino acids which can only be produced by bacteria and plants and have to be consumed by animals, non-essential amino acids that can be synthesized by any organism, and semi-essential amino acids that can be fabricated by metabolic pathways in the body, but probably not sufficiently and therefore may have to be supplied at least in part by the diet.

Amino acid metabolism includes catabolism and anabolism. Amino acids catabolism is composed of three main steps in order to generate energy: the removal of amino group by deamination, urea cycle to convert NH3 into urea, and metabolic breakdown of the carbon cytoskeleton. As for the amino acid anabolism, the biosynthetic pathways are organized by assembling the amino acids into different families depending on the metabolic precursor of each amino acid (Figure 1). Glutamine and glutamate, via transamination, confer amino groups in amino acid and carboxyl and hydroxyl groups are derived from monosaccharides such as glucose. Six metabolic precursors are involved in

this biosynthetic pathway and they derive from glycolysis, Krebs cycle (citrate acid cycle) and pentose phosphate pathway. Intermediate metabolites from glycolysis and pyruvate which is the end result of glycolysis enter the pentose phosphate pathway and the Krebs cycle respectively in order to induce the synthesis of amino acids (Figure 1). Serine, glycine, and cysteine arise from 3-phosphoglycerate, an intermediate in glycolysis and pyruvate gives alanine, valine, leucine and isoleucine. Pyruvate then enters the citrate acid cycle and α-ketoglutarate yields proline, glutamate, glutamine and arginine. Oxaloacetate synthesize aspartate and asparagine is then produced via the amidation of aspartate. Methionine, threonine, and lysine derive also from oxaloacetate. In the pentose phosphate pathway, ribose-5-phosphate is an intermediate in histidine synthesis and erythrose-4-phosphate along with phosphoenolpyruvate generate tryptophan, phenylalanine and tyrosine (Berg et al, 2002) (Figure 1).

Amino acid metabolism is altered in cancer and between a cancer cell and its surrounding environment, there is a complicated interplay of metabolic precursors. The main metabolic change that occurs in cancer is that, when oxygen is available, normal cells do not convert glucose to lactate, they only use anaerobic glycolysis, or the conversion of glucose to lactic acid, when oxygen is scarce or unavailable. However, cancer cells convert glucose to lactate even when oxygen is present (Kalyanaraman, 2017). Amino acid metabolism is becoming an increasingly attractive area in cancer research due to its broad impact on the cellular state. For instance, redox balance, energy management, biosynthetic support, and homeostatic maintenance are all key functions of amino acids in cancer (Lieu et al., 2020). The wide range of effects of amino acid metabolism in cancer also includes establishing the synthesis of non-essential amino acids for protein biosynthesis, conversion to glucose, lipids, and neurotransmitters such as glycine and serine (Wei et al., 2020). Additionally, amino acids are implicated in epigenetic modification, bioenergy supply, conversion of ammonia to urea for detoxification; and maintain intracellular redox state by, for example, synthesis of the main cellular non-enzymatic antioxidant glutathione, from glutamate, cysteine, and glycine (Wei et al., 2020). Finally, amino acids can also serve as opportunistic fuel sources for cells (Wei et al., 2020). For these reasons, aberrant amino acid metabolism plays a

variety of roles in cancer, and the effect of metabolic modulation in the tumor microenvironment is becoming highly significant (Wei et al., 2020).

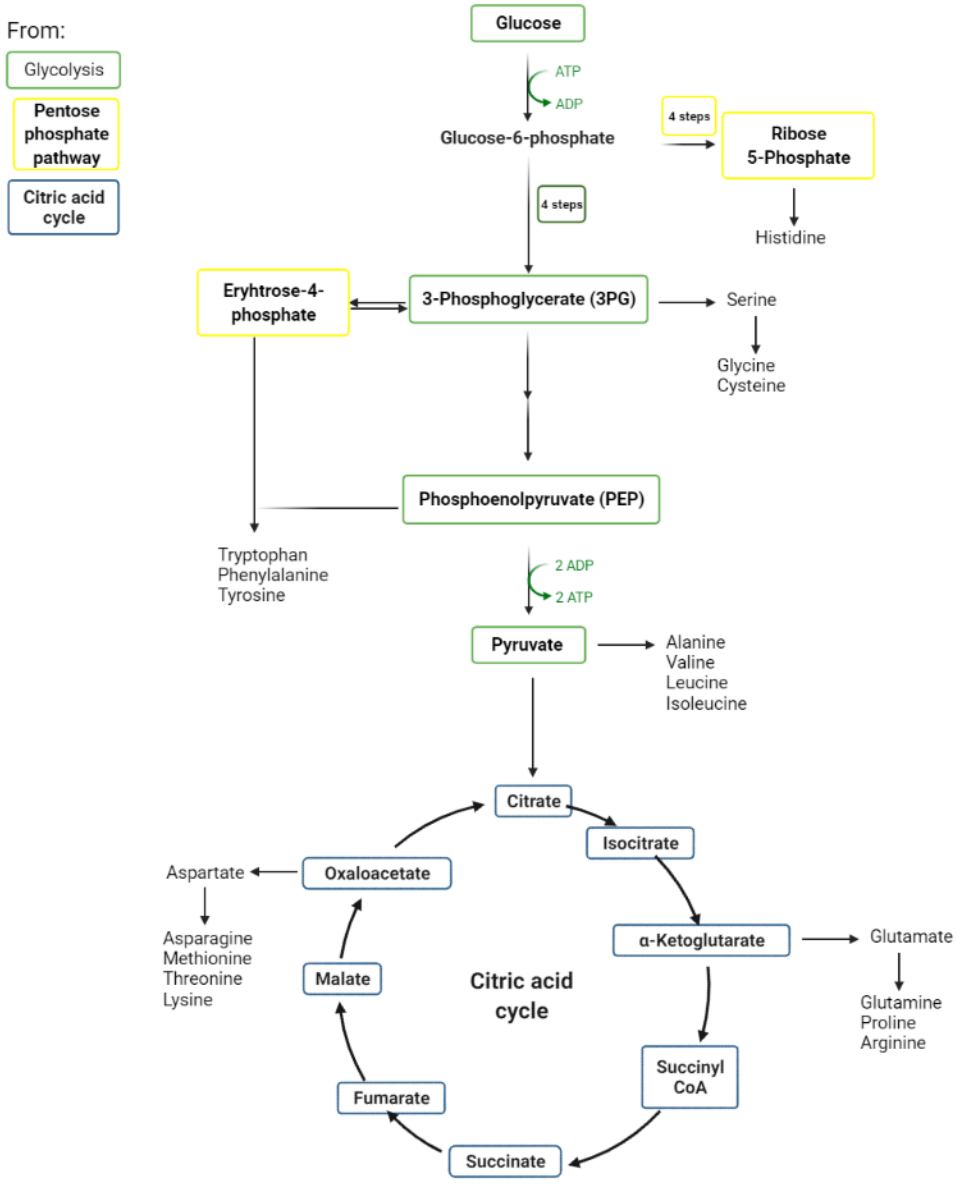

Figure 1. Amino acid synthesis. Six metabolic precursors are involved in this biosynthetic pathway and they derive from glycolysis (green), citrate acid cycle(blue) and pentose phosphate pathway(yellow). Intermediate metabolites from glycolysis and pyruvate which is the end result of glycolysis enter the pentose phosphate pathway and the citric acid cycle respectively in order to synthesize the amino acids.

1.3.2 Urea cycle

Amino acid degradation yields ammonia which is counted as waste. The urea cycle is a series of biochemical reactions that convert ammonia into urea, a nontoxic product which is then excreted. The urea cycle is also part of the anabolism of many amino acids (Figure 1). The carbamyl phosphate synthetase-I (CPS-I), ornithine transcarbamylase (OTC), argininosuccinate synthetase (ASS), argininosuccinate lyase (ASL), and arginase enzymes make up the urea cycle (Morris, 2002). The first two enzymes (OTC and CPS) are mitochondrial and the rest are cytosolic. One amino group from ammonia and a second one from aspartate and a carbon atom from bicarbonate (HCO_3^-) are converted to urea, and this happens in five main steps (Mew et al., 2015). Ammonia is transformed to carbamoyl phosphate before the urea cycle starts through CPS-I. The carbamoyl phosphate enters the urea cycle and is then transformed to citrulline by ornithine transcarbamylase. Next, the combination of amino group and carbonyl group of aspartate and citrulline respectively, forms argininosuccinate. The enzyme argininosuccinate synthetase catalyzes this ATP-dependent reaction. Argininosuccinate is cleaved by the enzyme ASL into arginine and fumarate. The arginase now breaks down arginine to formurea and ornithine. The latter will be carried back to the mitochondria to restart a new cycle (Haines et al., 2011; Cohen & Kuda, 1996; Meijer et al., 1990) (Figure 2).

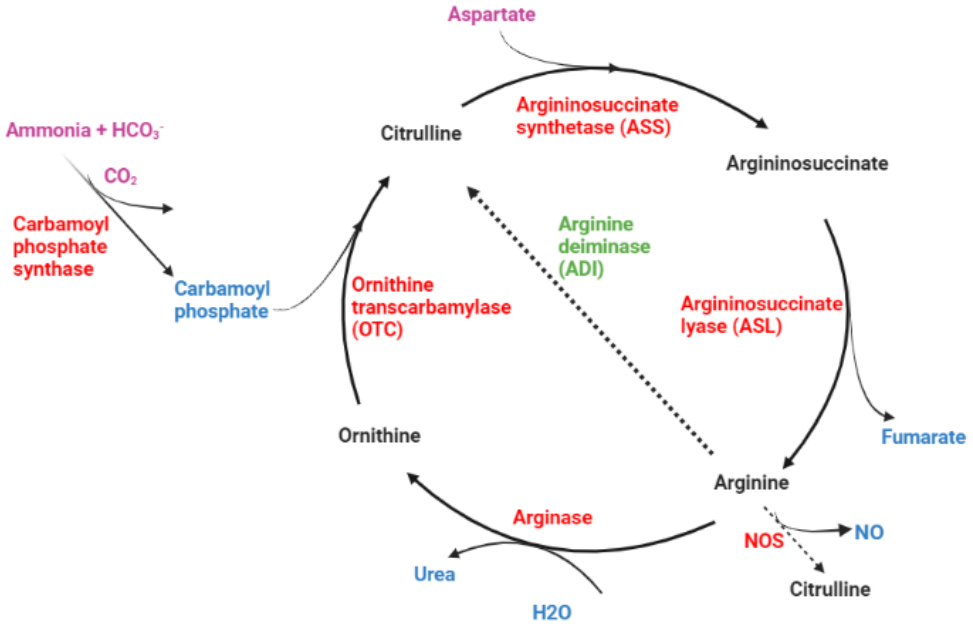

Figure 2. Urea cycle. Regular lines refer to the process that occurs in mammalians. Carbamoyl phosphate is obtained by the conversion of ammonia through carbamoyl phosphate synthase and it enters the cycle. Citrulline is converted to argininosuccinate via

ASS, the ASL converts argininosuccinate to arginine which will be converted to ornithine via arginase. Citrulline is then recycled by the conversion of ornithine through OTC. Dotted lines refer to the process that do not occur normally in mammalian where ADI converts arginine to citrulline. Red color refers to the enzymes implicated in mammalians. Green color for enzyme not used by mammalians. Black color refers to the main products implicated in this cycle.

1.3.3. The citric acid cycle

The citric acid cycle (CAC) (tricarboxylic acid cycle; TCA) is a sequence of chemical events that release stored energy by oxidizing acetyl-CoA generated from carbs, lipids, and proteins (Akram 2014). Organisms that respire use the TCA cycle to create energy, whether through anaerobic or aerobic respiration (Akram, 2014). Glycolysis produces pyruvate molecules, which are carried over the internal mitochondrial membrane and into the matrix. As in the regular cycle, they can be oxidized and coupled with coenzyme A to produce CO2, acetyl-CoA, and NADH via the enzyme pyruvate dehydrogenase. Krebs cycle is initiated with acetyl-CoA transferring a two-carbon acetyl group to the oxaloacetate to form citrate via the enzyme citrate synthase. The citrate then is transformed isocitrate via the enzyme aconitase. Next, isocitrate dehydrogenase will generate α-ketoglutarate that will be converted to Succinyl-CoA via the enzyme α-ketoglutarate dehydrogenase. Then succinyl-CoA is transformed into succinate by the enzyme succinyl-CoA synthetase and a molecule of ATP is produced. Succinate is converted to fumarate through Succinate dehydrogenase and fumarate yields malate through fumarase. Malate dehydrogenase converts malate to oxaloacetate and citrate will be regenerated by the conversion of oxaloacetate to citrate via citrate synthase and the cycle continues. Other than the ATP produced, most of the energy produced is captured by 3 molecules of NAD+ and one molecule of FAD that will be reduced to that will be reduced to NADH and $FADH_2$ respectively (Akram, 2014).

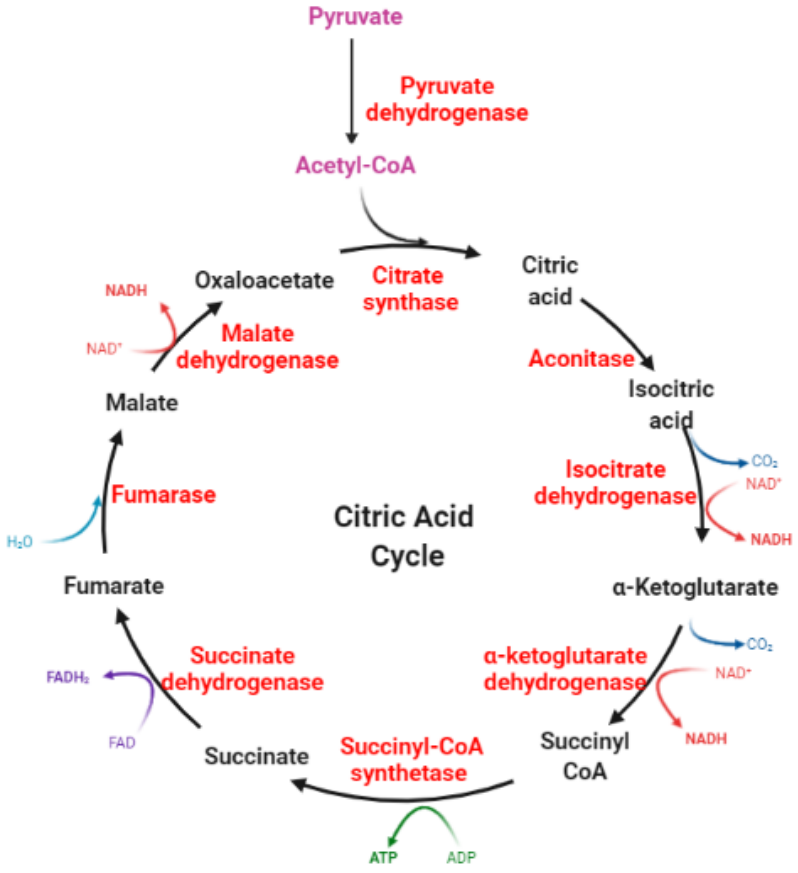

Figure 3. Citric acid cycle. A two-carbon acetyl group from Acetyl-CoA is transferred to the oxaloacetate to produce citrate via the enzyme citrate synthase. Citrate then is transformed isocitrate through the enzyme aconitase. Next, isocitrate dehydrogenase will generate α-ketoglutarate that will be trasnformed to succinyl-CoA via the enzyme α-ketoglutarate dehydrogenase. Then succinyl-CoA is transformed into succinate by the enzyme succinyl-CoA synthetase and a molecule of ATP is produced. Succinate is converted to fumarate through succinate dehydrogenase and fumarate yields malate through fumarase. Malate dehydrogenase converts malate to oxaloacetate and citrate will be regenerated by the conversion of oxaloacetate to citrate via citrate synthase and the cycle continues. Red color refers to the enzymes involved. Black color refers to the main products implicated in this cycle.

1.3.4 Auxotrophy

The inability of an organism to generate a certain organic component essential for its growth is known as auxotrophy. Auxotrophy is the opposite of prototrophy, which is defined by the ability to generate all of the necessary chemicals for growth (Morris et al., 2017). Organisms could be either fully auxotrophic or partially auxotrophic for a specific

component. For instance, a complete auxotrophic organism could never synthesize a specific nutrient, however a partially auxotrophic organism will be able to synthesize a specific nutrient if provided with a precursor, like in the case of citrulline rescuing arginine auxotrophy in some colorectal cancer cell lines that are considered to be partially auxotrophic (Al-Koussa et al., 2019).

1.4 Particularities of arginine metabolism

Arginine is a non-essential amino acid that becomes conditionally essential (semi-essential) when its production via endogenous biosynthesis becomes insufficient. This could happen in cases such as hemolytic anemias, asthma, pregnancy, and serious illness such as sepsis, burns, and trauma (Morris et al., 2017). Intracellular arginine can be obtained by *de novo* synthesis, uptake of extracellular arginine from diet, and protein turnover and degradation (Morris, Jr., 2016).

1.4.1 *De novo* arginine synthesis

De novo synthesis occurs through the urea cycle using citrulline as a primary substrate (Wu & Morris, 1998). The systemic supply of arginine through *de novo* synthesis comes from the small intestine and kidneys, collectively called the intestinal-renal axis of arginine synthesis. CPS-I and OTC are expressed in the enterocytes of the small intestine of adults, allowing for the production of citrulline from glutamine, proline, or ornithine. The latter is formed from arginine by the enzyme arginase. Citrulline is mainly synthesized in the small intestine and is gathered up by the kidney's proximal tubules, where it is efficiently transformed to arginine by ASS and ASL. The amount and activity of ASS and ASL enzymes determine the ability to recycle arginine from citrulline (Marini et al., 2014).

1.4.2 Cellular uptake of arginine

The organization and capability of arginine transporters localized to the plasma membrane depends on cellular arginine availability, and the transport is mainly conducted

by members of the solute carrier 7 (SLC7) family in mammals. SLC7 belongs to the amino acid polyamine cation (APC) superfamily, which is present in all species and is responsible for the transport of a wide variety of cell components towards intra and extracellular compartments of the cell, in same or opposite directions (Vastermark et al., 2014; Jack et al., 2000). SLC7 is divided into two groups: The cationic amino acid transporters (CAT) that function as monomers and recognize specifically and only cationic amino acids as their substrates, and the L-type amino acid transporters (LAT) that are obligate heterodimers. LAT's, in contrast to CAT's, catalyze the export of arginine in exchange for sodium and a neutral amino acid (NAA) (Closs et al., 2006; Verrey et al., 2004; Fotiadis et al., 2013) (Figure 3).

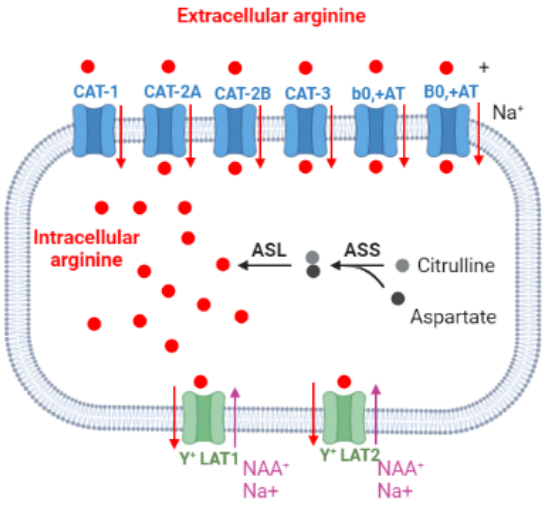

Figure 4. Arginine transport. Arginine may be uptaken through several transporters (colored blue). The two y+LAT isoforms (colored green) catalyze the export of arginine in exchange for sodium and a neutral amino acid (NAA).

1.5 Arginine metabolism enzymes in cancer

In response to their altered energy demands, cancer cells need to adapt their metabolism. Aside from increasing glucose uptake, cancer cells can rely on other nutrients such as amino acids. As a result, targeting amino acid metabolism could be a promising therapeutic strategy (Lukey et al., 2017). To target arginine metabolism, we must first

understand the expression and function of its regulatory enzymes OTC, ASS, ASL, and arginase.

1.5.1 Ornithine transcarbamylase (OTC)

OTC is responsible for controlling the reaction in which two compounds, carbamoyl phosphate and ornithine, combine to form a new compound known as citrulline (Haines et al., 2011) (Figure 2). OTC is differentially expressed between normal and tumor cells. For instance, in comparison to primary human hepatocytes, hepatocellular carcinoma cells (HCC) and tissues had lower OTC expression. Further research revealed that low OTC expression in HCC was linked to larger tumor size and advanced degree (He et al., 2019). OTC is downregulated in colorectal cancer and in most xenograft models of squamous cell lung carcinoma (Alexandrou et al., 2018)

1.5.2 Arginosuccinate synthetase (ASS)

ASS produces argininosuccinate, a precursor to arginine, by catalyzing the condensation of citrulline and aspartate (Haines et al., 2011) (Figure 2).
ASS1, which was first cloned from a carcinoma cell line in 1981 (Qiu et al., 2014), is expressed differently in a wide variety of tumor tissues when compared to corresponding normal epithelia, suggesting that ASS1 deregulation can play various roles in malignant disease. For instance, when compared to normal ovarian, gastric, and colonic epithelia, their respectively derived cancers have elevated levels of ASS1 mRNA and protein (Qiu et al., 2014). High ASS1 expression has been linked to poor disease-free survival in patients suffering from head and neck cancer (Huang et al., 2012). Conversely, Melanoma, HCC, mesothelioma, renal cell carcinoma, prostate cancer, and invasive breast carcinoma all display loss of ASS1 expression which associates with chemoresistance and a poor clinical outcome (Qiu et al., 2014). ASS1 expression lacks also in a variety of other tumors, including non-lymphoma, pancreatic cancer and multiple myeloma (Dillon et al., 2004). Tumors that lack ASS1 are chemoresistant, but they have a vital reliance on extracellular arginine for development, which is known as arginine auxotrophy. The causes of ASS1 deficiency are poorly understood. However, since it is a rate-limiting

enzyme that supplies arginine to several metabolic pathways, it is possible that its loss confers a biological advantage to these tumors. For example, 63.8% of breast tumors have low or loss of ASS1 (Qiu et al., 2014). A potential mechanism for ASS1 loss could be hypermethylation of the ASS1 promoter, which has been observed in malignant pleural mesothelioma, lymphoma, myxofibrosarcomas, and glioblastoma, and was found to be correlate with a poor patient survival in ovarian cancer patients. Interestingly, experimental demethylation reactivates ASS1 expression and leads to treatment failure (Kobayashi et al., 2010; Su et al., 1981; Szlosarek et al., 2006; Ensor et al., 2002; Szlosarek et al., 2007; Dillon et al., 2004; Kim et al., 2009; Yoon et al., 2007; Qiu et al., 2014).

Functionally, loss of ASS1 may promote specific cellular programs related to tumor growth, such as metastasis and drug resistance (Krummrei et al., 2003). In fact, a decreased ASS1 expression was connected to the development of lung metastasis and a poor prognosis in osteocarcinoma patients, whereas over-expression of the protein inhibited tumor growth *in vitro*. ASS1 shortage was also linked to a poor prognosis and *promoted proliferation and invasion in breast cancer (Qiu et al., 2014). In vitro, re-*expression of ASS1 hindered cancer angiogenesis, maturation, and migration, suggesting that ASS1 may be a new tumor suppressor (Huang et al., 2013).

1.5.3 Argininosuccinate lyase (ASL)

ASL catalyzes the cleavage of argininosuccinate, resulting in arginine and fumarate (Haines et al., 2011) (Figure 2). ASL is typically highly expressed in cancers. For example, in certain human liver cancer cell lines, ASL knockdown abrogated cell growth *in vitro*. *In vivo*, after implanting subcutaneously liver cancer cells whose ASL expression was knocked down, tumor growth was inhibited too. ASL expression was frequently upregulated in hepatocellular carcinoma (HCC) tissues and cell lines (Huang et al., 2013). In HCC cells, knocking down ASL inhibited cell proliferation and induced apoptosis (Gong et al., 2019). Furthermore, ASL was found to be caused by ER stress and to be substantially elevated in breast cancer tissues compared to normal tissues. Breast

cancer development and colorectal cancer formation was prevented *in vitro* and *in vivo* when ASL was downregulated (Huang et al., 2015; Huang HL et al., 2017).

1.5.4 Arginase

Arginase catalyzes the urea cycle's fifth and final step, which consists of converting L-arginine to L-ornithine and urea (Haines et al., 2011) (Figure 2). Two isoforms of human arginase exits (arginase I and arginase II), and their amino acid sequences have 60% commonality. Arginase I, found primarily in the liver, is the potent and remarkable subtype (Savoca et al., 1984). Patients with breast, lung, and colon cancer have elevated arginase activity, which may be responsible for maintaining required levels of polyamines for tumor growth and development (Chang et al., 2001; Singh et al., 2000; Elgün et al., 1999). In the cytoplasm of cancer cells, arginase was much more abundant when compared to normal cells. Consequently, serum arginase levels in colorectal adenocarcinoma patients are significantly higher than in control subjects (Leu & Wang, 1992). Arginase activity is elevated in prostate and ovarian cancer patients as well and it was linked to poor prognosis (Mumenthaler et al., 2008; Czystowska-Kuzmicz et al., 2019). In HCC, arginase was downregulated in small tumors and begins to be expressed when the tumor grows indicating that the higher the expression of arginase, the larger the tumor, the poorer the prognosis. Overexpression of arginase promoted cancer cell invasion and migration, enhanced their viability and caused an epithelial to mesenchymal transition and thus could be considered as an oncogene in the development of HCC (You et al., 2018).

1.6 Arginine regulation of cancer hallmarks

Arginine is involved in a number of key cellular metabolic pathways including the urea cycle (where it acts as an intermediate metabolite), the biosynthesis of polyamines, creatine, and nitric oxide, as well as the biosynthesis of nucleotides, proline, and glutamate and immunity. Arginine is an important nutrient needed by cancer cells to survive,

proliferate and migrate and it is thus involved in the regulation several cancer hallmarks (Morris, 2006).

1.6.1 Arginine promotes proliferation

ASS produces argininosuccinate, the precursor of arginine which is required for tumor development due to its involvement in the synthesis of polyamines that are known to be essential for tumor growth, metastasis and invasion (Patil et al., 2016) In fact, arginine deprivation by arginase reduces the proliferation of both HeLa cells and 3T3 cells *in vitro*, and rat Novikoff hepatoma *in vivo*, resulting in relative arginine deficiency (Umeda et al., 1968; Tanaka et al., 1988). Furthermore, arginine deficiency caused the cell lines to die much faster than when no other essential amino acids were present. In addition, when normal and tumor cells were co-cultured but not given arginine, the normal cells survived while the tumor cells died (Wheatley et al., 2000). According to these findings, arginine deprivation causes malignant cells in culture to die selectively. Arginine deficiency impairs the regulation of DNA synthesis at the G1 checkpoint, which usually prevents it from starting (Lamb & Wheatley, 2000). For these reasons, arginine auxotrophy (the inability of the cell to synthesize its own arginine) represents a therapeutic window whereby arginine deprivation, i.e. the introduction of arginine depleting agents, could abrogate tumor progression.

1.6.2 Arginine deficiency induces autophagy

In order to overcome nutrient deficiency, including that of essential and semi-essential amino acids such as arginine, many normal and tumor cells activate autophagy (Condello et al., 2019). Autophagy is a conserved catabolic mechanism, needed to preserve cellular homeostasis under stress conditions (Cooper, 2018). Autophagy involves the formation of special double-membraned autophagosomes that envelop intracellular components. Lysosomes bind to autophagosomes to form autolysosomes, and lysosomal hydrolases destroy isolated intra-autophagosomal components (Tanida et al., 2008) (Figure 4).

In addition to its role in autophagy, microtubule-associated proteins 1A/1B light chain 3 (LC3) regulates microtubule assembly and disassembly (Tanida et al., 2005). In cells, two types of LC3 molecules were discovered. LC3-I is present in the cytoplasm, and LC3-II is bound to the membrane and is converted from LC3-I to initiate autophagosome formation and extension (Kabeya et al., 2000). During the formation of autophagosomal membranes, pro-LC3 is cleaved by ATG4 into the cytosolic LC3 (LC3-I) which is then bound to phosphatidylethanolamine (PE) by two reactions catalyzed by the enzymes Atg7 and Atg3 for the formation of LC3-II. During the conjugation of autophagosomes and lysosomes, lysosomal proteases degrade LC3-II present inside the autophagosomes (Tanida et al., 2001; Tanida et al., 2002; Tanida et al., 2004; Kabeya et al., 2004; Sou et al., 2006) (Figure 4).

Various cancer treatments can activate autophagy, which can play tumor-protective function in certain cases, or cause tumor cell death in others (Nasreddine et al., 2020). For example, autophagy activation can protect resistant ovarian cancer cells treated, as well as induce cell death via autophagy in Burkitt's lymphoma cells treated and resistant to drugs (Zhang et al., 2010; Cloonan & Williams, 2011; Wang & Wu, 2014). As regulator of tumor suppression, human breast, prostate and ovarian cancer have a depletion in Beclin1, an autophagy related gene. Cervical squamous-cell carcinomas and hepatocellular carcinomas have a low expression of this gene too indicating that this autophagy-related gene works as a tumor suppressor. And also the depletion of autophagy regulators was linked to increase in oncogenesis (Qu et al., 2003). As a regulator of tumor promotion, in other cancer cells, autophagy helps tumor cells survive by increasing their stress tolerance and delivering nutrients to satisfy their metabolic demands, while inhibiting autophagy or knocking off autophagy genes can lead to tumor cell death (White, 2012).

In glioblastoma and AML, autophagy protects glioblastoma and acute myeloid leukemia cells exposed to arginine deprivation at early time points. Autophagy begins to mediate cell death in both glioblastoma and acute myeloid leukemia cells at longer arginine deficiency periods (Glick et al., 2010; Tanios et al., 2013). Arginine deprivation resulted in substantial and prolonged autophagy activation, which was detrimental to cell survival because its inhibition resulted in a major reduction in cytotoxicity. This suggests

that autophagy activation in response to arginine depletion, instead of being defensive, regulates cell cytotoxicity and autophagy-mediated death in ovarian cancer cells (Nasreddine et al., 2020). Autophagy can encourage cancer growth and dissemination under disrupted or uncontrolled conditions. For instance, ASS deficient melanoma cells undergo autophagy in response to arginine deprivation most likely as part of an adaptive mechanism to prevent cell death. These findings were confirmed in prostate cancer cell lines that do not express ASS. However, autophagy functions as a tumor suppressor during different phases of cancer growth by mediating the degradation and elimination of carcinogens and cancerous cells, allowing healthy cells to grow and develop (Delage et al., 2010; Kim, Coates, et al., 2009; Savaraj et al., 2010; Kim Bold, et al., 2009).

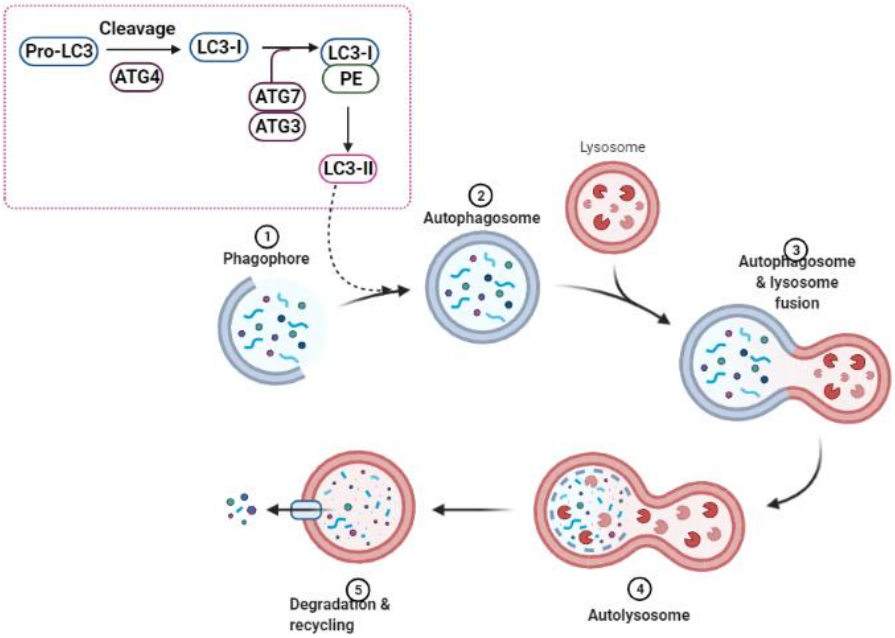

Figure 5. Autophagy. Atg4 degrades pro-LC3 to yield LC3-I which then binds to PE (via ATG7 and ATG3) for the production of LC3-II. LC3-II gets recruited to the autophagosomal membrane for helping membrane lengthening.

1.6.3 Arginine deficiency affects tissue architecture

In advanced tumors, loss of cell–cell adhesion and cell polarity via EMT and other mechanisms correlate with cell invasion into surrounding tissues, ultimately resulting in metastasis (Wodarz & Näthke, 2007). Cell junctions and cell-cell interaction are regulated

by polarity proteins (Rolli et al., 2010) and loss of their expression impairs morphogenesis (Rolli et al., 2010). Loss of apical–basal polarity is also a common early event in epithelial malignancies, and it can precede tumor invasion and dissemination (Rolli et al., 2010). The implication of arginine in disrupting tissue architecture was investigated in several studies. In pancreatic cancer cells depleted of arginine, an increase in E-cadherin's expression was observed. Thus, arginine depletion hinders the EMT-dependent invasion and aggregation (Wang et al., 2020). Arginine depletion inhibits also PI3K/Akt-GSK-3 pathway, which promotes EMT, by reducing cell adhesion and polarity, modifying cell shape with a decrease in cell-matrix adhesion, and inducing cell motility in pancreatic cancer cells (Lin et al., 2011).

1.6.4 Arginine deficiency disrupts ECM adhesion and migration

In solid tumors, the invasion of cancer cells into the surrounding tissue and blood vessels is the first step in tumor metastasis (Yamaguchi et al., 2005). Focal adhesions (FA) are massive protein complexes that bind a cell's cytoskeleton to the extracellular matrix (ECM) and help transmit regulating signals. Cell contractility is governed by stress fibers, which also affect cell shape, adhesion, and migration (Kreis & Birchmeier, 1980).

The majority of arginine-depleted cells grew elongated and did not produce a broad lamellipodium contrary to control cells. Significant morphological differences, fewer stress fibers and less intense cortical actin staining were seen in arginine deficient cells. Cells with depleted arginine showed way less aggregates and a higher number of cells compared with the control. Also, arginine deprivation affects organization of actin cytoskeleton and less actin filaments were seen in arginine deficient cells (Pavlyk et al., 2015). Arginine deprivation induces loss of cell structure, contractility as well as an increase in cell extension and flatness (El-Mais et al., 2021).

Arginine starvation can impair cell-ECM adhesion and migration. For instance, arginine deprivation drastically reduced cell migration of gastric and pancreatic cancer cells (Shan et al., 2015; Wang et al., 2020). In colorectal cancer cells, arginine starvation can reduce FAK activation, thereby slowing cell migration by preventing FA maturation and newly produced protrusions from adhering to the ECM (Rhoads et al., 2004).

Similarly, in ovarian cancer cells, arginine depletion abrogated cell spreading and invasion into the ECM (Al-Koussa et al., 2019; El-Mais et al., 2021). All of these events were happening in cell lines lacking the expression of ASS1 and this leads to the conclusion that ASS1 is implicated in cancer cell invasion and migration (Shan et al., 2015; Wang et al., 2020).

Autophagy-associated protein LC3B plays a role in cell-ECM adhesion and migration too. In fact, it is associated with podosome disintegration and osteoclast migration (Sharifi et al., 2016). In addition, LC3 can enhance focal adhesion disintegration in highly metastatic tumor cells thus driving cell spreading, migration, and invasion (Zhang et al., 2020).

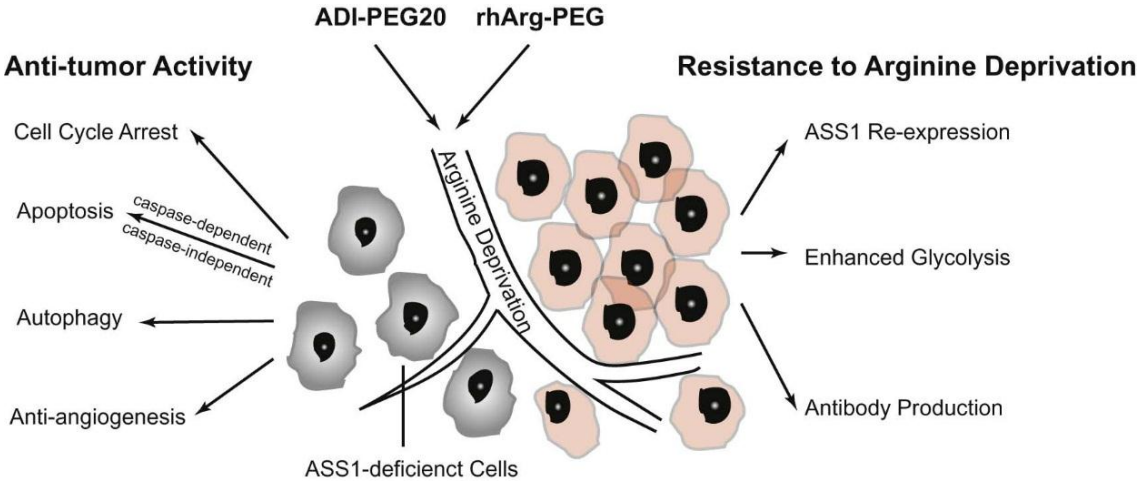

Figure 6. Anti-tumor activity and resistance to arginine deficiency. Promoting of cell cycle arrest, apoptosis, autophagy, and suppression of angiogenesis are all major mechanisms of arginine deprivation's anti-tumor efficacy. ASS1 re-expression, increased glycolysis, and synthesis of antibody, on the other hand, have been linked to arginine deprivation resistance (Qiu et al., 2015).

1.6.5 Arginine and stromal recruitment

On a cellular and metabolic level, cancers are exceedingly heterogeneous, containing populations of host and cancer cells exposed to various microenvironmental

conditions. The implication of the tumor microenvironment in modulating cancer behavior has been highlighted in different studies (Quail & Joyce, 2013).

Tumor microenvironment is composed of an extracellular matrix (ECM) formed of various cell types, fluids, gases, collagen, laminin, and other proteins, that constitutes a great support for cancer and stromal cells that interact closely and collaborate in the development of all cancer characteristics, including metastatic dissemination. A reciprocal tumor-stroma metabolic interaction results from that metabolic symbioses that induces the exchange of a variety of compounds capable of reprogramming anabolic and catabolic processes in recipient subpopulations (Pickup et al., 2014). Different cell types in the tumor stroma (e.g. endothelial cells, fibroblasts, adipocytes, and immune cells) are recruited and contribute to tumor growth and survival (Figure 6). Pancreatic cancer cells as well as ovarian cancer cells capture alanine and glutamine respectively, secreted by cancer associated fibroblasts (CAF) present around (Yang et al., 2016; Sousa et al., 2016). Specifically, aggressive breast carcinomas induce a metastatic phenotype by recruiting stromal fibroblasts and transforming them into CAF (Avgustinova et al., 2016). Breast cancer cells produce ATP that activates fibroblasts, which in turn stimulates cancer cell motility (Liu et al., 2018).

Adipose cells in the ovarian tumor microenvironment collaborate with ovarian cancer cells in a symbiotic metabolism pathway including arginine metabolism. Through inducible nitric oxide synthase, cancer cells use and degrade arginine to produce citrulline and nitric oxide. Nitric oxide stimulates glycolytic activity and proliferation while inhibiting oxidative phosphorylation. The citrulline produced by cancer cells is excreted into the extracellular environment, where it is absorbed by stromal adipocytes, transformed back to arginine, and expelled by cancer cells, perpetuating the symbiotic metabolic shuttle (Salimian Rizi et al., 2015).

1.7 Arginine deprivation as a cancer therapy

Arginine serves as a starting point for the production of a variety of metabolites that influence cancer cell development, invasion, and metastasis. As a result, therapies based on enzymes that catabolize amino acids, seem to work. In the case of arginine

auxotrophic tumors, arginine deprivation utilizing arginine-depriving enzymes has been proposed as a successful anti-cancer strategy (Qiu et al., 2015).

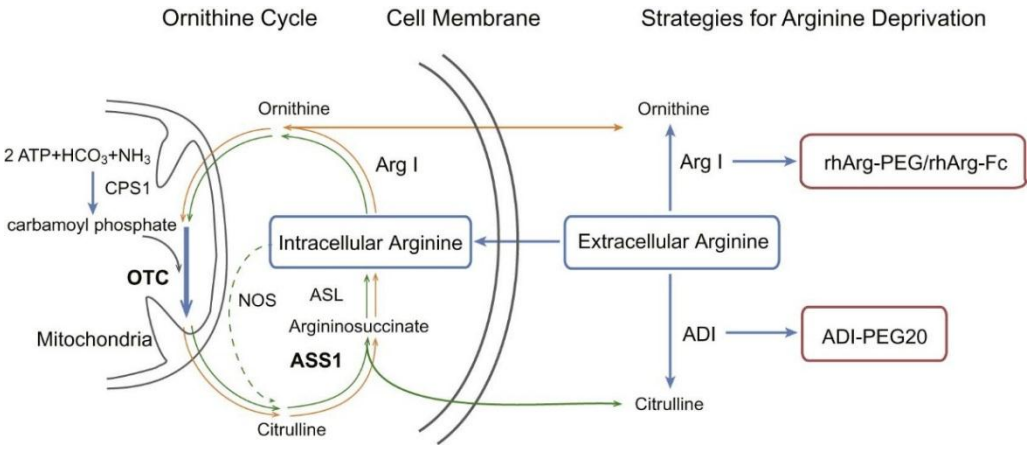

Figure 7. Strategies for arginine deprivation (Qiu et al., 2015). ASS1 and ASL catalyze the conversion of citrulline to arginine in two steps, with argininosuccinate as an transitional component. Arginase I can next breakdown arginine into ornithine and urea. OTC converts ornithine to citrulline, which is then recycled in mitochondria. Extracellular arginine could be depleted via enzymes such ADI and arginase I, which convert arginine to citrulline and ornithine, respectively. In vivo arginine deprivation can be achieved via the ADI-PEG20 and rhArg-PEG methods.

1.7.1 Arginine deiminase

ADI was first isolated from Mycoplasma and cannot be synthesized by humans. It is a metabolizing enzyme that converts arginine to citrulline (Figure 2). This enzyme has a very low half-life in circulation and is immunogenic. Due to its instability and quick clearance from the plasma, a more stable form of this enzyme known as ADI-polyethylene glycol 20 (ADI-PEG20) has been developed for therapeutic purposes. Even though both forms of this enzyme degrade arginine selectively *in vitro*, their potency *in vivo* is enormously dissimilar. For instance, ADI-PEG20 dramatically decreased tumor development and increased survival time in the case of multiple myeloma and hepatocellular carcinoma, but native ADI had minimal therapeutic efficacy. The effectiveness of ADI- PEG20 anticancer activity has been tested and proved in a variety of other cancers (pancreatic cancer, prostate cancer, breast cancer, …) (Kim Bold, et al., 2009).

Arginine deprivation mediated by ADI promotes a multiplex chain of mechanisms in the cells. It has the ability to cause autophagy and apoptosis (caspase-dependent or independent). ADI-anti-tumor PEG20's effect is autophagy-dependent and inversely correlated with ASS1 status in breast cancer cells *in vivo* and *in vitro* (Qiu et al., 2014). ADI's antiangiogenic property counts as another anticancer property of this enzyme. The inhibition of NO production, a well-known angiogenic factor, is thought to be one mechanism by which ADI exerts such an effect. Also, cell cycle arrest has been seen in HCC, mesothelioma, and melanoma patients treated with arginine depletors (Patil et al., 2016; Qiu et al., 2015; Mussai et al., 2015 ; Lam et al., 2011; Ensor et al., 2002).

ADI's anticancer efficacy in melanoma animal models *in vivo* has been investigated and several clinical studies were conducted (Takaku et al., 1992). As an example, in western countries, phase I/II clinical trials using ADI-PEG20 as a therapy for HCC and MM revealed encouraging outcomes. First, patients with metastatic HCC were treated with ADIPEG20 in phase I/II clinical studies. Clinical response was seen in 47% of enrolled patients, with two patients achieving a complete response. All patients' median overall survival was increased (Izzo et al., 2004). HCC patients received ADI-PEG20 therapy in another phase II clinical trial. All individuals had an increase mean overall survival (Glazer et al., 2010). Despite having successful results in the first 2 phases, the global phase III trial to confirm ADIPEG20's therapeutic efficacy in HCC failed (Abou-Alfa et al., 2018).

In an Italian phase I/II clinical trial, melanoma patients given ADI-PEG20 responded to treatment, and one of them had a complete response. Another clinical trial on melanoma patients, however, did not show a significant clinical response (Ascierto et al., 2005; Ott et al., 2013). In the first prospective investigation of ADI-PEG20 in mesothelioma with ASS1 deficiency, the drug extended progression-free survival (PFS) (Szlosarek et al., 2017). ADIPEG20 was well tolerated in all of these clinical investigations. Patients with HER2 negative breast cancer and other types of cancer such as AML, prostate cancer are being enrolled in many current clinical trials employing ADIPEG20.

1.7.2 Arginase I

HuArg-1 is conjugated to two manganese ions as cofactors, which are rapidly lost in the serum, resulting in inactivation of the enzyme and a limited life span of about four and a half hours. It also has an ideal PH of 9.6, thus it cannot work in a neutral PH environment like the plasma, which has a PH of roughly 7.4. For these reasons, several changes should be applied on HuArg-1 to achieve arginine depletion through it (Dillon et al., & Clark, 2002; Hsueh et al., 2012). First, Two Mn2+ ions are replaced with two Cobalt ions as part of HuArg's correction. When Co2+ is combined with Arg, the ideal pH is shifted to 7.5, making it compatible with the plasma's 7.4 pH. When compared to the native cofactor, this will boost the enzyme's total catalytic activity by tenfold and its serum stability by fivefold (Stone & Chantranupong, 2010; Stone & Glazer, 2010; Fultang et al., 2016).

Another change was HuArg's pegylation, which entails adding polyethylene glycol chains to the enzyme. This elevated the enzyme's molecular mass while also protecting it from proteolytic cleavages. As a result, pegylation improved the drug's pharmacokinetics, increased the HuArg half-life, and improved the drug's blood stability (Roberts et al., 2002; Harris & Chess, 2003; Yau et al., 2015). rhArg-PEG has a greatly better half-life time that was raised, as well as an enhanced arginine Km value allowing it to be used in clinical applications and thus rendering it more effective for cancer treatment (Cheng et al., 2007).

Like ADI, arginase is involved in inducing autophagy in several types of cancers such as human melanoma (Wang et al., 2014). rhArg-PEG successfully reduced arginine to a barely detectable level within 24 hours. rhArg-PEG eliminated leukemia cells *in vitro* and *in vivo*, as well as lengthened the survival period and exhibited symbiotic effects with chemotherapy medications (Hernandez et al., 2010).

In comparison with ADI, less clinical trials were done on rhArg-PEG. In advanced HCC, a phase I trial with pegylated recombinant human arginase 1 (Peg-rhArg1) was conducted and only 26.7 percent of patients had the best response, which was a stable illness (Yau et al., 2013).

A second clinical trial was conducted for patients suffering from advanced malignant melanoma. By response assessment in solid tumors criteria, no objective responses were identified, however nine patients out of 31 had stable disease, two of which were durable, more than months (Ott et al., 2013). rhArg-anti-tumor PEG's efficacy in leukemia and lymphoma collectively with other drugs is being studied in ongoing clinical trials.

1.8 Purpose of the study

Breast cancer is a disease of several subtypes defined by histological and molecular characteristics which correlate with distinct metastatic and treatment response profiles and, ultimately, with patient survival (Tsang & Tse, 2020). Given the importance of arginine metabolism to cancer progression and the potential impact of arginine-targeted therapies, the purpose of this study was to identify the cellular and molecular processes modulated by arginase in cell lines that belongs to different molecular subtypes of breast cancer.

Arginase-mediated arginine depletion affected cancer cell proliferation, adhesion abilities, cell motility and invasion as well as autophagy induction in several types of cancer.

Our hypothesis was that cell lines from distinct breast cancer subtypes would be more vulnerable to arginase treatment than others. We therefore investigated how cell-cell adhesion, cell-ECM adhesion and EMT markers of luminal (MCF-7) and triple-negative (MDAMB-231, UACC-2087) breast cancer cell lines were affected by treatment with HuArg1 (CO)-PEG 5000. In addition, we assessed the effect of arginase on LC3 (a marker of autophagy) localization, as well as focal adhesions and stress fiber formation, both important for motility.

Chapter 2

Materials and methods

2.1 Cell culture

Human breast cancer cell lines, MCF-7 and MDA-MB-231, were cultured in DMEM medium supplemented with 10% FBS, 1% PS, 1% L-Glutamine. Human breast cancer cell line UACC-2087 was cultured in DMEM medium supplemented with 10% FBS,1% PS, 1% L-Glutamine, and 0.01 mg/ml ITS. Cell were incubated at 37°C and 5% CO2.

2.2 Source of HuArg1(CO)-PEG 5000

Pegylated human recombinant Arginase I cobalt [HuArgI(Co)-PEG5000] was produced by Aeaglea Biotherapeutics Inc. (Austin, TX, USA) and obtained as a generous gift from Dr. Ralph Abi Habib (LAU).

2.3 Antibodies and reagents

The following primary antibodies were used in this study: Mouse monoclonal anti-LC3 (ab51520, abcam), Mouse monoclonal anti-E-Cadherin (14472S, cell signaling), Rabbit monoclonal anti-vimentin (8480S, cell signaling), and Mouse monoclonal anti-Vinculin (A129002, abcam), Rabbit monoclonal anti-S100A4 (13018S, cell signaling) and Mouse monoclonal anti-Pan-Keratin (454S, cell signaling). The following secondary antibodies were used in this study: Alexa Fluor 488 Goat anti-Rabbit IgG (H+L) (A11008), Alexa Fluor 594 Goat anti-Mouse IgG (H+L) (A11005), were obtained from Invitrogen. Alexa Fluor 564 Phalloidin (A12380, thermo) was also used.

2.4 Immunostaining and widefield microscopy

MCF-7 and UACC-2087 cells were plated on cover slips in a 24 well plate, and treated with the HuArgI(Co)-PEG5000 either at a 0.5 nM or 1.5 nM concentration. After 96 hours of plating, the cells were fixed with 4% paraformaldehyde for 10 minutes at 37°C, and permeabilized with 0.2% Triton-X 100 in PBS with Ca^{2+} and Mg^{2+} for 15 minutes. For blocking, cells were incubated with 0.1% Triton-X 100 in PBS with Ca and Mg, 10% FBS. Samples were then stained with primary antibodies for 1h 30 minutes at room temperature and with fluorophore-conjugated secondary antibodies for 1 hour. Fluorescent images were taken using a 63x objective lens on Zeiss Observer Z1 microscope.

2.5 Cell-ECM adhesion assay

A total of $3x10^5$ of MCF-7 and MDA-MB-231 cells were added in each well in a 96-well plate and incubated at 37°C in a CO2 incubator for 24 hours. Cells were then treated with trypsin and EDTA with or without HuArgI (Co)- PEG5000 at a concentration of 100 nM and incubated for 10 minutes at 37°C in a CO2 incubator, washed, and stained with crystal violet following the protocol from Feoktistova et al., 2016. Briefly, culture media were removed and well were filled with a crystal violet solution (0.5%) for 20 minutes on a benchrocker at room temperature (20 oscillations). Plates were then washed extensively with water, and left to dry completely for 2 hours. Crystal violet was subsequently solubilized by adding 200 µl of methanol to each well and incubated at room temperature on a benchrocker for 20 minutes. The absorption of the plates was read at 570 nm using a Thermo scientific Varioskan Flash Multimode reader (Thermo fisher scientific, USA).

2.6 Quantitative image analysis

Image J was used to quantify focal adhesions, stress fibers, mean intensity. Briefly, focal adhesions were counted in the vinculin channel and stress fibers from the F-actin channel. Mean intensity was measured by setting measurements on mean gray value, min and max and limit to threshold and by measuring the intensity of each marker beyond a visually determined signal threshold to eliminate background signal interference.

2.7 Cell-cell adhesion assay

A total of $3x10^5$ cell/ml of MCF-7, UACC-2087 and MDA-MB-231 were added to each well in a non-repellent 96-well plate. Cells were treated with HuArgI(Co)-PEG5000 at a concentration of 100 nM. The cells were then fixed with 28.5 µl of 32% PFA in 200 µl. The cells were transferred to 1.5 ml tubes, centrifuged for 4 seconds at 400 rcf and washed with 1 ml PBS. 50 µl of cells were placed on a petri dish and the clusters were counted.

2.8 Statistical analysis

GraphPad Prism was used for graphing and statistical analysis. For each experimental group, normality was tested, and the *p* values were calculated using the appropriate parametric or non-parametric test for normally and non-normally distributed datasets, respectively. For normally distributed data, t-test was used for comparisons of two groups and one-way ANOVA was used for three or more comparisons. For non-normally distributed samples, the Mann-Whitney test was used for comparisons of two groups and the Kurskal-Wallis test was used for three or more comparisons.

Chapter 3

Results

3.1 HuArgI(Co)-PEG5000 downregulates LC3 expression in luminal MCF-7 and triple negative UACC-2087 breast cancer cell lines.

Nutrient, including amino acid, deprivation can induce autophagy in normal and cancer cells. Given that LC3 is a known marker of autophagy, I utilized immunofluorescence to investigate the levels of LC3 in MCF-7 (luminal breast cancer) and UACC-2087 (triple negative breast cancer) and to see if these levels could be modulated by HuArgI(Co)-PEG5000 (Fig. 8A). Surprisingly, LC3 was predominantly localized to the nucleus, suggesting low activation levels of autophagy in all experimental conditions (Fig. 8A). Arginase treatment significantly lowered total LC3 expression in MCF-7 and UACC-2087 cells (Fig. 8B). Nuclear-to-cytoplasmic ratio of LC3 expression was higher in untreated MCF-7 cells compared to untreated UACC-2087 cells, and arginase treatment lowered this ratio only in MCF-7 cells without affecting in UACC-2087 cells (Fig. 8 C). These results suggest a possible degradation of nuclear LC3 in both cell lines, and indicates that these cell lines were either unable to induce autophagy in order to overcome arginine's deprivation, or underwent LC3-independent autophagy.

3.2 HuArgI(Co)-PEG5000 treatment affects epithelial and mesenchymal markers' expression in luminal and triple negative breast cancer cells

Given the importance of EMT to tumor invasion and metastasis, I investigated the effect of HuArgI(Co)-PEG5000 on epithelial (E-Cadherin, pan-Keratin**)** and mesenchymal (vimentin, FSP1) markers expression in HuArgI(Co)-PEG5000 treated and non-treated MCF-7 and UACC-2087 cells. Vimentin was not expressed in either cell type (Fig. 9A). The expression level of E-Cadherin, pan-Keratin and FSP1 decreased significantly upon arginine deprivation in both cell lines but was not totally lost (Fig. 9A,

B, C,D). These results suggest that treatment with HuArgI(Co)-PEG5000 did not trigger a clear EMT signature.

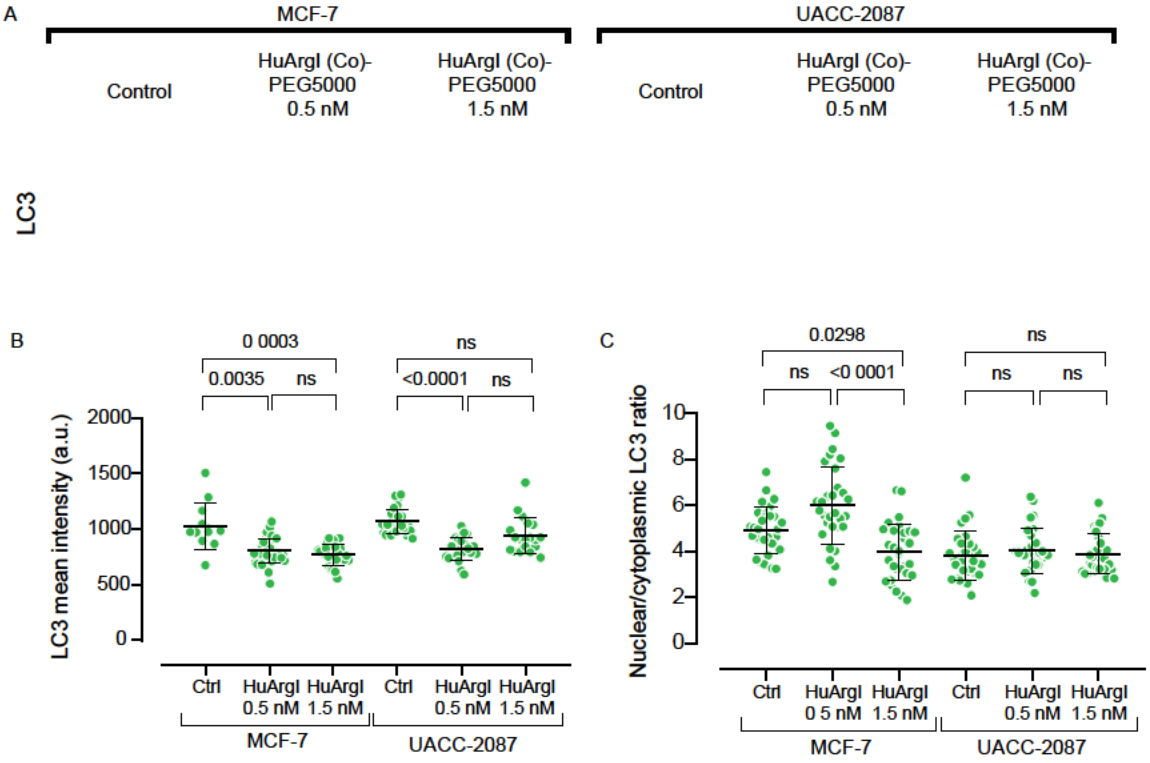

Figure 8. HuArgI(Co)-PEG5000 downregulates LC3 expression in luminal MCF-7 and triple negative UACC-2087 breast cancer cell lines. (A) Representative micrographs of LC3 and F-actin staining in control, HuArgI(Co)-PEG5000 (0.5 nM and 1.5 nM) treated MCF-7 and UACC-2087 cells. (B) Quantitation of LC3 mean intensity from micrographs in (A). (C) Quantitation LC3 nuclear/cytoplasmic ratio from mircographs in (A). Scale bar: 30 µm. Data is representative of 1 independent experiment and 555 cells quantified.

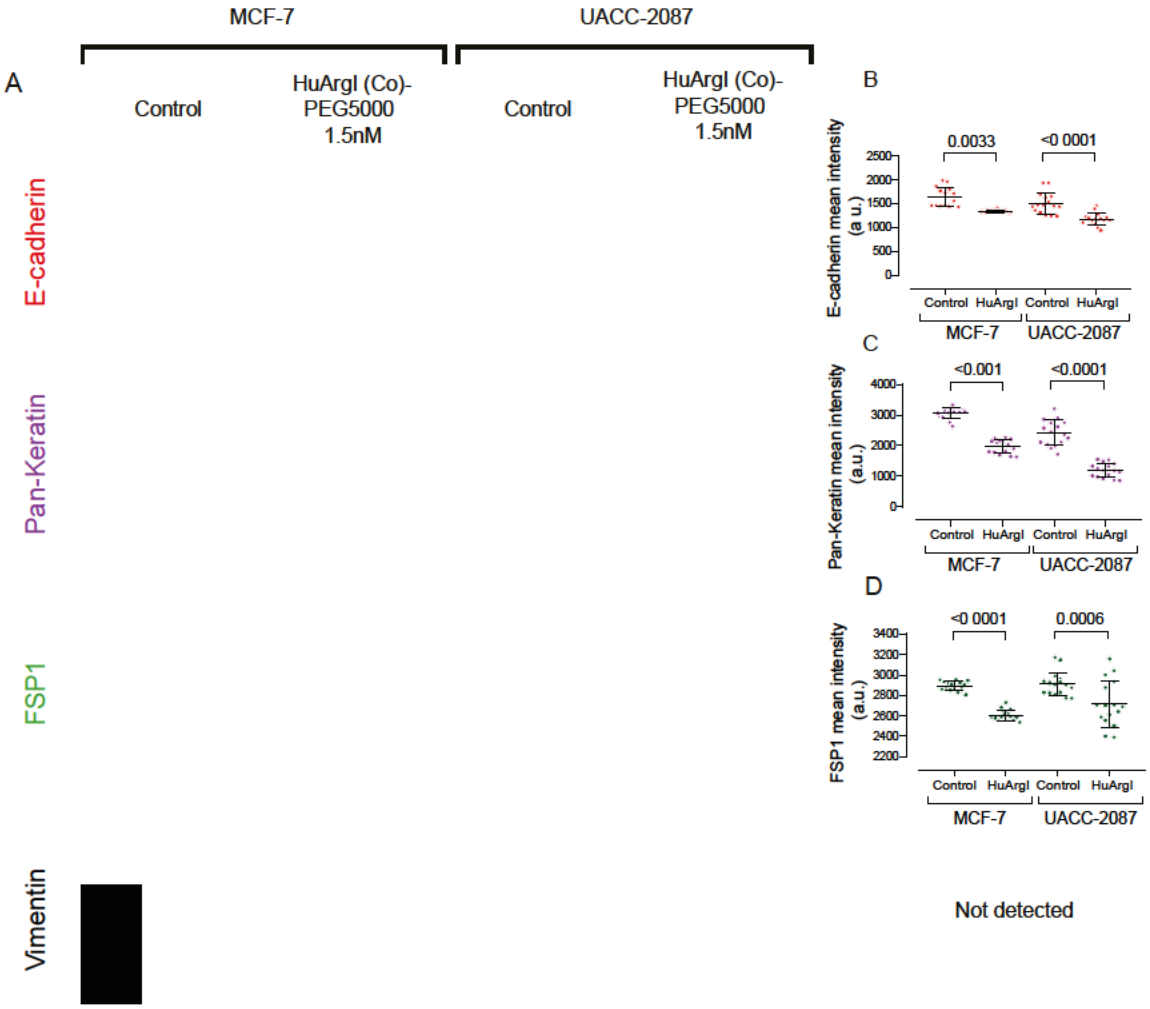

Figure 9. HuArgI(Co)-PEG5000 treatment affects epithelial and mesenchymal markers' expresssion in luminal and triple negative breast cancer cells. (A) Representative micrographs of E-cadherin, Vimentin, Pan-Keratin and FSP1 staining in control and HuArgI(Co)-PEG5000 1.5 nM treated MCF-7 and UACC-2087 cells. (B) Quantitation of E-cadherin mean intensity from micrographs in (A). (C) Quantitation of pan-keratin mean intensity from micrographs in (A). (D) Quantitation of FSP1 mean intensity from micrographs in (A).

3.3 HuArgI(Co)-PEG5000 decreases cell-cell adhesion in luminal breast cancer cells MCF-7 and triple negative breast cancer cells

The decrease of E-cadherin's expression levels could indicate a decrease in cell-cell adhesion. For this reason, a cell-cell adhesion assay was performed. Luminal (MCF-7) and triple negative (MDA-MB-231 and UACC-2087) breast cancer cells were plated on non-adherent culture dishes (which prevent cell-ECM adhesion) and allowed to cluster

in the presence or not of HuArgI(Co)-PEG5000 (Fig. 10, left panel micrographs). The number of clusters formed after 1h, 3h and 24h of incubation was then counted (Fig. 10, right panel graphs). In all three cell types, arginine deprivation significantly reduced cell-cell adhesion (clustering) ability. This trend was noticeable starting from the 1-h timepoint and reached statistically significant at the 3-h and 24-h timepoints (Fig. 10). These results suggest that arginine deprivation contributes to tissue disaggregation in breast cancer cell lines. Whether HuArgI(Co)-PEG5000-induced loss of tumor cell-cell adhesion promotes or reduces metastatic potential will need to be determined in future *in vivo* assays as cell detachment from the tumor can associate with increased local dissemination, while circulating tumor cell clusters can be an effective metastatic strategy in breast cancer.

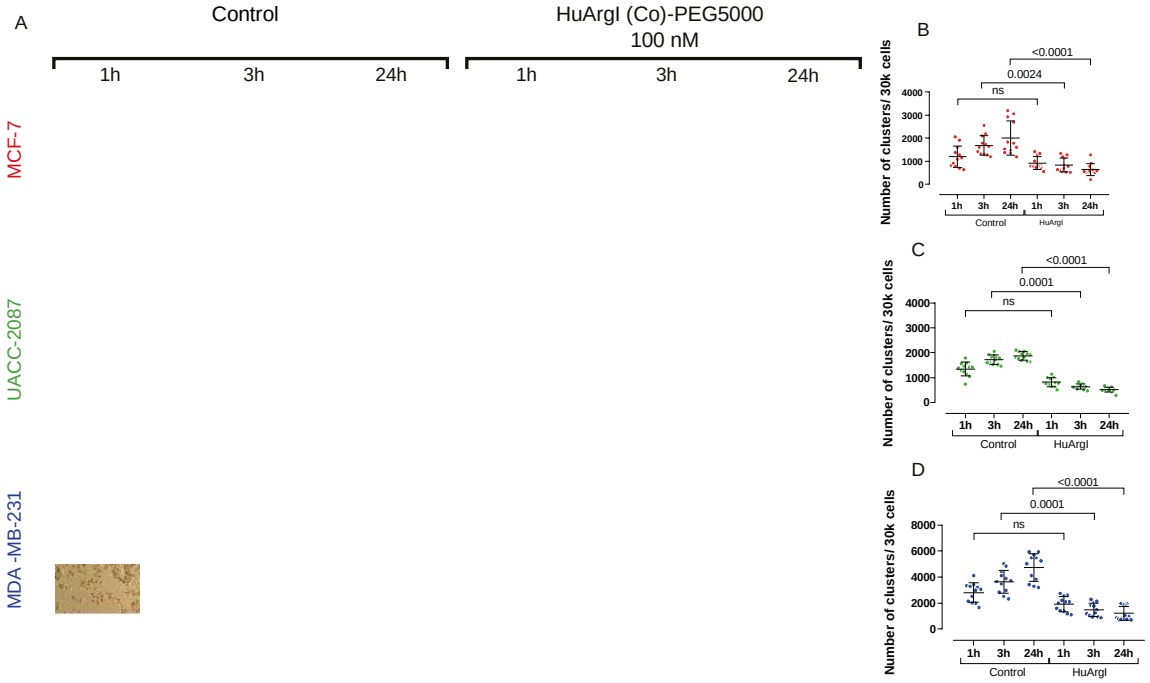

Figure 10. HuArgI(Co)-PEG5000 decreases cell-cell adhesion in luminal breast cancer cells MCF-7 and triple negative breast cancer cells UACC-2087 and MDA-MB-231. A) Representative micrographs of clusters formation in control and HuArgI(Co)-PEG5000 100 nM in MCF-7, UACC-2087 and MDA-MB-231 cell lines after 1h,3h and 24h of incubation B, C, D) Quantitation of the results in MCF-7, UACC-2087 and MDA-MB-231 respectively. Scale bar: 50 µm. Data is representative of 3 independent experiments.

3.4 HuArgI(Co)-PEG5000 does not contribute to cell-ECM deadhesion in luminal and triple negative breast cancer cell lines

To investigate whether arginine deprivation can modulate the ECM adhesion abilities of luminal and triple negative breast cancer cells, I designed a novel assay for trypsin/EDTA-induced cell-ECM deadhesion assay. Briefly, MCF-7 and MDA-MB-231 cells were treated with escalating concentrations of EDTA or trypsin-EDTA (forcing them to detach from the substrate) for 10 minutes, with or without treatment with HuArgI(Co)-PEG5000. Plates were then washed to remove detached cells and stained with crystal violet for quantification of remaining, adherent cells. As expected EDTA and trypsin-EDTA were effective in causing fast and dose-dependent deadhesion (Fig 11A-F). However, addition of HuArgI(Co)-PEG5000 treatment had no effect of cell-ECM deadhesion thus indicating the absence of short-term response of arginine deprivation on breast cancer cell binding to rigid substrates.

3.5 HuArgI(Co)-PEG5000 decreases focal adhesions formation in human triple negative breast cancer cells

Given that our short term (10 min) deadhesion assay did not reveal an effect of arginine deprivation on cell-ECM adhesion, we next turned to elucidate the long term (96-h) effect of HuArgI(Co)-PEG5000 treatment on actin cytoskeletal and adhesive structures in MCF-7 and UACC-2087 cells. After treatment, cells were stained for vinculin, a component of the FA structures (Humphries et al., 2007). Overall vinculin expression was not altered in either cell line which validated its use as a reliable marker of focal adhesion quantification in this assay (Fig. 12A, B). In the MCF-7 line, only a minority of cells had the ability to form focal adhesions and stress fibers in our experimental setting and these abilities where not modulated by arginine deprivation (Fig. 12A, C, D). In the UACC-2087 line, however, a different cytoskeletal arrangement was observed: the large majority of cells formed focal adhesions and stress fibers, and treatment with HuArgI(Co)-PEG5000 significantly reduced the number of focal adhesions per cell (Fig. 12A, C, D). This data suggests that UACC cell adhesion and motility might be negatively affected by arginine deprivation.

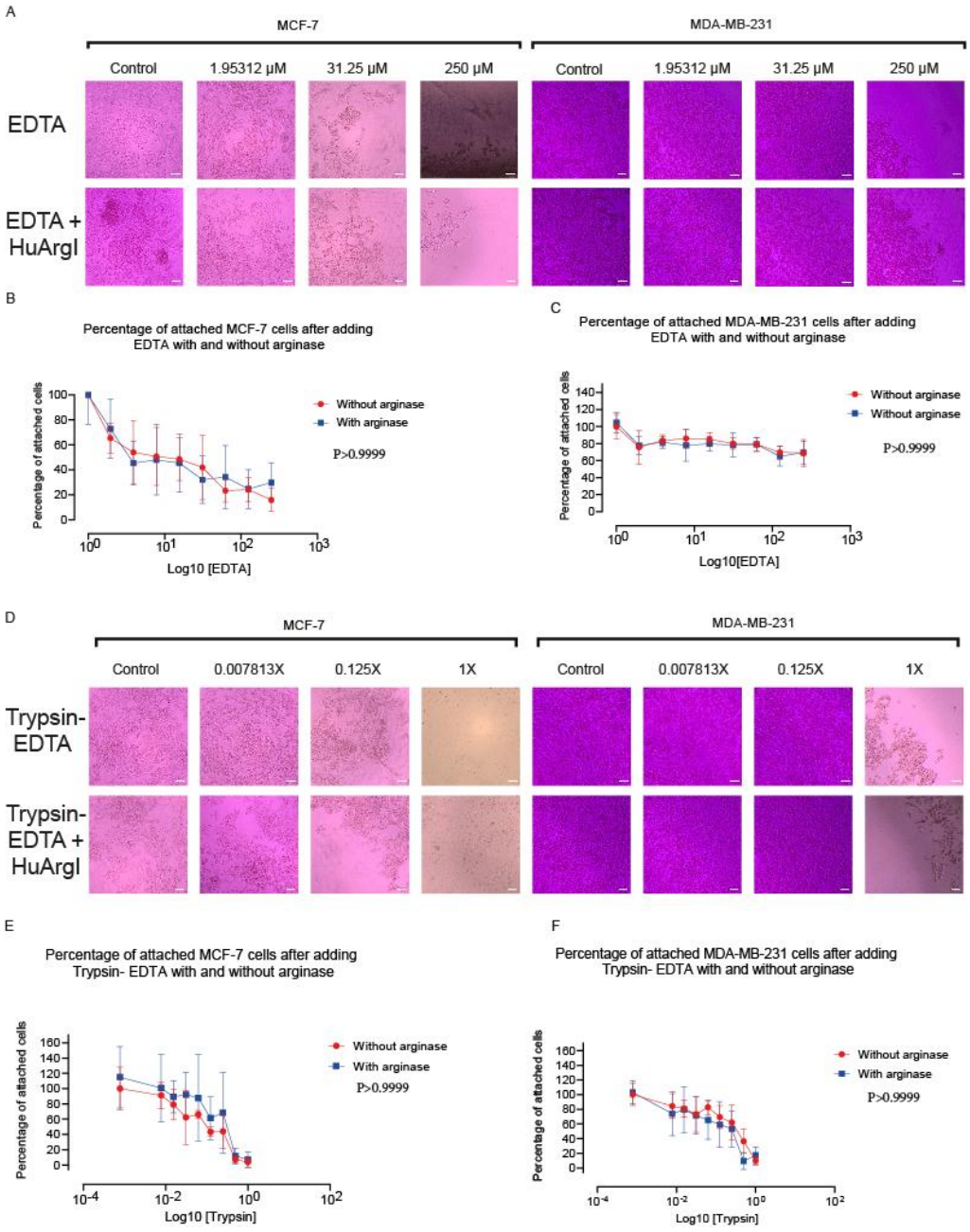

Figure 11. HuArgI(Co)-PEG5000 does not affect cell-ECM adhesion in luminal MCF-7 and triple negative MDA-MB-231 breast cancer cell lines. A) Representative micrographs of control, 1.95312 µM, 31.25 µM and 250 µM of EDTA (first, second, third, fourth column respectively) with and without HuArgI(Co)-PEG5000 100 nM. B) Quantitation of the results with EDTA in MCF-7. C) Quantitation of the results with EDTA in MDA-MB-231. D) Representative micrographs of control, 0.007813X 0.125X and 1X of Trypsin-EDTA (first, second, third, fourth column respectively) with and without HuArgI(Co)-PEG5000 100 nM. D) Quantitation of the results with Trypsin-EDTA in MCF-7. E) Quantitation of the results with Trypsin-EDTA in MDA-MB-231. Scale bar: 50 µm. Data is representative of 3 independent experiment and 30K cells per well quantified.

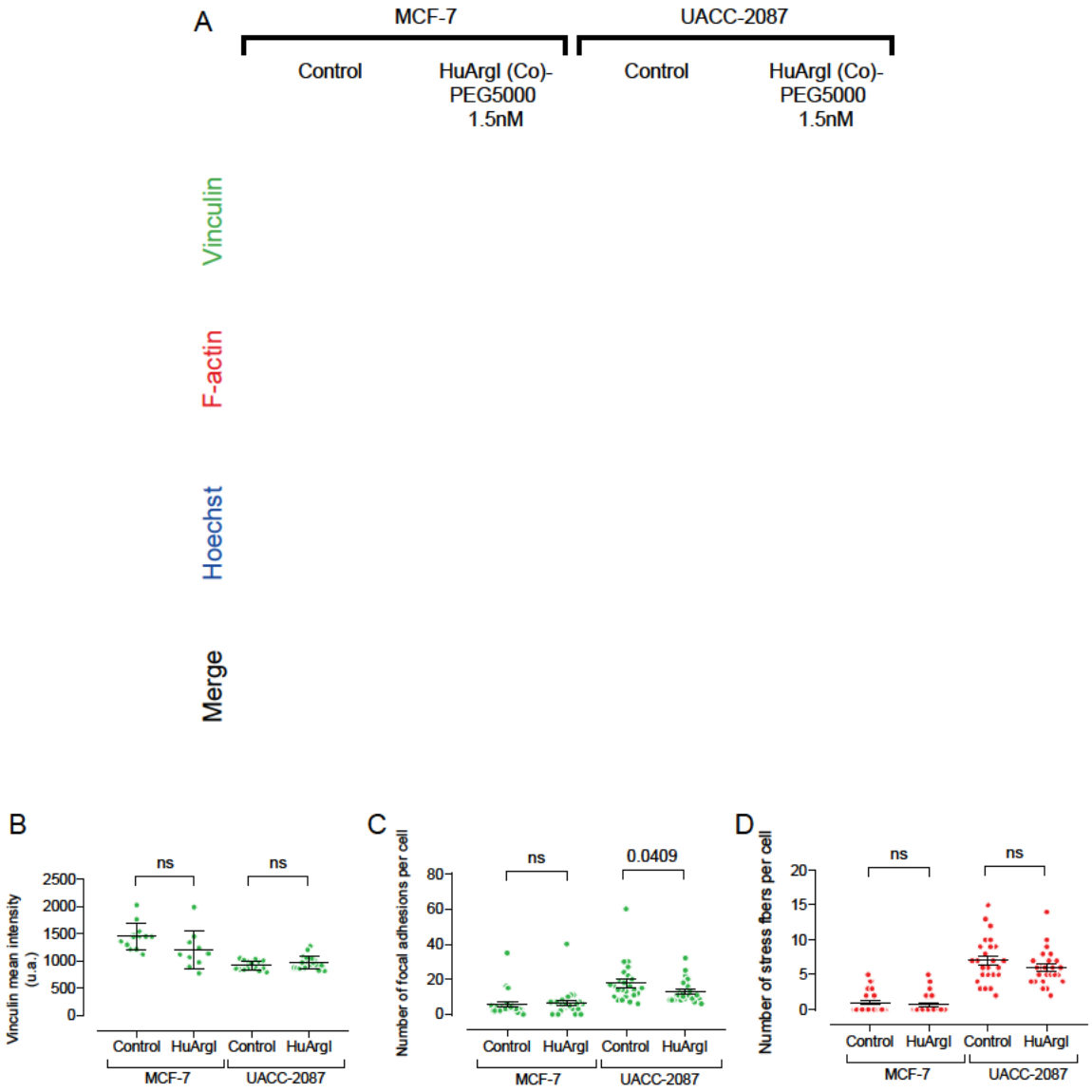

Figure 12. HuArgI (Co)-PEG5000 decreases focal adhesions formation in human triple negative breast cancer UACC-2087 cell line. (A) Representative micrographs of Vinculin and F-actin staining in control and HuArgI (Co)-PEG5000 1.5 nM treated MCF-7 and UACC-2087 cells. (B) Quantitation of Vinculin mean intensity from micrographs in (A). (C) Quantitation of percentage of cells with focal adhesions from micrographs (A). (D) Quantitation of number of focal adhesions per cell from micrographs (A). (E) Quantitation of percentage of cells with stress fibers from micrographs (A). (F) Quantitation of number of stress fibers per cell from micrographs (A). Scale bar: 10 µm. Data is representative of 1 independent experiment and 325 cells quantified.

Chapter 4

Discussion

Breast cancer is classified into molecular subtypes depending on the expression of estrogen, progesterone and human epidermal growth factor receptor 2 (HER2), which were later confirmed and categorized as basal-like (triple negative for ER, PGR, HER2), ErbB2+(HER2+), normal breast-like (ER, PGR positive; HER2 negative), luminal subtype A (ER, PGR positive; HER2 negative) and luminal subtype B (ER, PGR, HER2 positive) (Perou et al., 2000; Sørlie et al., 2001; Sorlie et al., 2003). These molecular subtypes have distinct therapeutic and clinical implications for patient. Previous reports have suggested a link between arginine metabolism and ER status in breast tumors. Arginine levels were lower in ER-positive BC patients than in ER-negative BC patients. In other words, arginine level varies among molecular subtypes of breast cancer. Arginine was linked to a molecular subtype. TNBC has the lowest arginine levels when compared to the other molecular subtypes. These findings suggested that a low arginine level was linked to the development of BC (Hu et al., 2016) suggesting that arginine deprivation using human recombinant arginase (e.g. HuArgI(Co)-PEG5000) could be a strategic therapeutic intervention in triple-negative breast cancer.

Three cell lines were investigated in thus study: MCF-7, a luminal A subtype that expresses estrogen and progesterone receptors. It is slightly aggressive and non-invasive with low metastatic abilities (Gunduz et al., 2011). UACC-2087 and MDA-MB-231, triple negative subtype, that do not express any hormone receptor. MDA-MB-231 is more invasive and metastatic than UACC-2087. In general, the TNBC subtype is the most aggressive among all subtypes (Huang et al., 2020). Arginine deprivation therapy for arginine auxotrophic cancers is the subject of numerous clinical trials. In this study, arginine deprivation using HuArgI(Co)-PEG5000 showed that cell lines from distinct breast cancer subtypes are more vulnerable to arginase treatment than others and that they are differentially affected.

First, a decrease in LC3 and in LC3 nuclear/cytoplasmic ratio during arginine deprivation could be due to nuclear LC3 degradation at a higher rate than LC3 degradation and this assay was not sufficient to detect induction of autophagy. Further markers implicated in autophagy should be studied to further confirm that breast cancer cell lines are not able to undergo canonical and non-canonical autophagy in order to overcome arginine deprivation.

EMT can correlate with metastatic and drug resistant properties in cancer (Wang et al., 2016). Arginine deprivation led to a concomitant decrease in epithelial and mesenchymal markers, suggesting the absence of clear EMT signature, and perhaps the absence of metastatic or chemoresistant response in breast cancer cells treated with HuArgI(Co)-PEG5000. We also observed a decrease but not complete loss in E-cadherin expression during arginine deprivation which explains the inhibitory effect HuArgI(Co)-PEG5000 on cell-cell adhesion and may affect metastatic incidence. We then looked for changes in cell-ECM adhesion and no effect of the drug was observed after 10 mins of incubation, this is a why a longer frame of time was applied when stained for vinculin. A notable difference between MCF-7 and UACC-2087 cell lines in baseline organization of the actin cytoskeleton, was detected. UACC-2087 cells indeed formed more stress fibers and focal adhesions, and this ability was decreased by HuArgI(Co)-PEG5000. This indicates a disruption in the cell-ECM adhesion and, potentially, motility and migration of triple negative breast cancer cells after arginine deprivation. Future live microscopy assays of migration should be done in order to further elucidate the effect of FA on migration.

Chapter 5
Conclusions

This study aimed to characterize the effect of arginine deprivation done by HuArgI(Co)-PEG5000 on breast cancer cell adhesion and cytoskeletal remodeling. Results showed that cell lines from distinct breast cancer subtypes are more vulnerable to arginase treatment than others. In fact, LC3 localization in the nucleus was downregulated in breast cancer cell lines, without an increase in the cytoplasm, suggesting lack of autophagy activation in breast cancer cells upon treatment. The decrease of focal adhesions and stress fibers in triple negative breast cancer UACC-2087 cells indicates a long-term disruption in ECM adhesion after treatment with arginase. Cell-cell adhesion assay indicated a decrease in the clustering (cell-cell adhesion) ability concomitant with decrease in E-cadherin in both luminal and triple-negative breast cancer cells after treatment with HuArgI(Co)-PEG5000. In future studies the effect of HuArgI(Co)-PEG5000 should be examined on a larger panel of breast cancer subtypes and *in vivo* studies should be conducted.

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