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Characterizing the Effect of Arginine Deprivation on Autophagic Response in Breast Cancer cells

By

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Characterizing the Effect of Arginine Deprivation on Autophagic Response in Breast Cancer cells

Fatemah Haidar

ABSTRACT

The metabolism of arginine, a semi-essential amino acid, is required for homeostasis and deregulated in various diseases. When deprived of arginine, several types of cancers can modulate arginine synthesis mediated by the enzyme ASS1 or employ broader response mechanisms such as autophagy. The co-regulation of arginine metabolism and autophagy in breast cancer is poorly understood. In this project, we first inspected *ASS-1* expression in human breast cancer patients and found that it decreases progressively with tumorigenesis and metastasis. Residual ASS1 tumor expression corelated with increased metastasis and decreased survival in patient luminal A, Her2+ and triple-negative breast cancer. We then utilize a pharmacological-grade formulation of human recombinant arginase (HuArgI(Co)-PEG5000) to characterize the effect of arginine deprivation in one luminal (MCF-7) and two triple negative (MDA-MB-231 and UACC-2087) models of breast cancer. Western blot analysis revealed that arginine deprivation was not sufficient to trigger a compensatory upregulation of ASS1 expression in any of the tested cell lines. However, arginine deprivation induced varying autophagic response in MCF-7, MDA-MB-231 and UACC-2087. Altogether, our findings suggest that the activation of autophagy could be a plausible mechanism for breast cancer cells to overcome arginine depletion. Future functional studies will reveal whether HuArgI(Co)-PEG5000-induced autophagy sustains long-term cancer cell survival, and whether combination with autophagy inhibitors constitutes a viable therapeutic strategy in breast cancer.

Key words: Arginine, Arginase, Breast Cancer, Autophagy, ASS1

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF ABBREVIATIONS

ADC: arginine decarboxylase

ADCD: autophagy-dependent cell death

ADI: arginine deiminase

AML: Acute myeloid leukemia

ARG1: arginase I

ARGII: arginase II

ASL: argininosuccinate lyase

ASS-1: argininosuccinate synthetase-1

ATG: autophagy related genes

BC: Breast cancer

CAAs: Cationic amino acids

CAT: cationic amino acid transporter

CP: carbamoyl phosphate

CPS: carbamoyl phosphate synthetase

CQ: chloroquine

ESCRT: endosomal sorting complex required for transport

FBS: Fetal Bovine Serum

hArg: human arginase

HATs: Heterodimeric amino acid transporters

HCC: hepatocellular carcinoma

HuArg I: human l-arginase I

HuArgI(Co)-PEG5000: pegylated human recombinant arginase I cobalt

NSCLC: non-small-cell lung cancer

NO: Nitric oxide

NOS: Nitric oxide synthase

Orn: Ornithine

OS: overall survival

OTC: ornithine transcarbamoylase

PEG: polyethylene glycol

ROS: reactive oxygen species

TCA: tricarboxylic acids cycle

TMZ: Temozolomide

TNBC: triple negative breast cancer

Chapter 1

Introduction

1.1 Breast Cancer

Breast cancer is a complex disease with significant tumor heterogeneity (Curtis et al., 2012). It is ranked amongst the most prevalent cancer types, with high frequency of mortality between women worldwide (Nagini, 2017). Breast cancer is classified on the molecular level based on the expression of three different receptors: estrogen receptor (ER), progesterone receptor (PR), and ERBB2 receptor (HER2). The different receptors categorize breast cancer into three major subtypes: (1) Luminal breast cancer representing 70% of breast cancer cases, and is divided into luminal A (ER^-/or PR^+ /$HER2^-$) with low expression of Ki67 protein, consequently low proliferation rate and luminal B (ER^-/or PR^+ / or $HER2^-$) with high expression of Ki67 protein, consequently high proliferation rate; (2) HER2+ breast cancer, HER2 enriched (ER^-/PR^-) representing 15-20% of cases; (3) Triple negative breast cancer (ER^-/PR^-/$HER2^-$) having the worse prognosis and is more common amongst women with *BRCA1* gene mutation, represents 15% of patients (Waks & Winer, 2019). Development and progression of breast cancer is highly affected by gene-environment interactions and epigenetic changes. Women with mutations in BRCA1 or BRCA2 genes are at increased risk of developing breast cancer after exposure to medical radiations (Berrington De Gonzalez et al., 2009). In addition, environmental chemicals can alter gene regulation involved in several signaling pathways affecting cell proliferation and apoptosis (Christopher Wild, 2013), which happens through epigenetic processes, such as DNA methylation and histone modification (Luzhna et al., 2015).

Breast cancer cell can break off from primary tissue, move into the blood and grow in secondary locations, a process referred to as metastasis. It begins with tumor cells in the breast

(primary) locally invading surrounding tissue until they are able to intravasate to the blood or into lymphatic vessels to dissemination into distant organs (Hunter et al., 2008). Consequently, the cell cycle is arrested in these tumor cells, and they adhere into secondary organs to produce secondary metastases (Talmadge & Fidler, 2010). In addition, the tumor cells need to evade the immune response inside the host to survive (Fidler, 1978).

1.2 Synthesis of Amino acids

1.2.1 Amino Acid Metabolism

Amino acids are organic substances that contain both an amino and a carboxyl group (Manna et al. 2009). There are 20 amino acids that serve as building blocks of proteins (Curis et al. 2007; Hu et al, 2008).

The various biochemical properties of amino acids are due to differences in their side chains. All 20 protein-building amino acids and their metabolites, including ammonia, nitric oxide (NO), urea, polyamines, and other nitrogenous substances are necessary for normal cell function (Montanez et al. 2008). Most amino acids undergo a biochemical process called endogenous or *de novo* synthesis by which they are synthesized by the human body from other compounds. Amino acids are synthesized through different patterns, and classified based on dietary need into 3 groups:

Essential amino acids, which must be provided by dietary intake as they cannot be synthesized *de novo* in the body. These include methionine (produced in the folate cycle), phenylalanine, lysine, threonine, and histidine (catabolized by the small intestine) (Stoll et al., 1998).

Non-essential amino acids, which are synthesized *de novo* in sufficient amounts to meet the body's requirements. These include glutamine, glutamate, and aspartate that is oxidized in the small intestine by enterocytes (Stoll et al. 1998). The intestinal lumen takes up glutamate and aspartate, while glutamine is utilized by the small intestine (Self et al. 2004). The nitrogenous

products of non-essential amino acids metabolism will include ornithine, citrulline, alanine, and arginine. The TCA cycle is an open cyclic process that serves as a precursor for aspartate and glutamate synthesis (required for amino acids synthesis) (Araújo et al., 2014). The TCA cycle is involved in gluconeogenesis, oxaloacetate, transamination, and α-ketoglutarate, that is responsible for the synthesis of the circulating glutamine (Self et al. 2004). Enterocytes uses that proline oxidase pathway to actively degrade proline for ornithine, citrulline, and arginine production (**Figure. 1)** (Wu, 2009).

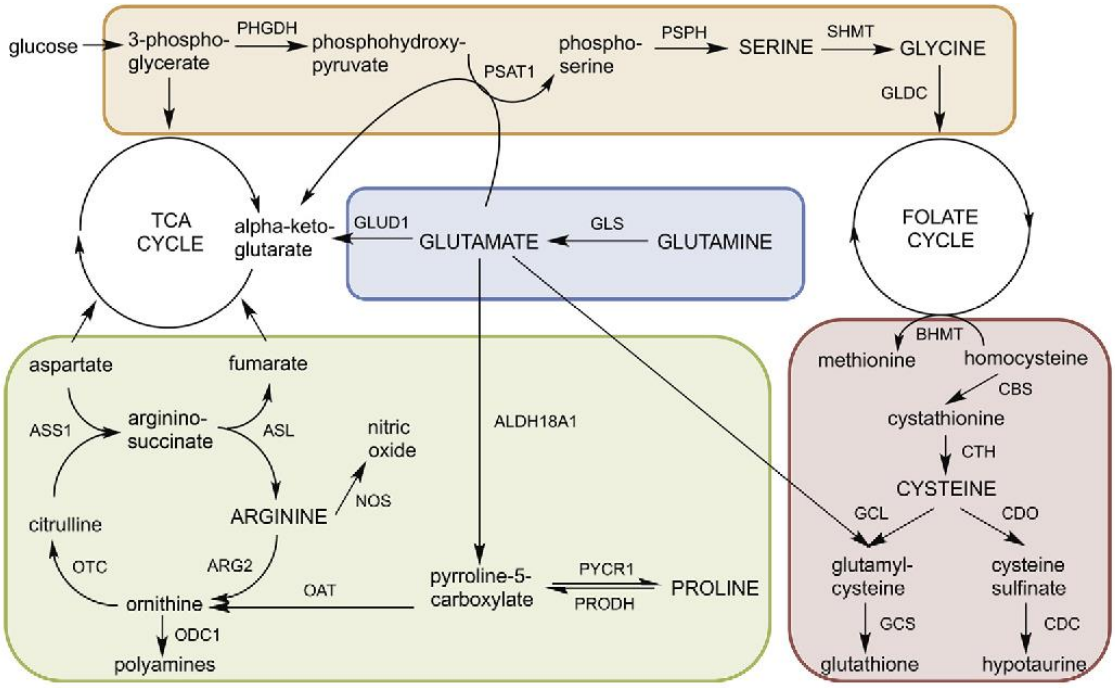

Figure. 1: Metabolism of nonessential amino acids. The metabolic pathways of nonessential amino acids are interconnected. α-ketoglutarate and other intermediates in the TCA cycle synthesize both glutamine and alanine (glutamine-glutamate metabolism in blue). After their release into the circulation, glutamine will be utilized to synthesize citrulline that can convert into arginine. In extrahepatic cells and tissues glycine, cysteine and glucose are synthesized from alanine (Geck & Toker, 2016).

Conditionally essential amino acids, which are normally synthesized *de novo* but, under conditions of high demand or growth, their dietary intake is required (Novelli and Tasker 2008;

Phang et al. 2008). The conditionally essential amino acids includes cystine and arginine (POHLANDT, 1974; Appleton, 2002). Normally, citrulline is released by the small intestine and converted into arginine in kidneys (Wu 1998). In cases of child development and adolescence, endogenous synthesis of arginine is no longer sufficient to meet metabolic demand making it a conditionally or semi-essential amino acid (Flynn et al., 2002).

When cells are unable to synthesize a certain amino acid they are considered auxotroph for this amino acid, and must obtain it from it from the environment for growth and survival (Carney, 2018). Auxotrophy can be helpful in terms of depriving tumor cells from amino acids required for growth, as forms of arginine depriving drugs was used to target cancer cells that are arginine auxotrophic (Nurzen, Tuba & Fatih, 2002).

1.2.2 Urea Cycle

The catabolism of proteins and amino acids in advanced eukaryotes results in releasing ammonia, a toxic metabolite (Jackson et al., 1986). Ammonia is a form of nitrogen that is excreted primarily as urea, a non-toxic, water soluble configuration. Urea synthesis is catalyzed through a series of five enzymatic reactions spanning two cellular compartments in liver cells. The first two steps of the urea cycle take place in the mitochondrial matrix, while the rest continues in the cytosol. The regulation of the urea cycle happens via modulation of the enzyme's expression. The five key enzymes in the urea cycle that can be subjected to considerable control includes, carbamyl phosphate synthetase (CPS), ornithine transcarbamylase (OT), arginosuccinate synthetase (ASS1), arginosuccinate lyase (ASL), and arginase, which exists in two distinct isozymes, arginase I and arginase II (Morris, 2002).

CPS catalyzes the conversion of ammonia into carbamoyl phosphate (CP) before the urea cycle begins, and it requires two ATP molecules. The carbamoyl phosphate group is denoted to ornithine (Orn) after catalysis by OTC. Carbamoyl phosphate will be converted to citrulline,

with the release of a phosphate group. The conversion is followed by an ATP dependent condensation reaction between the carbonyl group of citrulline and amino group of aspartates, catalyzed by ASS1 to form argininosuccinate. ASL will cleave argininosuccinate to form arginine and fumarate. Finally, arginine will be cleaved by arginase forming urea and ornithine. Urea will eventually be excreted in the urine, while ornithine will be transported back into the mitochondria to be used in the urea cycle **(Figure. 2)** (David & Michael, 2013).

The urea cycle enzymes are highly expressed in the liver. They also undergo similar developmental program, and similarly respond to changing levels in dietary protein and hormonal stimuli (Jackson et al., 1986). These enzymes differ in terms of tissue distribution and developmental expression. For instance, CPS and OTC expression is locally restricted to the liver (at high levels), and the intestine (at low levels) (Mary et al., 1961). The three cytosolic enzymes— ASS, ASL and arginase—are ubiquitously expressed and most active in the liver (Carritt et al., 1977).

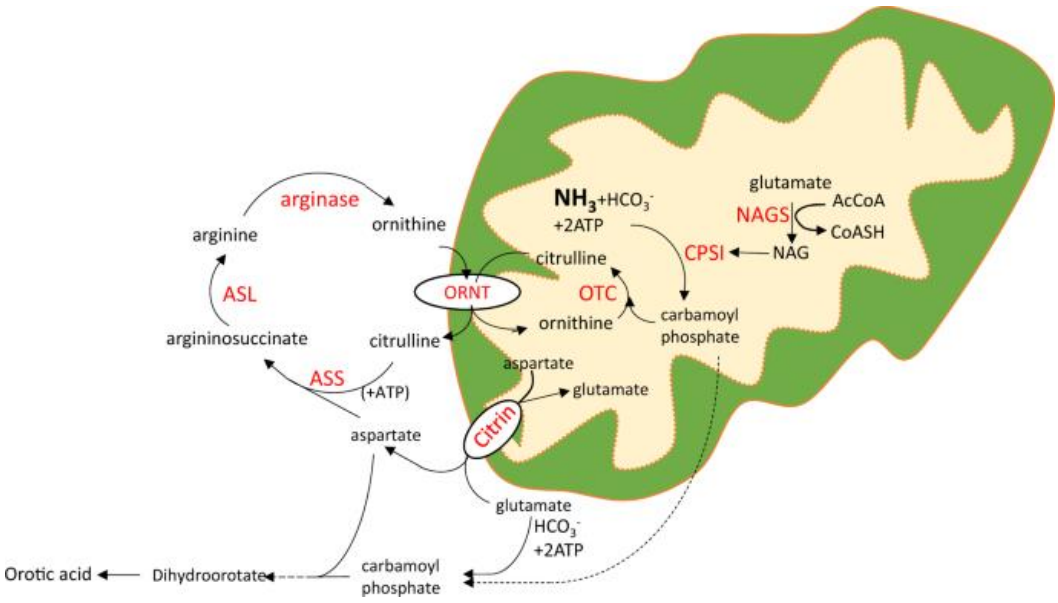

Figure 2. Metabolic cascade of the urea cycle in the liver. The first two steps of ures cycle are catalyzed by CPS and OT in the mitochondrial matrix. ASS1, ASL, and arginase continues in the cytosol leading to urea production. CPS, carbamyl phosphate synthase; OTC, ornithine

transcarbamylase; ASS1, argininosuccinate synthase 1; ASL, argininosuccinate lysase; (Matsumoto et al., 2019).

1.2.3 Arginine Synthesis

Arginine is a positively charged, semi-essential amino acid derived from the diet when endogenous synthesis is insufficient (Mori & Gotoh, 2000). Sources of human extracellular arginine include animal and plant proteins. Arginine is not only considered a substrate for protein synthesis, but also moderates several cellular processes by the conversion to different biologically active compounds including nitric oxide (NO), polyamines and citrulline (Morris, 2009). To produce these active compounds, particularly for growth, injury, and stress, arginine is processed through various metabolic pathways. Extracellular arginine can cross the plasma membrane via solute carrier (SLC) proteins in most cells (Fultang et al., 2016)

Arginine plays a role in immune modulation, hormones secretion, insulin response, wound healing, and endothelial function (Albaugh et al., 2017). The supply of arginine is primarily regulated through two enzymes: (1) Arginase, catalyze arginine conversion to ornithine and urea; (2) Nitric oxide synthase, catalyze arginine conversion to nitric oxide and citrulline. The ensuing NO pathway play a role in cellular functions including regulation of adhesion molecules expression, platelet aggregation, immune activation, wound healing, and carcinogenesis. Therefore, as a biologically active dietary compound, arginine has numerous physiological downstream roles (Albaugh et al., 2017; Husson et al., 2003)

Most of the endogenous synthesis of arginine occurs through a collaboration involving the epithelial cells of the small intestine and proximal tubule cells of the kidney (Windmueller & Spaeth, 1981).

The endogenous, or *de novo* synthesis of arginine occurs in many different cell types to a varying degree through the urea cycle from citrulline (a naturally-synthesized amino acid) via

ASS1 or ASL activity (Fernandes et al., 2017). Arginine synthesis happens in a two-step process. First, ASS1 catalyzes L-citrulline and aspartic acid conversion to argininosuccinate. Argininosuccinate will then be converted with the help of ASL into arginine and fumaric acid. Then, arginine will be degraded into L-ornithine and urea by arginase. OTC will then convert L-ornithine back into L-citrulline and recycle back by ASS1 and ASL into arginine (Fernandes et al., 2017; Feun et al., 2008; Riess et al., 2018) **(Figure. 3)**.

Arginine homeostasis depends on endogenous synthesis of arginine and its catabolism (Castillo et al., 1993). Insulin, glucagon, and glucocorticoids are key regulators of urea cycle enzyme's expression in the liver. Arginase II also maintains arginine homeostasis, knockout of arginase II in non-stressed adult mice increases arginine levels in the plasma (Shi et al., 2001).

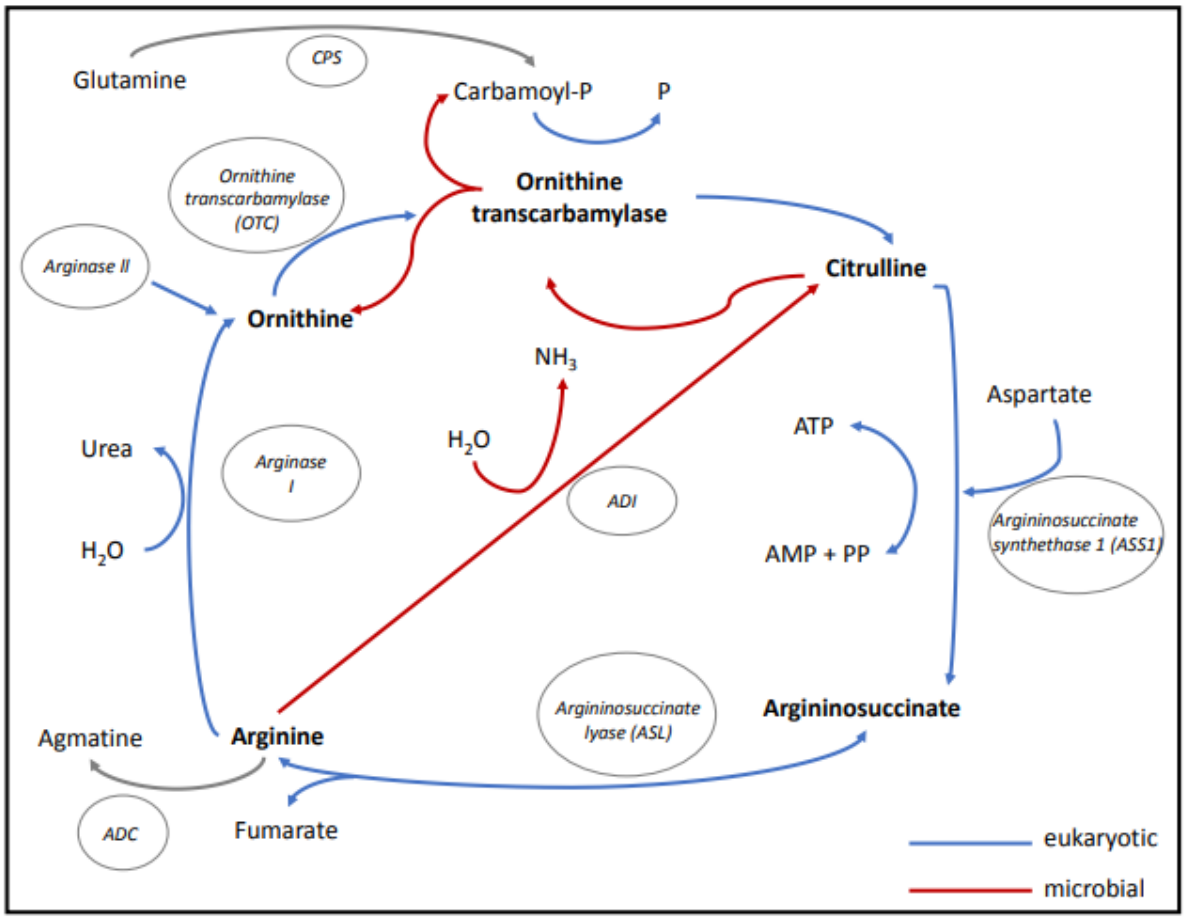

Figure 3. Arginine metabolism and *de novo* synthesis. Arginine is utilized from citrulline in a two-step process that is catalyzed by cytosolic urea cycle enzymes ASS-1 and ASL. Arginase

I then transform arginine into ornithine which is recycled back into citrulline by OTC, and urea that is excreted in the urine. *In vivo* arginine deprivation is achieved experimentally using ADI and HuArgI (Arginase I) by degrading extracellular arginine into citrulline and ornithine. ADI, arginine deiminase; Arg I, arginase I. (Riess et al., 2018).

1.2.4 Cellular Uptake of Arginine via Specialized Transporters

Besides dietary intake and endogenous synthesis, arginine transport through the mitochondrial and plasma membranes is an additional factor for arginine availability (Simon et al., 2003). The proteins involved in arginine transport belong to three gene families called solute carriers (SLC), which are SLC7, SLC3 and SLC6. SLC7 family consists of the CATs and the carrier proteins of the HATs. SLC3 family consists of the HAT glycoproteins. SLC6 family of containing $ATB^{0,+}$. These transporters are well characterized for facility of cellular arginine intake. Many of them function as exchangers rather than uniporters, and they are regulated by the composition of intracellular substrate (Closs et al., 2004). Except for hepatocytes, CAT-1 is expressed in all types of fast dividing cells, and its mRNA levels increase in response to insulin, glucocorticoids, and some tumor growth factors (Durante et al., 2001). Arginine absorption in the intestine is mediated by HATs (Roca et al., 2021) ATB0 are predominantly expressed in ciliated cells (Closs et al., 2004).

Cationic amino acids (CAAs) such as arginine, lysine, and ornithine share the same transport system termed ‘y’. The sodium independent transport system ‘y’, is a complex of different proteins that together mediate CAAs transport (Roca et al., 2021). Cationic amino acid transporters (CATs) are the main route for intracellular arginine. They are widely expressed, and any cell type in the body expresses at least one isoform (Hatzoglou et al., 2004). System ‘y’ activity is regulated by 3 different CATs: CAT-1, -2B, and -3. Failure in CAT-2 induction can limit arginine availability to certain degrees (Aulak et al., 1999).

HATs that are arginine-accepting work as exchangers, in which arginine uptake can only occur in exchange with CAA (Roca et al., 2021).

ATB0, a sodium-dependent transporter of arginine that mediates system $B^{0,+}$ activity. These transporters remove amino acids from the airway lumen contributing to protein clearance. The maintenance of low-nutrient environment thus plays a role in lung defense (Closs et al., 2004).

Arginine transport is regulated by various stimuli in specific subcellular compartments, such as the activation of NMDA receptor which reduce arginine transport by reducing CAT-1 and CATG-3 surface expression (Y. Huang et al., 2007).

1.3 Dysregulation of Amino Acid Metabolism

1.3.1 Amino Acids and Cancer

Metabolic dysregulation in several amino acids were linked to tumorigenesis through varying pathways by action of oncogene and tumor suppressor gene that sustain cell growth through metabolic rewiring. The reprograming of amino acids metabolism in cancer cells contributes to meeting the increased demand for enabling cell proliferation (Tsai et al., 2009). Mutations in mitochondrial and cytosolic key enzymes that lead to upregulation or downregulation of certain amino acids is largely associated with cancer (Hirschey et al., 2015).

Amino acids can be taken up from the tumor microenvironment or synthesized via different reactions inside cancer cells (Lieu et al., 2020). Since amino acid can cross the plasma membrane through transporter systems, their uptake is enhanced in cancer cells. One of the transporters that have broad selectivity to all essential amino acids is SLC6A14, and is upregulated in several epithelial cancers, including colon, cervical, and breast cancers (Gupta et al., 2006).

The catabolism of amino acids promotes the growth and survival of the tumor through the production of metabolic intermediates such as polyamines. Indeed, polyamine synthesis is

initiated by the arginase-mediated conversion of arginine to ornithine, and assists in tumor proliferation and aggressiveness (Pegg, 2009). Understanding the relationship between cancer and metabolic pathways can therefore assist in revealing novel biomarkers and therapeutic targets. Nonessential amino acids are a particularly attractive targets for starvation therapy as they become essential for growth of certain tumors but not that of normal tissues, as the onset of malignancy downregulates the enzyme needed to produce these amino acids. Indeed, studies on glutamine-glutamate, serine-glycine, cysteine, and arginine-proline metabolism revealed enzymes with promise to serve as targets for therapy by revealing the cell metabolic response to starvation (Geck & Toker, 2016).

1.3.2 Arginine Metabolism Enzymes in Cancer

Many tumors have dysregulated expression of enzyme components of the urea cycle, the primary source of nitrogen metabolism. This results in detectable changes of nitrogen metabolites, and profoundly impacts carcinogenesis, mutagenesis, and immunotherapy response (Joo Sang et al., 2018).

Carbamyl Phosphate Synthetases

CPS-I and CPS-II are necessary for carbanyl phosphate synthesis. CPS-I catalyzes the first step of the urea cycle. The localization of CPS-II happens in the cytosol and helps in pyrimidine biosynthesis (Jackson et al., 1986). In non-small cell lung carcinoma, CPS-1 will be suppressed, and its silencing reduces tumor growth, and induces cell death resulting from depletion of pyrimidine (Celiktas et al., 2017).

The synthesis of CPS-I happens mostly in the liver. The presence of CPS-I and ornithine transcarbamylase in the intestinal mucosa can synthesize citrulline, which is a significant location for citrulline production in human (Windmueller & Spaeth, 1981).

Ornithine Transcarbamylase

Ornithine transcarboxylase catalyzes citrulline synthesis from ornithine and carbamyl phosphate. It is expressed in the liver and intestinal mucosa while localizing in the mitochondrial matrix. OTC has low expression levels, making up 0.5-1.0% of the total mitochondrial proteins (Lusty et al., 1979).

OTC deficiency can lead to accumulation of ammonia that cause chronic liver damage, which is a known risk factor for one of the leading causes of cancer-associated deaths: hepatocellular carcinoma (HCC) (Wilson et al., 2012). OTC was found to be downregulated in HCC cells and tissues when compared to primary human hepatocytes. This low expression was associated with increase in tumor size and shorter overall survival time, while its overexpression led to cell proliferation inhibition. This highlights that OTC can provide a potential therapeutic target for HCC (He et al., 2019).

Argininosuccinate Synthestase

Argininosuccinate synthetase (ASS1) catalyzes aspartates and citrulline condensation to form argininosuccinate. In healthy tissues, ASS1 is ubiquitously expressed throughout the body (Delage et al., 2012) with 5-10-fold increase in expression in the liver compared to other tissues (Arthur et al., 1986). ASS-1 mediates citrulline-arginine conversion (Windmueller & Spaeth, 1981). If citrulline is provided, ASS-1 expression in cultured cells allows the cells to grow without arginine. This ability to grow in arginine-free medium was used as a selective growth system for ASS gene expression in cultured cells (Carritt et al., 1977)

The levels of ASS-1 are differentially regulated by various environmental conditions in cancer cells to benefit its progression. (Silberman et al., 2019). Primary tumors form several cancer types has downregulation of ASS-1 expression as compared to normal tissue. Thus, this downregulation supports cell proliferation by enhancing pyrimidine synthesis. Whereas high expression of ASS-1 supports the survival of cancer cells in nutrient-poor environments

(Keshet et al., 2020). Tumors with high ASS1 expression can enhance gluconeogenesis, which allows a metabolic shift towards the synthesis of serine and leading to purine synthesis addiction to sustain growth and proliferation of cancer cells (Villa & Ben-Sahra, 2020). Accordingly, cancer patients with high-ASS1 can be targeted for purine synthesis inhibition (Keshet et al., 2020). In glucose deprivation conditions, ASS1 expression is induced by c-MYC, which assists in survival through the increase in nitric oxide synthesis (Tsai et al., 2009).

Argininosuccinate Lyase

Argininosuccinic acid is converted to arginine and fumaric acid by argininosuccinate lyase (ASL). ASL has a detectable activity in all tissue types, highest present in the liver. ASL deficiency state in due to an autosomal recessive disease, mapped to chromosome 7 in humans (Naylor et al., 1978).

In vivo and *in vitro* tests of ASL downregulation inhibits breast cancer growth. The transfection of ASL shRNA-induced cell inhibition into MDAMMB-231 cells was rescued by exogenous cyclin A2, (a cell cycle related gene) that is reduced with ASL downregulation. ASL induced expression in breast cancer was due to ER stress The cells also underwent autophagy which played a survival role, as its inhibition reduced cell growth (H. L. Huang et al., 2015).

Arginase

Arginase catalyzes the cleavage of arginine into ornithine and urea. Being a source of ornithine it has a role in non-hepatic tissues, important for polyamine synthesis (Hölttä & Pohjanpelto, 1982). Arginase activity should be under constant regulation to assure arginine availability for protein synthesis (Kumar & Kalyankar, 1985; Spector et al., 1983). Arginase has two isozymes present in mammals, arginase I and arginase II. In the 1950s arginase 1 was used to target cancer cells that are arginine auxotrophic (Nurzen, Tuba & Fatih, 2002). Arginase II showed to be highly expressed in prostate cancer by which it helps in immune escape through mediation

of arginine consumption. This results in lower levels of arginine that will weaken and dysfunction the tumor infiltrating lymphocytes (Bronte et al., 2005)

1.4 Autophagy

1.4.1 Definition of Autophagy

Autophagy is the main process by which intracellular renovation can occur (Nakai et al., 2007). It involves the delivery of organelles, macromolecules and other cytoplasmic material to lysosomes (a major degradation system in eukaryotes) where they will be degraded (Chen & Klionsky, 2011) **(Figure 4)**. The dynamic system of autophagy allows the production of new building blocks and helps in maintaining cellular homeostasis and renovation through energy production (Mizushima & Komatsu, 2011). Under nutrient-rich conditions, 1%–1.5% of cellular proteins are catabolized in the liver by autophagy every hour. Autophagy is activated mainly under normal conditions at a basal rate for cellular renewal and homeostasis of different postmitotic cells such as neurons and hepatocytes. Basal autophagy is considered a quality control machinery for the components of the cytoplasm. There is selective and nonselective autophagy. Evidence indicates that the quality control machinery degrading proteins and organelles follows selective autophagy (Johansen & Lamark, 2011; Mizushima & Komatsu, 2011)

In addition, various physiological and pathological conditions can lead to upregulation of autophagy. Stressful environments, such as nutrient deprivation is a major activator of selective autophagy (Menzies et al., 2017). Starvation-induced autophagy occurs in fast growing tumors or when specific nutrients are depleted from the tumor microenvironment (Ravanan et al., 2017)

1.4.2 Mechanism of Autophagy

There are roughly three classes of autophagy divided according to the mechanism of delivery. Macroautophagy, microautophagy, and chaperone-mediated autophagy (Bhat et al., 2018).

Macroautophagy is typically referred to simply as autophagy. Macroautophagy is a multistep process that uses the autophagosome as an intermediate organelle. The autophagosome is formed from a phagophore, which is an isolation membrane that elongates sequestering a portion of the cytoplasm that includes soluble materials and organelles. The autolysosome is then formed autophagosome fuses with the lysosome enabling the degradation of the contained material **(Figure. 4)** (Nakamura & Yoshimori, 2017).

Microautophagy is when the invagination of the lysosome enables the engulfment of cytoplasmic components. This class has similar membrane dynamics to a process that occurs in the late endosome called endosomal sorting complex required for transport ESCRT (Sahu et al., 2011).

Chaperone-mediated autophagy is the third class of autophagy. This type does not include reorganization of the membrane. The proteins will directly be translocated across the membrane of the lysosome.

In the 1990s a series of 35 autophagy related genes (ATG) were identified through genetic studies in yeast (Klionsky et al., 2003; Nakatogawa et al., 2009). The Atg proteins were found to be conserved in mammals, acting in a similar hierarchical manner (Itakura & Mizushima, 2010). Several Atg proteins were found to govern the formation of autophagosome. The core Atg proteins include Atg 1-10,12-14, 16, and 18 (Nakatogawa et al., 2009). Atg core proteins are present in several other pathways related to autophagy including pexophagy, which is peroxisome degradation, and the cytoplasm to vacuole targeting pathway.

Since autophagy is induced by stress and starvation, this leads to a rapid increase in autophagosomes that are near the ER. (Mizushima et al., 2011; Tooze and Yoshimori, 2010).

Chloroquine, an autophagy inhibitor work by inhibiting the fusion between the autophagosome and the lysosome leading to autophagosome accumulation (Pascolo, 2016). Chloroquine can be used as a positive control for autophagosome formation and used as a treatment for cancer by activating apoptosis in tumors where autophagy is used as a cell survival process (Ye et al., 2016).

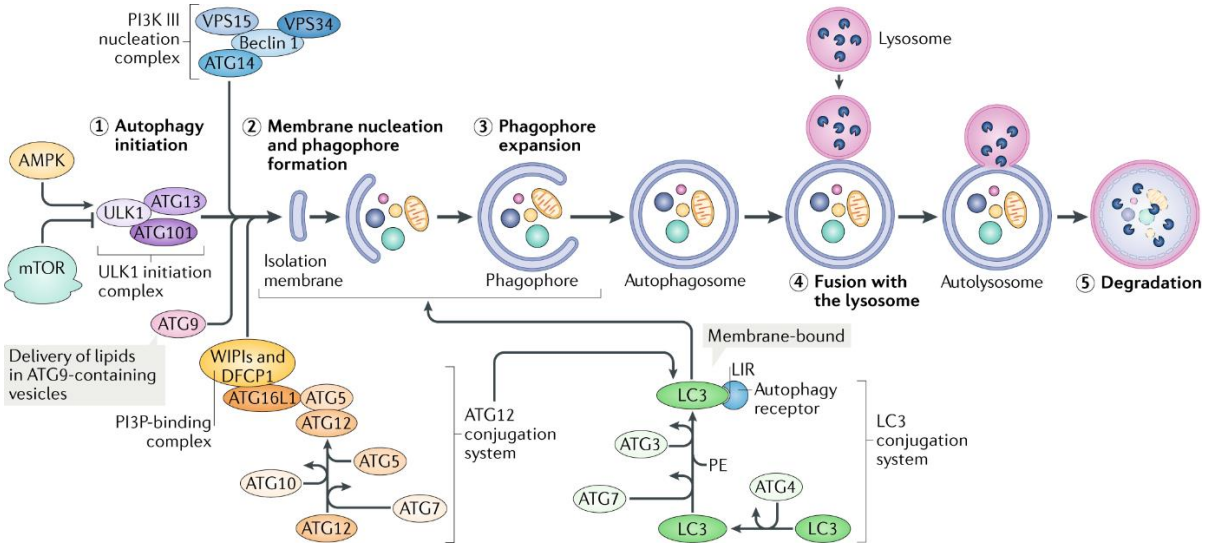

Figure 4. The macroautophagy pathway. (1) Autophagy is initiated from ULK1 initiation complex. (2) Membrane isolation, nucleation and phagophore formation. (3) Expansion of the phagophore to form the autophagosome. (4) Fusion of autophagosome with lysosome, to form the autolysosome. (5) Degradation of cellular materials. Atg12-conjugation (orange) and LC3-modification (green) key role in autophagosome formation. Cytosolic LC3I is activated by Atg7, transferred to Atg3, and converted to LC3II membrane bound form. The LC3 is essential part of the autophagosome and stay associated to it until fusion with the lysosome (Hansen et al.).

1.4.3 LC3

LC3 is a soluble protein called the microtubule-associated protein 1A/1B-light chain 3. Its molecular mass is approximately 17 kDa. In addition to the characterized ATG gene products, LC3 is involved in the two ubiquitylation-like modifications (Kabeya et al., 2003; Tanida et

al., 2001). The conjugation of Atg-12 is important in pre-autophagosome formation, while modification of LC3 is important for autophagosome formation (Mizushima et al., 2002). Atg12-conjugation and LC3-modification are two ubiquitylation-like modifications required during mammalian autophagosome formation.

LC3 has two forms, LC3-I and LC3-II. ProLC3 is first processed to LC3-I, which is the cytosolic form that exposes a carboxyl terminal glycine. Atg7 will activate LC3-I and then its transferred to a second E2-like enzyme Atg3. It is then modified to LC3II that is the membrane bound form **(Figure. 4)** (Tanida et al., 2001). The LC3II will be characterized as an autophagosomal marker as it localizes to the autophagosome. The intra-autophagosomal LC3II is then degraded after the fusion of autophagosome and lysosome through hydrolytic enzymes in the lysosome (Kabeya et al., 2003). The amount of cytosolic LC3I and membrane bound LC3II varies depending on tissue type, cell line and nutrient conditions.

Although these modifications occur independently at the enzymatic level, there is a cross talk between Atg12-conjugation and LC3-modification. Embryonic stem cells of Atg5 deficient mice lacked the Atg12 conjugation and impaired LC3I to LC3II modification (Mizushima et al., 2001). In mammalian cells starvation-induced autophagy is more complicated as Atg8 has three homologues, LC3, GABARAP, and GATE-16. After nutrient deprivation, the three homologues show uniform concentration when they are co-expressed in cultured cells. This suggest that the mammalian homologues undergo lipidation to allow recruitment to the autophagosme (Tanida et al., 2004). This is similar to yeast Atg8, where nutrient deprivation activates Atg8 mRNA transcription. Atg8 protein levels will be elevated and will be processed and conjugated, eventually recruited to the autophagosomal membrane (Kirisako et al., 2000).

Upon starvation LC3I is modified to LC3II and was found to localize on autolysosomal membranes (Kabeya et al., 2003). The GFP-LC3 fluorescence shows the LC3 role in

autophagy. Under nutrient deprivation conditions, the fluorescence is only detected in autophagosomes and autolysosomes (Mizushima et al., 2001). In contrast to the redistribution induced by starvation observed in heart and skeletal muscles, the nutrient deprivation in the brain had no response for LC3-GFP. This means that serum amino-acid levels do not control brain autophagy like it does to hepatocytes (Schworer, Shiffer, & Mortimore, 1981).

The autophagic activity at the cellular level is inversely correlated with malignancy, as autophagy is suppressed in many cancer cells, suggesting a relationship between malignancy and autophagy inactivation (Ogier-Denis & Codogno, 2003). Autophagy is stimulated in glioma cells by TMZ by increasing LC3-positive autophagic vacuoles (Kanzawa et al., 2004).

1.4.4 Advantages and Disadvantages of Autophagy

Cancer cell has a higher metabolic demand in terms of energy and requirement of amino acid compared to normal cells. Under nutrient deprived condition several factors are induced to as metabolic responses, such as autophagy (Kroemer et al., 2010). Autophagy plays a dual function in cancer progression and chemo-resistance of certain tumor types, having both negative and positive roles (Reyjal, et al., 2014; Lorin et al., 2013). Analysis of Ras-expressing cancer cells highlighted the importance of autophagy in TCA cycle metabolites, where conversion of amino acids into TCA intermediates can generate energy (Guo et al., 2011). Furthermore, the deletion of Atg7, an autophagy regulator, in the liver of mutant mice, significantly reduced the levels of glucose in the blood and amino acids after 24 hours starvation (Ezaki et al., 2011), which means that amino acids produced in the liver are needed for gluconeogenesis and the maintenance of the amino acids pool.

Although autophagy acts as a protective mechanism for the cell, it can also coincide with or induce non-apoptosis cell death (Kalil & Abi-Habib, 2019; Nasreddine et al., 2019; Yan et al., 2019). In cancer, the role of autophagy is complex: it can play either tumor suppressing or

promoting roles depending on various tissues. Ras mutated cancer heavily rely on autophagy induction for survival in ovarian cancer (Guo et al., 2011; Mariño et al., 2011). Furthermore, arginine deprivation can lead to cell death caused by autophagy in prostate cancer cells (Changou et al., 2014), ovarian cancer cells (Nasreddine et al., 2020), and pancreatic cancer cells (Khalil & Abi-Habib, 2020).

1.5 Arginine Deprivation

1.5.1 Arginine Deprivation as Cancer Therapy

Disordered arginine metabolism is a characteristic of several malignant diseases and plays a significant role in the development and progression of cancer cells. Arginine affects cell proliferation and carcinogenesis because of increased demand of polyamine and participation in various biosynthetic process (Wu, 2009). Some cancers even showed downregulation in pathways requiring energy that are necessary for arginine biosynthesis. This is in favor of importing arginine from exogenous sources to enable the consistent growth with minimum energy (DeBerardinis et al., 2008).

Arginine auxotrophy (inability to synthesize arginine) is a frequent feature of malignancies in human. It was originally identified in malignant melanoma and HCC, and then found in solid tumors including prostate, breast, pancreatic, small cell lung cancer and several others (Ascierto et al., 2005; Delage et al., 2012; L. Wu et al., 2013). Most of arginine auxotrophic tumors were found to be present at advanced stages, when diagnosed clinically. The underlying molecular mechanism happens through silencing of the enzymes involved in *de novo* arginine biosynthesis. The most common silencing is ASS1 genetic and epigenetic suppression. It is presented usually by promoter methylation of CpG islands (Delage et al., 2012; L. Wu et al., 2013).

A diagnostic point of view is important to determine tumor susceptibility to arginine depleting agents. Failure in expressing either ASS1 or ASL provides a therapy based on depriving cancer cells of arginine. This can render cells that are dependent on extracellular arginine leading to selective tumor starvation. Culturing tumors cells that are arginine auxotrophic in media that lacks arginine can lead to 80% death (Dillon et al., 2004; D. N. Wheatley & Campbell, 2003)

Standard main treatments for cancer include immunotherapy, surgery, radiation, and chemotherapy. In addition, there has been approaches for therapies by arginine deprivation that include dietary restriction of arginine, inhibition of transport and sensing of arginine, and arginine enzymatic degradation (Byus & Byus, 1991; Yeatman et al., 1991).

1.5.2 Arginine Deiminase

The catalysis of arginine decarboxylation to agmatine and carbon dioxide happen through arginine decarboxylase ADC. ADC can lead to arginine depletion in tumor cells and thus inhibiting cell growth in HeLa cells. It can also lead to cell cycle arrest and apoptosis (Patil et al., 2016; Philip et al., 2003; Denys N. Wheatley et al., 2000).

The most frequently used enzymes for therapeutic arginine depletion are recombinant bacterial ADIs. The first arginine deiminase (ADI) was isolated from *Bacillus pyocyaneus* (Qiu et al., 2015). It was subsequently identified in species of many genera such as, *Pseudomonas, Bacillus, Enterococcus, Lactobacillus, Streptococcus,* and *Mycoplasma* (Griswold et al., 2004; Lüthi et al., 1990; Maghnouj et al., 2000; Zúñiga et al., 1998). Most bacterial ADI shows low affinity to arginine, or difficult to apply in humans due to unfavorable pH or temperature.

The most experimentally and clinically characterized ADI is derived from *Mycoplasma*. This is due to its high affinity for arginine with a 1,000-fold increase than human arginase I affinity (Nurzen Sezgin, Tuba Torun, 2002). In addition, *Mycoplasma* ADI is highly immunogenic in humans, and can rapidly be cleared from the circulation due to its short half-life. ADI has been

modified for clinical application by conjugation with almost 16 polyethylene glycol molecules. The modification helps in reducing antigenicity and enhancing the circulatory half-life to 4 hours in serum (Holtsberg et al., 2002). ADI-PEG20 was approved by the US Food and Drug Administration, in March 1999 as a drug for HCC and malignant melanomas treatment (Ni et al., 2008). Autophagy was indicated to be the first stress response observed in tumor cells suffering from arginine depletion. Cells from the breast and prostate cancer showed an autophagy dependent process upon ADI treatment, referred to as mitochondrial dysfunction (Allen et al., 2014; Kim et al., 2009).

Although the data proposes that ADC can be used for arginine depletion, there has been significant drawbacks for it. As for arginase I, non-cancerous cells treated with ADC suffer severe side effects since arginine decarboxylation produces cytotoxic amine agmatine. The pegylation of such therapeutic enzymes increases their serum half-life but abolish ADC activity (Denys N. Wheatley & Campbell, 2002). In addition, tumor cells can re-acquire arginine *de novo* synthesis by re-expressing ASS1 thus becoming resistant to ADI (Bean et al., 2016). This conversion of tumor cells from negative to positive ASS1 expression status correlates with relapse in cancer patients (Kuo et al., 2010).

1.5.3 Human Recombinant Arginase I

Synthesized human arginase 1 (HuArgI) is an arginine deprivation agent. It was synthesized for targeting cancer cell lines that are auxotrophic to arginine. The drug had to undergo several modifications to overcome its limitations. First, it was enhanced by PEGylation, the addition of polyethylene glycol (Harris, 2003). Next, the substitution of the two Mn2+ initially present in human arginase I ions with Co2+ becoming HuArgI(Co)-PEG5000 (Everett et al,, 2011; Nasreddine et al., 2020). The substitution with Co2+ increased serum stability of the enzyme and displayed approximately 12-15-fold lower IC50 value for melanoma and human hepatocellular carcinoma cell death, Consistent with the measured kcat/KM values as it

improves cytotoxicity relative to Mn-hArgI. It is considered a promising new candidate for L-arginine auxotrophic tumors treatment that is even less exposed to the immune system (Everett et al., 2011; Glazer et al., 2011) The use of variants displaying more beneficial pharmacological properties is considered a major step forward in terms of treatment of urea cycle deficient tumors.

Arginine deprivation using HuArgI (Co)-PEG5000 in several ovarian cancer cell lines showed that some exhibit arginine auxotroph when addition of L-citrulline did not rescue the cells after arginine depletion, while others partial auxotrophic upon rescue by L-citrulline (Nasreddine et al., 2020), which means that different cells from the same cancer type can express different characteristics. In addition, treatment with HuArgI (Co)-PEG5000 was able to induce cell death by autophagy (Khalil & Abi-Habib, 2020; Nasreddine et al., 2020). Arginine deprivation was also capable of inducing cell cycle arrest in some cancer cells. Indeed, ovarian and pancreatic cancer cells treated with HuArgI(Co)-PEG5000 underwent C0/G1 cell cycle arrest (Al-Koussa et al., 2019; Khalil & Abi-Habib, 2020; Nasreddine et al., 2020).

1.5.4 Link between Arginine Deprivation and Autophagy

Arginine metabolism has been a target for preclinical and future clinical activity. Deficiency of the arginine synthesizing enzyme *ASS1* with ADI-PEG20 and HuArgI sensitizes tumors to arginine starvation (Allen et al., 2014; Szlosarek et al., 2006). A wide range of hematological and solid cancers are defective in urea cycle enzymes, specifically ASS1 and ASL (Phillips et al., 2013). Arginine withdrawals cause increased rate of protein turnover, by reducing synthesis and increasing breakdown. In addition, arginine depletion leads to induction of caspase-dependent and caspase-independent apoptotic cell death in a cell type-dependent manner (Allen et al., 2014; Kim et al., 2009). In arginine-dependent, auxotrophic prostate cancer cells, ROS-mediated mechanism involved in ADI-PEG20–induced cell death led to mitochondrial damage and chromatin autophagy (Changou et al., 2014). Nowadays, increasing evidence suggests that

early-onset autophagy plays a protective mechanism in arginine-auxotrophic tumor cells, otherwise they can undergo apoptosis via a caspase-independent mechanism (Kim et al., 2009). Growing evidence on the effects of arginine in cancer cells, and how arginine starvation can induce autophagy (Changou et al., 2014).

1.6 Objective

Breast cancer is a group of diseases with considerable inter- and intra- tumoral heterogeneity, ranking it as a complex disease (Curtis et al., 2012). The molecular classification of breast cancer is primarily based on the expression of three receptors: estrogen receptor (ER), progesterone receptor (PR), and ERBB2 receptor (HER2) (Perou et al., 2000), categorizing breast cancer into the 3 major subtypes: (1) Luminal breast cancer (ER and/or PR^+) which represents 70% breast cancer cases; (2) $HER2^+$ breast cancer, which represents 15-20% of cases; (3) Triple negative ($ER^-/PR^-/HER2^-$) breast cancer, which represents 15% of patients (Waks & Winer, 2019). Understanding the biology underlying tumor progression, metastasis, and therapeutic response in each subtype of breast cancer is the first step in discovering novel therapeutic solutions. Recently, L-arginine deprivation has shown therapeutic potential in cancer (Tawnya et al., 2008). Several human cancer cells have been found to be auxotrophic to arginine. More importantly, recombinant human arginase (rhArg) anticancer was reported to have anticancer effects in *in vitro* and *in vivo* models for lymphoblastic T-cell leukemia (Morrow et al., 2013), acute myeloid leukemia (Tanios et al., 2013) and solid tumors (Ziyu Wang et al., 2014).

In this study, our aim is to investigate how HuArgI (Co)-PEG5000, a formulation of human recombinant arginase, affects cell death and autophagic response in three breast cancer cell lines: MCF-7 (a model for luminal A, the most widespread subtype of breast cancer) and UACC-2087 and MDA-MB-231 (models for triple negative, the model aggressive subtype of breast cancer).

Chapter 2

Materials and Methods

2.1 Gene expression analysis in breast cancer patients

The TNMplot database and analysis engine (tnmplot.com) was used to compare normal, tumor and metastatic *ASS-1* expression. The data was generated by gene arrays from (NCBI GEO) or RNA-seq from (TCGA), (TARGET), and (GTEx) (Bartha & Győrffy, 2021). The altered expression between normal breast tissue (N=242), primary breast tumor (N=7569) and metastatic breast tumor (N=82) was analyzed separately using the Dunn's test. In addition, Kruskal–Wallis test was used for simultaneous comparison of ASS-1 mRNA expression.

2.2 Overall and distant-metastasis free survival analysis in breast cancer patients

The Kaplan–Meier plotter was used to analyze the prognostic value of *ASS1* mRNA expression in breast cancer patients through www.kmplot.com. The KM plotter uses gene expression data and survival information of cancer patients that is gathered from Gene Expression Omnibus (GEO), ArrayExpress or other relevant databases (Györffy et al., 2010). Samples were divided into high and low *ASS-1* expression using 'Auto select best cutoff option', and log-rank *p* value were calculated by the software and displayed on the graph (p value < 0.05 was considered as significant).

2.3 Cell lines

Human Breast cancer cell lines UACC-2087 (ATCC CRL-3180), MCF7 (ATCC HTB-22) and MDAMB-231 (ATCC HTB-26) were grown in DMEM (Sigma, UK) culture media with 10% FBS (Sigma-Aldrich), 1% penicillin-streptomycin (L0022, Biowest) 1% Glutamax (Thermo,

USA) and 0.005mg/ml ITS (Gibco, USA) added to the UACC-2087 medium. All cell lines were incubated at 37°C with 5% CO2.

2.4 Autophagosome accumulation assay

Cells were plated at 1.6 million/well in a flat-bottom 6-well plate. Cells were divided into four treatment conditions and treated for 72 hours: control (untreated), 50 nM chloroquine, and 100 nM HuArgI(Co)-PEG5000 with or without chloroquine. Autophagosome labeling was followed according to the company's manual (ENZO product manual - CYTO-ID® Autophagy Detection Kit, 2016 - Catalog No. ENZ-51031 - Part D. Live Cell Analysis by Flow Cytometry). Results were read on a C6 flow cytometer.

2.5 Western blotting

$3.2x10^6$ cells per well were plated in a flat-bottom 6-well plate. Cells were divided into four treatment conditions: control (untreated), 50 nM chloroquine, and 100 nM HuArgI(Co)-PEG5000 with or without chloroquine. Plates were incubated at 37^o C with 5% CO_2 for 72 hours. Cell lysis was performed using 1x Ripa buffer (ab156034) with Protease Inhibitor (Sigma fast, UK) on ice. Lysate clarification was performed by centrifugation at 17000 *x*g for 10 minutes at 4 C, and the supernatant was retained. Protein concentration was determined in a round-bottom 96-well plate using the *DC* protein quantification kit (Bio-Rad, USA) following the manufacturer's protocol. Lysates were heated at 95^o C with addition of 2X laemmli loading buffer (Sigma, USA) and loaded on 12% Gel for separation by SDS-PAGE at 150 V for 120 minutes with (Ab116028) protein ladder, transferred on PVDF membrane (Abcam, UK) at 250 mA for 80 minutes then blocked in 5% fat free milk in tris buffered saline (TBS) with 0.1% Tween (Bio-Rad, USA) for 1 hour at room temperature. Membranes were then incubated overnight at 4 C with rabbit anti-LC3 antibody (ab51520; 1:3000), mouse anti-Actin (3700S; 1:1000) or mouse anti-ASS1 (ab124465; 1:2000), washed with TBST and incubated with

secondary anti-mouse (W402B) and goat anti-rabbit (invitrogen 65-6120; 1:4000) in TBST with 5% milk for 2 hours at room temperature with gentle shaking at 30 RPM. Finally, membranes were washed again and incubated in enhanced chemiluminescence reagent ECL (Amersham Kit) for 2 minutes for imaging using ChemiDoc XRS+ (BioRad Laboratories Inc., UK). Protein quantification was done on ImageJ by obtaining the intensity level of each band and calculating the ratio relative to the loading control.

2.6 Statistical tests

For TNM plot data analysis, the Mann-Whitney U test was used by the TNMplot online platform to detect significant difference in *ASS-1* expression between normal and tumor RNA-Seq data across different tissues. Dunn's test was also used to separately look at expression difference of *ASS-1* in normal-tumor, tumor-metastatic, and normal-metastatic tissues. Kruskal-Wallis was used as non-parametric distribution, with Dunn's modification for multiple comparison between the conditions used in the study.

2.7 Pegylated human recombinant Arginase I

HuArgI(Co)-PEG5000 was produced by Aeaglea Biotherapeutics Inc. (Austin, TX, USA) and obtained as a generous gift from Dr. Ralph Abi Habib (LAU).

Chapter 3

Results

3.1 High *ASS1* expression is associated with poor survival in breast cancer

ASS1 is a rate limiting enzyme for arginine biosynthesis. ASS1 is expressed in many types of human malignancies, and its levels are differentially regulated in various cancer types.

I used the pan-cancer analysis to highlight the expression range of *ASS1* in normal and corresponding tumor tissues from patients. Interestingly, breast cancer showed a significant decrease in *ASS1* expression compared to normal breast tissue **(Figure.5 a)**. Loss of ASS1 can reduce breast tumor ability to make arginine, and consequently highlights the potential for arginase deprivation as breast cancer therapy.

I then sought to investigate how *ASS1* expression varies during breast cancer progression from local to metastatic disease. The TNM plot analysis allowed simultaneous comparison of the normal-tumor-metastatic breast tissue *ASS-1* expression **(Figure.5 b).** This analysis confirmed what we saw in Figure. 5a which is that *ASS-1* mRNA expression decreases in breast tumor vs normal breast. Additionally, this analysis now reveals that *ASS-1* expression again decreases in metastatic compared to primary tumors, which suggested that tumor have the potential to overcome arginine deprivation.

ASS1 upregulation support cell proliferation and is associated with poor prognosis in several common cancer. Given the decrease of *ASS-1* expression during breast carcinogenesis and metastasis, I next investigated the link between this expression and clinical outcomes, namely overall patient survival (OS) and distant-metastasis free survival (DMFS) **(Figure.5 c)** using the open-source KMplot database. In all breast cancer subtypes except luminal B, patients with high ASS-1 expression were more likely to have poorer overall survival and distant metastasis-

free survival compared to patients with low ASS-1 expression. These findings suggest that ASS-1, possibly by increasing arginine availability, could promote tumor progression in breast cancer.

Altogether, gene expression and patient survival analyses indicate that breast carcinogenesis is consistent with poor ASS-1 expression, which represents a therapeutic window for arginine deprivation in this disease. Such therapeutic intervention has the potential to enhance outcomes for subsets of luminal, Her2, and triple-negative breast cancer patients with relatively higher residual ASS-1 expression.

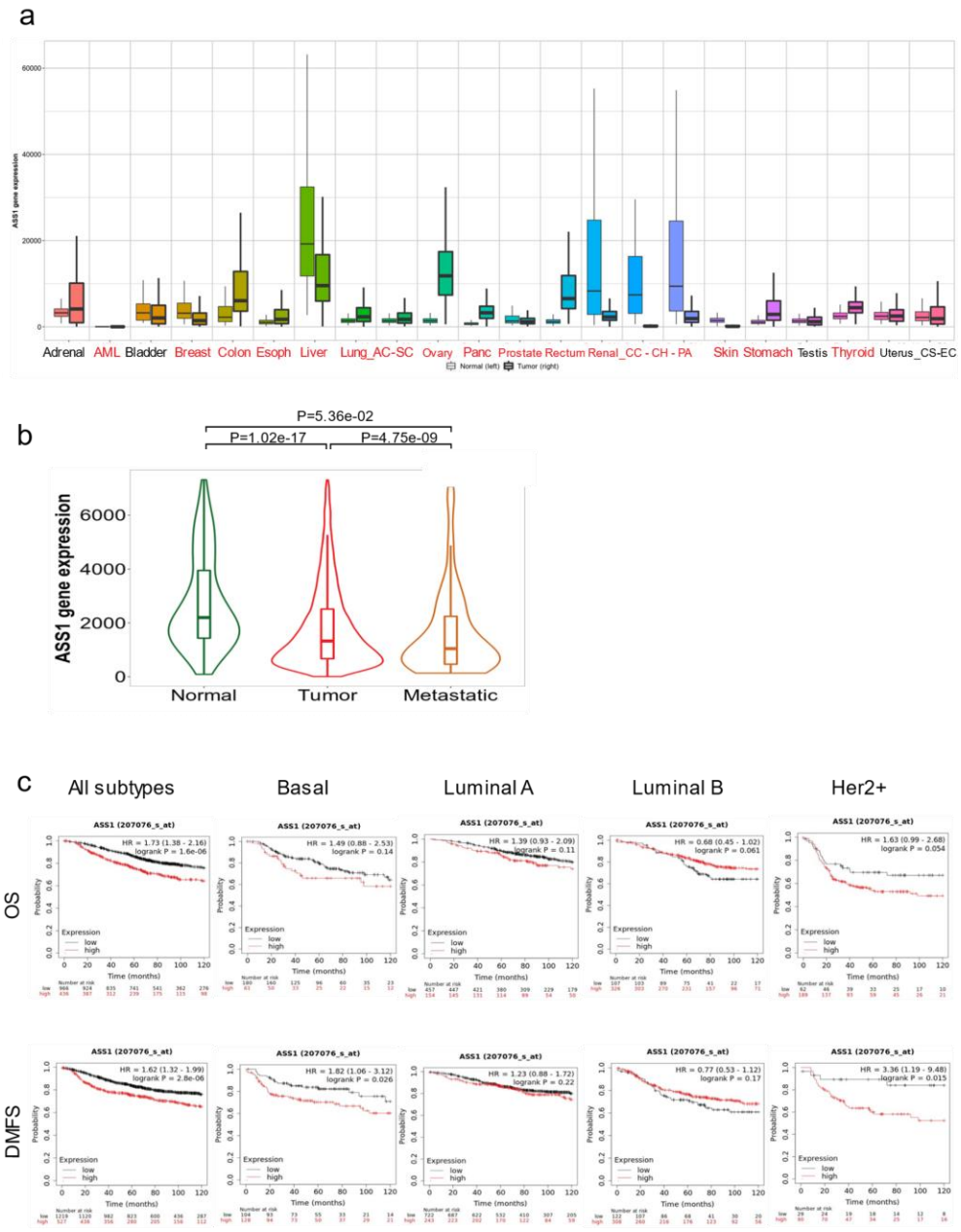

Figure 5. Correlation of ASS1 mRNA expression with breast cancer prognosis. **(a)** Boxplots of *ASS1* mRNA differentially expressed across 22 normal tissues and corresponding tumor tissues (* $p < 0.01$); determined by a Mann–Whitney U test. **(b)** Violin plots representing simultaneous comparison of *ASS1* mRNA expression in normal (N=242), tumor (N=7569) and metastatic (N=82) breast cancer. Statistical comparison was performed for using Mann-Whitney test with Dunn's correction for multiple comparisons. **(c)** Kaplan-Meyer graphs for overall survival (OS, upper panel) and distant-metastasis free survival (DMFS, lower panel) in patients with high or low *ASS1* expressing tumors. Data is shown for all breast cancers combined and for individual subtypes.

3.2 Metabolic stress can regulate ASS1 expression in slow proliferating triple-negative breast cancer cells

Arginine deprivation and inhibition of autophagy are two forms of metabolic stress that tumor cells (especially chemoresistant ones) could overcome by upregulating their ASS-1 expression (Silberman et al., 2019). ASS-1 downregulation makes tumors auxotrophic for arginine (Daylami et al., 2014)

To investigate the effect of arginine deprivation on ASS-1 expression using HuArgI(Co)-PEG5000, we examined ASS1 expression level in control cells and cells treated with 50 nM CQ or 100 nM HuArgI(Co)-PEG5000 or both for 72 hours **(Figure. 6)**. While arginine deprivation (with HuArgI(Co)-PEG5000) and, separately, autolysosome processing inhibition (with CQ) did not cause significant changes in ASS-1 expression, their combination increased ASS-1 expression in MCF-7 (luminal breast cancer) and UACC-2087 (triple negative breast cancer) cells but not in MDA-MB-231 (triple negative breast cancer) cells. This data underscores the existence of metabolic heterogeneity within the triple negative subset, a category of tumors simply grouped by simple lack of expression of three receptors. Additionally, our findings highlight the ability of some luminal (MCF-7) and triple negative breast cancer (UACC-2087) cells to overcome metabolic stress by upregulating ASS-1 when autophagy was blocked. Importantly, the more proliferative, metastatic, and overall aggressive triple negative cells (MDA-MB-231) is incapable of upregulating ASS-1 expression as a response to arginine starvation or autophagy inhibition or both. Together this suggests that the expression of ASS-1 maintained in all cell lines makes them targetable by arginine deprivation as semi auxotrophic cells, relying on extracellular arginine to maintain the high demand of the cell.

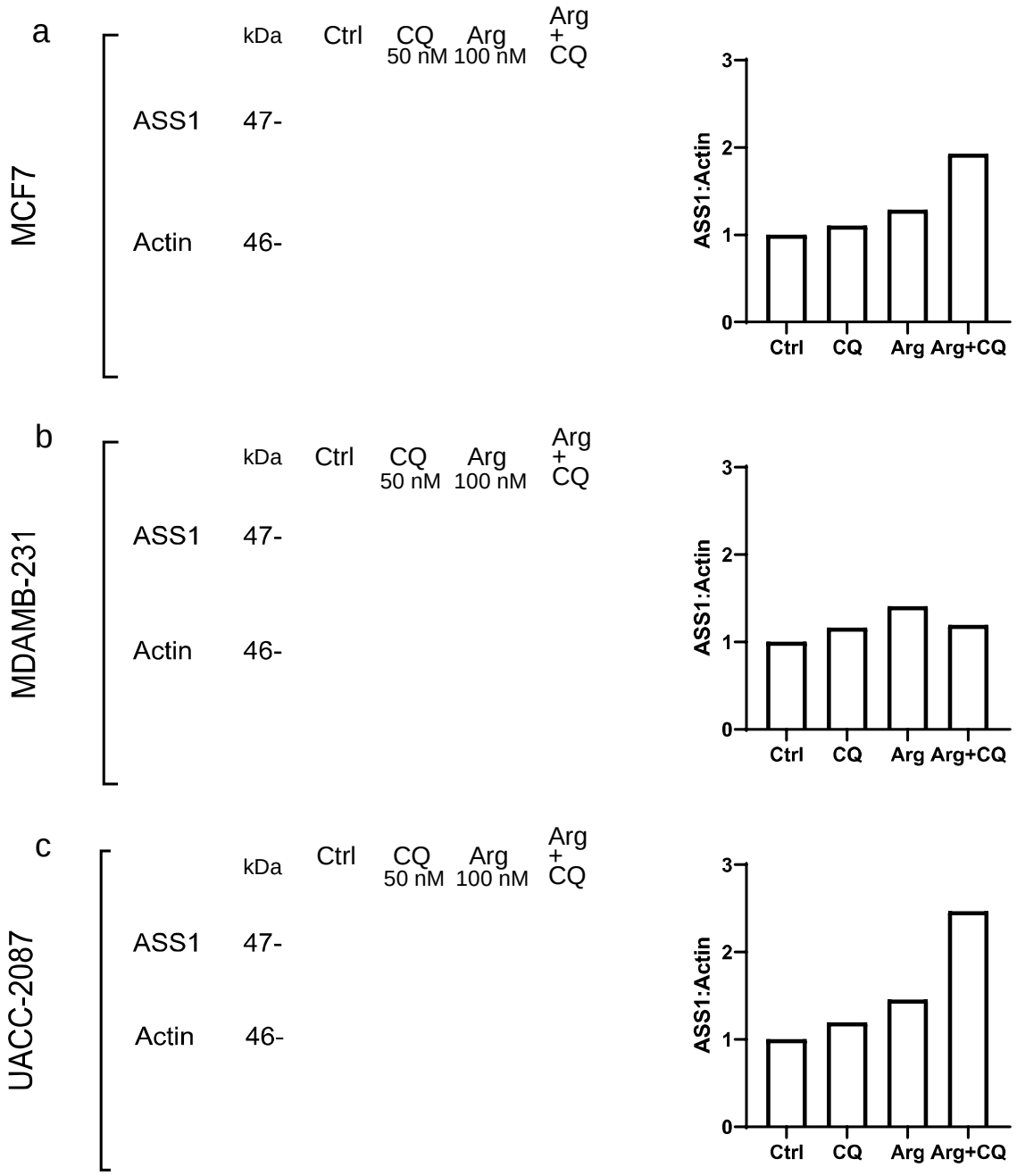

Figure 6. ASS1 protein expression level. ASS1 expression level in MCF7 (A), MDAMB-231 (B), and UACC-2087 (C) untreated, 50 nM of CQ, 100 nM HuArgI (Co)-PEG5000 alone and a combination of both 72 hours post-treatment. Bar graphs represent one independent experiment.

3.3 Arginine deprivation can induce cell line-specific autophagic responses

To investigate a plausible functional link between arginine deprivation and autophagic flux, we treated MCF-7, MDA-MB-231, and UACC-2087 cells with either 50 nM CQ, 100 nM HuArgI (Co)-PEG5000, or a combination of both. At 72 hours post-treatment, autophagy was

probed by two independent methods. We performed Cyto-ID staining followed by flow cytometry which allows to detect autophagosome formation and accumulation **(Figure 7a)**. We also performed western blot analysis of LC3-I conversion to LC3-II, a hallmark of canonical (LC3-dependent) autophagy **(Figure 7b)**. In both assays, CQ treatment, an inhibitor of late autophagosome/autolysosome processing, was used as a positive control for the detection of autophagy **(Figure 7a,b)**. In MCF-7 cells, HuArgI (Co)-PEG5000 treatment was sufficient to increase the percentage of autophagic cells **(Figure 7a)** and the LC3-II/LC3-I ratio **(Figure 7b)** compared to control cells, suggesting an overall activation of LC3-dependent autophagy. In MDA-MB-231 cells, HuArgI (Co)-PEG5000 treatment induced autophagosome formation **(Figure 7a)** and increased the LC3-II/LC3-I ratio when co-treated with CQ **(Figure 7b)** indicating autophagy. In UACC-cells, co-treatment of HuArgI (Co)-PEG5000 with CQ lead to increase accumulation of autophagosomes when compared to CQ alone, indicating activation of autophagy upon treatment with HuArgI (Co)-PEG5000 **(Figure 7a)** and increase in LC3-II/LC3-I ratio **(Figure 7b)**. Autophagy was not detected upon HuArgI (Co)-PEG5000 treatment alone in UACC-2087 due to autophagosomal lysis because of the high autophagic flux and rapid dissociation of autophagosome membrane-bound LC3-II. We have thus demonstrated that arginine deprivation can lead to distinct cell line-specific autophagic responses, ranging from low to high autophagic inductions.

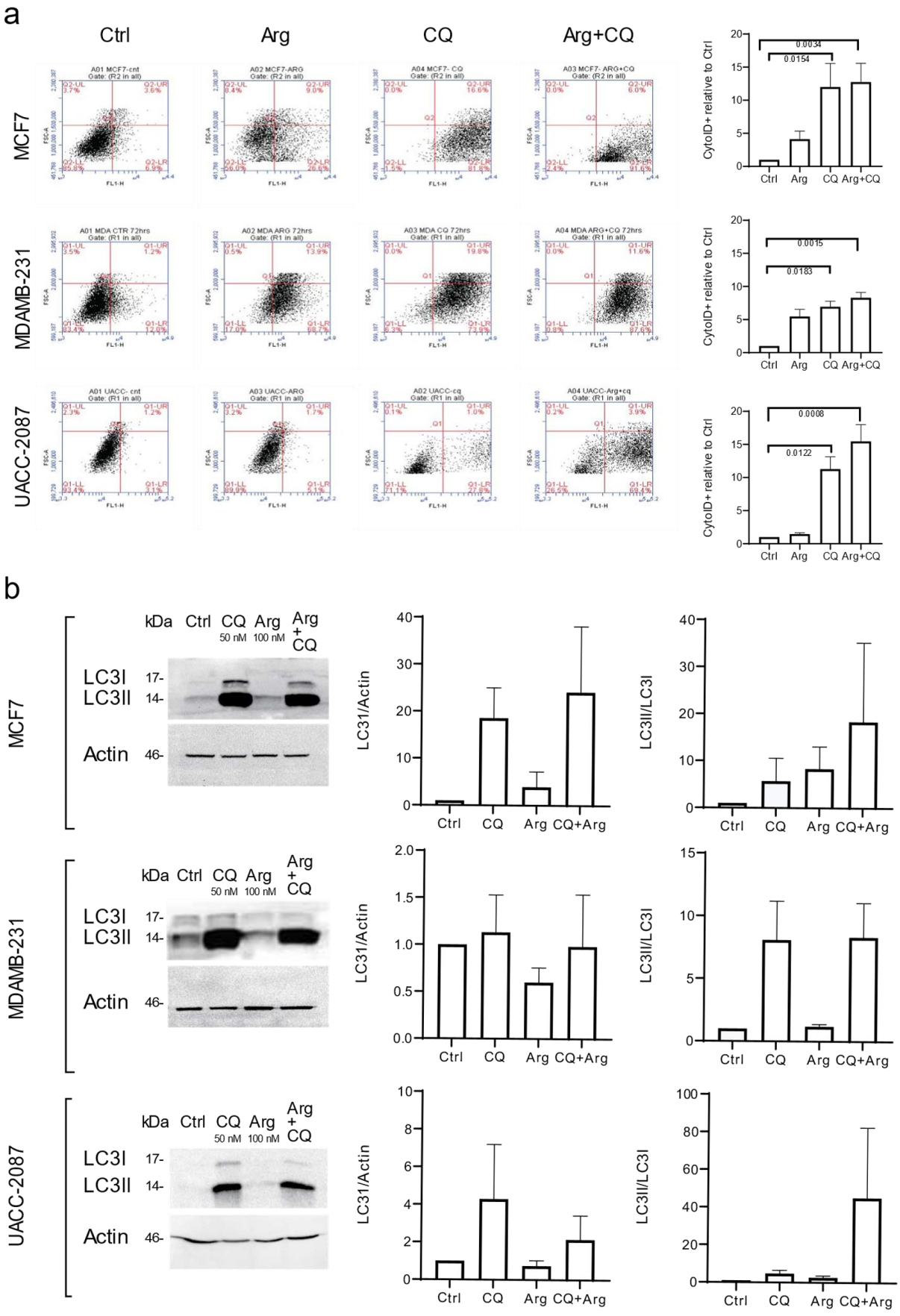
a
Ctrl
Arg
CQ
Arg+CQ
MCF7
MDAMB-231
UACC-2087
CytoID+ relative to Ctrl
0.0034
0.0154
0.0015
0.0183
0.0008
0.0122
b
kDa
Ctrl
CQ 50 nM
Arg 100 nM
Arg + CQ
LC3I
LC3II
Actin
17-
14-
46-
LC31/Actin
LC3II/LC3I
CQ+Arg

Figure 7. Autophagosome formation and LC3-I-to-II processing. (a) Autophagosome formation in MCF7, MDAMB-231, and UACC-2087 untreated cells, 100 nM HuArgI(Co)-PEG5000, 50 nM of cholorquine, and a combination of both HuArgI(Co)-PEG5000 and choloroquine 72 hours post-treatment. (Conditions ordered from left to right). Bar graphs represents the quantification analysis of 5 independent experiments for each cell line with significant p values, **(b)** LC3I and LC3II protein expression through western blot, and their conversion as a marker of autophagy. Bar graphs represent the ratio of LC3: Actin and LC3II:LC3I for each cell line based on 4 independent experiments. Bar graphs was quantified using Kruskal-Wallis test with Dunn's multiple comparison.

Discussion

Metabolic targeting of arginine is a developing novel anticancer therapy with no toxicity, and selectivity in controlling the growth of tumors deficient in arginine biosynthesis (Shuvayeva et al., 2014). Arginine deprivation in tumors induces several stress responses, including autophagy and change in ASS-1 expression. Autophagy, a process of degradation and recycling of cellular components, plays a fundamental role to preserve cell viability upon different stress conditions (Kroemer et al., 2010). One such metabolic stress is amino acid deprivation (Stipanuk, 2009). Deprivation of arginine triggers an autophagic response in tumor cells (Shvets et al., 2008; Viry et al., 2014). Additionally, modulation of ASS-1, a rate limiting enzymes in for arginine synthesis, has a role in responding to metabolic stress. In this work we investigated the effect of arginine deprivation-induced responses including ASS-1 expression and autophagy on 3 breast cancer cell lines (MCF-7, MDA-MB-231, and UACC-2087).

MCF-7, a fast proliferating and non-metastatic cell line was used as a model for luminal A breast cancer. This cell line's response to arginine deprivation included continuation of ASS-1 expression and upregulation of autophagy. From clinical data in lumina A breast cancer, ASS-1 expression correlates with for poor OS and DMFS. Altogether, this data motivates further inquiry into the conceptual framework whereby countering ASS-1 expression by arginase-based drugs in luminal A breast cancer could potentially enhance patient outcomes.

Metabolic stress responses were tested on two triple negative breast cancer models: UACC-2087 and MDA-MB-231. Both cell lines continued to express ASS-1 in response to arginine treatment. UACC-2087, a slow proliferating cell line with unknown metastatic potential, only indicated autophagy in response to arginine deprivation when co-treated with CQ due to high autophagic flux. MDA-MD-231, on the other hand, a fast proliferating and highly metastatic cell line, induced autophagy and was indicated upon treatment with HuArgI(Co)-PEG5000

alone. Interestingly, MDA-MB-231 showed conservation of LC3-I and LC3-II levels under arginine starvation, for which two plausible explanations are possible: (1) MDA-MB-231 cells are undergoing LC3 conversion upon treatment with HuArgI(Co)-PEG5000 but it's not being detected by western blot , as co-treatment with CQ showed increased conversion compared to CQ alone; (2) The kinetics of LC3-I synthesis, LC3-I-to-II conversion, and LC3 degradation are changing at the same rate. The LC3-II degrades inside the autolysosome, by autophagy (Mizushima & Yoshimori, 2007), while LC3-I is deconjugated from the outer membrane and returns to the cytosol (Tanida et al., 2005). This is consistent with previous work on triple negative breast cancer cells, where HuArgI induced increased autophagic flux in response to increased autophagic vacuole formation in MDA-MB-231 cells (Z. Wang et al., 2014). In this context, autophagy played a cytoprotective role in the treatment of HuArgI, as blocking autophagy enhanced HuArgI-induced cytotoxicity. Future *in vitro* and *in vivo* drug testing (e.g. with CQ and HuArgI(Co)-PEG5000) could thus benefit from quantitative assessment of early autophagosome versus late autolysosome dynamics by employing a live pH sensitive autophagy biosensor (mCherry-GFP-LC3 coupled reporter) (Yoshii & Mizushima, 2017).

We demonstrated that arginine deprivation can lead to distinct cell line-specific responses, with autophagy induction ranging from low-high in MCF-7, MDA-MB-231 and UACC-2087 after 72 hours. Our findings suggest a potential therapeutic benefit of HuArgI(Co)-PEG5000 treatment in luminal A and triple negative breast cancers which did not compensate for arginine deprivation by upregulating ASS-1. As MCF-7, MDA-MB-231 and UACC-2087 cells exposed to HuArgI(Co)-PEG5000 had a robust autophagic response, which could convey short-term survival but eventually lead to cell death, combinational treatments that which include autophagy inhibitors such as CQ should also be considered. Indeed, anti-autophagy drugs have shown stronger anticancer effect in chemo-resistant ovarian (Shuvayeva et al., 2014) and brain tumors (Levy, Towers, & Thorburn, 2017).

Although metabolic targeting through deprivation of arginine is nontoxic and selective, some limitations are still present. One such limitation is that upregulation of ASS-1 expression leads to relapse of ASS-1 positive tumors that are insensitive to therapy (Kuo et al., 2010). In addition, we were not able to identify the auxotrophic (or semi-auxotrophic) status of our cell lines by probing ASS-1 expression alone. Future studies including exploration of HuArgI(Co)-PEG500 toxicity and attempts to rescue by L-citrulline can be done to help determine auxotrophy of the investigated cell lines.

Conclusion

Arginine availability is critical to breast cancer progression. Here we show that expression of ASS-1, the key rate-limiting enzyme for arginine synthesis, decreases with oncognesis and metastasis, and residual expression correlates with poor patient outcomes. We also find that treatment of cultured MCF-7, MDA-MB-231 and UACC-2087 breast cancer cell lines with HuArgI(Co)-PEG5000, a pharmacologic formulation of arginase, induces a broad autophagy response without leading to an upregulation of ASS1 expression. Combined with our present findings, future characterization of the impact of HuArgI (Co)-PEG5000-induced autophagy on cancer cell death could motivate therapeutic testing in preclinical models of luminal A and triple-negative breast cancer.

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