The context-specific roles of urea cycle enzymes in tumorigenesis

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Summary

The expression of the urea cycle proteins is dysregulated in multiple cancers, providing metabolic benefits to tumor survival, proliferation, and growth. Here we review the main changes described in the expression of UC enzymes and metabolites in different cancers at various stages and suggest that these changes are dynamic and should hence be viewed in a context-specific manner. Understanding the evolvability in the activity of the UC pathway in cancer has implications for cancer-immune cell interactions and for cancer diagnosis and therapy.

Introduction

In 1932, Hans Krebs and his assistant Kurt Henseleit made the seminal discovery that ornithine, in the presence of ammonium salts, can boost the synthesis of urea when added on slices of animal liver maintained in a buffer that resembles blood composition (Krebs and Henseleit, 1932). They later demonstrated that ornithine and citrulline, in the presence of ammonia, were part of a cycle of reactions that converged on the generation of urea, which was initially called the 'ornithine cycle' and it is now referred to as the "Urea Cycle" (UC). The complete UC operates in the liver, and involves two transporters, five catalytic enzymes, and one cofactor-producing enzyme- N-acetyl glutamate synthetase (NAGS), to convert two nitrogen molecules, into one single disposable molecule of urea (Watford, 2003), (**Figure 1**). The first reaction occurs within the mitochondria, where CPS1 condensates ammonia and bicarbonate into carbamoyl-phosphate. The following step, catalyzed by ornithine transcarbamylase (*OTC*), produces citrulline from ornithine and carbamoyl-phosphate. At this stage, citrulline leaves the mitochondria through the ornithine transporter 1 (ORNT1, *SLC25A15*) to reach the cytosol. Then, through the subsequent cytosolic reactions, citrulline is first condensed with aspartate by argininosuccinate synthase (*ASS1*) to form argininosuccinate. For this reaction, the second mitochondrial transporter citrin (*SLC25A13*), provides ASS1 with the aspartate necessary for the reaction. Argininosuccinate is then broken down by argininosuccinate lyase (*ASL*) into arginine and fumarate. Finally, in the last step, arginine is hydrolyzed into urea and ornithine by *ARG 1*, regenerating the ornithine necessary to start another round of the cycle (Morris, 2002) (**Figure 1**). The generation of fumarate by ASL and the utilization of the aspartate derived from the mitochondria by ASS1 metabolically integrate UC with the tricarboxylic acid (TCA cycle).

UC enzymes account for the disposal of 85-95% of the waste nitrogen as urea in the urine (Morris, 2002). After birth, outside the liver, the expression of *ASS1* and *ASL* enables the kidneys to become the leading site for systemic *de novo* arginine generation through absorption of the citrulline produced by the small intestine (Ligthart-Melis et al., 2008). Still, when exposed to arginine deprivation, most cells can independently synthesize arginine *via* the arginine-citrulline cycle (van de Poll et al., 2007). In this cycle, arginine can be converted back to citrulline by Nitric Oxide (NO) Synthase (NOS), together with NO release. Arginine can also be used as a substrate to serve the cellular needs for multiple other metabolites such as agmatine, proline, ornithine, and polyamines (Nagamani et al., 2012).

The ARG enzyme competes with NOS over arginine and regulates both NO and ornithine levels. It exists in two forms; type 1 is a cytosolic form, highly expressed in the liver, and is

thought to be primarily involved in ureagenesis. Type 2 is the mitochondrial form, which is more widely expressed and involved in ornithine biosynthesis (Cederbaum et al., 2004). Thus, in extrahepatic tissues, the expression and composition of UC enzymes vary substantially.

The essentiality of arginine and its downstream metabolites to normal physiology necessitates several levels of regulation. Indeed, decreased arginine availability and increased cellular needs for its metabolites can upregulate the expression of the arginine-citrulline enzymes ASL and ASS1. ASL expression was also demonstrated to be regulated *via* lysine acetylation, which inhibits ASL activity; while amino acid supplementation decreased ASL acetylation, the addition of glucose increased it (Zhao et al., 2010). Recently, it was shown that the CLOCK protein directly acetylated and inactivated ASS1 *in vitro* and *in vivo*. ASS1 acetylation by CLOCK exhibits circadian oscillation in human cells and mouse liver, possibly caused by rhythmic interaction between the two proteins (Lin et al., 2017). Hence, it is logical to suggest that the essentiality of UC metabolites at different cellular states, the circadian rhythm, and glucose levels, all contribute to the canonical regulation of UC enzymes' expression in normal cells.

Undoubtedly, the ultimate proof for the necessity of the UC enzymes and metabolites to a healthy state is the severe clinical presentation of children with a malfunctioning UC due to germline mutations (Erez and DeBerardinis, 2015). In the most detrimental clinical scenario, these children can present with brain edema and even death due to ammonia accumulation (Erez et al., 2011b). Furthermore, the chronic remaining long-term complications in children with UC disorders even after liver transplant highlight the crucial roles of UC enzymes outside the liver (Erez et al., 2011; Lerner et al., 2019).

Recently, it became evident that alterations in the expression of the UC genes are also actively involved in tumorigenesis. Indeed, by supporting anabolic routes, favoring the appearance and selection of specific tumor mutagenic profiles, and affecting the activation of the immune response, alterations of UC were shown to drive cancer development and progression and affect the response to therapy (Keshet et al., 2018; Lee et al., 2018a; Lee et al., 2018b).

In this review, we summarize the roles of UC enzymes in cancer, highlighting the dynamic regulation of their expression in different tumor types and during cancer progression, and the potential diagnostic and therapeutic implications.

Alterations in expression of the UC genes and metabolites in cancer

Over the years, it was demonstrated that some genes of the UC are either overexpressed or silenced in different cancer types (**Figure 2 and Table 1**). These dysregulations were shown to benefit carcinogenesis by changing the availability of UC-related metabolites. Firstly, ammonia is commonly accumulated in cancer upon different types of dysfunction of UC enzymes. In human HepG2 derived cell lines, both UC enzymes ARG1 and OTC malfunctioning result in excess ammonia levels (Mavri-Damelin et al., 2007). Similarly, acute lymphocytic leukemia (ALL) cells also show downregulation of OTC expression (Fultang et al., 2016). Intriguingly, in contrast to the toxic effect of excess ammonia under normal physiological conditions, cancer cells can utilize ammonia and recycle it for amino acid and nucleic acids synthesis providing building blocks for proteins and nucleotides that support tumor proliferation (Spinelli et al., 2017) (Spinelli et al., 2017; Tardito et al., 2015).

A few recent papers demonstrated *CPS1* upregulation in several cancer types, including stomach, melanoma, sarcoma, B-cell lymphoma, lung, glioma, and glioblastoma (Lee et al., 2018a). High CPS1 levels were further shown to sustain tumor proliferation by increasing the synthesis of its product carbamoyl-phosphate. In non-small-cell lung cancer (NSCLC), defective for LKB1 (Liver kinase B1, gene *STK11*) and mutated in *KRAS, CPS1* upregulation generates an excess amount of carbamoyl-phosphate, which spills to the cytosol where it is used to boost pyrimidine biosynthesis *via* carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase (*CAD*) trifunctional protein. Silencing of *CPS1* in these cells resulted in cell death *in vitro* and inhibited tumor growth *in vivo* due to the depletion of pyrimidine (Kim et al., 2017). Moreover, the silencing of *CPS1* synergizes with erlotinib, an EGFR inhibitor, in suppressing NSCLC cells proliferation. The combined approach was shown to impair pyrimidine biosynthesis, glycolysis, cellular respiration, creatine, and polyamines production (Pham-Danis et al., 2019). Notably, the silencing of other UC genes such *ARG2*, *OTC,* and *ASS1* was also found to sensitize Non-small cell lung cancer (NSCLC) to EGFR inhibitors, suggesting that other intermediates of the UC may be essential for the proliferation of NSCLC. Beyond NSCLC, *CPS1* upregulation, together with *OTC* and *ARG1,* was linked to p53 loss in different cancer cells (Li et al., 2019), providing them with improved capability to maintain a high rate of polyamine biosynthesis.

Another UC enzyme found to be upregulated in cancer is citrin (Lee et al., 2018a; Rabinovich et al., 2020). In ASS1-deficient osteosarcoma cells, the silencing of citrin restricts aspartate availability for pyrimidine biosynthesis and, at the same time, reduces the mTOR-dependent activating phosphorylation of CAD and restricts proliferation (Rabinovich et al., 2015).

Additional studies in melanoma and osteosarcoma cells revealed that citrin could control not only other metabolic pathways, including glycolysis, OXPHOS, but also mitophagy, and invasiveness through intra-organelle regulation of NAD/NADH ratio (Rabinovich et al., 2020). The observation that citrin is upregulated in multiple cancer types, independent of ASS1 expression, provides further evidence for cancer dependence on the endogenous synthesis of aspartate (Birsoy et al., 2015; Sullivan et al., 2015). It is likely that in cancers with high ASS1 expression the increased usage of endogenous aspartate could result in increased dependancyon Glutamic-oxaloacetic transaminase, GOT1/2 for aspartate synthesis.

Other UC enzymes reported to be upregulated in specific tumor types are ASL and ASS1 for the synthesis of *de novo* arginine. High ASL levels are present in a few tumor types, including colon, liver, and breast, and are associated with poor survival (Gong et al., 2019; Huang et al., 2015, 2017; Huang et al., 2013b). In liver cancer, the silencing of *ASL* resulted in decreased proliferation and induction of apoptosis through BAX activation. Differently from ASL, high ASS1 levels were observed in a more significant cohort of cancers, including ovarian, thyroid, pancreatic, colorectal, gastric, esophageal, lung, cervical and squamous cell carcinoma (Bateman et al., 2017; Delage et al., 2010; Keshet et al., 2020; Shan et al., 2015; Szlosarek et al., 2007; Tsai et al., 2018). The upregulation of ASL and ASS1 in cancer is expected to increase NO's cellular availability *via* the arginine-citrulline cycle. In cancer, NO exerts both pro and anti-tumorigenic effects; e.g., it controls the activation of angiogenic (Jenkins et al., 1995), mitogenic (ERK, EGFR), and oncogenic signaling cascades (mTOR and WNT, EMT,p53), but it can also restrict tumor progression, activate apoptosis and the immune response against cancer cells (Albaugh et al., 2017; Keshet and Erez, 2018). We have shown that ASL expression in enterocytes alleviates colitis and that its deficiency decreases NO levels and consequently increases the severity of inflammation, contributing to inflammation-associated colon cancers (Stettner et al., 2018). Furthermore, we recently demonstrated that high *ASS1* expression contributes to cancer survival under glucose deprivation *in vitro* and *in vivo* due to increasing cGMP-mediated NO signaling and promoting gluconeogenesis by S-nitrosylation of PC and PCK2 enzymes (Keshet et al., 2020).

Conversely, multiple other tumor types and hematological malignancies display reduced expression of ASS1 and, to a lesser extent, of ASL, including myxofibrosarcoma, hepatocellular carcinoma (HCC), bladder cancer, ovarian cancer, and pancreatic cancers (Allen et al., 2014; Cao et al., 2019; Delage et al., 2010; Huang et al., 2013a; Kelly et al., 2012; Kim

et al., 2020; Syed et al., 2013; von Stechow et al., 2013). Furthermore, the expression of both *ASS1* and *ARG2* is lost in clear cell renal cell carcinoma (ccRCC), favoring tumor growth by preserving pyridoxal from consumption and avoiding the accumulation of polyamines (Ochocki et al., 2018). Another major metabolic consequence of the downregulation in ASS1 is the increase in the availability of its substrate aspartate to promote the activation of CAD, thus facilitating pyrimidine synthesis and supporting cancer proliferation (Rabinovich et al., 2015). Indeed, in different cancer types, the downregulation of ASS1 correlates with high proliferation, increased metastasis and worse prognosis (Kobayashi et al., 2010) (Allen et al., 2014) (Tan et al., 2014) (Tao et al., 2019).

Via ARG-mediated production of ornithine, the UC is intertwined with polyamine biosynthesis affecting proliferation, survival, DNA and protein stability in cancer cells (Casero et al., 2018). Indeed, ARG1 and ARG2 were both found to be expressed at different levels in prostate cancer cells, and their expression seems to be stimulated by androgens (Gannon et al., 2010; Mumenthaler et al., 2008; Weis-Banke et al., 2020). Silencing *ARG1* and *ARG2* in the prostate cancer line LNCaP impaired their proliferation without affecting NO levels (Gannon et al., 2010). *ARG1* upregulation has also been associated with poor prognosis in colorectal cancers (Krzystek-Korpacka et al., 2020; Ma et al., 2019), while *in vitro*, *ARG1* overexpression in HCC cells increased both cell migration and invasion through triggering of the epithelial-to-mesenchymal transition (EMT) (You et al., 2018). ARG2 was found upregulated in different tumor types, including breast basal-like tumors and occasionally in $Her2^{+}$ cancer cells (Roci et al., 2019), malignant follicular thyroid lesions (Cerutti et al., 2006), gastric adenocarcinomas (Takenawa et al., 2004), and acute myeloid leukemia (AML) (Mussai et al., 2013). In an elegant work by Roci et al., it was demonstrated that arginine uptake and ornithine synthesis are among the main metabolic reactions differentially active during the G_2/M phase in cancer cells compared to non-transformed cells. Intriguingly the knockdown of *ARG2* in these cells impairs polyamine synthesis and arrests proliferation in G_2/M (Roci et al., 2019). In a pancreatic cancer mouse model, *ARG2* loss was found to push the diversion of excess nitrogen towards producing other nitrogenous metabolites such as creatine, creatinine, aspartate, and glutamine (Zaytouni et al., 2017). Furthermore, in a mouse model of pancreatic cancer, the obesity-driven expression of *ARG2* and *ASS1* enables tumors to maintain low ammonia generation even in the presence of a high glutaminolysis rate (Zaytouni et al., 2017).

While the expression of some UC enzymes can be either upregulated or downregulated in different or even the same cancers, the expression of OTC and CPS1 was uniquely described

to be downregulated or upregulated, respectively. This is likely because both these changes increase the levels of carbamyl-phosphate. Similarly, citrin expression is mostly upregulated and is a prevalent phenomenon in many cancers to increase the availability of cytosolic aspartate. Notably, both cytosolic carbamyl-phosphate and aspartate promote pyrimidine synthesis by CAD. The observations that ARG and ASS1, and less often ASL, can be either upregulated or downregulated in cancers can support the notion that their expression is regulated by NO levels. Such flexibility in the expression of these enzymes occurs mainly in cancers originating from blood, epithelial or immune cells, likely because these cells need to dynamically regulate the expression of these enzymes in normal physiology. By analyzing the cancer genome atlas (TCGA) database, we further found in cancers a prevalent simultaneous dysregulation in expression of multiple UC enzymes that promotes pyrimidine synthesis by increasing UC metabolites availability for CAD enzyme, thus supporting cancer proliferation (Lee et al., 2018a).

Regulation of UC enzymes expression in cancer

The mechanisms for regulating the expression of UC enzymes are mostly shared between cancer types and seem to be dictated by the nutrient availability in the tumor microenvironment (**Figure 2 and Table 1**). Overall, the regulation of UC expression in tumors maximizes substrate availability for enhancing proliferation.

Increased expression of UC genes in cancer cells can be obtained through different molecular strategies. In NSCLC, it was demonstrated that *LKB1* loss removes the AMPK-mediated repression on FOXA1 binding to the *CPS1* gene (Kim et al., 2017). Additionally, silencing of neurotensin (NTS), IL6, or IL6 signal transducer (IL6ST) leads to suppression of *CPS1* in the same cells (Yue et al., 2021). p53 is also involved in the regulation of *CPS1*, *OTC*, and *ARG1* in colorectal, hepatic, and prostate cancer cells in which *TP53* is mutated or lost (Li et al., 2019). Of note, p53 was also shown to upregulate *ASS1* expression in multiple cancer types (Miyamoto et al., 2017). Other works proposed that *ARG* expression is under the control of some oncogenes such as RAS (Roci et al., 2019), and AKT (Zaytouni et al., 2017) and, as mentioned above, under the regulation of the androgen receptor in prostate cancer (Gannon et al., 2010).

In most cancer types such as myxofibrosarcomas, mesothelioma, ovarian cancer, bladder cancer, and brain tumors such as GBM, downregulation of *ASS1* and *ASL* is attributed to promoter hypermethylation (Allen et al., 2014; Huang et al., 2013a; Nicholson et al., 2009;

Syed et al., 2013; Szlosarek et al., 2006). In addition to the selection of ASS1 downregulation to promote pyrimidine synthesis and support cancer proliferation, under hypoxia, *ASS1* downregulation is controlled by a HIF1a-mediated induction of miR-224-5p, leading to increased levels of the upstream alkaline metabolites, ammonia, and glutamine. Thus, following a hypoxia-induced acidic environment, ASS1-depleted cancer cells can maintain a higher intracellular pH by increasing the levels of alkaline metabolites as glutamine and glutathione, which provide them with a survival advantage (Silberman et al., 2019).

Yet, arginine deprivation, either by nutritional starvation or exposure to arginine-depleting agents, induces an adaptive transcriptional upregulation of ASS1 and ASL in multiple tumors such as GBM, melanoma, and bladder cancer in *ex-vivo* cultures and cell lines, resulting in tumor resistance to arginine depleting treatment (Syed et al., 2013; Tsai et al., 2009; Yeon et al., 2018). More recently, the concept that epigenetic reprogramming linked to nutrient availability is responsible for regulating ASL and ASS1 expression levels has emerged. Indeed, in arginine-deprived glioblastoma multiform cells, *ASL* and *ASS1* are upregulated, likely through a DNA demethylation-mediated process since an artificial gain of methylation at the genetic locus of these genes prevents their activation by nutrient deprivation (Syed et al., 2013). Differently, in AML cells, arginine deprivation induces *ASS1* through activation of ATF4/ CEBPβ axis and requires chromatin remodeling (Crump et al., 2021), while glucose deprivation drives *ASS1* expression in a c-MYC-dependent way (Keshet et al., 2020). Finally, Long et al. showed that glutamine and arginine starvation indirectly boost *ASS1* expression relieving the transcriptional suppression mediated by HIF1A (Long et al., 2017).

It has been shown that arginine deprivation can induce an adaptive transcriptional upregulation of both ASS1 and ASL, for example, in glioblastoma multiform *ex vivo* cultures and cell lines (Syed et al., 2013). In addition, concurrent methylation in the CpG islands of both ASS1 and ASL was demonstrated in a subset of glioma cases, indicating that they are not mutually exclusive (Syed et al., 2013). The contrast between the multiple reports of changes in ASS1 expression in different cancers, and the scarce reports of cancers with changes in ASL expression, could hence imply that perhaps in comparison to ASS1, ASL basal expression level is more essential for cancer cells.

The multiple signaling molecules, cytokines and transcription factors that regulate UC enzymes expression in addition to nutrient availability, further support the notion that their expression is tightly regulated in a context dependent manner.

Tumor UC alterations have therapeutic implications

The observations that alterations in tumor UC affect metastasis formation suggest that changes in the expression of UC enzymes in cancer cells can modify the tumor microenvironemt (TME) finally affecting the immune system and metastasis initiation . In this context, several papers have shown that dysregulation in the expression of UC genes in cancer can affect arginine levels in the microenvironment and alter immune cells' activation. For instance, ovarian cancers release small extracellular vesicles containing ARG1, which inhibits T-cell proliferation (Czystowska-Kuzmicz et al., 2019), while the lower expression of *ARG2* in a subgroup of head and neck squamous carcinoma positively correlates with the infiltration of both $CD11c^+$ myeloid dendritic cells and $FOXP3^+$ T-cells (Bron et al., 2013). Similarly, in prostate cancers, androgen suppressive treatments boost T-cell activation and IFN-γ secretion through downregulation of ARG2 levels in the tumors (Gannon et al., 2010). Finally, AML cells that express high levels of *ARG2* release the enzyme both in the blood and *in vitro* leading to suppression of T-cell proliferation (Mussai et al., 2013). Since immune cells depend on arginine for their activity and survival, changes in the tumor-UC enzymes can consequently regulate the immune response against cancer (Geiger et al., 2016; Mussai et al., 2015; Mussai et al., 2019).

Beyond direct effects on immune cells' proliferation, activity, and survival, it has been demonstrated that cancer cells with dysregulated UC indirectly activate alternative strategies to evade the immune system, with therapeutic implications. For instance, *ASS1* highly expressing cells gain a purine-rich mutational bias signature that generates less immunogenic tumor-antigens (Keshet et al., 2020). In contrast, several UC changes occurring together in other cancers were found to augment pyrimidine synthesis and generate a unique genomic signature at the DNA, RNA, and protein levels. Intriguingly, inducing an imbalance between pyrimidine and purine pools in favor of pyrimidine for example by using a purine inhibitor, leads to an increased number of transversion mutations where purine is replaced by pyrimidine. This mutational bias is associated with increased numbers of hydrophobic and more immunogenic tumor antigens and a consequent better response to immune checkpoint inhibitors, independent of mutational load (Lee et al., 2018a).

Interestingly, Zhang et al. used multi-omic analysis to show that within HCC tumors, three different clusters of the distinct metabolic landscape were found, each harboring a unique immune response (Zhang et al., 2019). Such heterogeneity in enzymatic expression including the UC within the same tumor, can be explained by changes in the availability of different metabolites in different regions within the tumor, leading to various epigenetic manifestations.

Thus, the nutrient milieu can impose a phenotypic outcome beyond the cell-intrinsic metabolic preferences (Kim and DeBerardinis, 2019).

Another UC related therapeutic approach against cancer is to directly target the dysregulated UC enzyme, such as the upregulation of CPS1. Very recently, Yao et al. presenteda novel CPS1 inhibitor, H3B-120, that restricts pyrimidine biosynthesis in cancer cells that express high CPS1 levels (Yao et al., 2020). In the case of NSCLC cells refractory to standard EGFR inhibitors, the inactivation of *CPS1* synergizes with gemcitabine, pemetrexed, and AZD7762 treatments (Celiktas et al., 2017).

An alternative approach focuses on the secondary metabolic effects that UC dysregulation imposes on the surrounding cells. In this context, the dysregulated activity of some UC enzymes within the tumors that induce depletion of arginine from the tumor niche limits T-cells activation (Crump et al., 2021; Grzywa et al., 2020). To increase arginine availability and consequently to boost immune cell activation, it has been recently proposed to develop vaccines containing ARG2 epitopes (Weis-Banke et al., 2020). A few arginase second-generation inhibitors, with improved pharmacokinetics and efficacy, are currently tested *in vitro* and *in vivo* to treat cancers alone or in combination with other drugs (Grzywa et al., 2020). In addition, loss of function of ASS1 in cancers abolishes endogenous arginine synthesis, making the tumors arginine auxotrophic, i.e., depend on external supplementation of arginine. This discovery led to the emergence of therapies that can induce arginine deprivation selectively in cancer cells that do not express ASS1, in contrast to non-tumor cells that express ASS1 and synthesize arginine. A promising agent in advanced clinical trials is the mycoplasma-derived arginine deiminase (ADI-PEG20), a cloned arginine degrading enzyme conjugated with polyethylene glycol (PEG), (Alexandrou et al., 2018; Ji et al., 2020; Thongkum et al., 2017), alone or in combination with other drugs. For instance, Thongkum et al. found in an *in vitro* model of HCC that a combination of ADI-PEG20 with 5-fluorouracil (5-FU) affects pyrimidine synthesis, leading to tumor cells' apoptosis (Thongkum et al., 2017) while DNA damage inducing apoptosis was achieved when Kim et al. tested *in vitro* the usage of ADI-PEG20 with histone deacetylase inhibitor (HDACi) in a PDAC model (Kim et al., 2020). Additional evidence was provided by Hall et al. that conducted a phase-1 trial for high-grade glioma (HGG) patients combining ADI-PEG20, Cisplatin, and Pemetrexed, thinking that ADI-PEG20 disrupts pyrimidine pools in ASS1-deficient HGGs, thereby augmenting the sensitivity to the antifolate, Pemetrexed (Hall et al., 2019). Harding et al. described a successful

trial for patients with HCC and other gastro-intestinal malignancies combining treatment with ADI-PEG20 and modified FOLFOX6 (Harding et al., 2018) whileChang et al. combined ADI-PEG20 with anti-PD-1 immune checkpoint blockade in various advanced solid tumors and reported in a phase- 1b trial, encouraging results, further supporting the metabolic cross talk between cancer and immune cells (Chang et al., 2018). Another agent used to generate arginine deprivation is pegylated human arginase-1 (rhArg1peg5000, BCT-100). BCT-100 was preclinically tested and showed efficacy against ASS1-low solid and hematological cancers, as well as in several phase-1 clinical trials ((De Santo et al., 2018; Yau et al., 2015). ADI and human arginase (hArg) are currently the two major protein drugs undergoing clinical trials to induce arginine depletion as a strategy against cancer (Zhang et al., 2021). Both drugs share side effects like increased immunogenicity and a relatively short half-life, features that were alleviated with PEG usage. Although both agents deplete arginine levels, hArg and ADI play different roles in arginine metabolism, and hence the strategy of using these two enzymatic agents on cancer treatment varies. While ADI directly catalyzes arginine to citrulline and ammonia, hArg hydrolyses arginine to ornithine. Thus, for the re-generation of arginine after therapy, following ADI, recycling of accumulated citrulline to arginine requires expression of ASS1 and ASL, while following hArg, there is an additional requirement of OTC for the conversion of ornithine to citrulline. Indeed, compared to ADI, hArg displayed anti-cancer properties in cancers with OTC- negative expression even when ASS1 was expressed (Cheng et al., 2007). These findings indicate that choosing one drug over the other in the clinic should be based on expression profiles of cancers, especially for ASS1 and OTC (Zou et al., 2019).

Recently, an additional arginine depleting agent, Arginine decarboxylase (ADC), showed promising anti-cancer effects (Philip et al., 2003). ADC exists in the mammalian liver and is responsible for catalyzing the conversion of arginine into agmatine and carbon dioxide. The advantages of ADC are its low KM and the observation that in both normal and cancer cells, it can rapidly deplete arginine into agmatine without being converted back to arginine. Yet, the effects of agmatine on cancer cells require further investigation.

Notably, arginine depleting treatment can restore UC enzymes' expression (Syed et al., 2013; Tsai et al., 2009; Yeon et al., 2018). Indeed, tumor cells continuously adapt to their changing environment in order to survive and evade their targeting by drugs and the immune system. Therefore, all these informations should be taken in account before selecting a specific therapeutic agents.

Conclusions and future perspectives

In general, it has been shown that some metabolic genes are consistently expressed in both tumors and corresponding normal tissues, and their regulatory mechanisms maintained, such as those driven by epigenetic changes (Gaude and Frezza, 2016; Hu et al., 2013). Yet, a tumor is not a homogenous group of cells but rather a conglomerate of multiple "clones" of cells, each clone harboring a potential advantage for tumor survival in response to changing environmental cues. Following these notions, tumor express UC enzymes similarlyto their tissue of origin but there is also heterogeneity in the expression of UC enzymes between different clones within the tumor. Furthermore, there is a requirement for a new metabolic adjustment to support the evolving tumor needs at each carcinogenic stage (Allen et al., 2014; Tan et al., 2014).

The dynamic nature of alterations in the expression of UC enzymes can be used as a prism to understand metabolic changes during cancer evolution from the primary site to invasion and metastasis (**Figure 3**). It is important to note that such metabolic reprogramming uniquely promote the survival and growth of cancer cells. For example, high ammonia levels can lead to neurotoxicity, yet cancer cells recycle ammonia and reuse it for amino and nucleic acid synthesis. Additionally, although cancer hijacks physiologic-metabolic pathways, it rewires them continuously for its benefit. Cells with upregulation of UC enzymes' expression can be selected when UC products are needed for proliferation. Other clones with downregulation of UC enzymes' expression can be selected to increase the availability of UC substrates. At times, selection for both upregulation and downregulation of different UC enzymes can occur together within the same clone, indirectly affecting other metabolic pathways connected through the metabolic network. This dynamic process is constantly taking place within heterogeneous foci of the tumor, driven by the microenvironment, at different time points along with cancer progression.

These metabolic changes that provide tumors with the adaptability potential that enables their evolution in the changing microenvironment, also make tumors therapeutically challenging. Thus, treatment protocol needs to evolve alongside cancer progression. Alternatively, targeting metabolic pathways active in tumors can induce a metabolic vulnerability that will synchronize tumor heterogeneity and sensitize it to a specific drug. Hence, combining metabolic drugs with standard anti-cancer drugs can improve the therapeutic outcome.

Lastly, identifying and targeting this carcinogenic feature that enables metabolic adaptability holds promise for future therapeutic modalities.

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FIGURE LEGENDS

Figure 1. Schematic representation of the reactions catalyzed by the UC enzymes and the biological functions of UC intermediates. In hepatocytes, UC enzymes provide cells with the capability to remove waste ammonia in the form of urea. UC enzymes generate de novo arginine and ornithine in other cells to synthesize several metabolites, including NO and polyamines. Purple circles indicate UC enzymes and their associated biological functions, while pink ones indicate reactions and biological processes associated with the arginine-citrulline cycle. Dashed black arrows indicate metabolites exchanged between cytosol and mitochondria. ARG= arginase 1; ASA=argininosuccinate; ASL=Argininosuccinate Lyase; ASS1=Argininosuccinate Synthase; CP=carbamoyl-phosphate; CPS1=Carbamoyl-phosphate Synthase; NH_4^+=ammonia; NOS=Nitric Oxide Synthase; ORNT1=Ornithine Carrier 1; OTC=Ornithine Transcarbamylase.

Figure 2. Alterations of the UC enzymes in cancer. Upper panel- UC enzymes and associated reactions (purple circles) are upregulated in different cancer types. The consequent metabolic changes are highlighted in red and indicated by red dashed arrows. Transcriptional regulators responsible for the upregulation of the genes of the UC are also depicted (yellow= activator protein; blue=suppressors). **Lower panel**- UC enzymes are suppressed in several cancers (white circles). The UC metabolic reactions are highlighted with dark blue arrows, while dashed black arrows indicate the metabolites exchanged within different subcellular compartments. AKT=AKT Serine/Threonine Kinase; AR= androgen receptor; ARG= arginase 1; ASA=argininosuccinate; ASL=Argininosuccinate Lyase; ASS1=Argininosuccinate Synthase; ATF4=Activating Transcription Factor 4; CAD=Carbamoyl-phosphate Synthetase 2; aspartate transcarbamylase, and dihydroorotase cGMP= cyclic Guanosine monophosphate; c-MYC=MYC Proto-Oncogene; CP=carbamoyl-phosphate; CPS1=Carbamoyl-phosphate Synthase; HIF1A= Hypoxia-inducible factor 1- alpha; KRAS= KRAS proto-oncogenes; NH4+=ammonia; NO=nitric oxide; LKB1= Liver Kinase B1; IL6= interleukin 6; NOS=Nitric Oxide Synthase; NTS= neurotensin; ORNT1=Ornithine Carrier 1; OTC=Ornithine Transcarbamylase; PC= Pyruvate Carboxylase; PCK2= Phosphoenolpyruvate Carboxykinase 2; RAS= RAS proto-oncogenes; SNO= S-nitrosylation of cysteine thiol residues; TP53= Tumor Protein P53.

Figure 3: ASS1 levels dynamically change during tumor evolution. While in the primary tumor ASS1 downregulation promotes proliferation, as cancer grows and nutrients become limiting ASS1 expression increases. High ASS1 expression increases NO generation and

subsequently purine levels, supporting metastasis initiation by decreasing the anti-cancer immune response.

REFERENCES

Allen, M.D., Luong, P., Hudson, C., Leyton, J., Delage, B., Ghazaly, E., Cutts, R., Yuan, M., Syed, N., Lo Nigro, C*., et al.* (2014). Prognostic and Therapeutic Impact of Argininosuccinate Synthetase 1 Control in Bladder Cancer as Monitored Longitudinally by PET Imaging. Cancer Research *74*, 896-907.

Bateman, L.A., Ku, W.M., Heslin, M.J., Contreras, C.M., Skibola, C.F., and Nomura, D.K. (2017). Argininosuccinate Synthase 1 is a Metabolic Regulator of Colorectal Cancer Pathogenicity. ACS Chem Biol *12*, 905-911.

Birsoy, K., Wang, T., Chen, W.W., Freinkman, E., Abu-Remaileh, M., and Sabatini, D.M. (2015). An Essential Role of the Mitochondrial Electron Transport Chain in Cell Proliferation Is to Enable Aspartate Synthesis. Cell *162*, 540-551.

Bron, L., Jandus, C., Andrejevic-Blant, S., Speiser, D.E., Monnier, P., Romero, P., and Rivals, J.P. (2013). Prognostic value of arginase-II expression and regulatory T-cell infiltration in head and neck squamous cell carcinoma. Int J Cancer *132*, E85-93.

Cao, Y., Ding, W., Zhang, J., Gao, Q., Yang, H., Cao, W., Wang, Z., Fang, L., and Du, R. (2019). Significant Down-Regulation of Urea Cycle Generates Clinically Relevant Proteomic Signature in Hepatocellular Carcinoma Patients with Macrovascular Invasion. J. Proteome Res. *18*, 2032-2044.

Casero, R.A., Stewart, T.M., and Pegg, A.E. (2018). Polyamine metabolism and cancer: treatments, challenges and opportunities. Nat Rev Cancer *18*, 681-695.

Cederbaum, S.D., Yu, H., Grody, W.W., Kern, R.M., Yoo, P., and Iyer, R.K. (2004). Arginases I and II: do their functions overlap? Mol Genet Metab *81 Suppl 1*, S38-44.

Celiktas, M., Tanaka, I., Tripathi, S.C., Fahrmann, J.F., Aguilar-Bonavides, C., Villalobos, P., Delgado, O., Dhillon, D., Dennison, J.B., Ostrin, E.J*., et al.* (2017). Role of CPS1 in Cell Growth, Metabolism, and Prognosis in LKB1-Inactivated Lung Adenocarcinoma. Jnci-J Natl Cancer I *109*.

Cerutti, J.M., Latini, F.R., Nakabashi, C., Delcelo, R., Andrade, V.P., Amadei, M.J., Maciel, R.M., Hojaij, F.C., Hollis, D., Shoemaker, J*., et al.* (2006). Diagnosis of suspicious thyroid nodules using four protein biomarkers. Clin Cancer Res *12*, 3311-3318.

Chang, K.-Y., Chiang, N.-J., Yen, C.-J., Wu, S.-Y., Chen, S.-H., Johnston, A., Bomalaski, J.S., Wu, B.-W., and Chen, L.-T. (2018). A phase Ib study of ADI-PEG 20 plus pembrolizumab in advanced solid cancers. J. Clin. Orthod. *36*, 2556-2556.

Crump, N.T., Hadjinicolaou, A.V., Xia, M., Walsby-Tickle, J., Gileadi, U., Chen, J.-L., Setshedi, M., Olsen, L.R., Lau, I.J., Godfrey, L*., et al.* (2021). Chromatin accessibility governs the differential response of cancer and T cells to arginine starvation. Cell Reports *35*, 109101.

Czystowska-Kuzmicz, M., Sosnowska, A., Nowis, D., Ramji, K., Szajnik, M., Chlebowska-Tuz, J., Wolinska, E., Gaj, P., Grazul, M., Pilch, Z*., et al.* (2019). Small extracellular vesicles containing arginase-1 suppress T-cell responses and promote tumor growth in ovarian carcinoma. Nat Commun *10*, 3000.

De Santo, C., Booth, S., Vardon, A., Cousins, A., Tubb, V., Perry, T., Noyvert, B., Beggs, A., Ng, M., Halsey, C*., et al.* (2018). The arginine metabolome in acute lymphoblastic leukemia can be targeted by the pegylated-recombinant arginase I BCT-100. Int. J. Cancer *142*, 1490-1502.

Delage, B., Fennell, D.A., Nicholson, L., McNeish, I., Lemoine, N.R., Crook, T., and Szlosarek, P.W. (2010). Arginine deprivation and argininosuccinate synthetase expression in the treatment of cancer. Int J Cancer *126*, 2762-2772.

Erez, A., and DeBerardinis, R.J. (2015). Metabolic dysregulation in monogenic disorders and cancer - finding method in madness. Nat Rev Cancer *15*, 440-448.

Erez, A., Nagamani, S.C., Shchelochkov, O.A., Premkumar, M.H., Campeau, P.M., Chen, Y., Garg, H.K., Li, L., Mian, A., Bertin, T.K*., et al.* (2011). Requirement of argininosuccinate lyase for systemic nitric oxide production. Nat Med *17*, 1619-1626.

Fultang, L., Vardon, A., De Santo, C., and Mussai, F. (2016). Molecular basis and current strategies of therapeutic arginine depletion for cancer. Int. J. Cancer *139*, 501-509.
Gannon, P.O., Godin-Ethier, J., Hassler, M., Delvoye, N., Aversa, M., Poisson, A.O., Peant, B., Alam Fahmy, M., Saad, F., Lapointe, R.*, et al.* (2010). Androgen-regulated expression of arginase 1, arginase 2 and interleukin-8 in human prostate cancer. PLoS One *5*, e12107.
Gaude, E., and Frezza, C. (2016). Tissue-specific and convergent metabolic transformation of cancer correlates with metastatic potential and patient survival. Nature Communications *7*.
Geiger, R., Rieckmann, J.C., Wolf, T., Basso, C., Feng, Y., Fuhrer, T., Kogadeeva, M., Picotti, P., Meissner, F., Mann, M.*, et al.* (2016). L-Arginine Modulates T Cell Metabolism and Enhances Survival and Anti-tumor Activity. Cell *167*, 829-842.e813.
Gong, R., He, L., Zhou, H., Cheng, S., Ren, F., Chen, J., and Ren, J. (2019). Down-regulation of argininosuccinate lyase induces hepatoma cell apoptosis through activating Bax signaling pathway. Genes Dis *6*, 296-303.
Grzywa, T.M., Sosnowska, A., Matryba, P., Rydzynska, Z., Jasinski, M., Nowis, D., and Golab, J. (2020). Myeloid Cell-Derived Arginase in Cancer Immune Response. Front Immunol *11*.
Hall, P.E., Lewis, R., Syed, N., Shaffer, R., Evanson, J., Ellis, S., Williams, M., Feng, X., Johnston, A., Thomson, J.A.*, et al.* (2019). A Phase I Study of Pegylated Arginine Deiminase (Pegargiminase), Cisplatin, and Pemetrexed in Argininosuccinate Synthetase 1-Deficient Recurrent High-grade Glioma. Clin. Cancer Res. *25*, 2708-2716.
Harding, J.J., Do, R.K., Dika, I.E., Hollywood, E., Uhlitskykh, K., Valentino, E., Wan, P., Hamilton, C., Feng, X., Johnston, A.*, et al.* (2018). A phase 1 study of ADI-PEG 20 and modified FOLFOX6 in patients with advanced hepatocellular carcinoma and other gastrointestinal malignancies. Cancer Chemother. Pharmacol. *82*, 429-440.
Hu, J., Locasale, J.W., Bielas, J.H., O'Sullivan, J., Sheahan, K., Cantley, L.C., Vander Heiden, M.G., and Vitkup, D. (2013). Heterogeneity of tumor-induced gene expression changes in the human metabolic network. Nat. Biotechnol. *31*, 522-529.
Huang, H.-Y., Wu, W.-R., Wang, Y.-H., Wang, J.-W., Fang, F.-M., Tsai, J.-W., Li, S.-H., Hung, H.-C., Yu, S.-C., Lan, J.*, et al.* (2013a). ASS1 as a novel tumor suppressor gene in myxofibrosarcomas: aberrant loss via epigenetic DNA methylation confers aggressive phenotypes, negative prognostic impact, and therapeutic relevance. Clin. Cancer Res. *19*, 2861-2872.
Huang, H.L., Chen, W.C., Hsu, H.P., Cho, C.Y., Hung, Y.H., Wang, C.Y., and Lai, M.D. (2015). Argininosuccinate lyase is a potential therapeutic target in breast cancer. Oncol Rep *34*, 3131-3139.
Huang, H.L., Chen, W.C., Hsu, H.P., Cho, C.Y., Hung, Y.H., Wang, C.Y., and Lai, M.D. (2017). Silencing of argininosuccinate lyase inhibits colorectal cancer formation. Oncol Rep *37*, 163-170.
Huang, H.L., Hsu, H.P., Shieh, S.C., Chang, Y.S., Chen, W.C., Cho, C.Y., Teng, C.F., Su, I.J., Hung, W.C., and Lai, M.D. (2013b). Attenuation of argininosuccinate lyase inhibits cancer growth via cyclin A2 and nitric oxide. Mol Cancer Ther *12*, 2505-2516.
Jenkins, D.C., Charles, I.G., Thomsen, L.L., Moss, D.W., Holmes, L.S., Baylis, S.A., Rhodes, P., Westmore, K., Emson, P.C., and Moncada, S. (1995). Roles of Nitric-Oxide in Tumor-Growth. P Natl Acad Sci USA *92*, 4392-4396.
Kelly, M.P., Jungbluth, A.A., Wu, B.W., Bomalaski, J., Old, L.J., and Ritter, G. (2012). Arginine deiminase PEG20 inhibits growth of small cell lung cancers lacking expression of argininosuccinate synthetase. Br. J. Cancer *106*, 324-332.
Keshet, R., Lee, J.S., Adler, L., Iraqi, M., Ariav, Y., Lim, L.Q.J., Lerner, S., Rabinovich, S., Oren, R., Katzir, R.*, et al.* (2020). Targeting purine synthesis in ASS1-expressing tumors enhances the response to immune checkpoint inhibitors. Nature Cancer *1*, 894-908.
Keshet, R., Szlosarek, P., Carracedo, A., and Erez, A. (2018). Rewiring urea cycle metabolism in cancer to support anabolism. Nat Rev Cancer *18*, 634-645.
Kim, J., and DeBerardinis, R.J. (2019). Mechanisms and Implications of Metabolic Heterogeneity in Cancer. Cell Metab. *30*, 434-446.

Kim, J., Hu, Z.P., Cai, L., Li, K.L., Choi, E., Faubert, B., Bezwada, D., Rodriguez-Canales, J., Villalobos, P., Lin, Y.F.*, et al.* (2017). CPS1 maintains pyrimidine pools and DNA synthesis in KRAS/LKB1-mutant lung cancer cells. Nature *546*, 168-+.
Kim, S.S., Xu, S., Cui, J., Poddar, S., Le, T.M., Hayrapetyan, H., Li, L., Wu, N., Moore, A.M., Zhou, L.*, et al.* (2020). Histone deacetylase inhibition is synthetically lethal with arginine deprivation in pancreatic cancers with low argininosuccinate synthetase 1 expression. Theranostics *10*, 829-840.
Kobayashi, E., Masuda, M., Nakayama, R., Ichikawa, H., Satow, R., Shitashige, M., Honda, K., Yamaguchi, U., Shoji, A., Tochigi, N.*, et al.* (2010). Reduced argininosuccinate synthetase is a predictive biomarker for the development of pulmonary metastasis in patients with osteosarcoma. Mol. Cancer Ther. *9*, 535-544.
Krzystek-Korpacka, M., Szczesniak-Siega, B., Szczuka, I., Fortuna, P., Zawadzki, M., Kubiak, A., Mierzchala-Pasierb, M., Fleszar, M.G., Lewandowski, L., Serek, P.*, et al.* (2020). L-Arginine/Nitric Oxide Pathway Is Altered in Colorectal Cancer and Can Be Modulated by Novel Derivatives from Oxicam Class of Non-Steroidal Anti-Inflammatory Drugs. Cancers (Basel) *12*.
Lee, J.S., Adler, L., Karathia, H., Carmel, N., Rabinovich, S., Auslander, N., Keshet, R., Stettner, N., Silberman, A., Agemy, L.*, et al.* (2018a). Urea Cycle Dysregulation Generates Clinically Relevant Genomic and Biochemical Signatures. Cell *174*, 1559-+.
Lee, J.S., Carmel, N., Karathia, H., Auslander, N., Rabinovich, S., Keshet, R., Stettner, N., Silberman, A., Agemy, L., Helbling, D.*, et al.* (2018b). Mutagenicity of urea cycle dysregulation and its implications for cancer immunotherapy. Cancer Immunol Res *6*.
Lerner, S., Anderzhanova, E., Verbitsky, S., Eilam, R., Kuperman, Y., Tsoory, M., Kuznetsov, Y., Brandis, A., Mehlman, T., Mazkereth, R.*, et al.* (2019). ASL Metabolically Regulates Tyrosine Hydroxylase in the Nucleus Locus Coeruleus. Cell Rep *29*, 2144-2153 e2147.
Li, L., Mao, Y.X., Zhao, L.N., Li, L.J., Wu, J.J., Zhao, M.J., Du, W.J., Yu, L., and Jiang, P. (2019). p53 regulation of ammonia metabolism through urea cycle controls polyamine biosynthesis. Nature *567*, 253-+.
Long, Y., Tsai, W.B., Wang, D., Hawke, D.H., Savaraj, N., Feun, L.G., Hung, M.C., Chen, H.H., and Kuo, M.T. (2017). Argininosuccinate synthetase 1 (ASS1) is a common metabolic marker of chemosensitivity for targeted arginine- and glutamine-starvation therapy. Cancer Lett *388*, 54-63.
Ma, Z., Lian, J., Yang, M., Wuyang, J., Zhao, C., Chen, W., Liu, C., Zhao, Q., Lou, C., Han, J.*, et al.* (2019). Overexpression of Arginase-1 is an indicator of poor prognosis in patients with colorectal cancer. Pathol Res Pract *215*, 152383.
Mavri-Damelin, D., Eaton, S., Damelin, L.H., Rees, M., Hodgson, H.J.F., and Selden, C. (2007). Ornithine transcarbamylase and arginase I deficiency are responsible for diminished urea cycle function in the human hepatoblastoma cell line HepG2. Int. J. Biochem. Cell Biol. *39*, 555-564.
Miyamoto, T., Lo, P.H.Y., Saichi, N., Ueda, K., Hirata, M., Tanikawa, C., and Matsuda, K. (2017). Argininosuccinate synthase 1 is an intrinsic Akt repressor transactivated by p53. Sci Adv *3*, e1603204.
Mumenthaler, S.M., Yu, H., Tze, S., Cederbaum, S.D., Pegg, A.E., Seligson, D.B., and Grody, W.W. (2008). Expression of arginase II in prostate cancer. Int J Oncol *32*, 357-365.
Mussai, F., De Santo, C., Abu-Dayyeh, I., Booth, S., Quek, L., McEwen-Smith, R.M., Qureshi, A., Dazzi, F., Vyas, P., and Cerundolo, V. (2013). Acute myeloid leukemia creates an arginase-dependent immunosuppressive microenvironment. Blood *122*, 749-758.
Mussai, F., Egan, S., Hunter, S., Webber, H., Fisher, J., Wheat, R., McConville, C., Sbirkov, Y., Wheeler, K., Bendle, G.*, et al.* (2015). Neuroblastoma Arginase Activity Creates an Immunosuppressive Microenvironment That Impairs Autologous and Engineered Immunity. Cancer Res. *75*, 3043-3053.
Mussai, F., Wheat, R., Sarrou, E., Booth, S., Stavrou, V., Fultang, L., Perry, T., Kearns, P., Cheng, P., Keeshan, K.*, et al.* (2019). Targeting the arginine metabolic brake enhances immunotherapy for leukaemia. Int. J. Cancer *145*, 2201-2208.
Nagamani, S.C.S., Erez, A., and Lee, B. (2012). Argininosuccinate Lyase Deficiency. Genet Med.
Nicholson, L.J., Smith, P.R., Hiller, L., Szlosarek, P.W., Kimberley, C., Sehouli, J., Koensgen, D., Mustea, A., Schmid, P., and Crook, T. (2009). Epigenetic silencing of argininosuccinate synthetase confers

resistance to platinum-induced cell death but collateral sensitivity to arginine auxotrophy in ovarian cancer. International Journal of Cancer *125*, 1454-1463.
Ochocki, J.D., Khare, S., Hess, M., Ackerman, D., Qiu, B., Daisak, J.I., Worth, A.J., Lin, N., Lee, P., Xie, H.*, et al.* (2018). Arginase 2 Suppresses Renal Carcinoma Progression via Biosynthetic Cofactor Pyridoxal Phosphate Depletion and Increased Polyamine Toxicity. Cell Metab *27*, 1263-1280 e1266.
Pham-Danis, C., Gehrke, S., Danis, E., Rozhok, A.I., Daniels, M.W., Gao, D., Collins, C., Paola, J.T.D., D'Alessandro, A., and DeGregori, J. (2019). Urea Cycle Sustains Cellular Energetics upon EGFR Inhibition in EGFR-Mutant NSCLC. Mol Cancer Res *17*, 1351-1364.
Rabinovich, S., Adler, L., Yizhak, K., Sarver, A., Silberman, A., Agron, S., Stettner, N., Sun, Q., Brandis, A., Helbling, D.*, et al.* (2015). Diversion of aspartate in ASS1-deficient tumours fosters de novo pyrimidine synthesis. Nature *527*, 379-383.
Rabinovich, S., Silberman, A., Adler, L., Agron, S., Levin-Zaidman, S., Bahat, A., Porat, Z., Ben-Zeev, E., Geva, I., Itkin, M.*, et al.* (2020). The mitochondrial carrier Citrin plays a role in regulating cellular energy during carcinogenesis. Oncogene *39*, 164-175.
Roci, I., Watrous, J.D., Lagerborg, K.A., Lafranchi, L., Lindqvist, A., Jain, M., and Nilsson, R. (2019). Mapping Metabolic Events in the Cancer Cell Cycle Reveals Arginine Catabolism in the Committed SG2M Phase. Cell Rep *26*, 1691-1700 e1695.
Shan, Y.S., Hsu, H.P., Lai, M.D., Yen, M.C., Chen, W.C., Fang, J.H., Weng, T.Y., and Chen, Y.L. (2015). Argininosuccinate synthetase 1 suppression and arginine restriction inhibit cell migration in gastric cancer cell lines. Sci Rep *5*, 9783.
Silberman, A., Goldman, O., Boukobza Assayag, O., Jacob, A., Rabinovich, S., Adler, L., Lee, J.S., Keshet, R., Sarver, A., Frug, J.*, et al.* (2019). Acid-Induced Downregulation of ASS1 Contributes to the Maintenance of Intracellular pH in Cancer. Cancer Res. *79*, 518-533.
Spinelli, J.B., Yoon, H., Ringel, A.E., Jeanfavre, S., Clish, C.B., and Haigis, M.C. (2017). Metabolic recycling of ammonia via glutamate dehydrogenase supports breast cancer biomass. Science *358*, 941-946.
Stettner, N., Rosen, C., Bernshtein, B., Gur-Cohen, S., Frug, J., Silberman, A., Sarver, A., Carmel-Neiderman, N.N., Eilam, R., Biton, I.*, et al.* (2018). Induction of Nitric-Oxide Metabolism in Enterocytes Alleviates Colitis and Inflammation-Associated Colon Cancer. Cell Rep. *23*, 1962-1976.
Sullivan, L.B., Gui, D.Y., Hosios, A.M., Bush, L.N., Freinkman, E., and Vander Heiden, M.G. (2015). Supporting Aspartate Biosynthesis Is an Essential Function of Respiration in Proliferating Cells. Cell *162*, 552-563.
Syed, N., Langer, J., Janczar, K., Singh, P., Lo Nigro, C., Lattanzio, L., Coley, H.M., Hatzimichael, E., Bomalaski, J., Szlosarek, P.*, et al.* (2013). Epigenetic status of argininosuccinate synthetase and argininosuccinate lyase modulates autophagy and cell death in glioblastoma. Cell Death Dis *4*, e458.
Szlosarek, P.W., Grimshaw, M.J., Wilbanks, G.D., Hagemann, T., Wilson, J.L., Burke, F., Stamp, G., and Balkwill, F.R. (2007). Aberrant regulation of argininosuccinate synthetase by TNF-alpha in human epithelial ovarian cancer. Int J Cancer *121*, 6-11.
Szlosarek, P.W., Klabatsa, A., Pallaska, A., Sheaff, M., Smith, P., Crook, T., Grimshaw, M.J., Steele, J.P., Rudd, R.M., Balkwill, F.R.*, et al.* (2006). In vivo loss of expression of argininosuccinate synthetase in malignant pleural mesothelioma is a biomarker for susceptibility to arginine depletion. Clin. Cancer Res. *12*, 7126-7131.
Takenawa, H., Kurosaki, M., Enomoto, N., Miyasaka, Y., Kanazawa, N., Sakamoto, N., Ikeda, T., Izumi, N., Sato, C., and Watanabe, M. (2004). Differential gene-expression profiles associated with gastric adenoma. Br J Cancer *90*, 216-223.
Tan, G.S., Lim, K.H., Tan, H.T., Khoo, M.L., Tan, S.H., Toh, H.C., and Ching Ming Chung, M. (2014). Novel proteomic biomarker panel for prediction of aggressive metastatic hepatocellular carcinoma relapse in surgically resectable patients. J. Proteome Res. *13*, 4833-4846.
Tao, X., Zuo, Q., Ruan, H., Wang, H., Jin, H., Cheng, Z., Lv, Y., Qin, W., and Wang, C. (2019). Argininosuccinate synthase 1 suppresses cancer cell invasion by inhibiting STAT3 pathway in hepatocellular carcinoma. Acta Biochim Biophys Sin (Shanghai) *51*, 263-276.

Tardito, S., Oudin, A., Ahmed, S.U., Fack, F., Keunen, O., Zheng, L., Miletic, H., Sakariassen, P.O., Weinstock, A., Wagner, A.*, et al.* (2015). Glutamine synthetase activity fuels nucleotide biosynthesis and supports growth of glutamine-restricted glioblastoma. Nat Cell Biol *17*, 1556-1568.
Thongkum, A., Wu, C., Li, Y.-Y., Wangpaichitr, M., Navasumrit, P., Parnlob, V., Sricharunrat, T., Bhudhisawasdi, V., Ruchirawat, M., and Savaraj, N. (2017). The Combination of Arginine Deprivation and 5-Fluorouracil Improves Therapeutic Efficacy in Argininosuccinate Synthetase Negative Hepatocellular Carcinoma. Int. J. Mol. Sci. *18*.
Tsai, C.Y., Chi, H.C., Chi, L.M., Yang, H.Y., Tsai, M.M., Lee, K.F., Huang, H.W., Chou, L.F., Cheng, A.J., Yang, C.W.*, et al.* (2018). Argininosuccinate synthetase 1 contributes to gastric cancer invasion and progression by modulating autophagy. FASEB J *32*, 2601-2614.
Tsai, W.B., Aiba, I., Lee, S., Feun, L., Savaraj, N., and others (2009). Resistance to arginine deiminase treatment in melanoma cells is associated with induced argininosuccinate synthetase expression involving c-Myc/HIF-1α/Sp4. Mol. Cancer.
van de Poll, M.C.G., Siroen, M.P.C., van Leeuwen, P.A.M., Soeters, P.B., Melis, G.C., Boelens, P.G., Deutz, N.E.P., and Dejong, C.H.C. (2007). Interorgan amino acid exchange in humans: consequences for arginine and citrulline metabolism. American Journal of Clinical Nutrition *85*, 167-172.
von Stechow, L., Ruiz-Aracama, A., van de Water, B., Peijnenburg, A., Danen, E., and Lommen, A. (2013). Identification of cisplatin-regulated metabolic pathways in pluripotent stem cells. PLoS One *8*, e76476.
Watford, M. (2003). The urea cycle - Teaching intermediary metabolism in a physiological setting. Biochem Mol Biol Edu *31*, 289-297.
Weis-Banke, S.E., Hubbe, M.L., Holmstrom, M.O., Jorgensen, M.A., Bendtsen, S.K., Martinenaite, E., Carretta, M., Svane, I.M., Odum, N., Pedersen, A.W.*, et al.* (2020). The metabolic enzyme arginase-2 is a potential target for novel immune modulatory vaccines. Oncoimmunology *9*, 1771142.
Yao, S.H., Nguyen, T.V., Rolfe, A., Agrawal, A.A., Ke, J.Y., Peng, S.Y., Colombo, F., Yu, S., Bouchard, P., Wu, J.Y.*, et al.* (2020). Small Molecule Inhibition of CPS1 Activity through an Allosteric Pocket. Cell Chem Biol *27*, 259-+.
Yau, T., Cheng, P.N., Chan, P., Chen, L., Yuen, J., Pang, R., Fan, S.T., Wheatley, D.N., and Poon, R.T. (2015). Preliminary efficacy, safety, pharmacokinetics, pharmacodynamics and quality of life study of pegylated recombinant human arginase 1 in patients with advanced hepatocellular carcinoma. Invest. New Drugs *33*, 496-504.
Yeon, A., You, S., Kim, M., Gupta, A., Park, M.H., Weisenberger, D.J., Liang, G., and Kim, J. (2018). Rewiring of cisplatin-resistant bladder cancer cells through epigenetic regulation of genes involved in amino acid metabolism. Theranostics *8*, 4520-4534.
You, J., Chen, W., Chen, J., Zheng, Q., Dong, J., and Zhu, Y. (2018). The Oncogenic Role of ARG1 in Progression and Metastasis of Hepatocellular Carcinoma. Biomed Res Int *2018*, 2109865.
Yue, J., Dai, Q., Hao, S., Zhu, S., Liu, X., Tang, Z., Li, M., Fang, H., Lin, C., and Luo, Z. (2021). Suppression of the NTS-CPS1 regulatory axis by AFF1 in lung adenocarcinoma cells. J Biol Chem, 100319.
Zaytouni, T., Tsai, P.Y., Hitchcock, D.S., DuBois, C.D., Freinkman, E., Lin, L., Morales-Oyarvide, V., Lenehan, P.J., Wolpin, B.M., Mino-Kenudson, M.*, et al.* (2017). Critical role for arginase 2 in obesity-associated pancreatic cancer. Nat Commun *8*, 242.
Zhang, Q., Lou, Y., Yang, J., Wang, J., Feng, J., Zhao, Y., Wang, L., Huang, X., Fu, Q., Ye, M.*, et al.* (2019). Integrated multiomic analysis reveals comprehensive tumour heterogeneity and novel immunophenotypic classification in hepatocellular carcinomas. Gut *68*, 2019-2031.