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***Targeting amino acids transporters (SLCs)
to starve cancer cells to death***

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Nutrients, their capture, storage and metabolism are central for all forms of life providing energy and biomass (1,2). The pioneer obsessive idea of Judah Folkman and others of blocking tumor nutrient access by preventing tumor vascular development finally was achieved with great success (3). However, the anti-angiogenic benefits for patients have been modest and in some cases worse as a result of increased hypoxia favoring increased cancer stem cells, tumor migration and metastasis (4). Therefore, with the same logic it was reasonable to think that blocking essential nutrient transporters, overexpressed in cancers, will not have the adverse effects of directly targeting the vasculature.

Among the different class of nutrients, amino acids (AAs) are obligatory for survival of any cell. In addition of being the building blocks of proteins, AA also act as essential metabolites in the biosynthesis lipids nucleotides and antioxidant defenses. Thus, fast growing tumor cells require an enhanced uptake of AAs to face the increased demand of exacerbated anabolism. To date, approximately thirty AA transporters have been described for mammalian cells, which are expressed in a tissue-specific manner (5). However, among them, only a small group transporters is consistently up regulated in a large range of different cancer types (6,7). This, so-called 'minimal set of transporters', includes the L-type amino acid transporter 1 (LAT1, *SLC7A5*), alanine, serine, cysteine transporter 2 (ASCT2, *SLC1A5*), glutamate/cystine antiporter (xCT, *SLC7A11*), together with cell-surface antigen heavy chain (CD98, *SLC3A2*) that serves as chaperon for both LAT1 and xCT (8). In the last 5 years, through *SLC* genetic disruption in aggressive cancer cell lines (colon, lung, pancreas), our group demonstrated the strong potential development of these AA transporters as anticancer targets (9-11).

LAT1 is indispensable for EAA transport, general AA homeostasis and tumor growth

LAT1 is a Na⁺-independent obligatory exchanger (stoichiometry 1:1) that promotes essential AA (EAA) uptake combined with glutamine efflux (12). Overexpression of LAT1 has been reported as a negative prognostic factor in a wide range of tumor types (12,13). This global LAT1 overexpression pattern is explained by the fact that its expression is under control of

numerous signaling pathways that are deregulated during carcinogenesis (13,14). In 2016 we demonstrated that EAA transport activity of LAT1 is the key limiting step in cancer cell proliferation *in vitro* and *in vivo* by promoting EAA homeostasis and mTORC1 activity (11). Indeed, pharmacological inhibition or genetic disruption of LAT1 completely abolished leucine uptake, resulting in strong AA distress, and consequently, inhibition of mTORC1 and cell proliferation both *in vitro* and *in vivo*. These findings have been shown in six independent cell lines displaying different oncogenic mutations confirming the broad potential of targeting LAT1 for cancer therapy. Importantly, despite the existence of other EAA transporters in human physiology (i.e. SLC7a6-8, SLC43a1-2 and SLC6a14) (15), disruption of LAT1 activity revealed a lack of functional EAA transport redundancy. This study further increases the attractiveness of LAT1 as a therapeutic target and encourages continued development of specific inhibitors such as JPH203 (16). However, despite abolishing tumor growth, inhibition of LAT1 only displayed a cytostatic effect suggesting that this strategy alone would not be enough to obtain stable remission or cures in patients.

ASCT2-driven glutamine uptake promotes tumor growth independently of LAT1 activity

Following our line of research, we next investigated the role of another AA transporter, ASCT2. ASCT2 is a Na⁺-dependent transporter that exchanges small neutral AAs (Ala, Ser, Cys, Gln, and Asn – stoichiometry 1:1), and it has also been reported as a negative prognostic factor in multiple cancer types (17-20). ASCT2 has received a great interest in the last ten years as it has been described to be responsible for glutamine uptake in a large variety of cancer. Glutamine is a key metabolite required for anabolic growth of mammalian cells. Glutamine and its derivative glutamic acid are the obligate nitrogen donor during nucleotide and nonessential AA synthesis. Moreover, glutamine is used as carbon source in the mitochondria for the TCA cycle (anaplerosis). Precursors formed from glutamine are used for the synthesis of nucleotides, proteins, and lipids. In addition, a previous study proposed a model of functional coupling between ASCT2 and LAT1 by which ASCT2-driven glutamine export acts as a rate-limiting import for EAA by LAT1 (21). Therefore, according to these previous findings, we tested the hypothesis that targeting ASCT2 should have the dual advantage of starving cancer cells from both glutamine and EAA and therefore have a stronger therapeutic potential than targeting LAT1 alone. However, our results demonstrated that genetic disruption of ASCT2 in colon or lung adenocarcinoma cell lines neither affects LAT1 transport activity nor AA balance within the cell, suggesting that LAT1-ASCT2 functional coupling is not a general phenomenon across cancer types (21). Our findings corroborated a previous study showing that in osteosarcoma and triple negative breast cancer cell lines, glutamine uptake and cellular AA homeostasis could be achieved even in the absence of ASCT2 thanks to another set of Na⁺-dependent neutral AA transporter - SNAT1/2 (SLC38A1/2) (22). Although presented data argue that ASCT2 function in the cancer cells is dispensable for LAT1 activity and AA homeostasis, an important finding of this study showed that ASCT2 is yet a promising target for anticancer therapy. Namely, ablation of ASCT2 reduced tumor growth *in vivo* through mechanisms that seem not to involve LAT1

activity as no AA stress was observed in the ASCT2-KO tumor tissue analysis. These results suggest that targeting LAT1 and ASCT2 may have a synergistic effect and future efforts are required in order to uncover the true potential of these transporters as therapeutic targets.

The cystine transporter xCT, is critical for redox control, tumor growth and survival

Our most recent study focused on the role of the cystine/glutamate exchanger xCT. System Xc⁻ is Na⁺-independent exchanger of oxidized form of cysteine (cystine, CySSCy) and glutamate. xCT-dependent uptake of cystine has been reported to be fundamental not only for AA homeostasis but also redox balance within the cell, as cysteine is rate-limiting step for synthesis of the most important non-enzymatic antioxidant – glutathione (GSH). Numerous studies consistently reported that xCT inhibition inevitably leads to GSH depletion and to a new form of cell death called ferroptosis (23) (Figure 1). In addition, increased expression of xCT has been connected to poor patient prognosis and increased resistance to chemotherapy (24). However, still somewhere neglected remained the fact that many of these results are obtained in *in vitro* conditions, under 21% of oxygen, where the only form of cysteine (CySH) present is cystine (CySSCy). This is a strong caveat as in an *in vivo* setting both oxidized and reduced forms exist interchangeably (25-27). In our study, we investigate importance of xCT transporter for cancer cell growth and survival both *in vitro* and *in vivo*, in one of the most aggressive tumor types – pancreatic ductal adenocarcinoma (PDAC). Our study demonstrated that genetic invalidation of xCT *in vitro*: 1) completely abolishes ¹⁴C-CySSCy uptake and consequently induces AA stress, suggesting that xCT is the major transporter of oxidized form of cysteine, 2) collapses intracellular GSH pool, 3) increases sensitivity of the cancer cells to the cytotoxic effects of the chemotherapeutics such as cisplatin and gemcitabine, and finally 4) is lethal in two independent PDAC cell lines, inducing ferroptotic-cell death (9). xCT-KO phenotype was reverted by N-acetylcysteine, β-mercaptoethanol or GSH. On the other side, vitamin E prevented lipid peroxide accumulation and ferroptosis, but did not restore AA balance in the xCT-KO cells.

The most intriguing result of our *in vitro* study is that despite the lethal phenotype of xCT-KO, these cells were still capable to form tumor *in vivo*, although with a significant delay. Tumor-cell analysis suggested that *in vivo* reduced form of cysteine released, allowed survival and growth of xCT-KO cells *in vivo*. Recent reports of Wang and colleagues (28,29) showed that fibroblast present in cancer stroma can uptake CySSCy, reduced and transfer it to the cancer cells upon chemotherapy challenge. Interestingly, this collaboration seems to be interrupted by CD8⁺ T cell secreting interferon-γ, which down-regulates expression of xCT in fibroblasts and/or tumor cells through JAK-STAT1 signalling (28,29). Conversely, it has been shown that inhibition of xCT increases sensitivity of tumor cells to immune therapy, most likely by increasing their antigenicity (30). These findings highlight xCT as an AA transporter of great potential for future anti-cancer therapy.

In conclusion, our genetic approach provides a molecular basis pointing the key role of overexpressed LAT1, ASCT2 and xCT transporters in cancer cell homeostasis, growth, and survival. With the explosive knowledge of crystal structures of membrane transporter (31,32), our optimism is great to see a rapid pharmacology development and therapeutic success in this area of cancer.

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Figure 1: Genetic ablation of the Cystine/Glutamate Transporter xCT in PDAC cell lines, inhibits mTORC1, growth, survival and tumor formation. WT phenotype is restored by N-acetyl cysteine (NAC), or Glutathione (GSH). 1 μ M Erastin mimics xCT-KO cells inducing Ferroptotic cell death. (refs 9, 23, 28)