We append the most important highlights of our overall progress report in ascending chronological order.

- Proline Oxidation Supports Mitochondrial ATP Production When Complex I Is Inhibited

In this work we reported that proline catabolism in isolated mitochondria from murine tissues generate a sufficiently high membrane potential to maintain the F1-Fo-ATPase operation in the forward mode. This was observed in CI-inhibited mitochondria exhibiting high levels of proline oxidation and proline dehydrogenase activity. This action was not observed under anoxia or when either CIII or CIV were inhibited. Reduction of ubiquinone was directly shown by amperometric measurements using a three-electrode system with coenzyme Q2 as a mediator. The relevance of this work to the field of cancer metabolism lies on the strong association of proline supporting tumor growth, addressed widely in the literature (references 5, 46-48 in PMID: 35563503).

- Viability of HepG2 and MCF-7 cells is not correlated with mitochondrial bioenergetics

The most important finding of this study was that the viability of HepG2 cells (in 2D cultures, evaluated by high-content automated microscopy) was mostly unaffected by site-targeted inhibition of respiratory components or severe hypoxia. There, we established experimental protocols that we currently use in identifying targets in the oxidative decarboxylating branch of glutaminolysis. To this end, we are examining the effect of KGDHC inhibitors on HepG2 cell viability (see below under “work under revision/review/submission”), and we have also identified at least 5 high-affinity ligands for succinyl-CoA synthase using a DNA-encoded library.

- Residual Complex I activity and amphidirectional Complex II operation support glutamate catabolism through mtSLP in anoxia

Two concepts emerged from this work: A) even during complete anoxia, complex I is still able to oxidize NADH to NAD+ at a rate that can support KGDHC; this is of critical importance, because KGDHC is in the oxidative decarboxylating branch of glutaminolysis. B) for complex I to remain partially operational during anoxia, complex II is operating in reverse, oxidizing quinol to quinone, which in turn is provided to complex I. This step could only be maintained if complex II reverse operation is fueled by excess fumarate, that can originate from malate. The importance of this work in the cancer field is very high: considering that cancer cells perform glutaminolysis but at the same time experience hypoxia, our findings provide the missing link in understanding how glutamine catabolism may persist even in the absence of oxygen. Secondly, it underlines the potential importance of fumarate (and indirectly, malate) to this mechanism.

- Cell-specific expression of key mitochondrial enzymes limits OXPHOS in astrocytes of the adult human neocortex and hippocampal formation

In this work we reported that astrocytes of the adult human neocortex and hippocampal formation express barely detectable amounts of mitochondrial proteins critical for performing OXPHOS. Although our data were not directly linked to cancer, they are relevant from the following two points of view: A) OXPHOS inhibitors for the purpose of combating cancer may exert only limited side effects, as this pathway may not be operating in almost 50% of the human brain (in the human brain, the glia-to-neurons ratio is ~1:1). B) evidence of OXPHOS components (not necessarily related to OXPHOS itself, but side pathways, such as quinone and/or NAD+ metabolism) may be markers of cancer cells of glial origin.

- Reverse phase protein array-based investigation of mitochondrial genes reveals alteration of glutaminolysis in the parahippocampal cortex of people who died by suicide

In this work we showed that protein levels of DLD, OGDH, SDHB, SUCLA2, and SUCLG2 subunits were significantly elevated in the parahippocampal cortex but not in other cortical brain regions. Although this paper is not related to cancer in any way, we have established the workflow in quantitating the protein expression of several gene products participating in the glutaminolysis pathway, using RPPA technology.

Work under revision/review/submission

- Comparative Analysis and Correlation of Cancer Hotspot Proteins and Cell Markers in Tumor-Normal Adjacent Breast and Kidney Samples Using RPPA and LC-MS

In this work, we evaluated the expression of cancer-related proteins and cell markers, in human breast and kidney tumor-normal adjacent samples. To this end, we employed two distinct proteomic techniques—RPPA and Liquid Chromatography-Mass Spectrometry (LC-MS). Significant alterations in protein expression were found using both methods also revealing patient-specific proteomic profiles. Furthermore, both overlapping and unique findings between RPPA and LC-MS were observed, indicating their complementary nature. However, Spearman coefficient analysis demonstrated varying degrees of correlation between RPPA and LC-MS data. This integrated approach underscores the necessity of dual-platform analyses in cancer proteomics to improve diagnostic and therapeutic strategies.

- NQO1-mediated provision of NAD+ enables ATP export in Complex I-inhibited mitochondria oxidizing choline

By using a model established to assess ATP export produced by substrate-level phosphorylation in isolated mitochondria (mtSLP) with OXPHOS inhibited at the CI level, we discovered that addition of choline to substrate cocktails metabolized through KGDHC enhanced the rate of ATP export from mitochondria by the adenine nucleotide translocator (ANT). NQO1 could mediate the oxidation of NADH by suitable quinones, to a certain extent. Mechanistically, this work describes a metabolic rewiring through which choline reduces ubiquinone bypassing CI, allowing proton pumping through CIII and CIV, maintaining a mitochondrial membrane potential more negative than Erev_ANT. NAD+ regeneration facilitated by diaphorases—at least one being NQO1—enables these processes by supporting KGDHC activity, the latter fueling mtSLP.

- Arsenic-based antineoplastic drugs kill cancer cells that thrive without OXPHOS

In this work we demonstrate that several arsenic-based compounds -at clinically relevant concentrations- induce significant HepG2 cell death regardless of concomitant OXPHOS inhibition. However, experiments show that KGDHC (as part of glutaminolysis) is not the only target for the arsenicals, and branched chain amino acid dehydrogenase (BCAT) is probably an additional, important target for cancer cell survival.

Unpublished work

- The concept of ATP provision via MTHFD1L as a major pathway in the absence of OXPHOS has been rejected. This is because we were unable to establish a formate-induced MTHFD1L activity that would demonstrate ATP hydrolysis, considering that the enzyme has been reported to be reversible.

- Pathways yielding pyruvate and lactate from glucose bypassing pyruvate kinase have not been examined yet. We have been unable to meet the work quota that we intended, though we did purchase the necessary 13C-labelled metabolites for this work. Thus, the experiments will be performed and results will be submitted, but not within the time frame of the funded grant. However, it should be mentioned that these experiments were planned under the concept of cancer reductive stress, to which we have exhibited a more than adequate response as evident from the above published body of work.
- Gene silencing using CRISPR has failed. This was attributed to a meager level of transfection efficiency. We have exhausted our means for trying different transfection protocols, and we could not exceed the ~65% mark for almost any cell line that we examined. We are now in contact with a group in Brussels where they will alter our cell lines in a way that genes of our interest will be subject to on/off using exogenously added substances (i.e. tetracycline).