In the first year (2018-09-01 – 2019-08-31) of the funded KH_129567 grant we investigated the extent of contribution of the monofunctional C1-tetrahydrofolate synthase (MTHFD1L) which is known to produce mitochondrial ATP by interconverting 10-formyltetrahydrofolate to tetrahydrofolate and formate, and the effect of 2-Ketobutyrate (2-KB) on mitochondrial substrate-level phosphorylation. 2-KB is a common intermediate of threonine, serine and methionine catabolism converging towards succinyl-coA, but obligatorily passing through an ATP-consuming step substantiated by propionyl-CoA carboxylase.

In isolated mouse brain, liver and heart mitochondria, MTHFD1L did not exhibit the capacity of producing ATP under anoxia-mimicking conditions to an appreciable extent. As this was a negative finding -thus much harder to publish as such- we have not submitted it to a scientific journal; originally, we planned to incorporate these data to a much larger work benefited from the monetary support encompassing the role of residual complex I activity in anoxia within the first half of 2020. However, the data gathered from the "residual complex I activity in anoxia" project have not been submitted for publication yet, for the following three reasons: 1) we have teamed up with a GC-MS laboratory (Dr. Daniel Tennant in Birmingham, UK) because of the need that has arisen to follow 13C-labeled metabolites; this created the challenges of a) finding a suitable laboratory that could perform such an analysis -preferably free of charge, and accepting coauthorship- b) undergoing very extensive testing of the methodology used prior to performing experiments with 13C-labeled metabolites as they are extremely expensive, and c) due to the COVID pandemic there have been some obstacles in achieving work quota (personnel coming to the lab, deliveries of consumables, shipping of samples, etc). More specific details regarding the "residual complex I activity in anoxia" project appears below.

On the other hand, 2-KB was found to abolish mitochondrial substrate-Level phosphorylation in a dose-dependent manner and this was published in October 2019; this finding underlined the concept that provision of metabolites converging to 2-KB may be a useful way for manipulating mSLP without using pharmacological or genetic tools.

The additional work exploring the cell-specific expression of alpha-ketoglutarate dehydrogenase in the human brain has been published in Brain Structure and Function. There, we reported the exclusive neuronal detection of KGDHC-specific subunits in the adult human brain cortex despite pancellular protein lysine succinylation. Our findings were corroborated by data from the Human Protein Atlas as well as RNA-Seq data from the Allen Brain Atlas corresponding to genes coding for KGDHC components. This work has spurred the project of checking the expression of several other enzymes participating in major biochemical pathways in the adult human brain; more details on this appears below. Furthermore, an additional project emerged, that of searching for the origin of succinyl-CoA in the cytosol, which mediates the succinylation of cytosolic proteins. At the moment we are looking at peroxisomal biochemical pathways that may yield succinylcarnitine in the cytosol.

As stated above a large amount of work related to the extent of contribution of residual activity of complex I to matrical NAD+ production has been carried out, and this is absolutely critical to the proposal because it directly addresses NAD+ and quinone provision pathways converging to complex I. At the beginning of November 2019 we receive a next-generation Oroboros system which can record quinone/quinol ratio in an online manner. We have worked on such a demo machine in the winter of 2018, but several adjustments had to be made. Many more experiments have been performed, and within the next month or so we are receiving an additional Oroboros prototype that can measure NADH autofluorescence, in addition to Oxygen partial pressure. Metabolomics data from 13C-labeled metabolites will also be examined in parallel to using the two Oroboros prototypes, that measuring quinol/quinone ratio and that recording NADH autofluorescence.

We have also received fibroblasts from patients suffering from MDH2 deficiency, and this is important to this proposal because we have reasons to believe that reversal of MDH2 provides NAD+ to KGDHC during anoxia. Indeed, we have been evaluating novel MDH2 inhibitors and this seems to be the case. Furthermore, we are supplementing our data on the MDH2 project by generating 13C-labeled metabolites that can validate or reject the hypothesis that MDH2 reversal yields NAD+ for KGDHC supporting mitochondrial substrate-level phosphorylation.

In the second year (2019-09-01 – 2020-12-31) of the funded KH_129567 grant the following progress has been made:

9 scientific articles have been published. Out of these, 3 are reviews. Out of the 6 remaining papers, KH_129567 is acknowledged in 5. The PI is corresponding (or co-corresponding or single) author in 6 out of 9 papers.

Two more papers are under submission, one regarding the role of MDH2 reversal in providing NAD+ to KGDHC supporting mitochondrial substrate-level phosphorylation (mSLP) during OXPHOS dysfunction, and the other regarding the role of residual complex I activity during anoxia providing NAD+ to KGDHC supporting (mSLP), see above for details.

During the course of the experiments regarding complex I residual activity in anoxia we have also discovered that the FMN center of complex I is able to reduce safranine O under complete anoxic conditions. This has spun off a side-project in which we collaborate with a Polish institute; they provide yeast mitochondria genetically modified to lack FMN centers. This research is still ongoing; to this end, we have also discovered that safranine O can be reduced by NADH in a non-enzymatic manner, under completely anoxic conditions.

Furthermore, as an extension of the work where we published that KGDHC subunits are only found in human neurons but not glia in the adult brain (not in cultures), we have extended the search to all mitochondrial proteins. Regarding this work we collaborate with a bioinformatician in New York who mines the Allen human brain project database, and Dr. Gabor Nyiri, who expressed interest in performing in situ mitochondrial volume measurements in human hippocampi. In addition, Dr. Dobolyi Arpad has performed immunohistochemistry on several human hippocampi sections, validating antibodies directed against mitochondrial proteins participating in glycolysis, the citric cycle and the electron transport chain. Our preliminary data show that many mitochondrial proteins participating in basic energy-harnessing pathways are lacking in human glia, indicating that glial mitochondria work very differently from neurons described in classical biochemistry textbooks. This is absolutely crucial, because it will dictate how differently cells are expected to operate during anoxia.

Finally, we have engaged in a project in which we investigate the role of proline catabolism in bypassing the need of mSLP for rescuing mitochondria subjected to anoxic conditions. Our findings indicate that in tissues where proline dehydrogenase activity is sufficiently high –such as in the liver and kidney, but not in brain and heart- quinone reduction yields such a high electron flux in the ETC that renders the need of mSLP nearly obsolete. This is important because inhibition of mSLP in cancer cells (known to thrive during anoxia) may not bring the beneficial effect of energy failure if proline is available. This opens the new research avenue in investigating the role of proline catabolism during anoxia.

In 2021, only two articles have been published in which the PI is a co-author in unrelated studies. However, we anticipate to submit one paper from the “residual complex I activity in anoxia” project,

one from the action of safranine reducing NAD+ during anoxia, one on MDH2 yielding NAD+ during anoxia, one on proline catabolism, and one on cell-specific expression of mitochondrial proteins in the adult human brain. The PI has also been invited to write 3 reviews within 2021 on related topics regarding mSLP.