I thank Prof. Bay for the comments.

My responses inline (>Response).

Nevertheless, in my opinion the links to neoplasia, more precisely glioblastoma multiforme, were not evident from the introduction and the discussion of the results.

>Response: Indeed, in the dissertation there are no experimental data to support a definite link between mitochondrial substrate-level phosphorylation and any kind of neoplasia; this is based on data that remain unpublished and incomplete. When I was compiling the dissertation and before finalizing my publications records I was under the -false- impression that papers including these data would soon be accepted and I could include them. I did not wish to render such data public information prior to acceptance as the dissertation is publicly available through http://real-d.mtak.hu/1462/. An additional reason for withholding these data was to use them as preliminary results while applying for grants which I could not yet obtain.

Furthermore, there are many abbreviations that are not resolved, ambiguous or were not listed on the list of abbreviations. For example, what is the difference between mSLP and SLP or is it the same (i.e. substrate level phosphorylation)?

>Response: mSLP refers to mitochondrial-intrinsic substrate-level phosphorylation substantiated by succinate-CoA ligase (and to a minor extent by mitochondrial phosphoenolpyruvate carboxykinase, but as this contribution is very small it is ignored); SLP refers to substrate-level phosphorylation of non-mitochondrial origin, i.e. substantiated primarily by phosphoglycerate kinase and pyruvate kinase, both being glycolytic enzymes. In the dissertation, mitochondrial substrate-level phosphorylation (mSLP) is sometimes referred to as "matrix substrate-level phosphorylation" (pages 12, 76, 89, 90, 97, 101, 105, 198, 199, 211 213, 222) which may cause ambiguity because of the sub-term "substrate-level phosphorylation"; I have referred to mSLP as "mitochondrial substrate-level phosphorylation" or "matrix substrate-level phosphorylation" as mentioned in the pertinent publication. To this end, I should add that in many circumstances the Reviewers and/or our co-Authors had explicit requests as of how to refer to it, which led to multiple abbreviations. Non-mitochondrial substrate-level phosphorylation is not examined in the current dissertation.

Along the same lines, I have not identified a list of aims, neither a list of new scientific results. That latter is truly missing, I would like to ask Dr. Chinopoulos to introduce a slide with that content at the viva voce.

>Response: I chose to write the dissertation in chronological order of the experiments performed which does not yield a clear flow of aims, simply because I did not know where will the experimental findings lead me to; a turning point was the discovery that the mitochondrial F_O-F_1 ATP synthase and the adenine nucleotide translocase are not in obligatory directional synchrony, and that led to a very significant departure of research directives. A list of scientific results are rather addressed in the thesis booklet (as it was a formal requirement) and I did not wish to repeat myself on this in the dissertation, but I will introduce a slide with that content at the viva voce.

1. What was the rationale of selecting the metabolites for energizing mitochondria, as these were different between experimental setups and when mixtures were used not all individual components were assessed individually. As examples please see the upcoming two questions below.

>Response: The vast majority of experiments in which the choice of metabolites was a variable concerned the contribution of mitochondrial substrate-level phosphorylation substantiated by succinate-CoA ligase (to which I will refer to as mSLP in my responses) in mitochondria with either targeted OXPHOS defects (by pharmacological inhibition of the respiratory complexes or true anoxia), citric acid cycle constraints (by fueling specific segments of the cycle), presence of interfering metabolites (by itaconate, beta-hydroxybutyrate, 2-ketobutyrate, diaphorase substrates) or a combination thereof. Although all these lead to very elaborate substrate combinations with their own justification, the following simple distinction can be made: substrates were used that either favor mSLP (i.e. leading to formation of alpha-ketoglutarate) or antagonize mSLP (i.e. leading to formation of succinate and/or bypassing succinyl-CoA). Mindful of this question by Prof. Bay, I will be including a slide at the viva voce that will indicate entry points of various substrates to mitochondrial pathways and the rationale for being chosen in addressing particular aspects of mSLP.

2. On Figure 7.3 the mitochondria from the double heterozygous mice treated with malate+beta-hydroxybutyrate and glutamate+malate+beta-hydroxybutyrate is not displayed. What is the reason for that? In line with that, the representation of the lipidomics results often lacks the display of data from the double heterozygous mice.

>Response: Regarding the reason for not showing data obtained from the double heterozygous mice treated with malate+beta-hydroxybutyrate and glutamate+malate+beta-hydroxybutyrate: the double heterozygous mice were produced by breeding $DLD^{+/-}$ with $DLST^{+/-}$ mice that yielded a litter in which ≤25% exhibited the $DLD^{+/-}$ / $DLST^{+/-}$ genotype. $DLD^{+/-}$ / $DLST^{+/-}$ mice were very poor breeders, thus they were not inter-bred. As there was no visually discernible characteristic of the double transgenic mice, all litter had to be genotyped. This was increasing the costs of hosting the colony by a factor of ≥4 and we were running out of funds. Since the experiments with either malate and beta-hydroxybutyrate or glutamate and malate and beta-hydroxybutyrate did not show a statistically significant difference in ATP efflux rates among wild type and either $DLD^{+/-}$ or $DLST^{+/-}$ deficient mice, we deduced that it would not add a significant amount of new information if we performed the experiments with the double transgenic mice, thus, we did not have to breed more mice that would require further genotyping. Regarding the question on the representation of the lipidomics results, I am not sure I understand, as there were no lipidomics performed in this paper; perhaps prof. Bay refers to other kind of results from the double heterozygous mice often lacking display of data, and the reason for that is the same as above, i.e. if little amount of information was predicted to be obtained, the experiments were not performed so that we would not implement extensive genotyping in order to identify the double heterozygous mice.

3. In chapter 7.4 mutliple anaplerotic substrates are supplied to mitochondria. What is the reason for not assessing all substrates alone (e.g. glutamate was not assessed alone)? Given the potentiating effects, to me it remains an open question whether glutamate alone would be able to modulate mitochondrial oxidation.

>Response: There are two reasons for not assessing all substrates alone: i) substrates that were leading to succinyl-CoA formation but not to the extent of supporting an adequate amount of ATP

produced by mSLP was ignored, and ii) at least in liver mitochondria, endogenous substrates -even after isolation of mitochondria by extensive differential centrifugation- are abundant; thus, it is not really possible to 'choose' individual substrates, but to increase the availability and flux of some substrates over others. This is especially true if one wishes to use only beta-hydroxybutyrate (boH) or only acetoacetate (AcAc), and these are the only two substrates that have not been tested alone, individually. Glutamate (glut) alone has been tested only in brain, appearing in figure 7.4 (bottom, left panel) and is also referred to in the text in page 93...." glut+mal, glut+mal+AcAc, α-Kg, α-Kg+mal, glut+mal+bOH, glut), but not in liver ((supplementary figure 7 in (Kiss et al. 2013), reference given in page 259 of the dissertation), because in liver, glutamate alone is rather a poor substrate in terms of supporting mSLP.

4. p.66. "Assuming 1 microliter of matrix volume for every mg mitochondrial protein" what builds a case for this estimation?

>Response: This is an estimation by the group of Halestrap (Halestrap and Quinlan 1983) for rat liver mitochondria: they reported that the mean matrix volume of de-energized liver mitochondria in KCl medium estimated using mannitol was 0.46 microliter/mg of protein, whereas that obtained using sucrose was 1.68 microliters/mg of protein. We took an average of this value, i.e. ~1 microliter per mg protein, since we had similar, but not identical buffer conditions. I acknowledge that this should have been cited in the paper. For comparison, similar values (1-1.9 microliter/mg of protein) have been reported by Beavis, Brannan and Garlid (Beavis et al. 1985).

5. Fig.6.2E There seems to be a MW difference of SUCLA2 between brain synapses and the three other preps. Is it possible that there is a difference in the posttranslational modifications of SUCLA2 among these source of mitochondria?

>Response: The reason for MW difference of SUCLA2 between types of mitochondria could indeed be due to posttranslational modifications, in particular succinylations; an additional reason could be that there are two isoforms for SUCLA2; in one of them, amino acids in positions 26-47 are missing (Ota et al. 2004). It is possible that there is tissue-dependent distribution of the SUCLA2 isoforms.

6. p89. The hypothesis that other compartments may transfer phosphorylation potential is interesting. I have two questions on that issue. First, what does phosphorylation potential as a term refer to? Second, what compartments may be involved? Is the endoplasmic reticulum a potential compartment?

>Response: Strictly speaking, phosphorylation potential is defined as the amount of free energy (ΔG) released from one mole of ATP hydrolyzed to ADP and Pi; however, this potential can be transferred to other molecules that can harbor a significant amount of ΔG which can be subsequently passed on to adenine (or guanine) nucleotides. As such, transfer of phosphorylation potential can 'cross' membranes in the form of non-adenine nucleotides, whenever ATP or GTP cannot penetrate the membrane. Having said that, theoretically all intracellular compartments in which ATP or GTP is used should be able to accept compounds with a high phosphorylation potential, but most references are on nucleus, cytosol and mitochondria. For endoplasmic reticulum in particular, little is known, however, in 2019 it was reported ATP made in mitochondria enters the ER lumen through a cytosolic Ca^{2+}-antagonized mechanism, or *CaATiER* (Ca^{2+}-Antagonized Transport into ER) (Yong et al. 2019). To the best of my knowledge, there have been no further reports on this.

7. Chapter 8.2. is entitled "Identifying mitochondria as extramitochondrial ATP consumers during anoxia"; this title and the subsequent discussion seems to be contradiction in terms to me. How can mitochondria be extramitochondrial?

>Response: I acknowledge that the title may be slightly misleading, but with the term "extramitochondrial ATP consumers" I wished to address if mitochondria could hydrolyze ATP that was made outside mitochondria, i.e. by glycolysis. I used the term "extramitochondrial ATP" instead of "ATP made in glycolysis" because I did not want to narrow down to glycolysis; cytosolic ATP could have been recently made by normally polarized mitochondria, exported to cytosol, but then an abrupt mitochondrial depolarization would ensue that the ANT and the F_0-F_1 ATP synthase reverse and bring this ATP back into the matrix that would lead to its hydrolysis.

8. In terms of itaconate metabolism, figure 11.4 implicate that itaconate may reverse the succinyl-CoA → succinate reaction through succinate-CoA ligase. Is this possible or is it a wrong though based on the figure?

>Response: Itaconate does not directly reverse the succinyl-CoA → succinate reaction through succinate-CoA ligase, but addition of itaconate to mitochondria results as if it does: what actually happens is that itaconate is a preferred substrate over succinate by succinate-CoA ligase, so it leads to strong ATP or GTP hydrolysis and CoA entrapment in the form of itaconyl-CoA; the CoA entrapment leads to impairment of the function of KGDHC, the enzyme that produces succinyl-CoA. So, in effect, addition of itaconate to mitochondria exhibiting OXPHOS defects causes a further decrease in matrix ATP/ADP and GTP/GDP output from mSLP and decreases the availability of succinyl-CoA (because KGDHC is deprived of CoA), unfavoring mSLP; these all give the impression that itaconate reverses the succinyl-CoA → succinate reaction through succinate-CoA ligase, but in fact it does not inhibit the enzyme.

9. Apparently, SUCLA2 protein expression is specific for neurons, however, SUCLA2 mRNA expression appears in S100 positive glial cells. What is the reason for that? Can there be an issue of the SUCLA2 protein detection limit (i.e. SUCLA2 levels are below the detection limit in glial cells)? If so, it would be better to discuss a large difference in protein expression between glial cells and neurons.

>Response: SUCLA2 mRNA expression in S100 positive glial cells was addressed in panel D of figure 14.7. In the legend of figure 14.7 it is mentioned that positivity (i.e. presence of the mRNA) was acknowledged if the number of autoradiography grains was more than 4 (for the neurons). In panel D, there are indeed some autoradiography grains but their clustering levels did not exceed that of background (for the S100 positive astrocytes). Thus, in the same legend it was indicated that "mRNA expression of SUCLA2 is absent in S100-immunoreactive astrocytes". Having said that, it is still possible that there is some SUCLA2 mRNA expression in astrocytes that does not lead to a detectable expression of SUCLA2 protein. Relevant to this, it must be emphasized that the immunohistochemistry protocols applied in that manuscript harbored very strong amplification steps; thus, even if there is some SUCLA2 protein expressed in S100-positive astrocytes that we did not detect, it must be a very small amount.

10. With regard to the expression of OGDHL in neuros, it appears to me that the protein preferentially localizes to mitochondria close to the nucleus and the expression decreases in

mitochondria distant to the nucleus – at least based on the representative images in COS7 cells (Figure 16.4). Did the authors observe that, can this observation be generalized?

>Response: Yes, we have noticed that, but upon discussion with the confocal microscopy expert (Prof. Dobolyi Árpád, the first author of that study) it was suggested that this maybe because of the z-stacking of unevenly shaped cells; one can imagine that a sphere-shaped nucleus leads to a greater z-length, thus, around it there may be more perinuclear mitochondria; in the periphery, the cells are thinner, thus there is less space to be occupied by mitochondria. By applying even a minor z-stack, signal from the perinuclear region is summed from more layers harboring mitochondria, than in the periphery. Thus, we cannot be sure if the increased signal of OGDHL ICC was due to this, or, as Prof. Bay suggested OGDHL preferentially localizes to mitochondria close to the nucleus and the expression decreases in mitochondria distant to the nucleus. If the first concept were to be solely true, we should have observed that for all ICC subunits; but we only observed that with OGDHL and DLST (figure 16.7). To address this, we should perform confocal microscopy for OGDHL (and DLST) without any z-stacking and probably using very advanced confocal microscopy protocols and machinery.

REFERENCES CITED

Beavis AD, Brannan RD, Garlid KD. 1985. Swelling and contraction of the mitochondrial matrix. I. A structural interpretation of the relationship between light scattering and matrix volume. J Biol Chem. Nov 5;260:13424-13433.

Halestrap AP, Quinlan PT. 1983. The intramitochondrial volume measured using sucrose as an extramitochondrial marker overestimates the true matrix volume determined with mannitol. Biochem J. Aug 15;214:387-393.

Kiss G, Konrad C, Doczi J, Starkov AA, Kawamata H, Manfredi G, Zhang SF, Gibson GE, Beal MF, Adam-Vizi V, et al. 2013. The negative impact of alpha-ketoglutarate dehydrogenase complex deficiency on matrix substrate-level phosphorylation. FASEB J. Jun;27:2392-2406. Epub 20130308.

Ota T, Suzuki Y, Nishikawa T, Otsuki T, Sugiyama T, Irie R, Wakamatsu A, Hayashi K, Sato H, Nagai K, et al. 2004. Complete sequencing and characterization of 21,243 full-length human cDNAs. Nat Genet. Jan;36:40-45. Epub 20031221.

Yong J, Bischof H, Burgstaller S, Siirin M, Murphy A, Malli R, Kaufman RJ. 2019. Mitochondria supply ATP to the ER through a mechanism antagonized by cytosolic Ca(2). Elife. Sep 9;8. Epub 20190909.

March 1st, 2023

Christos Chinopoulos MD, PhD