Final report - Metabolic tissue heterogeneity related alterations in mTOR activity and their role in tumour biology

In the last decade, there were several new results and confirmatory publications about the important role of metabolic reprogramming in tumour cells and tissues. In our NKFI project, we concentrated on the mTOR hyperactivation as well as its remarkable regulatory role in signalling network and metabolic alterations of tumour tissues.

Regrading to the aims of our project, we have described many mTOR activity dysregulations in different tumour types. In correlation with these, we described further metabolic enzyme expression alterations in tumour tissues which could promote cancer progression.

Aims group 1.

In our pathological studies, **we further analysed mTOR activity and metabolic enzyme expressions and their tissues heterogeneity in different tumour types** – breast cancers, sarcomas, gliomas, lung malignancies, renal cancers and some rare tumour types. The mTOR and metabolic alteration-related important survival and pro-proliferative effects were studied in our in vitro and in vivo cancer model systems. We tested several metabolic targets using different metabolic inhibitors including mTOR inhibitors, drugs that target mitochondrial functions, antibiotics, lipid metabolism inhibitors, glutaminase inhibitors etc.

In the human tissues/cancers, **we established and tested many antibody stainings for metabolic characterisation. Finally, we could offer a staining panel for metabolic plasticity scoring** (1).

In our studied **breast cancer cohorts**, the statistical analyses of individual clinical data (including distant metastasis-free survival—DMFS—and overall survival—OS) and the heterogeneity of immunohistochemistry (IHC) staining patterns helped to detect prognostic correlations. For example, high p-S6 H-scores positively correlated with distant metastasis and older age. Furthermore, LDHA expression significantly correlated with subtype distribution, grade and stage. The most interesting observations were derived from multiple profile analyses. By combining mTOR activity and other enzyme expression scores, we could establish and suggest two categories after mTOR and metabolic protein expression analysis in human cancer tissues: cases associated with negative or positive metabolic plasticity. A high mTOR activity score (high p-S6 and/or Rictor H-score) along with high expression of at least two of four metabolic pathway elements (GLS, FASN, CPT1A and LDHA) were required as minimum criteria for positive metabolic plasticity. Patients with breast cancers showing positive metabolic plasticity had significantly worse prognosis in our study breast cancer tumour type independently (1). These stainings and/or metabolic plasticity scoring could be useful in many other tumour types. In our further published studies, we applied these stainings in gliomas (2,3), certain lung cancers (4), rhabdomyosarcomas (5), osteosarcomas (6) and renal cancers (7).

The in situ metabolic heterogeneity of high-grade human **glioblastoma cases** were analysed and the potential importance of the detected metabolic heterogeneity was tested in three glioma cell lines by protein expression analyses after temozolomide and metabolic inhibitor treatments. The importance of individual differences and metabolic alterations were observed in mono-therapeutic failures. In addition, we highlighted that rapamycin combinations with other metabolic inhibitors were the most effective in multi-targeting metabolic pathway strategies (2,3).

In Hungarian **small cell lung cancer cases, RICTOR amplification** was analysed, and detected in 15% of the cases. Regarding to our studies, we established Rictor amplification analyses using FISH and Droplet Digital PCR technology in our institute. Additionally, IHC positivity for Rictor and p-Akt was observed in 37 (37%) and 42 (42%) samples, respectively. These stainings were compared to FISH results as the

diagnostic standard of the amplification. The sensitivity and specificity of Rictor IHC were 93% and 73%, whereas the sensitivity and specificity of phospho-Akt IHC were 80% and 65%, respectively. However, there was no association between RICTOR amplification and clinical outcome – either Rictor or phospho-Akt positive staining result was associated with significantly decreased overall survival. Based on these, Rictor IHC can be used as a cost-effective method to select patients for RICTOR FISH and, potentially, for mTORC1/2 inhibitor therapy (4).

Metabolic characteristics of clear cell renal cell carcinoma (CCRCC) were described, however, the bioenergetic perturbances and metabolic adaptation possibilities of **papillary renal cell carcinoma (PRCC)** have not been detailed previously. We compared the in situ metabolic features of PRCC vs. CCRCC tissues and renal cancer vs. normal tubular epithelial cell lines. We observed higher protein expressions of the "alternative bioenergetic pathway" elements, in correlation with the possible higher glutamine and acetate consumption in PRCC cells instead of higher glycolytic and mTOR activity in CCRCCs. Increased expression of certain metabolic pathway markers correlates with the detected differences in metabolite ratios, as well. Moreover, both studied renal carcinoma cell lines showed higher mTOR activity than tubular epithelial cells, additionally, the metabolite ratio, the enzyme expression profiles, and the higher mitochondrial content also suggest increased importance of mitochondrial functions, including mitochondrial OXPHOS in PRCCs. The PRCC cells showed significant mTOR inhibitor sensitivity and the used metabolic inhibitors increased the effect of rapamycin in combined treatments. These underline the importance in the development of specific new treatment strategies, new mTOR inhibitors and other anti-metabolic drug combinations in PRCC therapy (7).

mTOR activation has been observed in **rhabdomyosarcoma** (RMS) as well, however, mTOR inhibitor therapies have limited success thus far. We analysed mTOR activation altering metabolic pathways, in Hungarian patients' materials. In total, 64% of the studied primary samples showed mTOR activity with an mTORC2 dominance (82%). These expression pattern of the studied mTOR markers can explain the inefficacy of mTORC1 inhibitor therapy. Elevated mTOR activity was associated with worse prognosis in relapsed cases. However, we could not detect that chemotherapy causes any relevant changes in mTOR activation level, and RICTOR amplification was not confirmed in any of the cases. Our metabolic enzyme expression study suggests the importance of the Warburg effect and the pentose-phosphate pathway beside glutamine demand in RMS cells. In an additional paediatric **osteosarcoma** patient cohort, mTOR activity profile and certain metabolic alterations were also studied. In total, 61% of the cases showed low mTOR activity, but higher p-mTOR expression was associated with poor histological response to chemotherapy and osteoblastic subtype. Rictor expression was higher in metastatic disease and older age at the time of diagnosis. Our findings suggest the importance of mTOR activation linked metabolic alterations, Warburg-effect, pentose-phosphate pathway, glutamine demand and fatty-acid beta oxidation in osteosarcoma cells. Therefore, we suggest performing a detailed investigation of the mTOR profile before administering mTORC1 inhibitor therapy and considering to target metabolic plasticity as an alternative therapy in these paediatric malignancies if it is necessary (5,6).

In some rare tumours including **SDH-mutant pheochromocytomas and paragangliomas,** we had additional confirmatory results from our intracellular and extracellular metabolite concentration analyses. These metabolic enzyme expression studies highlighted that metabolic alterations as consequences of oncometabolite producing mutations contribute to the survival and malignant progression in the mutation harbouring cells of these rare diseases. Our results and in vitro experiments also highlighted these metabolic alterations as potential targets in future therapies (8,9) in our collaboration work regarding SDH-mutant disease models.

Aims group 2.

In our further **in vitro and in vivo studies, we tested potential anti-metabolic drugs**. Based on our results and further published data from other research groups, some of these drugs are **suggestable for repositioning and using these in future combined oncology treatments** (GLS inhibitors, lipid oxidation inhibitors or mitochondria targeting antibiotics) (1,2,3,7,10). We described the tumour growth inhibitory effects of rapamycin+doxycycline treatments and its mechanism in vitro and in vivo treatments. This effect was characterised by mitophagy-dependent cell death mechanism (10). We have some additional unpublished results regarding to sensitivity differences in metabolic and mTORI treatments of small cell lung cancer cell lines (this manuscript is under preparation), as well.

In the last, more than 4 years, especially in the pandemic period, we had time to summarise our experiences and knowledge related to **the importance of metabolic heterogeneity and metabolic alterations in tumours, tumour progression and tumour evolution in several review publications.** We published these in Cancer Metastasis Reviews, where I, as the PI of this project, edited one special issue regarding to the importance of metabolic alterations and metabolic heterogeneity in progression and metastasis of cancers (11,12,13). We have some additional published reviews related to the previous subjects and this NKFI proposal in Pathology Oncology Research (14,15,16) and one recently published book chapter about ECM as a metabolic niche in The Extracellular Matrix and the Tumor Microenvironment. Biology of Extracellular Matrix, Springer (17). Additionally, In January 2022, Hanahan refreshed the previously summarised famous cancer hallmarks and supplemented with other new ones: non-mutational epigenetic reprogramming, polymorph microbiomes, the induced/reversed senescence and limitless phenotypic plasticity. These and especially, the last one are in nice harmony with our results and reviews published about the correlation between worse prognosis and high metabolic plasticity at tissue level in human cancers.

Aims group 3.

In 2004, the European Union (EU) prohibited testing end cosmetic products on animals, and finally in April of 2022 EU prohibited to place any new cosmetic product on the market which contains at least one new ingredient tested on animals, even if the final product was not tested on animals. Medical drug research is an extremely long and expensive process. Additionally, 95 percent of drugs fail after animal testing in human clinical safety and efficacy trials. In correlation with these, FDA and EMA modernisations also act for replaceing and lowering the number of animal testing. Growing evidences propagate that alternative new technologies (relevant human cell based – e.g. organ-on-chips or biofabricated models – or AI combined technologies) could help to predict human response and toxicity more accurately. The last pandemic situation, the COVID vaccines showed that faster and more efficient technologies are necessary in crisis and in drug/medical developments – in this case, they could leave and now suggest to minimalise the animal experiments and use as fast as possible clinical tests. The main problem in cancer therapy is the developing relapse, resistance during longer time in heterogeneous tissues of patients. The in vivo tissue heterogeneity and plasticity have many players in cellular microenvironment, including molecular genomic and immunologic as well as metabolic heterogeneity, which can be analysed in tissue sections using immune and molecular pathology methods but it can hardly be or cannot be represented in the recently applied cancer model systems.

Considering our metabolic expression studies and the detected tumour heterogeneity of human samples, in our experimental model systems – in in vivo xenograft models and in in vitro cell cultures, the metabolic enzymes expression alterations and the heterogeneity of cellular expressions were also analysed and compared after different treatments. **2D cell cultures, in vivo xenograft models and the established 3D spheroid cultures** (hanging drop and ultra-low-attachment plate cultured cells) of breast cancer and glioma cells were studied. Comparing the metabolic enzyme expressions, metabolite concentrations of the same cell line derived different cell cultures (2D, hanging drop, ULA plate) and

xenograft models, we confirmed that the traditional 3D cell culture systems differ from 2D and in vivo xenograft models of ZR75.1 cells. Moreover, the results in 2D and 3D cell cultures are more similar to each other than to xenograft experiments. The tissue slide cultures were established in the Semmelweis University 2nd Pathology Institute, and we built a **new 3D Bioprinter Unit** in our tissue and cell culture laboratory for live cell 3D bioprinting. Using this technology, we started to **bioprint with human tumour cells**, we applied breast cancer cell line derived bioink selection and finally, we built **new 3D bioprinted human breast cancer models** using ZR75.1, MD-MB-231 and another experimental breast cancer cell line mouse-derived 4T1 model, as well. We characterised the in vitro growth and tissue formation of these bioprinted rafts. For example, the tubular breast cancer derived ZR75.1 cells formed nice tubules in the printed materials, and these rafts were grown in vitro for 2-3 weeks continuously. We could compare the in situ distribution of metabolic markers and other protein expressions in these rafts ,and we could detect the in vivo situation mimicking heterogeneity in these. Additionally, the drug sensitivity and resistance were more similar to the in vivo results in our rapamycin combination experiments. Some of our 3D bioprinted results have been published in International Journal of Molecular Sciences and in Pathology Oncology Research regarding to ZR75.1 breast cancer models (18,19). Additionally, we are working on one new publication (it is under preparation) about using this technology for patient-derived technology. In these experiments, we tested an in vivo growing mouse tumour to prepare tissue-derived bioinks and 3D bioprinting. The already tested methods could forward to human patient-derived model development. The ethical improvement proposal has already been submitted to ETT-TUKEB. We are working on the metabolomics comparison of 2D – traditional 3D – 3D bioprinted – xenograft models of three breast cancer cell lines, and hopefully we can publish the results in manuscripts soon in collaboration with the Analytical Chemistry Department ELTE.

We are contributing in some other papers. In these, we could perform some experiments and measurements regarding metabolic alterations and/or mTOR activity differences, or WES Simple protein expression studies in exosome samples. These were very successful collaborations regarding to our NKFI project developments (20 ,21 ,22, 23, 24, 25). Additionally, our tumour metabolism and tumour model development studies related experiences helped us to contribute in some excellent other reviews, as well. For example, drug reposition possibilities of disulfuram or exosome studies (26, 27). We could publish some of our results and knowledge in Hungarian papers (28,29,30) and in educational book chapters (31,32).

Despite the pandemic situation reduced the number of conference attendances, our team members have presented their work in online or on-site conferences in recent years. In the abstract or on the slides and posters, the project number was indicated (more than 10 oral – including some invited speeches – and more than 15 poster presentations). The presented results are mainly published or near to publish (manuscripts are under preparation).

Additionally, in our research group several PhD dissertations were finalised and successfully completed in this period – Zoltán Hujber 2019, Gábor Petővári 2020, Ildikó Krencz 2020, Luca Felkai 2021 and Titanilla Dankó 2023 – and I also obtained my MTA doctoral degree.

Own reference lists (4 D1, 6 Q1 and 6 Q2 papers - ∑IF~83 - were published with indicated 128404 NKFI project number and there are some more additional review papers, collaboration works and book chapters)

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