**Final report on the research activities carried out from 2016 to 2022**

The main goals of this research project were to identify previously unknown, alternative pathways of biotic methane production, new elements in the mechanism of action of methane, and a methane-based approach, which might influence the outcome of hypoxia or ischemia-induced tissue reactions. Our plan was built on these largely interconnected avenues with "mechanistic" studies and "diagnostic-therapeutic" investigations, respectively and the key results are summarized along the main lines. The data are presented in detail in original publications, here the broader contexts and conclusions are discussed. It should be noted that the subject - and more specifically our work in this area - has been included in the narratives of recent perspective papers („*A number of reports on animals and cell culture have focused on the activity of methane as a gasotransmitter. Although the mode of action is still unknown, Boros et al. showed that the inhalation of 2.5% methane ameliorated the extent of ischaemia-reperfusion damage in dogs, or that exogenous methane inhibited leukocyte infiltration in vitro*" (Hoegenauer et al. Methanogenic archaea in the human gastrointestinal tract. **Nature Reviews Gastroenterol Hepatol** (01 Sept 2022). https://doi.org/10.1038/s41575-022-00673-z.

„*The detection of methane in the breath, blood or tissues could be used as a diagnostic test. … This is an encouraging sign of the potential value of understanding ROS-driven methane biology*" (Chang Liu & Jingyao Zhang. Methane might be made by all living organisms. **Nature** (09 March 2022) https://www.nature.com/articles/d41586-022-00206-3 News and Views).

It should be added that we had to initiate and authorize a 1-year postponement of the completion of the project during the COVID-19 pandemic, and further unforeseen difficulties, specific to university-based medical research have also been emerged. The introduction of the "health service employment" scheme in 2021 created many problems, scientists with MD degree, laboratory technicians, surgical assistants with medical skills experience were directed readily towards patient care (given the approx. 3-fold income gap in favour of clinical practice). Despite all these obstacles the research activity was kept up successfully, the studies were continued and completed without significant deviations from the plans.

## I. Mechanisms of generation - alternative, non-conventional methane production in rodents and humans

1. We have shown that l-alpha-glycerylphosphorylcholine (GPC) a potentially methane-donor compound can influence methane production and the mitochondrial respiratory activity (**Strifler et al. Plos One 2016**). GPC was proved to be protective against the inflammatory consequences of a hypoxic reaction as well. We have also demonstrated that this approach is beneficial in short-term to maintain the physiological balance of reactive oxygen species (ROS) production in ischemia-reperfusion (I/R) conditions, but in fact it can also have side effects if GPC is exposed to cells for a longer period of time (**Tuboly et al. Mol Cell Biochem 2019**).

2. Methane generation was detected in previously non-methane producer human volunteers consuming high doses of ethanol and a dose-response study was performed with non-methane producer rats as well. Ethanol intakes were followed by significant whole-body methane release in previously non-producer animals and in humans, the non-methane producer phenotype was transformed to methane-producer. The ethanol challenge decreased the complex II-linked oxygen consumption of rat liver mitochondria in a dose-dependent manner. The results provided the first evidence for increased in vivo methane output after such manipulations and suggested that non-bacterial factors and pathways should be considered in the background of mammalian methane generation (**Tuboly et al. Sci Rep 2017**).

3. We have investigated the methanogenic potential of several organosulfur compounds in model experimental systems of oxido-reductive stress. The impact of increased biothiol intake (organosulfur-enriched or SH-diet) was examined in association with established markers of redox imbalance in a rodent model of hepatic dysfunction induced by high oral doses of ethanol. The concentration of methane was measured with a purpose-built online photoacoustic laser spectroscopy system in a

reaction involving plant seed extracts in vitro and in vivo in hairless SKH1 mice fed with SH-diet with or without of daily oral ethanol doses. Liver NADPH oxidase activity, thiol concentrations, the reduced to oxidized glutathione (GSH/GSSG) ratios were measured and the methane emissions were recorded. On the one hand, the concentration of thiol-containing molecules in the liver was significantly increased, suggesting an influence on biothiol tissue storage. On the other hand, the SH-diet per se did not influence the net methane output which suggested that the onset of oxido-reductive imbalance is needed for an amplified in vivo methane release. Indeed, when these animals were involved in the high-dose ethanol-feeding protocol, methanogenesis was induced; the SH-diet remarkably increased the whole-body methane release after 24h of ethanol consumption, and this effect was not observed in animals kept on standard feeding. The methane production declined in the SH-enriched diet groups by the end of the ethanol protocol (due to pertaining tissue protective events), while in groups with no extra biothiol feeding, the highest methane production was reached by later time points. In summary, this study provided clear in vivo evidence for the alternative methanogenesis, the possibility of non-microbial methanogenesis during ethanol feeding (**Varga et al. Plos One 2020).**

**II. Mechanism of action**

1. Deformability of red blood cells (RBCs) and the microhemorheological properties of human RBCs were studied in vitro without the confounding in vivo effects of vasoactive metabolites. With methane treatment, the RBC deformability improved at low to moderate shear stress rates, suggesting a direct effect of methane on membrane fluidity and/or membrane cytoskeleton junctions (**Mészáros et al., Surgery 2017**).

2. Our previous data demonstrated that xanthine oxidoreductase (XOR), a major enzymatic source of reperfusion-induced superoxide formation can catalyze the reduction of nitrite to nitric oxide (NO) under hypoxic conditions in a pH-, nitrite-, and oxygen-dependent manner, while peroxynitrite formation might influence the function of nitrergic (nNOS-immunopositive) gut innervation. The role of methane and the mechanistic details of the nitrosative reaction was examined through the hypothesis that an association between XOR activity and the nitrergic neuron numbers might contribute to the differences in the susceptibility of the gastrointestinal (GI) tract to I/R damage. We have designed a complex research protocol to detect endogenous XOR activity changes and specific biomarkers of nitrogen-centered radical formation (nitrite/nitrate, NO and nitrotyrosine), in parallel with region-specific, quantitative parameters of nitrergic neurons (quantified by nNOS and HuC/HuD immunohistochemistry) during a standardized ischemic or I/R challenge, with or without exogenous methane administrations in rats. To quantify the main features of ischemia or reperfusion-induced changes, the intramural perfusion conditions of the main anatomical regions of the whole GI tract (duodenum, ileum and colon, respectively) were evaluated by in vivo microscopic methods. We used in vitro assays as well to corroborate the in vivo findings and to test another hypothesis that methane may directly modulate XOR and XOR-linked nitrate reductase activities. In control animals the exceedingly high duodenal XOR activity decreased to a five-times lower level in the ileum and then again in the colon, reaching the lowest values in the large intestine. The tissue nitrotyrosine content was closely associated with the endogenous XOR activity; it was high in the duodenum and the lowest in the colon. The levels of NO in intestinal tissue changed in the same direction, high local NO content was detected in the ileum, and tissue NO levels in the colon were much lower. The relative distribution of nitrergic neurons in the intestinal segments was relatively similar, but the number of nNOS-immune-positive neurons myenteric plexus increased from the duodenum towards the colon, and the tissue NOx content showed a similar proximal to distal gradient. We detected significant regional differences in the local NO levels, parallel to the region-specific arrangement of nitrergic myenteric neurons and XOR activity changes in the small and large intestines as well. In the ischemic groups, after the occlusion of the superior mesenteric artery, the intramural flow was fully stopped in the ileum during the initial ischemic episode, but the capillary perfusion did not stop completely in the duodenal and large intestinal parts, due to the compensatory flow redistributions through the intact collateral networks. The elevated XOR activities and the nitrotyrosine content were indicative of the presence of

the nitrosative response following the hypoxia-reoxygenation cycles, but the formation of nitrotyrosine was already started during the occlusion phase in the low-perfused duodenum, where the XOR activity was also significantly elevated during ischemia. These results provided evidence for a dynamic change in regional nNOS-expressing myenteric neurons in close association with the GI microcirculation and the XOR-dependent arm of the nitrosative stress. In addition, we provided new evidence that inhaled methane may influence XOR activity together with nitrogen biology: in this in vivo model, the elevated XOR activity was significantly decreased in all intestinal locations with an increase in the methane input; in parallel, nitrotyrosine formation was significantly suppressed. The reduced XOR activity was associated with a higher nNOS-immunopositive neuron ratio and increased NOx pool in the duodenum. Furthermore, normoxic methane administration significantly decreased tissue NO levels in the hypoxic duodenal tissue already during the ischemic phase. These phenomena were accompanied by reduced activation of an inflammatory response during reperfusion. The in vitro protocol provided further data on the inhibitory effect of methane-containing artificial air on the XOR-linked nitrate reductase activity. In conclusion, our data demonstrated segment-specific microcirculatory alterations in the GI tract, where the risk for nitrosative stress is highest in transiently hypoxic tissues with high endogenous XOR activities. Besides, we provided evidence that the XOR-inhibitory effect of methane can reduce nitroxidation and protects the nitrergic neuron population in such conditions - exogenous methane is neuroprotective by influencing XOR activity and its related effects on the pathways involved in the nitrosative stress response in the intestinal tract (**Poles et al. Free Rad Biol Med 2018**).

3. We have designed large animal models with extracorporeal circulation and veno-venous ECMO, where the aim was to investigate the methane bioactivity and the potential clinical benefits of methane gas administration in anesthetized minipigs. In this scheme we have investigated whether the systemic inflammatory response and kidney damage caused by extracorporeal circulation could be modified with exogenous methane-oxygen mixtures (despite the relative safety of circuits, this technique is still associated with potentially life-threatening post-operative inflammatory activation in cardiac surgery patients). We refined a previously employed experimental technique significantly and developed a clinically highly relevant animal model where the haemodynamic and microcirculatory consequences of extracorporeal circulation could be studied in sufficient detail. In this setup we demonstrated that methane administration through the oxygenator sweep increases the renal blood flow and diuresis in the post-cardiopulmonary bypass period. We also provided evidence that addition of methane is able to reduce the inflammatory activation (neutrophil accumulation and XOR activity) in peripheral tissues (i.e., heart, kidney, ileum). The inotropic demand was also significantly lower in methane-treated animals, which all together indicated that systemic methane administration may be a feasible way to modulate extracorporeal circulation-induced inflammatory and circulatory complications (**Bari et al. Eur J Cardio-Thoracic Surg 2019, Szabó-Biczók et al. Front Med 2022).**

4. The versatile activity of methane in various hypoxia-associated pathologies made it important to investigate cellular and subcellular reactions as well. Pilot experiments were conducted in order to analyse the effects of hypoxaemic hypoxia in a small animal model (anesthetized, mechanically ventilated rats) and to characterize the respiratory activity of mitochondria in such conditions. Secondly, and more directly, our aim was to examine the $Ca^{2+}$-activated mitochondrial permeability transition pore (mPTP) opening and mPTP-mediated $Ca^{2+}$ release, in association with oxygen consumption changes in isolated mitochondria, tissue homogenates and tissue biopsy samples, to be able to compare these data to those obtained in case of methane admixture. For this in-depth study, High-Resolution FluoRespirometry was used for the combined detection of $Ca^{2+}$ and oxygen fluxes, and Calcium Green-5N (CaGreen-5N), a single wavelength fluorescent dye, was employed to measure extramitochondrial $Ca^{2+}$. We provided evidence that the method is suitable to monitor simultaneous oxygen and $Ca^{2+}$ fluxes and the opening of mPTPs in various biological samples (in isolated mitochondria and tissue homogenate) after stimulation with external $Ca^{2+}$ (**Nászai et al. Sci Rep 2019).** Besides, we have demonstrated that measuring the duration of stimulated $Ca^{2+}$ fluxes provides a new parameter to evaluate the efficacy of mPTP inhibition – and that this approach can be used to monitor

methane-induced intramitochondrial changes in other studies as well **(Benke K et al. J Heart Lung Transplant 2021**).

**5.** We have designed new experimental models for methane-based diagnostics, as well because we hypothesized that exhaled methane levels can change in association with the flow conditions of the mesenteric microcirculation. In this context, we have employed strictly controlled in vivo tests to investigate the diagnostic value of real-time breath methane analysis for the detection of the actual status of the mesenteric perfusion in occlusive and non-occlusive models of splanchnic I/R (in methane producer pigs or after intraluminal methane supplementation in non-methane-producer rats). We have found a robust, dynamic correlation between the microcirculatory component of the mesenteric circulation and parallel changes in breath methane output. The decreases in mesenteric macro- and microcirculatory flow resulted in a reduction in exhaled methane concentration regardless of occlusive or non-occlusive forms of ischemia. Specifically, the level of exhaled methane did not change if ischemia persisted and increased rapidly as the mesenteric flow was restored. The results provided evidence for a significant correlation between exhaled methane and intestinal microcirculatory changes in methane-producing conditions when the range of baseline endogenous methane output exceeds the range of atmospheric methane concentration. The real-time analysis of exhaled methane levels merits attention toward the development of a new diagnostic concept for non-invasive detection of mesenteric ischemia **(Szűcs et al. Crit Care Med 2019).**

6. We continued the "diagnostic" protocol with real-time analysis of exhaled methane and our next goal was to validate and compare the sensitivity of the method with an established technique. We used sublingual microcirculatory monitoring (a diagnostic method already in clinical use) in controlled, graded hemorrhage and resuscitation conditions in anesthetized minipigs. In this large animal model gradual, relatively low-rate blood losses were followed by controlled, gradual and restricted (80% of the baseline mean arterial pressure) fluid resuscitation. As expected, the superior mesenteric artery (SMA) blood flow was significantly decreased after 5% blood withdrawal. Changes in the exhaled methane concentration followed the decrease with the same dynamics. Significant changes in serosal and mucosal components of the ileal microcirculation occurred slightly later, after a 10- or 20% blood loss, respectively, and the changes in exhaled methane levels strictly followed the mesenteric alterations. At the beginning of the resuscitation phase a sudden increase in the SMA flow and mucosal microperfusion parameters and a rise in exhaled methane levels were observed. Sublingual perfusion correlated with mucosal and serosal mesenteric microperfusion parameters during the hemorrhage phase but not during the resuscitation. In conclusion, changes in exhaled methane concentration may indicate bleeding at an early stage and follow changes in mesenteric perfusion during hemorrhage and resuscitation as well, with a diagnostic value comparable to the monitoring of the sublingual microcirculatory area. These data further supported the view that this technique might be a useful, additional non-invasive tool in cases where hemorrhagic complications might be expected in the GI tract (**Bársony et al. Front Med 2020).**

**III. Therapy**

1. We have designed protocols to establish and define the clinical value of methane-enriched transplantation solutions for static preservation of donor grafts. Firstly, we have carried out a sequential exploration of the mitochondrial effects of exogenous methane in normoxic and simulated ischemia-reperfusion (sI/R) environments. These experiments were performed in two main series using either intact neonatal rat cardiac myocytes or isolated mitochondria. The dynamics of the methane concentration changes were detected by photoacoustic spectroscopy and a High-Resolution Respirometry (HRR) system was used to examine the oxygen consumption of cardiac myocytes and mitochondria in various mitochondrial metabolic states. The mitochondrial hydrogen peroxide production and changes to mitochondrial membrane potential were recorded and cell viability and apoptosis were also detected as final outcomes. We have shown that the administration of methane reduces the sI/R-related mitochondrial ETC disturbances and mitigates the subsequent apoptotic consequences. Of importance, methane preserved mitochondrial membrane potential (a marker of

the integrity of the inner mitochondrial membrane) and decreased cytochrome c release (a sign of the integrity of the outer mitochondrial membrane) as well. Next, the role of Complex I and Complex II in the post-anoxic cardiac mitochondrial respiration was addressed in more detail. As a result of methane treatment, the mitochondrial respiration was inhibited when glutamate + malate was used as a Complex I substrate but not with succinate as a Complex II substrate. This finding suggested us that interaction with Complex I certainly occupies a key position in the protective mechanism of methane treatment against sI/R injury. (The addition of methane resulted in a decreased electron flux through Complex I but did not alter the succinate oxidation through Complex II. Based on these findings, the drop in net reactive oxygen species (ROS) production from mitochondria with preserved succinate oxidation in the presence of methane is most likely directly related to the inhibition of Complex I. It should be added that reversible deactivation of mitochondrial Complex I is an intrinsic mechanism, which provides a fast response of the mitochondrial respiratory chain to oxygen deprivation. However, subsequent reoxygenation leads to ROS generation due to the rapid burst of respiration. Under normoxic conditions, a high level of nicotinamide adenine dinucleotide hydride (NADH) can drive forward electron flow with superoxide generation at the flavin mononucleotide moiety located near the NADH binding subunit. During reoxygenation, reverse electron flow (RET) driven by a reduced ubiquinone (ubiquinol) pool and high proton motive force can generate ROS when electrons flow back from ubiquinol to Complex I). Therefore, it seems that methane treatment restricts the forward electron transfer within Complex I in control mitochondria while effectively inhibiting RET in post-ischaemic mitochondria. (**Jász et al. J Cell Mol Med 2021**)

2. Next we have used the generally employed organ storage solution (Custadiol - CS) with or without methane admixture in an isogenic rat model of heterotopic heart transplantation (HTX), devoid of immunological effects. Our additional goal was to better understand and explore the mechanisms underlying methane bioactivity in hypoxia-linked situations. It has already been demonstrated that methane can protect against I/R-induced apoptosis by inhibiting the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/glycogen synthase kinase-3β (GSK-3β) pathway and nuclear factor-erythroid2 p45-related factor 2 (Nrf2) activation and other evidence suggested us that methane can possibly limit endoplasmic reticulum (ER) stress as well. Therefore, we put special emphasis on the hypothesis that a cytoprotective action of methane enrichment may target ER stress and its functional links to mitochondria. We also hypothesized that structural and functional mitochondrial damage and ER stress are major upstream factors that govern the progression of graft dysfunction after organ procurement and replantation. In this study protocol the hearts of donor Lewis rats were explanted and stored in cold CS or in methane-saturated CS solution. In the control group donor rats underwent the same surgical procedure until the explantation, but the hearts were not subjected to cold ischemia and storage and were not transplanted. In another group the explanted grafts were stored in CS solution at 4°C during the 60-minute cold ischemic period, whereas in group 3 the grafts were stored in methane-enriched CS during the 60-minute cold ischemic period (the cold cardioplegic solution used to arrest the heart was also supplemented with methane). After HTX the left ventricular (LV) pressure-volume relations and coronary blood flow (CBF) were assessed to evaluate early post-transplant graft function. At the end of haemodynamic measurements, samples were taken for qPCR of ER stress and mitochondria-related apoptosis markers (CHOP, GRP78, GSK3b, VLDR, Caspase 3 and 9, Bcl2, Bax), and several biochemical parameters. Mitochondrial functional analysis was performed with HRR. The main results were as follows: LV contractility and active relaxation (dP/dtmin at 120 ml of LV volume) improved significantly after 60 minutes of reperfusion, while alteration of CBF (standardized to heart weight) was also significantly improved following methane pretreatment. The methane-enriched storage significantly reduced the transcription of pro-apoptotic proteins and the Bcl2/Bax ratios as compared to non-treated grafts. Increased mitochondrial oxidative phosphorylation, reduced leak respiration and cytochrome c release were also demonstrated in the methane-treated group and significantly lower plasma LDH, CK and troponin T levels were present as compared with CS storage alone. In summary these results provided evidence for the clinical benefits of a methane-enriched organ preservation solution during HTX. We have concluded that the mechanism involves the

inhibition of pro-apoptotic signals and ER stress, and therefore, a methane-enriched preservation solution could be a potential agent in the inventory of transplantation surgery and/or cardioprotective in cardiac surgical procedures requiring prolonged cardioplegia (**Benke K et al. J Heart Lung Transplant 2021**).

**IV. Summaries**

Review papers were published to put aerobic methane formation and the putative mechanisms into broader perspective (in **Gasotransmitters, Royal Soc Chem 2018**). Our results describing the novel medical aspects of interactions between methane and gas messengers (including NO) were discussed in **Intensive Care Med Exp** (2019) and in a special issue of **Frontiers** (2017) where we have reported on the biological functions and bioactivity of methane in various in vitro and in vivo experimental models. Besides, we have summarized the relevant literature on alternative methane-producing processes in eukaryotes, the results that established the bioactive role for methane, and the intracellular pathways influenced by exogenous methane (**Front Physiol 2019**). We have also summarized our findings on the organ protective activity of exogenous methane treatments with special emphasis on its versatile effects demonstrated in respiratory distress conditions (**Front Cell Dev Biol 2022**) and we have discussed the question if ROS-driven methane formation has a general physiological role in eukaryotes (**Clin Transl Med 2022**).

The presented results formed the basis of PhD theses as well (Mészáros András, 2017, Strifler Gerda 2017, Bari Gábor 2019, Szilágyi Ágnes 2021) and as planned, the results detailed above have been reported in national and international forums in the field.