Real-time detection of methane emission for monitoring intestinal microcirculatory changes

Anett Gabriella Bársony M.D.

Ph.D. Thesis

University of Szeged

Albert Szent-Györgyi Medical School

Doctoral School of Multidisciplinary Medical Sciences

Institute of Surgical Research

Szeged

2025

LIST OF PUBLICATIONS

Full papers related to the subject of the thesis

I. **Bársony A**, Vida N, Gajda Á, Rutai A, Mohácsi Á, Szabó A, Boros M, Varga G, Érces D. Methane Exhalation Can Monitor the Microcirculatory Changes of the Intestinal Mucosa in a Large Animal Model of Hemorrhage and Fluid Resuscitation. *Front Med* 2020; 7:567260. doi: 10.3389/fmed.2020.567260. eCollection 2020. **IF: 4.59**

II. Ugocsai M, **Bársony A,** Varga RA, Gajda Á, Vida N, Lajkó N, Rónaszéki B, Tóth G, Boros M, Érces D, Varga G. Conjugation with Tris decreases the risk of ketoprofen-induced mucosal damage and reduces inflammation-associated methane production in a rat model of colitis. *Pharmaceutics* 2023; 15 (9): 2329 **IF: 3.24**.

∑ Impact factor: 7.83

Full paper closely related to the subject but not included in the thesis

I. Vida N, Varga Z, Szabó-Biczók A, Bari G, Vigyikán G, Hodoniczki Á, Gajda Á, RutaiA, Juhász L, Tallósy SP, Turkevi-Nagy S, **Bársony A**, Öveges N, Szabó A, Boros M, Varga, G, Érces D. Methane administration during oxygenation mitigates acute kidney injury in a pig model of 24-hour veno-venous extracorporeal membrane oxygenation. *Shock* 2025; *in press* **IF: 2.7**

Full paper not related to the subject of the thesis

I. Lázár G, Paszt A, Simonka Z, **Bársony A**, Ábrahám S, Horváth G. A successful strategy for surgical treatment of Boerhaave's syndrome. *Surgical endoscopy*. 2011; 25(11), 3613-3619. DOI: 10.1007/s00464-011-1767-1 **IF: 4.013**

∑ Impact factor: 15.25

1. INTRODUCTION

1.1 Blood supply and perfusion of the intestinal tract

The vascular architecture of the intestinal tract consists of a complex network of arterioles that supply blood to the intestinal wall. These arterioles extend into the vasa recta within the mesentery, enveloping the intestinal musculature. They converge into an arterial plexus in the submucosal layer, enabling blood flow into the mucous membrane and subsequently branching into capillary networks within the longitudinal muscle layers. Approximately 20% of blood flow traverses the musculature, while 80% is directed through the mucous membrane. Intestinal contractile activity primarily occurs in the muscle layers, influencing blood flow dynamics within the mucosal capillary networks.

Variations in the capillary network exists across different intestinal segments, reflecting their unique microcirculatory architectures. In humans, arterioles supplying the small intestine mucosa are adapted to the villi's structure, featuring a rich capillary network at each villus apex, supplied by non-centrally located arterioles and venules. In contrast, colonic arterioles run parallel to the mucosal glands, and their surface capillaries are located closer to the epithelial layer. The capillary networks in the mucous membrane exhibit a honeycomb-like structure, with density diminishing from proximal to distal segments of the large intestine, indicating a perfusion gradient (Kvietys, 2010).

1.2 Inflammatory diseases in the intestinal tract

Inflammatory bowel disease (IBD) is an idiopathic condition marked by chronic, immune-mediated inflammation of the gastrointestinal (GI) tract, primarily categorized as Crohn's disease or ulcerative colitis. The incidence of IBD is rising, particularly in Western countries, necessitating improved management strategies (Ng et al, 2017). Pharmacotherapy includes traditional agents such as 5-aminosalicylates, corticosteroids, cytotoxic drugs, antibiotics, and probiotics, alongside biological therapies like anti-TNFα agents, anti-integrin agents, and monoclonal antibodies targeting IL-12/23 (Rutgeerts et al, 2004; Steigleder et al, 2020). However, many patients exhibit resistance to anti-TNFα antibodies, highlighting the urgency for alternative treatments. Promising future candidates include homing inhibitors, Janus kinase inhibitors, Th17 inhibitors, and sphingosine-1-phosphate receptor agonists.

IBD disproportionately affects young adults, placing a significant burden on healthcare systems. With appropriate therapeutic strategies, a favorable prognosis can be achieved, greatly improving the quality of life for patients, particularly the younger demographic. Ongoing

advancements in medical science, particularly the introduction of novel pharmacological agents, offer an increasingly optimistic outlook for those affected by IBD.

To further improve treatment outcomes, the development of innovative diagnostic tools is essential. Continuous, real-time monitoring of microperfusion in the lower GI tract could significantly deepen our understanding of the microcirculatory disturbances linked to IBD. Such monitoring would not only help assess treatment efficacy but also enable the early detection of disease flares, thereby allowing timely initiation of therapy. In this context, we propose the measurement of methane emissions as a promising, non-invasive method to support these diagnostic aims.

1.3 Acute changes in the intestinal macro- and microcirculation: Why and how to monitor?

Acute changes in GI circulation and microcirculation are significant medical issues often linked to conditions such as hemorrhage, inflammation, embolism, and low cardiac output. Impaired GI perfusion and reperfusion can lead to increased mortality and morbidity in vascular, reconstructive, and transplant surgeries. Studies indicate that macro-and microhemodynamics can change independently in stress conditions, making it difficult to predict microcirculatory changes based solely on macrohemodynamic assessments. Peripheral microcirculatory dysfunction may occur in critically ill patients, even when macrohemodynamic resuscitation goals are met (Dubin et al, 2009; Jhanji et al, 2009), contributing to organ failure (De Backer et al, 2010).

Monitoring GI microperfusion is vital for successful treatment, as early detection of altered GI perfusion can prevent mucosal damage and barrier loss. However, assessing microcirculatory changes is challenging due to difficult access to intraabdominal areas. While indirect tonometry has limited success, the only clinically useful non-invasive technique is sublingual circulation analysis via intravital microscopy, which has variable reliability across studies (Boerma et al, 2007; Qian et al, 2014).

1.4 Endogenous methane production: diagnostic importance

To address these challenges, we propose measuring exhaled methane concentrations. Endogenous methane production in humans has physiological significance, influencing cellular processes and membrane dynamics (Boros et al, 2012). Methane may accumulate at cell membrane interfaces, affecting the function of membrane-bound proteins, including those involved in reactive oxygen species (ROS) production (Boros and Keppler, 2019). All humans

produce methane, suggesting it may serve as a gasotransmitter with protective effects against oxidative stress (Keppler et al, 2016).

Methane production is predominantly associated with methanogenic archaea in the GI tract, particularly Methanobrevibacter smithii, which synthesizes methane from hydrogen produced during carbohydrate fermentation. Due to its properties, methane traverses membrane barriers and is exhaled through the lungs, making breath tests effective for diagnosing certain GI conditions. However, only 30-40% of the Western population are classified as methane producers based on exhaled levels, defined as more than 1 ppm above atmospheric concentration (Bond et al, 1971; Levitt et al, 2006).

Exhaled methane measurement has been used to assess malabsorption syndromes but is limited by traditional gas analysis methods (Levitt et al, 2006; Ligor et al, 2008), which risk artefacts and do not continuously reflect in vivo release (Yu and Pawliszyn, 2004). Notably, higher exhaled methane levels have been observed in porcine sepsis models, indicating its potential as a monitoring tool for microcirculation.

For continuous methane detection, high-sensitivity photoacoustic spectroscopy (PAS) offers an effective approach (Ngai et al, 2006). PAS utilizes near-infrared diode lasers to generate sound waves through light absorption by gas molecules, providing in situ, continuous measurements with high sensitivity (Bozóki et al, 2011). Our purpose-built PAS instrument has shown potential as a substitute for traditional gas chromatography (GC) in breath analysis (Tuboly et al, 2013).

2. MAIN GOALS

The overarching aim of this research was to thoroughly investigate the relationship between methane emission and GI microcirculation. More specifically, the primary objective of the first study was to explore the association between whole-body methane emission, colonic mucosal inflammation, and the microcirculation of the colonic and small intestinal serosa in experimental rat models.

1. Our goal was to demonstrate the connection between whole-body methane emission and the microcirculatory and inflammatory changes in the colon and small intestine induced by trinitrobenzene sulfonic acid (TNBS) induced experimental colitis.
2. Furthermore, we examined how drug-induced alterations in GI microcirculation affect methane emission levels.
3. An additional aim was to assess how whole-body methane production changes in response to colonic mucosal microcirculatory disturbances during TNBS-induced

colitis, and how these responses are modulated by ketoprofen (Ket) and ketoprofen- tris-hydroxymethyl-aminomethane conjugate (Ket-Tris) treatments.

In the second part of the research, our objective was to validate and compare the sensitivity of real-time exhaled methane monitoring against an established method - sublingual microcirculatory assessment - within a large animal model of controlled, graded haemorrhage followed by the early phase of fluid resuscitation.

4. We aimed to evaluate the diagnostic potential of real-time exhaled methane detection for identifying internal bleeding in a large animal model, correlating changes in methane output with superior mesenteric arterial blood flow and mucosal microcirculatory parameters.
5. Additionally, we sought to determine whether continuous breath methane monitoring could offer clinically relevant insights into the state of the mesenteric vascular bed during the early stages of fluid resuscitation.

3. MATERIALS AND METHODS

3.1. Experimental animals

In Study 1, experiments were performed on three separate but interlinked studies on male Sprague-Dawley rats (n=72; average weight: 200 g ± 10 g), housed in plastic cages. The rats were fed regular laboratory chow, followed by a carbohydrate-rich diet (bread rolls) for three days before the experiments. They were deprived of food (but not water) for 12 hours prior to anesthesia. All protocols adhered to EU directive 2010/63 on animal protection and were approved by the National Scientific Ethical Committee (license number V./148/2013).

In Study 2, experiments involved six male outbred Vietnamese minipigs (average weight: 40±3 kg), following EU Directive 2010/63 (approval number V/148/2013). The pigs underwent a 7 to 10-day acclimatization in conditions with natural light, having free access to water and food. Prior to experimentation, they were fasted for 12 hours, with water available.

3.2. In vivo experimental methods

The direct measurements of the microcirculation: In Study 1/1 and Study 2, the Cytocam-Incident Dark Field (IDF) imaging technique (CytoCam Video Microscope System, Braedius Medical, Huizen, the Netherlands) was used to visualize and evaluate the microcirculation. IDF imaging is optimized to visualize the hemoglobin-containing structures by illuminating the organ surface with linearly polarized light (Aykut et al, 2015). In Study 1/1

and 1/3, the microcirculation of colon serosa were measured. In Study 2, the micrucirculation of the ileal serosal and mucosal layers and the sublingual area were observed. The records were analysed off-line (AVA 3.0; Automated Vascular Analysis, Academic Medical Center, University of Amsterdam) and microvascular flow index (MFI), De Backer's Score (DBS) and heterogeneity index (HI) were determined (De Backer et al, 2007).

In Study 1/2, the orthogonal polarization spectral (OPS) imaging technique (Cytoscan A/R, Cytometrics, Philadelphia, PA, USA) was used for non-invasive visualization of the serosal microcirculation of the stomach, duodenum, jejunum, ileum or colon. This technique utilizes reflected polarized light at the wavelength of the isobestic point of oxy- and deoxyhaemoglobin (548 nm). As polarization is preserved in reflection, only photons scattered from a depth of 2–300 mm contributes to image formation. A 10x objective was placed onto the serosal surface of the stomach, and microscopic images were recorded with an S-VHS video recorder 1 (Panasonic AG-TL 700; Matsushita Electric Ind. Co. Ltd, Osaka, Japan). Quantitative assessment of the microcirculatory parameters was performed off-line by frame-to-frame analysis of the videotaped images. Red blood cell velocity (RBCV; μm/s) changes in the postcapillary venules were determined in three separate fields by means of a computer-assisted image analysis system (IVM Pictron, Budapest, Hungary).

Methane emission measurements: We employed a near-infrared laser technique-based PAS apparatus (Tuboly et al, 2013). PAS is a subclass of optical absorption spectroscopy that measures optical absorption indirectly via the conversion of absorbed light energy into acoustic waves due to the thermal expansion of absorbing gas samples. The amplitude of the generated sound is directly proportional to the concentration of the absorbing gas component. The gas sample passes through the photoacoustic cell, in which signal generation takes place, and a microphone then detects the photoacoustic signal produced. The gas samples were taken continuously from the exhalation outlet of the ventilator at a 150 mL min^{-1} rate during the experiments. In Study 1, whole-body methane level was measured, while in Study 2, exhaled methane level was measured.

3.3. In vitro experimental methods

Measurements of lactate level, total haemoglobin concentration and haematocrit: Changes in tHb, Hct and lactate concentration were analyzed with a cooximetry blood gas analyzer (Cobas b 123, Roche Ltd., Basel, Switzerland) from the arterial blood samples.

Evaluation of inflammatory markers: At the end of the experiments, we collected gastric, colonic samples, as well as blood samples from the animals. The tissue samples were

stored at -70°C until use. For the measurements, the tissue samples were homogenized and centrifuged at 24,000 g for 20 minutes at 4°C in Tris-HCl buffer (0.1 M, pH=7.4) containing 0.1 mM polymethylsulfonyl fluoride to inhibit tissue proteases. The myeloperoxidase (MPO) activity in the examined tissue reflects the accumulation of leukocytes and was determined from the sediment of the tissue homogenate according to the method of Kübler et al. (1996). The xanthine oxidoreductase (XOR) enzyme is a significant source of superoxide radical production in the tissues, and its level was measured in the supernatant of the homogenate using a fluorometric kinetic assay according to the method of Beckman et al. (1989). The malondialdehyde (MDA) level was used as an estimate of lipid peroxidation through the reaction with thiobarbituric acid reaction, and the values were corrected for the tissue protein content (Placer et al, 1966).

3.4. Experimental protocols

Study 1/1 - Connection beetwen whole-body methane emission microcirculatory change and inflammatory response induced by experimental TNBS-colitis.

In Study 1/1 the animals (n=30) were randomly allocated into 2 groups (n=15, each). In group 1 colitis was induced by the intracolonic (ic) administration of TNBS (40 mg/kg in 0.25 ml of 25% ethanol) through an 8 cm-long soft plastic catheter under transient light inhalation anaesthesia (Morris et al. 1989). In control group, the animals received enemas with a total volume of 0.25 ml containing 25% of ethanol (the solvent for TNBS). The animals were then returned to their cages and were fed ad libitum with standard laboratory chow. On days 1, 2 and 3 after colitis induction 5-5 animals of each group were anesthetized for invasive microcirculatory investigations and biochemical sample collection. Besides, whole-body methane detection was performed individually in the same timeframes.

Study 1/2: Connection between whole-body methane emission and drug induced GI microcirculatory changes.

In Study 1/2, the animals (n=18) were randomly allocated into 3 groups (n=6, each). Group 1 served as vehicle-treated control where 10 ml/kg buffered 0.11 M potassium hydroxide (KOH) was given orally. In group 2, high doses of Ket solution (0.56 mmol/kg, in a volume of 10 ml/kg) were gavaged via a flexible oesophageal tube to the animals. After the treatment, the animals were returned to their cages and were fed ad libitum with a carbohydrate-rich diet. Group 3 was treated with the Ket-Tris conjugate in equimolar doses to Ket (0.56 mmol/kg, in a volume of 10 ml/kg). At the beginning of the observations and before and after the treatments

on days 1 and 2 whole body methane generation was detected. On day 2, after methane output measurements, the animals were anaesthetized with sodium pentobarbital (50 mg/kg i.p.). For instrumentation, the animals were placed in a supine position on heating pads, and the trachea and right jugular vein were cannulated to secure spontaneous breathing and *iv* administration of fluids. After a midline abdominal incision, intravital videomicroscopy was performed to examine the microcirculatory changes on the serosal surfaces of stomach and colon. Whole-body methane detection was performed individually 24 h after the treatments.

Study 1/3: Connection between whole-body methane emission and TNBS-colitis induced GI microcirculatory changes with anti-inflammatory treatment.

In Study 1/3 (n=24) the control group (n=6) received enemas with a total volume of 0.25 ml containing 25% of ethanol (the solvent for TNBS). In groups 2, 3, and 4 (n=6, each) colitis was induced with a TNBS enema (40 mg/kg). In groups 3 and 4 Ket (Col+Ket; 0.56 mmol/kg, 20 mg/kg, in a volume of 10 ml/kg) or Ket-Tris conjugate (Col+Ket-Tris; 0.56 mmol/kg, (20 mg/kg, in a volume of 10 ml/kg), were gavaged to the animals 12 h after colitis induction. The animals in the control and the non-treated colitis groups were gavaged with the solvent for Ket (10 ml/kg buffered 0.11 M of potassium hydroxide). On day 2 (24 h after Ket or Ket-Tris treatments), the animals were anesthetized and surgery was performed. The animals were placed in a supine position on heating pads, the trachea and the right jugular vein were cannulated and after a midline abdominal incision. In each group, intravital videomicroscopy was performed on the colonic serosa to examine the changes in microcirculation. Whole body methane detection was performed individually 24 h after the treatments.

Study 2 - Diagnostic value of methane exhalation in a pig model of gradual, controlled haemorrhage

After the surgical preparation, a 30-min stabilizing period was provided, followed by baseline measurements. Gradual bleeding was then started. The protocol was divided into seven steps with haemorrhage (T_0–T_7) followed by gradual fluid resuscitation in five steps (T_8–T_{12}), until 80% of the baseline mean arterial pressure (MAP) value was reached. The total blood volume (BV) was estimated as 65 mL/kg and 5% of the estimated BV was withdrawn (129±8 mL) by the end of each bleeding step, and an equal volume of hydroxyethyl starch (HES; Voluven 6%, 130/0.4; Fresenius Kabi, Bad Homburg, Germany) was administered during each resuscitation step.

Every bleeding or resuscitation interval was started with microcirculatory recordings at the ileal mucosal and serosal surfaces and at the sublingual area. At each location, three, 20-sec video recordings were made. Following the intravital videomicroscopic investigations, MAP and SMA flow were recorded and finally blood samples were taken for lactate, total haemoglobin (tHb) and haematocrit (Hct) determinations. Methane values were continuously recorded throughout the observation period. At the end of the experiments the animals were sacrificed with an overdose of pentobarbital sodium (120 mg kg-1 iv; Sigma-Aldrich Inc, St. Louis, MO, USA). The timeline of measurement intervals is summarized.

3.6. Statistical analysis

Data analysis was performed with a statistical software package (SigmaStat for Windows, Jandel Scientific, Erkrath, Germany). Normality of data distribution was analyzed with the Shapiro–Wilk test. The Friedman on ranks or one-way repeated measures analysis of variance (ANOVA) was applied within groups. Time-dependent differences from the baseline for each group were assessed with Dunn's method or the Bonferroni t-test. Differences among groups were analyzed with the Kruskal–Wallis one-way analysis of variance on ranks, followed by Dunn's method. Median values and 75th and 25th percentiles are provided in the figures; P values <0.05 were considered significant. Correlations between two variables were examined using Pearson's correlation coefficient (r); regression lines and 95% confidence intervals are provided in the figures.

4. RESULTS

Study 1

Study 1/1 - Connection among whole-body methane emission microcirculatory change and inflammatory response induced by experimental TNBS-colitis.

On day 3, after colitis induction, significant elevation was demonstrated in the level of MPO, XOR activity and MDA levels relative to the control group values. A significant increase was observed in MFI and DBS values in the colitis group on days 1, 2 and 3 of colitis relative to the control values. Similarly, whole-body methane emissions were significantly elevated on days 2 and 3 after colitis induction in contrast to the control values and values of the control group.

Study 1/2: Connection between whole-body methane emission and drug induced GI microcirculatory changes.

RBCV of the mucosa was measured as a quantitative marker of GI microcirculatory condition. The RBCV in gastric serosa was significantly raised in the Ket-treated group as compared to the control group (Table 2), and a similar change was detected in the duodenum, jejunum and ileum, but not in the colon. Baseline microcirculatory values were measured in the entire GI tract in the Ket-Tris-treated group.

The whole-body methane level was measured before and 24 h after the treatments. In the control and Ket-Tris groups, no changes were detected at 24 h, while significant elevation was demonstrated 24 h after treatment in the Ket-treated group.

Study 1/3: Connection between whole-body methane emission and TNBS-colitis induced GI microcirculatory changes with anti-inflammatory treatment.

The RBCV in the colonic subserosa was significantly increased in colitis group as compared with the control group. Ket and Ket-Tris-treatment decreased the elevated RBCV by the end of the observation period.

Significant whole methane emission was detected after 24 h of colitis induction in colitis and Ket-treated colitis goups in contrast to the control group. The Ket-Tris-treatment did not result in a change in emission of methane.

Study 2 - Diagnostic value of methane exhalation in a pig model of gradual, controlled haemorrhage

After the bleeding, MAP significantly decreased by T_3 (20% of blood loss) and remained significantly lower until the end of the hemorrhage phase. During the resuscitation period, it remained significantly lower than the control values until T_{10}, at which the volume of fluid replacement was equal to 15% of the estimated BV. By the end of the resuscitation phase, MAP reached 80% of the baseline value, as planned.

The plasma lactate level was increased significantly by T_6 (30% blood loss) and remained significantly higher compared with the baseline value until T_{11}. The bleeding and fluid resuscitation caused a continuous decrease in both tHb and Hct values. A significant difference from the baseline values was observed at T_6 in the case of both parameters. tHb was decreased by more than 3 g dL^{-1} until the end of the hemorrhage phase (M=11.92; p25=9.7; p75=13.4 g dL^{-1} vs M=8.66; p25=7.43; p75=10.48 g dL^{-1}), which indicates severe bleeding.

The SMA flow decreased continuously during the hemorrhage phases. An early, significant drop was already noted at a 5% loss (T_1) of the estimated BV. After fluid resuscitation, the MAP started to increase steeply and reached its peak value at the second

resuscitation step at T_8. During the following parts of the resuscitation phase, it decreased gradually to the level of the baseline values.

The average for baseline exhaled methane was 60.9–90.1 ppm, which corresponds to the higher range of values measured in methane-producing humans (Levitt et al, 2006). The individual baseline data were subtracted from the test values to increase the comparability of measurements even in the case of larger individual variances (Szűcs et al, 2019).

The exhaled methane concentration decreased significantly after 5% blood loss, already at T_1, similarly to the SMA flow changes. After resuscitation was started, breath methane level rapidly increased to a significantly higher level than the baseline and reached a peak after a fluid volume equal to 10% of estimated BV, administered at T_8 period.

The DBS values decreased significantly as the bleeding progressed. The serosal DBS was lower than the baseline value from a 10% blood loss (T_2). This was followed by a deterioration of mucosal DBS from the loss of 20% of estimated BV (T_4), while the decrease of DBS in the sublingual area was statistically significant from a 25% blood loss only (T_5). Moreover, the sublingual DBS was significantly higher than the values in the ileal regions from T_5 to T_7, which marks the end of the hemorrhage phase and a loss of 35% of BV. When fluid resuscitation started, the mucosal DBS increased rapidly and was significantly higher as compared to the serosal and sublingual values after fluid replacement with a volume equal to 5% of BV (T_8), reaching the highest value at T_{10}. The serosal DBS values increased more gradually with a maximum at T_{12}.

Bleeding caused a decrease in the MFI in all three locations, and the first to reach significance was the MFI in the sublingual area at T_3. This was followed by a significant decrease in serosal MFI from T_4 and in mucosal MFI from T_5. The fluid resuscitation resulted in a significant improvement of the MFI in all investigated locations. Sublingual MFI was significantly higher than the MFI in the ileal mucosa and serosa from T_{10}.

The heterogeneity of the microcirculation increased during the hemorrhage phase as shown by the HI. The most important difference between the sublingual and ileal regions is that while the sublingual HI was restored during resuscitation, the HI in both the ileal mucosa and serosa remained significantly higher compared to the baseline and the sublingual values until the end of the experiments.

We compared the changes in the exhaled methane concentration during the hemorrhage and resuscitation phases with SMA flow data. In the hemorrhage phase, a significant correlation was found ($r=0.82$) and a significant relation could be demonstrated in the resuscitation phase as well.

When the possible links between the changes in exhaled methane levels and the DBS values of the two components of the ileal microcirculation during the hemorrhage and resuscitation were investigated, the DBS in the serosa correlated significantly with the exhaled methane values during bleeding (r=0.79) and fluid resuscitation (r=0.66). Similarly, a significant correlation was present in the case of the mucosal DBS values in both the hemorrhage (r=0.82) and resuscitation phases.

Phases are shown to demonstrate the changes in exhaled methane concentrations and the DBS of ileal mucosa on an original methane registration curve of a single animal and the simultaneous changes in the mucosal DBS in the same animal during hemorrhage and resuscitation.

Significant correlations were detected during the hemorrhage phase between the sublingual DBS and the serosal or mucosal DBS values (r=0.74 and r=0.66, respectively.

5. DISCUSSION

The primary objective of Study 1 was to investigate the relationship between whole-body methane emission and colonic mucosal inflammation, as well as serosal microcirculation in the colon and small intestine, using experimental rat models. We utilized a standardized TNBS method to induce varying degrees of colitis, resulting in significant mucosal injury, increased inflammatory enzyme activity, hyperemia in colonic microcirculation, and elevated whole-body methane emissions.

An additional goal was to explore the link between methane production and oxidative stress markers in inflamed intestines. The inflammatory response included hyperemia and the activation of XOR, an enzyme that generates ROS. Methane emissions were significantly higher in TNBS-treated rats compared to controls.

We proposed that increased methane output in this model stems from both inflammatory hyperemia and localized ROS generation in the bowel. Additionally, we examined drug-induced effects on GI microcirculation by administering Ket and Ket-Tris, expecting different impacts from the two non-steroid anti-inflammatory drugs (NSAIDs).

Traditionally, GI methane production was attributed solely to the enzymatic activity of anaerobic methanogenic archaea (Ellermann et al, 1988; Conrad 2009). However, recent evidence shows that eukaryotes can directly release methane in the presence of oxygen, independent of microbial activity (Boros and Keppler, 2019). Compelling findings indicate that biotic methane formation occurs across all multicellular organisms, even under aerobic conditions (Keppler et al, 2006; Wang et al, 2021). Research has demonstrated that methane

can be produced in chemical systems through the incubation of methyl group-containing compounds with ferric iron, ascorbate, and hydrogen peroxide without enzymatic involvement (Ghyczy et al, 2003).

Ernst et al. (2022) found that ROS-induced methyl radicals are key intermediates in methane production across eukaryotes, including human cell lines (Liu and Zhang, 2022). While the biological significance of this mechanism is not fully understood, it suggests that methane in mammalian breath may result from both intestinal bacterial fermentation and de novo production from metabolically active cells under oxidative stress (Tuboly et al, 2013; Ernst et al, 2022).

In Studies 1/2 and 1/3, inflammatory marker levels were significantly lower following Ket-Tris treatment compared to the original Ket formulation. These findings align with the established role of ROS in mediating gastric and intestinal hyperemia, which likely accounts for the observed differences in methane emissions during TNBS-colitis treatment. The elevated methane output in the Ket-treated colitis group, in contrast to the reduced levels following Ket-Tris administration, underscores the enhanced anti-inflammatory efficacy of the modified compound (Ugocsai et al, 2023). This improved inflammatory control may contribute to better colonic microcirculation and, consequently, lower methane emissions. Based on these findings, we propose that real-time monitoring of exhaled methane offers a valuable, non-invasive tool for tracking ROS-driven mucosal inflammation and evaluating therapeutic responses - particularly in rodent models.

In conclusion, the study confirms the specific protective effect of Tris against NSAID-induced GI damage. We observed that net methane production correlated closely with inflammatory status and microcirculation of the GI mucosa. In animals treated with Ket-Tris, methane levels were comparable to those of the sham-operated group, suggesting methane may act as a signal for pro-inflammatory responses and indicate anti-inflammatory interventions.

In Study 2, we used continuous, real-time monitoring of exhaled methane concentrations to explore its relationship with the macro- and microvascular components of mesenteric circulation during and after haemorrhagic events. Our findings show that changes in SMA flow occurred before alterations in systemic haemodynamics and haematocrit levels, with exhaled methane fluctuations reflecting changes in mesenteric circulation. Thus, monitoring exhaled methane could serve as an effective early warning tool for internal haemorrhage and provide insights into the microcirculatory status during bleeding episodes, including rapid changes during initial fluid resuscitation.

The methane breath test is a widely used diagnostic tool for GI disorders. Typically, breath methane levels are assessed using a lactulose test followed by GC, in which breath samples are collected in gas-tight bags and analysed with detectors such as flame ionization, thermal conductivity, or mass spectrometry (Costello et al, 2013). However, these methods have limited sampling frequency. In contrast, our study employs PAS, allowing real-time monitoring of methane concentration changes with a sensitivity of less than 1 ppm, compared to the 3-ppm sensitivity of conventional GC.

We used an anesthetized pig model to simulate a controlled haemorrhagic event with a gradual 35% BV loss, verifying the event's severity by measuring a corresponding decrease in tHb. This model facilitated high temporal resolution, allowing for multiple measurement intervals during haemorrhage and resuscitation phases. We chose HES as the resuscitation fluid, expecting it to induce significant macrohaemodynamic changes and restore intestinal microcirculation (Wu et al, 2015).

As anticipated, blood flow in the SMA decreased significantly with 5% BV loss, reflecting similar changes in exhaled methane concentration. Notable alterations in the serosal and mucosal blood flow were observed after 10% and 20% BV loss, respectively. The discrepancy between mesenteric macro- and microcirculation may be due to autoregulatory mechanisms (Pestel et al, 2010), while the delay in mucosal versus serosal microcirculation changes can be attributed to microcirculatory redistribution prioritizing mucosal oxygenation over serosal perfusion (Hiltebrand et al, 2003). The initial drop in exhaled methane levels likely corresponds to a reduction in perfused BV without a decrease in capillary density.

During the early resuscitation phase (T_8–T_9), there was a notable increase in SMA blood flow, mucosal blood flow (DBS), and exhaled methane levels. However, by the end of the experimental protocol, both exhaled methane concentrations and SMA flow had declined to baseline levels. Throughout the resuscitation phase, mucosal and serosal DBS remained stable, with no reductions during the last two measurements. Despite these findings, the HI increased by the end of the haemorrhage and remained elevated throughout resuscitation, indicating that microcirculatory perfusion was not fully restored, which may explain the decrease in methane levels after the initial rise.

The sublingual region is often studied for non-invasive assessment of microcirculation, based on the assumption that it reflects the status of distal GI areas like the ileum (Palágyi et al, 2015). However, in Study 2, we found no correlation between sublingual microcirculation and ileal serosal or mucosal perfusion during resuscitation, highlighting the differences in assessment techniques. While sublingual examinations provide broader insights over time, they

lack the frequent sampling capability seen with exhaled methane monitoring. The dynamics of exhaled methane closely matched those in mesenteric circulation, effectively capturing rapid changes during early resuscitation.

Exhaled methane concentration changes may indicate bleeding and reflect mesenteric perfusion during haemorrhage and resuscitation. This method offers diagnostic value similar to that of monitoring sublingual microcirculation and could serve as a valuable, non-invasive tool in clinical scenarios involving haemorrhagic complications. Moreover, this technique may enhance our understanding of mesenteric circulation in experimental settings.

6. SUMMARY OF NEW FINDINGS

1. Changes in whole-body methane emissions reflected alterations in small intestinal microcirculatory parameters induced by TNBS and
2. drug (NSAID) administration.
3. Whole-body methane production increased in parallel with GI inflammatory activation in TNBS-induced colitis. Therefore, real-time measurement of methane emission may serve as a non-invasive tool to estimate the progression of GI inflammation and monitor the effects of therapeutic interventions.
4. In a large animal model of controlled, gradual haemorrhage, exhaled methane levels showed a significant correlation with SMA blood flow as well as ileal mucosal and serosal microcirculation. Moreover, methane levels responded earlier to changes in SMA flow compared to sublingual microcirculation.
5. During the resuscitation phase, rapid shifts in small intestinal microcirculation were mirrored by changes in exhaled methane levels, whereas sublingual microcirculation failed to reflect these dynamic alterations.

7. ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to Andrea Szabó, Head of the Institute of Surgical Research, for the invaluable opportunity to work in his department and for his scientific guidance throughout this project.

Special thanks to Professor Mihály Boros, the formal Head of the Institute of Surgical Research, for his continuous support and guidance during my scientific work and supervising my papers.

I am grateful to Gabriella Varga and Dániel Érces for the basic research idea and their invaluable contribution to my experimental work and their guidance throughout the preparation of my thesis.

I am grateful to the staff at the Institute of Surgical Research for their outstanding technical assistance and to all my colleagues for their collaboration and support.

I sincerely thank Professor Gábor Tóth, Head of the Department of Medical Chemistry, University of Szeged for the conjugation of the newly developed NSAID derivatives.

This research was funded by the grants EFOP-3.6.2-16-2017-00006 (MB) and 2024-1.2.3-HU-RIZONT-2024-00033 which has been implemented with the support provided by the Ministry of Culture and Innovation of Hungary from the National Research, Development and Innovation Fund, financed under the 2024-1.2.3-HU-RIZONT funding scheme.

Finally, I wish to thank my Family and my Parents for their boundless love, patience, and trust, which have been my greatest sources of strength throughout this journey.