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Przepuszczalność bariery jelito-krew jako marker zaburzeń ogólnoustrojowych.

Rozprawa na stopień doktora nauk medycznych i nauk o zdrowiu

w dyscyplinie nauki medyczne

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Wykaz stosowanych skrótów

SHR – Spontaneously Hypertensive Rats (szczury hipertensyjne)

TMA - trimetyloamina

TMAO – tlenek trimetyloaminy

WKY – Wistar Kyoto (szczury normotensyjne)

Streszczenie w języku polskim

Przepuszczalność bariery jelito-krew jako marker zaburzeń ogólnoustrojowych.

Bariera jelito-krew pełni ważną funkcję selektywnego wchłaniania substancji odżywczych a jednocześnie ochrony przed przenikaniem szkodliwych cząsteczek ze światła jelita. Uszkodzenie bariery jelito-krew może mieć miejsce zarówno w chorobach jelit, jak i w chorobach układu krążenia i chorobach metabolicznych. Dochodzi w nich do upośledzenia perfuzji jelit w wyniku zmian hemodynamicznych lub naczyniowych. Zaburzone funkcjonowanie bariery jelito-krew może spowodować zwiększoną penetrację metabolitów bakteryjnych do krwiobiegu. W związku z powyższym upośledzona bariera jelito-krew i wzrost stężenia metabolitów we krwi może być markerem oraz potencjalnym punktem uchwytu terapii tych chorób.

Bakterie jelitowe produkują szereg związków o dużym potencjale biologicznym takich jak metyloaminy oraz krótkołańcuchowe kwasy tłuszczowe. Mogą one wywierać zarówno korzystny, jak i niekorzystny wpływ na organizm. Wykazano na przykład, że wzrost stężenia we krwi tlenku trimetyloaminy (TMAO), powstającego w wątrobie z trimetyloaminy (TMA, metabolitu bakterii jelitowych), jest pozytywnie skorelowany ze wzrostem ryzyka sercowo-naczyniowego. Mechanizmy prowadzące do wzrostu jego stężenia we krwi pozostają niejasne. Ważnym czynnikiem może być zwiększone przechodzenie TMA przez barierę jelito-krew. Dotychczas niewiele wiadomo, w jaki sposób zmiany morfologiczne i czynnościowe, które zachodzą w jelitach w chorobach ogólnoustrojowych wpływają na przepuszczalność bariery dla poszczególnych metabolitów.

Celem prezentowanych badań było stworzenie optymalnej metody oceniającej funkcję (przepuszczalność) bariery jelito-krew, która nie wymaga podawania substancji egzogennych i jest niezależna od czynników takich jak dieta. Kolejnym aspektem badań było poznanie zmian, jakie zachodzą w stężeniach metabolitów bakteryjnych z wiekiem oraz w nadciśnieniu tętniczym.

Opracowano metodykę badania bariery jelito-krew przy użyciu metabolitów bakteryjnych u szczurów (Publikacja nr 1). Następnie opracowana została nieinwazyjna metoda o potencjale klinicznym. Na podstawie stosunku stężenia metabolitów we krwi do stężenia w kale wyznaczany jest stopień przepuszczalności jelit. Metoda wymaga jedynie

pobrania próbki kału oraz krwi obwodowej, w związku z tym jest stosunkowo nieinwazyjna i prosta do wykonania. Jednocześnie, dzięki wykorzystaniu stosunku stężeń, jest ona niezależna od czynników zmiennych osobniczo, takich jak dieta czy skład flory bakteryjnej. Metodę tę przetestowano na modelu zwierzęcym zapalenia jelita oraz u pacjentów z nieswoistym zapaleniem jelit. Działanie metody potwierdził wzrost przepuszczalności bariery dla metabolitów bakteryjnych (krótkołańcuchowych kwasów tłuszczowych) u szczurów oraz pacjentów z zapaleniem jelita w porównaniu do grup kontrolnych. Dodatkowo, zmiany te były dodatnio skorelowane z klasycznie wykorzystywanymi markerami funkcji jelita (Publikacja nr 2).

Oceniono zmiany morfologiczne i czynnościowe, w tym przepuszczalność dla TMA, jakie zachodzą w jelicie wraz z wiekiem u szczurów szczepu Sprague Dawley i Wistar Kyoto, a także zmiany indukowane nadciśnieniem tętniczym (szczury szczepu SHR – Spontaneously Hypertensive Rats). Dodatkowo zbadano potencjał terapeutyczny enalaprilu, inhibitora konwertazy angiotensyny, w przywracaniu fizjologicznej funkcji bariery jelito-krew.

Zarówno szczury z nadciśnieniem tętniczym jak i szczury starsze wykazały zwiększoną przepuszczalność okrężnicy dla TMA, czemu towarzyszyły zmiany morfologiczne i hemodynamiczne w okrężnicy (Publikacja nr 3 i 4). Szczury pojone enalaprilem wykazały poprawę w zakresie zmian patologicznych w okrężnicy indukowanych nadciśnieniem tętniczym. W związku z tym bariera jelito-krew jest potencjalnym punktem uchwytu terapii inhibitorami konwertazy angiotensyny.

Podsumowując, zmiany funkcjonalne bariery jelito-krew mogą być skutecznie ocenione za pomocą opracowanej metody z użyciem stosunku stężeń metabolitów bakteryjnych. Ze względu na nieinwazyjny charakter metoda może być wykorzystana zarówno w warunkach eksperymentalnych, jak i klinicznych. Ponadto choroby sercowo-naczyniowe takie jak nadciśnienie tętnicze mogą charakteryzować się zwiększoną przepuszczalnością bariery jelito-krew dla metabolitów bakteryjnych, co może negatywnie wpływać na przebieg choroby podstawowej. Co więcej, wzrost ryzyka sercowo-naczyniowego wraz z wiekiem może być po części spowodowany wzrostem stężenia toksycznych metabolitów w osoczu z powodu uszkodzenia jelit.

Streszczenie w języku angielskim

Gut-blood barrier permeability as a marker of systemic disorders

The gut-blood barrier is a multilayer system that controls the passage of nutrients, bacterial metabolites, drugs, and other exogenous compounds from intestinal lumen to the bloodstream. The integrity of the barrier may be impaired in gastrointestinal, as well as in cardiovascular and some metabolic diseases where intestinal perfusion is impaired due to hemodynamic or vascular changes. This may result in easier access of biologically active compounds, such as gut bacterial metabolites, to the bloodstream and may affect functioning of entire organism. Therefore, the permeability of the gut-blood barrier and increase in bacterial metabolites concentration may be a marker of both intestinal and extraintestinal diseases.

Intestinal bacteria produce a number of biologically active compounds such as methylamines or short-chain fatty acids. They can have both beneficial and adverse effects on the body. For example, it has been shown that the increase in plasma level of trimethylamine oxide (TMAO), formed in the liver from trimethylamine (TMA, a bacterial metabolite), is positively correlated with cardiovascular risk. The mechanisms of the TMAO increase in blood remain unclear. Increased passage of TMA through the gut-blood barrier may be a key factor. So far, little is known how the morphological and functional changes that occur in the intestines in systemic disorders affect the gut-blood barrier permeability for bacterial metabolites.

The aim of presented studies was to develop an optimal method to assess the function (permeability) of the gut-blood barrier which does not require administration of exogenous marker and is independent of individual factors such as diet. Another aspect was to investigate the changes in bacterial metabolites concentration associated with aging and hypertension.

An in vivo method for evaluating the gut-blood barrier and liver metabolism of bacteria-derived products in rats has been developed (Publication No. 1). Furthermore, a non-invasive method was tested in the animal model of colitis and in patients with inflammatory bowel disease. Intestinal permeability was determined based on the ratio of metabolite concentration in blood to fecal concentration. The method only requires a sample of stool and peripheral blood, therefore it is relatively non-invasive and simple. At the same time it is independent of individual factors, such as diet or composition of the bacterial flora. The

increased permeability for bacterial metabolites (short-chain fatty acids) in rats and patients with colitis in comparison to control groups has been confirmed. In addition, these changes were positively correlated with standard intestinal function markers (Publication No. 2).

Morphological and functional changes in intestines, including permeability to TMA, which are associated with aging (in Sprague Dawley and Wistar Kyoto rats in different age), as well as hypertension-induced changes (in Spontaneously Hypertensive Rats) were assessed. Moreover, the therapeutic potential of enalapril (angiotensin converting enzyme inhibitor) in restoring the physiological function of the gut-blood barrier has been investigated.

Both hypertensive rats and older rats showed increased TMA colon permeability, which was accompanied by morphological and hemodynamic changes in the colon (Publication Nos. 3 and 4). Rats treated with enalapril showed improvement in pathological changes induced by hypertension. Therefore, the gut-blood barrier is a potential target for angiotensin converting enzyme inhibitors.

In conclusion, functional changes of the gut-blood barrier can be effectively assessed using the presented method by the ratio of bacterial metabolites concentrations. The method is non-invasive, therefore, it can be used in both experimental and clinical practice. In addition, cardiovascular diseases such as hypertension may be characterized by increased gut-blood barrier permeability to bacterial metabolites. This in turn may negatively affect the underlying disease. Furthermore, the increase in cardiovascular risk associated with aging may be partially caused by the increase in plasma concentration of toxic metabolites due to intestinal damage.

Wstęp uzasadniający połączenie wskazanych publikacji w jeden cykl

Przedstawiony cykl dotyczy prac, których tematem przewodnim było badanie kondycji bariery jelito-krew. Doświadczenia miały na celu określenie zmian zachodzących w przepuszczalności bariery dla metabolitów bakterii jelitowych w wyniku starzenia się organizmu oraz w chorobach gastroenterologicznych i chorobach układu krążenia. Ma to znaczenie ze względu na możliwość zastosowania w diagnostyce nieinwazyjnych metod badania bariery jelito-krew, a także ze względu na wartość poznawczą, jaką niesie ze sobą znajomość zmian stężeń we krwi metabolitów bakteryjnych, jako związków aktywnych biologicznie.

Jelito stanowi największą powierzchnię kontaktu ze środowiskiem zewnętrznym. Bariera jelito-krew to złożony układ, który kontroluje wchłanianie substancji odżywczych, a jednocześnie chroni przed nadmiernym przenikaniem cząsteczek szkodliwych. Składa się ona z wielu warstw, począwszy od powłoki śluzowej w świetle jelita, poprzez nabłonek jelitowy i tkankę łączną, kończąc na śródbłonku naczyń włosowatych jelita. Na funkcjonowanie bariery mogą mieć wpływ różnorodne czynniki wewnętrzne, np. prawidłowe ukrwienie jelit, stres, regulacja hormonalna, nerwowa, immunologiczna, jak i zewnętrzne: flora bakteryjna, leki, toksyny i inne[1]. W związku z tym upośledzenie funkcjonowania bariery jelito-krew może mieć miejsce zarówno w chorobach jelit (swoiste i nieswoiste choroby zapalne) jak i w wielu zaburzeniach ogólnoustrojowych, w których dochodzi do upośledzenia perfuzji jelit w wyniku zmian hemodynamicznych lub naczyniowych, np. w cukrzycy[2], niewydolności serca[3], nadciśnieniu tętniczym[4], otyłości i innych[5].

Zaburzone funkcjonowanie bariery jelito-krew może spowodować zwiększoną penetrację szkodliwych cząsteczek produkowanych przez bakterie jelitowe, tzw. metabolitów bakteryjnych, do krwiobiegu. W związku z powyższym upośledzona bariera jelito-krew i wzrost stężenia metabolitów bakterii jelitowych we krwi może być markerem oraz potencjalnym punktem uchwytu terapii tych chorób. Badania wchodzące w skład niniejszej rozprawy służyły między innymi opracowaniu optymalnej metody oceniającej stan bariery.

Obecnie istnieje kilka metod badania bariery jelito-krew, które są wykorzystywane klinicznie. Najczęściej przeprowadzane są testy wchłaniania cukrów, polimerów glikolu etylenowego lub znakowanych dekstranów[6]. Wszystkie te metody wymagają obciążenia organizmu substancjami egzogennymi - czynnymi osmotycznie lub znakowanymi radioaktywnie - co może mieć negatywny wpływ na przebieg choroby podstawowej. Wyklucza to stosowanie wymienionych powyżej metod w wielu przypadkach klinicznych,

takich jak niewydolność serca lub nerek. Inne, mniej inwazyjne metody, jak pomiar stężenia cytruliny lub zonuliny, oceniają natomiast uszkodzenia strukturalne jelit, co niekoniecznie odzwierciedla zmiany funkcjonalne[7]. Co więcej, różnorodne czynniki, takie jak zmiana w diecie, mogą wpływać na stężenie powyższych markerów.

W związku z powyższym, jednym z celów prezentowanych badań było stworzenie metody oceniającej funkcję (przepuszczalność) bariery jelito-krew, która nie wymaga podawania substancji egzogennych i jest niezależna od czynników zmiennych osobniczo, takich jak dieta.

Kolejnym aspektem prezentowanych badań było poznanie zmian, jakie zachodzą w stężeniach metabolitów bakteryjnych w zaburzeniach ogólnoustrojowych.

Bakterie jelitowe produkują szereg związków o dużym potencjale biologicznym. Po przekroczeniu bariery jelito-krew, związki te przedostają się do krwiobiegu, co umożliwia im działanie ogólnoustrojowe. Niektóre z nich, jak witaminy z grupy B, witamina K czy krótkołańcuchowe kwasy tłuszczowe są niezbędne dla prawidłowego funkcjonowania organizmu, ale inne mogą mieć działanie niekorzystne. Wykazano na przykład, że wzrost stężenia we krwi tlenku trimetyloaminy (TMAO), powstającego w wątrobie z trimetyloaminy (TMA, metabolitu bakterii jelitowych), jest pozytywnie skorelowany ze wzrostem ryzyka sercowo-naczyniowego[8-10]. Sugeruje się, że TMAO może być nowym markerem chorób układu krążenia, jednak mechanizmy prowadzące do wzrostu jego stężenia we krwi pozostają niejasne. Prace wchodzące w skład prezentowanego cyklu dotyczyły przepuszczalności bariery jelito-krew między innymi dla TMA. Pozwoliło to odpowiedzieć na pytanie, w jakim stopniu wzrost stężenia TMAO we krwi w chorobach układu krążenia może być spowodowany uszkodzeniem bariery jelito-krew.

Krótkołańcuchowe kwasy tłuszczowe, takie jak kwas masłowy, kwas propionowy i kwas walerianowy, będące produktami fermentacji bakteryjnej w okrężnicy, wydają się odgrywać korzystną rolę w organizmie i sugeruje się, że wzrost ich wytwarzania może zmniejszać ryzyko chorób metabolicznych. Ostatnie badania wskazują, że maślan, przez swój potencjał przeciwzapalny, może łagodzić związane z otyłością powikłania metaboliczne[11]. Zarówno w modelu eksperymentalnym, jak i u ludzi, zwiększenie ilości maślanu w jelicie grubym miało pozytywny wpływ na insulinowrażliwość[12,13].

Podsumowując, metabolity bakterii jelitowych mogą wywierać zarówno korzystny, jak i niekorzystny wpływ na organizm. Stężenie metabolitów bakterii jelitowych we krwi w dużej mierze zależy od funkcjonowania bariery jelito-krew. Z kolei wzrost stężenia metabolitów bakterii jelitowych może wpływać na przebieg i zmianę rokowania w chorobach

układu krążenia i innych, którym towarzyszy uszkodzenie jelit. Hipoteza ta została opublikowana w pracy, której jestem współautorem[14].

Dotychczas nie wiadomo, w jaki sposób zmiany morfologiczne i czynnościowe, które zachodzą w jelitach wraz z wiekiem oraz w chorobach gastroenterologicznych i układu krążenia, wpływają na przepuszczalność bariery jelito-krew dla poszczególnych metabolitów.

Przedstawiona rozprawa doktorska stanowi monotematyczny cykl 4 prac oryginalnych. Doktorant jest pierwszym autorem we wszystkich pracach. Łączna wartość wskaźnika cytowań (Impact Factor) czasopism, w których prace zostały opublikowane wynosi 11,209, natomiast suma punktów wg listy MNiSW z 2019 roku to 380.

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Założenia i cel pracy

Założenia i hipoteza badawcza

Związki wytwarzane przez bakterie jelitowe mogą wpływać zarówno korzystnie jak i niekorzystnie na organizm człowieka. Metabolity bakterii, aby przedostać się do krwioobiegu, muszą przekroczyć barierę jelito-krew. W wielu chorobach, głównie dotyczących przewodu pokarmowego, ale także zaburzeniach ogólnoustrojowych, np. chorobach układu krążenia i metabolicznych dochodzi do upośledzenia działania bariery jelito-krew. Dlatego też przepuszczalność bariery jelito-krew może być markerem zaawansowania zaburzeń ogólnoustrojowych.

Cel pracy

Określenie zmian w przepuszczalności bariery jelito-krew dla metabolitów bakterii jelitowych w zaburzeniach ogólnoustrojowych.

Cele szczegółowe

1. Opracowanie inwazyjnej metody badania bariery jelito-krew z wykorzystaniem metabolitów bakteryjnych jako markerów [Publikacja nr 1].
2. Opracowanie nieinwazyjnej metody badania bariery jelito-krew w zwierzęcym modelu zapalenia jelit oraz u pacjentów z nieswoistym zapaleniem jelit [Publikacja nr 2].
3. Zbadanie przepuszczalności bariery jelito-krew dla metabolitów bakteryjnych na zwierzęcym modelu nadciśnienia tętniczego [Publikacja nr 3].
4. Zbadanie na modelu zwierzęcym zmian jakie zachodzą wraz z wiekiem w przepuszczalności bariery jelito-krew dla metabolitów bakteryjnych [Publikacja nr 4]

PUBLIKACJA nr 1

An In Vivo Method for Evaluating the Gut-Blood Barrier and Liver Metabolism of Microbiota Products.

Kinga Jaworska

Tomasz Hutsch (Huć)

Marta Gawryś-Kopczyńska

Maksymilian Onyszkiewicz

Emilia Samborowska

Marcin Ufnal

Video Article

An *In Vivo* Method for Evaluating the Gut-Blood Barrier and Liver Metabolism of Microbiota Products

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Keywords: Medicine, Issue 140, Gut-blood barrier, intestinal permeability, liver clearance, portal blood sampling, gut bacteria, TMA, short chain fatty acids

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Abstract

The gut-blood barrier (GBB) controls the passage of nutrients, bacterial metabolites and drugs from intestinal lumen to the bloodstream. The GBB integrity is disturbed in gastrointestinal, cardiovascular and metabolic diseases, which may result in easier access of biologically active compounds, such as gut bacterial metabolites, to the bloodstream. Thus, the permeability of the GBB may be a marker of both intestinal and extraintestinal diseases. Furthermore, the increased penetration of bacterial metabolites may affect the functioning of the entire organism.

Commonly used methods for studying the GBB permeability are performed *ex vivo*. The accuracy of those methods is limited, because the functioning of the GBB depends on intestinal blood flow. On the other hand, commonly used *in vivo* methods may be biased by liver and kidney performance, as those methods are based on evaluation of urine or/and peripheral blood concentrations of exogenous markers. Here, we present a direct measurement of GBB permeability in rats using an *in vivo* method based on portal blood sampling, which preserves intestinal blood flow and is virtually not affected by the liver and kidney function.

Polyurethane catheters are inserted into the portal vein and inferior vena cava just above the hepatic veins confluence. Blood is sampled at baseline and after administration of a selected marker into a desired part of the gastrointestinal tract. Here, we present several applications of the method including (1) evaluation of the colon permeability to TMA, a gut bacterial metabolite, (2) evaluation of liver clearance of TMA, and (3) evaluation of a gut-portal blood-liver-peripheral blood pathway of gut bacteria-derived short-chain fatty acids. Furthermore, the protocol may also be used for tracking intestinal absorption and liver metabolism of drugs or for measurements of portal blood pressure.

Video Link

The video component of this article can be found at https://www.jove.com/video/58456/

Introduction

The gut-blood barrier (GBB), also known as the intestinal barrier, is a complex multilayer system that separates the gut lumen from the bloodstream in order to limit the passage of harmful compounds while allowing the absorption of nutrients[1]. It consists of the three main layers: the mucus layer, epithelium and lamina propria.

Numerous factors may affect the GBB integrity and function[2]. It has been shown that GBB is disturbed in both gastrointestinal and extraintestinal diseases, including cardiovascular and metabolic diseases[3], which may lead to an increased passage of gut bacterial metabolites to the bloodstream[4]. An increased penetration of gut bacterial metabolites may affect the functioning of the entire organism. For example, recent studies show a significant impact of bacterial metabolites, such as indoles, H2S, short-chain fatty acids (SCFA), and trimethylamine N-oxide, on the circulatory system functions[5,6,7,8,9]. Finally, it has been proposed that an increased GBB permeability may serve as a marker of cardiovascular and metabolic diseases which are associated with morphological and functional alterations in the intestines[10]. Therefore, tracking the gut-portal blood-liver-systemic blood pathway of bacterial metabolites may be of interest for both basic and clinical sciences.

Commonly utilized experimental methods for the evaluation of GBB permeability are performed *in vitro* using resected intestinal segments, fragments of mucosa, or artificial membranes[11,12]. The accuracy of those methods is compromised by the fact that proper functioning of the GBB requires constant intestinal blood flow. On the other hand, the available *in vivo* methods are based on the evaluation of urine or peripheral blood concentrations of exogenous markers[13]. However, peripheral blood and urine concentration of exogenous compounds is influenced by kidney function, *i.e.,* glomerular filtration rate and tubular excretion, as well as by liver metabolism, *i.e.,* first pass metabolism. Both parameters may differ significantly between study subjects independently of the GBB function.

This paper describes a direct measurement of the GBB permeability in rats using portal blood sampling. This *in vivo* method preserves the intestinal blood flow and is virtually not influenced by liver and kidney function. The described approach is not commonly used, possibly because

of some methodological difficulties. We describe in detail the catheterization of the portal vein and inferior vena cava just above the hepatic vein confluence. Blood sampling from the portal vein and inferior vena cava allows evaluation of the GBB permeability and liver clearance as well as tracking of gut-portal blood-liver-systemic blood pathway of molecules of interest, such as gut bacterial metabolites or medicines. We also present several applications of the method that were tested in our laboratory. These include the evaluation of the colon permeability to TMA, a gut bacterial metabolite, evaluation of liver clearance of TMA, and evaluation of a gut-portal blood-liver-systemic blood pathway of SCFA.

To evaluate gut-blood barrier permeability, the following protocol steps should be followed, in order: **1** (insertion of the line for intraintestinal administrations), **3** (portal vein catheterization), **4** (portal vein blood sampling), **6** (administration of a gut permeability marker), **4**.

To evaluate liver clearance and a gut-portal blood-liver-systemic blood pathway, the following protocol steps should be followed, in order: **1** (insertion of the line for intraintestinal administrations), **2** (inferior vena cava catheterization), **3** (portal vein catheterization), **4** (portal vein blood sampling), **5** (inferior vena cava blood sampling), **6** (administration of a gut permeability marker), **4**, **5**, **7** (calculation of liver clearance).

Protocol

The experiments were performed on male Wistar Kyoto rats according to Directive 2010/63 EU on the protection of animals used for scientific purposes and were approved by the I Local Bioethical Committee in Warsaw.

1. Insertion of the Line for Intraintestinal Administration

NOTE: Here we propose intracolonic administration of a marker using a catheter. It may be modified by oral administration or gavage at various levels of the digestive tract *e.g.* stomach or duodenum. Remember to use disposable surgical clothing, including surgical gown, hood and gloves, and ensure to follow the safety precautions related to the sharp tools used in surgery (needles, *etc.*) during procedures 1-6.

1. Fast animals overnight before the procedure. Perform all procedures during general anesthesia, *i.e.,* obtained by injection of urethane 1.5 g/kg bw i.p. Assess proper anesthetization by the lack of palpebral and corneal reflexes, and by toe-pinch and tail-pinch method.
2. Use a pediatric Foley catheter (10F or 8F) as a colonic catheter. Mark the catheter to indicate the part that will be inserted into the colon (approximately 8 cm).
3. Check the anal region and the stool content in the rectum before inserting the catheter into the colon. If stool is present, empty the rectum by massaging the rectal area.
4. Put a lubricant (*e.g.* glycerin or petrolatum) along the catheter. Moisten the anus and its surroundings with the lubricant.
5. Insert the catheter with a guide wire approximately 8 cm through the external anal sphincter. Make slow forward-backward and circular movements.
   NOTE: Keep on checking the location of the catheter by abdominal palpation while inserting the catheter.

2. Inferior Vena Cava Catheterization

1. Shave fur in the groin. Alternately disinfect the skin with alcohol and povidone iodine 3 times and cover the groin area with surgical drapes.
2. Try to feel the pulse on the femoral artery and cut the skin longitudinally for the length of about 2.0 cm in the place where the pulse is palpable.
3. Dissect the fascia and muscles to visualize the neurovascular bundle.
4. Dissect the femoral vein from the neurovascular bundle: first nerves, then the femoral artery, and then the vein.
   NOTE: Be careful during the dissection of the neurovascular bundle, since tiny branches of the femoral vein may easily be damaged, producing bleeding.
5. Put two ligatures on the femoral vein. Do not tie the knots yet. Catch the ends of the proximal ligature with a needle holder.
6. Carefully pull the ligature ends with the holder upwards to close the proximal part of the vein. Wait until the vein is filled with blood and tie the distal knot.
7. Make a small incision (*ca.* 1 mm) on the vein between the knot and proximal ligature, using microsurgical scissors. Insert the catheter using tweezers or the needle with the curved end.
   NOTE: Puncture the vein and use the bended tip of the needle as a guide for the catheter. Loosen the proximal ligature while inserting the catheter. Insert the catheter for 6-7.0 cm.
8. Secure the catheter in the femoral vein with two single surgical knots. Tie the proximal ligature as well.
9. Check the patency of the catheter by attempting to draw blood with a syringe. Rinse the catheter with 0.3 mL of the heparinized saline (100 units/mL).
10. Close the surgical wound with two layers of single stitches.

3. Portal Vein Catheterization

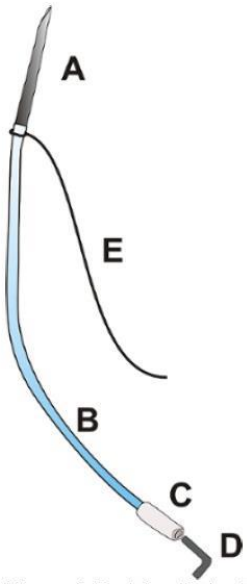

Figure 1: Portal catheter. The portal catheter consists of a needle OD: 0.9 mm with a length of about 25.0 mm [A], a flexible polyurethane catheter OD: 0.025", length about 100.0 mm [B], a flexible polyethylene tip of the catheter OD: 0.040", approximately 15.0 mm long [C], a plug [D], and a ligature 3/0 with a length of 100.0 mm [E]. Please click here to view a larger version of this figure.

1. Prepare the portal catheter according to **Figure 1**.
    1. Insert the cut end of the needle (OD: 9 mm) into the polyurethane catheter OD: 0.025".
    2. Tie the ligature 3/0 at the junction of the needle and catheter.
       NOTE: Ensure that the longer part of the ligature is at least 6 cm long.
    3. Insert the end of the catheter OD: 0.025" into the polyethylene catheter OD: 0.040".
    4. Close the catheter with a metal or plastic plug.

2. Midline laparotomy
    1. Shave fur in the abdomen, alternately disinfect the skin with alcohol and povidone iodine 3 times, and cover the area with surgical drapes.
    2. Cut the skin longitudinally from the xiphoid of the sternum to the navel.
    3. Cut the muscles of the abdominal wall along the white line.
    4. Expand the cut rostrally in the Y shape so that the xiphoid cartilage is between two cuts.

3. Portal vein dissection
    1. Moisten the surgical swabs with saline.
    2. Exteriorize the cecum, ascending and transverse colon, and small intestine loop. Put the intestines on the left side to expose the root of the mesentery.
       NOTE: Cover the intestines with gauze moistened with a physiological saline to protect the intestines from drying.
    3. To expose the portal vein, carefully move the hepatic lobes to the sides or upwards towards the diaphragm with the moistened swabs.
    4. Localize the part of the portal vein that is not covered with the mesentery (in the hepatic hilum, about 5 mm long) and pass the ligature 3/0 (15 cm long) under the portal vein.
       NOTE: To protect tissues from damage while placing the ligature, moisten the ligature with a physiological saline solution.
    5. Clamp the ends of ligature with forceps and tighten it gently to stabilize the vessel.

4. Insertion and stabilization of the catheter
    1. Pass the longer part of the portal catheter's ligature under the free part of the portal vein and pull it so that the catheter is located just next to the portal vein.
    2. Insert the needle into the upper mesenteric vein 3 mm below the junction of upper mesenteric vein and the portal vein. Hold the needle at a 30° angle and, after entering into the vein, reduce the angle and advance the needle almost horizontally, in parallel to the portal vein.
       NOTE: Insert the needle for a length of approximately 6-7 mm. The stabilizing ligature should gently tighten the portal vein while inserting the catheter.
    3. Apply 1-2 drops of tissue glue at the place where the needle is inserted. Remove the swabs that cover the liver.
    4. Put the intestines back into the abdominal cavity.
    5. Moisten the intestines with a warmed up saline solution and cover it with moistened sterile gauze.
    6. Check the patency of the catheter and rinse the catheter with 0.3 mL of the heparinized saline (100 units/mL).
       NOTE: Venous blood spontaneously backflows in the catheter.

5. Ending of the surgery
    1. After 5 minutes, check the color of the intestines and peristaltic movements, make sure that the proper mesenteric blood flow is maintained.
    2. Close the abdominal cavity with 3 stitches: wall peritoneum with the inner layer of the abdominal wall muscles - a continuous, absorbable suture; remaining muscles of the abdominal wall - a continuous, absorbable suture; skin and subcutaneous tissue - single, non-absorbable sutures.
    NOTE: Exteriorize the distal part of the catheter around the navel.

4. Portal Vein Blood Sampling

1. Sample portal vein blood at times according to the specific testing protocol used; see **Table 1**.

Short protocol	Long protocol
t_0 – baseline (before intracolonic administration)	t_0 – baseline (before intracolonic administration)
t_1 – 5 min after intracolonic administration	t_1 – 30 min after intracolonic administration
t_2 – 30 min after intracolonic administration	t_2 – 60 min after intracolonic administration

Table 1: Portal blood sampling protocols for gut permeability assessment.

NOTE: The time between consecutive blood sampling depends mainly on the bioavailability of the tested substances and the site of administration (colon, stomach, *etc.*).

2. Open the portal catheter plug and let the blood flow freely.
3. Use syringe (vol. 2 mL) and blunt needle OD: 0.9 mm. Collect no more than 0.7 mL of blood.
4. Rinse the catheter with 0.3 mL of heparinized saline (100 units/mL) and close the catheter plug.

5. Inferior Vena Cava Blood Sampling

1. Sample inferior vena cava blood at times according to the specific testing protocol used; see **Table 2**.

Portal vein	Inferior vena cava
t_0 – baseline (before intracolonic administration)	t_0 – baseline (before intracolonic administration)
t_1 – 30 min after intracolonic administration	t_1 – 30 min after intracolonic administration

Table 2: Protocol of blood sampling for liver clearance measurement and tracking the gut-portal blood-liver-systemic blood pathway.

2. Open the inferior vena cava catheter plug and let the blood flow freely.
3. Collect no more than 0.7 mL of blood using syringe (vol. 2 mL) and broken needle OD: 0.9mm.
4. Rinse the catheter with 0.2-0.3 mL of heparinized saline (100 units/mL) and close the catheter plug.

6. Administration of a Gut Permeability Marker

1. Remove the guide wire and inflate the colonic catheter balloon, using adequate volume of sterile water (usually 1 mL but check actual balloon size before insertion).
NOTE: The balloon diameter should not exceed 1 cm.
2. Place the rat head down (inclination about 15%) to minimize the risk of the outflow of the administered solution from the colon.
3. Slowly administer the tested substance (*e.g.* trimethylamine, 100 mg/kg bw) using a drainage port in colonic catheter.
NOTE: Do not exceed the volume of 0.75 mL of the administered solution and the feeding speed of 0.5 mL/min to prevent the outflow of the administered solution from the anus.
4. After 10 min deflate the catheter balloon.
5. Sample blood from the inferior vena cava and the portal vein according to the specific testing protocol used; see **Table 1 and Table 2**.
6. Euthanize animal via approved method.

7. Calculation of Liver Clearance

1. Express liver clearance, understood as hepatic extraction, by the difference between portal blood concentration and inferior vena cava blood concentration or by the ratio of inferior vena cava to portal blood concentration, (1 - (inferior vena cava concentration/portal vein concentration)).

8. Evaluation of the Test Substance Concentration n Blood Samples

1. Depending on the test substance and test methodology, subject the sample to appropriate laboratory procedures (centrifugation, *etc.*). In the proposed protocols, we evaluate TMA/TMAO and SCFA concentration using liquid chromatography coupled with triple-quadrupole mass spectrometry. Please find a detailed description of the method in Supplemental Material.

Representative Results

We have successfully measured the GBB permeability and liver clearance of TMA in rats. We have demonstrated that hypertensive rats have an increased colon permeability to TMA in comparison to normotensive rats (**Figure 2**)[4]. In another study we found that high salt intake does not affect the GBB permeability and liver clearance of TMA (**Figure 3**)[14].

Measuring the concentration of SCFA in stools, portal blood, and peripheral blood, we traced the path of the molecules from the intestine to the peripheral blood. The exemplary results for those experiments are presented in **Table 3**.

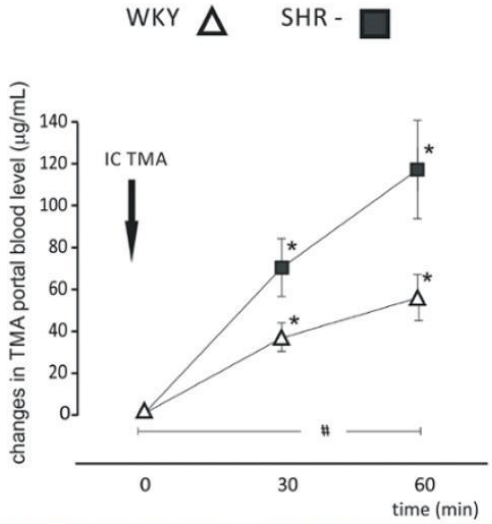

Figure 2: Hypertension-associated changes in gut-blood barrier permeability. Intracolonic administration of TMA produced a significant increase in portal blood TMA in each group (n=12 for each group). The increase in portal blood TMA in the hypertensive (SHR) group was significantly higher than in normotensive (WKY) group. We used the long protocol consisting of blood sampling 30 min and 60 min after TMA administration (IC TMA). Values are means, + SE, *p < 0.05 vs baseline, #p < 0.05 WKY vs SHR. This figure has been modified from Jaworska *et al.*[4] Please click here to view a larger version of this figure.

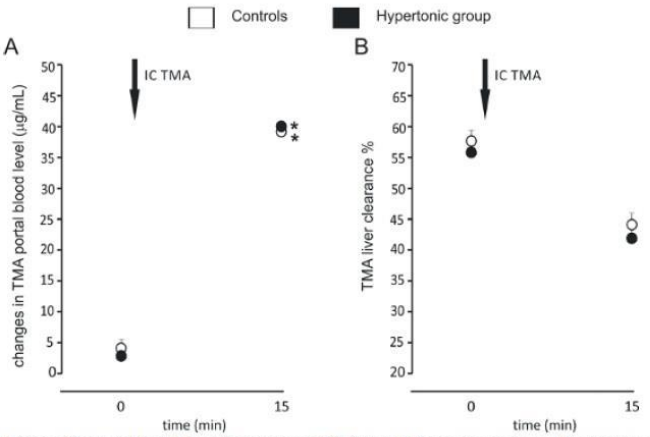

Figure 3: Gut-blood barrier permeability and liver clearance after high salt intake. (A) Intracolonic administration of TMA produced a significant increase in portal blood TMA. The size of the increase was similar between the groups (n=7 for each group). We used a simplified protocol, taking blood samples at baseline (0) and 15 min after administration of TMA (IC TMA). (B) TMA liver clearance was similar between the groups at baseline, and 15 min after the intracolonic administration of TMA. Values are means, + SE. *p < 0.05 vs baseline. This figure has been modified from Bielinska *et al.*[14] Please click here to view a larger version of this figure.

SCFA	Stool concentration (µM)	Portal blood concentration (µM)	Peripheral blood concentration (µM)
AA- acetic acid (C2)	15998.40 ± 4317.58	564.22 ± 155.34	149.89 ± 31.74
IPA- propionic acid (C3)	5390.70 ± 1016.19	138.25 ± 55.50	5.36 ± 3.25
IBA- isobutyric acid (C4)	191.20 ± 123.87	4.51 ± 1.60	1.14 ± 1.16
BA- butyric acid (C4)	4159.80 ± 3141.68	143.14 ± 68.42	6.43 ± 4.18
2MeB- 2 methylbutyric acid (C5)	80.90 ± 59.86	2.02 ± 0.88	1.14 ± 1.42
IVA- isovaleric acid (C5)	109.10 ± 56.05	2.59 ± 1.07	0.90 ± 1.22
VA- valeric acid (C5)	281.9 ± 158.20	8.55 ± 3.56	0.72 ± 1.02
ICA- isocaproic acid/ 4-methylvaleric acid (C6)	5.9 ± 2.95	0.61 ± 0.15	1.76 ± 0.87
CA- caproic acid (C6)	287.00 ± 309.68	11.19 ± 4.94	1.12 ± 0.93

Table 3: SCFA concentration in stool, portal blood, and peripheral blood (n=7).

Test substance	Possible application
Bacterial metabolites: trimethylamine (TMA), short chain fatty acids (SCFA), hydrogen sulfide, etc.	GBB permeability studies Tracking a gut-portal blood-liver-systemic blood pathway Hepatic clearance studies
Classic permeability markers: FITC-dextran, polysaccharides, PEG, etc.	GBB permeability studies
Drugs	absorption and hepatic clearance studies

Table 4: Exemplary test substances with possible applications.

Discussion

The described direct, *in vivo*, method of measuring the GBB permeability maintains closetophysiological conditions in the gastrointestinal system (preserves the intestinal blood flow), and is virtually not influenced by liver and kidney function.

The critical step of this technique is the insertion of the portal catheter. This must be done gently and decisively at the same time. A mild, short bleeding may occur from the correctly performed puncture of the portal vein; however, it stops when the needle is inserted into the vessel. Persistent bleeding indicates that the portal vein is perforated. To facilitate the catheter insertion, the portal vein should be well exposed. After exteriorizing the intestines, when the mesenteric root is well exposed, the upper mesenteric vein should also be visible (mesenteric vein enters cranially into the portal vein). The portal vein is usually covered by the hepatic lobes, which have to be moved to the sides. Also, the proper stabilization of the portal catheter is crucial for a successful procedure, since the catheter's movement may produce portal vein rupture and bleeding, especially in longer experiments. Additional stabilization of the catheter may be achieved by attaching the catheter to mesentery by sticking it to a mesentery with tissue glue or by applying two single stitches (thread 6/0). After closing the abdominal cavity to secure the placement of the catheter, a purse-string suture may be applied on the catheter.

There are several minor difficulties that may occur during the experiment. After catheterization of the femoral vein, if the venous blood does not backflow in the catheter, try the following solutions: flush the catheter with heparinized saline, gently pull the catheter 1-2 mm from the vein, remove the surgical knots, and tie a new one, pull the catheter out and reinsert, or replace with a new catheter. Remember to confirm the proper placement of the catheter after the experiment. The catheter should be inserted for 6-7 cm, depending on the size of the animal, to place the proximal tip of the catheter in the inferior vena cava just above the hepatic vein confluence. When it comes to colon catheterization, if you have problems with advancing the catheter you may inject 0.3-0.5 mL of saline or leave the catheter in the colon for 5-10 minutes, and try again. Do not use force while inserting a catheter to avoid perforation of the intestine.

In our studies, we used a gut bacteria-derived molecule, trimethylamine (TMA), as a marker of the colon GBB permeability, as TMA is produced mostly by colonic bacteria. However, many other substances, including classic permeability markers like FITC-dextran or sugars, may be used as well (see **Table 4**). When preparing a solution of the test substance, take into account its irritating effect on the intestinal mucosa and appropriately choose the concentration of the substance. Further laboratory procedures of the blood samples must be adjusted to the selected marker.

In our protocol, we propose intracolonic administration of a marker; however, it may be modified by oral administration or gavage at various levels of the digestive tract. The variable speed of peristalsis and possible interactions with enzymes and gastric acid should be taken into account while administering a marker into upper parts of the gastrointestinal tract *e.g.* stomach or duodenum. Accordingly, time of blood sampling after administration of a marker needs to be adjusted.

There are several limitations of the presented method, including adverse effects of anesthesia and fasting overnight, that may both influence GBB function. It should be taken into account as the procedure is terminal and involves blood sampling during not fully physiological conditions. However, as mentioned before, it has still many advantages over other experimental methods assessing GBB permeability, especially performed *in vitro*[11]. For example, an Ussing chamber measures the conductance and particle flux through the intestinal epithelial cells. The main weakness

of this technique lies in its excessive simplification. It is difficult to describe the complex physiological system of the intestinal mucosa using a small number of measurements on epithelial cell layer alone. Some researchers use whole-thickness intestine for Ussing chamber studies, but this procedure is accompanied by several methodological complications[15]. Furthermore, the accuracy of the method is compromised by a limited viability of tissues isolated from the organism. Some *in vitro* methods used in pharmacokinetic studies use artificial membranes as a model of the intestinal barrier[12]. However, those methods, similarly to the Ussing chamber, do not reflect the complexity of the GBB structure and functions.

There are also *in vivo* permeability assays available in experimental and clinical studies. They are mostly based on urine or peripheral blood sampling after oral or colonic administration of various markers[13]. The widely used sugar test involves oral intake of mono- and oligosaccharides, which are not metabolized in mammalian organism, *e.g.* mannitol and lactulose. The method is non-invasive and may be employed in both experimental and clinical use[16,17]; however, the results are affected by first-pass liver metabolism and kidney function, which may differ significantly between the study subjects. In contrast to the above mentioned indirect methods, collecting blood from the portal vein allows direct evaluation of the GBB permeability[12]. This method is not dependent on liver and kidney function and virtually preserves physiological conditions in the intestines which is an important advantage over *ex vivo* or *in vitro* methods.

The techniques described in this paper also allow for a relatively accurate liver clearance evaluation, as the blood is collected from the portal vein and inferior vena cava just above the hepatic vein confluence. The representative results for the hepatic extraction are presented in **Figure 3** (for TMA) and **Table 3** (for SCFA). Our data suggest that three main SCFA, acetate, propionate, and butyrate, are characterized by different hepatic clearance, which is supported by previous studies, where Bloemen *et al.* shown that intestinal release of butyrate and propionate, but not acetate, is almost equaled by hepatic uptake[18]. Therefore, the presented protocol is suitable for tracking intestinal absorption and liver metabolism of drugs, which can be used in pharmacokinetic studies.

The techniques may also be adjusted to other experimental purposes. Catheterization of the portal vein may be used to measure portal blood pressure or for administration of drugs directly to the portal vein, in order to study hepatic circulation. For instance, in our previous work, we administered hydrogen sulfide donors to the portal vein to assess its influence on hepatic circulation and portal pressure[19].

Disclosures

The authors have nothing to disclose.

Acknowledgements

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PUBLIKACJA nr 2

Inflammatory bowel disease is associated with increased gut-to-blood penetration of short-chain fatty acids: A new, non-invasive marker of a functional intestinal lesion.

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RESEARCH PAPER

Inflammatory bowel disease is associated with increased gut-to-blood penetration of short-chain fatty acids: A new, non-invasive marker of a functional intestinal lesion

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Abstract

Intestinal diseases, such as inflammatory bowel disease (IBD), are characterized by an impaired gut–blood barrier commonly referred to as 'leaky gut'. Therefore, functional evaluation of the gut–blood barrier is a promising diagnostic marker. We hypothesized that short-chain fatty acids (SCFAs) produced by gut bacteria might serve as a marker in IBD. Animal experiments were performed on male Sprague–Dawley rats with acetic acid-induced colitis and in sham control animals. The gut–blood barrier permeability was determined by assessing the ratios of the following: (i) portal blood concentration of SCFAs (C_p) to faecal concentration of SCFAs (C_f); (ii) systemic blood concentration of SCFAs (C_s) to faecal concentration of SCFAs (C_f); and (iii) C_p and C_s of fluorescein isothiocyanate (FITC)–dextran administered into the colon. As a clinical study, we evaluated C_s, C_f and the C_s/C_f ratio of SCFAs in six paediatric patients with IBD, assessed as mild/moderate/severe by the Paediatric Ulcerative Colitis Activity Index (PUCAI) and the Paediatric Crohn's Disease Activity Index (PCDAI) at the time of sample collection, and nine age-matched healthy control subjects. Rats with histologically confirmed IBD had significantly increased ratios of C_p/C_f and C_s/C_f for SCFAs. This was positively correlated with the plasma FITC–dextran concentration. Likewise, IBD patients showed a significantly higher C_s/C_f ratio for SCFAs, including acetic, valeric, isocaproic, caproic and propionic acids, in comparison to control subjects. In conclusion, in the rats and in paediatric patients with IBD we found an increased blood-to-stool ratio of SCFAs, suggesting an increased gut-to-blood penetration of SCFAs. These findings pave the way for a new, non-invasive diagnostic tool in IBD and other diseases accompanied by intestinal barrier malfunction.

KEYWORDS
gut bacteria, leaky gut, short-chain fatty acid

1 | INTRODUCTION

The gut–blood barrier (GBB) is a complex system that protects an organism against the external environment. Intestinal wall integrity and the proper functioning of the GBB depend on the interaction of the circulatory system, immunological system, hormonal system and other factors (Farhadi, Banan, Fields, & Keshavarzian, 2003). An increased GBB permeability, also referred to as 'leaky gut', has been reported in a variety of intestinal and extra-intestinal diseases, including cardiovascular and metabolic diseases (Bischoff et al., 2014; Sandek et al., 2007).

Disturbances in GBB function are present in gastrointestinal diseases such as inflammatory bowel disease (IBD), coeliac disease, necrotizing enterocolitis (NEC) and irritable bowel syndrome (Camilleri, Madsen, Spiller, Greenwood-Van Meerveld, & Verne, 2012). The possibility of using permeability assessment as an indicator of disease severity in ulcerative colitis (UC) was often raised in the past (Arslan, Atasever, Cindoruk, & Yildirim, 2001; Gerova, Stoynov,

Katsarov, & Svinarov, 2011; Welcker, Martin, Kolle, Siebeck, & Gross, 2004), but it has not been established in clinical practice. This is attributable in part to significant limitations of the currently used methods. The most commonly used methods for assessing GBB are based on non-functional biochemical markers of epithelial lesions, such as zonulin or claudin-3, which may reflect the intestinal function poorly (Galipeau & Verdu, 2016). In contrast, functional methods are based on oral administration of exogenous compounds, such as sugars or labelled dextrans, with risks for the patients that are not well defined, especially for paediatric patients and those with concomitant kidney and cardiovascular diseases.

Nevertheless, functional assessment of the GBB using non-invasive methods may be of great clinical importance for an early ambulatory diagnosis and for monitoring the treatment of diseases such as IBD or functional bowel disorders. Furthermore, some clinical conditions, such as NEC in preterm infants or acute pancreatitis, still lack proper diagnostic methods (Niemarkt et al., 2015). Therefore, new methods for assessing GBB in clinical practice are needed.

We hypothesized that gut bacterial products, specifically a blood-to-stool ratio of bacterial metabolites, might be a good indication of the GBB permeability. We decided to use short-chain fatty acids (SCFAs) because they are naturally present in the intestines, which eliminates the need for administration of exogenous markers. The absorption of SCFAs in the colon is concentration dependent (Ruppin, Bar-Meir, Soergel, Wood, & Schmitt, 1980) and may be influenced by intestinal motility, gut bacterial metabolism, differences in diet or the concomitant treatment. Therefore, we evaluated the blood-to-stool ratio of the bacterial products (and not only the blood concentration), which virtually eliminates problems associated with interindividual variations in SCFA stool concentrations. Finally, we have chosen SCFAs because these bacterial products are commonly evaluated in numerous laboratories.

New Findings

- **What is the central question of this study?**
  'Leaky gut' has been found in intestinal and extra-intestinal diseases. However, functional evaluation of intestinal permeability is not widely used as a diagnostic marker, possibly owing to significant limitations of currently used permeability assays. There is an unmet need for development of a new, non-invasive test to assess intestinal function.
- **What is the main finding and its importance?**
  We show that an increased blood-to-stool ratio of the concentration of gut bacteria-produced short-chain fatty acids may be used as a marker of gut permeability. Our findings lay the groundwork for establishing a new, non-invasive, risk-free diagnostic tool in diseases associated with intestinal barrier malfunction, such as inflammatory bowel disease.

2 | METHODS

2.1 | Ethical approval

The experiments on animals were carried out according to Directive 2010/63/EU and were approved by the local bioethical committee (616/2018). We understand the ethical principles under which the journal operates, and our work complies with the animal ethics checklist.

All subjects in the clinical study gave written informed consent. The study conformed to the standards set by the latest revision of the *Declaration of Helsinki*, except for registration in a database, and was approved by the local ethics committee (KB/125/2018).

2.2 | Animal study

2.2.1 | Induction of colitis in rats

Male, 24- to 26-week-old Sprague–Dawley rats were fasted overnight before the procedure. General anaesthesia was achieved with a ketamine–xylazine cocktail at a dose of 90 mg [kg body weight (BW)]$^{-1}$ of ketamine (Bioketan 100 mg ml^{-1}; Vetoquinol Biowet, Gorzow Wielkopolski, Poland) and 5 mg (kg BW)$^{-1}$ of xylazine (Xylapan 20 mg ml^{-1}; Vetoquinol Biowet). Next, rats received 1 ml of either saline (sham, control group) or 4% (v/v) acetic acid (pH 2.3) (colitis and recovery group) administered into the colon via a paediatric Foley catheter (10 French) inserted through the external anal sphincter, as previously described (Jaworska et al., 2018). After 30 s of exposure, excess fluid was withdrawn, and the colon was flushed with 1.5 ml of PBS. Afterwards, rats received 50 mg (kg BW)$^{-1}$ of tramadol (Tramal; Grünenthal GmbH, Aachen, Germany) and were placed in separate cages with food and water *ad libitum*.

After the experiments described below, the IBD was confirmed post-mortem by histological examination. Tissues sections were fixed in 10% buffered formalin and dehydrated by means of graded ethanol and xylene baths and embedded in paraffin wax. Sections 3–4 μm thick were stained with Haematoxylin and Eosin (HE). General histological examination was performed at magnifications of ×10, ×40 and ×100 (objective lens) and ×10 (eyepiece), and photographic documentation was made. The mucosa and submucosa of the colon, intestinal crypts and its cell composition, blood vessels of the mucosa and the submucosa of the colon were assessed.

2.2.2 | Gut–blood barrier permeability to SCFAs and a classic permeability marker (FITC–dextran)

The study was conducted on three experimental groups: (i) control, sham group ($n = 9$); (ii) acute colitis group (24 h after the induction of colitis, $n = 9$); and (iii) recovery group (1 week after the induction of colitis, $n = 9$).

The rats were anaesthetized with urethane (Sigma-Aldrich, Poznan, Poland) at a dose of 1.5 g (kg BW)$^{-1}$. Polyurethane catheters were

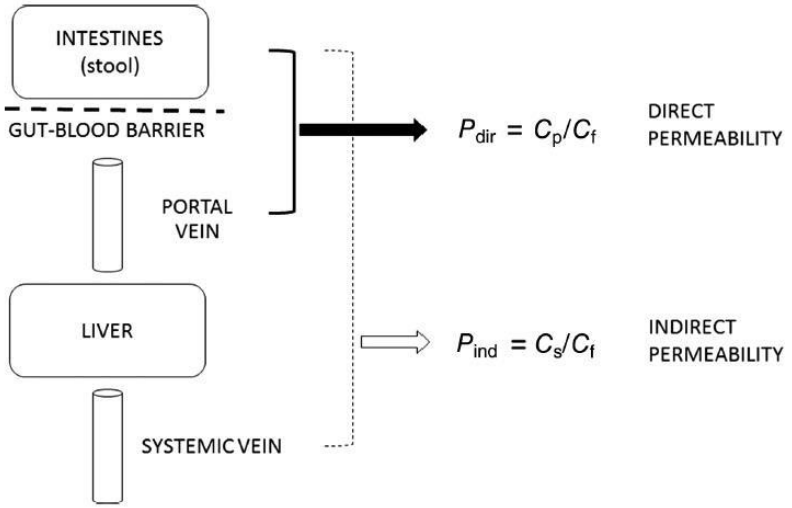

FIGURE 1 Scheme of the method. Abbreviations: C_f, concentration of the marker in stool; C_p, concentration of the marker in portal blood; C_s, concentration of the marker in systemic blood; P_{dir}, direct gut–blood barrier permeability; and P_{ind}, indirect gut–blood barrier permeability

inserted into the portal and femoral veins as previously described (Jaworska et al., 2018). Blood samples were collected from the veins 30 min after the intracolonic administration of 4 kDa fluorescein isothiocyanate-conjugated dextran (FITC–dextran) (10 mg dissolved in 1 ml of PBS; Sigma-Aldrich). After the blood sampling, rats were killed by decapitation, and 0.5 cm^3 of stool was collected from the middle part of the colon. The stool sample was weighed and homogenized with 1 ml of 0.9% NaCl in a closed 2 ml laboratory tube by vortexing it for 5 min. Afterwards, the sample was centrifuged for 12 min at 2,000 g, and the supernatant obtained was transferred to a laboratory tube and again centrifuged for 12 min. All procedures were performed at a temperature of 2–5°C. The supernatant was collected into an Eppendorf tube and frozen at −20°C.

Evaluation of plasma concentration of FITC–dextran

The plasma samples (0.1 ml) were mixed with 1.9 ml of PBS (pH 7.4). The concentration of FITC–dextran was analysed by fluorescence spectrometry. Fluorescence measurements were made at an excitation wavelength of 480 nm and an emission wavelength of 520 nm (spectrofluorometer F-7000; Hitachi, Japan). Standard curves were prepared from dilutions of FITC–dextran in PBS (pH 7.4).

Evaluation of SCFA concentration in plasma and stool

Plasma and stool concentrations of SCFAs were evaluated using liquid chromatography coupled with triple-quadrupole mass spectrometry. The concentration of the following SCFAs has been measured: AA, acetic acid (C2); PA, propionic acid (C3); IBA, isobutyric acid (C4); BA, butyric acid (C4); 2MeB, 2-methylbutyric acid (C5); IVA, isovaleric acid (C5); VA, valeric acid (C5); ICA, isocaproic acid/4-methylvaleric acid (C6); CA, caproic acid (C6); and 3MeVA, 3-methylvaleric acid (C6). The instrumentation consisted of a Waters Acquity Ultra Performance Liquid Chromatograph coupled with a Waters TQ-S triple-quadrupole mass spectrometer. The mass spectrometer operated in the multiple-reaction monitoring (MRM)– positive electrospray ionization (ESI) mode, as we have described in detail previously (Jaworska et al., 2018).

Calculation of direct and indirect GBB permeability

Given that the production of bacterial metabolites depends on stool bacterial composition and their metabolic activity, which is affected by numerous interindividual differences, such as diet and demographic factors (Bourriaud et al., 2005; Weir et al., 2013), the proposed GBB permeability assessment is based on the measurement of portal blood (rats), systemic blood (rats and humans) and stool (rats and humans) concentrations of SCFAs. Relative to these parameters, we have determined direct and indirect permeability. The direct permeability was determined by the ratio of portal metabolite concentration to faecal SCFA concentration (C_p/C_f), whereas the indirect permeability was determined by the ratio of systemic blood SCFA concentration to faecal SCFA concentration (C_s/C_f) (Figure 1).

2.3 | Clinical study

The study was conducted in the Department of Pediatric Gastroenterology and Nutrition in Warsaw, Poland. Children with IBD, both UC and Crohn's disease (CD) ($n = 6$) and healthy control subjects ($n = 9$) were included in this study (Table 1). The diagnosis of IBD was established according to the Porto criteria. The severity of UC and CD was evaluated using the Paediatric Ulcerative Colitis Activity Index (PUCAI) and the Paediatric Crohn's Disease Activity Index (PCDAI), which incorporate symptoms, physical examination findings and laboratory test results. Median disease activity was 30 (12.5–65). A PCDAI score ≤10 for CD and a PUCAI score <10 for UC were defined as remission. All patients were treated with mesalamine and immunomodulators (azathioprine or methotrexate). Additionally, four of six were on biologic therapy (infliximab/adalimumab). Control subjects were healthy children with a history of no gastrointestinal symptoms or chronic diseases.

Blood plasma and stool samples were collected near simultaneously (maximum within 1 h). Faecal calprotectin concentration was evaluated using the particle-enhanced turbidimetric immunoassays (PETIA) method with the Bühlmann fCAL Turbo test (Bühlmann Laboratories AG, Schönenbuch, Switzerland) on the Vitors 5600

TABLE 1 Characteristics of patients and healthy control subjects

Characteristic	Inflammatory bowel disease patients	Healthy control subjects
Total number of patients	6	9
Age [years; median (range)]	13.2 (5.2–17.9)	9.4 (4.6–13.8)
Sex [female/male; *n* (%)]	2/4 (33/67)	7/2 (78/22)
Time from IBD diagnosis [months; median (range)]	15 (10–61)	–
Type of IBD		–
Ulcerative colitis [*n* (%)]	2 (33)	
Crohn's disease [*n* (%)]	4 (67)	
Disease activity (PUCAI/PCDAI) [*n* (%)]		–
Mild	3 (50)	
Moderate	2 (33)	
Severe	1 (17)	
Diarrhoea [*n* (%)]	4 (67)	–

Abbreviations: IBD, inflammatory bowel disease; PCDAI, Paediatric Crohn's Disease Activity Index; and PUCAI, Paediatric Ulcerative Colitis Activity Index.

integrated system (Ortho Clinical Diagnostics, Raritan, NJ, USA). All assays were performed following the manufacturer's instructions. The concentration of SCFAs was evaluated as described above for the animal study.

2.4 | Data analysis and statistics

Differences between the groups were evaluated by one-way ANOVA, followed by Duncan's new multiple range test (MRT) or Student's unpaired *t* test where appropriate. The relationships between the concentration of FITC–dextran and calculated indirect permeability in rats and between calprotectin concentration and permeability in patients were determined by Pearson's correlation coefficient. A value of two-sided $P < 0.05$ was considered significant. Analyses were conducted using Dell Statistica, v.13 (Dell Inc., Tulsa, OK, USA).

3 | RESULTS

3.1 | Experimental study in rats

3.1.1 | Induction of colitis in rats

Histological examination of the colon revealed moderate swelling of the mucosa and submucosa, mucosal erosions reaching subepithelial connective tissue, minor subepithelial extravasations and focal, extensive extravasations over the mucosa and submucosa, with epithelial detachment, mononuclear cell infiltration and features of necrosis in the colitis group (Figure 2b). Colons of control and recovery groups had no signs of inflammation (Figure 2a,c).

3.1.2 | Gut–blood barrier permeability to SCFAs and a classic permeability marker (FITC–dextran)

Gut–blood barrier permeability to SCFAs

Rats with colitis showed significantly higher GBB permeability to SCFAs. Specifically, there were significant differences in the direct GBB permeability between the groups for the following SCFAs: AA ($F_{2,17} = 6.69$, $P < 0.01$), PA ($F_{2,17} = 5.55$, $P < 0.05$), IBA ($F_{2,18} = 5.60$, $P < 0.05$), IVA ($F_{2,19} = 4.04$, $P < 0.05$) and ICA ($F_{2,19} = 7.27$, $P < 0.01$) (Figure 3).

Likewise, there were significant differences in the indirect permeability between the groups for AA ($F_{2,17} = 4.11$, $P < 0.05$) and ICA ($F_{2,19} = 4.35$, $P < 0.05$). There was also a trend towards higher permeability values in the colitis group for the following SCFAs: PA ($P = 0.10$), IBA ($P = 0.07$), VA ($P = 0.08$) and IVA ($P = 0.14$) (Figure 4).

Gut–blood barrier permeability to FITC–dextran

After the administration of FITC–dextran into the colon, there was a significant difference between the groups in portal blood FITC–dextran concentration ($F_{2,20} = 7.13$, $P < 0.01$). The colitis group showed a significantly higher portal blood concentration of FITC–dextran than the control ($P < 0.001$) and recovery groups ($P < 0.001$).

Likewise, there was a significant difference between the groups in FITC–dextran concentration in systemic blood ($F_{2,20} = 3.63$, $P < 0.05$).

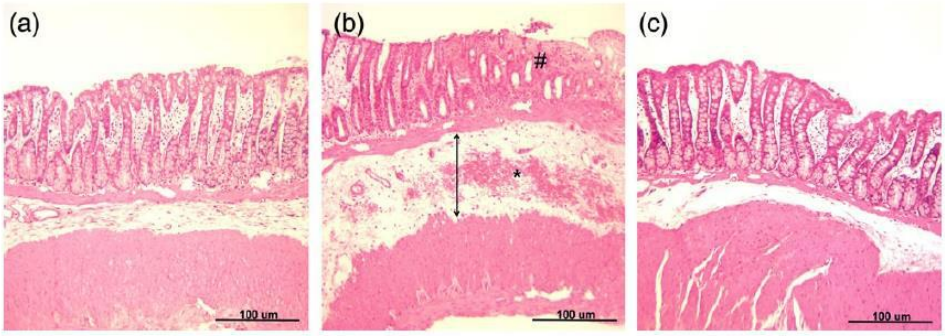

FIGURE 2 Histopathological examination of the colon in rats (Haematoxylin and Eosin staining). Colons were obtained from the control (a), acute colitis (b) and recovery (c) groups. Examination revealed moderate swelling of the mucosa and submucosa (double-headed arrow), mucosal erosions reaching subepithelial connective tissue (#), and extravasations over the mucosa and submucosa with epithelial detachment (*)

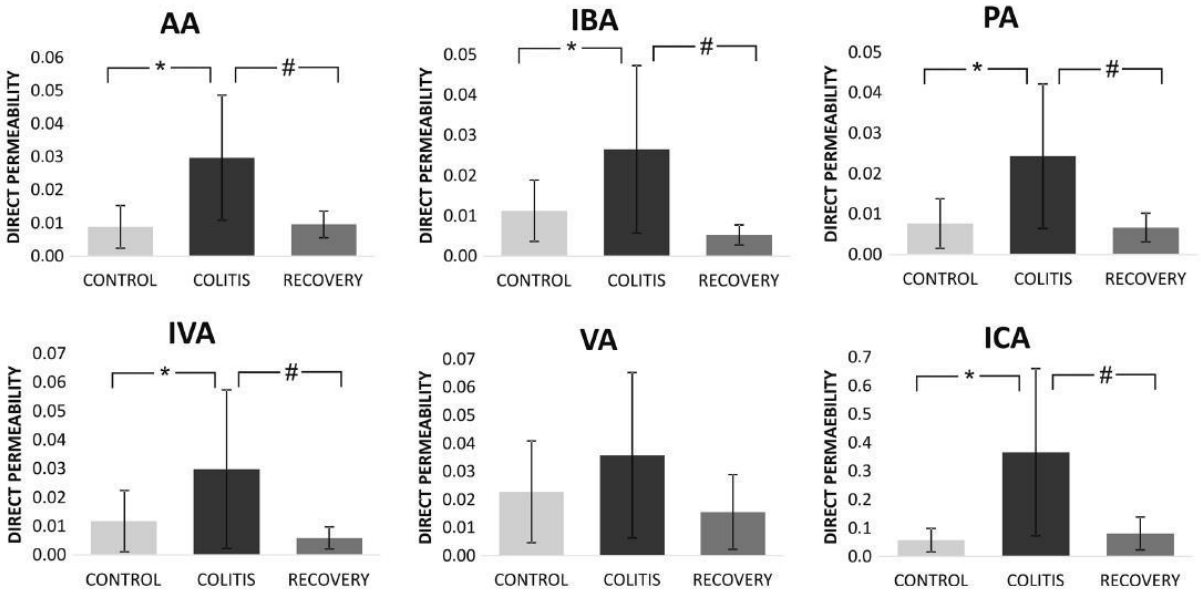

FIGURE 3 Direct permeability to short-chain fatty acids in acute colitis, control and recovery groups of rats. Abbreviations: AA, acetic acid; IBA, isobutyric acid; ICA, isocaproic acid/4-methylvaleric acid; IVA, isovaleric acid; PA, propionic acid; and VA, valeric acid. Values are means ± SD; $^{*}P < 0.05$ colitis *versus* control and $^{\#}P < 0.05$ colitis *versus* recovery, by ANOVA followed by Duncan's test

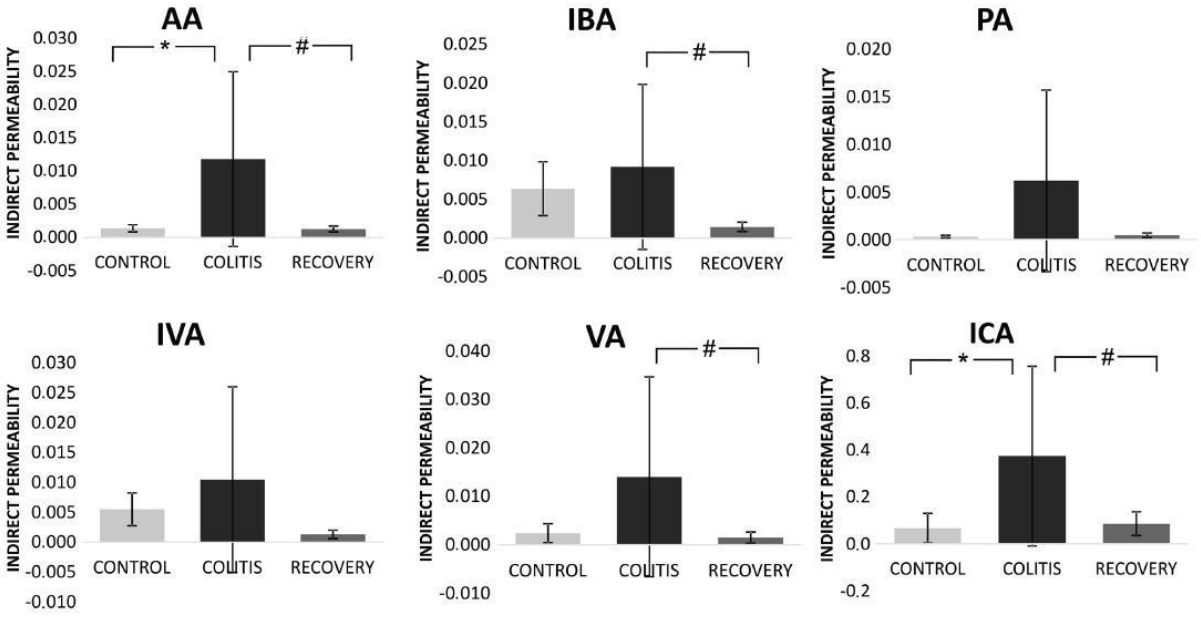

FIGURE 4 Indirect permeability to short-chain fatty acids in acute colitis, control and recovery groups of rats. Abbreviations: AA, acetic acid; IBA, isobutyric acid; ICA, isocaproic acid/4-methylvaleric acid; IVA, isovaleric acid; PA, propionic acid; and VA, valeric acid. Values are means ± SD; $^{*}P < 0.05$ colitis *versus* control, $^{\#}P < 0.05$ colitis *versus* recovery, by ANOVA followed by Duncan's test

In the colitis group, the concentration was significantly higher in comparison to the control ($P < 0.05$) and the recovery groups ($P < 0.05$).

Correlation of the new method (indirect permeability to SCFAs) with a classic permeability assay

There was a significant correlation between the indirect permeability to SCFAs and the FITC–dextran systemic concentration (classic method). Results are presented in Figure 5.

3.2 | Clinical study

3.2.1 | Gut–blood barrier permeability to SCFAs

There were pronounced interindividual variations in faecal (C_f) SCFA concentrations (Table 2). Nevertheless, there were significant differences in the calculated C_s/C_f (indirect permeability) ratio.

Patients with IBD showed significantly increased GBB permeability (C_s/C_f) in comparison to the control group with the use of the following

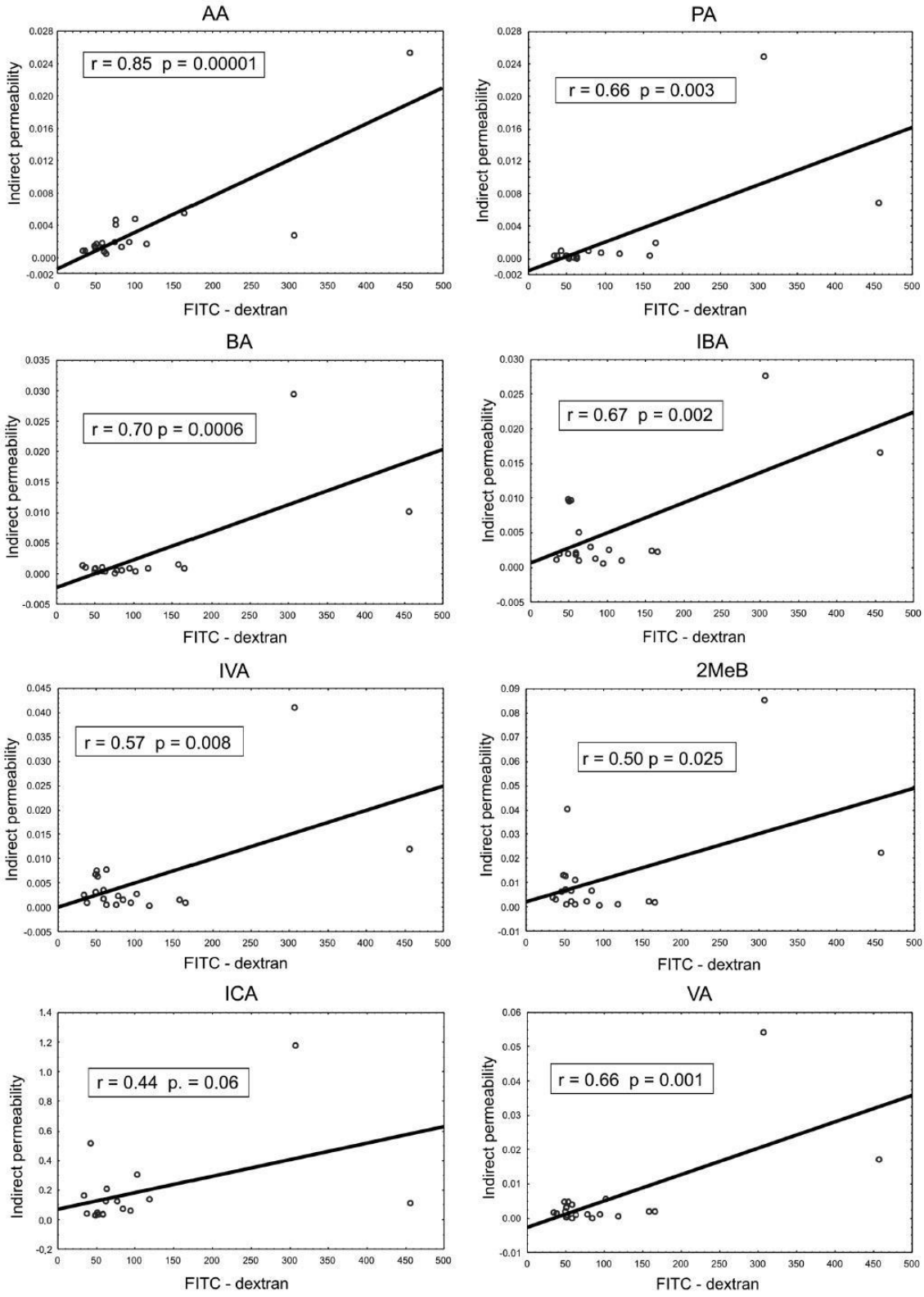

FIGURE 5 Correlation of indirect permeability to short-chain fatty acids with FITC–dextran (classic permeability marker) in rats. Abbreviations: AA, acetic acid; BA, butyric acid; IBA, isobutyric acid; ICA, isocaproic acid/4-methylvaleric acid; IVA, isovaleric acid; 2MeB, 2-methylbutyric acid; PA, propionic acid; *r*, Pearson's correlation coefficient; and VA, valeric acid

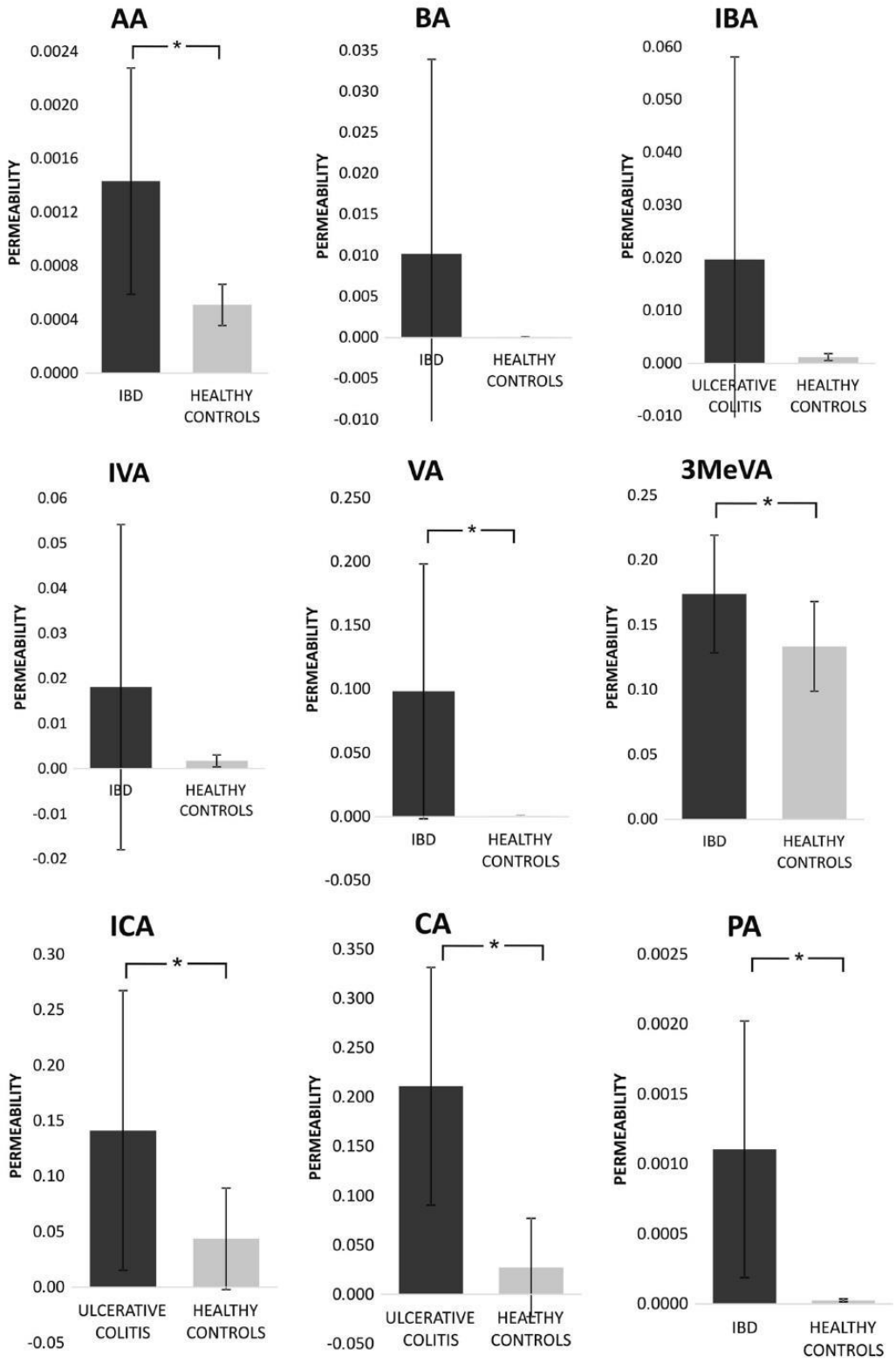

FIGURE 6 Gut–blood barrier permeability to short-chain fatty acids in patients with inflammatory bowel disease (IBD) and healthy control subjects. Abbreviations: AA, acetic acid; BA, butyric acid; CA, caproic acid; IBA, isobutyric acid; ICA, isocaproic acid/4-methylvaleric acid; IVA, isovaleric acid; 3MeVA, 3-methylvaleric acid; PA, propionic acid; and VA, valeric acid. Values are means ± SD; $^{*}P < 0.05$, by Student's unpaired *t* test

TABLE 2 Stool and plasma concentrations of short-chain fatty acids in patients with IBD and healthy control subjects

SCFA	Group	Faecal concentration (μM)	Plasma systemic concentration (μM)
AA	IBD	21,471.4 (9154.4)*	31.03 (22.21)
	Control	77,522.3 (32,603.5)	27.09 (6.11)
PA	IBD	2921.4 (2340.8)*	2.20 (0.70)*
	Control	77,522.3 (32,603.5)	1.56 (0.32)
IBA	IBD	779.5 (643.4)	2.13 (0.96)*
	Control	1478.4 (837.8)	1.28 (0.17)
BA	IBD	3126.9 (2418.3)	2.00 (0.63)
	Control	29,583.0 (13,608.0)	1.52 (0.36)
2MeB	IBD	386.1 (301.9)	2.10 (0.64)*
	Control	865.7 (578.1)	1.36 (0.15)
IVA	IBD	692.5 (493.9)	2.04 (0.74)*
	Control	1034.4 (600.3)	1.14 (0.16)
VA	IBD	30.9 (23.6)*	1.87 (0.62)*
	Control	1661.9 (318.9)	0.89 (0.11)
ICA	IBD	259.0 (539.2)	2.10 (0.83)*
	Control	50.8 (47.8)	1.12 (0.14)
CA	IBD	11.1 (3.4)*	2.48 (0.74)*
	Control	764.5 (804.7)	1.46 (0.22)
3MeVA	IBD	11.5 (1.3)	1.91 (0.76)*
	Control	9.2 (3.2)	1.14 (0.11)

Abbreviations: AA, acetic acid; BA, butyric acid; CA, caproic acid; IBA, isobutyric acid; IBD, inflammatory bowel disease; ICA, isocaproic acid/4-methylvaleric acid; IVA, isovaleric acid; 2MeB, 2-methylbutyric acid; 3MeVA, 3-methylvaleric acid; PA, propionic acid; and VA, valeric acid. Values are means (SD); $^{*}P < 0.05$ IBD *versus* control, by Student's unpaired *t* test.

SCFAs as markers: AA ($P = 0.025$), VA ($P = 0.01$), ICA ($P = 0.049$), PA ($P = 0.003$) and CA ($P = 0.001$), by Student's unpaired *t* test (Figure 6).

Additionally, patients with IBD had significantly higher systemic blood concentrations of IBA ($P = 0.02$), 2MeB ($P = 0.005$), IVA ($P = 0.003$), VA ($P = 0.001$), 3MeVA ($P = 0.01$), ICA ($P = 0.004$), CA ($P = 0.001$) and PA ($P = 0.03$) in comparison to healthy controls, by Student's unpaired *t* test (Table 2).

There was also a significant positive correlation between the permeability to SCFAs and faecal calprotectin concentration (Figure 7).

4 | DISCUSSION

In this study, we found an increased gut-to-blood penetration of SCFAs both in an animal model and in paediatric patients with IBD. These findings lay the groundwork for establishing a new, non-invasive diagnostic tool in diseases associated with intestinal barrier malfunction, such as IBD and NEC, or extra-intestinal diseases associated with GBB dysfunction.

The current strategy to diagnose and monitor IBD includes frequent assessment of gut inflammation, which is based on clinical symptoms, laboratory markers and radiological, endoscopic and histological evaluation. If taken separately, none of the diagnostic methods is good enough. Moreover, colonoscopy requires special bowel preparation and sedation or general anaesthesia, especially in children. Finally, colonoscopy is contradicted in severe disease. Therefore, there is an unmet need for developing a new, non-invasive and easy-to-perform test that assesses IBD activity.

An increased permeability of the GBB, or a 'leaky gut', is a pivotal symptom of gut malfunction and is present in numerous intestinal diseases, such as IBD, NEC or acute pancreatitis (Camilleri et al., 2012; Ravisankar et al., 2018; Sonika et al., 2017), and in extra-intestinal diseases, including heart failure (Sandek et al., 2007), hypertension (Jaworska et al., 2017; Santisteban et al., 2017), obesity (Bischoff et al., 2014) and diabetes (Carratu et al., 1999). Therefore, non-invasive evaluation of the GBB might be a promising method for diagnosis and treatment monitoring of diseases associated with GBB malfunction.

Clinical assessment of the GBB is constrained by numerous limitations of the currently available methods. Frequently used tests are based on the plasma concentration of fatty acid-binding proteins, citrulline or tight junction proteins, such as zonulin and claudin-3. Unfortunately, those markers reflect either epithelial loss or tight junction damage, which may correspond poorly with functional alterations of the GBB (Galipeau & Verdu, 2016). This is because the GBB is a complex multilayer system, the functioning of which depends on numerous factors, such as intestinal perfusion, immunological and neurohormonal activity, mucous secretion and the composition of microbiota (Farhadi et al., 2003). In contrast, the currently available functional tests evaluating permeability of the GBB use orally administered exogenous, radio-labelled, highly osmotic markers, such as polysaccharides or dextrans, and their safety is uncertain, especially for paediatric patients and those with concomitant kidney and cardiovascular diseases (Nomani et al., 2014). Moreover, the blood concentration of orally administered agents varies in time depending on concomitant diet or duration of fasting and gastrointestinal motility.

Our study shows that an increased blood-to-stool ratio of gut bacteria-produced SCFAs is an indicator of increased permeability of the GBB. This method circumvents problems associated with the above-mentioned methods for evaluation of the GBB. First, this is a functional method, i.e. it evaluates the GBB function and not only structural/morphological alterations. Second, SCFAs are naturally present in the intestines, which eliminates the need for administration of exogenous, radio-labelled markers. Third, assessment of the blood-to-stool ratio and not only the blood concentration of those molecules virtually eliminates problems associated with inter-individual variations in intestinal motility or metabolism of gut bacteria caused by differences in a diet or the concomitant treatment. Hence, the proposed method does not require any special preparation of the patient and is risk free.

To establish the method, we initially conducted experiments on an animal model of IBD confirmed by histological examination. We evaluated the concentration of SCFAs in the colon content (stool masses sampled from the colon), in portal blood and in systemic blood. Comparisons between the colitis group and the sham group showed

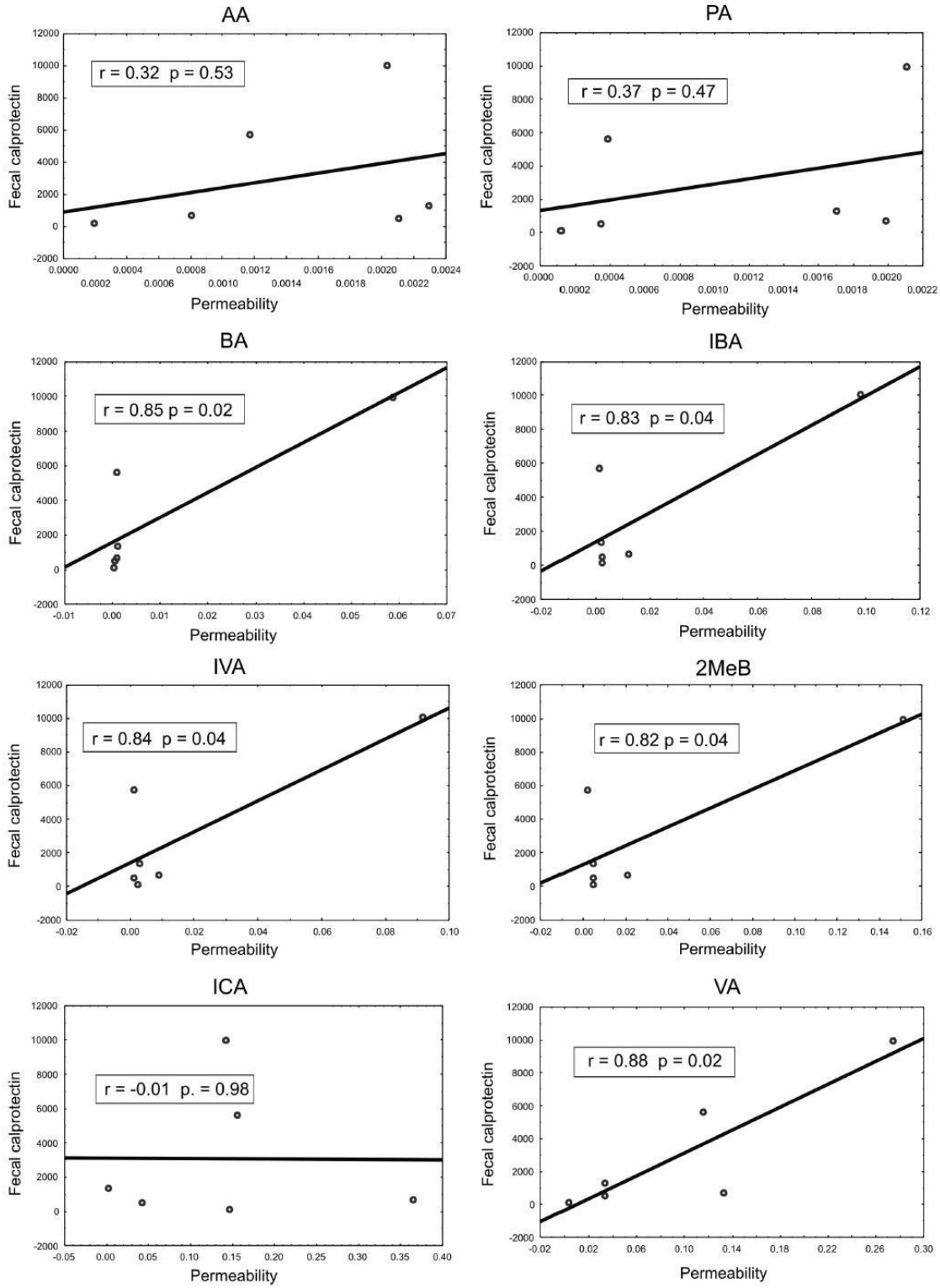

FIGURE 7 Correlation of gut–blood barrier permeability to short-chain fatty acids with faecal calprotectin in patients with inflammatory bowel disease. Abbreviations: AA, acetic acid; BA, butyric acid; IBA, isobutyric acid; ICA, isocaproic acid/4-methylvaleric acid; IVA, isovaleric acid; 2MeB, 2-methylbutyric acid; PA, propionic acid; *r*, Pearson's correlation coefficient; and VA, valeric acid

that the colitis group had a significantly higher ratio of portal blood to colon concentration of SCFAs (direct permeability; Figure 3) and a significantly higher ratio of systemic blood to colon concentration of SCFAs (indirect permeability; Figure 4). These findings imply an increased gut-to blood penetration, or increased permeability of the GBB, in the colitis group. This notion is also supported by other findings of the present study, i.e. a significantly higher gut-to-blood penetration of fluorescent-labelled dextran in rats with colitis. The accuracy of SCFAs as permeability markers was confirmed by correlation of the indirect permeability with the classic method (Figure 5).

To check the feasibility of this method in clinical settings, we assessed a systemic blood-to-stool ratio of SCFA concentrations in paediatric patients with IBD and age-matched healthy control subjects. Analogously to animal experiments, IBD patients showed a significantly higher blood-to-stool ratio of SCFAs, including AA, VA, ICA, CA and PA (Figure 6). Furthermore, we found a significant positive correlation between the permeability to SCFAs and faecal concentration of calprotectin, a biochemical marker of intestinal inflammation (Figure 7). The latter provides further evidence for an association between inflammation and a 'leaky gut'.

To the best of our knowledge, this study is the first to propose a blood-to-stool ratio of bacterial metabolites as a marker for GBB permeability. Nevertheless, there are some previous studies reporting an increased blood concentration of gut bacterial products in diseases associated with 'leaky gut'. For example, an increased blood concentration of bacteria, endotoxins (LAL assay) and anti-endotoxin immunoglobulins (EndoCAb assay) have been suggested to reflect increased permeability of the GBB (Grootjans et al., 2010). Furthermore, Ktsoyan et al. (2016) showed that infectious and autoinflammatory diseases elevated SCFA concentrations in plasma. Also, in our previous studies we showed an increased concentration of bacterial metabolites in portal and systemic blood in animal models of portal hypertension (Huc et al., 2018) and systemic hypertension (Jaworska et al., 2017). However, the concentration of bacterial metabolites in the blood depends strongly on gut bacterial composition and their metabolic activity shaped by diet, drugs, etc. To circumvent this problem in the present study, we used a blood-to-stool ratio of SCFA concentrations, which virtually eliminates the problem of interindividual differences in metabolic activity of the gut bacteria.

A limitation of this research is the small size of the clinical study. Nevertheless, taking the clinical and animal findings of this proof-of-concept study together, we postulate that the blood-to-stool ratio of SCFAs is a marker of leaky gut in IBD. Further, larger clinical studies are needed to assess whether the proposed method provides additional diagnostic and prognostic information to the methods currently used for diagnosis and treatment monitoring of IBD and other diseases associated with 'leaky gut'. It also needs to be stated that in IBD rats and IBD patients an increased blood-to-stool ratio of SCFAs might result, in part, from reduced metabolism of SCFAs by colonocytes owing to the IBD-related damage. Nevertheless, if that factor contributes to our findings it does not belittle the diagnostic importance of an increased blood-to-stool ratio of SCFAs in IBD.

In conclusion, our research shows an increased blood-to-stool ratio of SCFAs both in an animal model and in patients with IBD, which suggests an increased gut-to-blood penetration of those bacterial metabolites. We strongly believe that our findings lay the groundwork for establishing a new, non-invasive, risk-free diagnostic tool in diseases associated with intestinal barrier malfunction, such as IBD or NEC. Further studies are needed to assess whether the blood-to-stool ratio of bacterial metabolites might provide important diagnostic information in addition to the methods currently used for diagnosis and treatment monitoring.

COMPETING INTERESTS

None declared.

AUTHOR CONTRIBUTIONS

Conception and design of the work: K.J., A.B. and M.U. Acquisition, analysis and interpretation of animal experimental data: K.J., M.K., K.B., T.H. and M.U. Acquisition, analysis and interpretation of the clinical data M.D., A.B., K.J. and M.U. Drafting the manuscript or revising it for important intellectual content: K.J., M.K., K.B., T.H., A.B., M.D. and M.U. All authors approved the final version of the manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacteria metabolite.

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RESEARCH ARTICLE

Hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacteria metabolite

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Abstract

An increased blood trimethylamine N-oxide (TMAO) has emerged as a marker of cardiovascular mortality, however, the mechanisms of the increase are not clear. We evaluated if hypertension was associated with changes in the colon permeability to trimethylamine (TMA), a TMAO precursor. We did experiments on male, 24-26-week-old normotensive Wistar-Kyoto rats (WKY), spontaneously hypertensive rats (SHR) and SHR treated with enalapril, an antihypertensive drug (SHR-E). To check the colon permeability and liver TMA clearance, blood was collected from the portal vein and hepatic veins confluence, at baseline and after the intracolonic administration of TMA. Arterial blood pressure (BP) and intestinal blood flow (IBF) recordings and histological assessment of the colon were performed. SHR showed an increased gut-blood barrier permeability to TMA. Namely, at baseline SHR had a higher BP and portal blood TMA, but a lower IBF than WKY. After the intracolonic administration of TMA, SHR had a significantly higher portal blood TMA and higher TMA liver clearance than WKY. In SHR the arteriolar walls of the colon mucosa were significantly thicker than in WKY. Furthermore, SHR showed a significant decrease in the height of the mucosa. In contrast, SHR-E had lower portal blood TMA, lower BP and smaller thickness of arteriolar walls, but higher IBF than SHR, which indicates improved function of the gut-blood barrier in SHR-E. All groups had similar immunostaining of occludin and zonula occludens-1, markers of tight junctions. In conclusion, hypertensive rats show an increased permeability of the colon to TMA, which is accompanied by morphological and hemodynamic alterations in the colon. Therefore, cardiovascular diseases may be characterized by an increased permeability of the gut-blood barrier to bacterial metabolites such as TMA.

Introduction

Increasing evidence suggests that gut microbiota produce biologically active compounds that enter the circulation and affect the circulatory system homeostasis [1–3]. To enter the circulation, gut bacteria metabolites need to pass the gut-blood barrier (GBB). The integrity and permeability of the GBB is dependent on numerous factors, including intestinal blood flow [4]. Hypertension is a major risk factor for heart failure, coronary artery disease and stroke, causing high morbidity and mortality. Hypertension is known to produce pathological changes in vasculature, such as microangiopathy in the retina, kidneys and in other organs [5]. However, there are scant data on the effect on hypertension on intestinal vasculature.

A positive correlation between an elevated fasting plasma trimethylamine N-oxide (TMAO), a gut bacteria metabolite, and an increased risk of major adverse cardiovascular events has been suggested [3, 6], however, the diagnostic value of blood TMAO level in cardiovascular diseases is debatable [7, 8].

In mammals, blood TMAO concentration increases after ingesting choline and L-carnitine which are absorbed from the small intestine. However, if the concentration of the nutrients exceeds the transport capacity of small intestine, they reach the large bowel and are metabolised by intestinal bacteria producing trimethylamine (TMA) [9, 10]. The bacteria-derived TMA is absorbed from the large bowel and goes with portal blood to the liver where it is oxidized to TMAO by flavin-containing monooxygenase-3 (FMO3) [11]. TMA and TMAO in humans are excreted mainly with the urine, but also with the sweat and the exhaled air [12, 13]. Therefore, the blood TMAO concentration may depend on several factors, including diet, gut microbiota activity, the GBB permeability to TMA, the oxidation of TMA by the liver, and TMA and TMAO excretion.

To the best of our knowledge, the effect of hypertension on the permeability of the colon, a major site of gut bacteria activity, has not been investigated. Therefore, in this study, we evaluated the permeability of the colon to TMA, as well as other factors that may influence blood TMA/TMAO concentration, including TMA liver clearance, TMA/TMAO excretion, and TMA stool concentration in normotensive and hypertensive rats.

Materials and methods

The experiments were performed according to Directive 2010/63 EU on the protection of animals used for scientific purposes and approved by the I Local Bioethical Committee in Warsaw.

We did the study on male, 24-26-week-old, normotensive Wistar Kyoto (WKY, n = 30) rats, spontaneously hypertensive rats (SHR, n = 30) maintained on a standard laboratory diet and tap water, and on SHR, maintained on standard laboratory diet and treated with enalapril (Polpharma, Poland), an antihypertensive drug, dissolved in drinking water 100 mg/L (a dose of 12.9 ± 0.5 mg/kg BW) for 8 weeks (SHR-E, n = 30). Animals were provided by the Central Laboratory of Experimental Animals, Centre for Preclinical Research and Technology, Warsaw, Poland. All surgical procedures were performed under general anaesthesia with urethane (Sigma-Aldrich, Poland) at a dose of 1.5 g/kg of body weight (BW).

Gut-blood barrier permeability

The experiments were performed on WKY (n = 12), SHR (n = 12) and SHR-E (n = 12). Rats were implanted with a polyurethane catheter inserted into the portal vein that collects blood from the intestines. Blood samples from the portal vein were collected at baseline and 30 and 60 min after the intracolonic administration of TMA (TMA intracolonic challenge test) or 0.25 mL of 0.9% NaCl saline (controls). Namely, half of the rats from the WKY, SHR and

SHR-E groups were given saline and the other half were given TMA (100 mg/kg BW) dissolved in 0.25 mL of saline via a flexible polyurethane tube inserted 9 cm from the external anal sphincter.

Liver clearance of TMA

The experiment was performed on WKY (n = 6), SHR (n = 6) and SHR-E (n = 6) rats implanted with polyurethane catheters inserted into the portal vein and into the inferior vena cava, just above the hepatic veins confluence. Blood samples from the veins were collected 30 min after the intracolonic administration of TMA (100 mg/kg BW) as described above.

TMA liver clearance was defined as the difference between portal blood TMA and hepatic vein blood TMA, and as the ratio of hepatic vein blood TMA and portal blood TMA, $(1-\frac{\text{hepatic vein TMA}}{\text{portal vein TMA}})$.

Assessment of blood pressure and intestinal blood flow

WKY (n = 6), SHR (n = 6), and SHR-E (n = 6), were implanted with a polyurethane arterial catheter inserted into the aorta via femoral artery to measure blood pressure (BP) with Biopac MP 150 unit (Biopac Systems, Goleta, USA). Next, the upper mesenteric vein collecting the blood from the colon was separated from the surrounding tissue for a distance of ca. 10 mm, to enable placement of a flow probe (ID 1 mm). The access to the vein was obtained by performing midline laparotomy with a cut from the xiphoid to the navel. The intestines were lined outside the abdominal cavity and wrapped with moistened gauze, protecting them from drying. Measurements of upper mesenteric vein blood flow (intestinal blood flow—IBF) were conducted using the transit time ultrasound set-up which consists of a noncannulating acoustic (20 kHz) flow probe connected with a dedicated flowmeter (type T106, Transonic System Inc., Ithaca, N.Y., USA). The IBF and BP were measured at baseline, for 30 minutes after the intracolonic administration of 0.25 mL of saline, and 30 min after the intracolonic injection of TMA (100 mg/kg BW) dissolved in 0.25 mL of saline.

24-hr TMA balance, metabolic and biochemical data

WKY (n = 6), SHR (n = 6), and SHR-E (n = 6) were maintained for 2 days in metabolism cages to evaluate 24-hr TMA, water, food balance and to collect urine for TMA excretion study. Samples from the second day were analysed. After the experiments, blood from the right ventricle of the heart, stool, and intestine samples were collected as described below.

Blood biochemical tests. Rats were anaesthetized with urethane at a dose of 1.5 g/kg BW. Next, blood was taken to the chilled EDTA tubes. The sample was centrifuged for 5 minutes at 5000 rpm at 4˚C. The plasma was collected into Eppendorf tubes and frozen at -20˚C. Biochemical blood analyses (creatinine, urea, AST, ALT) were performed on the *Cobas 6000 analyzer* (Roche Diagnostics, Indianapolis, USA).

Evaluation of TMA, TMAO and indoxyl sulfate concentration. Blood plasma, urine and stool concentration of TMA/TMAO and indoxyl sulfate was evaluated using liquid chromatography coupled with triple-quadrupole mass spectrometry as previously described [1]. Instrumentation consisted of Waters Acquity Ultra Performance Liquid Chromatograph coupled with Waters TQ-S triple-quadrupole mass spectrometer. The mass spectrometer operated in the multiple-reaction monitoring (MRM)- positive electrospray ionization (ESI) mode. The ion transitions were m/z 76.076> 57.97 and m/z 76.076> 58.97 for TMAO, m/z 60.08> 44.05 and m/z 60.08> 45.057 for TMA, m/z 64.09> 48.05 for TMA-13C315N, m/z 85.13> 68.2 for TMAO-D9, 212.00>79.96 and 212.00>132.04 for indoxyl sulfate and 216.11>135.76 for

indoxyl sulfate-D4. Only first was used as quantification transition. The calibration curve ranges were 0.1–60 μg/mL for TMAO, 0.99–120 μg/mL for TMA and 0.1–50 μg/mL for indoxyl sulfate. The limits of quantification (LOQ) were 0.1 μg/mL, 0.99 μg/mL and 0.1 μg/mL for TMAO, TMA and indoxyl sulfate, respectively.

Preparation of stool samples for TMA and TMAO analysis. After the blood taking, anaesthetized rats were killed by decapitation. A 5–6 cm long segment of the colon (a middle part between the cecum and the rectum) was closed with sutures and removed. The isolated fragment of the colon was dissected and the tissues were used for histological and immunohistochemical evaluation.

A sample of 0.5 mL of stools from the removed colon was weighted and homogenized with 1.0 mL of 0.9% NaCl in a closed 2 mL laboratory tube by vortexing it for 5 min. Afterwards, the sample was centrifuged for 5 minutes at 5000 rpm, and 1 mL of the obtained supernatant was transferred to a tube with a 0.45 μm pore size filter (Ultrafree-CL, Merck KgaA, German) and again centrifuged for 5 minutes at 5000 rpm. All procedures were performed at the temperature of 2–5˚C. The supernatant was collected into Eppendorf tubes and frozen at -20˚C.

Histology and morphometry of the colon

Histological assessment was performed on tissues harvested from WKY, SHR and SHR-E. Tissues sections fixed in 10% buffered formalin were dehydrated by means of graded ethanol and xylene baths and embedded in paraffin wax. Sections of 3–4 μm were stained with haematoxylin and eosin (HE) and van Gieson stain (for connective tissue fibres). General histopathological examination was evaluated at magnification of 10x, 40x and 100x (objective lens) and 10x (eyepiece) and photographic documentation was made. The mucosa and submucosa of the colon, intestinal crypts and its cell composition, blood vessels of mucosa and submucosa of the colon were examined. The morphometric analysis included: the height of the mucous membrane—measured at magnification of 10x (objective lens) and 10x (eyepiece), and the thickness of walls of arterioles which were visible in submucosa—measured at magnification of 40x (objective lens) and 10x (eyepiece). Measurements of the width of the vascular wall were made for two types of vessels: in diameter 25–50 μm—smaller arterioles or in diameter 75–100 μm larger arterioles. Both types of arterioles were in the colonic submucosa. The height of the colonic mucosa was measured from the top of the crypt to the beginning of the muscularis mucosae. The microscopic evaluation was performed in a blinded fashion, using a standard light microscope Olympus BX41 and CellSens software (Olympus Corporation, Tokyo, Japan).

Immunohistochemistry staining of occludin and ZO-1

Tissue was fixed in 4% paraformaldehyde in PBS (phosphate-buffered saline) (4˚C, 24 hours), impregnated with 30% sucrose solutions (w/v in PBS) and frozen in dry-ice cold heptane. Next, the tissue was cut with cryotome into 12 μm thick sections and mounted on adhesion slides (Superfrost™ Ultra Plus, Thermo Scientific).

Sections were blocked with 5% goat serum in PBST (phosphate-buffered saline with 0.1% Triton X-100) and incubated overnight at 4˚C with primary antibodies (anti-ZO-1, Thermo Fisher, catalog number: 61–7300 or anti-occludin, Thermo Fisher, catalog number: 71–1500, Waltham, USA). Next, sections were washed with PBST and incubated with Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, catalog number: 111-165-144) secondary antibody at room temperature for 2 h. After washing with PBST, mounting medium (Vectashield with DAPI, Vector, catalog number: H-1200) sections were coverslipped. Anti-

ZO-1 staining or anti-occludin staining of all three experimental groups were processed simultaneously.

Immunofluorescence measurements were performed with an Olympus X1000 confocal microscope (Olympus Corporation, Tokyo, Japan), 20x objective. The same parameter settings for all three experimental groups were applied for measurements of anti-ZO-1 staining signal or anti-occludin staining signal. Images were analyzed with Fiji software [14]. Mean signal of fluorescence was measured for ROIs (regions of interest) which were manually selected within epithelial cells of the mucous membrane of the colon. Background signal was measured in area of slide with no tissue and was subtracted from epithelium fluorescence signal.

Statistical analyses

The Kolmogorov-Smirnov test was used to test normality of the distribution. Arterial blood pressure (BP) and heart rate (HR) were calculated by AcqKnowledge 4.3.1 Biopac software (Biopac Systems, Goleta, USA). For evaluation of systolic and diastolic BP, HR and IBF within the series, the average over 5-minute before intracolonic infusion was compared with the averages over 5 minutes after the intracolonic infusion by ANOVA for repeated measures. Differences between the groups were evaluated by one-way or multivariate ANOVA, followed by Tukey's post hoc test. A value of two-sided $p<0.05$ was considered significant. Analyses were conducted using STATISTICA 12.0 (Stat Soft, Krakow, Poland).

Results

Gut-blood barrier permeability

Gut-blood barrier permeability at baseline. At baseline, spontaneously hypertensive rats (SHR, n = 12, 4.20±0.19 μg/mL) had a significantly higher portal blood TMA than normotensive Wistar Kyoto rats (WKY, n = 12, 3.09 ± 0.25 μg/mL) and SHR treated with enalapril, an antihypertensive drug (SHR-E, n = 12, 3.11 ± 0.21 μg/mL), ($p<0.05$). In addition, SHR (5.95 ± 0.62 μg/mL) had a significantly higher hepatic blood indoxyl sulfate level than WKY (3.55 ± 0.29 μg/mL) and SHR-E (4.49 ± 0.44 μg/mL), ($p<0.05$).

TMA intracolonic challenge test. At baseline, SHR (n = 6) had a significantly higher portal blood TMA than WKY (n = 6) and SHR-E (n = 6), i.e. SHR: 4.23 ± 0.35 μg/mL, WKY: 2.69 ± 0.20 μg/mL, and SHR-E: 3.05 ± 0.20 μg/mL, ($p<0.05$). The intracolonic administration of TMA produced a significant increase in portal blood TMA in all the groups [SHR ($p<0.05$), WKY ($p<0.05$), SHR-E ($p<0.05$), by one-way ANOVA for repeated measures]. The increase in the SHR group was significantly higher than in WKY and SHR-E groups ($p<0.05$, by ANOVA for repeated measures, followed by post-hoc Tuckey test), (Fig 1A). The intracolonic administration of saline did not produce a significant change in portal blood TMA (Fig 1B).

Liver clearance of TMA

After the intracolonic administration of TMA, SHR had a significantly higher portal blood TMA than WKY and SHR-E (Table 1). TMA liver clearance was defined as the difference between portal blood TMA and hepatic vein blood TMA, and as the ratio of hepatic vein blood TMA and portal blood TMA ($1-\frac{\text{hepatic vein TMA}}{\text{portal vein TMA}}$). SHR had a significantly higher TMA liver clearance in comparison to WKY and SHR-E, (Table 1).

Assessment of blood pressure and intestinal blood flow

At baseline, there were significant differences between WKY, SHR and SHR-E in intestinal blood flow (IBF) ($p<0.05$), systolic blood pressure (SBP) ($p<0.05$), diastolic blood pressure

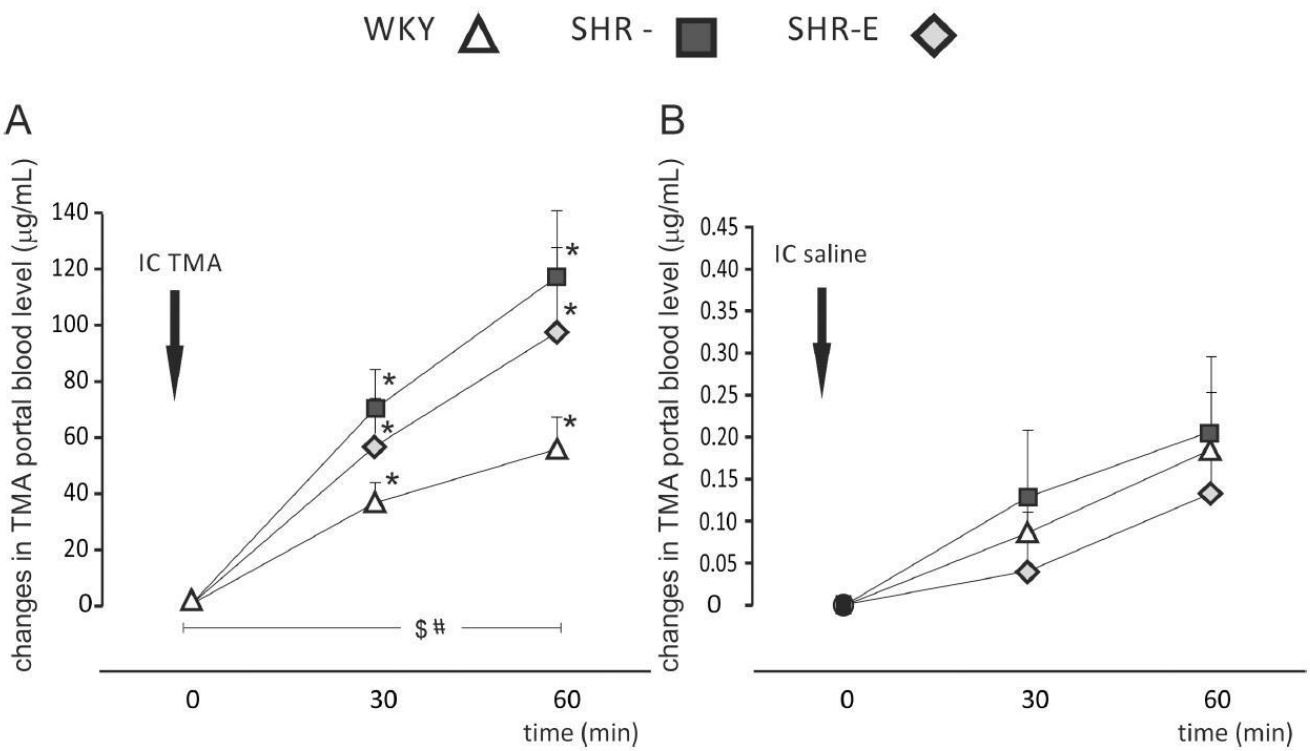

Fig 1. TMA intracolonic challenge test. Changes in trimethylamine (TMA) portal blood level (µg/mL) after the intracolonic administration of **A)** TMA (100 mg/kg BW) dissolved in 0.25 mL of 0.9% NaCl (IC TMA) or **B)** 0.25 mL of 0.9% NaCl (IC saline) in normotensive Wistar Kyoto rats (WKY) (n = 6), spontaneously hypertensive rats (SHR) (n = 6), and enalapril-treated SHR (SHR-E) (n = 6). Values are means, + SE, *—p<0.05 vs baseline, $- p<0.05 WKY vs SHR, #- p<0.05 WKY vs SHR-E (repeated measures ANOVA followed by post hoc Tuckey test).

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Table 1. TMA and TMAO liver clearance.

Group/ parameter	WKY (n = 6)	SHR (n = 6)	SHR-E (n = 6)	ANOVA
Portal vein TMA (µg/mL)	41.84 ± 5.78	71.34 ± 5.67	42.1 ± 5.52	($F_{2,15}$ = 10.1, p<0.05), *,†
Hepatic vein TMA (µg/mL)	27.68 ± 3.31	29.17 ± 2.52	25.6 ± 3.66	NS
Liver TMA clearance (Portal—Hepatic veins difference) (µg/mL)	14.15 ± 4.06	42.17 ± 5.98	16.5 ± 6.31	($F_{2,15}$ = 8.2, p<0.05), *,†
Liver TMA clearance $(1-\frac{\text{hepatic vein TMA}}{\text{portal vein TMA}})$.	0.32± 0.05	0.58 ± 0.04	0.33 ± 0.10	($F_{2,15}$ = 3.9, p<0.05), *,†
Hepatic vein TMAO (µg/mL)	5.61 ± 0.41	15.27 ± 1.24	10.86 ± 1.73	($F_{2,15}$ = 12.8 p<0.05), *,‡

Portal and hepatic vein blood TMA and TMAO (µg/mL) 30 min after the intracolonic administration of TMA (100 mg/kg BW) dissolved in 0.25 mL of 0.9% NaCl in Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and enalapril-treated SHR (SHR-E). Values are means, ± SE. One-way ANOVA followed by post-hoc Tuckey-test. NS—not significant differences between groups.

*- p<0.05 for WKY vs SHR

†- p<0.05 for SHR vs SHR-E

‡- p<0.05 for WKY vs SHR-E.

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(DBP) ($p<0.05$) and heart rate (HR) ($p<0.05$) as analysed with one-way ANOVA. Namely, SHR had a significantly lower IBF but higher SBP, DBP and HR in comparison to WKY and SHR-E (Fig 2). Such between-group differences were also present after the intracolonic administration of saline ($p<0.05$, for IBF), ($p<0.05$, for SBP), ($p<0.05$, for DBP) and ($p<0.05$ for HR) as analysed with ANOVA for repeated measures. Similar between-group differences were found after the intracolonic administration of TMA ($p<0.05$, for IBF), ($p<0.05$, for SBP), ($p<0.05$, for DBP) and ($p<0.05$, for HR), (Fig 2). There was no significant within-group difference in neither of the parameters after the intracolonic infusion of saline and TMA as analysed for 5 min pre-administration vs 20 min post-administration period by ANOVA for repeated measures. However, in all the groups there was a mild, non-significant decrease in IBF during the experiment.

24-hr TMA balance, metabolic and biochemical data

There were no significant differences between WKY, SHR and SHR-E in body mass and food intake. SHR-E showed a higher water intake and 24-hr urine output in comparison to WKY and SHR. There was a significant within group variation in stool TMA level (TMA concentration in stool sample extracts in 3 of 6 WKY, in 1 of 6 SHR and 2 of 6 SHR-E was below the limit of quantification), consequently statistical analysis showed no significant differences between the groups, (Table 2).

There was no significant difference in peripheral blood TMAO level (blood taken from the right ventricle of the heart) between the groups (WKY: 0.17 ± 0.02 μg/mL, SHR: 0.23 ± 0.03 μg/mL and SHR-E: 0.19 ± 0.03 μg/mL), while blood TMA level was below the limit of quantification. 24-hr urine TMAO and TMA output was higher in SHR than in WKY, however, the difference was not significant. Plasma creatinine and urea were higher in SHR and SHR-E than in WKY. AST and ALT levels were higher in SHR-E rats in comparison to WKY, (Table 2).

Histology and morphometry of the colon

In SHR (n = 6), the walls of larger arterioles in the mucosa and submucosa membrane were significantly thicker than in WKY (n = 6) and SHR-E (n = 6), ($p<0.05$). Similar changes were found in smaller arterioles ($p<0.05$). In general, in the SHR group the lumen of arterioles was smaller, while the veins were more expanded and filled with blood in comparison to WKY and SHR-E, (Fig 3).

Morphometric measurement showed a significant decrease in the height of the mucosa in SHR ($p<0.05$). SHR showed also fewer goblet cells which were present only in the lower part of the intestinal crypts and had no contact with the light of the colon. There were no significant differences between SHR and SHR-E in the height of mucosa. SHR-E showed a small increase in the number of goblet cells in comparison to SHR, (Figs 3 and 4).

In SHR in the van Gieson staining, the hyperplasia of connective tissue of mucosa and submucosa was observed. There was no fibrosis in the walls of arterioles, however, the increase in thickness of adventitia and the amount of connective tissue around arterioles were present, (Fig 3). Besides, SHR showed infiltration of mucosa by mononuclear cells and their clusters under the epithelium. Additionally, numerous lymphoid follicles of GALT (*gut-associated lymphoid tissue*) and cryptopatches were found, but there was no increased infiltration of epithelium by migrating lymphocytes or other leukocytes (Fig 4).

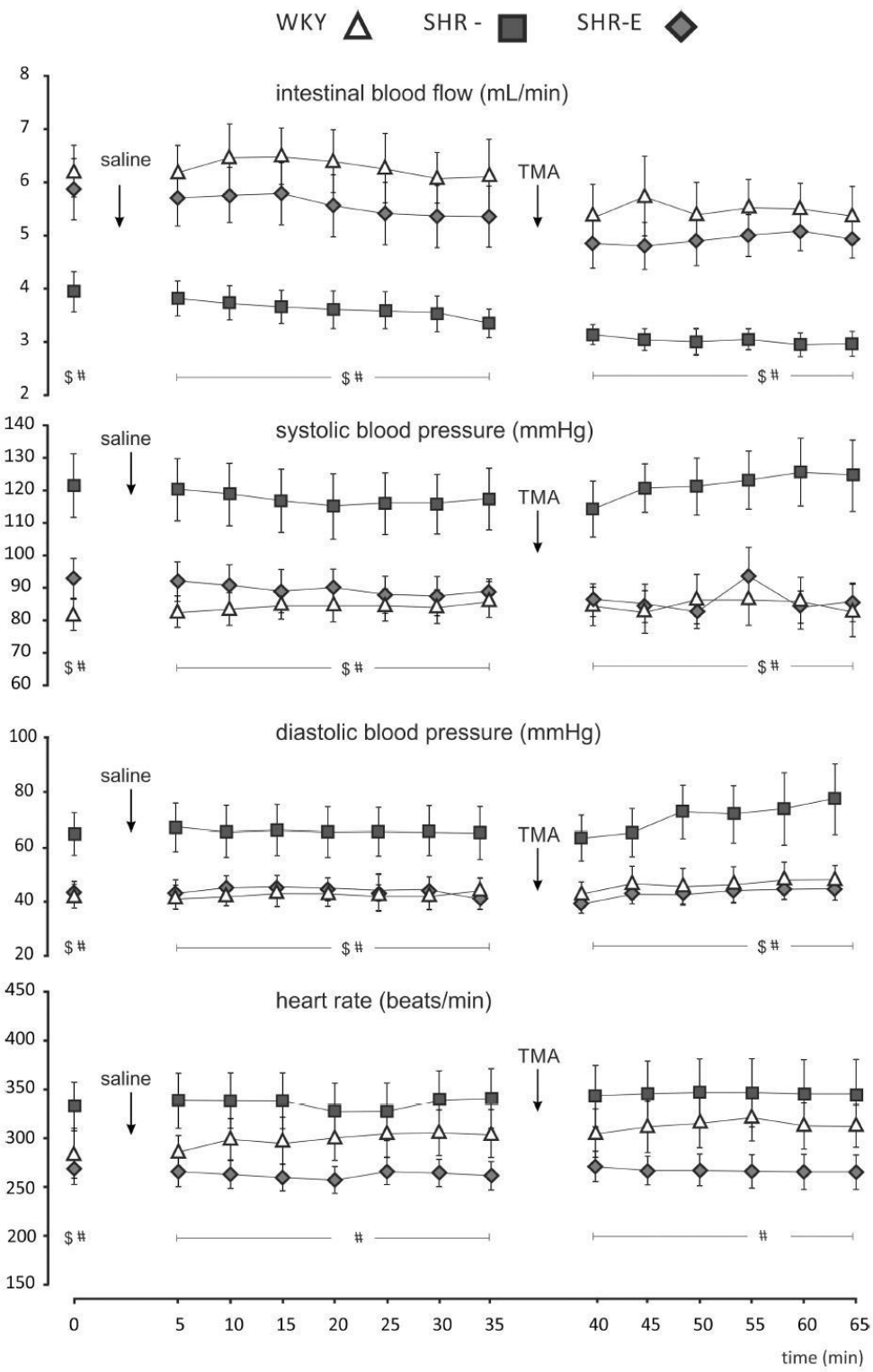
WKY
SHR -
SHR-E
intestinal blood flow (mL/min)
saline
TMA
systolic blood pressure (mmHg)
diastolic blood pressure (mmHg)
heart rate (beats/min)
time (min)

Fig 2. Assessment of intestinal blood flow and blood pressure. Intestinal (upper mesenteric vein) blood flow, systolic arterial blood pressure, diastolic arterial blood pressure and heart rate in anaesthetized normotensive Wistar-Kyoto rats (WKY) (n = 6), spontaneously hypertensive rats (SHR) (n = 6) and enalapril-treated SHR (SHR-E) (n = 6) at baseline (0) and after the intracolonic administration of 0.25 mL of 0.9% NaCl (↓ saline) and TMA (100 mg/kg BW) dissolved in 0.25 mL of 0.9% NaCl (↓ TMA). $^{\$}$- p<0.05 for SHR vs WKY, $^{\#}$- p<0.05 for SHR vs SHR-E comparison.

https://doi.org/10.1371/journal.pone.0189310.g002

Immunohistochemistry studies of occludin and ZO-1

There was no significance difference between the groups in mean fluorescent signal for ZO-1 and occludin. However, there was a tendency for higher values for ZO-1 staining in WKY than in SHR and SHR-E (p = 0.13, by one-way ANOVA), (Fig 5).

Discussion

A new finding of our study is that hypertensive rats show an increased permeability of the colon to TMA, a gut bacterial metabolite and the precursor of TMAO. This suggests that hypertension facilitates penetration of gut microbiota metabolites to the circulation, and that diagnostic value of blood TMAO level, a new marker of cardiovascular risk, may depend on the colon permeability.

Gut bacterial metabolites such as H_2S and TMAO affect the functions of the circulatory system [1, 15]. Recent clinical studies showed a positive correlation between an elevated blood TMAO and an increased cardiovascular risk [3, 10, 16] and some experimental evidence shows potentially negative effects of TMAO on the circulatory system [3, 17]. Since TMAO is a metabolite of phosphatidylcholine and L-carnitine, TMAO has been proposed to constitute a

Table 2. Metabolic data and TMA/TMAO excretion.

Group/ Parameter	WKY (n = 6)	SHR (n = 6)	SHR-E (n = 6)	ANOVA
Stool TMA (μg/mL)	1.62 ± 0.16	2.04 ± 0.66	2.46 ± 0.81	NS[1]
Stool TMAO (μg /mL)	LQQ	LQQ	LQQ	-
Stool density (g/mL)	1.13 ± 0.09	1.06 ± 0.13	1.15 ± 0.08	NS
24-hr urine TMA output (μg)	137.0 ± 15.9	160.6 ± 21.4	161.6 ± 44.0	NS
24-hr urine TMAO output (μg)	436.8 ± 88.2	474.0 ± 74.4	426.6 ± 90.0	NS
Blood creatinine (mg/dL)	0.41 ± 0.05	0.45 ± 0.03	0.76 ± 0.04	($F_{2,15}$ = 15.2, p<0.05)†,‡
Blood urea (mg/dL)	38.8 ± 1.9	52.5 ± 1.6	63.0 ± 1.44	($F_{2,15}$ = 46.1, p<0.05)*,†,‡
AST (U/L)	83.0 ± 6.7	134.2 ± 21.7	214.6 ± 34.7	($F_{2,15}$ = 7.6, p<0.05)*
ALT (U/L)	51.7 ± 7.4	79.0 ± 14.9	99.6 ± 13.4	NS
Body mass (g)	344.45 ± 4.9	347.9 ± 13.4	359.6 ± 5.9	NS
24-hr water intake (mL)	25.6 ± 1.4	28.3 ± 1.4	46.2 ± 2.1	($F_{2,15}$ = 41.3, p<0.05)†,‡
24-hr food intake (g)	20.2 ± 0.9	20.3 ± 0.8	20.8 ± 0.5	NS
24-hr urine output (mL)	10.7 ± 0.7	8.6 ± 0.8	22.9 ± 1.7	($F_{2,15}$ = 44.2, p<0.05)†,‡

Metabolic data and TMA/TMAO excretion in normotensive Wistar Kyoto rats (WKY), spontaneously hypertensive rats (SHR), and enalapril-treated SHR (SHR-E). Values are means, ± SE. ANOVA followed by post-hoc Tuckey-test. NS–not significant differences between the groups.

[1] –WKY (n = 3), SHR (n = 5), SHR-E (n = 4), TMA stool level in the remaining samples WKY (n = 3), SHR (n = 1), SHR-E (n = 2) was below the limit of quantification (LQQ).

*- p<0.05, WKY vs SHR

†- p<0.05, SHR vs SHR-E

‡- p<0.05, WKY vs SHR-E

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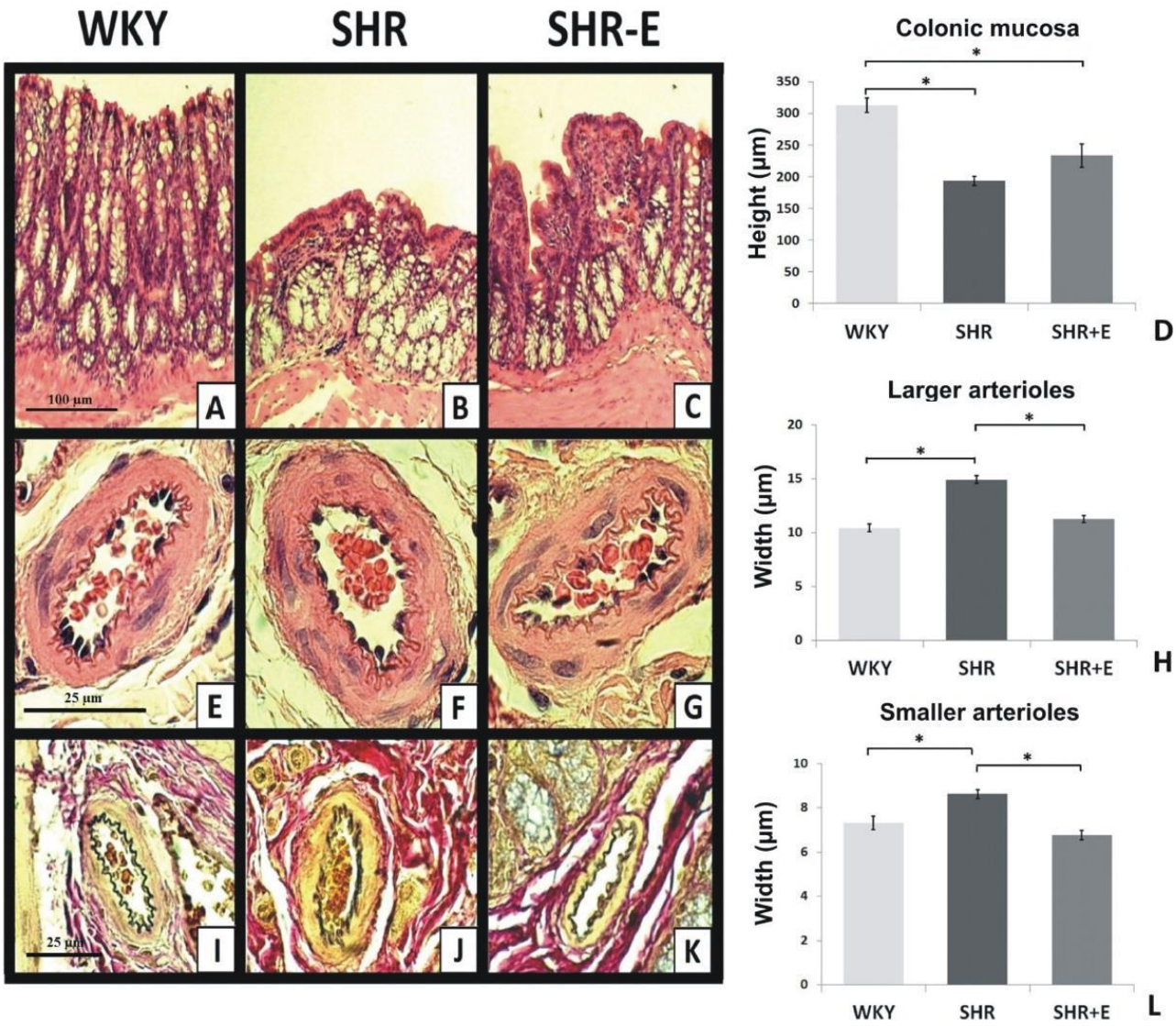

Fig 3. Histopathological and morphometric parameters in the colon of WKY (Wistar Kyoto rats), SHR (spontaneously hypertensive rats) and SHR-E (enalapril-treated SHR). A, B, C–The mucosa of the colon with haematoxylin and eosin (x10 objective lens). **E, F, G**–Larger arterioles of mucosa with haematoxylin and eosin (x100 objective lens). **I, J, K**–Larger arterioles of mucosa with van Gieson stain (x100 objective lens). **D**–The height of the colonic mucosa– μm (x10 objective lens). **H**–The width of the tunica media of larger arterioles– μm (x40 objective lens). **L**—The width of the tunica media of smaller arterioles– μm (x100 objective lens). *- $p<0.05$.

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link between the diet and cardiovascular diseases [10]. However, due to high interindividual variety of blood TMAO [8, 10] and the beneficial role of TMAO in some animals [18], the relevance of TMAO as a diagnostic marker and/or a causative factor in cardiovascular diseases is debatable [7]. Furthermore, the mechanism of an increased blood TMAO in cardiovascular diseases has been not elucidated.

Blood TMAO level may depend on numerous factors such as gut microbiota activity (production of TMA, a TMAO precursor), the GBB permeability to TMA, the oxidation of TMA

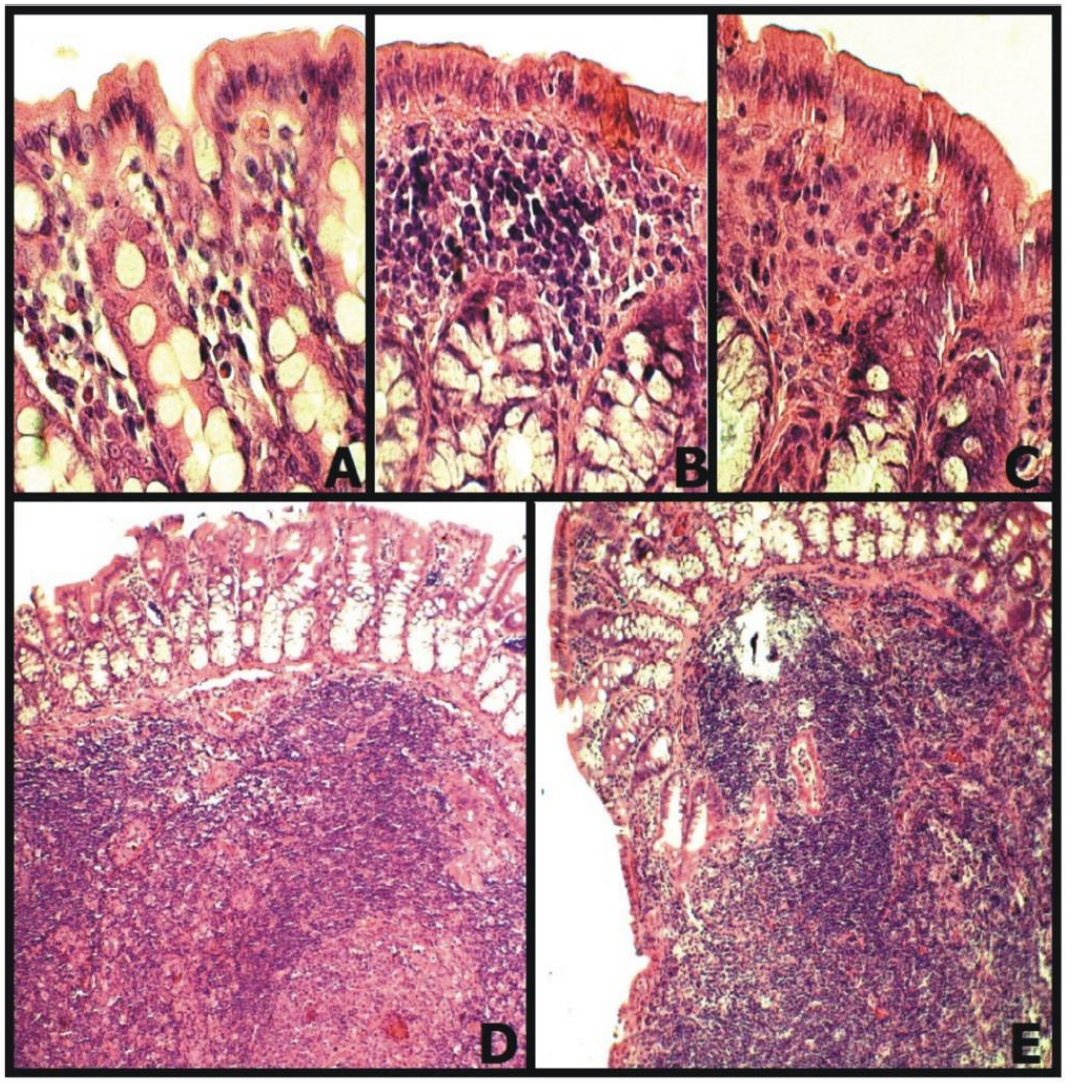

Fig 4. Inflammatory changes in the mucosa of the colon in WKY (normotensive Wistar Kyoto rats), SHR (spontaneously hypertensive rats) and SHR-E (SHR treated with enalapril). A, B, C–The colonic mucosa in WKY, SHR and SRE-E, respectively, (x 40 objective lens). **B**—Significant infiltration of mononuclear cells under the epithelium, similar to cryptopatches, (x 40 objective lens). **D, E**—Active GALT lymph nodes (Peyer's patches) were present only in SHR, (x 4 objective lens).

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by the liver and the excretion of TMA and TMAO [19]. In general, there are scant data on TMA/TMAO balance in mammals, and, to the best of our knowledge, the influence of hypertension on the factors affecting TMAO blood level has not yet been studied.

The access of TMA and other gut bacteria products to the circulation is protected by the GBB, a complex multilayer system [20, 21]. Interestingly, there is some clinical evidence that cardiovascular diseases such as heart failure may impair the function of the GBB [22]. Recently, Santisteban at al. found that hypertensive rats showed morphological changes in the small intestine and higher systemic blood level of orally administered 4kDa dextran [23].

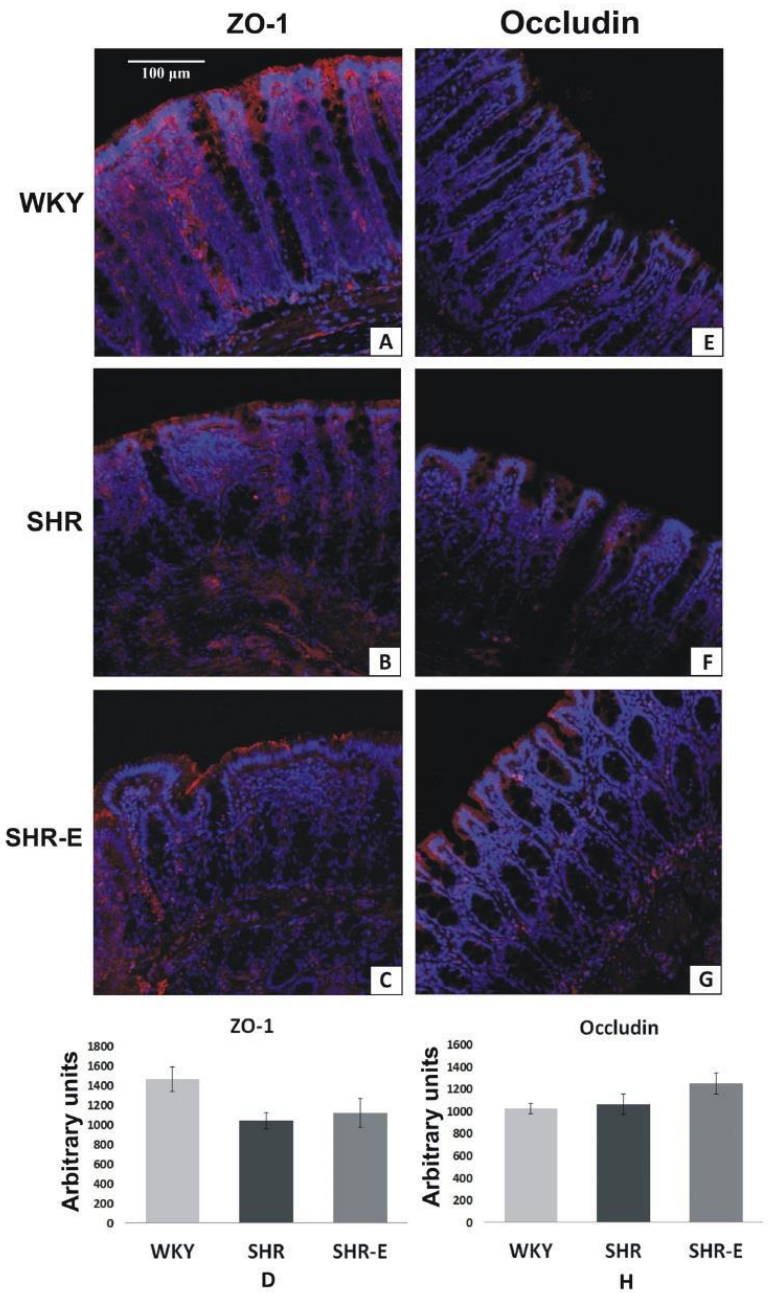

Fig 5. Immunohistochemistry stain of zonula occludens-1 (ZO-1) and occludin in colon mucosa. A, B, C–The colonic mucosa with the immunostaining of ZO-1 in WKY (Wistar Kyoto rats), SHR (spontaneously hypertensive rats) and SHR-E (enalapril-treated SHR). **E, F, G**—The colonic mucosa with the immunostaining of occludin in WKY, SHR and SHR-E. **D, H**–Changes of mean signal of fluorescence in epithelium of the colonic mucosa (arbitrary units of fluorescence).

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However, systemic blood concentration of orally given compounds depends on numerous factors including gastrointestinal motility, intestinal absorption, liver metabolism, and kidney excretion. Furthermore, dextran is an exogenous compound, therefore changes in the gut permeability to dextran is of unknown physiological and pathological significance. Therefore, in this study we measured portal blood concentration of TMA, a mammalian gut bacteria-derived compound. This approach has allowed us to asses directly the GBB permeability, excluding the abovementioned confounding factors. In addition, we measured hepatic vein blood concentration of indoxyl sulfate, a gut bacterial metabolite of tryptophan [24].

Our study shows that SHR had a significantly higher blood TMA and indoxyl sulfate levels than WKY at baseline. Furthermore, SHR had a significantly higher portal blood TMA than WKY after the intracolonic TMA challenge test. These findings suggest that hypertension increases the colon permeability to gut bacterial metabolites.

It has been found that functions of the GBB are compromised by decreased IBF [25–27]. In our study, in comparison to normotensive WKY, hypertensive SHR had a decreased IBF and decreased height of the mucosa. The latter was the likely cause of an increased permeability of the colon to TMA in SHR. Decreased IBF in hypertensive rats was also reported by others [28]. Furthermore, in our study SHR showed a significant increase in the thickness of arterioles walls, resulting from hyperplasia of smooth myocytes in the tunica media and from the hyperplasia of connective tissue of the adventitia. Such changes usually reflect the adaptation of arterioles to elevated blood pressure. Thicker walls and reduced lumen of arterioles increase the vascular resistance and could be responsible for a decreased IBF in SHR. Additionally, we found a decreased number of goblet cells, increased inflammatory cells infiltration and GALT stimulation in the SHR's colonic mucosa. The similar histological findings were reported in the small intestine [23].

In comparison to SHR, SHR treated with enalapril (SHR-E) showed a significantly lower portal blood TMA level at baseline, and a trend for lower portal blood TMA level in the intracolonic TMA challenge test. Furthermore, SHR-E showed a lower arterial blood pressure, higher IBF and smaller morphological changes in the colon than SHR. This may suggest that enalapril treatment improved the GBB function by reducing the hypertension-induced hemodynamic and morphological changes in the colon.

The mean of gut bacterial metabolites transportation through the gut's wall is unknown. The regulation of the paracellular transport is provided by tight junctions consisting of several transmembrane and cytoplasmic proteins, such as occludin and zonula occludens-1 (ZO-1) [29], which are often used as tight junctions markers [30]. In our study, SHR and WKY had a similar immunofluorescent signal of occludin and ZO-1 suggesting no significant effect of a decreased intestinal blood flow on tight junction composition with regard to occludin and ZO-1 level. Therefore, we think that an increased permeability of the colon to gut bacterial metabolites in SHR was caused by the reduced height of the colon mucosa, a major structural and functional layer of the GBB, rather than by alterations in tight junctions.

Another factor that may affect blood concentration of gut bacterial metabolites is gut microbiota metabolic activity. Here, we attempted to check the TMA concentration in SHR and WKY stools, but our results do not allow to draw clear conclusions. The processing of the stools reduced the concentration of TMA, a strongly volatile compound. Therefore, in several samples, mostly in WKY, the TMA level was below the limit of quantification. Further studies are needed to elucidate this issue.

Finally, we have found that SHR had a higher TMA liver clearance than WKY. Namely, after the intracolonic administration of TMA, more TMA was converted to TMAO by SHR than by WKY. This may suggest higher activity of enzymes oxidizing TMA to TMAO in SHR

than in WKY, which may result from an increased GBB permeability to TMA and thereby increased TMA delivery to the liver in SHR.

In conclusion, the present study shows that hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacterial metabolite, which is accompanied by morphological and hemodynamic alterations in the colon. This suggests that hypertension increases the penetration of gut microbiota products to the circulation.

Several clinical trials showed a positive correlation between an elevated plasma TMAO level and an increased cardiovascular risk. Furthermore, it has been speculated that an increased plasma concentration of TMAO may contribute to the etiology and symptoms of cardiovascular diseases. Our findings imply that high plasma TMAO concentration in cardiovascular diseases may depend on increased permeability of the colon to TMA, a TMAO precursor. Therefore, the alterations in the colon permeability, but not plasma TMAO level, may be a marker of cardiovascular risk.

Author Contributions

Conceptualization: Tomasz Huc, Marcin Ufnal.

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Formal analysis: Kinga Jaworska, Tomasz Huc, Emilia Samborowska, Leszek Dobrowolski, Klaudia Bielinska, Maciej Gawlak, Marcin Ufnal.

Funding acquisition: Marcin Ufnal.

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Project administration: Marcin Ufnal.

Writing – original draft: Kinga Jaworska, Tomasz Huc, Emilia Samborowska, Maciej Gawlak, Marcin Ufnal.

Writing – review & editing: Marcin Ufnal.

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Trimethylamine But Not Trimethylamine Oxide Increases With Age in Rat Plasma and Affects Smooth Muscle Cells Viability

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Abstract

It has been suggested that trimethylamine oxide (TMAO), a liver oxygenation product of gut bacteria-produced trimethylamine (TMA), is a marker of cardiovascular risk. However, mechanisms of the increase and biological effects of TMAO are obscure. Furthermore, the potential role of TMAO precursor, that is TMA, has not been investigated. We evaluated the effect of age, a cardiovascular risk factor, on plasma levels of TMA and TMAO, gut bacteria composition, gut-to-blood penetration of TMA, histological and hemodynamic parameters in 3-month-old and 18-month-old, male, Sprague–Dawley and Wistar–Kyoto rats. Cytotoxicity of TMA and TMAO was studied in human vascular smooth muscle cells. Older rats showed significantly different gut bacteria composition, a significantly higher gut-to-blood TMA penetration, and morphological and hemodynamic alterations in intestines. In vitro, TMA at concentration of 500 µmol/L (2-fold higher than in portal blood) decreased human vascular smooth muscle cells viability. In contrast, TMAO at 1,000-fold higher concentration than physiological one had no effect on human vascular smooth muscle cells viability. In conclusion, older rats show higher plasma level of TMA due to a "leaky gut". TMA but not TMAO affects human vascular smooth muscle cells viability. We propose that TMA but not TMAO may be a marker and mediator of cardiovascular risk.

Keywords: Circulatory system, Gut–blood barrier, Trimethylamine, Trimethylamine oxide, Bacterial metabolites

Increasing evidence shows that gut bacteria-produced molecules may be involved in cardiovascular and metabolic diseases (1–4).

To enter the circulation, gut bacterial products need to pass the gut–blood barrier, which limits the passage of harmful substances to systemic circulation from the intestinal lumen. Next, bacterial metabolites are carried with portal blood to the liver. In the liver, some bacterial products are metabolized or excreted with bile (liver clearance), whereas some pass the liver without a change. In turn, bacterial products and their liver metabolites enter the systemic circulation and may be used by tissues or excreted with urine (renal clearance), saliva, sweat, and expired air (5–7).

Trimethylamine oxide (TMAO), a liver oxidation product of gut bacteria-produced trimethylamine (TMA), has attracted large attention after the publication of several clinical articles, suggesting that increased plasma TMAO is associated with increased cardiovascular risk (8–10). The number of publications on TMAO is growing rapidly; only in the last 3 years, nearly 500 articles have been published according to PubMed. Very recently, we found that a chronic, low-dose, oral TMAO treatment reduced diastolic dysfunction in pressure-overloaded heart in hypertensive rats (11). However, data whether TMAO is beneficial or harmful for a living organisms are contradictory (12,13). These discrepancies may result from the fact

that only few studies evaluated the effect of TMAO at concentrations close to physiological level in mammals. Finally, the potential role of TMAO precursor, that is, TMA has not been investigated.

The aim of the study was to evaluate the effect of age, a cardiovascular risk factor, on gut bacteria composition, and other factors that affect plasma TMA and TMAO levels, including the functions of the gut–blood barrier, the liver, and kidneys. Furthermore, we evaluated in vitro the effect of TMA and TMAO on human vascular smooth muscle cells (hVSMCs) at concentrations found in rats and higher.

Methods

The data that support the findings of this study are available from the corresponding author on request.

Animal Study

Animals

This study was performed in accordance with Directive 2010/63 EU on the protection of animals used for scientific purposes and approved by the I Local Bioethical Committee in Warsaw (permission: 747/2017 and 555/2018). The study was performed on male, normotensive Wistar–Kyoto (WKY) and Sprague–Dawley (SD) rats, Central Laboratory for Experimental Animals, Medical University of Warsaw, Poland.

Rats were housed in groups of three animals, in polypropylene cages with environmental enrichment, 12 hours light/12 hours dark cycle, temperature 22–23°C, humidity 45%–55%, food, and water ad libitum.

The effect of age on gut bacteria composition, intestinal and metabolic parameters

Metabolic and hemodynamic parameters

WKY (n = 16) and SD (n = 16) were maintained on the same diet and in the same environmental conditions. Three-month-old WKY (WKY-3mos, n = 8) and SD (SD-3mos, n = 8) and 18-month-old WKY (WKY-18mos, n = 8) and SD (SD-18mos, n = 8) were maintained for 2 days in metabolism cages for energy and water balance evaluation and urine collection. Data from the second day were analyzed. Rats were anesthetized i.p. with urethane 1.5 kg/BW (Sigma–Aldrich) for a direct arterial blood pressure and intestinal blood flow measurements, as we described previously (14). In short, arterial blood pressure was measured via a polyurethane arterial catheter inserted into the aorta via femoral artery with Biopac MP 150 unit (Biopac Systems, Goleta, CA). Intestinal blood flow was evaluated by measuring blood flow in the upper mesenteric vein that collects blood from the colon using the transit time ultrasound set-up, which consists of a noncannulating acoustic (20 kHz) flow probe connected with a dedicated flowmeter (type T106, Transonic System Inc., Ithaca, NY). Hemodynamic parameters were recorded for 10 minutes. Next, the blood samples were taken from the right ventricle of the heart. Afterward, rats were killed by decapitation. A segment of the colon (7–8 cm, a middle part between the cecum and the rectum) and jejunum (5 cm, a middle part between the duodenum and the cecum) was closed with sutures and removed. The isolated fragments of intestines were dissected, and the tissues were used for histological evaluation. The colon content (stool masses) was collected for analysis.

Creatinine, urea, Na, K concentrations in plasma and 24-hour urine samples were measured using Cobas 6000 analyzer (Roche Diagnostics, Indianapolis, IN).

TMA level in stool

The stool sample assigned for bacterial metabolites concentration analyses was weighted and homogenized with 1 mL of 0.9% NaCl in a closed 2 mL laboratory tube by vortexing it for 5 minutes. Next, the sample was centrifuged for 12 minutes at 8,000 rpm, and 1 mL of the obtained supernatant was transferred to a laboratory tube and again centrifuged for 12 minutes. All procedures were performed at the temperature of 2–5°C. The supernatant was collected into Eppendorf tubes and frozen at –20°C.

16S rRNA metagenomics (gut bacteria composition)

A sample of stool allocated for metagenomic evaluation was frozen at –80°C immediately after collection. DNA was extracted from approximately 60 mg of stool using Nucleospin DNA Stool Kit (Macherey-Nagel; Germany) and suspended in 100 µL of water. Amplification of the V3–V4 region of the 16S rRNA gene and sequencing was performed based on 16S Metagenomic Sequencing Library Preparation protocol designed by Illumina (San Diego, CA). Briefly, V3–V4 region was amplified using 12.5 ng of microbial genomic DNA with 2× KAPA HiFi HotStart Rady Mix (0.5 U); (Roche, Basel, Switzerland), 16S PCR forward primer 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAG ACAGCCTACGGGNGGCWGCAG-3′ and 16S PCR reverse primer 5′-GTCTCGTGGGCTCGGAGATGTGTAT-3′. Dual-indexed PCR products were sequenced on Illumina MiSeq (300 nt; paired-end reads).

Adaptor removal was performed using cutadapt 1.18 (15). Next, reads were filtered and removed using FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/) if they were below the threshold of phred quality score of 23. Chimeras were checked and removed with ChimeraSlayer (16). Reads were subjected to OTU (operational taxonomic unit) picking using open-reference process against Greengenes database (97% similarity threshold) using Qiime (17). Normalization of reads was performed with metagenomeSeq, v1.22.0 package in R (18). Alpha, beta diversity, and taxonomical comparisons between groups were assessed using Phyloseq, v1.8.2 (19).

Histology and morphometry of the intestines

Tissues sections fixed in 10% buffered formalin were dehydrated with graded ethanol and xylene baths and embedded in paraffin wax. Sections of 3–4 µm were stained with hematoxylin and eosin (HE). Samples were evaluated at magnification of 10×, 40×, and 100× (objective lens) and 10× (eyepiece). The height of the villi and depth of crypts in the jejunum—measured at magnification of 10× (objective lens) and 10× (eyepiece), and the height of the mucosa in the colon jejunum—measured at magnification of 40× (objective lens) and 10× (eyepiece). The microscopic evaluation was performed in a blinded fashion, using a standard light microscope Olympus BX41 and CellSens software (Olympus Corporation, Tokyo, Japan).

The effect of age on gut-to-blood penetration of TMA, liver, and renal clearance of TMA and TMAO

In separate experiments, 3-month-old SD (3mos group, n = 7) and 18-month-old SD (18mos group, n = 7) were maintained for 2 days in metabolism cages for urine collection and food and water balance. Sodium benzoate was used to prevent bacterial proliferation in urine collecting tanks. Data from the second day were analyzed. Next day, anesthetized rats were implanted with catheters inserted into the portal vein and into the inferior vena cava, just above the hepatic veins confluence as we have described previously (20) and blood samples from the veins were collected. Afterward, rats were killed and a sample of stool from the colon for bacterial metabolites concentration analyses was prepared as described earlier.

Gut permeability to a metabolite was calculated as a $P = \frac{CP}{CC}$, where P is a gut permeability, CP concentration of a metabolite in the portal blood, and CC is the concentration of the metabolite in the colon content.

Liver clearance of a metabolite was calculated as a $Lc = \frac{CP-CH}{CP}$, where Lc is a liver clearance, CP concentration of a metabolite in portal blood, and CH concentration of a metabolite in inferior vena cava (just above hepatic vein confluence).

Renal clearance was calculated as a $Rcl = \frac{CU \times VU}{CP}$, where Rcl is a renal clearance of a metabolite, CU is a concentration of the metabolite in urine, VU is the urine output (mL/min), and CP is plasma concentration of the metabolite.

In Vitro Study

Cells and treatment

Human primary vascular smooth muscle cells (hVSMCs) were purchased from Lonza (Switzerland), ATCC (LGC Standards, Lomianki, Poland), and Gibco (Thermo Fisher Scientific, Poland). Cells were cultured in a SmGM (smooth muscle growth medium, LGC Standards, Lomianki, Poland). Cells at passage 5–8 were used for experiments. TMA and TMAO solutions were dissolved in cell culture medium and diluted to obtain the desire concentrations.

Metabolic activity assay in human VSMCs (MTT assay)

The MTT assay was used to check viability of human VSMCs treated with different concentrations of TMA and TMAO. Cells were seeded in 96-well plate at a density 1,500 cells per well. After 1 day the medium was exchanged for those containing TMA or TMAO. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma–Aldrich) solution (5 mg/mL) was added at indicated time points and cells were incubated for 2 hours at 37°C in a humidified atmosphere (5% CO_2). Formazan formed in living cells was dissolved in dimethyl sulfoxide (Sigma–Aldrich) and absorbance of the solution was measured at 570 nm using a microplate reader (Reader 400 SFC, LabInstruments, Hamburg, Germany).

Osmolality analysis

Osmolality of the cell culture medium with different concentrations of TMA and TMAO was measured by determining freezing-point depression using a semi-micro osmometer (Osmomat 030, Gonotec, Germanywith).

Gut Bacterial Metabolites Analysis

TMA and TMAO levels in blood plasma, urine, and stool supernatant were evaluated using Waters Acquity Ultra Performance Liquid Chromatograph coupled with Waters TQ-S triple-quadrupole mass spectrometer, as we described previously (20).

Statistics

The Kolmogorov–Smirnov test was used to test normality of the distribution. Differences between the groups were evaluated by two-way ANOVA (age × strain design) followed by Tukey's post hoc test or by *t*-test (for comparison between two age groups within one rat strain group). Kruskal–Wallis test was used for data that were not normally distributed. To analyze differences in the viability of TMA/TMAO treated cells by MTT *t*-test was used. A value of two-sided $p < .05$ was considered significant. Analyses were conducted using Dell Statistica, version 13 (Dell Inc., Tulsa, OK).

Results

Animal Study

The effect of age on gut bacteria composition, intestinal, and metabolic parameters

Metabolic and hemodynamic parameters

Body weight, water intake, urine output, plasma creatinine, and 24-hour sodium urine excretion were significantly higher in older than in younger rats. Plasma potassium was significantly higher in younger than in older group. Creatinine clearance was not significantly lower in older than in younger rats. There was no significant difference between the groups in mean arterial blood pressure. In contrast, intestinal blood flow was significantly lower in older than in younger rats.

The results are collected in Table 1.

TMA level in stool

Two-way ANOVA (age × strain design) revealed no significant difference between the groups for age (3mos vs. 18mos), strain (WKY vs. SD), and age × strain interaction in the concentration of TMA in stool specimens obtained from the colon; however, older rats tend to have higher concentration of TMA (μmol/L; WKY-3mos: 261 ± 18, WKY-18mos: 315 ± 39, SD-3mos: 290 ± 27, SD-18mos: 352 ± 72).

16S rRNA metagenomics (gut bacteria composition)

There was a significant difference in gut bacteria composition between older and younger rats in WKY and SD groups. Specifically, a total of 6,850,402 reads were obtained from all samples resulting in average number of 289,187 reads per sample. After quality filtering and chimera removal 5,127,396 reads remained (approximately 213,641 reads per sample). The mean number of reads in SD-18mos samples was 219,300 whereas in SD-3mos 211234. In WKY-18mos and WKY-3mos samples, the average number of reads were 189,153 and 234,877, respectively. As a result of open-reference OTU picking process in SD rats, we obtained an average number of 2,339 (18mos) and 2,407 (3mos) OTUs, whereas in WKY rats 1,874 (18mos), 2,312 (3mos; Supplementary Figure 1). There was no significant difference in observed OTUs between analyzed groups. Using Shannon diversity index, we did not observe any significant changes in species richness between groups (Supplementary Figure 1). Beta diversity was assessed using principal coordinates analysis (PcoA; Figure 1). PCoA found a significant divergence in microbial communities between groups 18mos and 3mos in SD rats (ANOSIM: $p = .03$) and WKY rats (ANOSIM: $p = .03$). In both groups, the most abundant bacterial family in all analyzed groups was Ruminococcaceae (appx. 25;Supplementary Figure 2). Among bacterial genera with TMA producing capacity only genus *Clostridium* was present but without significant difference between groups.

Histology and morphometry of intestines

We have compared the morphology of jejunum and colon in both age groups of animals (Supplementary Figures 3 and 4). In jejunum, we have observed that independently from rat strain, the height of villi and enterocytes as well as the depth of crypts were significantly lower in older in comparison with younger rats. The height of the colon mucosa was significantly lower in older than in younger rats (Table 1; Supplementary Figures 3 and 4).

The effect of age on gut-to-blood penetration of TMA, liver, and renal clearance of TMA and TMAO

In general, older rats showed significantly higher portal and systemic blood concentration of TMA, which was associated with not significantly higher stool level of TMA and significantly increased gut-to-portal blood penetration of TMA (a significantly higher portal blood-to-stool ratio of TMA concentrations). Finally, older rats showed mildly lower systemic blood TMAO level, which was associated with moderately lower liver clearance of TMA (TMA to TMAO oxidation; Table 2).

Table 1. Metabolic Data, Water-Electrolyte Balance and Hemodynamic Parameters in Rats

Group/parameter	WKY-3mos	WKY-18mos	SD-3mos	SD-18mos
General metabolic parameters				
Body mass (g)*·†·‡	311.2 ± 8.9	396.9 ± 13.7§	300.5 ± 6.1	478.8 ± 9.5‖
24 h food intake (g)	23.97 ± 1.79	23.58 ± 1.19	25.84 ± 1.34	20.67 ± 1.08
24 h water intake (mL)*	30.38 ± 0.94	33.13 ± 1.61	28.73 ± 1.46	31.98 ± 1.67
24 h urine output (mL)*	10.14 ± 0.58	16.77 ± 2.32§	10.85 ± 1.02	18.08 ± 1.03‖
Biochemical parameters				
Plasma sodium (mmol/L)	141.3 ± 0.43	137.83 ± 1.08	139.6 ± 1.06	140.6 ± 0.93
Plasma potassium (mmol/L)*	5.38 ± 0.18	4.23 ± 0.14§	5.52 ± 0.18	4.51 ± 0.13‖
Plasma creatinine (μmol/L)*·†·‡	61.94 ± 4.42	77.88 ± 4.43§	37.13 ± 1.50	61.88 ± 3.98‖
24 h sodium urine excretion (mmol)*	1.59 ± 0.12	2.08 ± 0.33	1.51 ± 0.17	2.29 ± 0.04‖
24 h potassium urine excretion (mmol)‡	4.37 ± 0.36	2.33 ± 0.33§	5.52 ± 0.18	4.51 ± 0.13
Creatinine clearance (mL/min)‡	0.38 ± 0.02	0.26 ± 0.02	0.86 ± 0.09	0.61 ± 0.09
Hemodynamic parameters in anesthetized rats				
MABP (mmHg)	80.83 ± 4.45	85.61 ± 4.53	80.82 ± 11.0	90.92 ± 3.49
HR (beats/min)	294 ± 27	318 ± 21	307 ± 25	351 ± 27
IBF (ml/min)*·†	6.88 ± 0.41	4.49 ± 0.44	8.57 ± 1.35	5.27 ± 0.75‖
Histological parameters of intestines				
The height of jejunum villi (μm)*·‡	400.24 ± 7.55	378.70 ± 6.67§	651.54 ± 23.18	561.97 ± 21.49‖
Depth of jejunum crypts (μm)*	186.37 ± 3.06	172.82 ± 2.92§	205.11 ± 7.25	166.17 ± 4.49‖
The height of jejunum enterocytes (μm)*	33.22 ± 0.62	28.58 ± 0.72§	30.64 ± 1.49	27.74 ± 0.69
The height of colonic mucosa (μm)*	287.52 ± 4.81	246.83 ± 4.97§	264.63 ± 14.73	230.59 ± 13.29 ‖
The height of colonic enterocytes (μm)	24.77 ± 0.53	22.35 ± 0.49	27.27 ± 0.94	23.13 ± 1.21

Note. 3-month-old Wistar–Kyoto rats (WKY-3mos), 18-month-old Wistar–Kyoto rats (WKY-18mos), 3-month-old Sprague–Dawley rats (SD-3mos), and 18-month-old Sprague–Dawley rats (SD-18mos). HR = heart rate; IBF = intestinal blood flow through upper mesenteric vein; MABP = mean arterial blood pressure. Values are means, ± *SE*. Two-way ANOVA for age (3mos × 18mos) × strain (WKY × SD) design: *$p < .05$ for age, †$p < .05$ for strain and ‡$p < .05$ for age × strain interaction. §$p < .05$ vs WKY-3mos, ‖$p < .05$ vs SD-3mos (post hoc Tukey's test).

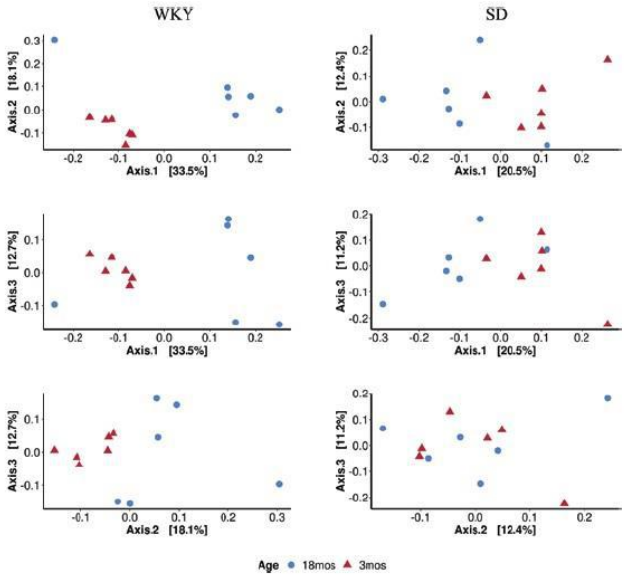

Figure 1. Beta diversity parameters in rats. Principal coordinate analysis (PCoA) based on 97% similarity Weighted Unifrac distance matrices for Wistar Kyoto (WKY, on the left) Sprague–Dawley (SD, on the right) rats. Circles—18-month-old rats (18mos), triangles—3-month-old (3mos) rats. rats. Percentage value (%) on each axis indicates percentage of the captured variation in the input data. PCoA analysis indicates that samples ordinated closer to one another are more similar than those ordinated further away. PCoA shows how examined samples differ between each other in tri-dimensional space, forming distinct clusters.

In Vitro Study

Impact of TMA and TMAO on hVSMCs viability (MTT assay)

We have analyzed metabolic activity of hVSMCs treated with different concentrations of TMA and TMAO in vitro using MTT assay. Changes in the level of this parameter result from differences in the rate of metabolism, proliferation, and survival of TMA/TMAO treated versus untreated, control cells. Thus, MTT assay serves as a general indicator of cell viability.

It was revealed that TMA at a concentration of 500 μmol/L reduced cell viability after 24 hours of treatment. In contrast, TMAO at the same concentration had no impact on the cells even after 72 hours of treatment. Importantly, treatment with 100 mmol/L TMAO was not cytotoxic whereas TMA at the same concentration killed cells within 24 hours (Figure 2A). The MTT findings were confirmed by a morphological observation of TMA or TMAO-treated cells (Figure 2B). TMA at a concentration of 100–200 mmol/L caused cell shrinkage and detachment from the bottom of the plate whereas TMAO used at the same concentration did not influence cell morphology but only lowered the density of cell culture, indicating that that only cell proliferation but not viability was affected.

Osmolality analysis

With increasing concentrations of TMA and TMAO in the cell culture medium, there was a marked increase in osmolality (Supplementary Table 1).

Discussion

A new finding of our study is that older rats show a significantly higher plasma TMA level than younger rats, which seems to result from a "leaky gut," that is, increased gut-to-blood penetration of TMA. Furthermore, we found that TMA, but not its liver metabolite TMAO, exerts a cytotoxic effect.

Ample evidence indicates that products of intestinal bacteria affect the function of the circulatory system (21). The concentration of bacterial metabolites in the blood depends on numerous factors, including gut bacteria activity, gut-to-blood penetration of bacterial metabolites (gut–blood barrier permeability), liver metabolism of

Table 2. Factors Affecting Blood Concentration of TMA and TMAO in 3-Month-Old (3mos Group) and 18-Month-Old (18mos Group) Sprague–Dawley Rats

Group/parameter	3mos Group	18mos Group	*t*-test
TMA			
Stool level (μmol/L)	301.5 ± 29.5	357.2 ± 42.4	Ns
Portal blood level (μmol/L)	146.53 ± 14.25	233.18 ± 5.55	$p < .05$
Systemic blood level (μmol/L)	64.32 ± 9.18	109.61 ± 6.78	$p < .05$
Intestinal permeability	0.42 ± 0.04	0.65 ± 0.07	$p < .05$
Liver clearance	0.61 ± 0.05	0.50 ± 0.05	Ns
Renal clearance	0.31 ± 0.002	0.33 ± 0.007	Ns
TMAO			
Stool level (μmol/L)	<LOQ	<LOQ	—
Portal blood level[a] (μmol/L)	7.69 ± 0.27	5.99 ± 0.55	$p < .05$
Systemic blood level (μmol/L)	7.31 ± 0.53	6.50 ± 0.61	Ns
Renal clearance	0.32 ± 0.003	0.37 ± 0.005	Ns

Note. LOQ = below the limit of quantification; Ns = not significant; TMA = trimethylamine; TMAO = trimethylamine oxide. Values are means, ± SE, *p*-value calculated with t-test for independent samples. Intestinal permeability was calculated as a CP/CC; CP = concentration of the metabolite in the portal blood, CC = concentration of the metabolite in the colon content. Liver clearance was calculated as a (CP-CH)/CP; CP—concentration of a metabolite in portal blood, CH = concentration of a metabolite in inferior vena cava (systemic blood). Renal clearance was calculated as a metabolite concentration in the urine x urine output (mL/min) / plasma concentration of the metabolite.

[a]The presence of TMAO in portal blood is caused by TMAO arriving with systemic arterial blood to intestines.

bacterial products (liver clearance), and excretion of metabolites by the kidneys (renal clearance) (22). In this context, it is well established that intestinal, liver, and kidney functions deteriorate with age and that older age is a cardiovascular risk factor. Therefore, it may be hypothesized that plasma level of gut-bacteria metabolites may be one of the links between increased cardiovascular risk and aging.

The Effect of Age on Gut Bacteria and TMA Stool Level

We found that the composition of gut bacteria significantly differs between 3-month-old and 18-month-old rats. However, despite changes in gut bacteria composition, there was only a trend toward higher TMA level in stools of the older rats. It is worth stressing that changes in gut bacteria composition may or may not translate to changes in production of bacterial metabolites. This is because various bacterial genera produce similar metabolites (23). On the other hand, one type of bacteria may produce various metabolites dependently on availability of substrates (24).

The Effect of Age on Plasma TMA and TMAO

Our study shows that older rats have significantly increased plasma TMA and mildly lower plasma TMAO level than younger rats. As depicted in Figure 3 plasma TMA and TMAO levels depend on numerous factors (22,25,26). Given that all rats in our study were on the same diet and had similar TMA stool level, it seems that higher plasma TMA in older rats was mostly due to increased gut-to-blood penetration of TMA. Specifically, older rats showed a significantly higher concentration of TMA in portal blood and a significantly higher ratio of TMA concentration in portal blood to TMA concentration in stools.

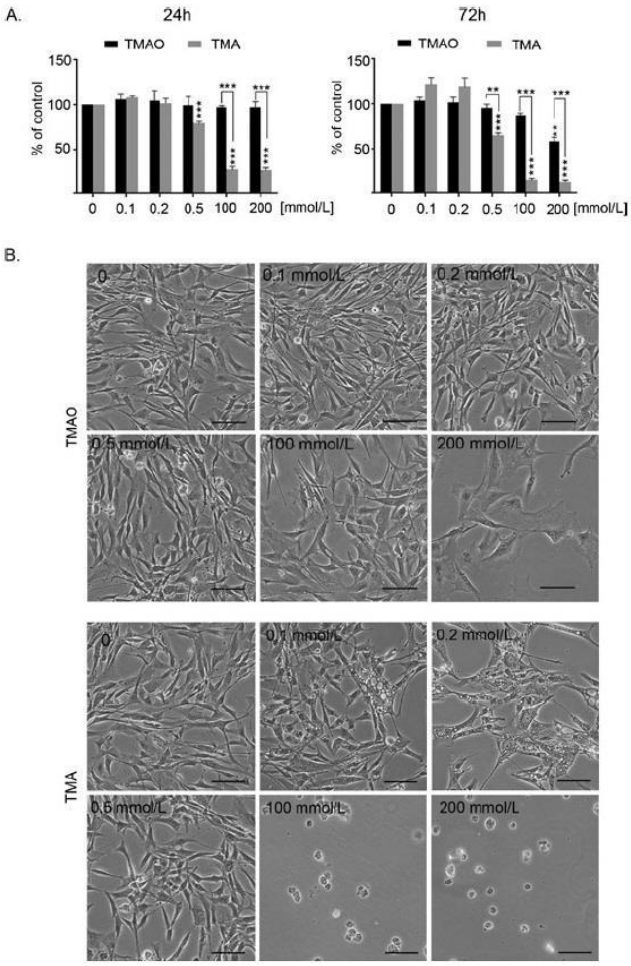

Figure 2. The influence of TMA and TMAO on hVSMCs in vitro. (**A**) Comparison of viability of human vascular smooth muscle cells (hVSMCs) treated with trimethylamine oxide (TMAO) or trimethylamine (TMA). MTT test was performed after 24 and 72 hours of treatment. Results were normalized to the control (untreated) cells; Graphs show mean ± *SE*, $^{**}p < .01$, $^{***}p < .001$; (**B**) Representative pictures showing changes in the number and morphology of cells after 72 hours of treatment with of trimethylamine oxide (TMAO) or trimethylamine (TMA), bar 100μm.

This was associated with decreased intestinal blood flow and reduced thickness of colonic mucosa, which constitutes the physical barrier for gut-to-blood penetration of bacterial products (27).

The Effect of TMAO and TMA on hVSMCs In Vitro

Here, we have found that in vitro TMA, but not TMAO, at close to physiological concentrations, decreased proliferation and viability of hVSMCs in vitro. Specifically, TMA affected cell viability at concentrations only 2- to 3-fold higher than those in portal blood in rats. In contrast, TMAO did not exert cytotoxic effects at concentrations exceeding its physiological level by 3 orders of magnitude.

At present, Ke and collaborators suggested that TMAO accelerates cell senescence and vascular aging (28). In our study, TMAO at concentrations tested by Ke and colleagues (200–500 mmol/L) also decreased cell proliferation and viability. However, we checked that such concentration of TMAO increased osmolality of cell culture medium from 277 to 385, that is approximately 40%. Therefore, it could be a dramatic increase in osmolality of the medium and not biological properties of TMAO which

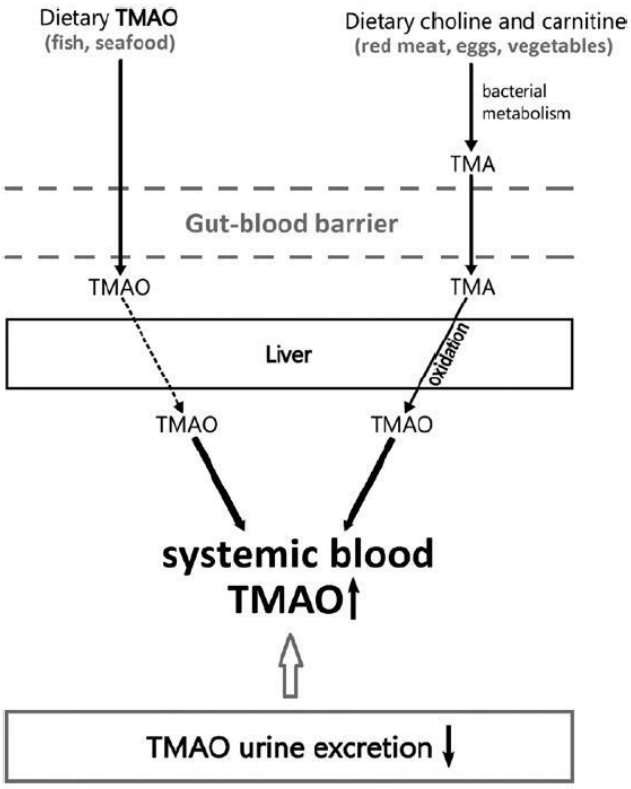

Figure 3. Factors affecting plasma trimethylamine (TMA) and trimethylamine oxide (TMAO) levels. Plasma TMAO levels depend on numerous factors including ingestion of a direct dietary TMAO (eg, fish, seafood), ingestion of substrates for bacterial production of TMA (choline, l-carnitine), intestinal absorption of TMAO and penetration of TMA from the colon to the circulation (the gut–blood barrier), liver metabolism of TMA (TMA to TMAO oxidation), and finally TMAO excretion by the kidneys.

affected cell viability and proliferation in the studies by Ke and colleagues and in our experiments. Notably, the earlier-mentioned concentrations of TMAO are 1,000 times higher than physiological plasma level of TMAO in rats and humans (29). It needs to be stressed that several in vivo studies in rats and mice reported negative effect of TMAO. However, doses used in those studies increased plasma TMAO level by 15–25 times (30,31). Therefore, physiological relevance of such findings is questionable. Although plasma TMA levels were not reported in the cited studies, it seems plausible that overloading the organism with TMAO that is the product the oxidation reaction (TMA ↔ TMAO) may shift the reaction to the left, that is, increase TMA level, which may produce deleterious effects.

TMA But Not TMAO Is Associated With Increased Cardiovascular Risk, A Hypothesis

At present, it has been found that increased plasma TMAO is associated with increased cardiovascular risk (8,10,32). However, the potential role of TMAO precursor, that is, TMA has not been investigated.

This study reveals that TMAO at 1,000-fold higher concentration than physiological one is neither cytotoxic nor cytostatic for vascular smooth muscle cells. In contrast, TMA at close to physiological concentration decreases cell proliferation. Therefore, we propose that it is TMA but not TMAO that exerts deleterious effects on the circulatory system and may be a cardiovascular risk marker.

Our hypothesis may also be supported by the following indirect evidence. First, plasma TMAO originates not only from TMA, a gut bacterial metabolite but also from dietary TMAO, that is TMAO-rich seafood (33). With this regard, it is accepted that humans consuming fish have lower risk of age-associated diseases such as cardiovascular and metabolic diseases (34–36). Second, numerous biophysical studies show that TMAO exerts positive effects, that is, protects cell proteins from denaturants such as high osmotic pressure, hydrostatic pressure, NaCl, urea, or a high temperature (37–40). The importance of TMAO has been recognized in marine animals, which are exposed to high hydrostatic stress (deep water) and osmotic stress (salty water) (41,42). Third, as one of major functions of the liver is detoxification, it seems plausible that toxic TMA is converted to nontoxic TMAO. In addition, in contrast to neutrally smelling TMAO, TMA has a strong unpleasant smell of decaying fish. In this regard, the sense of smell has evolved to alert animals to the poisons and to avoid ingestion of foods that are harmful (43).

Finally, our hypothesis is indirectly supported by evidence from numerous clinical studies documenting a positive association between elevated levels of plasma TMAO and an increased cardiovascular risk. Although those studies did not evaluate plasma TMA, it is very likely that increased plasma TMAO levels were accompanied by increased level of its precursor, that is TMA. Therefore, our study suggests that future clinical studies determining the association between cardiovascular risk and plasma TMAO should obligatory evaluate TMA level.

Limitations

A limitation of our study is that we used 3-month old and 18-month old rats, which may correspond to young adults (less than 18 years) and 50 years-old of human, respectively (44). Though the latter age is already associated with increased cardiovascular risk in male, arguably, experiments on older rats could show even more pronounced changes in plasma TMA and/or TMAO levels. In addition, further studies with respect to sex differences are needed as sex-specific impact of aging on the circulatory system has been well documented (45,46).

Conclusions

Older rats show higher blood plasma TMA level. This is associated with significantly changed gut bacteria composition, structural, and functional alterations in the colon and increased penetration of TMA from the colon to portal blood. Importantly, TMA at concentrations close to physiological levels decreases human VSMCs proliferation and viability. In contrast, TMAO does not exert cytotoxic effects at concentrations exceeding its physiological level by 1,000-fold. Therefore, we hypothesize that it is TMA, not TMAO, that exerts detrimental effects on cardiovascular system and contributes to increase in cardiovascular risk with aging. Future clinical and experimental studies evaluating cardiovascular effects of TMAO should involve assessment of TMAO precursor, that is TMA.

Supplementary Material

Supplementary data are available at *The Journals of Gerontology, Series A: Biological Sciences and Medical Sciences* online.

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Conflict of Interest

Authors declare no conflict of interest.

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Materiały multimedialne związane z pracą doktorską

Film instruktarzowy dotyczący metodyki badania bariery jelito-krew, stanowiący integralną część Publikacji nr 1:

https://drive.google.com/file/d/1DUcdWGgDTT9Km0ROuNV_zorxMRXmHHAU/view?usp=sharing

Animacja przygotowana przez Brytyjskie Towarzystwo Fizjologiczne (The Physiological Society) promująca wyniki przedstawione w Publikacji nr 2:

https://www.youtube.com/watch?v=I5ha-1Pte-0&feature=youtu.be

Podsumowanie i wnioski

Wyniki pracy pozytywnie zweryfikowały hipotezę badawczą zakładającą, że przepuszczalność bariery jelito-krew może być markerem zaburzeń ogólnoustrojowych.

Zrealizowano wszystkie zaplanowane cele główne i szczegółowe, tj. opracowano metodę badania bariery jelito-krew z wykorzystaniem metabolitów bakteryjnych jako markerów (Publikacja nr 1 i 2). Zbadano przepuszczalność bariery jelito-krew dla metabolitów bakteryjnych na zwierzęcym modelu nadciśnienia tętniczego (Publikacja nr 3). Oceniono na modelu zwierzęcym zmiany jakie zachodzą wraz z wiekiem w przepuszczalności bariery jelito-krew dla metabolitów bakteryjnych (Publikacja nr 4).

Najważniejsze wyniki i wnioski

Publikacja nr 1 i 2. Opracowano metodykę badania bariery jelito-krew przy użyciu metabolitów bakteryjnych u szczurów. Dokładny opis procedur wraz z instruktażowym materiałem video został opublikowany w czasopiśmie JOVE (Publikacja nr 1). Następnie opracowana została nieinwazyjna metoda o potencjale klinicznym. Stopień przepuszczalności jelit wyznaczany jest na podstawie stosunku stężenia metabolitu bakteryjnego we krwi do jego stężenia w kale. Metoda wymaga jedynie pobrania próbki kału oraz krwi obwodowej, w związku z tym jest stosunkowo nieinwazyjna i prosta do wykonania. Jednocześnie, dzięki wykorzystaniu stosunku stężeń, jest ona niezależna od czynników zmiennych osobniczo, takich jak dieta czy skład flory bakteryjnej. Metodę tę opracowano na modelu zwierzęcym zapalenia jelita, a następnie we współpracy z Kliniką Gastroenterologii i Żywienia Dzieci Warszawskiego Uniwersytetu Medycznego oceniono przepuszczalność jelita u pacjentów z nieswoistym zapaleniem jelit. Działanie metody zarówno w warunkach eksperymentalnych jak i klinicznych potwierdził wzrost przepuszczalności bariery dla metabolitów bakteryjnych (krótkołańcuchowych kwasów tłuszczowych) w porównaniu do zdrowych grup kontrolnych. Dodatkowo, zmiany te były dodatnio skorelowane ze standardowo wykorzystywanymi markerami funkcji jelita, tj. dekstranem znakowanym fluorescencyjnie i kalprotektyną. Metoda ta spotkała się z zainteresowaniem środowiska naukowego. Wyniki zostały opublikowane w Experimental Physiology (Publikacja nr 2), a praca została wyróżniona przez Brytyjskie Towarzystwo Fizjologiczne (The Physiological Society) i rozpropagowana na kilkunastu międzynarodowych portalach naukowych.

Wnioski:

- Zmiany funkcjonalne bariery jelito-krew mogą być skutecznie ocenione za pomocą opracowanej metody z użyciem stosunku stężeń metabolitów bakteryjnych.
- Ze względu na nieinwazyjny charakter metoda może być wykorzystana zarówno w warunkach eksperymentalnych, jak i klinicznych.

Publikacja nr 3. U szczurów z nadciśnieniem tętniczym (SHR) wykazano zwiększoną przepuszczalność bariery jelito-krew dla trimetyloaminy (TMA) w porównaniu do szczurów normotensyjnych (WKY). Zarówno przed, jak i po obciążeniu dookrężniczym TMA, SHR miały istotnie wyższe stężenie TMA we krwi wrotnej i wyższy klirens wątrobowy TMA niż WKY. Ponadto zaobserwowano zmiany morfologiczne w okrężnicy szczurów SHR, którym towarzyszyło zaburzenie przepływu jelitowego. Zarówno zmiany morfologiczne, jak i czynnościowe w jelicie były mniejsze u szczurów SHR pojonych inhibitorem konwertazy angiotensyny, enalaprilem, przez 4 tygodnie. Grupa ta miała niższe stężenie TMA w żyle wrotnej, niższe ciśnienie tętnicze, a większy przepływ krwi przez jelito niż SHR, co wskazuje na lepszą kondycję bariery jelito-krew u szczurów pojonych enalaprilem.

Wnioski:

- Szczury z nadciśnieniem tętniczym wykazują zwiększoną przepuszczalność okrężnicy dla TMA, czemu towarzyszą zmiany morfologiczne i hemodynamiczne w okrężnicy.
- Wzrost stężenia tlenku trimetyloaminy (TMAO) we krwi obserwowany w chorobach układu krążenia może być po części spowodowany uszkodzeniem bariery jelito-krew i wzrostem przepuszczalności dla TMA, prekursora TMAO. Ma to znaczenie pod kątem stratyfikacji ryzyka sercowo-naczyniowego z użyciem TMAO.
- Choroby sercowo-naczyniowe mogą charakteryzować się zwiększoną przepuszczalnością bariery jelito-krew dla metabolitów bakteryjnych, jak TMA, co może wpływać na przebieg choroby podstawowej.
- Szczury pojone enalaprilem wykazują poprawę w zakresie morfologicznych i czynnościowych zmian w okrężnicy indukowanych nadciśnieniem tętniczym. Bariera jelito-krew jest potencjalnym punktem uchwytu terapii inhibitorami konwertazy angiotensyny.

Publikacja nr 4. Oceniono wpływ wieku na przepuszczalność bariery jelito-krew dla TMA, oraz parametry histologiczne i hemodynamiczne u 3-miesięcznych i 18-miesięcznych szczurów szczepu Sprague Dawley i Wistar Kyoto. Wykazano, że starsze szczury miały istotnie zwiększoną przepuszczalność bariery jelito-krew dla TMA oraz charakteryzowały się zmianami morfologicznymi i hemodynamicznymi w jelitach.

Wnioski:

- Starsze szczury wykazują większą przepuszczalność bariery jelito-krew dla TMA, czemu towarzyszą zmiany morfologiczne jelit związane ze spadkiem ukrwienia jelit.

Opinia Komisji Etycznej

UCHWAŁA NR 616/2018

z dnia 22 maja 2018 r.

I Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Warszawie

§ 1

Na podstawie art. 48 pkt. 1 ustawy z dnia 15 stycznia 2015r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266) zwanej dalej „ustawą" po rozpatrzeniu wniosku pt. **„Bariera jelito-krew jako nowy marker oraz cel terapii w chorobach układu krążenia i cukrzycy"** z dnia **27.04.2018 r.** złożonego przez **Warszawski Uniwersytet Medyczny, Wydział Lekarsko-Dentystyczny ul. Banacha 1B, 02-091 Warszawa** zaplanowanego przez **dr hab. Marcina Ufnala** lokalna komisja etyczna

WYRAŻA ZGODĘ

Na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku.

§ 2

W wyniku rozpatrzenia wniosku o którym mowa w § , Lokalna Komisja Etyczna ustaliła, że:

1. Wniosek należy przypisać do kategorii: **DOŚWIADCZENIA NA ZWIERZĘTACH (A)**
2. Najwyższy stopień dotkliwości proponowanych procedur to: **DOTKLIWY**
3. Doświadczenia będą przeprowadzane na gatunkach lub grupach gatunków:

Szczur, szczep Sprague Dawley	**18 tygodni**	**36 os.**
Szczur, szczep SHR	**18 tygodni**	**27 os.**
Szczur, szczep Wistar Kyoto	**18 tygodni**	**72 os.**
Szczur, szczep SH-HF	**18 tygodni**	**27 os.**

4. Doświadczenia będą przeprowadzane przez: **Ufnal Marcin, Huć Tomasz, Jaworska Kinga, Bielińska Klaudia**
5. Doświadczenie będzie przeprowadzane w terminie[1] **01.07.2018 –01.07.2023 r.**
6. Doświadczenie będzie przeprowadzone w ośrodku:
7. Doświadczenie będzie przeprowadzone poza ośrodkiem w:
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez:
9. Doświadczenie **zostanie poddane ocenie retrospektywnej w terminie do 3 miesięcy** od dnia przekazania przez użytkownika dokumentacji, mającej stanowić podstawę dokonania oceny retrospektywnej. Użytkownik jest zobowiązany do przekazania ww. dokumentacji niezwłocznie, tj. w terminie, o którym mowa w art. 52 ust. 2 ustawy.

[1] Nie dłużej niż 5 lat

§ 3

Uzasadnienie:

Celem projektu jest zbadanie przepuszczalności bariery jelito-krew w nadciśnieniu tętniczym, niewydolności serca i cukrzycy oraz potencjału terapeutycznego enalaprilu w stanach zaburzonej funkcji bariery jelito-krew. Projekt obejmuje opracowanie nowej metody badania bariery jelito-krew, która będzie pozbawiona ograniczeń dotychczasowych metod. Projekt zalicza się do badań podstawowych obejmujących wiele układów. Ze względu na złożoność badanych procesów będących wypadkową współdziałania wielu narządów, tkanek i hormonów nie jest możliwe przeprowadzenie opisanych badań na innym modelu zwierzęcym o niższym poziomie ewolucyjnym oraz z wykorzystaniem metod alternatywnych, takich jak hodowle komórkowe lub tkankowe. Badania poszerzą wiedzę na temat funkcjonowania bariery jelito-krew w cukrzycy i chorobach układu krążenia oraz będą ważnym krokiem w poszukiwaniu możliwych działań terapeutycznych związanych z przywróceniem fizjologicznej przepuszczalności jelit, jako elementu leczenia w tych chorobach.

Kategoria dotkliwości procedur, uzasadnienie ich przeprowadzenia oraz liczebność grup doświadczalnych zostały określone prawidłowo. Członkowie Komisji nie zgłosili zastrzeżeń do procedur oraz czynności doświadczalnych opisanych w przedłożonym wniosku i uznali, że realizacja tego projektu badawczego jest dopuszczalna.

Ze względu na najwyższy stopień dotkliwości proponowanych procedur przeprowadzona zostanie ocena retrospektywna 6 miesięcy po zakończeniu doświadczenia.

Niniejsza uchwała wchodzi w życie z dniem wydania i jest ważna do 01.07.2023 r.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1

I LOKALNA KOMISJA ETYCZNA
ds. Doświadczeń na Zwierzętach
przy Wydziale Biologii UW
ul. Ilji Miecznikowa 1, 02-096 Warszawa
tel. 022 5541028, e-mail: lke1waw@biol.uw.edu.pl

(Pieczęć lokalnej komisji etycznej)

Podpisy przewodniczącego komisji

Pouczenie:

Zgodnie z art. 33 ust. 3 i art. 40 ustawy w zw. z art. 127 § 1 i 2 oraz 129 § 2 ustawy z dnia z dnia 14 czerwca 1960 r. Kodeks postępowania administracyjnego (Dz. U. 2017, poz. 1257 – t.j.; dalej KPA) od uchwały Lokalnej Komisji Etycznej strona może wnieść, za jej pośrednictwem, odwołanie do Krajowej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach w terminie 14 od dnia doręczenia uchwały.

Na podstawie art. 127a KPA w trakcie biegu terminu do wniesienia odwołania strona może zrzec się prawa do jego wniesienia, co należy uczynić wobec Lokalnej Komisji Etycznej, która wydała uchwałę. Z dniem doręczenia Lokalnej Komisji Etycznej oświadczenia o zrzeczeniu się prawa do wniesienia odwołania przez ostatnią ze stron postępowania, decyzja staje się ostateczna i prawomocna.

Otrzymuje:

1) Użytkownik,
2) Organizacja społeczna dopuszczona do udziału w postępowaniu (jeśli dotyczy)
3) a/a

Użytkownik kopie przekazuje:

- Osoba planująca doświadczenie
- Zespół ds. dobrostanu

UCHWAŁA NR WAW2/185/2018

z dnia 12 grudnia 2018 r.

II Lokalnej Komisji Etycznej do spraw doświadczeń na zwierzętach w Warszawie

§ 1

Na podstawie art. 48 ust. 1 pkt. 1[1] ustawy z dnia 15 stycznia 2015r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266), zwanej dalej „ustawą" po rozpatrzeniu wniosku pt.: „Wpływ starzenia się organizmu oraz nadciśnienia tętniczego na aktywność metaboliczną flory jelitowej oraz funkcje bariery jelito-krew" z dnia 27 listopada 2018 roku, złożonego przez Warszawski Uniwersytet Medyczny, Wydział Lekarsko-Dentystyczny, adres: ul. Banacha 1B, 02-091 Warszawa, zaplanowanego przez Marcina Ufnala[2]

przy udziale[3] -

Lokalna Komisja Etyczna:

WYRAŻA ZGODĘ

Na przeprowadzenie doświadczeń na zwierzętach w zakresie wniosku.

§ 2

W wyniku rozpatrzenia wniosku o którym mowa w § , Lokalna Komisja Etyczna ustaliła, że:

1. Wniosek należy przypisać do kategorii: badania podstawowe obejmujące wiele układów.
2. Najwyższy stopień dotkliwości proponowanych procedur to: umiarkowana.
3. Doświadczenia będą przeprowadzane na gatunkach lub grupach gatunków[4]:

Gatunek	Wiek/stadium	Liczba
Szczur wędrowny, szczep SPRD	3 miesiące	18
Szczur wędrowny, szczep SPRD	18 miesięcy	18
Szczur wędrowny, szczep WKY	3 miesiące	18
Szczur wędrowny, szczep WKY	18 miesięcy	18
Szczur wędrowny, szczep SHR	3 miesiące	18
Szczur wędrowny, szczep SHR	18 miesięcy	18

4. Doświadczenia będą przeprowadzane przez: Marcin Ufnal, Tomasz Huć, Marta Gawryś, Klaudia Bielińska.
5. Doświadczenie będzie przeprowadzane w terminie[5] od 01.01.2019 do 01.01.2024r.
6. Doświadczenie będzie przeprowadzone w ośrodku[6]: -.
7. Doświadczenie będzie przeprowadzone poza ośrodkiem, w: -.
8. Użyte do procedur zwierzęta dzikie zostaną odłowione przez: nie dotyczy.

---

[1] Niewłaściwy zapis usunąć
[2] imię i nazwisko osoby, która zaplanowała i jest odpowiedzialna za przeprowadzenie doświadczenia
[3] Wypełnić w przypadku dopuszczenia do postępowania organizacji społecznej.
[4] Podać liczbę, szczep/stado, wiek/stadium rozwoju
[5] Nie dłużej niż 5 lat
[6] Podać jeśli jest to inny ośrodek niż użytkownik

9. Doświadczenie ~~zostanie/~~nie zostanie poddane ocenie retrospektywnej ~~w terminie do 6 miesięcy od dnia przekazania przez użytkownika dokumentacji, mającej stanowić podstawę dokonania oceny retrospektywnej. Użytkownik jest zobowiązany do przekazania ww. dokumentacji niezwłocznie, tj. w terminie, o którym mowa w art. 52 ust. 2 ustawy~~.

§ 3

Uzasadnienie:

Komisja oceniła wniosek zgodnie z kryteriami zawartymi w art. 47.1. ustawy z dnia 15 stycznia 2015 r. o ochronie zwierząt wykorzystywanych do celów naukowych lub edukacyjnych (Dz. U. poz. 266). Po zapoznaniu się z problematyką badawczą przedstawioną we wniosku komisja stwierdza, że przedstawiony projekt spełnia zasady dopuszczenia doświadczeń na zwierzętach.

Na podstawie art. 107 § 4 ustawy z dnia 14 czerwca 1960 r. – Kodeks postępowania administracyjnego z późniejszymi zmianami (Dz. U. z 2017 poz. 1257) odstąpiono od sporządzania uzasadnienia decyzji, gdyż uwzględnia ona w całości żądanie strony.

§ 4

Integralną część niniejszej uchwały stanowi kopia wniosku, o którym mowa w § 1.

(Pieczęć lokalnej komisji etycznej)

PRZEWODNICZĄCA

(Podpis Przewodniczącej komisji)

Pouczenie:

Zgodnie z art. 33 ust. 3 i art. 40 ustawy w zw. z art. 127 § 1 i 2 oraz 129 § 2 ustawy z dnia z dnia 14 czerwca 1960 r. Kodeks postępowania administracyjnego (Dz. U. 2017, poz. 1257 – t.j.; dalej KPA) od uchwały Lokalnej Komisji Etycznej strona może wnieść, za jej pośrednictwem, odwołanie do Krajowej Komisji Etycznej do Spraw Doświadczeń na Zwierzętach w terminie 14 od dnia doręczenia uchwały.

Na podstawie art. 127a KPA w trakcie biegu terminu do wniesienia odwołania strona może zrzec się prawa do jego wniesienia, co należy uczynić wobec Lokalnej Komisji Etycznej, która wydała uchwałę. Z dniem doręczenia Lokalnej Komisji Etycznej oświadczenia o zrzeczeniu się prawa do wniesienia odwołania przez ostatnią ze stron postępowania, decyzja staje się ostateczna i prawomocna.

Otrzymuje:
1) Użytkownik,
2) Organizacja społeczna dopuszczona do udziału w postępowaniu (jeśli dotyczy)
3) a/a

Użytkownik kopie przekazuje:
- Osoba planująca doświadczenie
- Zespół ds. dobrostanu

Oświadczenia wszystkich współautorów publikacji

PUBLIKACJA nr 1

An In Vivo Method for Evaluating the Gut-Blood Barrier and Liver Metabolism of Microbiota Products.

Kinga Jaworska

Tomasz Hutsch (Huć)

Marta Gawryś-Kopczyńska

Maksymilian Onyszkiewicz

Emilia Samborowska

Marcin Ufnal

dr n. med. Tomasz Hutsch

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „An In Vivo Method for Evaluating the Gut-Blood Barrier and Liver Metabolism of Microbiota Products" oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przygotowaniu manuskryptu oraz współudział w tworzeniu filmu instruktarzowego. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przygotowaniu manuskryptu oraz tworzeniu filmu instruktarzowego (60%).

Tomasz Hutsch

(podpis współautora)

mgr Marta Gawryś-Kopczyńska Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „An In Vivo Method for Evaluating the Gut-Blood Barrier and Liver Metabolism of Microbiota Products" oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przygotowaniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przygotowaniu manuskryptu oraz tworzeniu filmu instruktarzowego (60%).

Marta Gawryś-Kopczyńska

(podpis współautora)

Lek. Maksymilian Onyszkiewicz

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „An In Vivo Method for Evaluating the Gut-Blood Barrier and Liver Metabolism of Microbiota Products" oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przygotowaniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przygotowaniu manuskryptu oraz tworzeniu filmu instruktarzowego (60%).

Maksymilian Onyszkiewicz

(podpis współautora)

Mgr Emilia Samborowska

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „An In Vivo Method for Evaluating the Gut-Blood Barrier and Liver Metabolism of Microbiota Products" oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przygotowaniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przygotowaniu manuskryptu oraz tworzeniu filmu instruktażowego (60%).

Emilia Samborowska

(podpis współautora)

Prof. dr hab. n. med. Marcin Ufnal

Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „An In Vivo Method for Evaluating the Gut-Blood Barrier and Liver Metabolism of Microbiota Products" oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w opracowaniu koncepcji i w przygotowaniu manuskryptu. (20%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przygotowaniu manuskryptu oraz tworzeniu filmu instruktarzowego (60%).

(podpis współautora)

Kierownik Zakładu Fizjologii i Patofizjologii Eksperymentalnej
prof. dr hab. n. med. Marcin Ufnal

Oświadczenia wszystkich współautorów publikacji

PUBLIKACJA nr 2

Inflammatory bowel disease is associated with increased gut-to-blood penetration of short-chain fatty acids: A new, non-invasive marker of a functional intestinal lesion.

Kinga Jaworska

Marek Konop

Klaudia Maksymiuk (Bielińska)

Tomasz Hutsch

Marcin Dziekiewicz

Aleksandra Banaszkiewicz

Marcin Ufnal

dr n. med. Marek Konop

Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Inflammatory bowel disease is associated with increased gut-to-blood penetration of short-chain fatty acids: A new, non-invasive marker of a functional intestinal lesion." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przygotowaniu manuskryptu oraz przeprowadzeniu doświadczeń. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń, przygotowaniu manuskryptu oraz wykonaniu figur zawartych w pracy (50%).

Marek Konop

(podpis współautora)

lek. wet. Klaudia Maksymiuk (Bielińska) Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko, nazwisko rodowe)

OŚWIADCZENIE

Jako współautor pracy pt. „Inflammatory bowel disease is associated with increased gut-to-blood penetration of short-chain fatty acids: A new, non-invasive marker of a functional intestinal lesion." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przygotowaniu manuskryptu oraz przeprowadzeniu doświadczeń. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń, przygotowaniu manuskryptu oraz wykonaniu figur zawartych w pracy (50%).

Klaudia Maksymiuk

(podpis współautora)

dr n. med. Tomasz Hutsch

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „Inflammatory bowel disease is associated with increased gut-to-blood penetration of short-chain fatty acids: A new, non-invasive marker of a functional intestinal lesion." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to przeprowadzenie analizy histologicznej tkanek i współudział w tworzeniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń, przygotowaniu manuskryptu oraz wykonaniu figur zawartych w pracy (50%).

Tomasz Hutsch

(podpis współautora)

dr n. med. Marcin Dziekiewicz

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „Inflammatory bowel disease is associated with increased gut-to-blood penetration of short-chain fatty acids: A new, non-invasive marker of a functional intestinal lesion." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przeprowadzeniu klinicznej części badania i w przygotowaniu manuskryptu. (10%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń, przygotowaniu manuskryptu oraz wykonaniu figur zawartych w pracy (50%).

dr n. med. Marcin Dziekiewicz
lekarz
specjalista pediatrii
i gastroenterologii dziecięcej
2310005
Warszawa

(podpis współautora)

dr hab. n. med. Aleksandra Banaszkiewicz Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „Inflammatory bowel disease is associated with increased gut-to-blood penetration of short-chain fatty acids: A new, non-invasive marker of a functional intestinal lesion." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przeprowadzeniu klinicznej części badania i w przygotowaniu manuskryptu. (10%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń, przygotowaniu manuskryptu oraz wykonaniu figur zawartych w pracy (50%).

(podpis współautora)

Prof. dr hab. n. med. Marcin Ufnal

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „Inflammatory bowel disease is associated with increased gut-to-blood penetration of short-chain fatty acids: A new, non-invasive marker of a functional intestinal lesion." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w opracowaniu koncepcji i przygotowaniu manuskryptu. (15%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń, przygotowaniu manuskryptu oraz wykonaniu figur zawartych w pracy (50%).

(podpis współautora)

Kierownik Zakładu Fizjologii i Patofizjologii Eksperymentalnej
prof. dr hab. n. med. Marcin Ufnal

Oświadczenia wszystkich współautorów publikacji

PUBLIKACJA nr 3

Hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacteria metabolite.

Kinga Jaworska

Tomasz Hutsch (Huć)

Emilia Samborowska

Leszek Dobrowolski

Klaudia Maksymiuk (Bielińska)

Maciej Gawlak

Marcin Ufnal

dr n. med. Tomasz Hutsch

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „ Hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacteria metabolite." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w opracowaniu koncepcji, przeprowadzeniu doświadczeń i w tworzeniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń oraz przygotowaniu manuskryptu (55%).

Tomasz Hutsch

(podpis współautora)

Mgr Emilia Samborowska

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „ Hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacteria metabolite." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to wykonanie oznaczeń metabolitów bakteryjnych w próbkach i współudział w tworzeniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń oraz przygotowaniu manuskryptu (55%).

Emilia Samborowska

(podpis współautora)

Dr hab. n. med. Leszek Dobrowolski Warszawa, 22.05.2020 r.

(Stopień/tytuł, imię, nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. *Hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacteria metabolite* (opublikowanej *w PLoS One.* 2017 Dec 13; 12(12):e0189310) oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przeprowadzeniu doświadczeń na zwierzętach i w tworzeniu manuskryptu (5%).

Jednocześnie wyrażam zgodę na przedłożenie ww. pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń oraz przygotowaniu manuskryptu (55%).

L. Dobrowolski

(podpis współautora)

Lek.wet. Klaudia Maksymiuk (Bielińska) Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko, nazwisko rodowe)

OŚWIADCZENIE

Jako współautor pracy pt. „ Hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacteria metabolite." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przeprowadzeniu doświadczeń na zwierzętach i w tworzeniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń oraz przygotowaniu manuskryptu (55%).

Klaudia Maksymiuk

(podpis współautora)

Dr n.biol. Maciej Gawlak

Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „ Hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacteria metabolite." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przeprowadzeniu doświadczeń na zwierzętach i w tworzeniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń oraz przygotowaniu manuskryptu (55%).

M. Gawlak

(podpis współautora)

Prof. dr hab n.med. Marcin Ufnal

Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „ Hypertension in rats is associated with an increased permeability of the colon to TMA, a gut bacteria metabolite." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to opracowanie koncepcji, analiza danych i współudział w tworzeniu manuskryptu. (20%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, przeprowadzeniu doświadczeń oraz przygotowaniu manuskryptu (55%).

(podpis współautora)

Oświadczenia wszystkich współautorów publikacji

PUBLIKACJA nr 4

Trimethylamine but not trimethylamine oxide increases with age in rat plasma and affects smooth muscle cells viability.

Kinga Jaworska

Marek Konop

Tomasz Hutsch

Karol Perlejewski

Marek Radkowski

Marta Grochowska

Anna Bielak-Żmijewska

Grażyna Mosieniak

Ewa Sikora

Marcin Ufnal

dr n.med. Marek Konop

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „ Tma but not tmao increases with age in rat plasma and affects smooth muscle cells viability." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przeprowadzeniu doświadczeń i w tworzeniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, analizie danych oraz przygotowaniu manuskryptu (35%).

Marek Konop

(podpis współautora)

dr n.med. Tomasz Hutsch

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „ Tma but not tmao increases with age in rat plasma and affects smooth muscle cells viability." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to analiza histologiczna tkanek i współudział w tworzeniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, analizie danych oraz przygotowaniu manuskryptu (35%).

Tomasz Hutsch

(podpis współautora)

dr n.med. Karol Perlejewski

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „ Tma but not tmao increases with age in rat plasma and affects smooth muscle cells viability." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przeprowadzeniu doświadczeń i w tworzeniu manuskryptu. (10%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, analizie danych oraz przygotowaniu manuskryptu (35%).

(podpis współautora)

21.05.20

Karol Perlejewski

Prof. dr hab. n.med. Marek Radkowski

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „ Tma but not tmao increases with age in rat plasma and affects smooth muscle cells viability." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przeprowadzeniu doświadczeń i w tworzeniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, analizie danych oraz przygotowaniu manuskryptu (35%).

KIEROWNIK
Zakładu Immunopatologii
Chorób Zakaźnych i Pasożytniczych
prof. dr hab. n.med. Marek Radkowski

(podpis współautora)

Mgr Marta Grochowska

Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „ Tma but not tmao increases with age in rat plasma and affects smooth muscle cells viability." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przeprowadzeniu doświadczeń i w tworzeniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, analizie danych oraz przygotowaniu manuskryptu (35%).

Marta Grochowska

(podpis współautora)

dr hab. Anna Bielak-Żmijewska

(Stopień/tytuł, imię, nazwisko)

Warszawa, 21.05.2020

OŚWIADCZENIE

Jako współautor pracy pt. „ Tma but not tmao increases with age in rat plasma and affects smooth muscle cells viability." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przeprowadzeniu doświadczeń i w tworzeniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, analizie danych oraz przygotowaniu manuskryptu (35%).

Anna Bielak-Żmijewska

(podpis współautora)

dr hab. Grażyna Mosieniak Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „ Tma but not tmao increases with age in rat plasma and affects smooth muscle cells viability." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w przeprowadzeniu doświadczeń i w tworzeniu manuskryptu. (5%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, analizie danych oraz przygotowaniu manuskryptu (35%).

(podpis współautora)

Prof. dr hab. Ewa Sikora Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko)

OŚWIADCZENIE

Jako współautorka pracy pt. „ Tma but not tmao increases with age in rat plasma and affects smooth muscle cells viability." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to współudział w planowaniu części doświadczeń i przygotowaniu ostatecznej wersji manuskryptu. (5%).

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, analizie danych oraz przygotowaniu manuskryptu (35%).

Sikora

(podpis współautora)

Prof. dr hab n.med. Marcin Ufnal

Warszawa, 21.05.2020

(Stopień/tytuł, imię, nazwisko)

OŚWIADCZENIE

Jako współautor pracy pt. „ Tma but not tmao increases with age in rat plasma and affects smooth muscle cells viability." oświadczam, że mój wkład merytoryczny w przygotowanie publikacji to opracowanie koncepcji, analiza danych i współudział w tworzeniu manuskryptu. (20%)

Jednocześnie wyrażam zgodę na przedłożenie w/w pracy przez lek. Kingę Jaworską jako części rozprawy doktorskiej w formie spójnego tematycznie zbioru artykułów opublikowanych w czasopismach naukowych.

Oświadczam, że samodzielna i możliwa do wyodrębnienia część niniejszej pracy wykazuje indywidualny wkład lek. Kingi Jaworskiej w opracowaniu koncepcji, analizie danych oraz przygotowaniu manuskryptu (35%).

(podpis współautora)

Kierownik Zakładu Fizjologii i Patofizjologii Eksperymentalnej
prof. dr hab. n. med. Marcin Ufnal