

16S rRNA gene amplicon-based gut microbiome signature in cerebral vasospasm and delayed cerebral ischemia after aneurysmal subarachnoid hemorrhage: A prospective nested case-control pilot study

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Abstract

A recent systematic review indicated that gut-microbiota-brain axis contributes to growth and rupture of intracranial aneurysms. However, gaps were detected in the role of intestinal microbiome in cerebral vasospasm (CVS) after aneurysmal subarachnoid hemorrhage (aSAH). This is the first pilot study aiming to test study feasibility and identify differences in gut microbiota between subjects with and without CVS following aSAH.

A prospective nested case-control study with 1:1 matching was conducted recruiting subjects with aSAH: cases with CVS; and controls without CVS based on the clinical picture and structured bedside transcranial Doppler (TCD). Fecal samples for microbiota analyses by means of 16S rRNA gene amplicon sequencing were collected within the first 96 hours after ictus. Operational taxonomic unit tables were constructed, diversity metrics calculated, phylogenetic trees built, and differential abundance analysis (DAA) performed.

At baseline, the groups did not differ significantly in basic demographic and aneurysm-related characteristics ($p > 0.05$). Alpha-diversity (richness and Shannon Index) was significantly reduced in cases of middle cerebral artery (MCA) vasospasm ($p < 0.05$). In DAA, relative abundance of genus *Acidaminococcus* was associated with MCA vasospasm ($p = 0.00013$). Two butyrate-producing genera, *Intestinimonas* and *Butyricimonas*, as well as [*Clostridium*] *innocuum* group had the strongest negative correlation with the mean blood flow velocity in anterior cerebral arteries ($p < 0.01$; $\rho = -0.63$; -0.57 , and -0.57 , respectively). In total, 16 gut microbial genera were identified to correlate with TCD parameters, and two intestinal genera correlated with outcome upon discharge.

In this pilot study, we prove study feasibility and present the first preliminary evidence of gut microbiome signature associating with CVS as a significant cause of stroke in subjects with aSAH.

Introduction

Cerebral vasospasm (CVS) is one of the dreadful sequelae of aneurysmal subarachnoid hemorrhage (aSAH). Clinically, it might lead to delayed cerebral ischemia (DCI) with delayed ischemic neurologic deficits (DIND). The latter one has been defined as a new focal deficit (hemiparesis, dysphasia) or decrease in Glasgow Coma Scale by two points not attributable to hydrocephalus, rebleeding, seizures, or hyponatremia. It accounts for death or disability of approximately 20% of the aSAH survivors¹. Despite its significance, cerebral vasospasm fundamentals remain obscure and it lacks effective prophylaxis or treatment.

Gut-microbiota-brain axis (GMBA) is a bidirectional route of signaling between the digestive tract and central nervous system (CNS) that modulates health and disease and was shown to play a role in a plethora of neurological disorders such as ischemic stroke, multiple sclerosis, Alzheimer's disease, or others²⁻⁴. Moreover, our group recently published a systematic review with a narrative synthesis which highlighted the role of gut microbiota in growth and rupture of intracranial aneurysms⁵. However, as we indicated, currently there are no studies searching associations between intestinal microbiota and CVS following aSAH. Since CVS poses a significant risk of permanent neurological disability or death, it is of great concern to seek new diagnostic and therapeutic targets to prevent or counteract this phenotype.

Therefore, we aimed to investigate taxonomic components of gut microbiome as well as its ecological indices (alfa-diversity, beta-diversity) and their association with the presence of CVS and DIND after aSAH or with its surrogate markers in transcranial Doppler ultrasound examination as it is the only non-invasive and bedside modality to visualize in-vivo CVS. The primary objective of this pilot study was to assess feasibility at a small scale with recruitment rates, ultimate study completion rates, and troubleshooting as outcomes. The secondary objective was to search for preliminary clinical associations between microbiota and CVS utilizing the following outcomes: alfa-diversity, beta-diversity, differential abundance at a genus level, and correlation matrix of the microbial abundance.

Results

Patient characteristics

26 subjects with aSAH met the eligibility criteria and completed the entire study protocol. There were 18 women (69.2%) and eight men (30.8%), a proportion concordant with the female sex being a risk factor for aSAH (hazard ratio: 1.90 [95% CI, 1.47–2.46] PMID 31521957). The most prevalent locations of aneurysms were anterior communicating artery (n = 6; 23.1%), middle cerebral artery (n = 6; 23.1%) and internal carotid artery (n = 6; 23.1%), followed by posterior communicating artery (n = 5; 19.2%). Posterior circulation aneurysms comprised 30.8% (n = 8). A group of 13 patients developed CVS with DIND and 13 patients did not. Upon discharge, subjects with CVS had worse clinical outcome defined as an unfavorable (3–6) modified Rankin scale (62% versus 15%; p = 0.041, Fisher's exact test). Table 1 and Table 2 provide study group characteristics. All elements of the STROBE statement were addressed and can be viewed in Supplementary Material 1. Table 3 summarizes the measurements of transcranial Doppler ultrasound examination.

Table 1
Continuous variables of study groups. CVS – cerebral vasospasm. BMI – Body Mass Index. DIND – delayed ischemic neurologic deficit. GCS – Glasgow Coma Scale. SD – standard deviation. WFNS – World Federation of Neurological Surgeons grade. *Wilcoxon test.

	No CVS or DIND			CVS with DIND			
Variable	N	Mean	SD	N	Mean	SD	P*
Age	13	55	12	13	50	14	0.166
BMI	13	26.7	3.2	13	26.2	5	0.798
Size of aneurysm	13	8	6.5	13	7.1	4.7	0.837
Hunt-Hess grade	13	2	0.71	13	2.4	0.87	0.248
WFNS before repair	13	1.7	1.1	13	1.9	1.1	0.369
GCS on admission	13	14	0.88	13	14	2.9	0.257

Table 2
Categorical variables of the study groups. CVS – cerebral vasospasm. DIND – delayed ischemic neurologic deficit. F – females. IVH – intraventricular hemorrhage. M – males. *Fisher's exact test. **significant p value

	No CVS		CVS with DIND		
Variable	N	Percent	N	Percent	P*
Sex	13		13		0.202
F	7	54%	11	85%	
M	6	46%	2	15%	
Active smoker	13		13		0.253
No	7	54%	3	23%	
Yes	6	46%	10	77%	
Alcohol abuser	13		13		0.676
No	10	77%	8	62%	
Yes	3	23%	5	38%	
Aneurysm location	13		13		0.202
Anterior circulation	11	85%	7	54%	
Posterior circulation	2	15%	6	46%	
Any comorbidities	13		13		1.0
No	4	31%	4	31%	
Yes	9	69%	9	69%	
Hypertension	13		13		0.441
No	5	38%	8	62%	
Yes	8	62%	5	38%	
Multiple comorbidities (≥ 2)	13		13		1.0
No	10	77%	10	77%	
Yes	3	23%	3	23%	
IVH	13		13		0.694
No	7	54%	5	38%	
Yes	6	46%	8	62%	
Type of repair	13		13		1.0
Endovascular	13	100%	12	92%	
Surgical	0	0%	1	8%	
Modified Rankin on discharge	13		13		0.041**
Favorable (0–2)	11	85%	5	38%	
Unfavorable (3–6)	2	15%	8	62%	

Table 3

Transcranial Doppler ultrasound measurements. Velocities are presented in centimeters per second. Group A – without delayed cerebral ischemia (DCI) or delayed ischemic neurological deficit (DIND); Group B – with DCI or DIND. ACA – anterior cerebral artery; BA – basilar artery; CVS – cerebral vasospasm; MCA – middle cerebral artery. NA – not available. *mean from both sides. **mean from the side with higher velocity

Case #	Group	MCA mean velocity	MCA pulsatility index	MCA resistance index	ACA mean velocity*	ACA mean velocity 2**	ACA pulsatility index	ACA resistance index	BA mean velocity	BA pulsatility index
1	A	55	1,11	0,63	32	49	1,38	0,61	28	1,02
2	A	53	1,3	0,68	72	80	0,77	0,52	20	1,65
3	B	77	0,91	0,59	48	68	0,94	0,59	81	0,73
4	A	24	1,28	0,69	NA	NA	NA	NA	37	1,43
5	B	103	0,9	0,57	67	67	0,92	0,58	61	1,07
6	B	282	0,32	0,26	111	111	1,47	0,94	37	0,84
7	B	77	1,05	0,61	57	76	1,02	0,6	21	2,08
8	B	92	1	0,62	125	125	0,8	0,54	21	1,51
9	B	89	1,31	0,71	52	52	1,68	0,79	58	1,4
10	B	112	0,72	0,51	98	98	0,62	0,45	61	1,02
11	A	119	119	0,75	72	84	0,71	0,51	67	0,97
12	B	211	0,88	0,56	62	76	0,83	0,55	60	0,96
13	B	134	0,97	0,6	58	61	1,1	0,63	41	1,32
14	A	79	0,72	0,5	51	78	0,62	0,45	52	0,71
15	A	88	1,18	0,67	81	81	1,08	0,63	21	2,02
16	A	88	0,85	0,55	44	44	1,38	0,73	37	1,15
17	B	228	0,57	0,41	70	74	0,99	0,6	63	1,11
18	A	74	0,72	0,51	62	67	0,65	0,48	63	0,78
19	B	188	0,62	0,45	67	100	1,12	0,64	59	1,22
20	A	96	0,52	0,4	54	60	0,61	0,45	43	0,82
21	B	67	1,2	0,67	56	75	1,11	0,65	60	1,21
22	A	57	0,64	0,46	72	72	0,56	0,42	34	1,51
23	A	115	0,75	0,52	76	76	0,73	0,51	45	0,77
24	A	50	0,56	0,45	NA	NA	NA	NA	34	0,94
25	A	54	1,56	0,75	NA	NA	NA	NA	29	1,46
26	B	210	0,6	0,44	54	84	0,92	0,58	47	1,48

Microbiota analyses

A significant decrease in alpha diversity was observed in subjects with MCA vasospasm ($p = 0.031$ for richness and $p = 0.045$ for Shannon index) – see Fig. 2. There was no difference in beta diversity for any group comparison (MCA vasospasm, ACA vasospasm, DIND) – see Supplementary Material 2.

In differential abundance analysis, Wilcoxon test with Total Sum Scaling (TSS) normalization indicated that genus *Acidaminococcus* was relatively abundant in cases of MCA vasospasm ($p = 0.0001296$). All subjects without MCA vasospasm had nonexistent abundance of genus *Acidaminococcus* whereas subjects with MCA vasospasm presented with significantly larger abundance (in MCA vasospasm group median = 0.000268, interquartile range = 0.0003195) – Fig. 3.

The strongest negative linear correlation was found between butyrate-producing genus *Intestinimonas* and mean velocity in ACA ($\rho = -0.63$; $p = 0.003$; $Q = 0.16$), butyrate-producing genus *Butyricimonas* and ACA mean velocity ($\rho = -0.57$; $p = 0.008$; $Q = 0.246$), as well as genus *[Clostridium]_innocuum_group* and ACA mean velocity ($\rho = -0.57$; $p = 0.004$; $Q = 0.246$). See correlation matrix in Fig. 4. Positive correlations were weaker than the negative but initial significance threshold was observed for genus *Sellimonas* and ACA mean velocity, genus NK4A214_group and ACA mean velocity, genus *Flavonifractor* and basilar artery mean velocity ($\rho = 0.51$; $p = 0.025$; $Q = 0.508$), genus *Bifidobacterium* and MCA mean velocity ($\rho = 0.42$; $p = 0.035$; $Q = 0.920$) – see Fig. 4. In total, 16 gut microbial genera were identified to correlate with TCD parameters and two intestinal genera correlated with outcome upon discharge as determined by modified Rankin scale – negative correlation with genus *Megasphaera* ($\rho = -0.45$; $p = 0.034$; $Q = 0.778$) and positive correlation with genus *Eisenbergiella* ($\rho = 0.47$; $p = 0.021$; $Q = 0.781$).

Results that did not reach the significance threshold are displayed in Supplementary Material 2.

Feasibility assessment of the pilot study

Of 31 consecutive subjects with subarachnoid hemorrhage, 27 met the eligibility criteria, which produces a recruitment rate of 87,1%. Ineligibility was due to lack of acoustic bony window in two subjects, an aneurysm within the arteriovenous malformation in one, and Hunt-Hess grade V with early death in one. Of the 27 eligible participants, one did not complete the study because of the early transfer to another hospital in a different city upon request from the family (a completion rate of 96,3%). Stool sample collection, DNA isolation, library preparation, 16S rRNA gene amplicon sequencing, and bioinformatics were uneventful. The final outcome of the feasibility assessment for a future trial is: feasible with close monitoring.

Discussion

We conducted the very first study to find the link between the intestinal microbiome and CVS following aSAH. Thereby, we demonstrated that the methodology used to conduct this study was appropriate and can be used in the future to validate research hypotheses on the relationship between the microbiome and CVS after aSAH conducted in a larger group of patients. Here we present that alpha-diversity metrics were significantly reduced in cases of MCA vasospasm. Our study also showed that relative abundance of genus *Acidaminococcus* was associated with MCA vasospasm. Butyrate-producing genera, namely *Intestinimonas* and *Butyricimonas*, along with *[Clostridium]_innocuum_group* had the strongest negative correlation with the mean blood flow velocity in anterior cerebral arteries. At last, we identified 16 gut microbial genera correlating with TCD parameters and two with outcome upon discharge.

MCA vasospasm is linked to reduced microbial diversity

In our study, patients with MCA CVS had significantly lower alpha diversity of intestinal microbiome. It is accepted that composition of the microbiota fluctuates strongly and constantly with age, gender, genetic factors of the host, environmental conditions, and lifestyle¹⁴. Therefore, there is no single component that would be universally characteristic of healthy human gut microbiota. Rather, a broader term of high diversity was introduced and is considered desirable. Indeed, most of the human diseases of the westernized regions are associated with reduction in microbial diversity¹⁵. However, according to Shade and colleagues¹⁶ diversity is neither good nor bad, it simply “is”, and its deviations can be utilized in clinical settings. In terms of CVS, there is evidence that gut microbiome alterations including bacterial sub-population diversity might result in endothelial dysfunction, which in turn is strongly linked to vasospasm¹⁷. Also, bacterial diversity alters levels of plasma trimethylamine N-oxide (TMAO) of bacterial origin¹⁸. Interestingly, in ischemic stroke TMAO is usually elevated, whilst in aSAH it is reduced^{19–22}. The question remains what levels of TMAO are observed in DCI after aSAH. Also, what needs further studies is whether modulation of microbial alpha diversity and TMAO levels could impact course of CVS after aSAH.

Butyrate-producing bacteria in cerebral ischemia

We presented that butyrate-producing genus *Intestinimonas* and genus *Butyricimonas* negatively correlate with mean flow velocity in anterior cerebral artery which is used as a diagnostic parameter of in-vivo cerebral vasospasm. Such vasospasm is a leading cause of stroke in subjects after aSAH. Moreover, it has been shown that abundance of butyrate-producing bacteria in gut could be a protective factor against the primary ischemic stroke²³. Butyrate is a four-carbon short-chain fatty acid that readily absorbs from intestines into the portal circulation and modulates inflammation by exerting antioxidant and anti-inflammatory effects. It is also a potent neuroprotectant and modulates immune function as well as energy metabolism²⁴. Importantly, this SCFA has been found to cross blood-brain barrier and its involvement in neural signaling has been proven²⁵. There are data indicating that low levels of butyrate-producing bacteria are associated with a variety of cardiovascular disorders such as acute ischemic stroke²⁶, hypertension²⁷, or chronic kidney disease²⁸. Moreover, an in vitro study elegantly demonstrated that mice orally supplemented with butyrate improved myocardial ischemia/reperfusion injury via a gut-brain neural circuit²⁹.

However, we here provide the first preliminary evidence indicating negative correlation between two butyrate-producing genera, *Intestinimonas* and *Butyricimonas*, and mean flow velocity in anterior cerebral arteries, one of the surrogate markers of the in-vivo CVS following aneurysmal subarachnoid hemorrhage. Interestingly, in May 2023 Meng et al. found that a relative decrease in abundance of *Intestinimonas* could play a role in the pathomechanism of both large artery and small vessels ischemic strokes, conditions which share many similarities with the CVS and delayed cerebral ischemia³⁰. Although the delayed cerebral ischemia and atherosclerotic ischemic stroke are of different etiologies, their end points are congruent – decreased cerebral blood flow leading to a cascade of responses including local hypoxia, excitotoxicity, release of free radicals, mitochondrial death pathways, protein misfolding, apoptosis, and necrosis³¹. Whether promoting the growth of butyrate-producing genera such as *Intestinimonas* or *Butyricimonas* and restoring levels of butyrate could be a protective factor or serve as a complementary therapy for subjects with ACA vasospasm deserves further studies.

Acidaminococcus and inflammatory fundamentals of CVS

In our cohort, subjects with vasospasm in middle cerebral artery had higher relative proportions of genus *Acidaminococcus* as detected in DAA approach using Wilcoxon test with Total Sum Scaling (TSS). TSS is considered the best normalization method reducing experimental bias regarding statistical power and effect on false discovery rate (FDR) compared to other normalization schemes such as ALDEx2 or CLR (centered log ratio)³². Thus, we consider this finding significant, however, it requires external validation with larger samples. CVS and *Acidaminococcus* could be linked via its role in modulation of inflammation. Even though the exact etiology and pathomechanism of CVS are unknown, one of the accepted theories is the antecedent inflammatory cascade of leukocytes, cytokines (interleukin-1 and interleukin-6), and cell adhesion molecules^{33,34}. In fact, the abundance of *Acidaminococcus* has been observed in a number of inflammatory disorders such as ankylosing spondylitis, rosacea, oral mucositis after autologous hematopoietic stem cell transplantation, or primary biliary cholangitis^{35–38}. Thus, we hypothesize that the so-called dysbiosis involving genus *Acidaminococcus* associates with CVS throughout the inflammatory mediators in the vessel wall and could corroborate the inflammatory theory of CVS. Indeed, *Acidaminococcus* was negatively correlated with plasma levels of omega-6-derived oxylipins³⁹, and importantly, oxylipins such as 20-hydroxyeicosatetraenoic acid were reported to be involved in cerebral vasospasm following aSAH⁴⁰ – see Fig. 5. Moreover, only recently was the relative abundance of genus *Acidaminococcus* found to be also associated with lacunar cerebral infarcts⁴¹. Crucially, the lacunar pattern of infarct is seen in approximately 20% of MCA vasospasm-related cerebral ischemia⁴². Finally, this genus has been observed abundantly in obese subjects with increased visceral adipose tissue which could constitute a potential confounding factor^{43,44}. However, the groups in our sample did not differ significantly in terms of Body Mass Index, so we do not consider it a confounder (27,97 kg/m² in case group versus 25,43 kg/m² in control group; p = 0,21). Overall, we provide preliminary evidence of association between *Acidaminococcus* and MCA vasospasm after aSAH, yet it remains unclear whether a true causal relationship exists, or it is merely an epiphenomenon.

Limitations & strengths

Although the sample size appears small, it is an inherent feature of pilot studies exploring no man's land of research and, whatsoever, it was sufficient to detect significant differences and provide preliminary evidence of gut microbiome-based signature in CVS. Nonetheless, the generalizability of the study results is limited and it is supposed that this study will appeal to researchers to conduct external validation of the findings on larger cohorts. By the same token, only strong correlations were identified, whereas weaker but potentially significant correlations might remain undetected. A strong point of this investigation is the multidisciplinary expertise of the researchers involved in planning, microbiota analysis, and neurosurgical clinical evaluation of the subjects. The homogenous sample comes from the single center, whereas the expertise and assistance were obtained throughout multiple centers.

Another limitation refers to the radiographic measures of vessel narrowing. TCD was utilized because it is noninvasive, repeatable and can be performed bedside. The authors acknowledge that digital subtraction angiography (DSA) could visualize the vasospasm more accurately. However, DSA is more invasive, not repeatable at a bedside, and is not performed routinely at our institution in every single subject to determine CVS or lack thereof.

Future perspectives and clinical implications

Considering strengths and limitations of the study, the results presented here, after external validation, have potential to impact further research and clinical practice in several ways. In future, prediction models might include microbial signature to aid in early diagnosis and prevention of DCI. Ideally, a test from the stool sample collected upon admission indicating a subject at a high risk of CVS could guide commencement of therapy, for instance permissive euvolemic hypertension, which is currently the mainstay of treatment⁴⁵. Moreover, recognizing interventions that impact intestinal microbiome, prospective randomised placebo-controlled clinical trials with administration of probiotics promoting growth of butyrate-producing bacteria could be initiated to analyze whether the risk of CVS could be reduced by alteration of the gut microbiota. Thereby, more profound understanding of the CVS can introduce multidisciplinary approach to the individualized treatment.

Conclusions

In this pilot study, we confirmed that the used methodology was appropriate and we have presented the first preliminary evidence of gut microbiome-based signature associating with CVS as a significant cause of stroke in subjects with aneurysmal subarachnoid hemorrhage. It is the only attempt in the literature of sequencing 16S rRNA gene of the intestinal microbiota in subjects with CVS and DCI, which provides insights into its metagenomic landscape. Moreover, these findings could fuel further research on gut microbiome as a potential biomarker in diagnosis or a target for prevention and treatment of cerebral vasospasm.

Methods

Study design and eligibility

This is a prospective observational nested case-control pilot study evaluating the association between CVS after aSAH and gut microbiota structure. Recruitment started in June 2022 once Institutional Review Board of Pomeranian Medical University in Szczecin approval had been obtained (resolution no. KB-0012/61/2021-A-4897) and the pilot phase ended in July 2023. The authors confirm that all methods were performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from the participants. Inclusion criteria were as follows: (1) subarachnoid hemorrhage identified in non-enhanced computed tomography (CT), (2) culprit intracranial aneurysm detected on CT angiogram or digital subtraction angiography, (3) age > 18 years old, (4) successful management of the aneurysm. On the other hand, we dismissed subjects with (1) non-aneurysmal pretruncal subarachnoid hemorrhage, (2) antibiotics usage within the previous two months, (3) under the age of 18 years, (4) mycotic aneurysms or aneurysms within arteriovenous malformations, (5) admitted to hospital beyond postbleeding day 5, (6) remaining in Hunt Hess grades IV-V from admission up to postbleeding day 14 as this would preclude obtaining informed consent (consent from the next of kin was not attempted as it is not legally valid for observational studies in Poland), or (7) no acoustic bony window. Cases were eligible subjects with cerebral vasospasm as determined by transcranial Doppler (TCD) ultrasonography whereas controls were eligible patients who did not develop cerebral vasospasm (see section '*Determination of cerebral vasospasm and delayed cerebral ischemia*'). Matching was performed with 1:1 control-to-case ratio. STROBE

(Strengthening the Reporting of Observational Studies in Epidemiology) Statement was applied to maintain appropriate structure of the manuscript.

Stool sample collection

Between postbleeding days 1 to 4 (prior to cerebral vasospasm development), a single stool sample was collected with a swab into 5ml Eppendorf tubes filled with 1 ml of ultraclean high-purity 96% ethyl alcohol (EMSURE®). The sample was then transferred to the laboratory refrigerator and stored at a temperature of -25°C until analyses. Upon collection of the entire batch, DNA isolation took place.

DNA isolation

Auto-Pure Mini (Allsheng, Hangzhou) extraction machine was utilized for DNA isolation along with MagnifiQ (A&A Biotechnology) reagents and consumables kits. All samples were processed at the same time as a single batch in order to minimize the batch effect. Isolation was performed by trained and certified laboratory technicians in accordance with standard protocol of genomic DNA isolation provided by the manufacturer.

Quantification and quality assessment

Quantity of the isolated genomic DNA and purity of the samples were determined by means of spectrophotometry using DeNovix DS-11 machine by trained and certified laboratorians.

Library preparation

DNA libraries were prepared according to Illumina procedure with PCR amplification of V3-V4 fragment of 16S rRNA gene. Length of the fragment was 460 base pairs (bp). Next-generation sequencing of 16S rRNA gene amplicons was conducted with MiSeq system of Illumina generating fragments of 300 bp in pair-end reads (2x300 bp) obtaining approximately 100,000 reads per sample.

Determination of cerebral vasospasm and delayed cerebral ischemia

Radiological CVS was determined by transcranial Doppler ultrasound examination, which is the only non-invasive, safe, surrogate measure of the real in-vivo vasospasm. Moreover, TCD was proven to be a suitable intermediate measure for genetic association studies ⁶. Measurements were performed by a neurosurgical resident (T.K.) and validated by a senior radiologist (M.S.) with over 20 years of experience. Primary hardware in use was Dolphin IQ Transcranial Doppler platform (Viasonix, Ra'anana, HaMerkaz, Israel) using 2 MHz linear probe whereas validation was conducted with Multi-Dop T DIGITAL (Compumedics DWL, Germany). Serial assessments were done up to three times on a structured basis on postbleeding days 3–5, 7–10, and 11–14, the schedule grounded upon the pathophysiology of cerebral vasospasm ⁷. ALARA (as low as reasonably achievable) principle was applied to reduce ultrasound exposure. Care was taken to maintain the insonation angle below 30° to ensure measurement bias of the reflector speed below 15%. All measurements were conducted with a patient lying flat in bed. Middle cerebral arteries (MCAs) were found in transtemporal acoustic window at a depth between 40 to 62mm with flow towards the probe – see Fig. 1. Extracranial segment of internal carotid artery (for calculation of the Lindegaard ratio and Sloan ratio) was found through the submandibular window in the carotid triangle of the neck. MCA vasospasm was declared if both conditions were met: Lindegaard ratio > 3 and MCA mean velocity > 120 cm/s. Anterior cerebral arteries (ACAs) could be found via the transtemporal acoustic window at a depth between 63 to 80 mm with flow away from the probe. ACA vasospasm was declared if both conditions were met: ACA mean velocity > 80 cm/s and Sloan ratio > 3. In case of hypoplastic ACA on one side, only the other side was insonated. Due to less sensitivity of TCD in ACA flow assessment, the measured ACA velocity was curated in two ways: ACA mean velocity from both sides and ACA mean velocity from the side with higher velocity. Basilar artery (BA) was found in suboccipital region at a depth 70 to 90 mm and its vasospasm was declared if BA mean flow velocity was > 70 cm/s and Svir ratio > 2. Acknowledging the fact that in about 10% of the population no acoustic window can be identified ⁸ those patients were excluded from the study. Clinically, delayed cerebral ischemia or delayed ischemic neurologic deficit was defined as a new neurological deficit and/or drop in Glasgow Coma Scale by 2 points not attributable to hydrocephalus, rebleeding, hyponatremia or other causes, lasting more than one hour, or the appearance of new infarct in the affected territory on CT or magnetic resonance imaging (MRI) scan.

The 16S sequence processing bioinformatics pipeline was initiated with Illumina paired-end sequencing, specifically targeting the V3-V4 region of the 16S rRNA gene. To ensure data quality, we conducted an initial rigorous assessment using FastQC (version 0.12.1) and MultiQC (version 1.12) tools. Subsequently, all data preprocessing procedures were executed using QIIME2 software⁹. The 16S rRNA gene sequences underwent processing through the DADA2 (Divisive Amplicon Denoising Algorithm 2) algorithm, as introduced by Callahan et al¹⁰. DADA2 is primarily designed for identifying and correcting errors in high-throughput sequencing data. It employs a denoising approach to distinguish genuine biological sequence variants (amplicon sequence variants or ASVs) from sequencing errors. This is achieved by implementing a denoising algorithm that detects and removes errors inherent in sequencing reads. Before applying DADA2, primer trimming on the V3-V4 region was performed using the cutadapt plugin. Representative sequences resolved by DADA2 underwent filtering to exclude low-abundance features, specifically ASVs with a frequency below 10. This resulted in 1,572 unique ASVs, each with abundance estimates within every sample. Metrics per sample included a minimum frequency of 21,393.0, a 1st quartile at 39,060.5, a median frequency of 43,536.00, a 3rd quartile at 50,829.0, a maximum frequency of 60,817.0, and an overall mean frequency of 43,477.6. Additionally, taxonomic profiling of microbial communities was conducted using the scikit-learn method in QIIME 2, employing a naive Bayes machine learning classifier¹¹. The scikit-learn classifier was trained on the Silva 138 – 99 reference dataset of 16S rRNA gene sequences. Query sequences were associated with predicted taxonomic assignments at different taxonomic ranks (phylum, class, order, family, and genus). Following taxonomic profiling, ASVs assigned to categories such as Eukaryota, Archaea, mitochondria, and chloroplasts were excluded due to their likely representation as contaminants or non-bacterial sequences.

Statistical analysis

All analyses were performed in R (R Core Team, 2021), and, where necessary, False Discovery Rate (FDR) correction was applied to calculate Q value (FDR-adjusted P value). We predefined the following potential baseline confounders to be checked between the analyzed groups: age, sex, hypertension presence, BMI, aneurysm location and size, smoking cigarettes, alcohol abuse, multiple comorbidities, Hunt-Hess and WFNS grades. Fisher's exact test was applied for categorical variables. For continuous variables we used unpaired two-sample Wilcoxon-Mann-Whitney test. Alpha diversity measures, including Shannon's diversity, Pielou's evenness, and richness, were calculated from rarefied samples to a minimum frequency depth of 21393. The indices were compared between groups using a Wilcoxon test. Beta-diversity was calculated using Bray-Curtis distance at the genus level, and pairwise relations between samples were visualized through Principal Coordinate Analysis (PCA). Beta-diversity distances were compared between groups using Permutational Analysis of Variance (PERMANOVA). Differential abundance analysis was conducted using four different methods: ALDEx2¹², the Wilcoxon test on rarefied data (WilcoxRarefied), center-log-transformed data (WilcoxCLR), and data transformed using total sum scaling (WilcoxTSS). This approach, suggested by Nearing et al.¹³ served as a workaround for the lack of reproducibility and consistency among various differential abundance techniques. Correlations with numerical clinical variables and microbiome data were assessed using the ALDEx2 package. Missing data were handled by omission from the given analysis.

Declarations

Data availability statement

The datasets generated and analysed during the current study are available in the BioProject repository, accession number: PRJNA1075509, accession hyperlink: www.ncbi.nlm.nih.gov/bioproject/PRJNA1075509. Taxonomic data are available as Supplementary Material 3.

Disclosures

None

Author contribution statement

T.K., K.S.Ż and I.Ł. wrote the main manuscript. T.K. and K.S.Ż. prepared figures 1 and 5. M.K. prepared figures 2-4. M.S., W.P., N.A. and C.O. provided expertise on cerebral vasospasm diagnosis. L.S. provided neurosurgical expertise. T.K. collected stool samples. T.K. performed transcranial Doppler examination. M.S. validated transcranial Doppler examination. A.W.W., I.Ł., K.S.Ż. and M.K. provided resources for 16S rRNA sequencing. J.P. assisted with sample collection and handling. M.K. performed biostatistical analysis. T.K. prepared all tables. K.S.Ż., M.K., S.P. and D.T. provided expertise on metagenomics. T.K. and L.S. acquired national funding. E.S. and L.S. supervised the entire project. All authors reviewed the manuscript.

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Figures

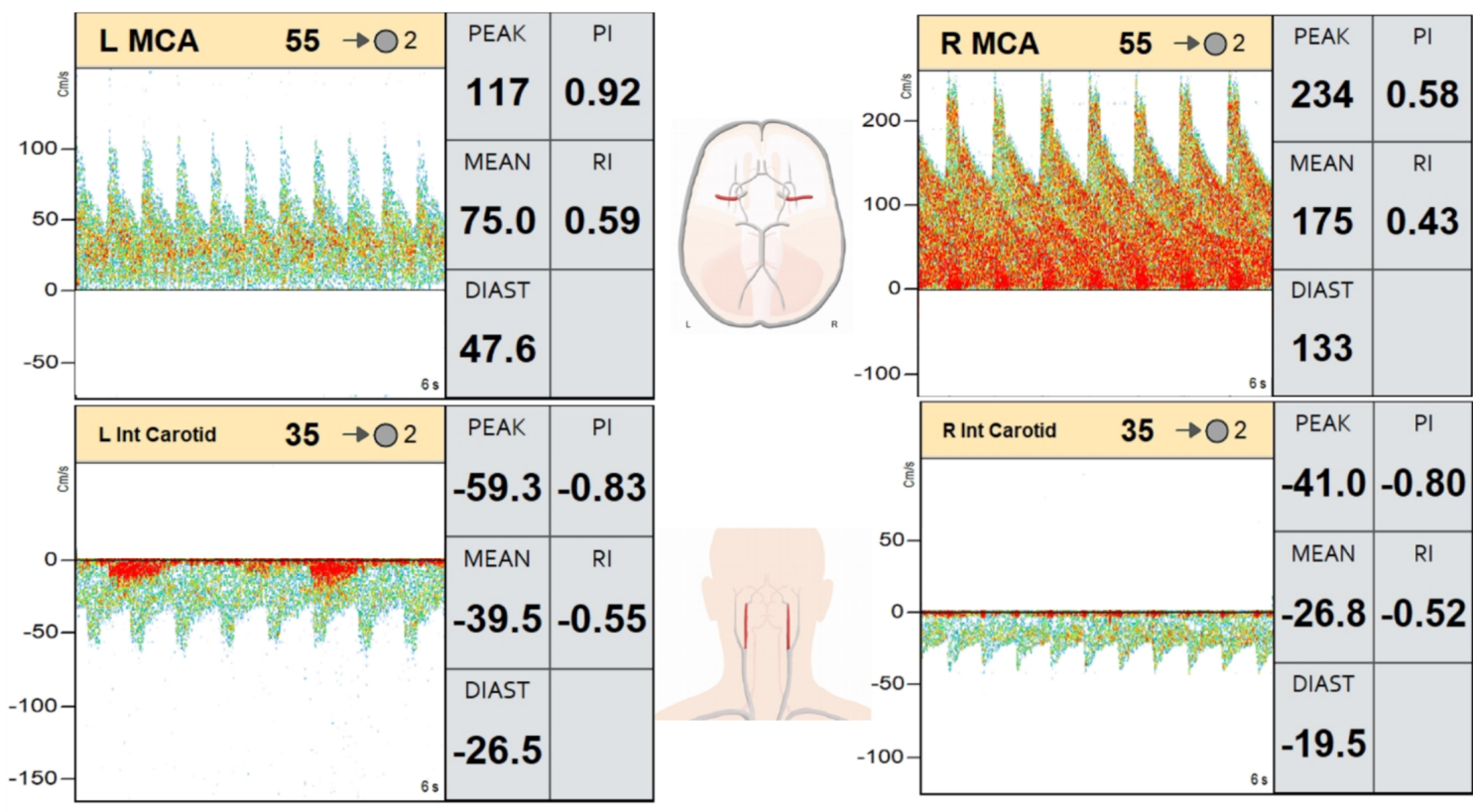


Figure 1

An example of transcranial Doppler examination report demonstrating normal mean blood flow velocity in the left middle cerebral artery with Lindegaard ratio of 1.9 and increased mean blood flow velocity due to posthemorrhagic vasospasm in the right middle

cerebral artery with Lindegaard ratio of 6,53. L – left; R – right; MCA – middle cerebral artery; Int Carotid – internal carotid artery; PI – pulsatility index; RI – resistance index;

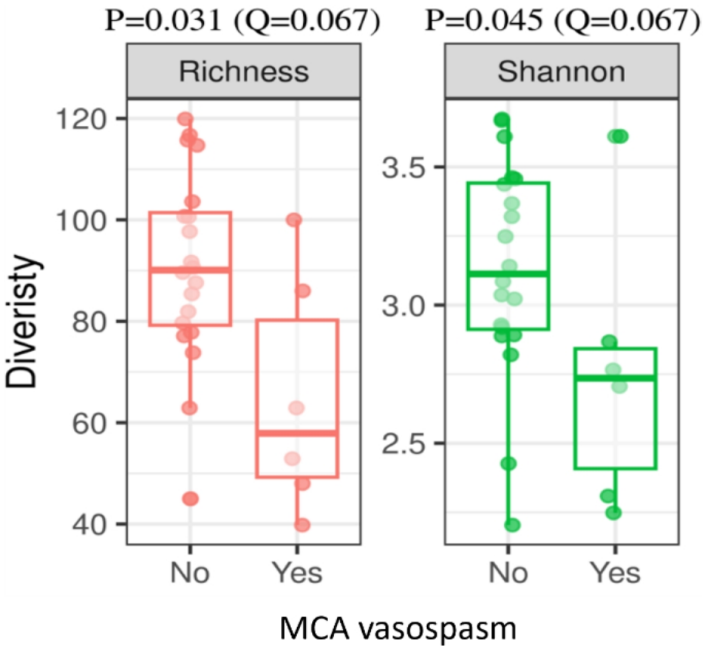


Figure 2

Indices of alfa diversity: richness and Shannon index that reached the initial threshold of significance. MCA - middle cerebral artery; Q - false discovery rate-adjusted p-value

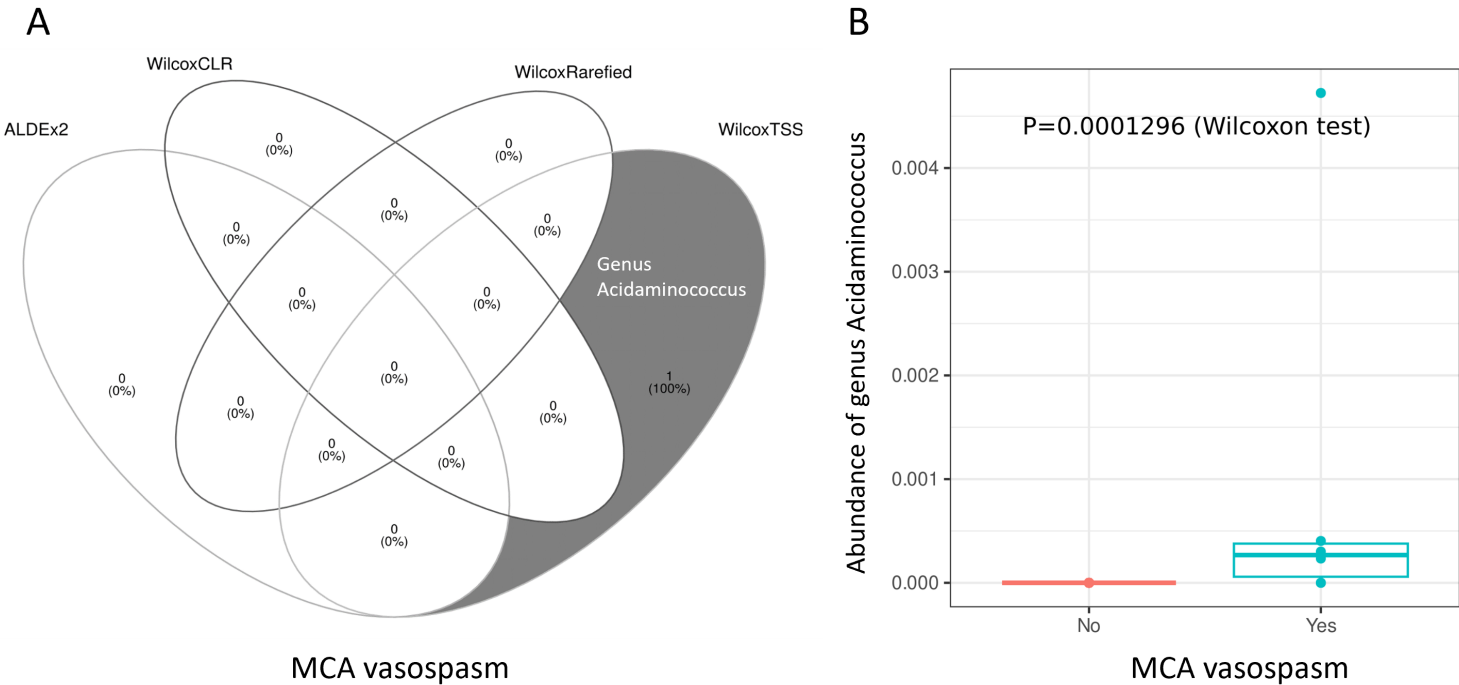


Figure 3

(A) Venn's diagram illustrating the results of differential abundance analysis with four different normalization methods. Wilcoxon test with total sum scaling (WilcoxonTSS) displayed that genus Acidaminococcus is more abundant in middle cerebral artery vasospasm. TSS is considered the best normalization method reducing experimental bias regarding statistical power and effect

on false discovery rate (FDR) compared to other normalization schemes such as ALDEx2 or centered log ratio (CLR) (PMID: 36074761). **(B)** A box plot illustrating difference in abundance of genus *Acidaminococcus* between subjects without MCA vasospasm and with MCA vasospasm. All subjects without MCA vasospasm had nonexistent abundance of genus *Acidaminococcus* whereas subjects with MCA vasospasm presented with significantly larger abundance (in MCA vasospasm group median: 0.000268, interquartile range: 0.0003195)

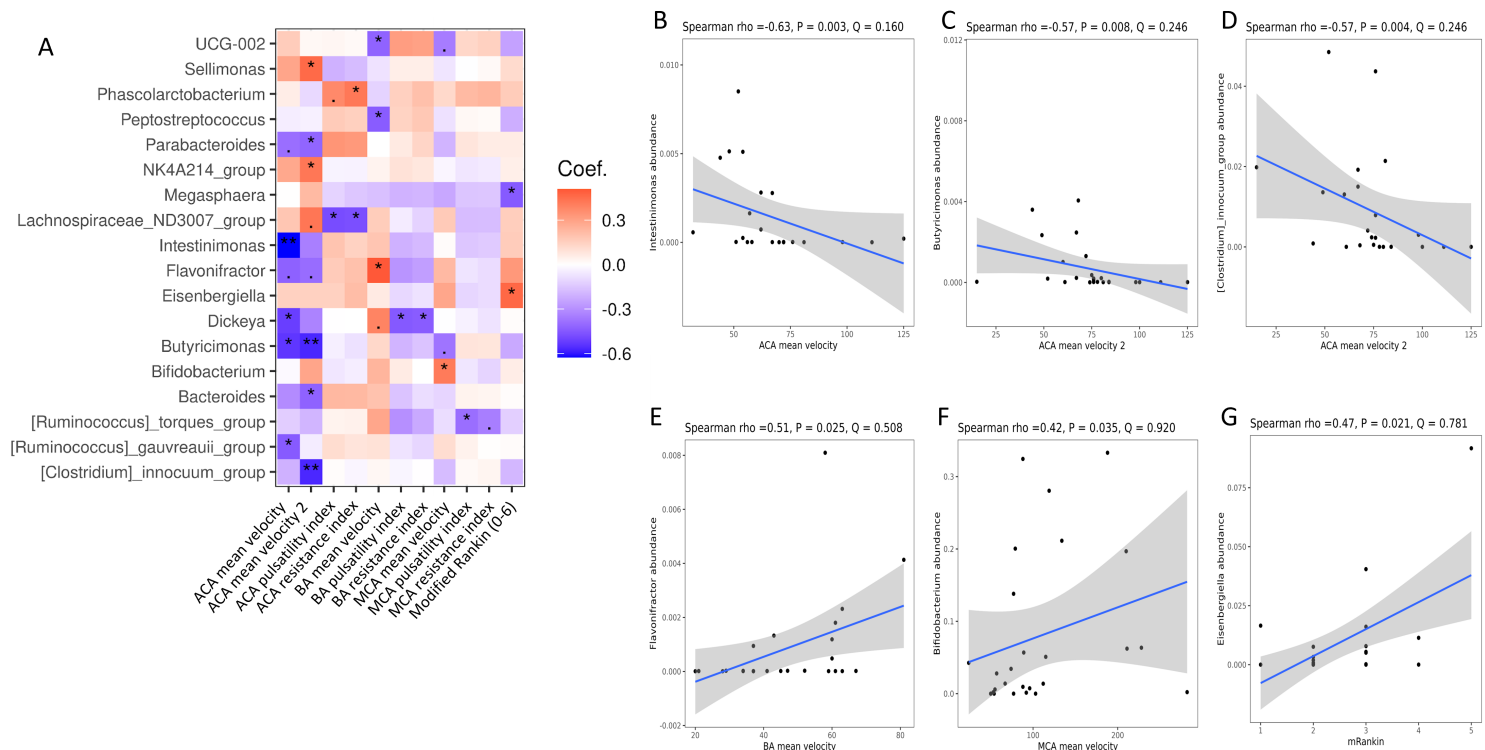


Figure 4

(A) Correlation matrix summarizing Spearman's correlation testing of the microbial abundance at the genus level with parameters of transcranial Doppler ultrasound examination in subjects with aneurysmal subarachnoid hemorrhage. The single asterisk (*) indicates correlations with p value in range 0.01-0.05; The double asterisk (**) - p value 0.001-0.01; The dot (.) – p value with tendency to significance 0.05-0.1. ACA mean velocity was obtained as a mean from both sides whereas ACA mean velocity 2 as a mean velocity from the side with higher velocity. Direction of correlation for both ACA velocities remains the same. B-G show graphs illustrating Spearman linear correlation testing. **(B)** Genus *Intestinimonas* and ACA mean velocity; **(C)** Genus *Butyricimonas* and ACA mean velocity 2; **(D)** Genus *[Clostridium] innocuum* group and ACA mean velocity 2; **(E)** Genus *Flavonifractor* and basilar artery mean velocity; **(F)**Genus *Bifidobacterium* and MCA mean velocity; **(G)** Genus *Eisenbergiella* and modified Rankin scale upon discharge. ACA – anterior cerebral artery; BA -basilar artery; MCA – middle cerebral artery; mRankin – modified Rankin scale upon discharge; Q – False discovery rate-adjusted p-value

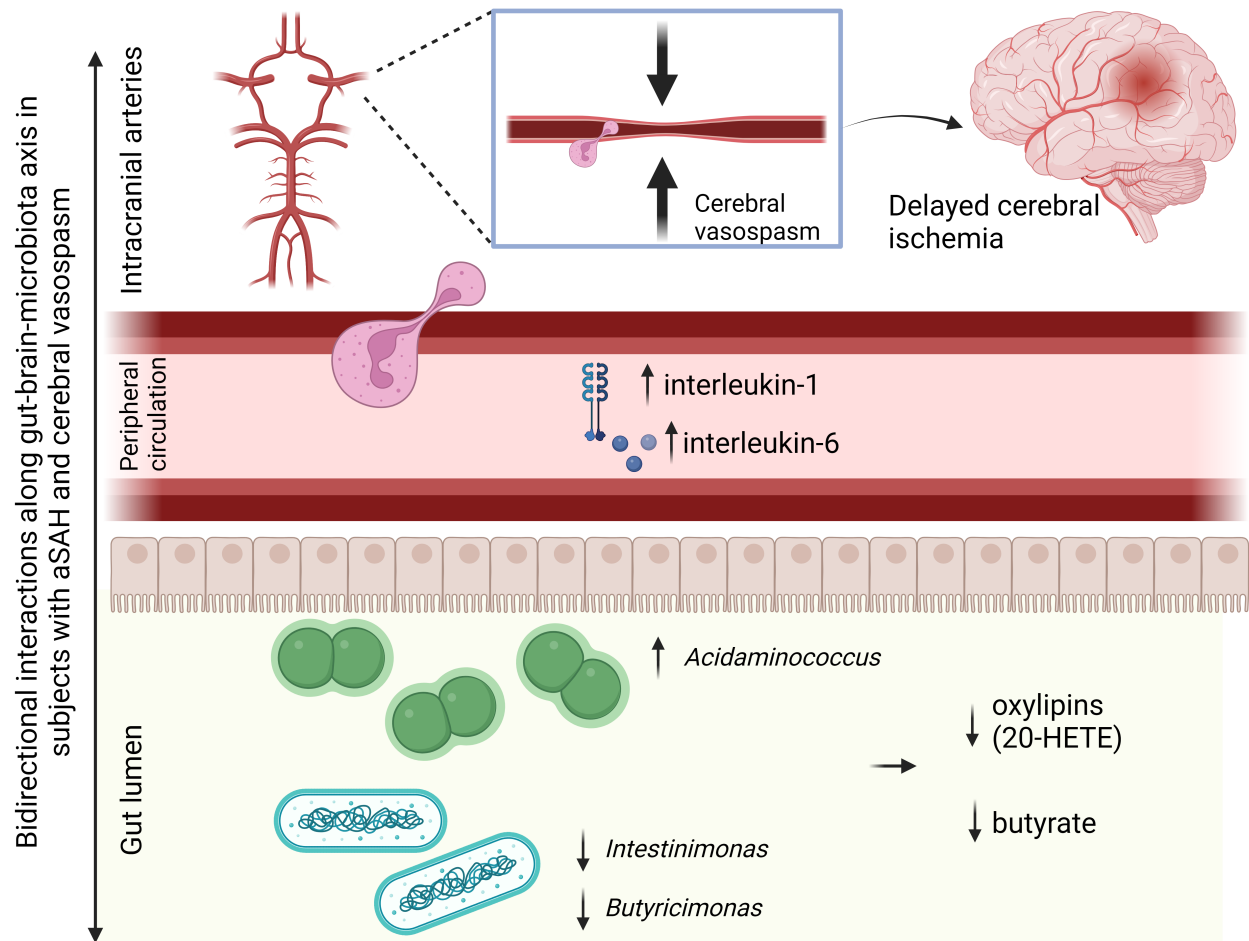


Figure 5

The hypothetical role of gut microbiota in the cerebral vasospasm and purportedly in delayed cerebral ischemia, based on the findings of this study and literature.

Supplementary Files

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