SUPPLEMENTAL MATERIAL

Distinct commensal bacterial signature in the gut is associated with acute and long-term protection from ischemic stroke

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Supplemental Methods

Mouse husbandry and antibiotic treatment. C57BL/6 4 weeks old male mice were housed in a 12-h light cycle in autoclaved microisolator cages, with individual sterile air supply, on autoclaved bedding and drinking water, with sterilized standard chow *ad libitum*. Upon one week of arrival, mice were randomly allocated to water alone or antibiotic treatment filtered sterilized in autoclaved water (A: ampicillin 2.7 mmol/L (1 g/L) #A9518, M: metronidazole 5.8 mmol/L (1 g/L) #M3761, N: neomycin sulfate 1.1 mmol/L (1 g/L) #N5285, and V: vancomycin 0.3 mmol/L (0.5 g/L) #V2002, all purchased from Sigma). Antibiotic-containing bottles (AMNV) were inverted every day, the antibiotic solution was changed twice a week together with the cage bedding for 4 weeks. AMNV treatment was discontinued 3 days before inducing transient occlusion of the middle cerebral artery (MCAO) to avoid off-target effects of antibiotics[1-3]. In some AMNV animals, wild-type (WT) microbiota was re-established by co-housing a WT untreated mouse with AMNV mice for 2 weeks. In another set of experiments, 200 µL of AMNV mixture was orally gavaged one day prior inducing stroke and until sacrifice.

Quantification of infarct volume and tissue loss. Brains were frozen on dry ice and serially sectioned (30-µm thick and 600-µm intervals) for Nissl staining[4,5]. Quantification of the infarct volume (corrected for swelling[6]), and tissue loss (percentage of the volume decrease in the ipsilateral in comparison to the contralateral hemisphere) was performed using an image analysis software (MCID, Imaging Research Inc., Ontario, Canada) by an investigator blinded to the treatment.

N-methyl-D-aspartate (NMDA) microinjection in neocortex. Mice were treated for 4 weeks with AMNV in the drinking water. AMNV treatment was discontinued 3 days before NMDA lesion was induced into the cortex as described previously[7]. In brief, NMDA (20 nmol) or vehicle (0.1 mol/L phosphate-buffered saline) was injected in the parietal cortex. Mice were euthanized 24 h after NMDA injection. Coronal forebrain sections were serially sectioned (30-µm thick and 180-µm intervals) and stained with cresyl violet for determination of lesion volume by MCID image analyzer.

Neurobehavioral testing. The sequence in which mice from different treatment groups were assessed was randomly assigned. Antibiotic-treated mice cannot be blinded to the investigator because they show an enlarged abdomen and their stool pellets appear distinguishable from control mice. **Open field test.** All procedures were carried out in a square open field chamber (27.3 X 27.3 x 20.3 cm LxWxH; Med Associates, St. Albans,

VT). Behavior was monitored via a grid of invisible infrared light beams mounted on the sides of the walls of the arena. To examine activity levels in the presence of a foreign mouse (intruder), each animal was first exposed to the test chamber for 15 min and allowed to move freely. After the 15 min trial, an intruder mouse – placed in a ventilated Plexiglass box – was positioned at the right corner of the open field area and stereotypic behavior (time and count of repetitive behaviors including circling, scratching, jumping or repetitive back and forth motion) was recorded for an additional 15 min in 60 second time blocks. The arena was cleaned with 70% ethanol after each mouse completed a session. Data was collected and analyzed via SPSS statistical software (IBM Corp., Armonk, NY).

Cell isolation from the lamina propria (LP) and FACS analysis. *Il17a-eGFP* (C57BL/6-Il17atm1Bcgen/J; purchased from JAX) were sacrificed by pentobarbital overdose (Sigma; 200 mg per kg body weight (mg/kg); i.p.). Cells from the LP layer were isolated as previously described[5]. Briefly, the small intestine was isolated, Peyer's patches were excised, and intestines were carefully cleaned of mesenteric fat and intestinal contents. Intraepithelial cells were removed by mechanical disruption (3 cycles of shaking at 250 r.p.m. in an incubator at 37 °C for 20 min in cell media containing 4 mM EDTA and 0.5 mM dithiothreitol). The remaining tissue was enzymatically dissociated to obtain the LP cell suspension (0.2 mg/ml collagenase D (Sigma), in an incubator at 250 r.p.m., at 37 °C for 20 min and vigorously vortexed for 20 s). LP cells were filtered through a 40-μm cell strainer and the cell suspensions were collected at 500*g* for 10 min at 4 °C. Cells were stained for flow cytometric analysis as previously described[5].

Stool sample collection and DNA extraction. Stool pellets were collected at different time points and stored at −80 °C. Frozen stool samples (~100 mg) were placed in sterile polypropylene microvials (BioSpec Products) containing 1 mL InhibitEX Buffer (QIAGEN), 1 mL of 0.1-mm diameter zirconia-silica beads (BioSpec Products) and one 3.5-mm diameter glass bead (Biospec Products). Samples were homogenized for 2 min using a Mini-BeadBeater 16 (BioSpec Products). DNA was purified with the QIAamp Fast DNA Stool Mini Kit (QIAGEN) according to the manufacturer's instructions.

Quantification of 16S ribosomal (r16S) DNA copy numbers. For quantification using qPCR, stool samples were adjusted to a 3 μg/μl stool weight equivalent. r16S DNA sequences were amplified from stool DNA using 0.2 μmol/L of the universal bacterial r16S gene primers 16S-V2-101F (5′-AGYGGCGIACGGGTGAGTAA-3′) and 16S-V2-361R (5′-CYIACTGCTGCCTCCCGTA G-3′) in conjunction with Maxima SYBR Green/ROX qPCR Master mix 2× (Thermo Scientific). Amplifications were performed on a Chromo 4 Detector (Bio-Rad, Hercules, CA) using a two-step cycling protocol consisting of: 50 °C for 2 min, 95 °C for 10 min, followed by 45 cycles of 95 °C for 15 s, 60 °C for 1 min. The standard curve was prepared using a plasmid containing an *Escherichia coli* r16S V2 DNA fragment.

16S rRNA gene amplification and multiparallel sequencing. For each sample, duplicate 50-μL PCR reactions were performed, each containing 50 ng of purified DNA, 12.5 μL Phusion Hot Start Flex 2X Master Mix (New England Biolabs), 2.5 μL of each primer designed to amplify the V4 universal region: 515F

(5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACNVGGGTWTCTAAT-3′), and 25 µL ddH_2O. Cycling conditions were 98 °C for 30 s, followed by 35 cycles of 98 °C for 10 s, 54°C for 30 s, and 72 °C for 45 s. A condition of 72 °C for 10 min was used for the final elongation step. PCR amplification products were detected by 2% agarose gel electrophoresis. The target fragments were recovered using the AxyPrep PCR Cleanup Kit (Thermo Fisher Scientific). The PCR product was purified using the Quant-iT PicoGreen dsDNA Assay Kit and quantified (Thermo Fisher Scientific). Libraries with the concentration above 2 nmol/L were pooled, loaded on Illumina MiSeq and the MiSeq Reagent Kit v2 (500 cycles) was used for paired-end sequencing (2 x 250 bp).

Sequence analysis and data visualization. Demultiplexed sequence reads were processed with the QIIME2 (vers. 2019.4) pipeline[8]. Reads were denoised with Dada2[9]. OTUs were called using the Greengenes database v. 13_8[10] with a similarity cutoff of 97%. Microbial community composition data were analyzed using the web-interface Calypso (http://cgenome.net/calypso/). Uploaded data were normalized and transformed to account for the generally non-normal distribution of microbial community composition data. Number of excluded taxa was set to 0.001% relative abundance across all samples. OTU-based microbial diversity was estimated by calculating the α-index estimators of diversity including the nonparametric Shannon index, richness, and evenness. The Shannon's diversity index "H" is expressed as "–Summation[P(i) * lnP(i)]" with i=species, P(i) = proportion of each species. The species richness "S" is the number of species of the taxonomy of interest. Evenness is calculated as "H" divided by the natural logarithm of species richness ln(S)". Species evenness ranges from 0 to 1, with zero signifying no evenness and one, a complete evenness. The Circos (http://circos.ca/) online software was used to represent interactions between individual samples and bacteria detected in stool samples. To infer metabolic pathways from the taxonomic composition we used the QIIME2 implementation of PICRUSt[11]. Data were visualized using STAMP[12]. Random Forest analysis was performed with the randomForest (vers. 4.6-14[13]) and randomForestExplainer (vers. 0.9[14]) packages in the R statistical environment (vers. 3.5.4).

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Supplemental Tables

Tables related to Figure 1

	Infarct volume Fig. 1D, left			
	Mean (mm^3)	SD	n	t-test
CTR	56.2	21.4	10	NA
AMNV	33.9	22.4	9	0.0404

	Lesion volume Fig. 1D, right			
	Mean (mm^3)	SD	n	t-test
CTR	2.96	1.26	7	NA
AMNV	3.52	0.92	5	0.4152

Tables related to Figure 2

	Infarct volume Fig. 2B, right				
	Mean (mm^3)	**SD**	**n**	**ANOVA**	**p value**
CTR	65.1	17.6	8	CTR versus AMNV short	0.969
AMNV long	28.2	5.5	5	CTR versus AMNV long	0.0006
AMNV short	66.9	10.2	5	AMNV long versus AMNV short	0.001

	Infarct volume Fig. 2D, right				
	Mean (mm^3)	**SD**	**n**	**t-test**	**p value**
AMNV	43.4	15.9	6	AMNV versus Recolonization	0.0089
Recolonization	71.8	17.6	8		

Tables related to Figure 4

	Infarct volume Fig. 4B			
	Mean (mm^3)	SD	n	ANOVA versus CTR
CTR	55.6	21.2	10	NA
N	52.4	20.2	10	NS
V	35.1	19.4	10	*p<0.05
A	19.8	5.7	10	***p<0.001

	Tape test Contact time at d+3 Fig. 4D left			
	Mean (relative to d-1)	SD	n	t-test at d+3 in comparison to d-1
CTR	6.8	2.8	8	***p<0.001
N	8.4	4.3	8	***p<0.001
V	3.7	3.6	7	0.106
A	3.0	2.9	9	0.0773

	Tape test Remove time at d+3 Fig. 4D right			
	Mean (relative to d-1)	SD	n	t-test at d+3 in comparison to d-1
CTR	4.9	0.9	8	****p<0.0001
N	8.3	0.9	8	****p<0.0001
V	2.8	1.4	7	0.0683
A	4.2	3.5	9	*p<0.05

Tables related to Figure 5

	Stereotypic time at d14 Fig. 5B left			
MCAO	Mean	SEM	n	ANOVA versus CTR
CTR	217.6	2.71	12	NS
N	213.2	3.52	7	0.872
V	201.2	5.28	13	0.025
A	199.9	5.35	10	0.023

	Stereotypic time at d14 Fig. 5B left			
SHAM	Mean	SEM	n	ANOVA versus CTR
CTR	203.3	10.50	4	NS
N	208.7	5.27	7	0.889
V	197.5	3.86	4	0.899
A	206.9	5.93	8	0.959

	Stereotypic count at d14 Fig. 5B right			
MCAO	Mean (%)	SEM	n	ANOVA versus CTR
CTR	3068	53.88	12	NS
N	3089	97.31	7	0.997
V	2783	94.86	13	0.0357
A	2802	85.36	10	0.0760

	Stereotypic count at d14 Fig. 5B right			
SHAM	Mean	SEM	n	ANOVA versus CTR
CTR	2827	122.0	4	NS
N	2941	73.81	7	0.7262
V	2607	75.39	4	0.3623
A	2892	90.58	8	0.9235

	Volume tissue loss Fig. 5C			
MCAO	Mean (%)	SD	n	ANOVA versus CTR
CTR	–17.3	7.8	11	NA
N	–19.5	10.8	7	0.8877
V	–10.4	6.4	12	0.0806
A	–4.5	5.0	10	0.001

Tables related to cerebral blood flow (CBF)

CBF 10 min reperfusion as % from baseline (100%)

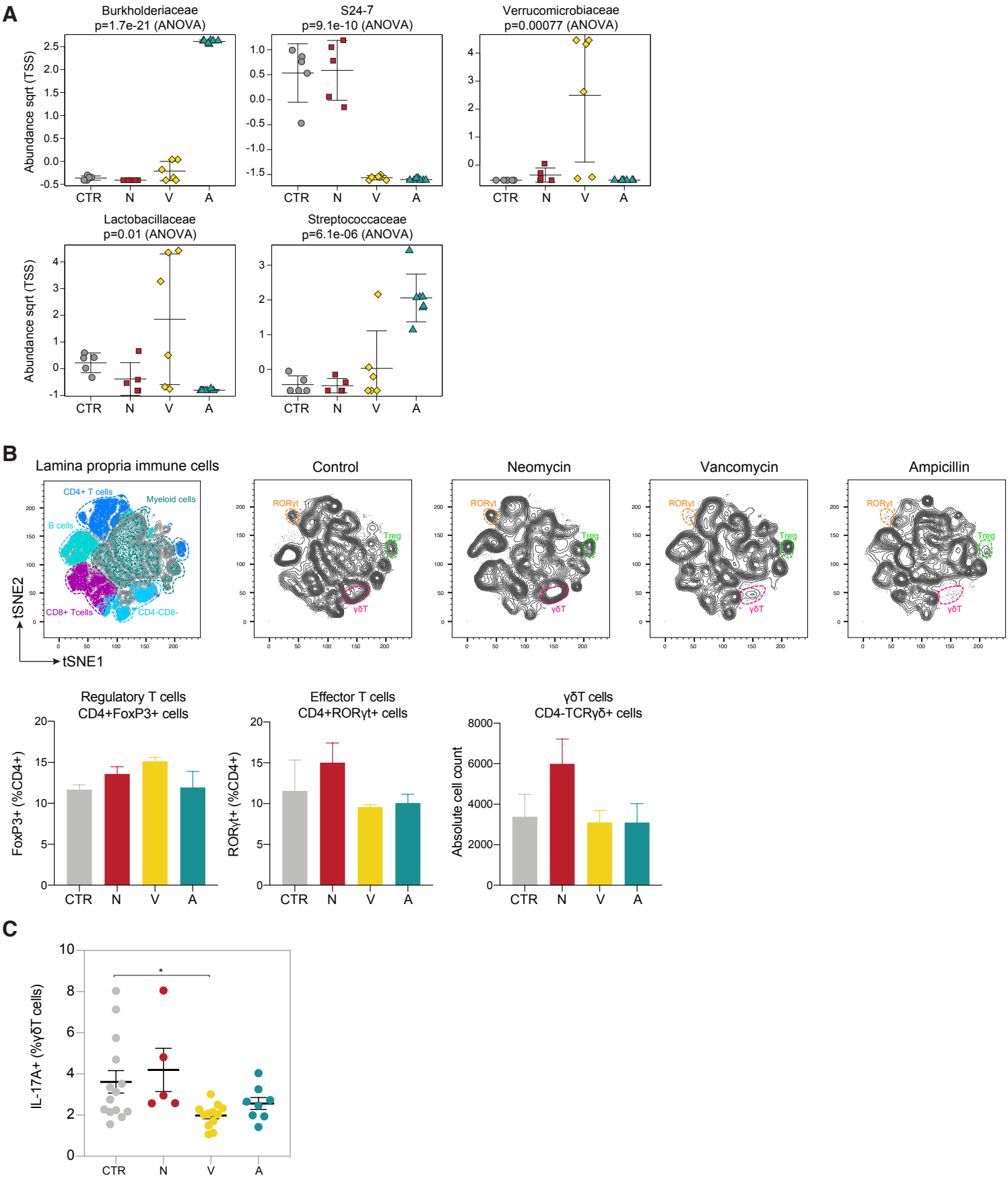
	Mean (%)	SD	n	ANOVA versus CTR
CTR	113.5	27.3	10	NA
N	111.0	8.8	10	0.9913
V	131.6	25.8	10	0.2404
A	132.4	29.2	10	0.2111

CBF 20-30 min reperfusion as % from baseline (100%)

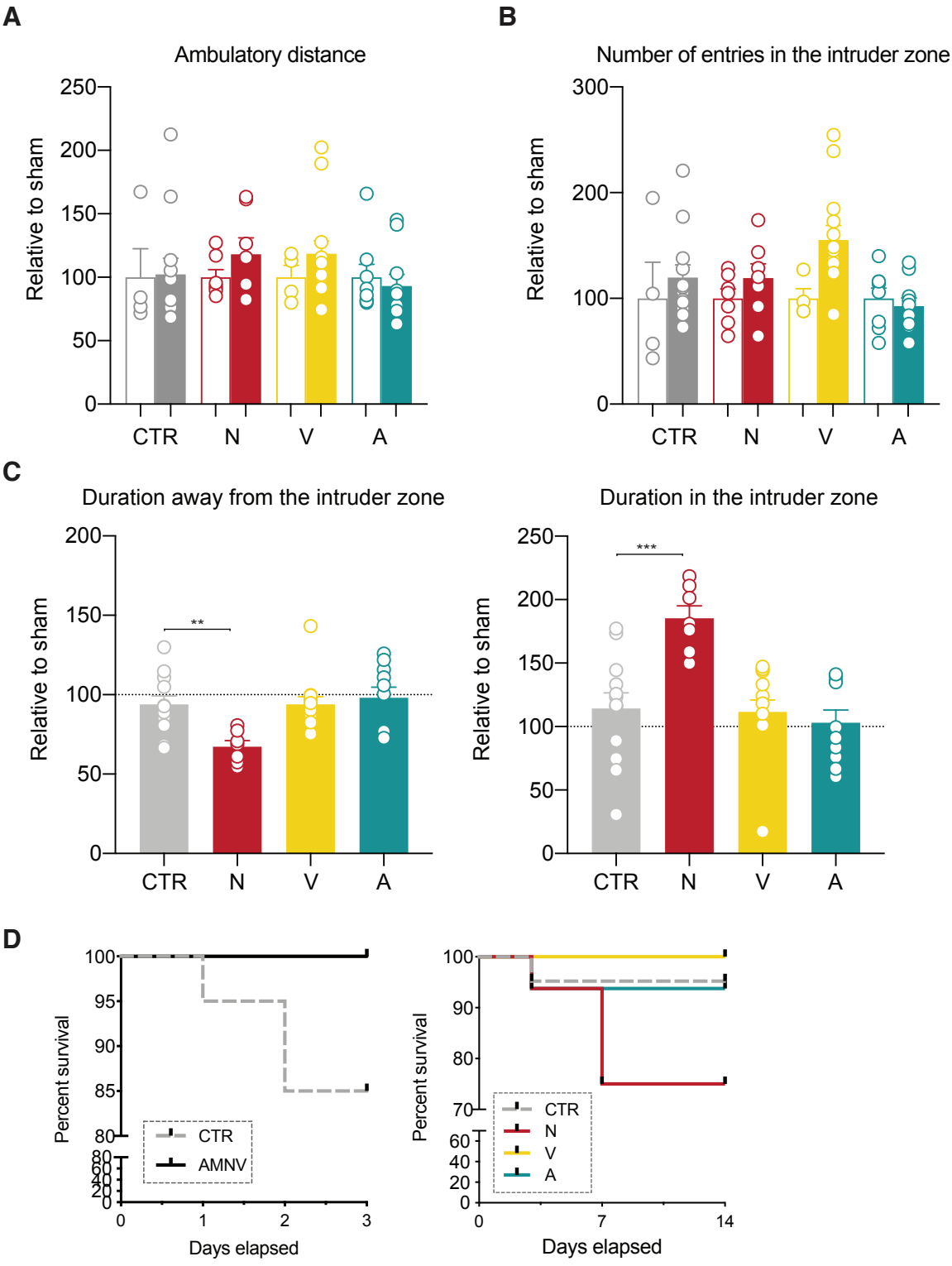
	Mean (%)	SD	n	ANOVA versus CTR
CTR	113.5	34.8	10	NA
N	108.3	20.02	10	0.9705
V	123.8	37.6	10	0.8241
A	127.6	35.8	10	0.6560

CBF during occlusion as % from baseline (100%)

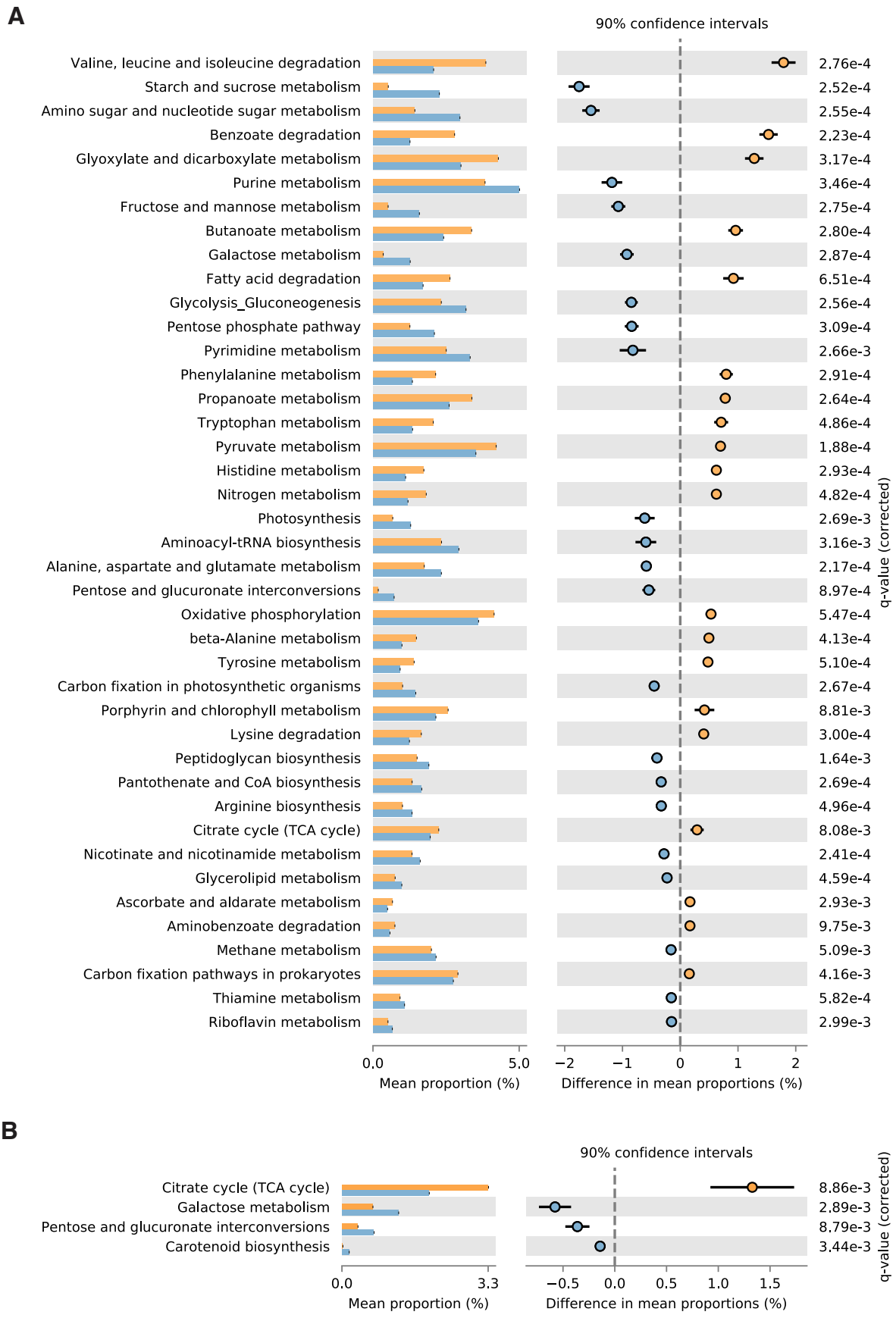
	Mean (%)	SD	n	ANOVA versus CTR
CTR	6.3	3.5	10	NA
N	7.9	3.4	10	0.5235
V	7.0	2.8	10	0.9221
A	7.2	2.6	10	0.8531



Supplementary Figure I. Specific bacteria families are enriched in mice treated with antibiotics and are associated with an anti-inflammatory milieu in the gut. A) Abundance of bacteria at the family level in mice treated with single antibiotic for 4 weeks. **B)** Top row, t-Stochastic eighbor embedding (t-SNE) representation of immune cells in the lamina propria of control and antibiotic-treated mice. Plot 1 shows clusters identifiying immune cell subtypes based on the expression of specific antibody markers analyzed by FACS. Plots 2 to 4 show cell density for the different clusters shown in plot 1. Clusters for the anti-inflammatory regulatory T cells (Treg, green), the pro-inflammatory effector T cells CD4+RORγt+ (RORγt, orange) and γδ T cells (magenta) in control and antibiotic-treated mice. Bottom row indicates quantification of the different T cell poulation indicated on the t-sne plots (n=3-5, no significant difference between groups). **C)** Quantification of IL-17 producing γδT cells in the lamina propria of the small intestine in control mice and in mice treated for 4 weeks with either neomycin (N), vancomycin (V) or ampicillin (A).(CTR: 3.62%±0.54, n=14; N: 4.19%±1.05, n=5; V: 1.98%±0.16, n=12; A: 2.56%±0.29, n=8; CTR versus N: p=0.9442; CTR versus V: p=0.0328; CTR versus A: p=0.2692).



Supplementary Figure II. Mouse behavior in the open field test following antibiotic treatment in sham and stroke mice. Distance travelled **(A)** and number of entries in the intruder zone **(B)** assessed by the open field during the introduction of a foreign foreign mouse (intruder) 14 days after stroke (plain bars) or sham surgery (empty bars). Ambulatory distance relative to sham: CTR: 102±9, n=12; N: 118±13, n=7; V: 119±10, n=13; A: 93±9, n=10; mean±SEM, p=0.334; number of entries in the intruder zone relative to sham: CTR: 119±12, n=12; N: 119±13, n=7; V: 155±14, n=13; A: 93±8, n=10; mean±SEM, ANOVA **p=0.007, multiple comparisons: non-significant). **(C)** Duration spent in the zone away (left) and close to the intruder (right) as relative to sham (ANOVA: **p<0.01, ***p<0.001, mean±SEM). **D)** Survival curve after MCAO. Left graph indicates the percent survival in control (CTR, survival proportion: 85%) and AMNV (100%) mice before sacrifice at 3 days post-MCAO. Right graph indicates the percent survival in CTR (95.2%), N (75%), V (100%) and A-treated mice (93.8%) for the 14 days before sacrifice.



Supplementary Figure III. Enzymatic pathways regulated in the gut of antibiotic treated mice. Significantly regulated enzymatic pathways in **A)** ampicillin-treated mice and **B)** vancomycin-treated mice (orange) in comparison to control (blue). Data were analyzed with the STAMP software (vers. 2.1.3).

*** Preclinical Checklist**

Preclinical Checklist: Prevention of bias is important for experimental cardiovascular research. ***This short checklist must be completed, and the answers should be clearly presented in the manuscript.*** *The checklist will be used by reviewers and editors and it will be published. See "Reporting Standard for Preclinical Studies of Stroke Therapy" and "Good Laboratory Practice: Preventing Introduction of Bias at the Bench" for more information.*

This study invovles animal models:
Yes

Experimental groups and study timeline

The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study:	Yes
An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated:	Yes
An overall study timeline is provided:	N/A

Inclusion and exclusion criteria

A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article:	Yes

Randomization

Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided:	Yes
Type and methods of randomization have been described:	Yes
Methods used for allocation concealment have been reported:	Yes

Blinding

Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible:	Yes
Blinding procedures have been described with regard to masking of group assignment during outcome assessment:	Yes

Sample size and power calculations

Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided:	Yes

Data reporting and statistical methods

Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups:	Yes
Baseline data on assessed outcome(s) for all experimental groups have been reported:	Yes
Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms:	Yes
Statistical methods used have been reported:	Yes
Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures:	Yes

Experimental details, ethics, and funding statements

Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described:	Yes
Different sex animals have been used. If not, the reason/justification is provided:	Yes

Statements on approval by ethics boards and ethical conduct of studies have been provided:	Yes
Statements on funding and conflicts of interests have been provided:	Yes

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