SUPPLEMENTAL MATERIAL

Gut microbiota-dependent TMAO predicts risk of cardiovascular events in patients with stroke and is related to proinflammatory monocytes

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Major Resources Tables

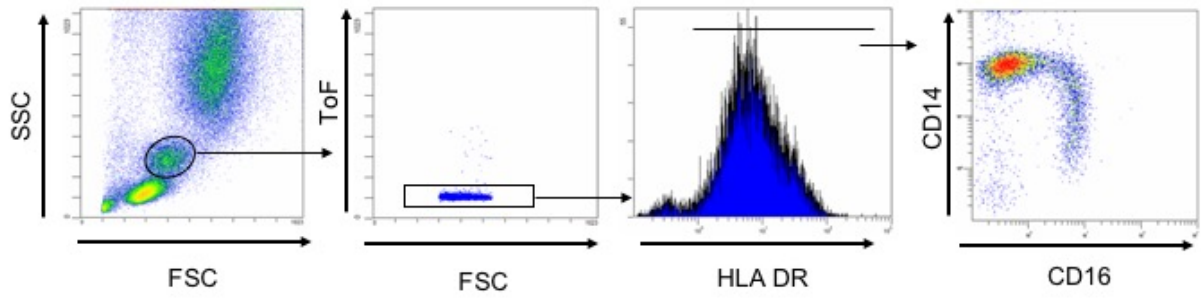
Animals

Species/Strain	Vendor or Source	Background Strain	Sex
C57BL/6J	Charles River	C57BL/6J	male

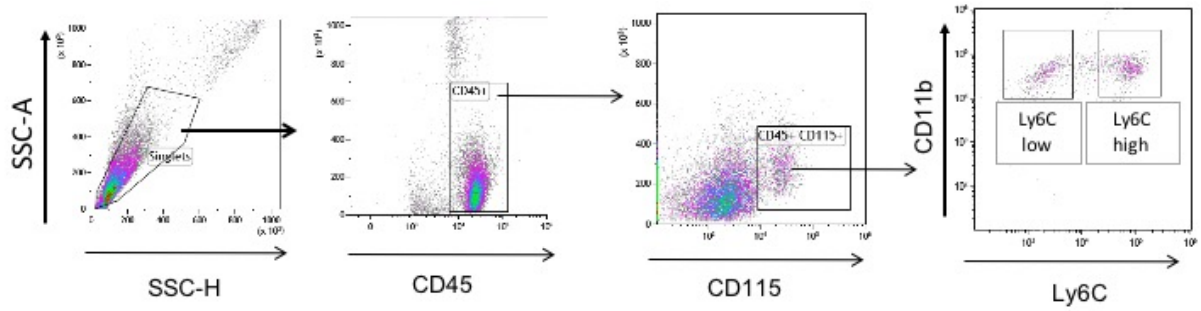
Antibodies

Target antigen	Vendor or Source	Catalog #	Working concentration	Lot # (preferred but not required)
anti-CD14-APC-H7	BD Biosciences	561384	5 µl/100µl sample	
HLA-DR-FITC	Biolegend	307604	10 µg/ml	
anti-CD16 eVolve™ 605	eBioscience	83-0168-42	5 µl/100µl sample	
anti-mouse Ly6C (Gr1) PerCp/Cy5.5	Biolegend	108427	4 µg/ml	B194615
anti-mouse CD45 PE/Cy7	Biolegend	103113	4 µg/ml	B205174
anti-mouse CD115 AF488	Biolegend	135511	10 µg/ml	B180483
anti-mouse CD11b BV711	Biolegend	101241	4 µg/ml	B203911
anti-mouse CD117(c-kit) APC	Biolegend	105811	4 µg/ml	B217854
anti-mouse Lineage Cocktail(CD3/Ly-6G(Ly-6C)/CD11b/CD45R(B220)/Ter-119) Pacific Blue	Biolegend	133305	2 µl/50µl sample	B234789
Anti-mouse Ly-6A/E(Sca-1) APC/Cy7	Biolegend	108125	4 µg/ml	B234287
anti-mouse CD135 (FLT3) PE Antibody	Biolegend	135305	4 µg/ml	B241121

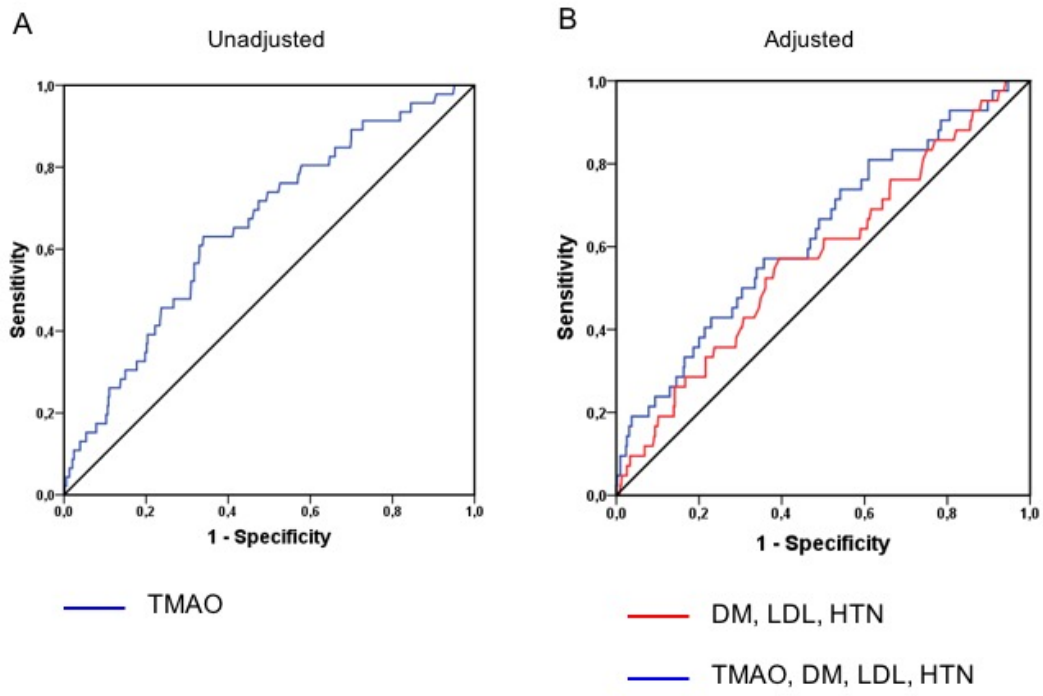
Supplemental Figures and Figure Legends



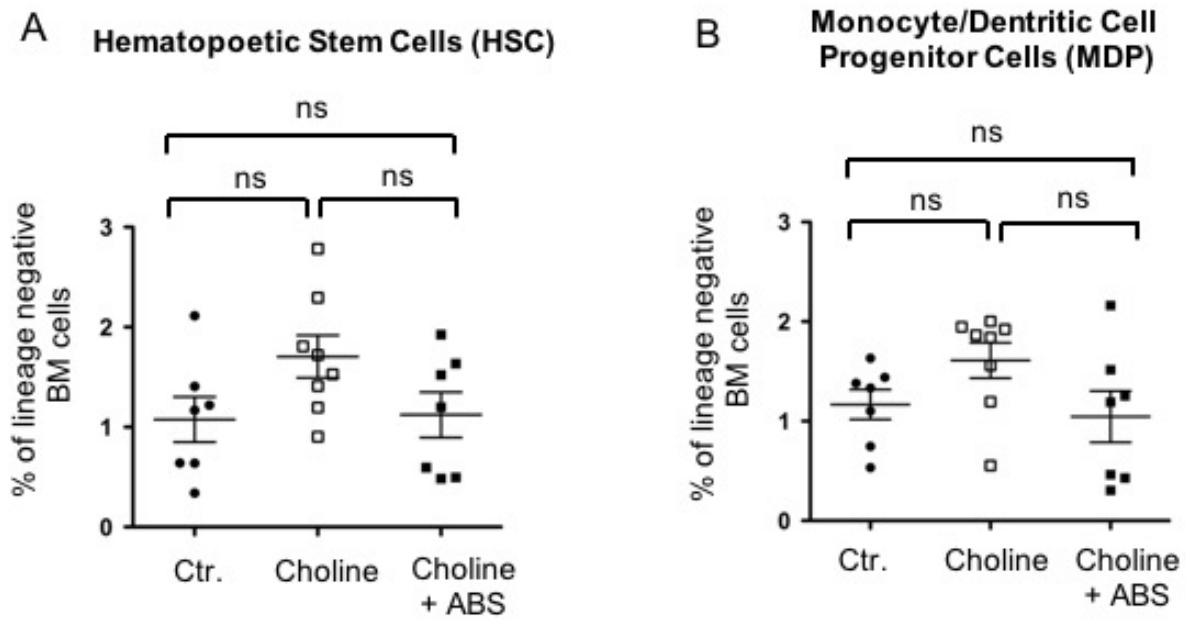
Supplemental Figure I. Gating strategy for identification of human monocyte subsets. Monocytes were identified as HLA DR^{+} after pre-selection in side scatter (SSC) vs. forward scatter (FSC) dot plot and FSC vs. Time-of-Flight (ToF) dot plot. Afterwards, monocytes were further classified according to CD14/DC16 expression as classical ($CD14^{++}CD16^{-}$), intermediate ($CD14^{++}CD16^{+}$) and non-classical monocytes ($CD14^{+}CD16^{++}$).



Supplemental Figure II. Gating strategy for identification of murine monocyte subsets. After doublet exclusion CD45 positive cells were selected and further stratified by CD115 expression. CD115 positive cells were further stratified according to CD11b and Ly6C expression.



Supplemental Figure III. ROC curve analyses relating TMAO levels to 1-year incident cardiovascular risk. (A) illustrates the AUC for unadjusted TMAO and (B) depicts combined ROC curve analyses of diabetes, hypertension, LDL with (blue) and without (red) TMAO. Note that adding TMAO to classical cardiovascular risk factors results in higher AUC. DM: diabetes mellitus; HTN: hypertension.



Supplemental Figure IV. Impact of choline supplemented diet on hematopoietic stem and progenitor cells levels in the bone marrow. Graphs depicting proportions of HSC (left) and monocyte/dentritic cell progenitor cells (right) in mice with normal chow diet (ctr.) and in choline supplemented diet with intact gut microbiome (choline) or with depleted gut microbiota (choline + ABS), respectively.

Detailed Methods

Study populations and study design

Two prospective cohorts of patients with first-ever ischemic stroke were examined. The primary outcome measure of both cohorts was a combined vascular end-point composed of myocardial infarction (MI), recurrent stroke and cardiovascular death during 1-year follow-up on a structured interview with the patient or their relatives and, if indicated, with additional information from the hospital or physician.

The inclusion criteria of both studies were: (1) Age 18 years; (2) first ever acute stroke that occurred with stroke onset in the last seven-days and (3) written informed consent by patient or legal guardian prior to study participation. The exclusion criteria were: (1) Prior stroke; (2) patients presenting brain tumor or brain metastasis and (3) participation in an intervention study. Both studies were approved by the respective local research ethics committee. All participants with adjudicated diagnosis of ischemic stroke provided written informed consent and were prospectively enrolled. For evaluation of acute ischemic stroke, diffusion weighted magnetic resonance imaging (Siemens Verio 3.0 Tesla) was performed.

In the first cohort, 78 patients with complete data on primary outcome were enrolled. The relation of the gut microbe-dependent metabolite TMAO with the risk of subsequent (1-year) cardio- and cerebrovascular events (CVE) including myocardial infarction, recurrent stroke and cardiovascular death was examined.

After finding a significant relation between TMAO with the cardiovascular risk in the first cohort, a second larger independent validation cohort including 608 patients from the 'Prospective Cohort with Incident Stroke' study (PROSCIS) with first-ever stroke conducted at the Center for Stroke Research Berlin in Germany (PROSCIS-B: Center for Stroke Research Berlin, Charité University Hospital) was examined with 1-year of follow-up to evaluate if the observed relation persists in an independent non-overlapping patient population. Study protocol and design of the PROSCIS study were described previously[1]. Of these patients 593 patients with complete data on the primary outcome and all variables required for adjustment for co-morbidities and cardiovascular risk factors including diabetes, arterial hypertension, low-density lipoprotein (LDL) cholesterol and history of peripheral or coronary artery disease or myocardial infarction were subjected to TMAO analyses.

Based on the primary outcome data of the first study and given a test significance level a of 0.05 and a 90% (α=0.1) rectangular confidence, an 80% power would be attained with a minimum of 556 patients (139 patients per TMAO quartile) with 1-year of follow-up. Accordingly, the validation cohort consisting of 593 patients was of adequate size.

Blood tests

Laboratory workup was performed for total cholesterol, low- and high-density lipoproteins, triglyceride, C-reactive protein, and full blood count. Blood samples were immediately processed and frozen at −80°C for further analyses. Levels of choline and TMAO were quantified by stable isotope dilution liquid chromatography with online tandem mass spectrometry as previously described[2], using a Shimadzu LCMS-8050 CL Triple Quadrupole Mass Spectrometer interfaced with a Nexera LC-30AD CL Ultra High Performance Liquid Chromatograph (UHPLC) system. With every batch of 30 samples tested, three different concentrations of a TMAO quality control were included throughout the analyses of all samples for the study. The analytical intraday and inter-day coefficients of variance observed for the quality controls were < 3.3% and 5.0%, respectively, across the range of TMAO levels. Investigators performing analyses were blinded to sample identity and the patients` clinical characteristics and primary outcome.

Analysis of monocyte subsets in humans

Flow cytometry was used to determine human monocyte subsets as previously described[3]. Blood samples were taken by venipuncture and transferred immediately on ice to the lab and prepared directly without time delay for flow cytometry analysis. Absolute monocyte counts were determined using an automated hematology analyzer (XT-2000i, Sysmex). Whole blood (100 µl) was incubated with antibodies for 20 minutes at room temperature in the dark. Versalyse Lysing Solution1 (Beckman Coulter) was used to lyse red blood cells. After pre-selection (side scatter and forward scatter) monocytes were identified as HLA-DR positive and classified according to CD14 and CD16 expression: $CD14^{++}CD16^{-}$ as classical monocytes, $CD14^{+}CD16^{++}$ as non-classical monocytes and $CD14^{++}CD16^{+}$ as intermediate monocytes (Supplementary Figure S1). Data were acquired on a GalliosTM flow cytometer and analyzed with GalliosTM software (Beckman Coulter). The following antibodies were used (please see the Major Resources Table in the Supplemental Material): anti-CD14-APC-H7 (BD Biosciences, 561384), anti HLA-DR-FITC (Biolegend, 307604); anti-CD16 eVolve™ 605 (eBioscience, 83-0168-42).

Mouse experiments

All animals were bred, raised and housed in the facilities of the "Forschungseinrichtungen für Experimentelle Medizin" (FEM, Charité – University Medicine Berlin, Germany) under specific pathogen-free (SPF) conditions. Adult (10 weeks of age) C57BL/6J mice (Charles River) were placed on normal chow (0.2% choline) or choline supplemented diets (1.3% choline) (Ssniff, Soest, Germany) for 3 weeks prior to monocyte subset analysis. At the end of the 3 weeks period mice were sacrified for collection of blood and bone marrow (BM). Mouse experiments were approved by the research advisory committee and permitted by LAGeSo (Landesamt für Gesundheit und Soziales Berlin). We performed the experiments only in mice of one sex to avoid sex-related variations of the microbiota related effects on metabolism of choline and TMAO production with potential impact on monocyte subset regulation.

Generation of secondary abiotic (gnotobiotic) mice

Secondary abiotic mice were generated through quintuple antibiotic treatment (+ABS) for 6 weeks via the drinking water. To achieve this, mice were transferred to sterile cages and treated with a mix of ampicillin plus sulbactam (1 g/L), vancomycin (500 mg/L), ciprofloxacin (200 mg/L), imipenem (250 mg/L) and metronidazol (1 g/L) in autoclaved tap water (ad libitum). The intestinal colonization status of the mice was controlled once a week by highly sensitive cultural analysis of fecal samples as described earlier[4]. As early as three weeks of broad-spectrum antibiotic treatment quality controls indicated virtually complete eradication of the intestinal microbiota, as demonstrated by negative results derived from both, culture and molecular detection of bacteria using real-time PCR targeting of bacterial 16S rRNA genes[5]. To avoid contaminations, mice were continuously kept in a sterile environment (autoclaved food and drinking water, sterile filtered antibiotic cocktail) and handled under strict aseptic conditions.

Analysis of blood monocyte subsets in mice

Blood samples were collected by cardiac puncture using EDTA-coated syringes. Whole blood (100 µl) or BM cell suspension was incubated with antibodies for 20 minutes at room temperature in the dark. After doublet exclusion monocytes were identified as CD45 positive and CD115 positive and further selected by highly positive

expression of CD11b, a marker of dendritic cells, monocytes and granulocytes, shown in the CD11b/Ly6C plot. Monocytes were further classified according to Ly6C expression. The following antibodies were used (please see the Major Resources Table in the Supplemental Material): anti-mouse Ly6C (Gr1) PerCp/Cy5.5 Antibody (Biolegend, B194615); anti-mouse CD45 PE/Cy7 Antibody (Biolegend, B205174); anti-mouse CD115 AF488 Antibody (Biolegend, B180483); anti-mouse CD11b BV711 Antibody (Biolegend, B203911). Detailed gating strategy for identification of monocyte subsets is shown in Supplemental Figure II.

Analysis of bone marrow hematopoietic stem cells and progenitor cells in mice
BM cells were collected from both femur and tibia of mice by flushing the BM with Dulbecco's PBS. Harvested cells were filtered through a 70mm cell strainer and washed with PBS. Erythrocytes from all samples were lysed with red blood cell (RBC) lysis buffer. BM cell suspension was incubated with antibodies for 20 minutes at room temperature in the dark. After pre-selection (side scatter and forward scatter) the Lineage negative cells were gated in a histogram and hematopoietic stem cells (HSC) were identified as the C-kit positive and Sca1 positive population and further selected by positive expression of CD135 and CD115 as monocyte and dendritic cell progenitors (MDP). The following antibodies were used (please see the Major Resources Table in the Supplemental Material): anti-mouse CD117(c-kit) APC Antibody (Biolegend, B217854), anti-mouse Lineage Cocktail(CD3/Ly-6G(Ly-6C)/CD11b/CD45R(B220)/Ter-119) Pacific Blue Antibody (Biolegend, B234789), anti-mouse Ly-6A/E(Sca-1) APC/Cy7 Antibody (Biolegend, B234287), anti-mouse CD135 PE Antibody (Biolegend, B241121), anti-mouse CD115 AF488 Antibody (Biolegend, B180483).

Statistical Analyses
Database management and statistical analyses were performed with PRISM version 5.0a (GraphPad Software Inc., USA) and SPSS version 23 (IBM SPSS, USA).
Mouse studies:
Continuous data were subjected to the Kolmogorov–Smirnov test to determine their distribution and were expressed as mean±standard deviation (SD) or median and range. Comparison of means was performed by independent t-test. and Wilcoxon rank-sum test of medians was used if data were not normally distributed. For continuous variables, univariate correlation analyses were performed.
Human studies:
For comparison of basic patients' characteristics continuous data were subjected to the Kolmogorov–Smirnov test to determine their distribution and were expressed as mean±standard deviation (SD) or median and range. Comparison of means was performed by independent t-test and Wilcoxon rank-sum test of medians was used if data were not normally distributed. Kaplan–Meier survivor curves were examined to assess the relationship between quartiles of TMAO or choline levels timing of events during follow-up, and the log-rank test for statistical assessment. Cox regression forward selection was used to further characterize the relation of TMAO and choline to outcome. To determine whether TMAO was independently related to outcome, three cox regression models were used: In model 1 the outcome was adjusted for sex and age, in model 2 outcome was adjusted for model 1 and cardiovascular risk factors including hypertension, diabetes, LDL cholesterol and eGFR and in model 3 the outcome was adjusted for model 1 and additionally for stroke severity, stroke etiology (TOAST)[6], cardiovascular risk factors including hypertension, diabetes, LDL cholesterol, history of peripheral or coronary artery disease or myocardial infarction.

For additional comparison of the prognostic value of TMAO with regard to CVE or stroke at 1-year, receiver operating characteristic (ROC) curves were generated, and the areas under the curves (AUC) were calculated. To assess the prognostic value of TMAO in patient subgroups Chi-Square and Odds ratio analyses were performed. Additionally, combined ROC curve analyses of the cardiovascular risk factors diabetes, hypertension and dyslipidemia with and without TMAO were performed to assess the additive prognostic value of TMAO over classical cardiovascular risk factors.
Parametric correlation analyses were used to evaluate the correlation between TMAO and intermediate $CD14^{++}CD16^{+}$ monocytes. Multiple linear regression analysis was performed to further assess the correlation between TMAO levels and intermediate monocyte subsets after adjustment for age, sex and the cardiovascular risk factors including diabetes, hypertension, dyslipidemia and smoking. Significance was assumed at a two-sided value of $P \leq 0.05$.

References:

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