Supplemental Experimental Procedures

Materials, general procedures and ethical considerations
All animal procedures were approved by the Cleveland Clinic Institutional Animal Care and Use Committees (IACUC). Mice were housed in a facility fully accredited by AALAC and in accordance with all federal and local regulations. Mouse plasma total cholesterol and triglycerides were measured using Abbott ARCHITECT platform model ci8200 (Abbott Diagnostics). The Cleveland Clinic Institutional Review Board (IRB) approved all human subject studies. All human study samples used were collected from consented subjects.

Platelet preparation and aggregometry assays in humans
Whole blood was collected from consenting healthy donors using 0.109 M sodium citrate as anticoagulant. Platelet rich plasma (PRP) was separated by centrifuging at 100 x g for 10 min at 22°C. Platelet poor plasma (PPP) was prepared by further centrifugation at 11,000 x g for 2 min. Platelets were counted using a hemocytometer and for aggregometry assays, concentrations were adjusted to 2 x 10^8/ml with PPP. To prepare washed platelets for studies, 100 nM prostaglandin E1 (PGE-1) was added to PRP and the PRP was then centrifuged at 500 xg for 20 min at 22°C. The platelet pellet was gently washed with a modified phosphate buffer saline (137 mM NaCl, 2.7 mM KCl, 12 mM Na[PO_4], 1 mM $MgCl_2$, and 5.5 mM glucose, pH 7.4) with 100 nM PGE-1, and centrifuged again at 500 xg for 20 min. Platelet pellets were then re-suspended in modified Tyrode's buffer (137 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.4 mM NaH_2PO_4, 5 mM HEPES, 5.6 mM glucose, 0.35% BSA, pH 7.4). ADP (up to 15 μM) and thrombin (up to 0.2 units) were used as indicated to initiate aggregation with constant stirring (600 rpm). In some experiments, platelets were pre-incubated with TMAO (100 μM final or the indicated concentration) for 30 minutes at 22°C before platelet aggregation was performed.

Measurement of inositol phosphates
Washed platelets (5×10^8 platelets/mL) were gently resuspended in stimulation buffer (10 mM HEPES, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, 4.2 mM KCl, 146 mM NaCl, 5.5 mM glucose, and 50 mM LiCl, pH7.4). Platelets were pre-treated with the indicated final concentration of TMAO (either 0 (vehicle), or 3.1, 10, 31, or 100 μM) for 30 minutes at room temperature prior to addition of thrombin (0.2 U/mL) on a 24-well plate for 1 h at 37 °C. Platelets were lysed with lysis reagent at 37°C for 30 min, followed by immediate measurement of inositol phosphate levels according to the manufacturer's protocol for the IP-One ELISA kit (Cisbio Bioassays, Bedford, MA).

Whole blood *in vitro* thrombosis assay:
Microfluidics experiments were performed using the Cellix Microfluidics System (Cellix Ltd., Dublin, Ireland). Following addition of fluorescent label, blood was incubated for 30 min at 22ºC in the presence vs. absence (saline control) of additional TMAO (100 μM), before being perfused over chips coated with or without immobilized type 1 collagen (150 μg/ml). Images of fluorescently labeled platelets adhering to the collagen coating were captured every 2 seconds on a Leica DMI6000 inverted microscope equipped with an environmental chamber and cooled CCD camera, and computer assisted image quantification used for analyses. Where indicated, each micro channel of a Vena8 Fluoro+ biochip was coated with 15 μl of type 1 collagen (150 μg/ml) and the biochip was then place in a humidified box overnight at 4ºC. Each channel of the Vena8Fluoro+ biochip was washed with 1X PBS using the Mirus Nanopump before placing the biochip on the microscope. Images were collected using an HC Plan Apo 20X/0.7NA lens on a Leica DMI6000 inverted microscope equipped with an environmental chamber and a Hamamatsu ImagEM cooled CCD camera. Whole blood collected from consented healthy volunteers was fluorescently tagged with Calcein AM and was pretreated with TMAO (100 μM final, pH 7.4) or normal saline control for 30 min at 22ºC. After the incubation, blood was then perfused over chips coated with or without immobilized type 1 collagen (150 μg/ml) at physiological shear rate (67.5 dynes/cm^2) using a multi-channel microfluidic device for 3 minutes. Images of fluorescent platelets adhering to the collagen coating were captured every 2 seconds during that time. At the end of the experiment, the tube containing the platelets was removed and the 1X PBS in the biochip reservoir was drawn through the channel at 2.5 dynes. Ten images were captured along the length of the channel during that time. Platelet activation and adherence to the collagen surface was then quantified with computer assisted tomographic analyses as previously described (Srikanthan et al., 2014).

Intracellular Ca^{2+} measurements:
Ratiometric fluorescence measurements were conducted with 8x10^8 washed human platelets re-suspended in Hank's buffered salt solution supplemented with BSA and glucose (HBSS-BSA-glucose; 0.137 M NaCl, 5.4 mM KCl, 0.25

mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 1.0 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 5 mM glucose and 0.1% BSA) and incubated with 1 μM Fura 2-AM at 22°C for 30 min. Excess Fura 2-AM was removed by centrifuge at 100 xg for 5min. Changes in $[Ca^{2+}]_i$ was monitored by measuring Fura 2-AM fluorescence using 340/380 nm dual–wavelength excitation and an emission of 510nm at 37°C with constant stirring in a temperature controlled spectrofluorometer (Photon Technology International). In some experiments, Ca^{2+} release and entry were measured, respectively, in Ca^{2+}-free (0 mm Ca^{2+} and 0.1 mM EGTA) for 10 minutes, and then in 1 mM Ca^{2+} for another 15 min. (Regodón et al., 2009; Zhu et al., 2012).

Mouse *ex vivo* platelet aggregometry studies:
Mice were anesthetized with ketamine (90 mg/kg) and xylazine (15 mg/kg). Whole blood (600 μl) was collected from the inferior vena cava into 0.109 M sodium citrate (100 μl) and then diluted with an additional 500 μl Ca^{2+}/Mg^{2+} free modified Tyrode's buffer. Diluted platelet rich plasma was separated by centrifuging at 100 xg for 10 min at 22°C. Diluted platelet poor plasma was prepared by further centrifugation at 11,000 x g for 2 min. In some experiments, the platelet poor plasma went through an additional ultracentrifugation (100,000 x g, 2 hour) spin to remove all microparticles and exosomes. Platelets were counted using a hemocytometer and concentrations adjusted to 2 x 10^8/ml with platelet poor plasma. $CaCl_2$ and $MgCl_2$ (both 1 mM final concentration) were added immediately before platelet aggregation studies. Platelet aggregation in response to 1 μM ADP was assessed at 37°C in a dual channel Type 500 VS aggregometer (Chrono-log Corporation, Havertown, PA) with stirring at 1200 rpm.

$FeCl_3$ carotid artery injury thrombosis model
Typically, 4 week old C57BL/6J female mice were placed for 6 weeks on the indicated chemically defined diets (± oral broad-spectrum antibiotics (ABS) provided in the drinking water, where indicated) as previously described (Wang et al., 2011). Mice were subjected to common carotid artery injury by application of 10% FeCl3 for 1 min as previously described (Chen et al., 2008). Rhodamine 6G (100μl; 0.5mg/ml) was injected directly into the right jugular vein to label platelets and thrombus formation was observed in real time using intravital fluorescence microscopy and video image capture. Time to complete cessation of blood flow was determined by visual inspection of computer images and end points were set as cessation of blood flow for more than 30 sec or observation of 30 min (Chen et al., 2008).

Rose Bengal photochemical injury thrombosis model
C57BL/6J female mice (13 weeks old) were anaesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Rose Bengal (10 mg/ml in saline) was injected directly into jugular vein to a dose of 50 mg/kg. The mid portion of common carotid artery was isolated and a Doppler ultrasound flow probe (model 0.5VB; Transonic System, Ithaca, NY) was placed around it. The probe was connected to a flow meter (Model TS420, Transonic System, Ithaca, NY). Then the artery was illuminated 5cm from the artery with 1.5mW green light laser source (540nm; Melles Griot, Carlsbad, CA). Blood flow was monitored by the flow meter. The time to occlusion was determined after the vessel remained closed for 10 min (Furie and Furie, 2005; Wang Y et al., 2014).

Germ-free and conventionalization studies
C57BL/6J female mice (4 week old) were placed for 6 weeks on the sterilized chemically defined diets including chow (0.08% total choline) versus chow diet supplemented with 1% choline. Chow and choline diets were sterilized by autoclave. Because the autoclave settings needed to sterilize the food for germ free studies were found to cause deterioration of TMAO, to supplement mice with TMAO, mice were instead placed on sterile chemically defined chow diet (0.08% total choline) and TMAO (0.12% v/v) was provided in sterile drinking water. For conventional mice studies, TMAO supplementation (0.12 gm%) was provided in the chemically defined diet and the food irradiated (analyses showed this did not degrade the TMAO, but was insufficient sterilization for the germ free studies). The content of total choline and TMAO in food was determined using established LC/MS/MS methods (Wang et al., 2011). On the day of *ex vivo* platelet functional analysis with platelet aggregometry, or *in vivo* thrombosis assay, blood (less than 100 μl) was draw from the saphenous vein and plasma was recovered for TMAO analyses (Wang et al., 2014). For conventionalization studies, 16 to 22 week-old female C57BL/6J germ-free mice were used. Immediately following removal from the germ-free microisolator shipper, germ-free mice were conventionalized by being housed in cages spread with conventional C67BL/6J female feces and fed with chemically defined chow (0.08% total choline) versus chow diet supplemented with 1% choline for 3 weeks before the evaluation of plasma TMAO and platelet function.

Microbiota studies and statistical analyses

Microbial community composition was assessed by sequencing of the V4 hypervariable region of the 16S rRNA gene derived from mouse cecal samples as previously described (Gregory et al., 2015). Briefly, cecal microbial DNA was isolated using the MoBio PowerSoil DNA isolation kit according to the manufacturer's instructions (MO BIO Laboratories, Carlsbad, CA) and sequenced according to previously described methods (Caporaso et al., 2012) using the Illumina MiSeq platform at the GenoSeq Facility at the University of California, Los Angeles. De-multiplexing sequences, quality control and operational taxonomic unit (OTU) binning were performed using the open source pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0 (Caporaso et al., 2010; Bokulich et al., 2013). Sequences that passed quality control were binned into OTUs based on 97% identity using UCLUST (Edgar, 2010) against the Greengenes reference database (version 13-08) (McDonald et al., 2012). For Principal Coordinate Analyses, each sample's sequences were rarefied to 6000 reads to reduce the effect of sequencing depth. To find OTUs that exhibited significant differences between groups the Linear discriminate analysis effect size (LEfSe) analysis was used (Segata et al 2011). All data were analyzed using R software version 2.15 (JMP, SAS Institute). False discovery rates (FDRs) of the multiple comparisons were estimated for each taxon based on the p values resulting from Spearman correlation estimates. The Wilson rank-sum test was used for two-group comparison.

Metagenomic Analyses

To determine whether the transcriptional activity of microbiota contribute to variation in TMA synthesis, we performed shotgun sequencing of the gut microbes in mice fed a diet containing choline, TMAO or a control chow diet (5 mice per group). Microbial DNA from mouse cecum was extracted using PowerSoil DNA Isolation Kit (MoBio Laboratories). Microbial genomic sequences were analyzed using the Illumina HiSeq2000 (2 lanes). FastQC (Babraham Bioinformatics) was used to check quality and identify adapter contamination. Cutadapt (Python.org) was used to trim adapter sequences and remove the 101st base from each read, discarding any resulting reads less than 50 bp, leaving from 10.5 million to 21 million reads per sample. The resulting sequence data was aligned to the 2015 NCBI-NR (non-redundant) protein database (Benson et al., 2005) using the DIAMOND (Buchfink et al., 2015) alignment tool, resulting in 4-7 million reads per sample with alignments. MEGAN5 (Huson et al., 2011) was used to assign taxonomy (LCA-assignment) and function [KEGG (Kanehisa and Goto, 2000) and SEED (Overbeek et al., 2005) via gi numbers], with the following parameters: maxMatches=100 minScore=50.0 maxExpected=1.0 topPercent=10 minSupport=5 minComplexity=0.44 useMinimalCoverageHeuristic=false useSeed=true useCOG=false useKegg=true paired=false useIdentityFilter=false textStoragePolicy=0 blastFormat=BlastTAB mapping='Taxonomy:GI_MAP=true,KEGG:GI_MAP=true,SEED:GI_MAP=true'. Additionally, MetaPhlAn (Segata et al., 2012) was used to get a less sensitive, but more accurate representation of the taxa present. MetaPhlAn uses a smaller reference database of carefully selected markers which correctly discriminate between species and are single copy per cell. Finally, the sequences were functionally characterized using MG-RAST on-line server (Meyer et al., 2008). The abundances of the various groups are presented as reads per sample following correction for differences in the number of reads per sample.

Cecal microbial transplant study

Germ-free C57BL/6J mice bred and housed at the National Gnotobiotic Rodent Resource Center University of North Carolina (UNC) were used as recipients of cecal microbes from two selected donor inbred strains maintained in conventional cages, C57BL/6J and NZW/LacJ. For at least 3 weeks preceding the start of the study, all mice (both donors and germ-free recipients) were maintained on the chemically defined chow diet (total choline 0.08%). Following donor mouse euthanization, cecal contents were removed and suspended in reduced PBS. Cecal content slurry was either flash frozen in sterile 10% glycerol in physiological saline and sent to UNC, or freshly prepared from newly arrived donor mice sent from CCF to UNC and used immediately for cecal content harvest and gavage. Cecal contents from one donor mouse were used to gavage approximately 5-10 mice. Prior to the first gavage, recipients were separated into two separate experimental isolators, one for the C57BL/6J recipients and the other for NZW/LacJ recipients. Mice were given three serial gavages of freshly prepared homogenized cecal contents over the first week (baseline, day 3 and day 7) and placed on sterile chow vs. choline-supplemented diet for 6 weeks, at which point mice were shipped to Cleveland Clinic in sterile microisolators, and immediately upon receipt, *ex vivo* enzyme activity assays on tissues, platelet aggregometry and *in vivo* thrombosis assays were carried out. TMA lyase activity was determined by the amount of d9-TMA released after incubation of bisected (lengthwise) mouse cecum *ex-vivo* with synthetic d9-choline under anaerobic conditions in gas tight vials as previously reported (Koeth et al., 2014).

TMA lyase activity assay
TMA lyase enzyme activity was determined by quantifying d9-TMA production from d9-choline substrate using stable isotope dilution LC/MS/MS analyses (Koeth R.A., et al, 2014). Briefly, at the time of mouse euthanization either the cecum was isolated, time permitting, or the entire intestine was excised, snap frozen and stored at -80°C until time of experiment. Each tissue was thawed at 4°C and the cecum was bisected lengthwise, and approximately 100 mg of cecum was incubated in gas tight sealed reaction vial in a 10 mM HEPES, pH 7.4 reaction buffer containing 400 μM d9-choline at 37°C for 20 hours. The reaction was stopped by placing the reaction vessels on ice and adding internal standard mix (10 μM of d4-choline and $^{13}C_3$,$^{15}N_1$TMA) in methanol containing 0.2N formic acid. Reaction mixtures were prepared for analyses and run on an AB SCIEX API 5000 triple quadrupole mass spectrometer. Unique parent-> daughter ion transitions for d9-TMA and $^{13}C_3$,$^{15}N_1$TMA isotopologues were monitored, integrated, and the amount of d9-TMA formed determined. Activity is shown as pmol d9-TMA / mg tissue / hour.

Liver FMO activity assay
Liver total FMO activity was determined by quantifying flavin dependent d9-TMAO production from d9-TMA substrate using stable isotope dilution LC/MS/MS analyses (Wang Z. et al, 2011). Briefly, livers (100 mg of tissue) were weighed and homogenized in 10 mM HEPES, pH 7.4, containing protease inhibitor cocktail. Protein concentration was determined from supernatants by BCA protein assay. FMO activity was measured in liver homogenates (200 μg protein buffered with 10mM HEPES, pH7.4) in a 250 μl reaction mixture containing d9-TMA (100 μM final) in the presence vs. absence of freshly prepared NADPH (100 μM final) for 8 hours at 37°C. Reactions were stopped by adding 50 μl 0.2N formic acid containing 100 μM d4-choline as internal standard. The concentration of d9-TMAO was quantified by stable isotope dilution LC/MS/MS, and FMO activity is shown as nmol d9-TMAO / mg protein / hour.

Statistical analysis
All experiments or animal studies were repeated at least 3 times and values were expressed as mean ± SE or SEM as indicated. The Kruskal Wallis test was used for multiple group comparison. The Wilson rank-sum test was used for two-group comparison. The Wilcoxon Signed-Rank Test was used for paired data analyses. Linear mixed effect models were used for repeated measure data analyses. Loglikelihood ratio tests were used to estimate the effect of different treatments. All data were analyzed using R 3.1.0 (Vienna, Austria) and JMP (SAS Institute). Pearson correlation coefficient for normally distributed data, and the Spearman rank correlation for non-normally distributed data, was used to analyze association between quantitative variables. Multilogistic regression analyses were performed for human clinical studies. Kaplan–Meier analysis with Cox proportional hazards regression was used for time-to-event analysis to determine Hazard ratio (HR) and 95% confidence intervals (95%CI). The Cox model was adjusted for age, gender, BMI, systolic blood pressure, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, triglyceride, estimated creatinine clearance, smoking, diabetes, medications (lipid lowering medication and antiplatelet therapy), and history of documented cardiovascular disease. For all statistical tests $p < 0.05$ was considered significant.

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Table S1: Effect of diet and antibiotics on platelet counts and other metabolic parameters, related to Figure 3.

	Chow	Chow +TMAO	Chow + choline	Chow + choline +ABS	Chow + ABS
Total Cholesterol(mg/dL)	46.3±7.8	66.5±9.2 (p=0.06)	62.3±2.4 (p=0.04)	61.0±8.0 (p=0.1)	52.6±5.7 (p=0.3)
Triglyceride(mg/dL)	27±5.8	47.1±3.4 (p=0.01)	26.0±2.0 (p=0.43)	33.5±6.0 (p=0.21)	54.3±7.3 (p=0.01)
Platelet count ($X10^9$/ml)	992±4	973±8 (p=0.01)	982±11 (p=0.05)	999±5 (p=0.03)	1002±5 (p=0.01)

C57BL/6J (4 week old) female mice were placed on the indicated diets (chemically defined normal chow (0.08% total choline) vs. same diets supplemented with either 0.12% TMAO, or 1% choline) at time of weaning for 6 weeks. In addition, where indicated, gut microbiota were suppressed by addition of a cocktail of poorly absorbed antibiotics (ABS) in the drinking water (Wang Z., et al, 2011). Blood platelet counts and plasma levels of total cholesterol and triglycerides were determined as described under Experimental Procedures. Data shown are mean ± SD; n=5 each group. A two-sample permutation test was used for two group comparisons using chow as the comparator group.

Table S2: **Gut microbial taxa whose proportions in cecum were significantly associated with plasma levels of TMAO and occlusion time, related to Figure 4.**

	TMAO		Occlusion Time	
Cecum: Taxonomic class	r	p-value	r	p-value
Family: *Lachnospiraceae*	-0.445	0.034	0.569	0.002
Order: *Clostridiales*	-0.428	0.039	0.479	0.006
Family: *Lachnospiraceae*	-0.413	0.046	0.434	0.015
Genus: *Candidatus Arthromitus*	-0.394	0.061	0.520	0.002
Family: *Mogibacteriaceae*	-0.368	0.080	0.396	0.032
Genus: *Roseburia*	-0.308	0.172	0.520	0.003
Class: *Clostridia*	-0.286	0.233	0.472	0.007
Genus: *Oscillospira*	-0.260	0.286	0.364	0.047
Genus:*Odoribacter*	-0.239	0.283	0.549	0.002
Genus: *Rikenella*	-0.193	0.425	0.416	0.022
Order: *Bacteroidales*	-0.168	0.461	0.355	0.053
Family: *Erysipelotrichaceae*	0.251	0.289	-0.386	0.036
Family: *Clostridiaceae*	0.343	0.097	-0.371	0.043
Family:*Coriobacteriaceae*	0.369	0.09	-0.293	0.142
Genus: *Sutterella*	0.460	0.033	-0.437	0.016
Genus: *Allobaculum*	0.492	0.026	-0.527	0.003

C57BL/6J female mice (4 week old) were placed on the chemically defined chow (0.08% total choline) or choline supplemented (total choline 1.0%) diets for 6 weeks. Plasma TMAO levels were determined by stable isotope dilution LC/MS/MS methods, and in vivo thrombosis potential assessed using the $FeCl_3$ carotid artery injury model, as described under Experimental Procedures. Shown are Spearman correlations (r) and FDR-adjusted p values for the cecal taxa (from DNA encoding 16S ribosomal RNA analyses) whose proportions were significantly associated with either plasma TMAO levels or in vivo occlusion time (time to blood flow cessation) among all mice.

Table S5: Gut microbial taxa whose proportions in cecum of microbial transplant recipients were significantly associated with plasma levels of TMAO and occlusion time, related to Figure 6.

	TMAO		Occlusion Time	
Cecum: Taxonomic class	r	P-value	r	P-value
Family: *Peptococcoaceae*	-0.302	0.026	0.306	0.447
Order: *Clostridiales*	-0.289	0.026	-0.070	0.860
Genus: *Aeroplasma*	-0.289	0.026	0.312	0.447
Genus: *Oscillospira*	-0.222	0.122	0.517	0.118
Family: *Lachnospiraceae*	-0.215	0.134	0.430	0.345
Family: *Erysipelotrichaceae*	0.209	0.142	-0.368	0.433
Family: *Rikenellaceae*; Genus: *AF12*	0.234	0.097	0.088	0.830
Order: *Bacteroidales*; Family: *S24.7*	0.269	0.043	-0.203	0.645
Family: *Coriobacteriaceae*	0.289	0.026	0.070	0.860
Genus: *Akkermansia*	0.299	0.026	-0.516	0.118
Genus: *Parabacteroides*	0.308	0.026	0.241	0.555
Genus: *Allobaculum*	0.320	0.025	-0.261	0.527
Phylum: *Cyanobacteria*; Family: *4C0d*; Order: *YS2*	0.360	0.007	-0.629	0.034
Genus: *Sutterella*	0.375	0.006	-0.329	0.447
Genus: *Prevotella*	0.382	0.006	-0.374	0.433

Cecal contents from either conventional C57BL/6J or NZW/LacJ donor mice were used for microbial transplantation into germ-free C57BL/6J recipient mice and maintained individually in microisolators on sterile chemically defined chow (0.08% total choline) or choline supplemented (total choline 1.0%) diets for 6 weeks. Then, plasma TMAO levels, *in vivo* thrombosis potential (monitored by time to blood flow cessation in the *in vivo* carotid artery injury thrombosis model) and cecal microbial composition in mice were determined as described under Experimental Conditions. Cecal microbial taxa whose proportions are significantly associated with either plasma TMAO or in vivo thrombosis potential in recipient mice following the cecal microbial transplantation study are shown. Table is sorted by spearman rho (r) value for TMAO least to greatest. p values are false discovery rate adjusted.