Supplemental Materials

Supplemental Methods

Sample Preparation and LC-MSMS Methods for Plasma TMAO Quantification

The methods used consisted of quantification of 330 plasma samples for their concentrations of trimethylamine oxide (TMAO) using multiple reaction monitoring (MRM) analysis. Liquid chromatography tandem mass spectrometry (LC-MSMS) of the transitions for TMAO and a spiked internal standard of a known amount of d9-TMAO was performed with a Thermo Scientific Dionex Ultimate 3000 HPLC used for separation and a Thermo Scientific TSQ Quantiva Triple Quadrupole Mass Spectrometer used for transition monitoring. Given the small size of each of the targeted compounds, one transition was used for each. The internal standard d9-TMAO was monitored at precursor 85.1 m/z and product 66.1 m/z, light TMAO was monitored at 76.1→59.1 m/z with collision energies of 25 eV. The Heated Electrospray ionization (HESI) source on the TSQ Quantiva Triple Quadrupole Mass Spectrometer was set to a capillary voltage of 3.5 kV with a capillary temperature of 300 °C, a vaporizer temperature of 50 °C, a sheath gas of 8, and auxiliary gas of 3.

TMAO standards in water (calibration curve solutions) were treated identically to plasma samples, with pure cold methanol precipitation 1:1 followed by 0.2 µm centrifugation filtering (13,000 G). Calibration curves for TMAO ratio to internal standard are shown in Figure 1S, while the standard chromatogram peak areas for TMAO and internal standard are provided for water matrix, plasma, and urine in Figure 1S (C-E), respectively. All results were collected on a TSQ Quantiva Triple Quadrupole Mass Spectrometer and 1-5 µL of each sample was injected onto the Imtakt Scherzo SM-C18 column (100 mm x 1 mm, 3 µm particle size). Sample amounts injected were optimized with regard to the final concentration amounts of each sample/standard

analyzed and the sensitivity of the triple quadruple MS instrument being used. With these methods, quantitation with limits of quantification down to 0.13 µM (TMAO) (LOD 0.013 µM) with standard addition were achieved. The intra assay reproducibility was 3% for plasma TMAO. The inter assay reproducibility was calculated to be 10% by averaging the low (0.1 µM) and high (330 µM) QC signals for three separate calibration trials.

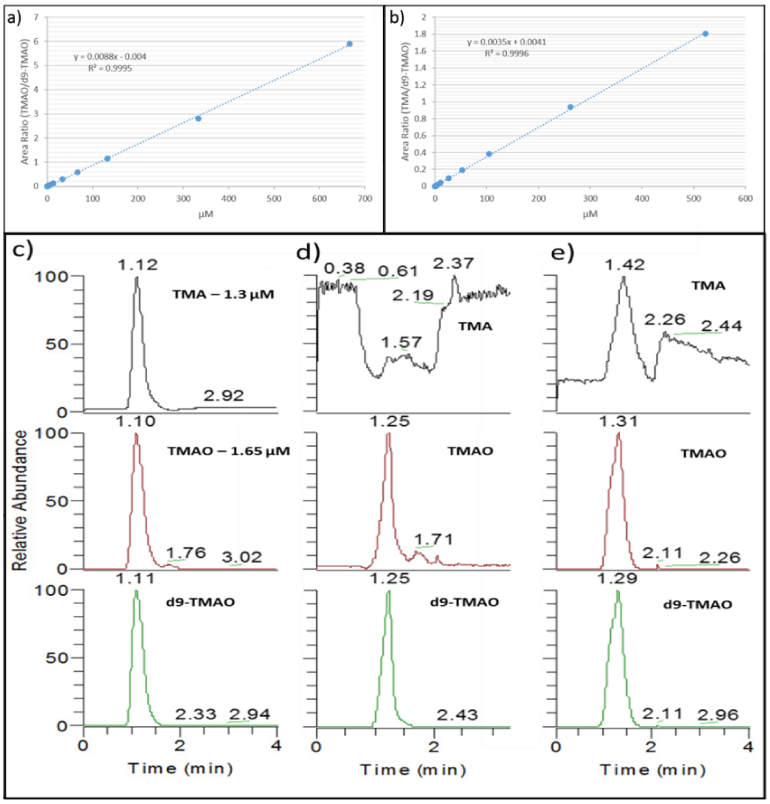

Figure 1S. Representative calibration curves of the concentrations of a) TMAO and b) TMA standards taking the area ratios to d9-TMAO shown in c) in an aqueous matrix following identical treatment to plasma samples (with traces for TMA, TMAO, and IS in (d)) and urine samples (with traces for TMA, TMAO, and IS in (e)).

Assay detail for CRP, ferritin, and iron measurements

High Sensitive C-Reactive Protein

C-reactive protein was measured using a chemiluminescence methodology using the Immulite 1000 (Siemens Healthcare Diagnostics, Inc., 1717 Deerfield Rd., Deerfield, IL). Analytical sensitivity for this assay is 0.01 mg/L. Functional sensitivity is 0.3 mg/L. Intra-assay coefficient of variation is 3.1% and inter-assay coefficient variation is 7.3%.

The kit uses a solid phase chemiluminescence immunometric assay. The solid phase (bead) is coated with anti-ligand, the liquid phase consists of ligand labeled anti-CRP murine monoclonal antibody and alkaline phosphatase (bovine calf intestine) conjugated to rabbit polyclonal anti-CRP antibody in buffer. This is manufactured specifically for this equipment.

Ferritin

Ferritin was measured using a chemiluminescence methodology using the Immulite 1000 (Siemens Healthcare Diagnostics, Inc., 1717 Deerfield Rd., Deerfield, IL). Analytical sensitivity for this assay is 1.5 ng/mL. Intra-assay coefficient of variation is 5.2% and inter-assay coefficient variation is 8.2%.

The kit uses a solid-phase, enzyme-labeled chemiluminescent immunometric assay. The solid phase (bead) is coated with monoclonal murine anti-ferritin antibody. The liquid phase consists of alkaline phosphatase (bovine calf intestine) conjugated to polyclonal goat anti-ferritin antibody.

Iron

Plasma samples were analyzed in singular using the Dimension Xpand Clinical Chemistry System (Siemens Medical Diagnostics, Decatur, GA). The analytical sensitivity is 5 µg/dL.

Table S1. Cohort Characteristics at Baseline According to Hard MACE Status

		All Subjects (n = 158)	Case (n = 79)	Control (n = 79)	*p*
Age, years		62.1 (58.9-66.9)	62.7 (58.9-67.1)	61.9 (58.7-66.6)	0.959*
Female, N (%)		73 (46)	37 (47)	36 (46)	1.000†
Race	White, N (%)	108 (68)	53 (67)	55 (70)	0.530‡
	Black, N (%)	23 (15)	12 (15)	11 (14)	
	Hispanic, N (%)	10 (6)	7 (9)	3 (4)	
	Other, N (%)	17 (11)	7 (9)	10 (13)	
BMI, kg/m^2		32.2 ± 5.6	32.0 ± 5.5	32.3 ± 5.7	0.703§
Total Cholesterol, mg/dL		191 ± 45	195 ± 45	188 ± 45	0.385 §
HDL Cholesterol, mg/dL		40 (35-50)	39 (33-47)	41 (37-51)	0.192 *
LDL Cholesterol, mg/dL		109 ± 37	111 ± 39	107 ± 36	0.559 §
Triglycerides, mg/dL		171 (121-250)	183 (143-261)	156 (111-236)	0.040 *||
Systolic Blood Pressure, mmHg		138 ± 18	139 ± 20	138 ± 16	0.824 §
HbA1c, %		8.3 (7.7-9.2)	8.4 (7.8-9.5)	8.1 (7.6-8.9)	0.038 *||
10-Year ASCVD Risk, %		23.8 ± 11.9	23.8 ± 11.3	23.8 ± 12.5	0.938 §
eGFR, mL/min		86.6 ± 25.7	86.7 ± 28.1	86.6 ± 23.4	0.777 §
Fasting Plasma Glucose, mg/dL		170 (143-204)	168 (142-207)	171 (143-204)	0.437 *
Smoking Status	Never, N (%)	71 (45)	36 (46)	35 (44)	0.973‡
	Former, N (%)	62 (39)	31 (39)	31 (39)	
	Current, N (%)	25 (16)	12 (15)	13 (16)	
ACEI and/or ARB, N (%)		103 (65)	46 (58)	57 (72)	0.112 †
β-blocker, N (%)		34 (22)	18 (23)	16 (20)	0.839 †
Statin, N (%)		74 (47)	34 (43	40 (51)	0.429 †
Antiplatelet, N (%)		76 (48)	35 (44)	41 (52)	0.391 †

Note: Variables expressed as mean±SD, median (IQR range), or N (%). * Wilcoxon signed rank test for paired subjects. † McNemar's test for paired subjects. ‡ Marginal homogeneity test for paired subjects. § Paired *t*-test. || $p < 0.05$ considered significant. BMI: Body Mass Index; HDL: High-Density Lipoprotein; LDL: Low-Density Lipoprotein; HbA1c: Hemoglobin A1c; ASCVD: Atherosclerotic Cardiovascular Disease; eGFR: estimated Glomerular Filtration Rate; ACEI: Angiotensin Converting Enzyme Inhibitor. ARB: Angiotensin II Receptor Blocker.

Table S2. Biomarker Profile Overall and According to Hard-MACE Status

	All Subjects (n = 158)	**All Cases (n= 79)**	**All Controls (n = 79)**	***p***
hsCRP, mg/L	6.9 ± 7.8	7.8 ± 9.0	5.9 ± 6.2	0.638
Ferritin, ng/mL	224.6 ± 206.4	238.3 ± 242.2	210.9 ± 163.6	0.802
Iron, µg/dL	78 ± 30	77 ± 33	79 ± 26	0.169
ALT, mg/dL	26 ± 11	25 ± 8	28 ± 14	0.276
Potassium, mmol/L	4.48 ± 0.44	4.50 ± 0.42	4.45 ± 0.46	0.437
Serum Creatinine, mg/dL	0.91 ± 0.23	0.92 ± 0.24	0.91 ± 0.23	0.686
Urinary Creatinine, mg/dL	129.5 ± 72.3	128.6 ± 77.5	130.4 ± 67.3	0.545
Urinary Albumin, mg/DL	15.23 ± 37.34	23.00 ± 49.12	7.46 ± 16.50	0.001*
Plasma TMAO, µmol/L	**7.75 (6.17-9.45)**	**7.94 (6.38-9.66)**	**7.67 (5.95-9.07)**	**0.373**

Note: Variables expressed as mean±SD, or median (IQR range). Paired *t*-tests or Wilcoxon signed rank tests used for statistical comparisons. * $p < 0.05$ considered significant. † µmol/L converted to mmol/L by factor of 10^{-3}, mg/dL converted to mol/L by factor of 8.842×10^{-5}.

hsCRP: High-Sensitivity C-Reactive Protein; ALT: Alanine Aminotransferase; TMAO: Trimethylamine N Oxide.