Supplemental Files:

Untargeted metabolomics identifies trimethyllysine, a TMAO-producing nutrient precursor, is associated with incident cardiovascular disease risk

Xinmin S. Li[1], Zeneng Wang[1], Tomas Cajka[2], Jennifer A. Buffa[1], Ina Nemet[1], Alex G. Hurd[1,#], Xiaodong Gu[1], Sarah M. Skye[1], Adam B. Roberts[1], Yuping Wu[3], Lin Li[1], Christopher J. Shahen[1], Matthew A. Wagner[1], Jaana A. Hartiala[4], Robert L. Kerby[5], Kymberleigh A. Romano[5,*], Yi Han[4], Slayman Obeid[6], Thomas F. Lüscher[6,7], Hooman Allayee[4], Federico E. Rey[5], Joseph A. DiDonato[1], Oliver Fiehn[2,8], W.H. Wilson Tang[1,9], Stanley L. Hazen[1,9]

[1] Department of Cellular & Molecular Medicine, Lerner Research Institute, Cleveland Clinic, Cleveland, Ohio, USA
[2] Department of Molecular and Cellular Biology and Genome Center University of California, Davis, Davis, California, USA
[3] Department of Mathematics, Cleveland State University, Cleveland, Ohio, USA
[4] Department of Preventive Medicine and Biochemistry and Molecular Medicine, USC Keck School of Medicine, Los Angeles, California, USA
[5] Department of Bacteriology, University of Wisconsin-Madison, Madison, Wisconsin, USA
[6] University Heart Center, Department of Cardiology, University Hospital Zurich, Switzerland
[7] Royal Brompton and Harefield Hospitals and Imperial College, London, United Kingdom
[8] Department of Biochemistry, King Abdulaziz University, Jeddah, Saudi-Arabia
[9] Department of Cardiovascular Medicine, Heart and Vascular Institute, Cleveland Clinic, Cleveland, Ohio, USA

Current address: [#]Department of Biostatistics, University of Pittsburgh, Pittsburgh, Pennsylvania, USA; *Department of Cellular & Molecular Medicine, Cleveland Clinic, Cleveland, Ohio, USA

Address for Correspondence:
Stanley L. Hazen, MD PhD
Department of Cellular & Molecular Medicine, Lerner Research Institute
Cleveland Clinic, 9500 Euclid Avenue, NC-10, Cleveland, OH 44195
Phone: (216) 445-9763, email: hazens@ccf.org

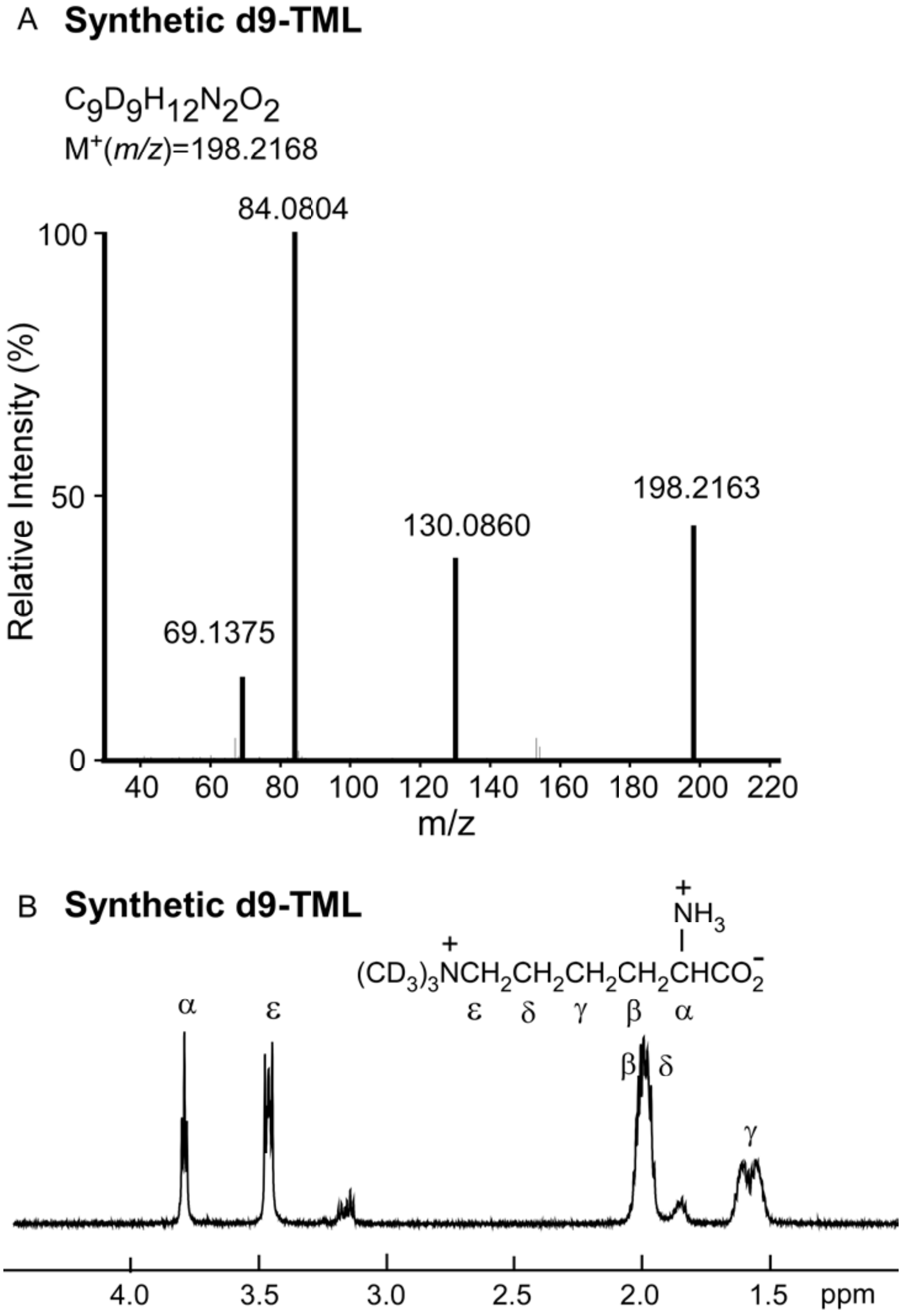
Supplemental Methods

Chemical synthesis of [d9-N, N, N-trimethyl] lysine and trimethyllysine

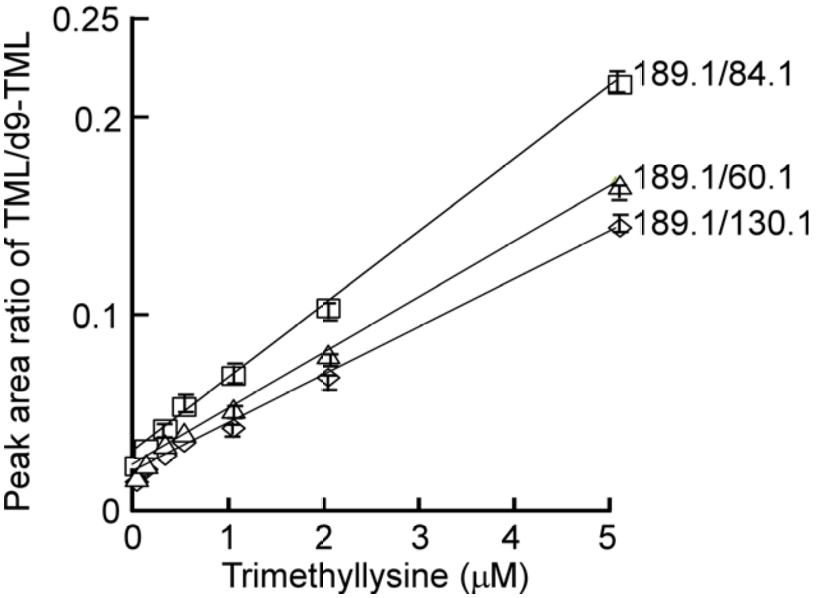
[d9-N, N, N-trimethyl] lysine(d9-TML) and trimethyllysine(TML) were prepared as described previously (Chen and Benoiton, 1976) but with some modifications[1]. In brief, N (α) *Boc*-Lysine (9.85 g, 40 mmol) was dissolved into 400 ml methanol. Sodium hydroxide (5.28 g, 132 mmol) was added. Either d3-methyl iodide (for preparing d9-TML) or methyl iodide (for preparing TML) (140 mmol) was added in one portion. The reaction mixture was stirred at room temperature overnight. The reaction was followed by TLC on plastic backed Silica Gel plate in methanol with1% v/v formic acid, developed with I_2 fumes. The reaction mixture was transferred onto silica gel bed (90 mm diameter) equilibrated in methanol in a coarse fritted Buchner funnel. Sodium iodide was removed by elution with 1.5 liters of methanol. The product (d9-TML or TML) was eluted from the column with 3 liters of 30% water v/v in methanol. Rotary evaporation of this methanol portion gave the crude product. *Boc* group of the crude product was removing by adding 100 ml concentrated HCl (1.2 moles) and incubation at 90 – 100 °C for one hour. Rotary evaporation of the product solution at 60 °C under 60 mBar vacuum was performed until a viscous oil was obtained. The remaining HCl was removed by adding 100 ml of water and evaporated. The crude product was dissolved in 100ml water and titrated to pH 6 with 1 M sodium hydroxide, and was concentrated by rotary evaporation in reduced pressure. The crude product was again dissolved in absolute ethanol and was filtered to remove residual inorganic salt. Residual water was removed by rotary evaporation and any final moisture was removed azeotropically by sequential rotary evaporation after adding absolute ethanol under vacuum. The crude product was re-dissolved in a minimal amount of boiling 2-propanol, the solution was cooled to room temperature, and gave the recrystallized d9-TML or TML recovered. High resolution mass spectrometry (*m/z*) for d9-TML: $[M]^+$ (*m/z*) calculated exact mass for ${}^2H_9C_9H_{12}N_2O_2$, 198.2168; found, 198.2163; TML: calculated exact mass for $C_9H_{21}N_2O_2$, 189.1603; found, 189.1595. d9-TML and TML were also confirmed by high-resolution NMR (**Supplementary Figure 1 and 4**).

References:

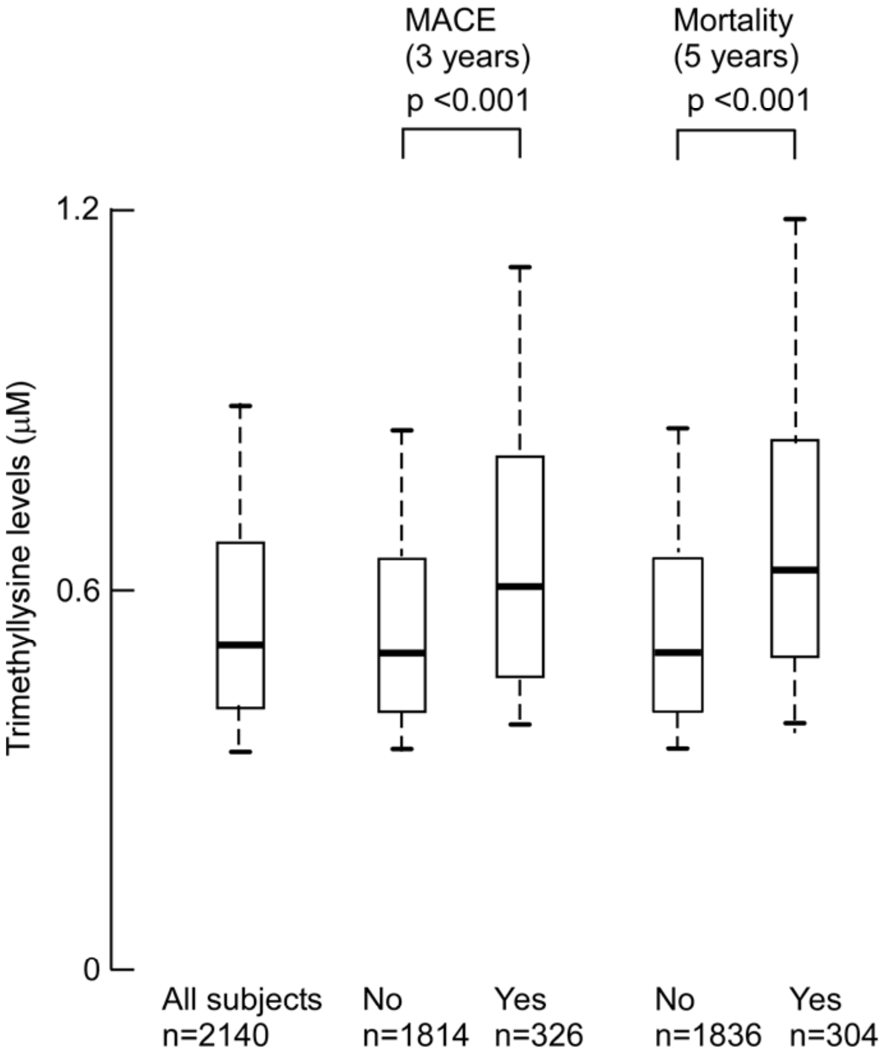
1. Chen FCM and Benoiton NL. A new method of quaternizing amines and its use in amino acid and peptide chemistry. *Can J Chem*. 1976;54:3310-3311.



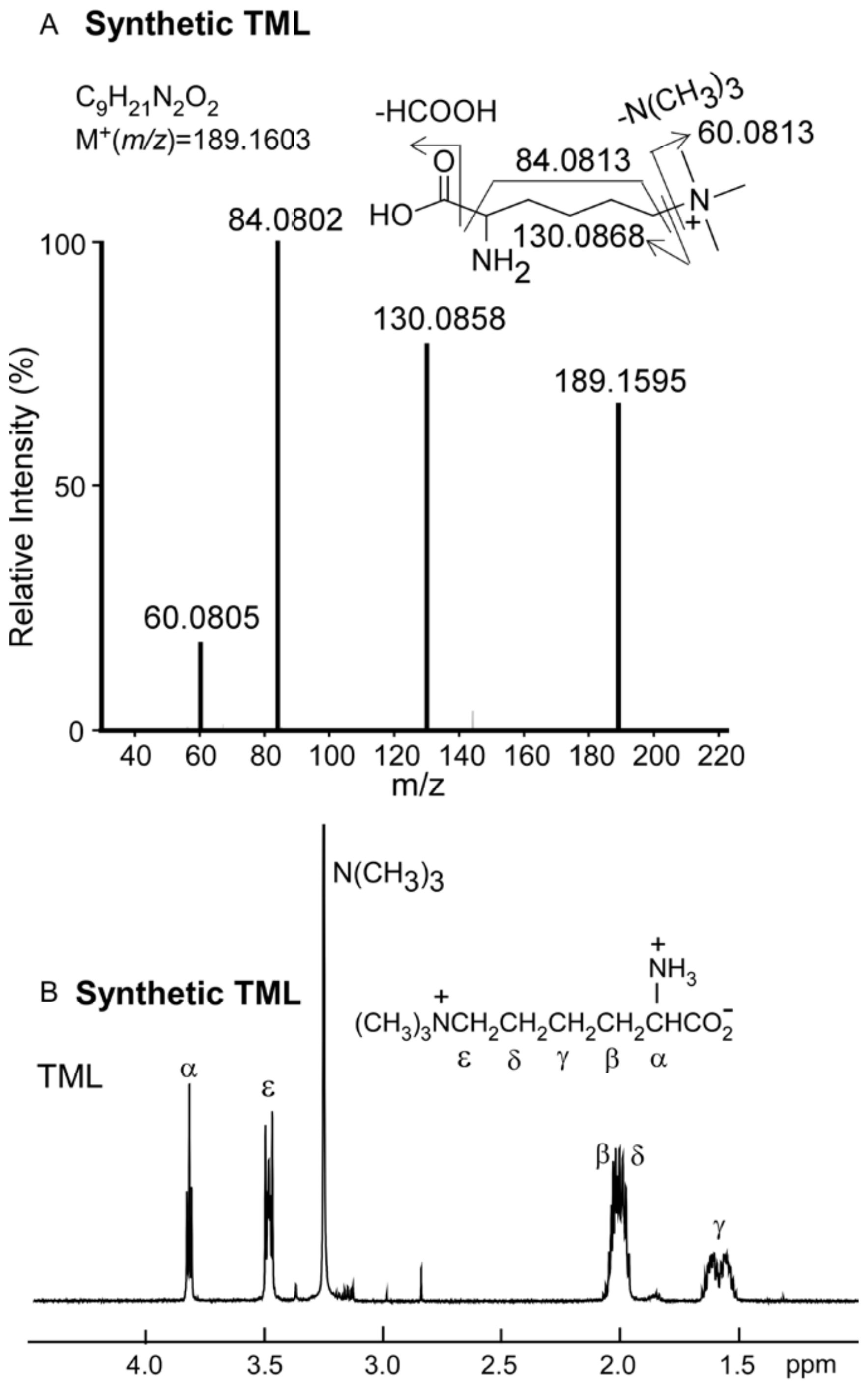
Supplemental Figure 1. Structural confirmation of synthetic d9(trimethyl)-trimethyllysine. d9-TML was synthesized and purified as described under Methods. Its structure was confirmed by both high resolution MS and ^{1}H NMR. **(A)** MS/MS CID spectrum in positive mode of d9(trimethyl)-TML. **(B)** ^{1}H NMR spectrum of synthetically prepared d9-TML.



Supplemental Figure 2. Standard curves for LC/ESI/MS/MS analysis of trimethyllysine (TML) within plasma matrix. All data are presented as means ± standard error (n=3). Numbers to the right of the curves indicate the parent -> daughter ion transitions monitored in multiple reaction monitoring mode.



Supplemental Figure 3. Plasma TML levels in sequential stable subjects undergoing elective diagnostic cardiac evaluations. Subjects (n=2140 total) were divided into groups as indicated based on whether (Yes) or not (No) they experienced an incident major adverse cardiac event (MACE; MI, stroke or death), or died over the indicated period of follow-up. In the box-whisker plot, the upper and lower boundaries of the box represent the 25th and 75th percentiles, the median is marked by a horizontal line inside the box, and the TMAO in the cohort.



Supplemental Figure 4. Structural confirmation of synthetic trimethyllysine. TML was synthetized and purified as described under Methods. TML structure was confirmed by both high resolution MS and ^{1}H NMR. **(A)** MS/MS CID spectra in positive mode of TML; **(B)** **^{1}**H NMR spectra of TML, $N(CH_3)_3$ at 3.25 ppm was observed.

Supplemental Table 1. High resolution mass spectrometry analysis of synthetic d9-TML

Analyte	Elemental Composition	Theoretical m/z	Measured m/z	Delta (ppm)
d9-TML	$C_9D_9H_{12}N_2O_2$	198.2168	198.2163	2.52
Daughter 1	C_3D_9HN	69.1378	69.1375	4.34
Daughter 2	$C_5H_{10}N$	84.0813	84.0804	10.70
Daughter 3	$C_6H_{12}NO_2$	130.0868	130.0860	6.15

The full scan CID mass spectrum of synthetic d9-TML was analyzed on an LC Triple TOF as described under Methods. The experimentally measured m/z of both parent and daughter ions are shown, along with the theoretical m/z and calculated difference (in ppm).

Supplemental Table 2. TMAO and TML provide independent additive clinical prognostic value for prediction of MACE risks

	Range	TMAO (µM)		
		< 2.5 µM (low)	2.5-5.99 µM (intermediate)	≥ 6 µM (high)
TML (µM)	< 0.5 µM (low)	2.8%/yr 1.0	3.5%/yr 1.2(0.8-2.0) P=0.40	5.2%/yr 1.9(1.1-3.4) P=0.03
	0.5-0.79 µM (intermediate)	4.7%/yr 1.7(1.01-2.9) P=0.047	3.9%/yr 1.4(0.9-2.2) P=0.18	7.7%/yr 2.9(1.8-4.5) P<0.001
	≥ 0.8 µM (high)	7.2%/yr 2.7(1.1-7.1) P=0.04	6.4%/yr 2.4(1.4-4.2) P=0.002	10.0%/yr 4.0(2.6-6.2) P<0.001

The entire cohort (N=2,140) was stratified into low, intermediate and high level subgroups of TML and TMAO using the cutoffs indicated. Within each cell, the annual absolute incident event rate for MI, stroke or death (MACE) is shown at the top. The middle line depicts the calculated Hazard Ratio (95% Confidence Interval) for risk of incident MACE relative to subjects with low levels of both TML and TMAO as reference group. And the bottom line provides the p value for comparison relative to subjects with low levels of both TML and TMAO as reference group.

Supplemental Table 3. High resolution mass spectrometry analysis of comparison of measured synthetic TML

Analyte	Elemental composition	Theoretical m/z	Measured m/z	Delta (ppm)
TML	$C_9H_{21}N_2O_2$	189.1603	189.1595	4.23
Daughter 1	$C_3H_{10}N$	60.0813	60.0805	13.30
Daughter 2	$C_5H_{10}N$	84.0813	84.0802	13.08
Daughter 3	$C_6H_{12}NO_2$	130.0868	130.0858	7.69

The full scan CID mass spectrum of synthetic TML was analyzed on an LC Triple TOF as described under Methods. The experimentally measured m/z of both parent and daughter ions are shown, along with the theoretical m/z and calculated difference (in ppm).