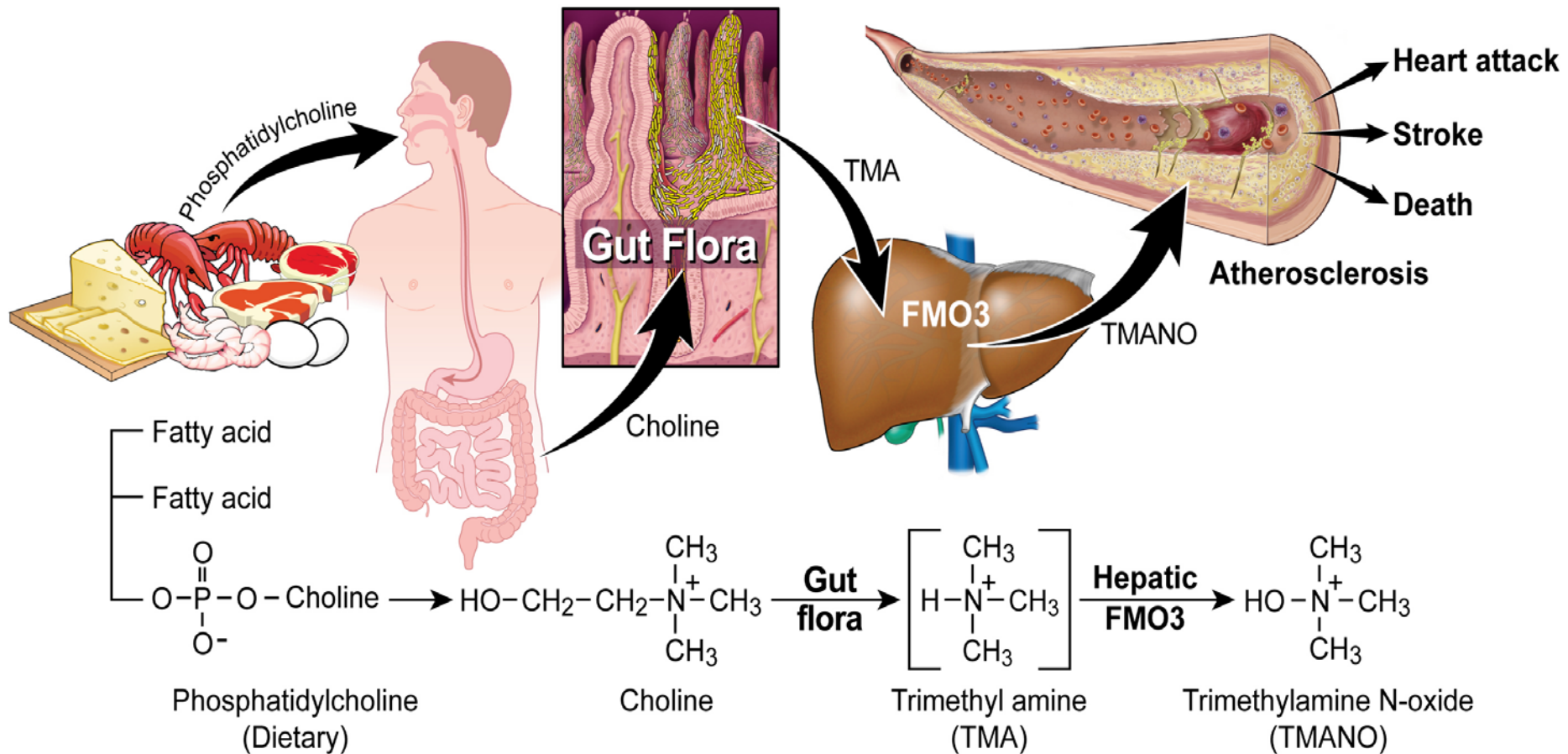# Gut flora dependent metabolism of phosphatidylcholine and the pathogenesis of cardiovascular disease

Stanley Hazen MD PhD

Take home summary:
Gut flora participates in atherosclerosis in the presence of specific dietary exposures

Phosphatidylcholine

Gut Flora

TMA

FMO3

TMANO

Atherosclerosis

Heart attack
Stroke
Death

Choline

Fatty acid
Fatty acid

$$O-P-O-Choline$$

Phosphatidylcholine (Dietary)

$$HO-CH_2-CH_2-N^+(CH_3)_3$$

Choline

Gut flora →

$$\left[H-N^+(CH_3)_3 \right]$$

Trimethyl amine (TMA)

Hepatic FMO3 →

$$HO-N^+(CH_3)_3$$

Trimethylamine N-oxide (TMANO)

Wang Z et al, *Nature* (2011)

Phase 1: Discovery-based investigations
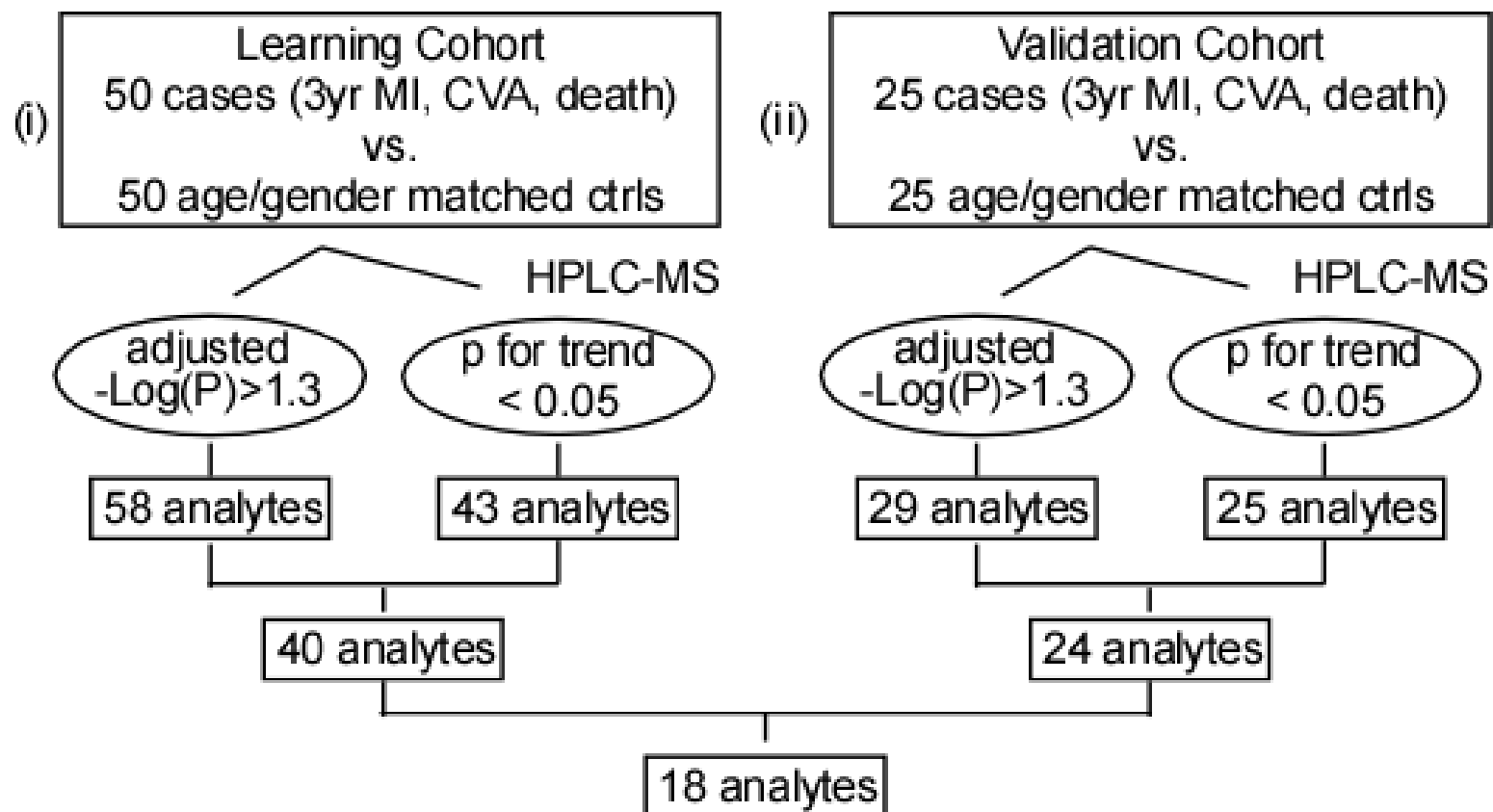**Metabolomics screening and structural identification**

Phase 2: Clinical validation

Demonstration of clinical utility

Phase 3: Mechanistic studies

Demonstration of causality for novel pathway

Strategy of metabolomics study design for identifying unbiased small molecule profiles predictive of incident risks for major adverse cardiovascular events

(i)

Learning Cohort
50 cases (3yr MI, CVA, death)
vs.
50 age/gender matched ctrls

(ii)

Validation Cohort
25 cases (3yr MI, CVA, death)
vs.
25 age/gender matched ctrls

HPLC-MS

adjusted -Log(P)>1.3

p for trend < 0.05

HPLC-MS

adjusted -Log(P)>1.3

p for trend < 0.05

58 analytes

43 analytes

29 analytes

25 analytes

40 analytes

24 analytes

18 analytes

(iii) Structural identification of analytes

(iv) Confirm clinical prognostic utility in Independent Prospective Cohort (N>1000)
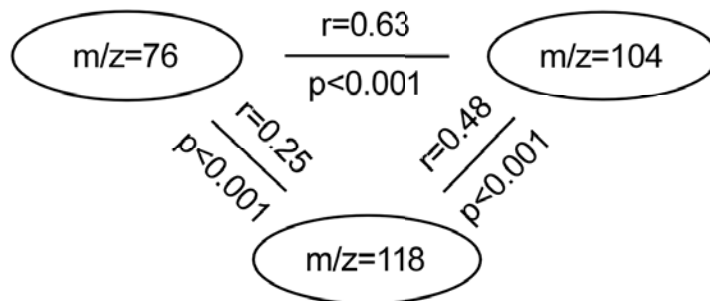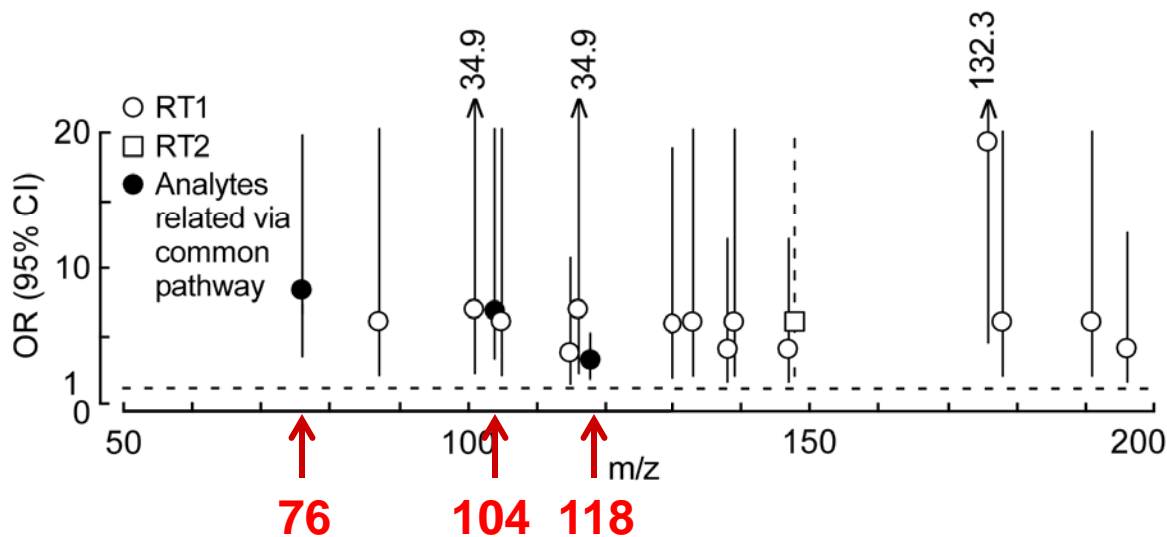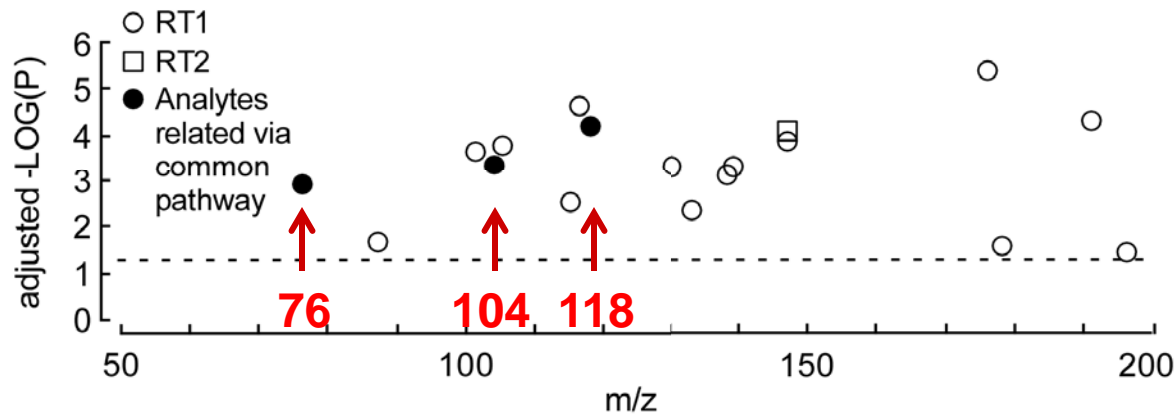
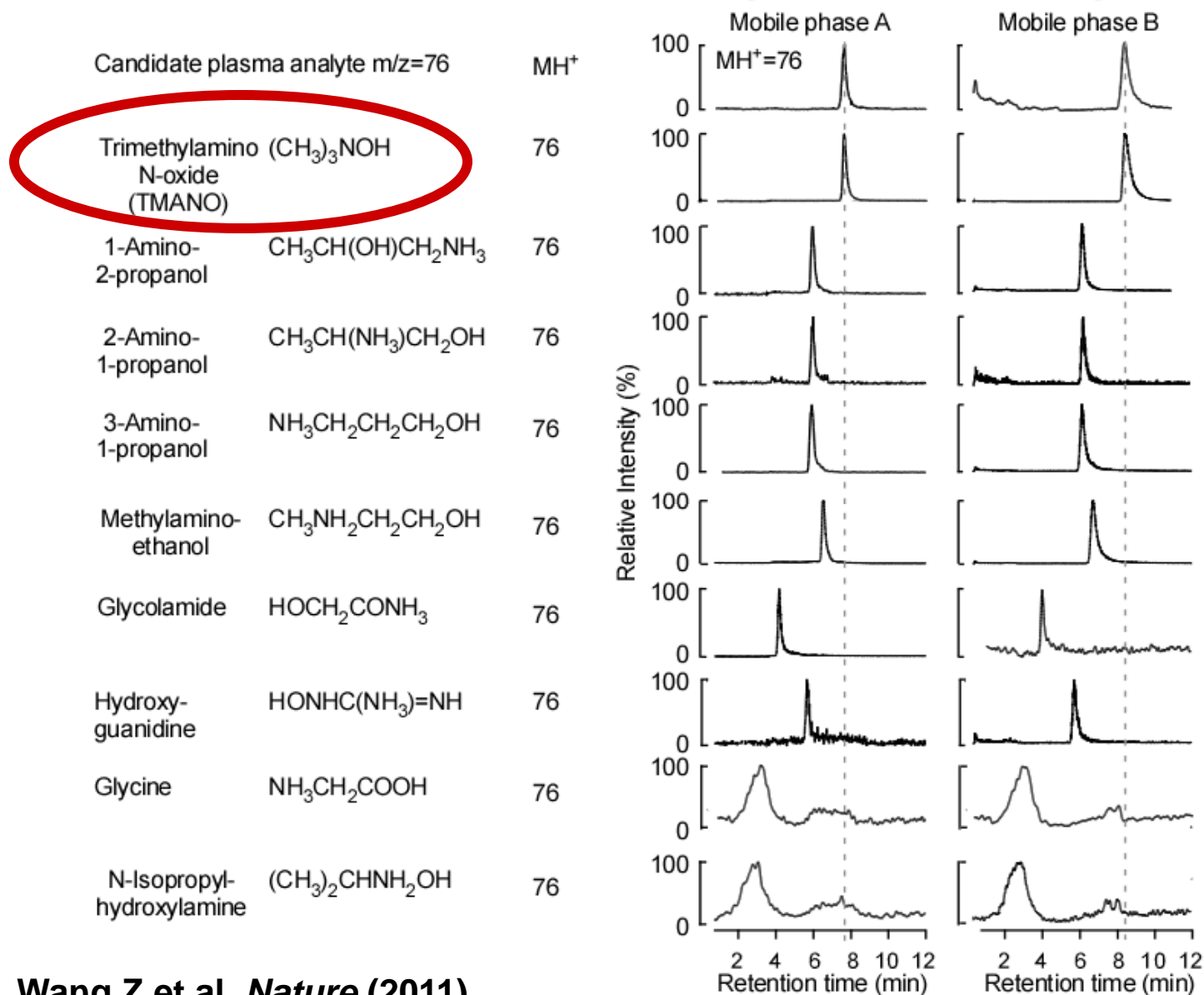Plasma analytes with m/z **76**, **104**, and **118** are associated with CVD, show a dose-response relationship with MACE and are correlated, suggesting participation in a common pathway

Choline, betaine and trimethylamine *N*-oxide are the plasma analytes associated with CVD
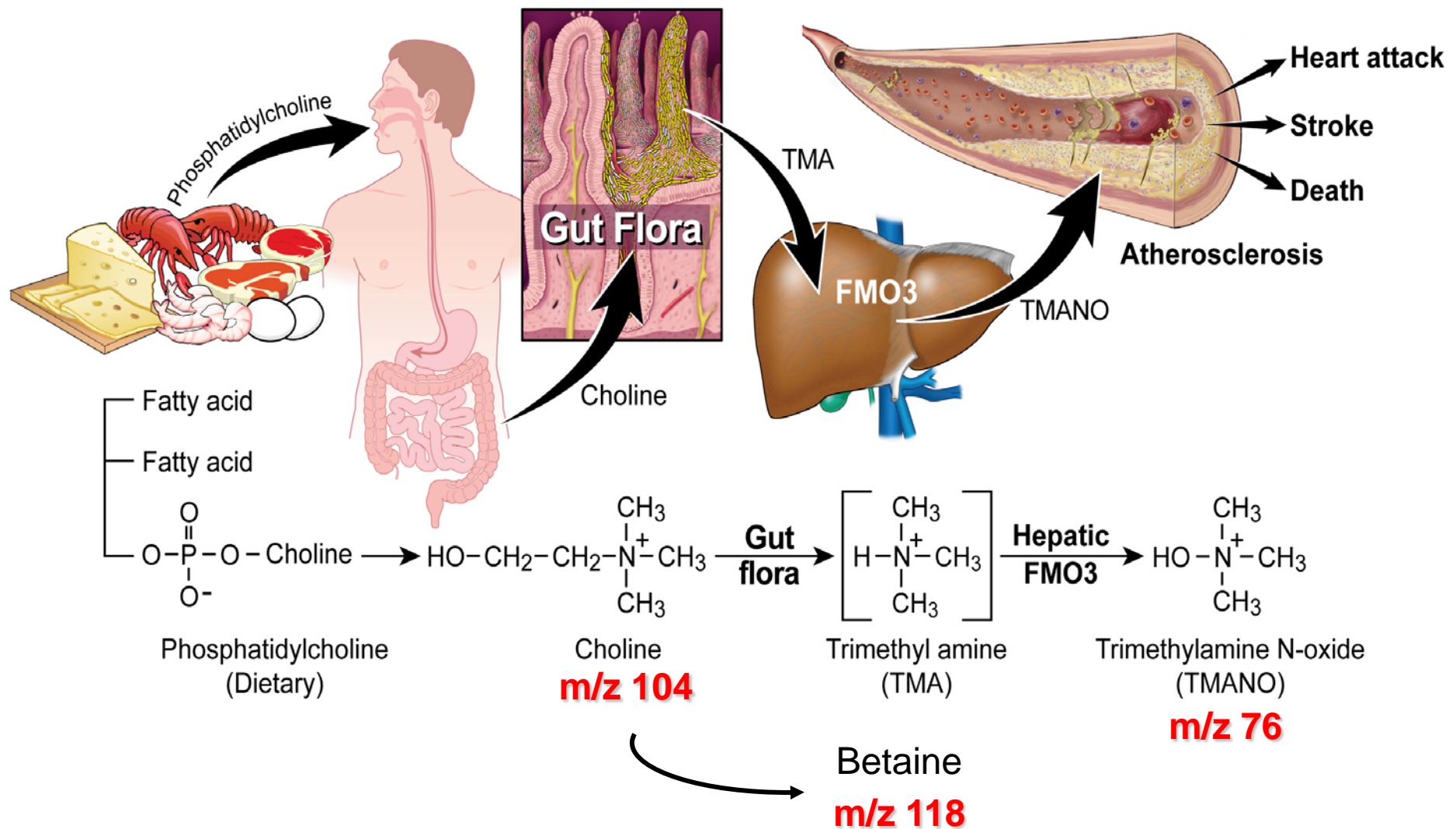
But the metabolomics library says its "glycine"…
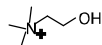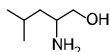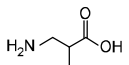## Candidate structures of plasma analyte m/z=76

Candidate plasma analyte m/z=76		MH⁺
Trimethylamino N-oxide (TMANO)	$(CH_3)_3NOH$	76
1-Amino-2-propanol	$CH_3CH(OH)CH_2NH_3$	76
2-Amino-1-propanol	$CH_3CH(NH_3)CH_2OH$	76
3-Amino-1-propanol	$NH_3CH_2CH_2CH_2OH$	76
Methylamino-ethanol	$CH_3NH_2CH_2CH_2OH$	76
Glycolamide	$HOCH_2CONH_3$	76
Hydroxy-guanidine	$HONHC(NH_3)=NH$	76
Glycine	NH_3CH_2COOH	76
N-Isopropyl-hydroxylamine	$(CH_3)_2CHNH_2OH$	76

Wang Z et al, *Nature* (2011)

Plasma analyte m/z=104

MH+

MS1, m/z=104

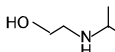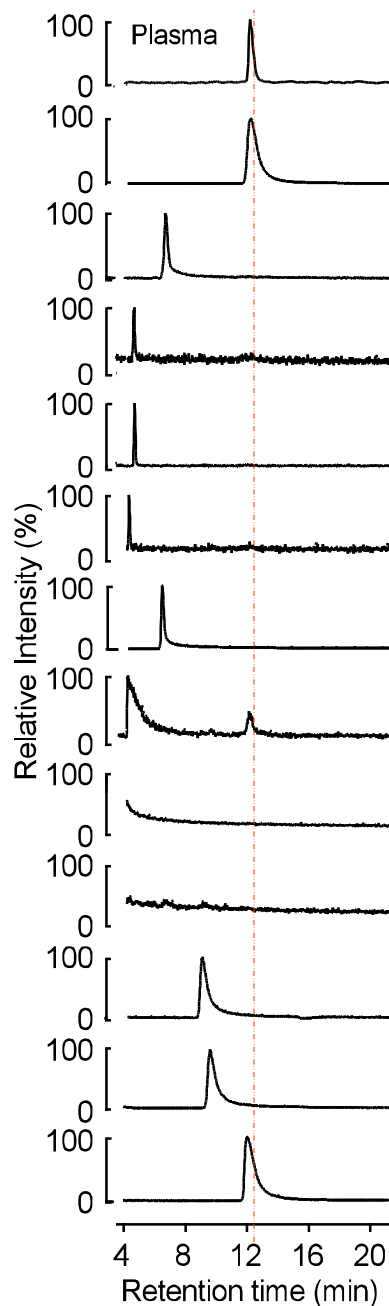| Analyte	MH+
choline	104
2-amino-3-methyl-1-butanol	104
3-aminoisobutyric acid	104
2-aminobutyric acid	104
biuret	104
N,N-dimethyl glycine	104
benzonitrile	104
diethylenetriamine	104
ethyl-N-hydroxyl acetimidate	104
2-isopropyl-aminoethanol	104
2-propyl-aminoethanol	104
1-dimethyl-amino-2-propanol	104
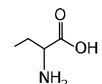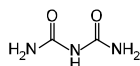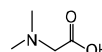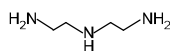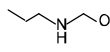
Relative Intensity (%)

Plasma

Retention time (min)
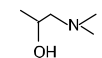
Candidate plasma analytes linked to CVD risks with m/z 104

Identity as Choline was confirmed by:

LC-MS^n

GC/MS/MS

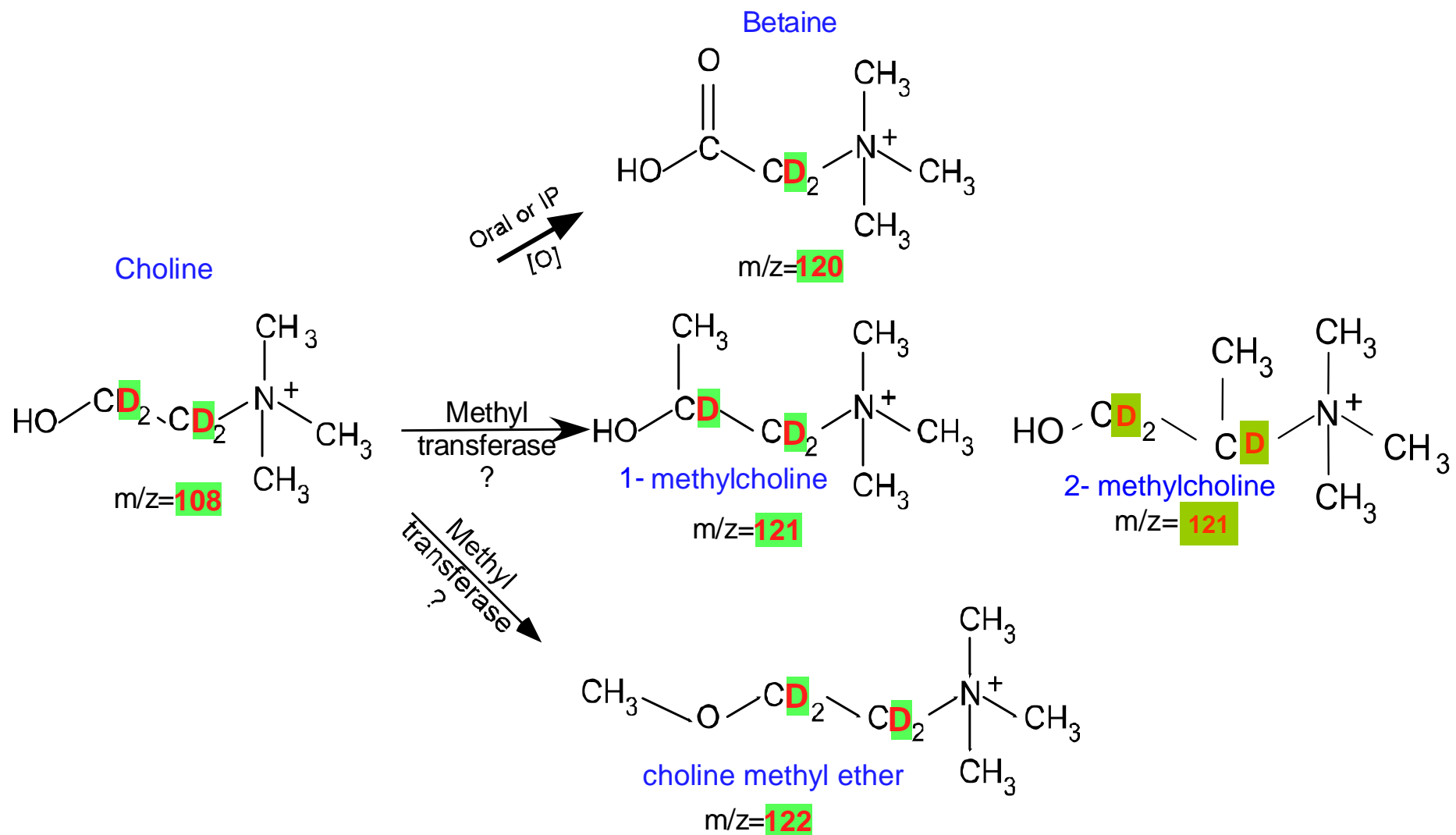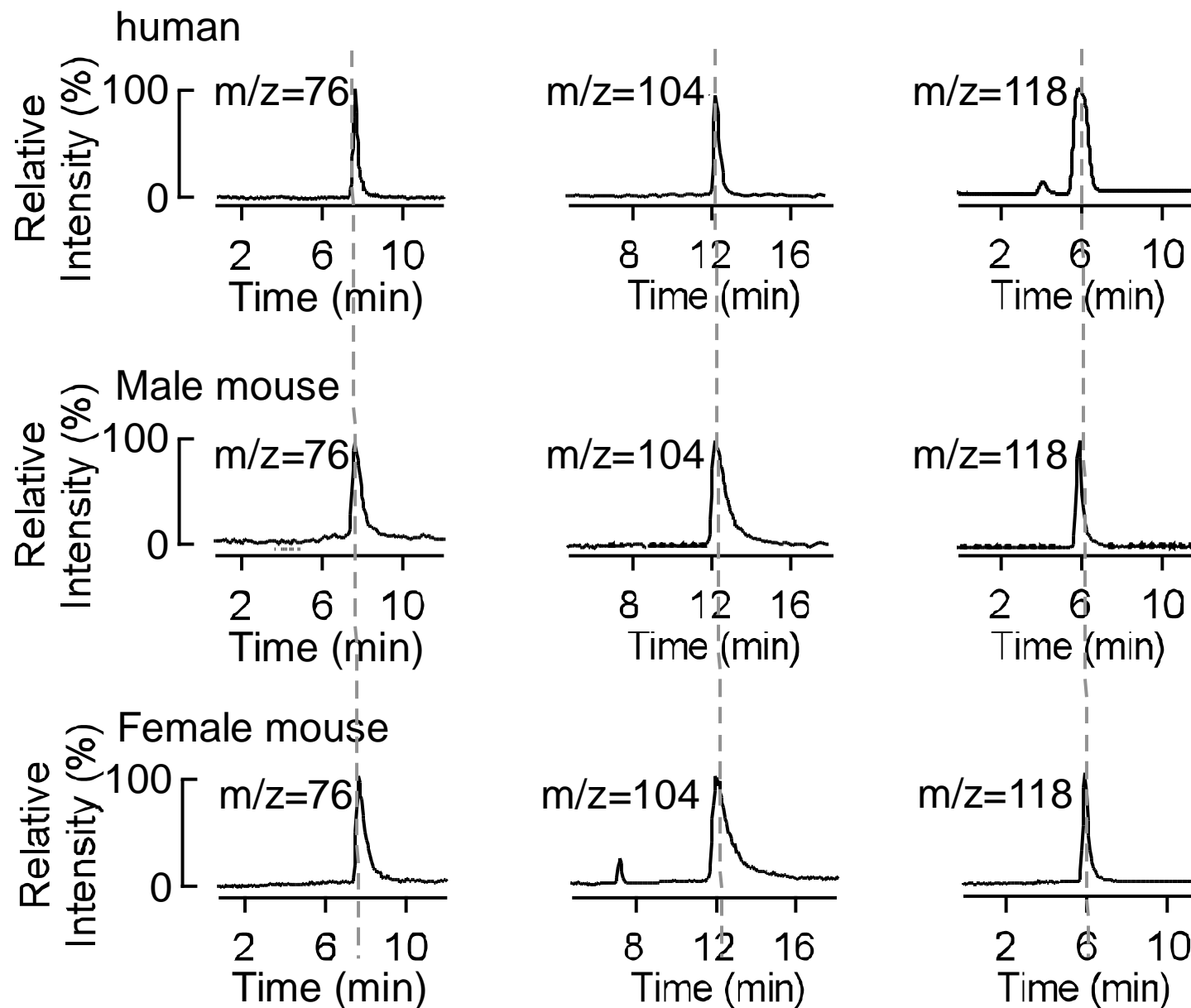**^{1}H, ^{13}C, and ^{15}N NMR**

Isotope tracer studies: d9-choline and d4-choline

Strategy to determine the analyte at m/z=118 by choline deuterated isotopologue feeding study
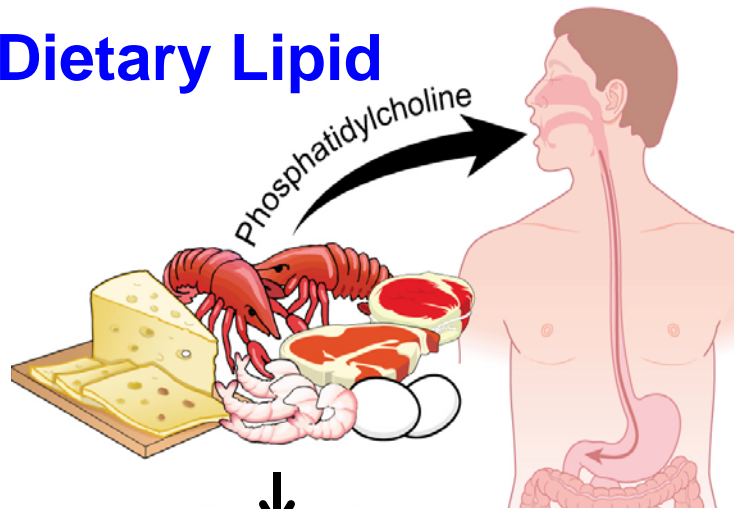

Wang Z et al, *Nature* **(2011)**

Dietary egg yolk PC produces increases in analytes with m/z 76, 104,and 118 in both human and mouse plasma

Dietary Lipid

Phosphatidylcholine
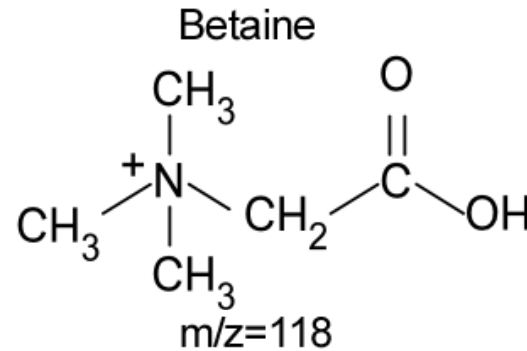
What is the role of gut flora?

Choline

m/z=104

Oral or i.p. [O]

Betaine

m/z=118
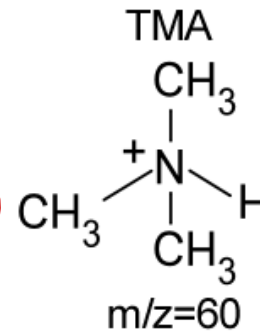d4 metabolite m/z=120
d9 metabolite m/z=127

Oral only

Gut Flora

TMA

m/z=60
d4 metabolite m/z=60
d9 metabolite m/z=69

presumed FMO3 [O]
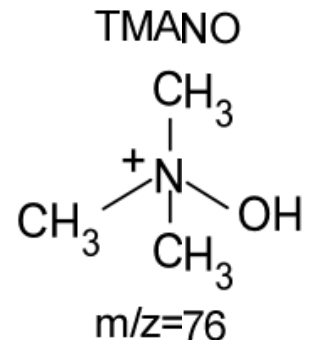
TMANO

m/z=76
d4 metabolite m/z=76
d9 metabolite m/z=85

d4-choline: $(CH_3)_3{}^+NCD_2CD_2OH$

d9-choline: $(CD_3)_3{}^+NCH_2CH_2OH$

Wang Z et al, *Nature* (2011)

Intestinal Microbial Organisms Play an Obligatory Role in TMANO Generation from Dietary Egg Yolk PC
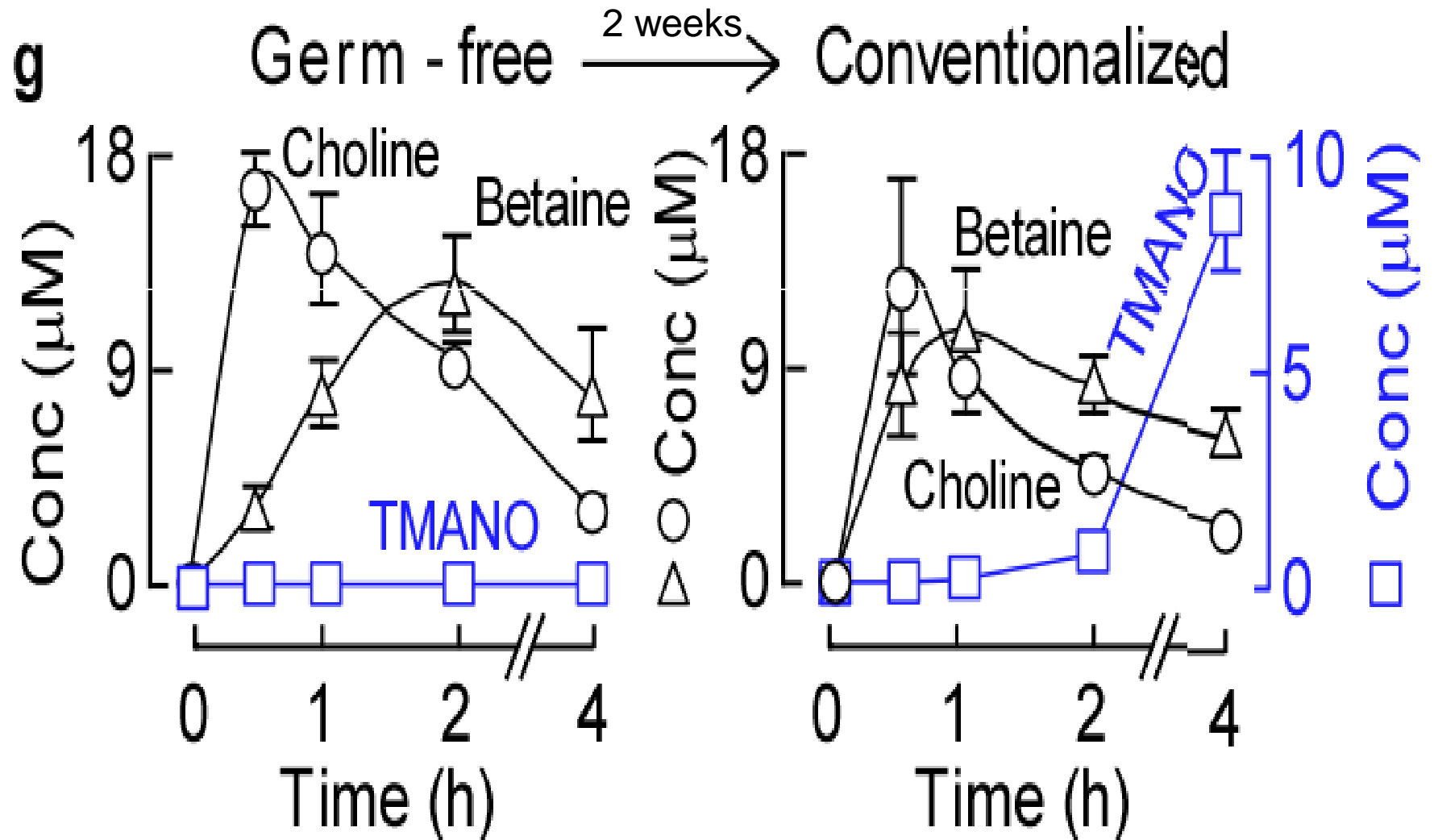
Wang Z et al, *Nature* (2011)

Phase 1: Discovery-based investigations

Metabolomics screening and structural identification

Phase 2: Clinical validation
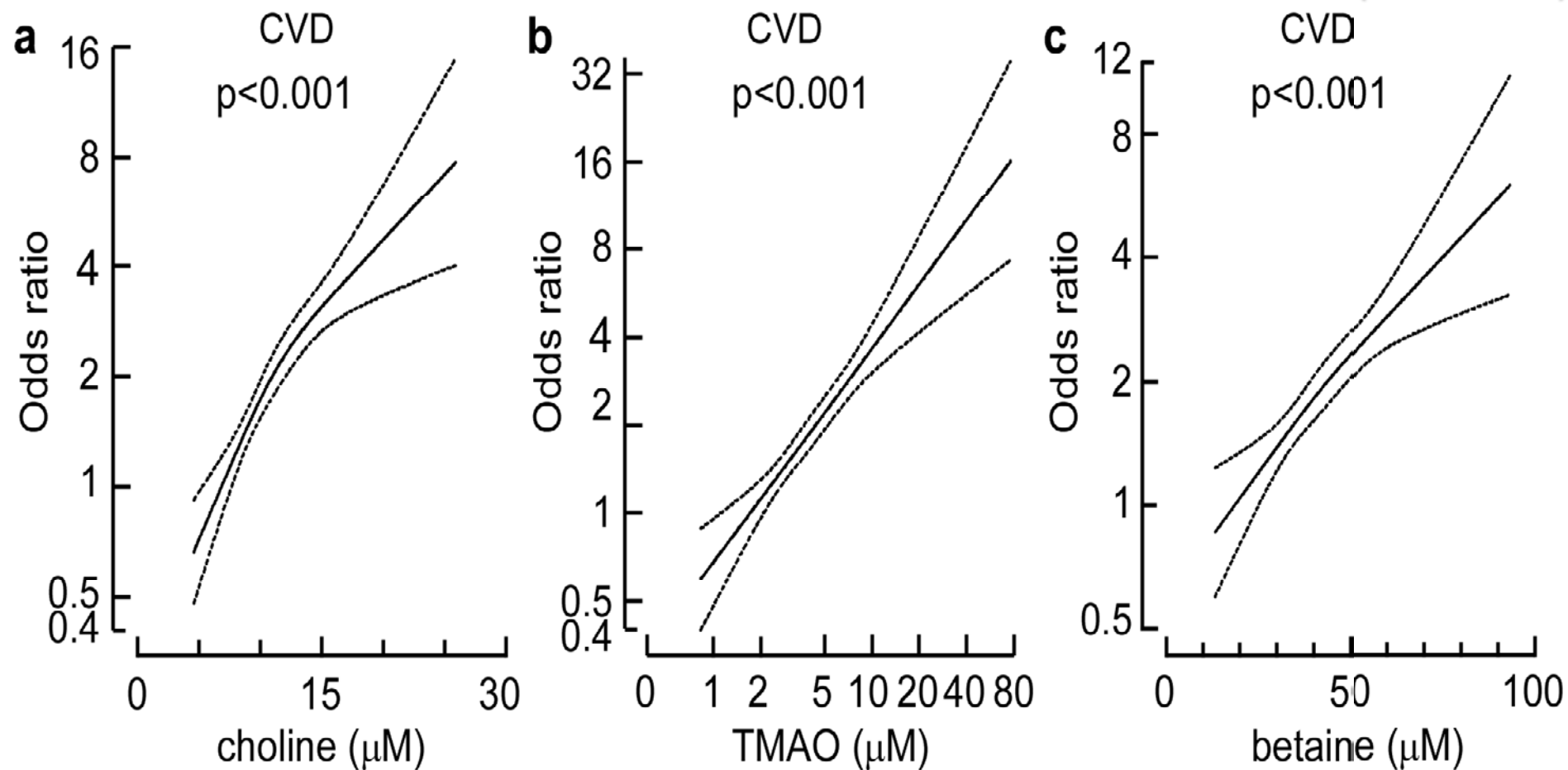## Demonstration of clinical prognostic utility

Phase 3: Mechanistic studies

Demonstration of causality for novel pathway

Prospective Cohort: N=1865 Sequential Cardiology Patients

a. Demographics of CVD prevalence

Characteristic	Non-CVD	CVD	P value
Age, mean (SD), y	61.2 (7.8)	65.3 (9.8)	<0.001
Women, %	52.5	49.8	0.59
Diabetes, %	11.1	32.8	<0.001
Hypertension, %	65.3	81.7	<0.001
History of smoking, %	52.2	56.7	0.36
Current smoking, %	5.3	5.9	0.80
LDL cholesterol, median (IQR), mg/dL	108 (85-132)	95 (78-122)	<0.001
HDL cholesterol, median (IQR), mg/dL	50 (40- 63)	42 (34-53)	<0.001
Triglycerides, median (IQR), mg/dL	116 (81-163)	139 (103-200)	<0.001
CRP, median (IQR), mg/dL	2.2 (1.2-4.6)	3.3 (1.5-7.3)	<0.001
Framingham Risk Score, median (IQR)	7.0 (5.0-9.0)	9.0 (6.0-12.0)	<0.001
MDRD (GFR), median (IQR)	75.5 (64.7-87.9)	71.6 (56.8-84.8)	0.004
ACE, % / Statin, % / Aspirin, %	34.7/29.8/55.1	56.4/64.9/75.5	each <0.001
TMANO, median (IQR), μM	3.3 (2.2-5.0)	4.4 (2.8-7.4)	<0.001
Choline, median (IQR), μM	10.9 (8.6-13.5)	12.3 (9.8-15.4)	<0.001
Betaine, median (IQR), μM	5.6 (4.4-7.0)	5.9 (4.6-7.2)	0.02

Wang Z et al, *Nature* (2011)

Plasma choline, TMANO and betaine levels predict CVD risks
(N=1865)

Odds ratio (95%CI) adjusted for age, sex, DM, HTN, smoking, LDL, HDL, TG, CRP, eGFR

Z Wang et al, *Nature* (2011)

Phase 1: Discovery-based investigations

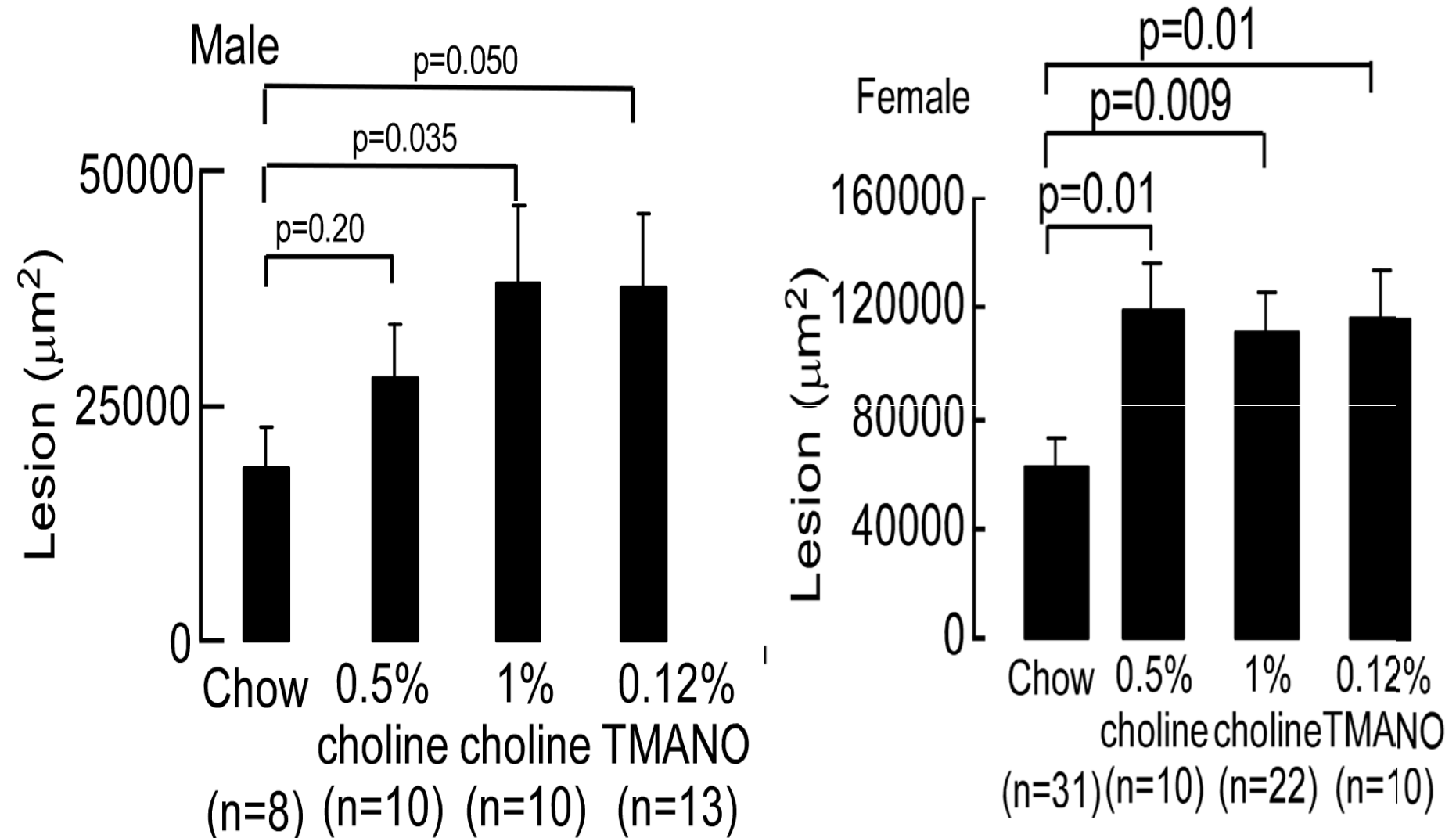Metabolomics screening and structural identification

Phase 2: Clinical validation

Demonstration of clinical utility

Phase 3: Mechanistic studies

Demonstration of causality for a novel pathway

Dietary supplementation with choline or TMANO promotes atherosclerosis ApoE $^{-/-}$ mice

Normal chow diet + indicated supplement weeks 4-20

Wang Z et al, *Nature* (2011)

Dietary supplementation with choline or TMANO promotes atherosclerosis without dyslipidemia or hepatic steatosis

	Lipids	Chow (n=9)	0.5% choline (n=10)	1.0% choline (n=10)	0.12% TMAO (n=13)
Plasma	Triglyceride (mg/dL)	114±16	150±22 (p=0.20)	99±13 (p=0.46)	104±8 (p=0.58)
	Cholesterol (mg/dL)	426±28	387±25 (p=0.32)	346±10 (p=0.02)	352±12 (p=0.04)
	Glucose (mg/dL)	238±28	290±18 (p=0.15)	191±33 (p=0.29)	248±20 (p=0.78)
Liver	Triglyceride (mg/g protein)	34.9±4.4	42.9±8.2 (p=0.47)	48.0±5.3 (p=0.07)	47.2±8.5 (p=0.22)
	Cholesterol (mg/g protein)	10.1±0.8	9.6±0.6 (p=0.64)	10.4±0.4 (p=0.72)	12.0±1.2 (p=0.18)
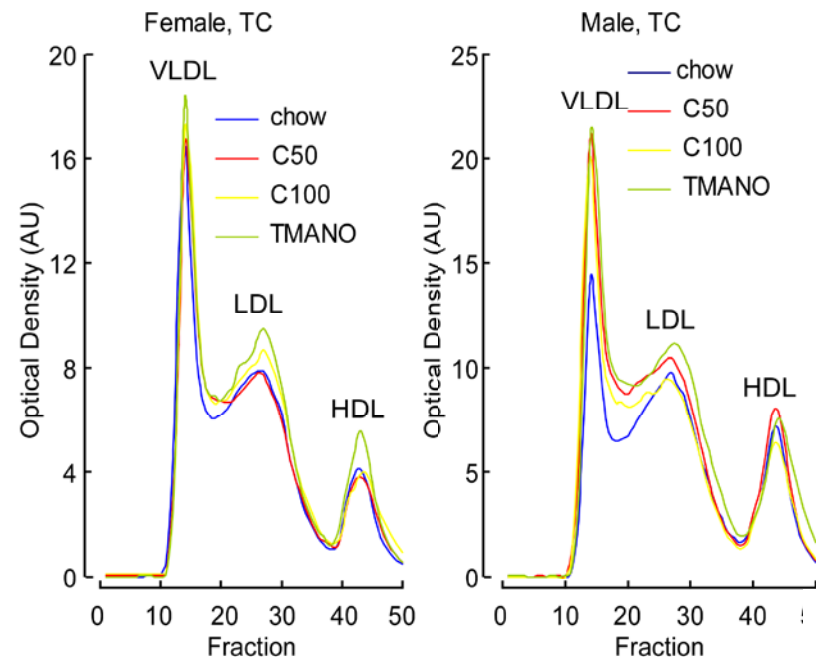
Liver oil-red-o staining
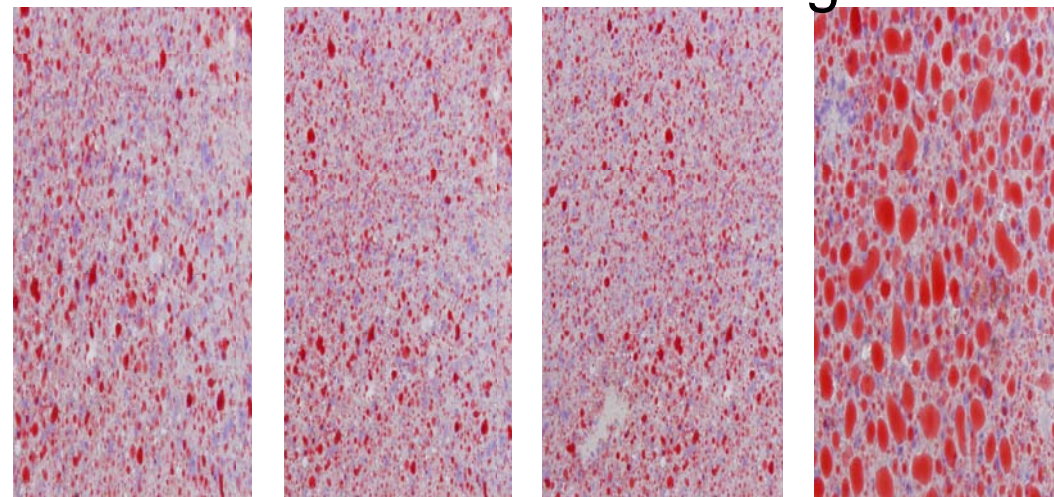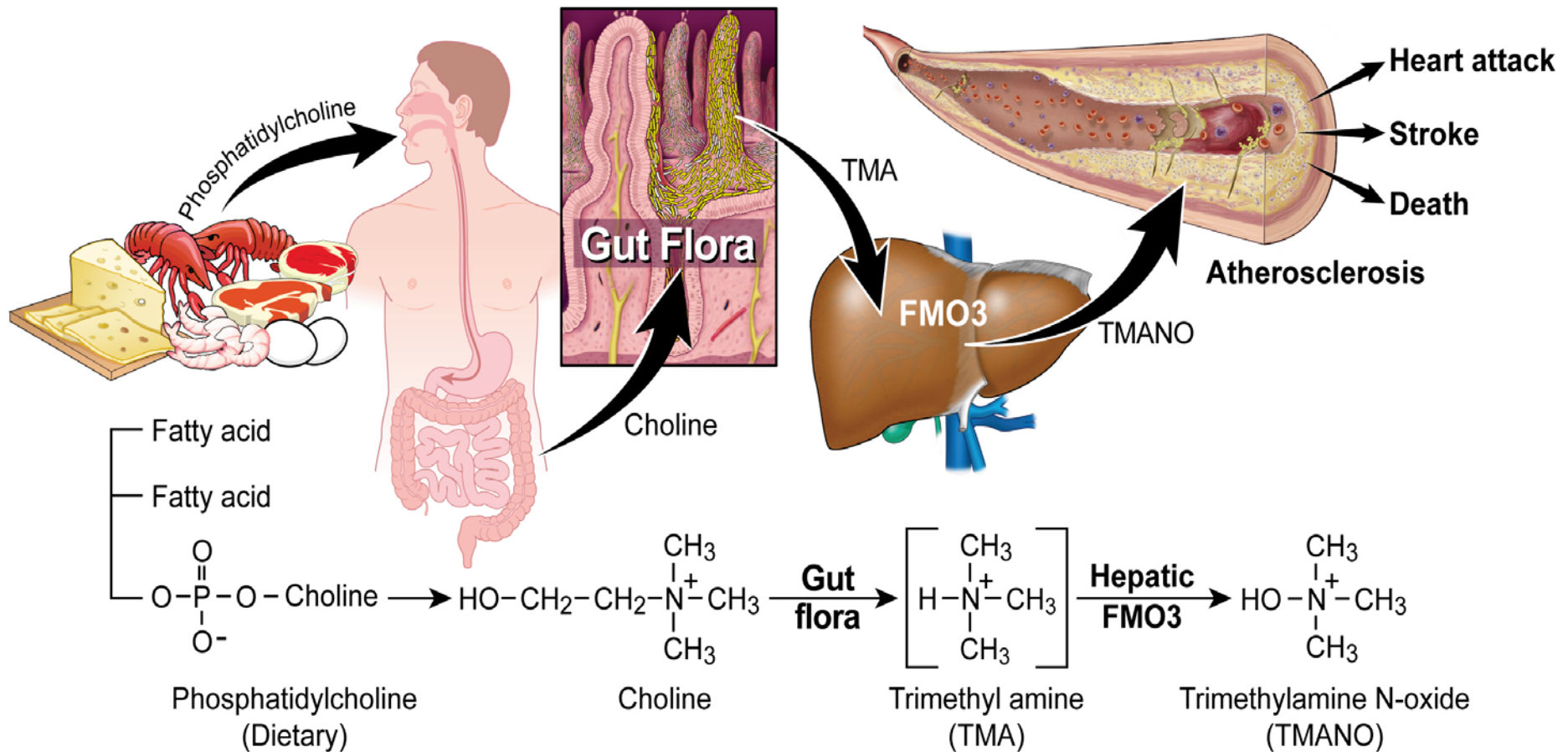
Chow   1% Choline   0.12% TMAO   MCD

Plasma TMANO levels are correlated with aortic plaque in apoE-/-

Wang Z et al, *Nature* (2011)
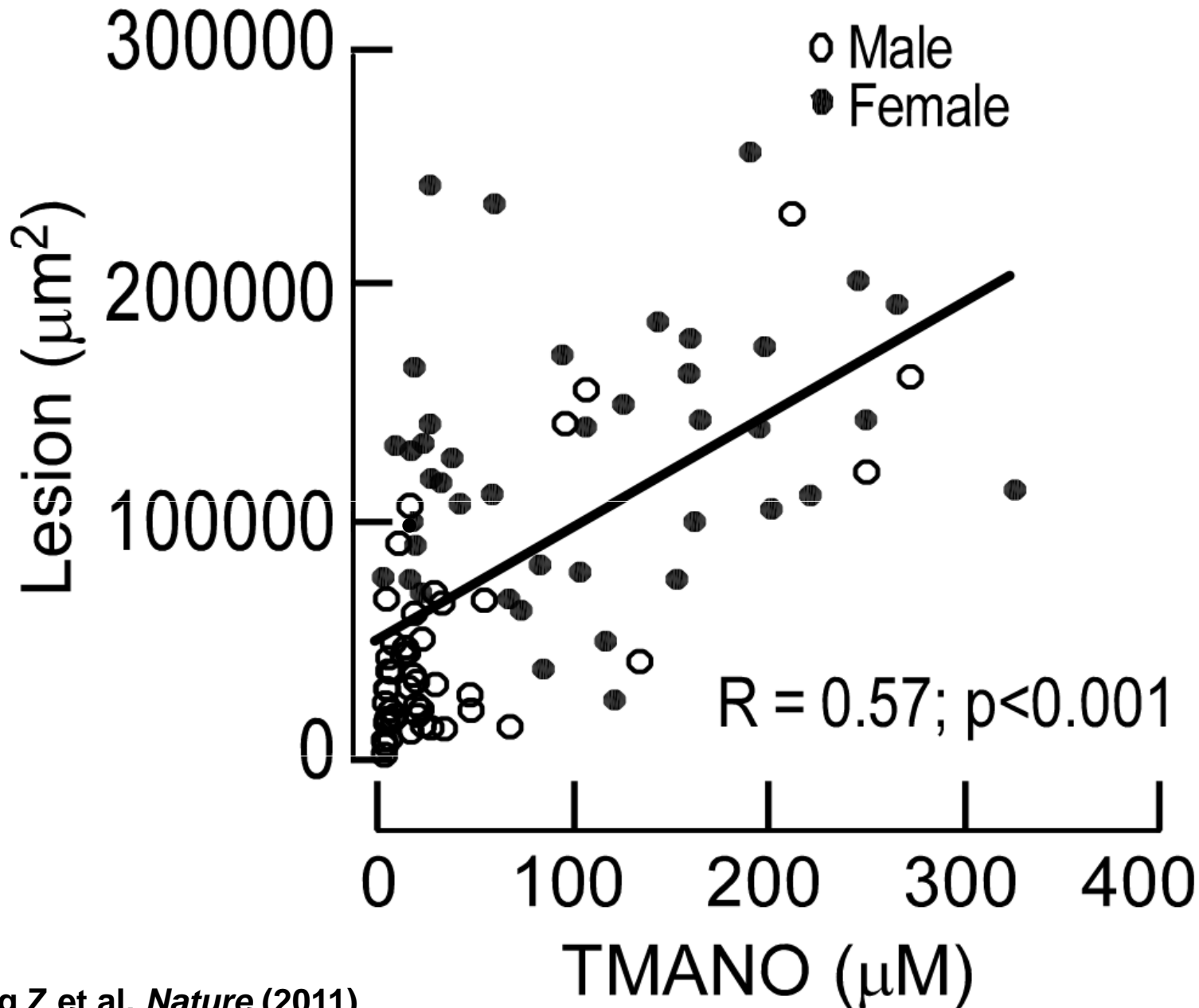
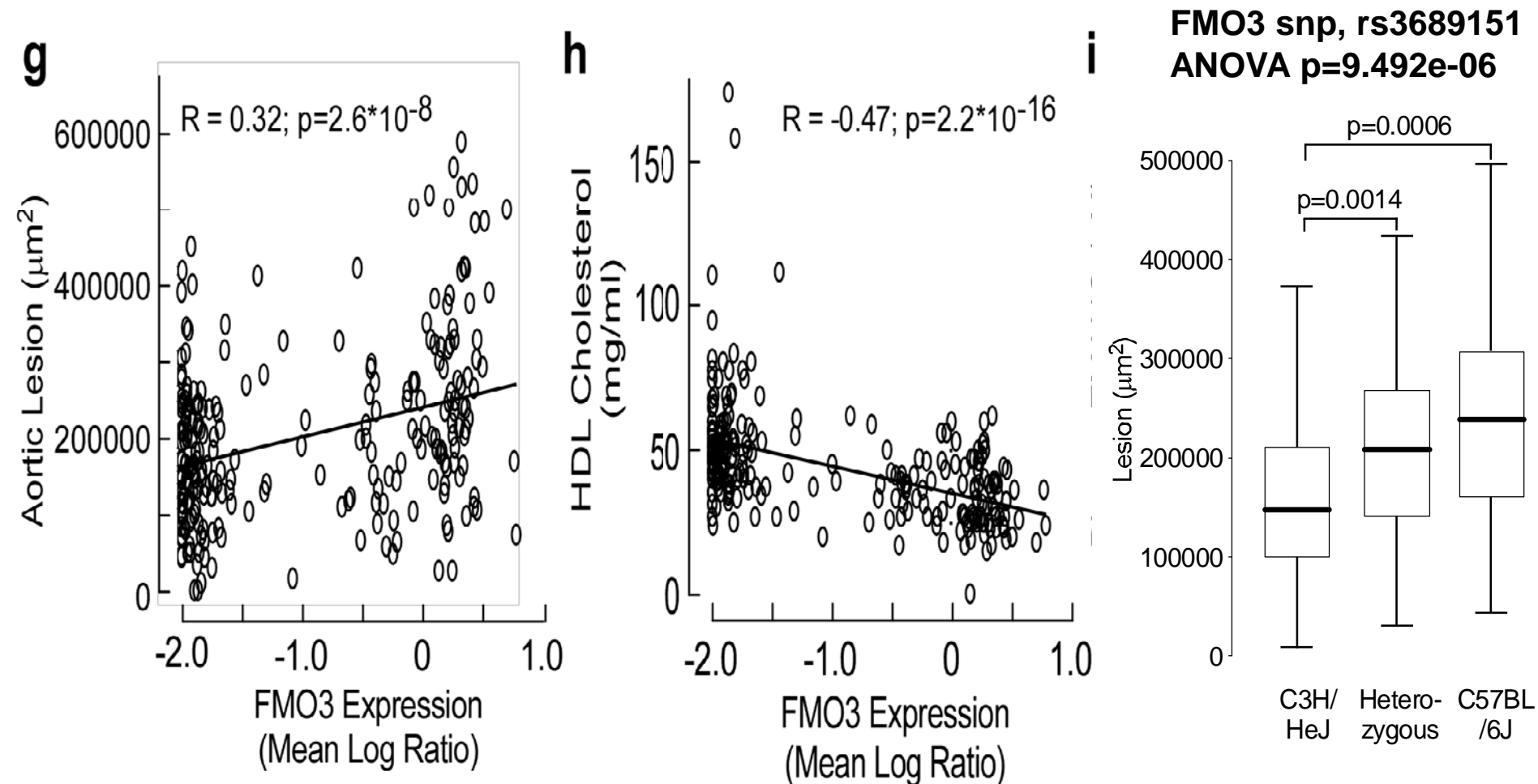# Plasma TMANO levels are correlated with angiographic coronary artery disease severity in subjects (N=1020)

p<0.0001
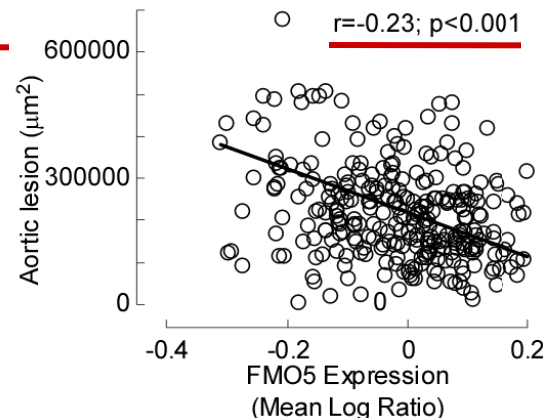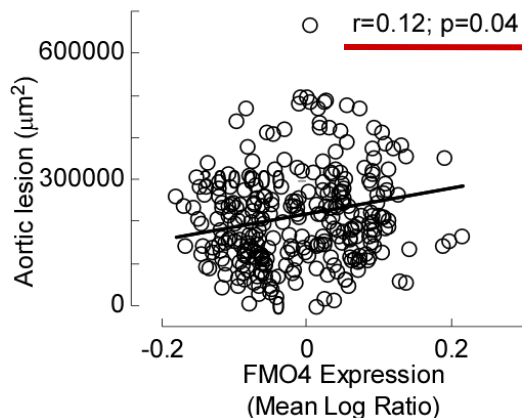Kruskal Wallis test

Wang Z et al, *Nature* **(2011)**
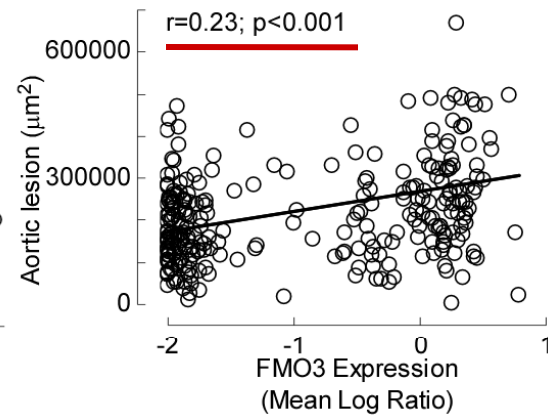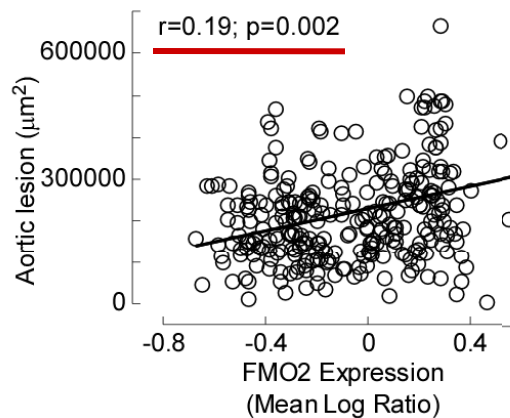
What of FMO3 ?

Wang Z et al, *Nature* (2011)
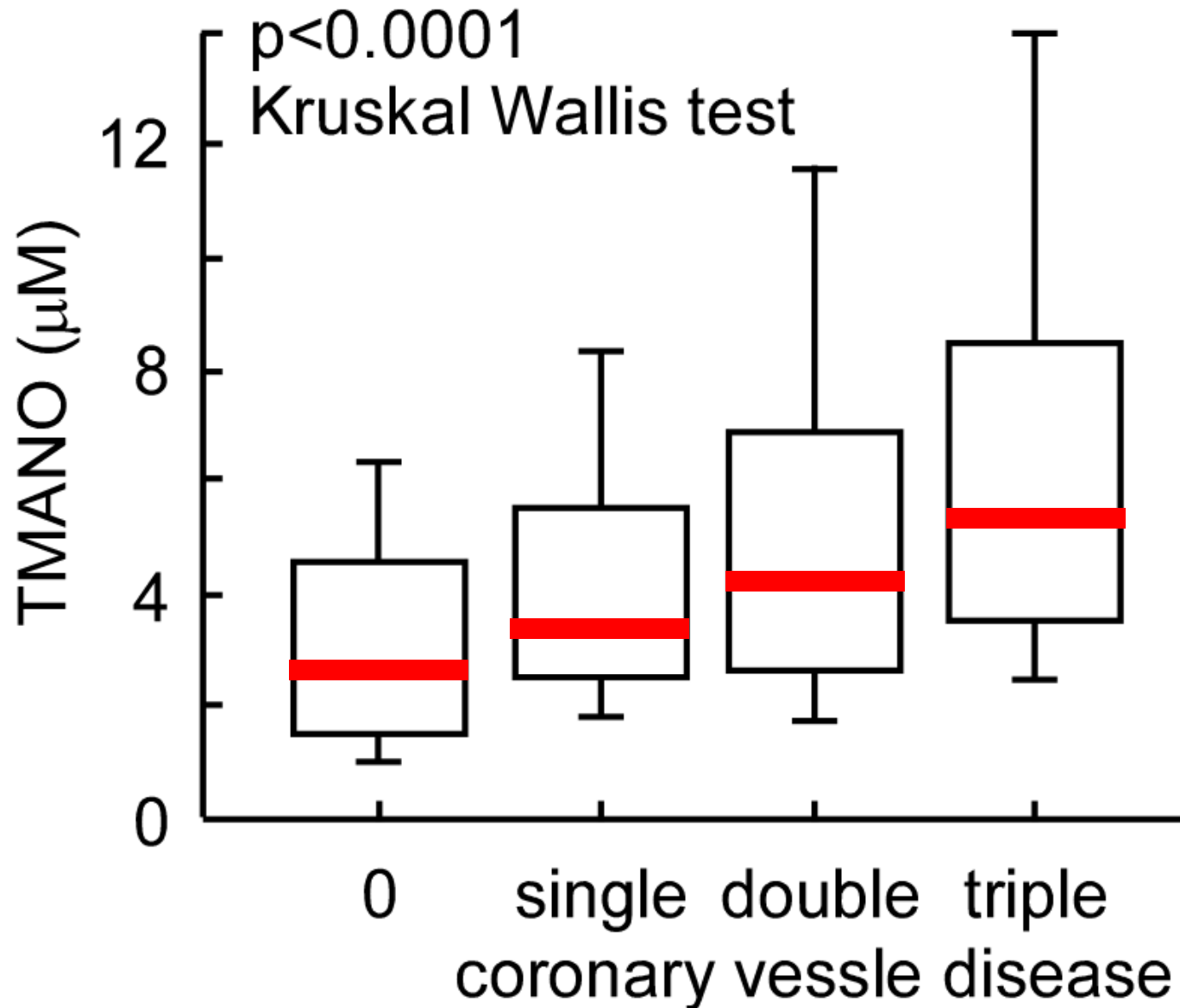
Integrative genetic studies in mice show FMO3 is linked to atherosclerosis susceptibility

FMO3 snp, rs3689151
ANOVA p=9.492e-06
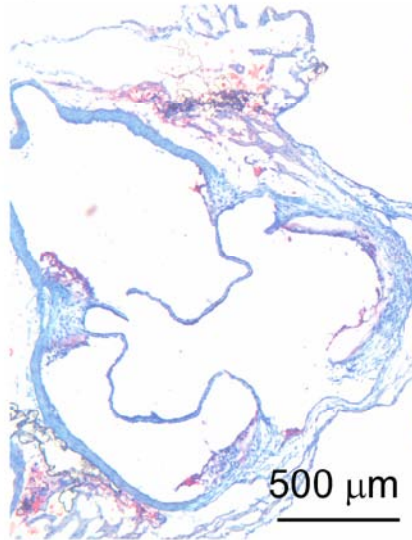
Wang Z et al, *Nature* (2011)
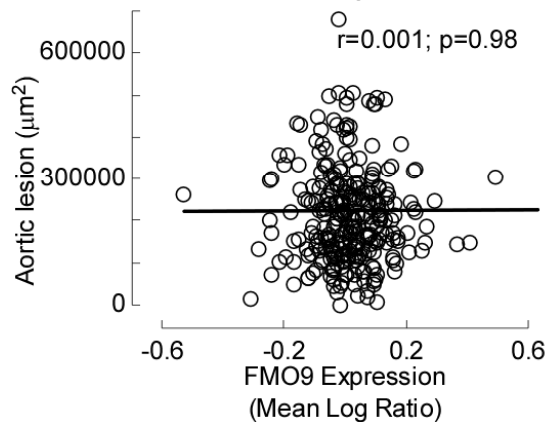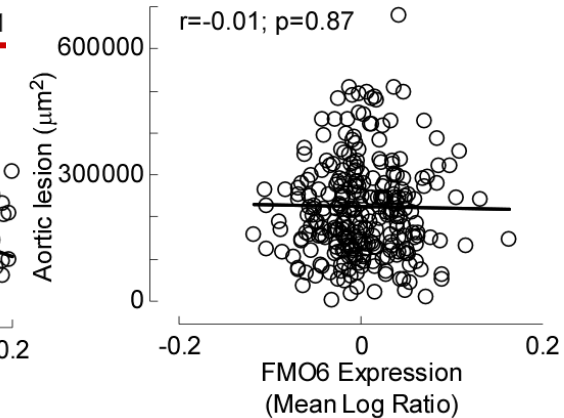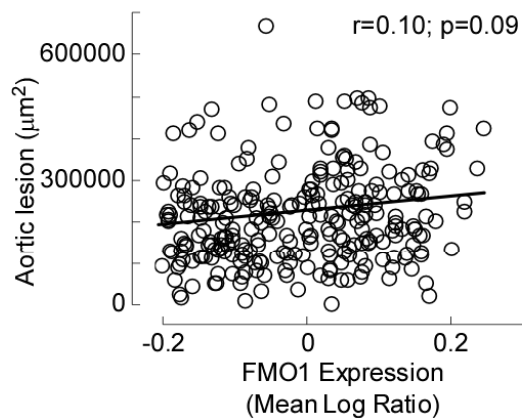
Hepatic expression of multiple FMO in the FMO gene cluster are correlated with aortic atherosclerotic lesion area (and TMANO and HDL)

Dietary choline and gut flora promote a pro-atherogenic macrophage phenotype *in vivo*
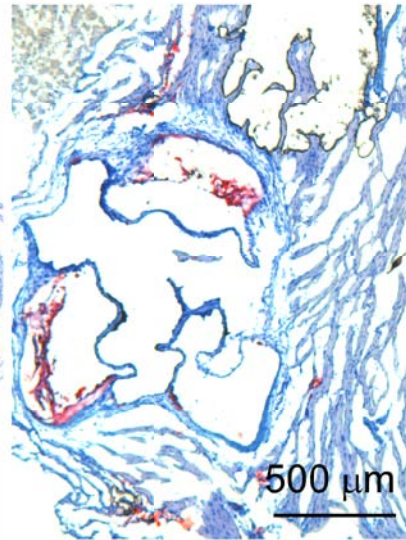

Wang Z et al, *Nature* (2011)
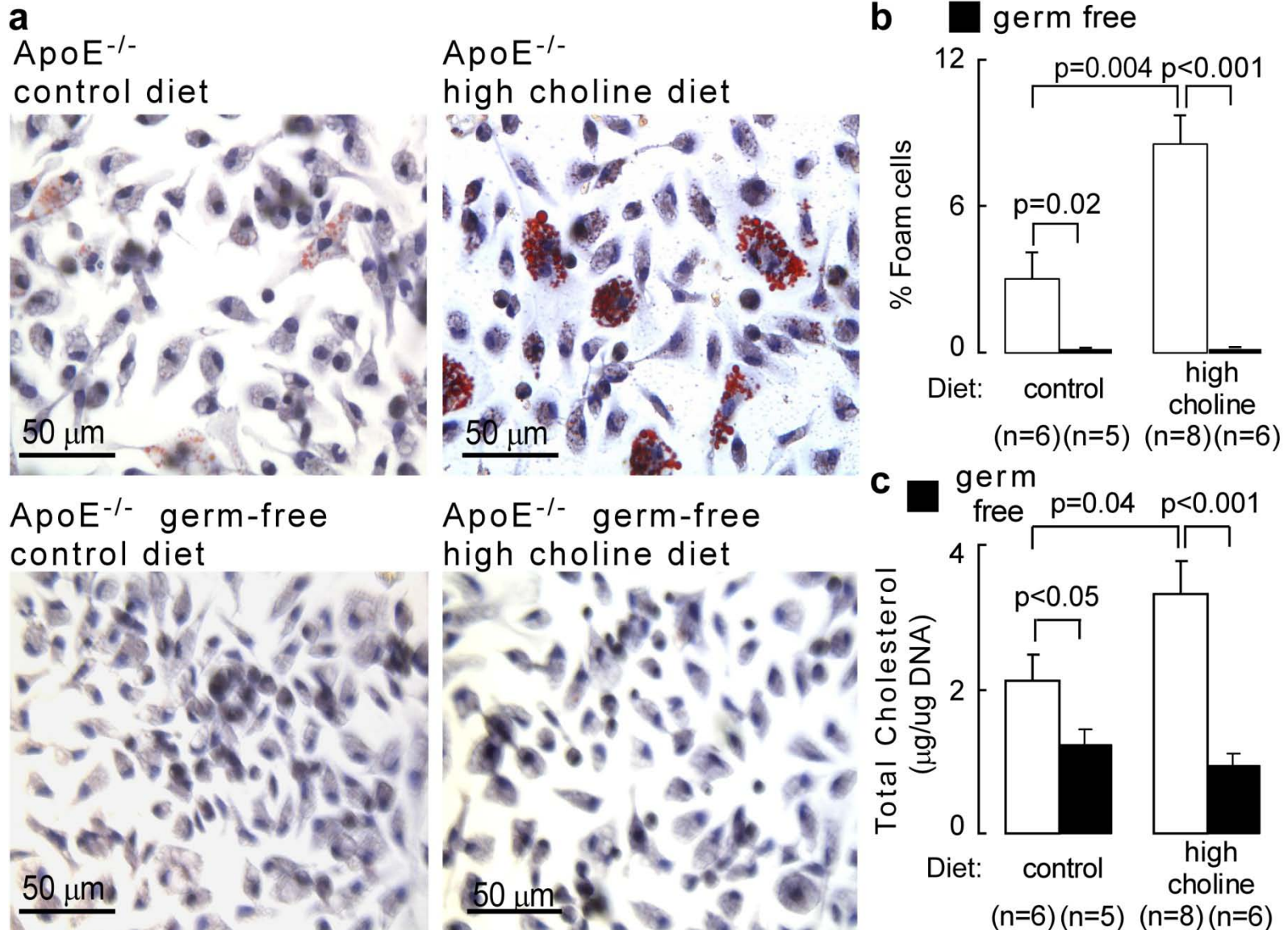
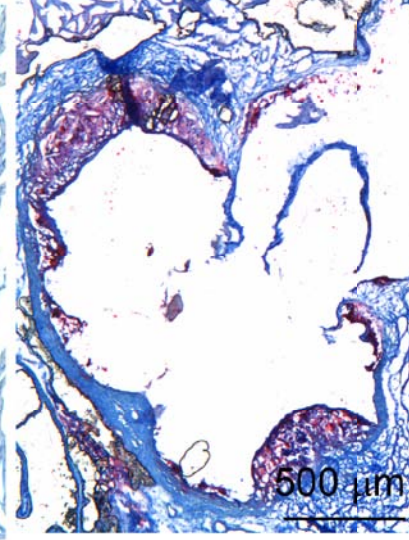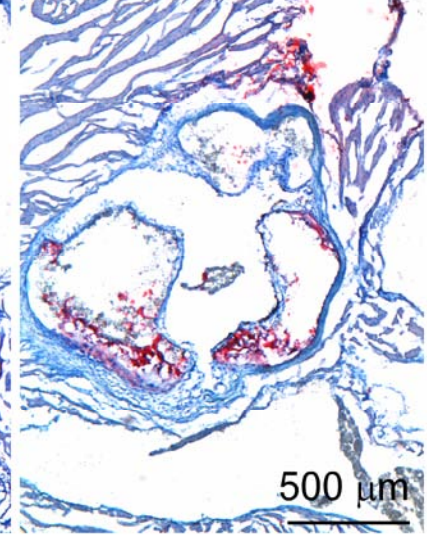# Suppression of gut flora inhibits dietary choline induced pro-atherogenic phenotype

F, control   F, control/Abx   F, choline   F, choline/Abx
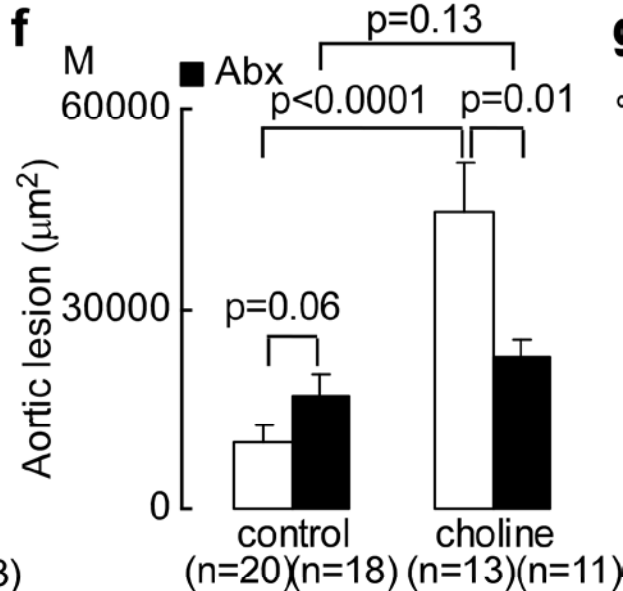
500 μm   500 μm   500 μm   500 μm

e
F ■ Abx
p=0.53
p=0.002   p=0.04
p=0.33

Aortic lesion (μm²)
90000
60000
30000
0
control   choline
(n=24)(n=21) (n=13)(n=13)

f
M ■ Abx
p=0.13
p<0.0001   p=0.01
p=0.06

Aortic lesion (μm²)
60000
30000
0
control   choline
(n=20)(n=18) (n=13)(n=11)

g
p=0.56
p=0.01 p=0.04
p=0.09

Aorta F4/80 positive (μm²)
120000
80000
40000
0
control   choline
n=   13 17   8 7
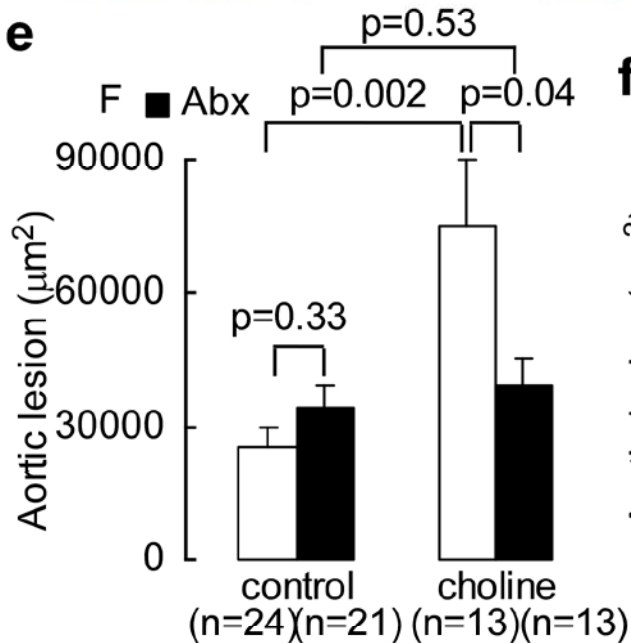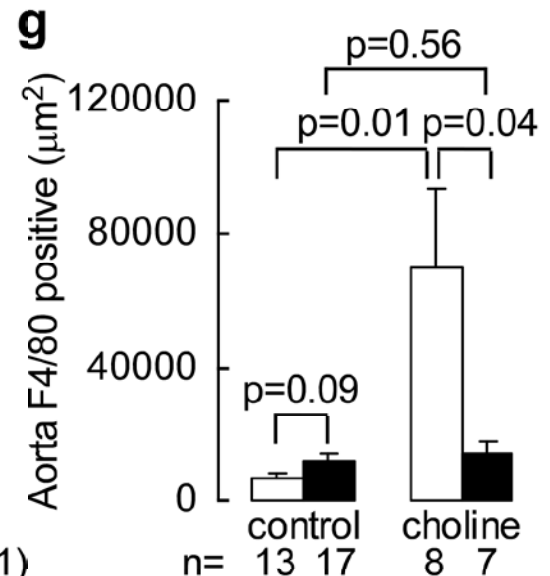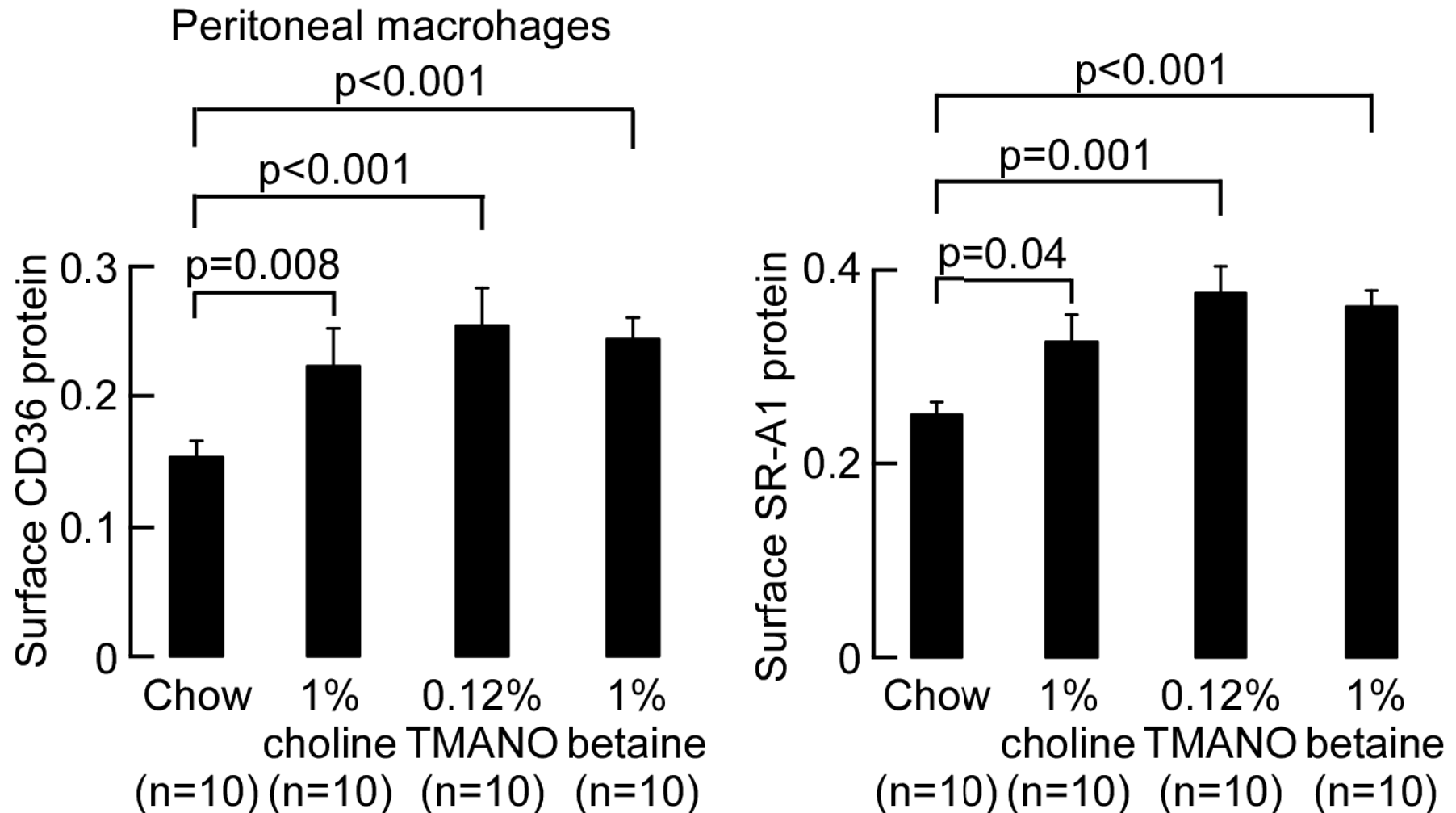
Effect of trimethylamine compound supplementation on macrophage scavenger receptor surface levels *in vivo*

Wang Z et al, *Nature* (2011)

A new (additional) nutritional basis of atherosclerotic heart disease……suggests new diagnostic and therapeutic approaches for the detection/treatment of CVD