Serum $N'\text{-Methylnicotinamide}$ is Associated With Coronary Artery Disease in Chinese Patients

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**Background**—We previously reported that serum $N'\text{-methylnicotinamide}$ (me-Nam), an indicator of nicotinamide N-methyltransferase activity, is associated with obesity and diabetes mellitus in Chinese patients. However, whether nicotinamide N-methyltransferase plays a role in human coronary artery disease (CAD) remains to be elucidated. We aim to investigate the associations of serum me-Nam with CAD in Chinese patients.

**Methods and Results**—Serum me-NAM was measured by liquid chromatography-mass spectrometry in patients with ($n=230$) or without ($n=103$) CAD as defined by coronary angiography. The severity of CAD was expressed by number of diseased coronary arteries. Serum me-Nam was higher (7.65 ng/mL versus 4.95 ng/mL, $P<0.001$) in patients with CAD than in those without. Serum me-Nam was positively correlated with high-sensitivity C-reactive protein and negatively correlated with high-density lipoprotein before and after adjustment for potential confounding variables ($P\leq0.002$). In multivariable logistic regression analyses, compared with those in the lowest tertile of serum me-NAM levels, patients in the top tertile had the highest risks for CAD (odds ratio, 4.21; 95% CI, 1.97–8.97 [$P<0.001$]). After adjustment for potential confounding variables, serum me-NAM was also increased from 0- to 3-vessel disease ($P$ for trend=0.01).

**Conclusions**—Serum me-Nam is strongly associated with presence and severity of CAD, suggesting nicotinamide N-methyltransferase as a potential target for treating atherosclerosis in humans. (*J Am Heart Assoc.* 2017;6:e004328. DOI: 10.1161/JAHA.116.004328.)

**Key Words:** coronary angiography • coronary artery disease • inflammation • $N'\text{-methylnicotinamide}$

Nicotinamide N-methyltransferase (NNMT) gene is abundantly expressed in adipose tissue and liver and lower expressed in other organs.1,2 NNMT behaves as an important methyltransferase to catalyze the transfer of a methyl group from s-adenosylmethionine to nicotinamide (vitamin B3) to generate $N'\text{-methylnicotinamide}$ (me-NAM) and s-adenosylhomocysteine, a precursor of homocysteine,1,2 and is therefore considered to play a unique role in the development of coronary artery disease (CAD) because it has long been known that elevated homocysteine is associated with cardiovascular disease.3

Recent studies have suggested that NNMT activity was increased in obesity and type 2 diabetes mellitus in mice and humans.4–6 Our recent epidemiological study also revealed that serum me-NAM, an indicator of NNMT activity, was associated with obesity and diabetes mellitus in a Chinese population.7 Because obesity and diabetes mellitus were independent risk factors for the development of CAD,8,9 we hypothesized that increased serum me-NAM levels might be associated with CAD. Indeed, upregulation of hepatic NNMT activity and subsequent increase in plasma me-NAM levels were associated with the progression of atherosclerosis in an animal model.10 Evidence for the association of me-NAM with CAD is still lacking in humans. Therefore, the present case-control study aims to...
investigate the relationship between serum me-NAM levels and CAD in Chinese patients.

Methods

Study Population

From April 2015 to July 2015, we invited 460 consecutive patients who were suspected of having CAD with chest pain and were referred for diagnostic coronary angiography to the Department of Cardiology, Jiangsu Province Hospital of Traditional Chinese Medicine, Nanjing, China. Of the 460 invited, 342 (74.3%) participated. We excluded 9 patients from the present analyses because of inadequate blood samples for me-NAM measurements (n=2) or missing information on angiographic data (n=2), plasma glucose (n=2), or serum lipids (n=3). Thus, the total number of participants in the present analyses was 333. All patients gave written informed consent. The study protocol was approved by the ethics committee of Jiangsu Province Hospital of Traditional Chinese Medicine.

Anthropometric and Biochemical Measurements

Sociodemographics, medical history, smoking and drinking habits, and the use of medications were documented with a standardized questionnaire. Body weight, body height, and blood pressure (BP) were measured by an experienced nurse. Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Three sitting BP measurements taken consecutively at 5-minutes intervals using an automated electronic device (Omron HEM 7130, Kyoto, Japan) were averaged for analysis. Venous blood samples were taken after overnight fasting for the measurement of plasma glucose, serum creatinine, total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglycerides (TGs), alanine aminotransferase (ALT), ß-glutamyltransferase (GGT), and high-sensitivity C-reactive protein (hs-CRP). Serum samples were subsequently stored in aliquots at −80°C for measurements of me-NAM.

As previously described,7 serum me-NAM concentrations were determined by liquid chromatography with tandem mass spectrometry (LC/MS/MS, Agilent 6430 Triple Quad LC/MS, USA) in the Clinical Pharmacology Laboratory, Jiangsu Province Hospital of Traditional Chinese Medicine. The intra- and inter-assay coefficients of variance were 3.69% and 4.95% for me-NAM, respectively. The analytic sensitivity of the assays for measuring me-NAM was 2.5 ng/mL. See Data S1 for further details (Tables S1 through S3).

Hypertension was defined as a BP of at least 140 mm Hg systolic or 90 mm Hg diastolic or as the use of antihypertensive drugs. Diabetes mellitus was defined as a fasting plasma glucose of at least 7.0 mmol/L or as the use of antidiabetic agents. Dyslipidemia was defined according to guideline of the National Cholesterol Education Program (Adult Treatment Panel III).11

Coronary Angiography and Analysis

Coronary angiography was performed via the radial or femoral access with 6-F diagnostic catheters. The angiographic assessment was performed independently by two interventional cardiologists (H.B.S., R.S.Z.) who were both blinded to the concentrations of me-NAM, according to lesion classification scheme of the American College of Cardiology/American Heart Association.12 In case of disagreement, the difference in interpretation was resolved by a third reviewer (Z.X.W.). CAD was defined as luminal diameter narrowing estimated visually at least 50% in any epicardial coronary artery. We then defined 3 patient groups based on the degrees of CAD: single-vessel disease, double-vessel disease, and triple-vessel disease.

Statistical Methods

Statistical analyses were performed using SAS software, version 9.1 (SAS institute, Cary, NC). Data were presented as mean±SD or median (25th and 75th percentiles) for continuous variables or as percentage for categorical variables. Departure from normality was tested by the Shapiro-Wilk statistic. Serum me-NAM, TGs, ALT, GGT, and hs-CRP concentrations were not normally distributed and were, therefore, logarithmically transformed for statistical analyses. Means and proportions were compared with the Student t test and Fisher exact test, respectively. Relationships among me-NAM, age, BMI, BP, liver enzymes, renal function, fasting plasma glucose, serum lipids, and hs-CRP were examined by calculation of partial correlation coefficients. Multivariable logistic regression analyses were employed to evaluate the odds ratios (ORs) and 95% CIs of having CAD for higher tertiles of me-NAM compared with the lowest tertile. Linear regression analysis was used to test for trend of the changes of serum me-NAM concentrations across the severity of coronary angiography (normal to triple-vessel disease). Comparisons between groups were performed with ANOVA, and Bonferroni correction was used for post hoc analysis. Sensitivity analyses stratified by sex or after exclusion of diabetic patients were performed. A 2-sided value of P<0.05 was considered statistically significant.

Results

Characteristics of the Study Participants

The 333 participants (mean age 65.6±10.6 years) included 193 men (57.9%), and 233 had hypertension (70.0%), 103 had type 2 diabetes mellitus (30.9%), and 173 had dyslipidemia.
(52.0%). Table 1 summarizes the characteristics of the study participants with or without CAD. There were no statistically significant differences between CAD and control patients with respect to BMI, systolic/diastolic BP, rates of alcohol intake, plasma glucose, total and LDL cholesterol, and TG, ALT and GGT, and hs-CRP levels. However, CAD patients, compared with control patients, had higher proportions of male sex and current smoking ($P \leq 0.02$); higher rates of hypertension or taking antihypertensive drugs, diabetes mellitus or taking antihyperglycemic drugs, and dyslipidemia or taking hypolipidemic drugs ($P \leq 0.001$); and higher serum creatinine and lower serum HDL cholesterol concentrations ($P \leq 0.03$). In addition, CAD patients, compared with controls, had higher serum me-NAM concentrations (7.65 ng/mL versus 4.95 ng/mL; $P < 0.001$).

### Association of me-NAM With Clinical Parameters

We next investigated the relationship of serum me-NAM levels with various anthropometric and laboratory parameters.

#### Table 1. Characteristics of Control Subjects and Patients With CAD

| Characteristic                              | Control (n=103) | CAD Patients (n=230) | $P$ Value |
|---------------------------------------------|----------------|----------------------|-----------|
| Age, y                                      | 61.8±9.7       | 67.2±10.5            | <0.001    |
| Male, n (%)                                 | 50 (48.5)      | 143 (62.2)           | 0.02      |
| Body mass index, kg/m$^2$                    | 24.7±3.5       | 24.9±3.3             | 0.65      |
| Systolic blood pressure, mm Hg              | 134.3±18.8     | 137.9±17.9           | 0.10      |
| Diastolic blood pressure, mm Hg             | 81.7±11.5      | 81.4±11.7            | 0.84      |
| Current smoking, n (%)                      | 18 (17.5)      | 73 (31.7)            | 0.007     |
| Alcohol intake, n (%)                       | 17 (16.5)      | 57 (24.8)            | 0.09      |
| Hypertension, n (%)                         | 56 (54.4)      | 177 (77.0)           | <0.001    |
| Diabetes mellitus, n (%)                    | 12 (11.7)      | 91 (39.6)            | <0.001    |
| Dyslipidemia, n (%)                         | 37 (35.9)      | 136 (59.1)           | <0.001    |
| Taking antihypertensive drugs, n (%)        | 47 (45.6)      | 159 (69.1)           | <0.001    |
| Taking antihyperglycemic drugs, n (%)       | 11 (10.7)      | 70 (30.4)            | <0.001    |
| Taking hypolipidemic drugs, n (%)           | 20 (19.4)      | 86 (37.4)            | 0.001     |
| Plasma glucose, mmol/L                      | 5.50±1.40      | 5.85±1.70            | 0.07      |
| Total cholesterol, mmol/L                   | 4.10±0.77      | 4.13±1.16            | 0.80      |
| LDL cholesterol, mmol/L                     | 2.21±0.58      | 2.29±0.75            | 0.36      |
| HDL cholesterol, mmol/L                     | 1.24±0.31      | 1.16±0.33            | 0.03      |
| Triglycerides, mmol/L                       | 1.04 (0.80–1.75) | 1.23 (0.88–1.69) | 0.40      |
| Alanine aminotransferase, U/L               | 19 (15–27)     | 20 (15–33)           | 0.13      |
| γ-glutamyltransferase, U/L                  | 19 (13–35)     | 21 (15–33)           | 0.91      |
| Serum creatinine, μmol/L                    | 76.9±16.9      | 86.9±20.3            | <0.001    |
| High-sensitivity C-reactive protein, mg/L*  | 5.0 (3.0–11.0) | 6.0 (2.5–12.0)       | 0.88      |
| $N^\prime$-methyl nicotinamide, ng/mL       | 4.95 (3.31–8.74)| 7.65 (4.57–11.59) | <0.001    |

Data are mean ± standard deviation, median with interquartile range in parenthesis, or number with percentage in parenthesis. CAD indicates coronary artery disease; LDL, low-density lipoprotein; HDL, high-density lipoprotein.

*High-sensitivity C-reactive protein was measured in 71 control and 60 CAD patients.

Serum me-NAM concentrations were positively associated with BMI ($r=0.14; P=0.03$) and hs-CRP ($r=0.39; P<0.001$) but negatively associated with HDL cholesterol ($r=-0.17; P=0.002$) in the CAD group. All of these correlations remained statistically significant ($P \leq 0.01$) after adjustments for age, current smoking, alcohol intake, BMI, systolic BP, serum ALT and GGT, creatinine, fasting plasma glucose, total and LDL cholesterol, and TGs, as appropriate. In the control group, there was no correlation between me-NAM and these clinical parameters.

### Association of me-NAM With Presence and Severity of CAD

In logistic regression analyses, compared with patients in the lowest tertile of serum me-NAM levels, patients in the highest tertile were associated with an increased OR of CAD both before and after adjustment for potential confounders (ie, age; sex; BMI; systolic BP; current smoking and alcohol intake; hypertension;...
diabetes mellitus; dyslipidemia; use of antihypertensive, antihyperglycemic, and hypolipidemic drugs; and fasting plasma glucose, total and low-density lipoprotein cholesterol, and triglycerides. CAD indicates coronary artery disease; me-NAM, N\(^\text{\textsuperscript{1}}\)-methyl nicotinamide.

In the adjusted model, odds ratio (95% CI) were adjusted for age, sex, body mass index, systolic blood pressure, current smoking and alcohol intake, hypertension, diabetes mellitus, dyslipidemia, use of antihypertensive, antihyperglycemic and hypolipidemic drugs, and fasting plasma glucose, total and low-density lipoprotein cholesterol, and triglycerides. CAD indicates coronary artery disease; me-NAM, N\(^\text{\textsuperscript{1}}\)-methyl nicotinamide.

Table 2. Associations of Serum me-NAM Concentration Tertiles With CAD

| Serum me-NAM, ng/mL (Tertile 2 vs Tertile 1) | Odds Ratio (95% CI) | P Value |
|--------------------------------------------|--------------------|---------|
| Crude model                                | 2.06 (1.18–3.59)   | 0.01    |
| Adjusted model                             | 2.41 (1.23–4.72)   | 0.01    |

In adjusted model, odds ratio (95% CI) were adjusted for age, sex, body mass index, systolic blood pressure, current smoking and alcohol intake, hypertension, diabetes mellitus, dyslipidemia, use of antihypertensive, antihyperglycemic and hypolipidemic drugs, and fasting plasma glucose, total and low-density lipoprotein cholesterol, and triglycerides. CAD indicates coronary artery disease; me-NAM, N\(^\text{\textsuperscript{1}}\)-methyl nicotinamide.

We next performed linear regression analysis to investigate whether me-NAM levels were associated with severity of CAD. In unadjusted analyses, serum me-NAM concentrations were increased across the severity of CAD (Table 3). After adjustment for the aforementioned covariates, the trends of serum me-NAM concentrations across the severity of CAD remained statistically significant (Figure and Table 3).

Separating all patients into men and women did not materially change the results (Table S4 and Figure S1). In further analyses, similar results were observed when we performed analyses in 230 patients without diabetes. Indeed, after exclusion of diabetic patients, patients in the highest tertile compared with the lowest tertile of serum me-NAM were associated with higher risks for CAD after adjustment for potential confounders (OR: 4.45; 95% CI: 1.95–10.15 [P<0.001]). Similarly, the trends of serum me-NAM concentrations across the severity of CAD remained statistically significant after adjustment for potential confounders (P<0.001).

Discussion

The main finding of our study is that serum me-NAM, an indicator of NNMT activity, is strongly associated with CAD, independent of conventional CAD risk factors. Among 333 Chinese adults in our study, patients in the highest compared with those in the lowest tertile of serum me-NAM were associated with a ≥3-fold increase in the risk for CAD. Another interesting finding of the present study is that me-NAM concentrations are also associated with severity of CAD.

Until now, there has been only one animal-based study that examined the effects of hepatic NNMT activity and plasma me-NAM levels in the development of atherosclerosis.\(^1\) Mateuszuk et al\(^1\) found that hepatic NNMT activity and plasma me-NAM levels increased by more than 2-fold in apolipoprotein-E/LDL double knockout mice compared with controls, and they were associated with the progression of atherosclerosis. These findings support our results that serum me-NAM concentrations were not only associated with the risk for CAD, but also with the severity of CAD. However, it is not clear whether increased me-NAM concentrations is a compensatory response or only a marker of atherosclerosis. Notably, although traditionally considered as an inactive or toxic metabolite of terminal nicotinamide clearance,\(^13,14\) some publications suggest that, at pharmacological doses, me-NAM has biological activity and affects endothelial cell function.\(^15–17\) Recently, Domagala et al\(^18\) reported that me-NAM ameliorated endothelial dysfunction via increasing endothelial NO synthase–mediated NO release from endothelial cells. In addition, Mateuszuk et al\(^19\) demonstrated that me-NAM displayed antiatherosclerotic effects in apolipoprotein-E/LDL double knockout mice via improving endothelial function, inhibiting platelet activation and inflammation. Therefore, we speculate that the increase in serum me-NAM in CAD might be attributable to a defensive
response, which may represent an ability to adapt to atherosclerosis by preventing endothelial dysfunction. However, we cannot exclude that the elevated me-NAM levels may be associated with other unknown factors of CAD, such as diabetes mellitus, which was widely recognized as a CAD risk equivalent. Indeed, Kanten’s and our recent studies reported a significant association between me-NAM and diabetes mellitus. To exclude the role of diabetes mellitus on the relationship between me-NAM and atherosclerosis, we further investigated the association of me-NAM with CAD in nondiabetic patients. Although there was no statistical difference, the effect of me-NAM on CAD was slightly larger after exclusion of diabetes mellitus (OR, 4.45 versus 4.21). Further studies are needed to delineate the mechanisms and independent role of me-NAM in CAD and atherosclerosis. Of note, serum me-NAM levels in the current study was slightly lower than that in our previous study. Although the exact reasons for the differences are unclear, several potential factors including study design, sample size, ethnicity and patient characteristics may explain the differences.

Our finding on the association between serum me-NAM concentrations and HDL is consistent with the result of our previous epidemiological study but not with a recent study. Hong et al reported that serum me-NAM was not significantly associated with HDL. The disparities may be due to the different sample size in Hong’s study (n=51) compared with ours (n=333), and the patients in Hong’s study were of different ethnicity and those with morbid obesity who needed elective gastric bypass surgery. Hong’s study did not find the association between me-NAM and HDL perhaps due to the severity of obese patients because serum me-NAM levels were affected by obesity. However, hepatic NNMT activity was inversely associated with HDL in an animal model in their study, which is consistent with our finding.

Another interesting observation of the current study is a positive correlation between me-NAM and hs-CRP, independent of other risk factors, which indicates that the association between me-NAM and CAD is probably through inflammatory pathways because hs-CRP, a well-known marker of inflammation, may contribute to the development of CAD. Indeed, hepatic NNMT activity and me-NAM plasma concentrations increased with the development of vascular inflammation in the aorta. However, we cannot rule out that increased me-NAM concentrations in our study may represent a compensatory mechanism in response to inflammation because me-NAM possesses anti-inflammatory properties by inhibiting the generation of reactive oxygen species.

Study Limitations

Our study should be interpreted within the context of its limitations. The cross-sectional design does not allow causal inference. In addition, we only investigated me-NAM levels in Chinese patients, and therefore our findings need to be confirmed in other ethnicities.

Conclusions

Our first case-control study shows significant associations of serum me-NAM with the presence and severity of CAD in Chinese patients. Although prospective and interventional studies are necessary to investigate whether elevated NNMT activity may play a causal role in CAD in humans, our findings provide novel insights into the potential role of NNMT in CAD.

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SUPPLEMENTAL MATERIAL
Data S1.

Supplemental Methods

Quantification of serum N1'-methylnicotinamide

Serum me-NAM was measured by liquid chromatography with tandem mass spectrometry (LC/MS/MS) using an electrospray ionization-triple quadrupol mass spectrometer (Agilent G6430, USA) coupled to a liquid chromatography system (Agilent 1290, USA) controlled by MassHunter workstation software with version B. 05.00. Chromatographic separation was achieved on a Spherisorb CNRP 5 µm, 4.6x150 mm analytical column (Waters, USA) at 30 °C with a flow rate of 200 µL/min. A sample volume of 3 µL was injected onto the column. Eluents consisted of 5 mM ammonium formate/0.1% formic acid aqueous solution (A) and 100% acetonitrile (B). The gradient program of the mobile phase was set as follows: a 55/45 (v/v) mixture of solvents A and B from 0.00 to 3.2 min, a 90/10 mixture from 3.3 to 3.6 min, and a 55/45 mixture from 3.7 to 5.5 min, resulting in a total run time of 5.5 min per sample (Table S1). The column flow was directly converted into the electrospray ionization (ESI) source of the mass spectrometer, which was operated in the positive ion mode. The optimal MS parameters were as follows: capillary voltage 4000 V; gas temperature 350 °C, gas flow 10 L/min and nebulizer 20 pounds per square inch (psi). The compound dependent parameters like fragmentor and collision energy for N1'-methylnicotinamide (me-NAM) were optimized at 100 V and 20 V, respectively, and for internal standard N'-methylnicotinamide were 100 V and 25 V, respectively (Table S2). Quantification was performed via peak area ratios (multiple reaction monitoring m/z 137.1 -> m/z 94.1) applied to internal standard N'-methylnicotinamide (multiple reaction monitoring m/z 137.1 -> m/z 80.1) in an external calibration curve.

Samples were prepared using deproteinization with acetonitrile. 20 µL of N'-methylnicotinamide (internal standard, IS) working solution (30 ng/mL in methanol) and 160 µL of acetonitrile were sequentially added to 50 µL serum sample and vortex-mixed for 2 min. After centrifugation at 12,000 rpm for 10 min at 4 °C, an aliquot of 3 µL of the supernatant was injected into the LC-MS/MS system.
Calibration curves were prepared by spiking pooled blank serum with an appropriate amount of working solution to produce the calibration curve points equivalent to 80, 40, 20, 10, 5, 2.5 ng/mL of me-NAM (Sigma-Aldrich, USA). Samples were made in five replicates and each of them also contained 20 µL of the IS working solution. The results (peak-area ratio of analytes to IS) versus concentration were fitted to the linear equation. The peak intensities of the mean blank serum of me-NAM should be subtracted from the calibration standards response. The results show that the calibration curves were linear over the concentration range of 2.5–80 ng/mL for me-NAM with the linear regression equation of $f = 0.0630 \times C + 0.0268$, $r = 0.9997$ ($n = 5$), where $f$ represented the peak-area ratio of analyte to IS and $C$ represented the serum concentrations of analyte.

The stability of the analytes was validated in analyte-spiked plasma samples under five different storage conditions (Table S3). Three different concentrations of me-NAM (3.75, 15 and 60ng/mL) were used for calculating the variability of the analyte according to the criteria limits defined in FDA Bioanalytical Method Validation Guidance for Industry. The samples for calculating the intra- and inter-assay variability of each concentration of me-NAM is 5 and 15, respectively, resulting in a total sample size of 15 and 45 for calculating the intra- and inter-assay variability, respectively.
Table S1. HPLC Conditions for measuring \( N' \)-methylnicotinamide

| HPLC Conditions |
|-----------------|
| **Column**      | Waters Spherisorb CNRP (4.6×150 mm, 5 µm) |
| **Column temperature** | 30°C |
| **Mobile phase** | a 55/45 (v/v) mixture of solvents A and B from 0.00 to 3.2 min, a 90/10 mixture from 3.3 to 3.6 min, and a 55/45 mixture from 3.7 to 5.5 min |
| **Flow rate**   | 200 µL/min |
| **Run time**    | 5.5 min |
| **Injection volume** | 3 µL |
Table S2. MS Conditions for measuring $N'$-methylnicotinamide

| Ion Source                  | MS Conditions |
|-----------------------------|---------------|
| ESI                         |               |

| Polarity        | Positive     |
|-----------------|--------------|

| Scan Type       | ESI          |
|-----------------|--------------|
| MRM             |              |

| Compound name              | Precursor Ion (amu) | Product Ion (amu) | Dwell time (msec) | Fragmentor (v) | Collision energy (v) |
|---------------------------|---------------------|-------------------|-------------------|----------------|---------------------|
| $N'$-methylnicotinamide    | 137.1              | 94.1              | 200               | 100            | 20                  |
| $N'$-methylnicotinamide (IS)| 137.1             | 80.1              | 200               | 100            | 25                  |

| Capillary voltage | 4000 v |
|-------------------|--------|
| Gas temperature   | 350°C  |
| Gas flow          | 10 L/min |
| Nebulizer         | 20 psi |

ESI indicates electrospray ionization; MRM, multiple reaction monitoring; psi, pounds per square inch.
### Table S3. Stability of $N'$-methylnicotinamide (me-NAM) under different storage conditions (n=3)

| Condition | Spiked concentrations of me-NAM (ng/mL) | 3.75 | 15 | 60 |
|-----------|-----------------------------------------|------|----|----|
| **Condition 1 (as control)** | Mean ± SD (ng/mL) | 3.31 ± 0.14 | 13.74 ± 0.25 | 56.40 ± 1.47 |
| | RSD (%) | 4.28 | 1.84 | 2.60 |
| | Accuracy (%) | 88.37 | 91.63 | 93.99 |
| **Condition 2 (4 °C for 24 h)** | Mean ± SD (ng/mL) | 3.40 ± 0.32 | 14.86 ± 0.34 | 66.37 ± 1.59 |
| | RSD (%) | 9.45 | 2.32 | 2.40 |
| | Accuracy (%) | 90.76 | 99.04 | 110.62 |
| **Condition 3 (25 °C for 4 h)** | Mean ± SD (ng/mL) | 3.45 ± 0.06 | 13.78 ± 0.22 | 58.05 ± 1.61 |
| | RSD (%) | 1.84 | 1.57 | 2.77 |
| | Accuracy (%) | 92.67 | 91.47 | 98.68 |
| **Condition 4 (-20 °C for 25 days)** | Mean ± SD (ng/mL) | 3.53 ± 0.24 | 13.78 ± 0.14 | 60.62 ± 2.03 |
| | RSD (%) | 6.87 | 1.00 | 3.34 |
| | Accuracy (%) | 94.02 | 91.84 | 101.04 |
| **Condition 5 (3 freeze/thaw cycles at -20 °C)** | Mean ± SD (ng/mL) | 3.83 ± 0.42 | 15.22 ± 0.34 | 62.10 ± 1.13 |
| | RSD (%) | 10.89 | 2.25 | 1.82 |
| | Accuracy (%) | 102.03 | 101.46 | 103.49 |

Stability of the analytes were validated in analyte-spiked samples under five different storage conditions. Condition 1 represents sample immediately extracted and assayed after the analytes spiked (as control); Condition 2, sample assayed after storage in autosampler at 4 °C for 24 h; Condition 3, sample extracted and assayed after the analytes spiked at 25 °C for 4 h; Condition 4, parallel-prepared sample stored at –20 °C for 25 days; Condition 5, parallel-prepared sample stored after three freeze/thaw cycles at –20 °C. RSD indicates relative standard deviation.
Table S4. Associations of serum $N'$-methylnicotinamide concentration tertiles with coronary artery disease by sex

|                     | Serum $N'$-methylnicotinamide, ng/ml (Tertile 2 vs. Tertile 1) | Serum $N'$-methylnicotinamide, ng/ml (Tertile 3 vs. Tertile 1) |
|---------------------|---------------------------------------------------------------|---------------------------------------------------------------|
|                     | Odds ratio (95% CI)                                           | Odds ratio (95% CI)                                           |
|                     | $P$                                                           | $P$                                                           |
| **Men (n=193)**     |                                                               |                                                               |
| Crude model         | 2.19 (1.01–4.75)                                              | 3.07 (1.35–6.96)                                              |
| Adjusted model      | 2.34 (1.01–4.82)                                              | 2.67 (1.06–6.77)                                              |
| **Women (n=140)**   |                                                               |                                                               |
| Crude model         | 2.19 (0.96–5.06)                                              | 3.22 (1.35–7.71)                                              |
| Adjusted model      | 3.31 (1.09–10.09)                                             | 4.99 (1.49–16.72)                                             |

In the adjusted model, odds ratio (95% CI) were adjusted for age, body mass index, systolic blood pressure, current smoking and alcohol intake, hypertension, diabetes, dyslipidemia, use of antihypertensive, antihyperglycemic and hypolipidemic drugs, and fasting plasma glucose, total and LDL cholesterol, and triglycerides.
Figure S1. Association between serum $N'$-methylnicotinamide and severity of coronary artery disease in men (left) and women (right), respectively.

**Male**

- Controls
- CAD patients

$P$ for trend = 0.02

$n=50$ (Normal), $n=51$ (1-vessel), $n=53$ (2-vessel), $n=53$ (3-vessel)

**Female**

- Controls
- CAD patients

$P$ for trend = 0.009

$n=41$ (Normal), $n=21$ (1-vessel), $n=25$ (2-vessel), $n=25$ (3-vessel)

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*Figure S1.* The analysis was adjusted for age, body mass index, systolic blood pressure, current smoking and alcohol intake, hypertension, diabetes, dyslipidemia, use of antihypertensive, antihyperglycemic and hypolipidemic drugs, and fasting plasma glucose, total and HDL
cholesterol, and triglycerides. The $P$ value for test for trend of the changes of serum me-NAM concentrations across the severity of coronary angiography is given. * $P \leq 0.01$ vs. normal.