The Associations of Gut Microbiota Metabolites with Blood Lipids and the Effect of Rosuvastatin Therapy on Them

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Research Article

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Abstract

Background: Gut microbiota is contributed to the variations of blood lipids, and statins are found to affect the gut microbiota compositions. The aims of this study were to evaluate the associations of trimethylamine N-oxide (TMAO) and related precursors with blood lipids and the effect of rosuvastatin therapy on them.

Methods: Totally 112 patients with suspected cardiovascular disease and received regular rosuvastatin therapy were enrolled in this study previously. The TMAO, choline, carnitine, betaine and γ-butyrobetaine (γBB) levels of the patients were analyzed by stable isotope dilution liquid chromatography-tandem mass spectrometry (LC/MS/MS), and the associations between them and blood lipids were analyzed by statistical methods.

Results: Plasma TMAO was correlated with triglyceride positively (r=0.303, p<0.05) and high density lipoprotein cholesterol (HDL-c) negatively (r=-0.405, p<0.001). Plasma betaine was correlated with low density lipoprotein cholesterol negatively (r=-0.308, p<0.01). After adjustment of sex, age, body mass index, blood lipids and concomitant diseases, the association between TMAO and HDL-c was still significant (p<0.05). Besides, the correlation between TMAO and HDL-c still existed after rosuvastatin therapy (r=-0.253, p<0.01). Rosuvastatin therapy could decrease TMAO levels and increase carnitine, betaine and γBB levels significantly when it lowering the blood lipids.

Conclusions: These results indicated that TMAO and related precursors were associated with blood lipids significantly, especially HDL-c. Rosuvastatin therapy not only affects the blood lipids, but also influences the levels of TMAO and related precursors.

Trail registration: This study was retrospectively registered at http://clinicaltrials.gov/ (NCT02305862).

Introduction

Atherosclerosis (AS) is a kind of chronic inflammatory disease characterized by lipids disorders, lipids-related inflammations take part in the whole process of AS formation and development [1, 2]. Based on this, statins are the footstone of cardiovascular disease (CVD) therapy because of their lipids-lowering and anti-inflammation effect [3–5]. Even though, traditional and hereditary risk factors cannot explain all the atherosclerotic lesions [6, 7], there are still some residual cardiovascular risks after standard secondary prevention therapy in coronary heart disease (CHD) patients. An increasing body of evidence indicated that gut microbiota and related metabolites play an important role in various metabolic diseases, including obesity, hyperlipidemia, diabetes mellitus (DM) and CVD [1, 8–11]. Studies had found that gut microbiota metabolites trimethylamine N-oxide (TMAO) and related precursors choline, carnitine, betaine or γ-butyrobetaine (γBB) were associated with the development of CVD, the occurrence of stroke, and the risk of major adverse cardiovascular events (MACEs)[1, 12]. Recent studies found that gut microbiota was associated with the variation of blood lipids [9], and statin therapy could influence the compositions of gut microbiota[13, 14]. However, there is little information about the relationships between gut microbiota

metabolites and blood lipids. The aims of this study were to investigate the associations of plasma TMAO and related precursors with blood lipids and the effect of rosuvastatin therapy on them.

Methods

Study population

We conducted a retrospective study based on a registered clinical trial at http://clinicaltrials.gov/ (No.02305862). Patients with suspected CHD and cerebrovascular disease, and willing to receive coronary angiography and carotid magnetic resonance imaging (MRI) were consecutively and randomly recruited in Beijing Tiantan Hospital between January 2013 and December 2016. The demographic characteristics, personal history and diagnosis were based on personal statements, clinical records, current drug usages, and auxiliary examinations in hospital. The exclusion criteria were patients with age < 18 years, malignant tumor, acute myocardial infarction, severe heart failure, active infections, severe liver or kidney dysfunction, stroke, diabetes mellitus (DM), familial hyperlipidemia or antibiotics usage. Patients who received statins therapy regularly more than one month were also excluded in order to avoiding potential effect on gut microbiota metabolites and lipids. In this study, 135 patients were recruited, and 121 patients received rosuvastatin therapy because of hyperlipidemia, carotid plaque, CHD, and/or other proper purposes. After more than three months' regular therapy, 112 patients with complete follow-up data constituted the study cohort finally. The study flowchart is displayed in Figure 1. The differences of lipids levels between patients with statin therapy less than one month and without statin therapy were not significant (**Supplementary Table S1**). This study was abide by the principles of Declaration of Helsinki, and approved by the Ethics Committee of Beijing Tiantan Hospital (Approval No: KY2014-020-02). All patients in this study signed the informed consent. The data can be achieved from the corresponding author after the permission of the Ethics Committee of Beijing Tiantan Hospital.

Laboratory Testing

Fasting venous bloods were collected using EDTA tubes, centrifuged at 4℃, 3000rpm for 10min, plasma samples were obtained and stored at -80℃ until analysis. The TMAO, choline, carnitine, betaine and γBB levels were measured by stable isotope dilution liquid chromatography-tandem mass spectrometry (LC/MS/MS) according to previous methods [15]. Briefly, plasma samples were mixed with internal standards of TMAO-trimethyl-d9 (d9-TMAO), choline-trimethyl-d9 (d9-choline), carnitine-d3 (methyl-d3), betaine-trimethyl-d9-methylene-d2 (d11-betaine) and γBB N-(carboxypropyl)-N,N,N,trimethyl-d9) in methanol respectively. Proteins were precipitated and the supernatant were recovered after centrifugation at 20000g at 4℃ for 10 minutes. Supernatants were analyzed with an API 5500Q-TRAP mass spectrometry (AB SCIEX, Massachusetts, USA). Analytes were monitored in positive liquid chromatography-tandem mass spectrometry (MRM) mode by using characteristic precursor-production ion transitions: TMAO at *m/z* 76→58, choline at *m/z* 104→60, betaine at *m/z* 118→58 and γBB at *m/z* 146→87, respectively. Lipids parameters including triglyceride (TG), total cholesterol (TC), high density lipoprotein cholesterol (HDL-c), low density lipoprotein cholesterol (LDL-c), apolipoprotein A1 (ApoA1) and apolipoprotien B (ApoB) were

measured by a biochemistry auto-analyzer (Beckman Coulter DxC 800, California, USA) in the clinical laboratory center of Beijing Tiantan Hospital.

Statistical analysis

Categorical variables were compared by Chi square and expressed as frequency (n) and percentage (%). Continuous variables were compared by using t-test, non-parametric test or paired-test, and expressed as mean ± standard deviation (SD). Correlations analysis was performed by Spearman or Pearson analysis, as appropriate. The association analysis was performed by multivariate linear regression analysis. The statistical analysis was performed by using SPSS version 17.0 (SPSS Inc., Chicago, IL). A p value < 0.05 was considered statistically significant.

Results

Baseline characteristics

The baseline characteristics of the patients are listed in Table 1. Compared with male patients, female patients with suspected cardio- or cerebrovascular disease were older ($p < 0.05$), and they also had lower rates of smoke and alcohol use ($p < 0.05$), the results were consistent with epidemiological characteristics. The lipids and gut microbiota metabolites levels between two patients groups were not different ($p > 0.05$). Further analysis indicated that (**Supplementary Table S2**), compared with patients in low-TMAO group (TMAO ≤ 3.92μM), patients in high-TMAO group (TMAO > 3.92μM) had higher TG levels (2.19±1.15mmol/l vs 1.69±0.68mmol/l, $p < 0.05$), but lower HDL-c levels (1.09±0.32mmol/l vs 1.32±0.42mmol/l, $p < 0.05$).

Table 1
Baseline characteristics of the study cohort

Variables	Total patients (n = 112)	Male (n=65)	Female (n=47)	*P*
Age, years	62.12±8.91	60.34±9.77	64.57±6.94	0.008
BMI, kg/m^2	26.22±3.50	25.87±3.26	26.71±3.79	0.215
Smoke, n (%)	55 (49.1)	49 (75.4)	6 (12.8)	<0.001
Alcohol, n (%)	28 (25)	27 (41.5)	1 (2.1)	<0.001
Hypertension, n (%)	85 (75.9)	49 (75.4)	36 (76.6)	0.882
Anti-hypertension, n (%)	82 (73.2)	47 (72.3)	35 (74.5)	0.799
Hyperlipidemia, n (%)	58 (51.8)	31 (47.7)	27 (57.4)	0.308
Anti-hyperlipidemia, n (%)	27 (24.1)	14 (21.5)	13 (27.7)	0.455
CHD, n (%)	94 (83.9)	54 (83.1)	40 (85.1)	0.773
TG, mmol/l	1.94±0.97	1.86±0.84	2.05±1.13	0.296
TC, mmol/l	4.30±1.01	4.19±0.96	4.45±1.06	0.171
HDL-c, mmol/l	1.21±0.39	1.18±0.39	1.24±0.39	0.407
LDL-c, mmol/l	2.55±0.88	2.59±0.73	2.62±0.76	0.847
ApoA1, mmol/l	1.26±0.18	1.25±0.18	1.28±0.19	0.310
ApoB, mmol/l	1.10±0.35	1.13±0.36	1.07±0.33	0.376
TMAO, μM	5.49±4.56	4.91±3.56	6.62±5.48	0.066
Choline, μM	13.25±3.03	13.46±2.70	12.97±3.45	0.406
Carnitine, μM	78.89±15.93	79.71±13.96	77.76±18.41	0.544
Betaine, μM	38.66±10.47	39.77±10.18	37.12±10.78	0.187
γBB, μM	0.10±0.02	0.10±0.02	0.09±0.02	0.099

Data are expressed as mean ± standard deviation or n (%). BMI, body mass index; CHD, coronary heart disease; TG, triglyceride; TC, total cholesterol; HDL-c, high density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; TMAO, trimethylamine N-oxide; γBB, γ-butyrobetaine.

Associations Between Gut Microbiota Metabolites And Blood Lipids

As Table 2 indicates, plasma TMAO was correlated with TG positively and HDL-c negatively (r = 0.303 and -0.405 respectively, $p < 0.05$), and betaine was correlated with LDL-c negatively (r = -0.308, $p < 0.05$). There were no significant correlations between choline, carnitine or γBB and lipids parameters ($p>0.05$). After adjustment of sex, age, body mass index (BMI), blood lipids and the concomitant diseases hyperlipidemia, CHD and hypertension, the associations between TMAO and TG or HDL-c, or between betaine and LDL-c were still significant (**Supplementary Table S3 and S4**, $p<0.05$). After rosuvastatin therapy (Table 3), the positive correlation between TMAO and TG and the negative correlation between betaine and LDL-c were disappeared, but the negative correlation between TMAO and HDL-c was still existed (r = -0.253, $p < 0.05$). Besides, TMAO was shown negative correlation with ApoB (r = -0.241, $p < 0.05$), and carnitine was shown negative correlations with LDL-c and ApoB after rosuvastatin therapy (r = -0.296 and -0.245 respectively, both $p < 0.05$). After adjustment of potential effect of the factors mentioned above, the association between TMAO and HDL-c was still significant after rosuvastatin therapy (**Supplementary Table S5**, $p<0.05$).

In addition we found that (Figure 2), plasma TMAO was correlated with patients' age and BMI positively (r=0.265 and 0.247 respectively, both $p<0.05$; Figure 2A, B), betaine was correlated with BMI negatively (r= -0.285, $p<0.05$; Figure 2C). And the associations between TMAO and age and between betaine and BMI were still significant after rosuvastatin therapy (r= 0.260 and -0.201 respectively, both $p>0.05$). There were no significant associations among other TMAO related metabolites and age or BMI (**Supplementary Table S6**, $p>0.05$).

Finally we analyzed the associations among TMAO and related precursors and found that, TMAO was associated with choline and γBB positively (r= 0.268 and 0.221 respectively, both $p<0.05$), and the associations among TMAO related precursors were significant, except betaine and γBB (**Supplementary Table S7**, $p<0.05$).

Table 2
Correlations of TMAO and related precursors with lipids before rosuvastatin therapy (n = 112)

Variables	TMAO		Choline		Carnitine		Betaine		γBB	
	r	*P*	*R*	*P*	*R*	*P*	*R*	*P*	*r*	*P*
TG	0.303	0.001	-0.025	0.792	-0.052	0.589	-0.060	0.531	-0.138	0.146
TC	0.026	0.787	0.052	0.584	0.051	0.593	-0.138	0.146	0.044	0.642
HDL-c	-0.405	<0.001	-0.104	0.275	-0.007	0.945	-0.059	0.534	0.004	0.967
LDL-c	-0.052	0.585	-0.079	0.407	-0.104	0.275	-0.308	0.001	0.000	0.999
ApoA1	-0.075	0.430	-0.084	0.379	0.061	0.522	-0.078	0.416	-0.054	0.570
ApoB	000	0.998	0.102	0.284	0.044	0.646	-0.006	0.947	0.080	0.403

TMAO, trimethylamine N-oxide; TG, triglyceride; TC, total cholesterol; HDL-c, High density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; γBB, γ-butyrobetaine.

Table 3
Correlations of TMAO and related precursors with lipids after rosuvastatin therapy (n = 112)

Variables	TMAO		Choline		Carnitine		Betaine		γBB	
	r	*P*	*R*	*P*	*r*	*P*	*R*	*P*	*r*	*P*
TG	0.050	0.599	0.082	0.392	-0.018	0.854	-0.124	0.193	-0.163	0.086
TC	-0.181	0.057	0.025	0.796	-0.098	0.304	-0.061	0.523	0.174	0.066
HDL-c	-0.253	0.007	-0.039	0.686	0.063	0.510	0.138	0.145	0.050	0.604
LDL-c	-0.172	0.069	-0.074	0.436	-0.296	0.002	-0.175	0.065	0.011	0.908
ApoA1	-0.025	0.791	0.051	0.592	-0.023	0.810	0.058	0.544	0.007	0.940
ApoB	-0.241	0.011	-0.089	0.350	-0.245	0.009	-0.154	0.104	-0.063	0.507

TMAO, trimethylamine N-oxide; TG, triglyceride; TC, total cholesterol; HDL-c, High density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; γBB, γ-butyrobetaine.

The Effect Of Rosuvastatin Therapy On Gut Microbiota Metabolites

As Table 4 shows, rosuvastatin therapy decreased TG, TC, LDL-c and ApoB, and increased HDL-c and ApoA1 levels significantly ($p<0.05$). Meanwhile, the TMAO levels were decreased and the carnitine, betaine and γBB levels were increased significantly after rosuvastatin therapy ($p < 0.05$).

Table 4
The effect of rosuvastatin therapy on TMAO and related precursors (n = 112)

Variables	Rosuvastatin therapy		*P*
	Before	After	
TG, mmol/l	1.94±0.97	1.43±0.83	<0.001
TC, mmol/l	4.30±0.99	3.54±0.87	<0.001
HDL-c, mmol/l	1.21±0.39	1.39±0.42	<0.001
LDL-c, mmol/l	2.60±0.74	1.99±0.62	<0.001
ApoA1, mmol/l	1.26±0.18	1.43±0.29	<0.001
ApoB, mmol/l	1.10±0.35	0.89±0.23	<0.001
TMAO, μM	5.63±4.52	3.82±2.72	<0.001
Choline, μM	13.51±3.03	13.03±2.89	0.470
Carnitine, μM	78.89±15.93	83.23±12.80	0.003
Betaine, μM	38.66±10.47	44.67±12.62	<0.001
γBB, μM	0.10±0.02	0.11±0.03	<0.001

Data are expressed as mean ± standard deviation. TMAO, trimethylamine N-oxide; TG, triglyceride; TC, total cholesterol; HDL-c, High density lipoprotein cholesterol; LDL-c, low density lipoprotein cholesterol; ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; γBB, γ-butyrobetaine.

Discussion

In this study, we systematically investigated the associations between the metabolites of gut microbiota and blood lipids and the effect of statin therapy on them. We found that plasma TMAO and related precursors were associated with blood lipids significantly, especially TG, HDL-c and LDL-c. And the association between TMAO and HDL-c was not influenced by personal characteristics and rosuvastatin therapy. Besides, rosuvastatin therapy could decrease plasma TMAO levels but increase related precursors such as carnitine, betaine and γBB levels significantly when it lowering blood lipids.

The associations between the metabolites of gut microbiota and chronic metabolic diseases were focused in recent years. Early studies found that TMAO can lead to AS in animal model [16, 17], and patients with elevated TMAO were correlated with increased risk of MACE in clinical [11]. Since then, TMAO and related precursors were found to be associated with the risks and extent of CVD and cerebrovascular disease in many studies later [10, 18–21]. TMAO was found to be pro-inflammatory and mediate inflammation by up-regulating inflammatory factors [22, 23]. Recent studies also reported that the gut microbiota contributed the variation of blood lipids and associated with hyperlipidemia [9, 24], and TMAO could reduce the reverse cholesterol transport and cholesterol absorption [17]. All the studies stated above indicated that TMAO had a similar biological effect like disordered lipids, and there may be some associations between gut

microbiota metabolites and blood lipids. In this study, we systematically analyzed the associations of TMAO and related precursors with blood lipids and the effect of rosuvastatin therapy on them. We found that plasma TMAO was correlated with TG positively and HDL-c negatively, betaine was associated with LDL-c negatively, and these associations were still significant after adjustment of the potential effect of sex, age, BMI, blood lipids and concomitant diseases hyperlipidemia, hypertension and CHD. In further analysis we found that the correlation between TMAO and HDL-c was still existed after rosuvastatin therapy, and this association was still significant after adjustment of potential effect of factors mentioned above. Firstly, our findings supported a recent study conducted by Fu, J. et al. indirectly, which found that the gut microbiota contributed the variations of blood lipids, especially HDL-c [24]. Secondly, the results also indicated that not only the gut microbiota was associated with the variation of blood lipids, but also its metabolites were associated with the lipids levels, especially HDL-c. However, we found that the positive correlation between TMAO and TG, and the negative correlation between betaine and LDL-c disappeared after rosuvastatin therapy. The disturbed gut microbiota metabolites and lipids levels may be responsible for the disappeared associations between them after statin therapy. In addition as we have known, HDL-c was found to have anti-atherogenic effect in previous studies [25–27], and betaine was also found to be associated with non-HDL-c and the risk of CVD inversely [28, 29]. So, the results stated above tended to support the pro-atherogenic effect of TMAO, and the anti-atherogenic effect of betaine. Although gut microbiota was associated with the lipids variations and hyperlipidemia, and there were some associations between TMAO related parameters and blood lipids, we failed to find significant differences of TMAO, choline, betaine and γBB, except carnitine between patients' with hyperlipidemia and non-hyperlipidemia in this study.

Previous studies found that drugs like proton pump inhibitors (PPIs) and statins could affect the gut microbiota compositions profoundly [30, 31], but there was scarce information about the effect of drugs on the gut microbiota metabolites. Recently, a small cohort study found that rosuvastatin therapy not only affected the genetic compositions of gut microbiota, but also may affect the gut microbiota metabolites [13]. Patients in rosuvastatin therapy group had higher betaine and γBB levels than placebo group, and the TMAO and carnitine levels in rosuvastatin therapy group also tended to decrease and increase separately, although the differences were not significant [13]. In this study we found that, rosuvastatin therapy could decrease TMAO, but increase carnitine, betaine and γBB levels significantly while it lowering the blood lipids levels. So, the results of our study further supported the findings of this previous study. As information about the potential bad effect of TMAO, carnitine and γBB, and the protective effect of betaine listed above, the TMAO-lowering and betaine-increasing effect of rosuvastatin therapy may be part of the pleotropic protective effect of statins therapy in CVD patients, and the increased carnitine and γBB may be responsible for the residual cardiovascular risks. However, it's still difficult to explain the contradictory phenomenon for the pathway between TMAO production and related precursors. Although some studies had found the influence of drugs on the gut microbiota and related metabolites [30, 31], the associations between the changes of gut microbiota and related metabolites are still not clear, especially the decreased TMAO and increased related precursors. More experimental studies are needed to clarify the potential mechanisms of them.

As we known, TMAO is mainly produced by related precursors choline, carnitine and/or γBB under the effect of gut microbiota and hepatic flavin-containing monooxygenases (FMOs) [32]. Many factors, including

food, gut microbial flora and FMOs functions can influence their metabolisms and productions [33, 34]. Previous studies found that there is not a one-to-one relationship between TMAO and related precursors [33, 35]. In this study we also analyzed the associations between TMAO and related precursors, and their associations with personal characteristics. We found that TMAO was positively associated with related precursors choline and γBB, but had no associations with carnitine and betaine. We found that plasma TMAO was correlated with patients' age positively, and this correlation was still existed after rosuvastatin therapy. This phenomenon was previously reported in an early study, which found that TMAO levels increased with advancing age [36]. Besides, we found that TMAO or betaine was correlated BMI positively or negatively, and the association between betaine and BMI was still existed after rosuvastatin therapy. Previous studies had reported that gut microbiota was associated with patients' BMI and obesity [24], and the gut microbiota transplantation could lead to obesity in animal model [37, 38]. The results of our study supported that the gut microbiota and related metabolites TMAO and betaine may play active role in the pathogenesis of obesity.

There are also some limitations in our study. Firstly, enrolled patients with suspected CHD and relatively old age inevitably make some selection bias. Secondly, the usage of multiple drugs may lead to potential effect on the gut microbiota metabolites. Thirdly, the modest sample size may influence the power of the results. Finally, although all the patients were ordered to eat low-salt and low-lipid diet, the non-uniform diet also may affect the levels of gut microbiota metabolites potentially. So a large and universal patient cohort with less drugs usage and standard diet is needed in further study.

Conclusions

Our study suggested that the gut microbiota metabolite TMAO and related precursors have significant associations with blood lipids, especially HDL-c. Rosuvastatin therapy could decrease the blood lipids and TMAO levels, but increase the levels of TMAO related precursors significantly.

Abbreviations

AS
Atherosclerosis
CVD
Cardiovascular disease
CHD
Coronary heart disease
DM
Diabetes mellitus
TMAO
Trimethylamine N-oxide
γBB
γ-butyrobetaine
MACE

Major adverse cardiovascular events
TG
Triglyceride
TC
Total cholesterol
HDL-c
High density lipoprotein cholesterol
LDL-c
High density lipoprotein cholesterol
ApoA1
Apolipoprotein A1
ApoB
Apolipoprotein B
BMI
Body mass index
PPIs
Proton pump inhibitors
FMOs
Flavin-containing monooxygenases.

Declarations

Ethics approval and consent to participate

This study was registered at http://clinicaltrials.gov/ (NCT02305862), abided by the principles of Declaration of Helsinki, and approved by the Ethics Committee of Beijing Tiantan Hospital (Approval No: KY2014-020-02). All patients in this study signed the informed consent.

Consent for publication

Not applicable.

Availability of data and materials

The data can be achieved from the corresponding author after the permission of the Ethics Committee of Beijing Tiantan Hospital.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Xiaowei Xiong and Jian Zhou took part in study design, recruiting the patients, data analysis and writing the manuscript. Qiang Fu, Xiaowei Xu, Shaobin Wei and Shenghua Yang recruited the patients, isolated the blood samples, and assayed the levels of plasma TMAO and related precursors. Buxing Chen initiated and designed the study. All authors read and approval the final version of this manuscript.

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Figures

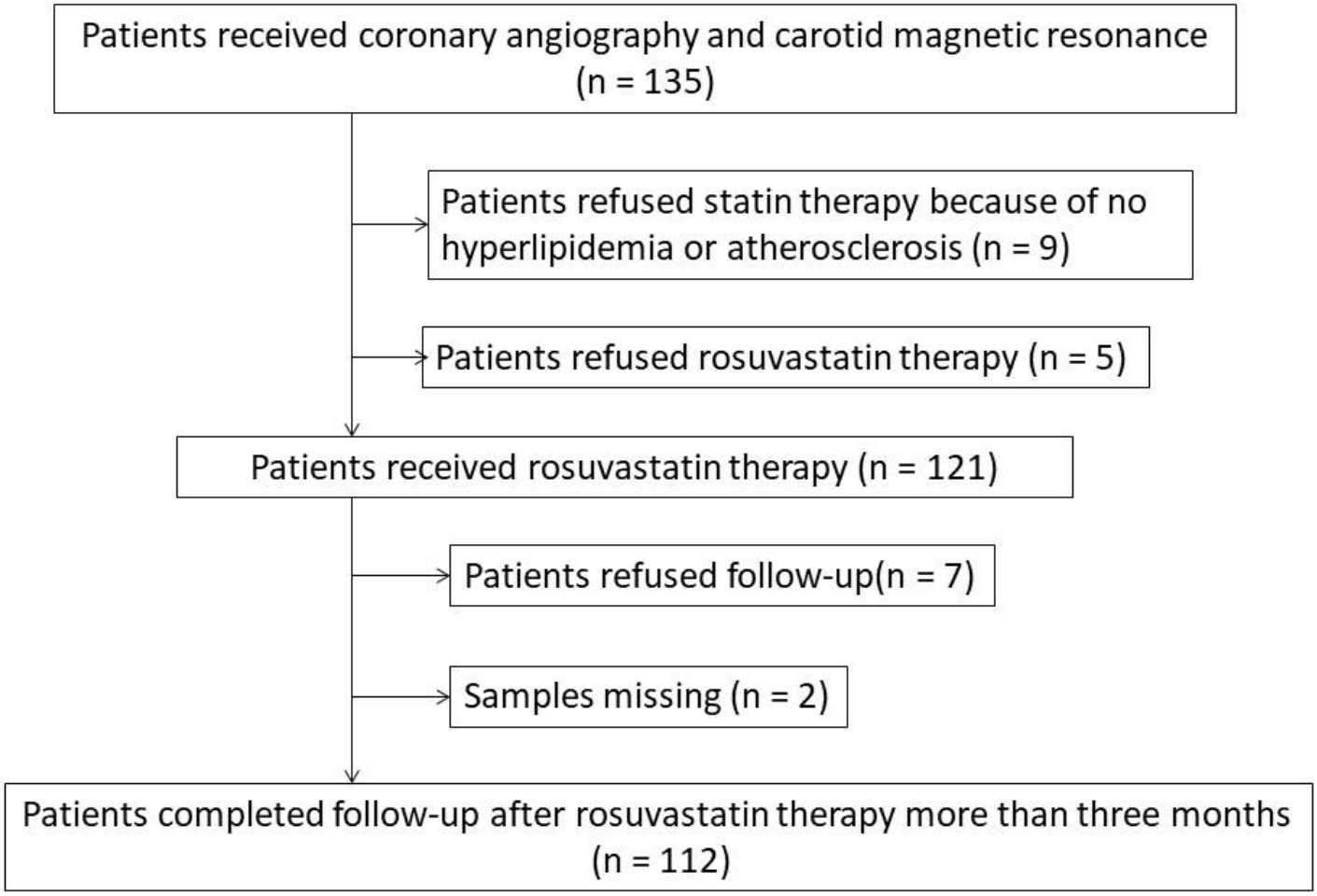

Figure 1

Study flowchart

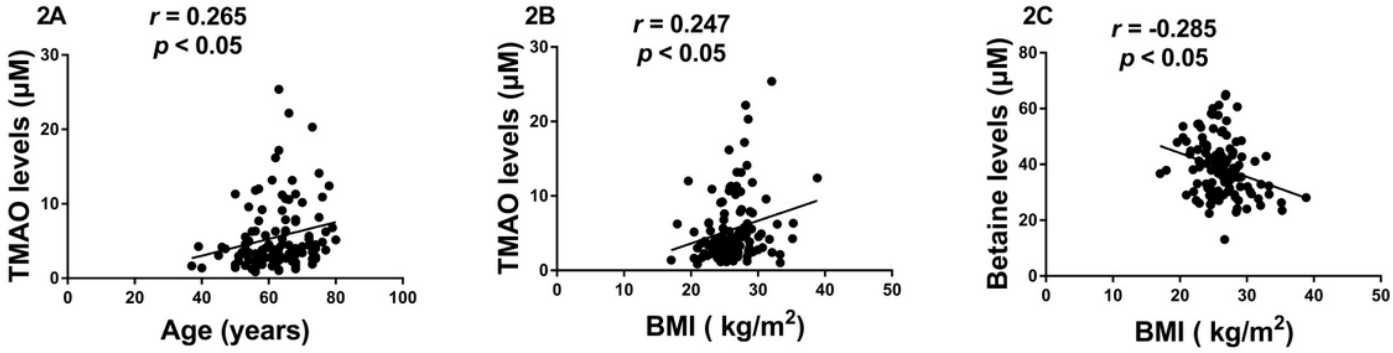

Figure 2

Correlations between TMAO and personal characteristics TMAO, trimethylamine N-oxide; BMI, body mass index.

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