Gut microbiota-dependent TMAO predicts risk of cardiovascular events in patients with stroke and is related to proinflammatory monocytes

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Abstract

Objective—Gut microbiota-dependent metabolites, in particular trimethylamine N-oxide (TMAO), have recently been reported to promote atherosclerosis and thrombosis. Here, we examined for the first time the relation of TMAO and the risk of incident cardiovascular events (CVE) in patients with recent first-ever ischemic stroke in two independent prospective cohorts. Moreover, the link between TMAO and proinflammatory monocytes as a potential contributing factor for cardiovascular risk in stroke patients was studied.

Approach and Results—In a first study (n=78), higher TMAO plasma levels were linked with an increased risk of incident CVE including myocardial infarction, recurrent stroke and cardiovascular death (Quartile (Q)4 vs Q1; HR: 2.31; 95% CI: 1.25–4.23; P<0.01). In the second independent validation cohort (n=593), high TMAO levels again heralded marked increased risk of adverse CVE (Q4 vs Q1; HR: 5.0; 95% CI: 1.7–14.8; P<0.01), and also after adjustments for cardiovascular risk factors including hypertension, diabetes, LDL cholesterol and eGFR (HR: 3.3 95% CI: 1.2–10.9; P=0.04).

A significant correlation was also found between TMAO levels and percentage of proinflammatory intermediate $CD14^{++}CD16^{+}$ monocytes (r=0.70; P<0.01). Moreover, in mice fed a diet enriched with choline to increase TMAO synthesis, levels of proinflammatory murine $Ly6C^{high}$ monocytes were higher than in the chow-fed control group (choline: $9.2\pm0.5 \times 10^{3}$ per ml vs. ctr.: $6.5\pm0.5 \times 10^{3}$ per ml; P<0.01). This increase was abolished in mice with depleted gut microbiota (choline + ABS: $5.4\pm0.7 \times 10^{3}$ per ml; P<0.001 vs. choline).

Conclusions—The present study demonstrates for the first time a graded relation between TMAO levels and the risk of subsequent CVE in patients with recent prior ischemic stroke. Our data support the notion that TMAO-related increase of proinflammatory monocytes may add to elevated cardiovascular risk of patients with increased TMAO levels.

Keywords

gut microbiota; trimethylamine *N*-oxide; stroke; cardiovascular risk; proinflammatory monocyte

Introduction

The risk of secondary cardiovascular events (CVE) varies across the spectrum of patients with ischemic stroke. Despite guideline-based optimal treatment of patients with TIA and stroke including urgent management in specialized units, broad diagnostic work-up, rapid treatment with antithrombotic agents and other stroke-prevention strategies, recent studies suggest a considerable residual risk of 6.2% for CVE and 5.1% for stroke within 1-year follow-up[1]. In view of these data, there is still great need to identify additional factors impacting the patients' risk beyond traditional risk factors that may help to more accurately estimate patient vulnerability for subsequent CVE improving preventive concepts.

Recently, an important contribution of gut microbiota in pathobiological processes including metabolic[2], immunological[3], and cardiovascular diseases[4] has been suggested. Notably,

metabolomics combined with mechanistic animal model studies have led to a proposed potential link between dietary nutrients, such as choline and carnitine, and their gut microbiota-dependent metabolism to trimethylamine *N*-oxide (TMAO), and atherosclerosis[4, 5]. There is considerable evidence linking the metaorganismal TMAO pathway to the development of atherosclerosis and thrombosis in animal models and incident cardiovascular disease risks. In particular, proatherogenic effects of microbe-dependent TMAO formation include enhanced macrophage cholesterol accumulation and subsequent foam cell formation[4], proinflammatory changes in the artery wall[6], and promotion of both platelet hyperreactivity and enhanced arterial thrombosis potential[7].

With regard to recent observations on the relation between TMAO and the risk for CVE in patients with acute coronary syndrome[8] we sought to evaluate the prognostic value of TMAO levels in patients with recent first ever ischemic stroke and investigate a potential interaction between TMAO and proinflammatory circuits that promote thrombotic complications of patients at high risk.

In recent years a critical role of monocyte subsets with distinct expressions of the lipopolysaccharide (LPS) receptor CD14 and the FcγIII receptor CD16 has been unveiled for formation, propagation and vulnerability of atherosclerotic lesions[9, 10]. In particular the intermediate CD14++CD16+ monocytes have been identified to independently predict future cardiovascular events in large patient cohorts of coronary artery disease, chronic kidney disease and peripheral artery disease[11–13]. The intermediate CD14++CD16+ monocytes are considered as a subset with particular proinflammatory functions[14] known to secrete high amounts of inflammatory cytokines, such as TNF-α, upon activation[15]. In patients with ischemic stroke the increased number of the intermediate monocyte subset has been strongly correlated with the progression and severity of brain infarction[16]. Based on advanced cluster analysis the intermediate subset has been closely related to proinflammatory murine Ly6C^{high} monocytes[10, 15, 17] allowing to investigate the biology of these monocytes in experimental settings.

Here, we evaluated for the first time the relationship between circulating TMAO levels and incident CVE in two independent prospective cohorts of patients with ischemic stroke. Moreover, we examined the relation between TMAO and the level of proinflammatory monocyte subsets as a potential contributing factor to trigger cardiovascular events both in patients and in a mouse model of antibiotics-induced depletion of the gut microbiota. The results may provide novel insights into the relationship between the gut microbiota-related metabolite TMAO, proinflammatory monocytes and clinical prognosis in patients with stroke.

Materials and Methods

The data that support the findings of this study are available from the corresponding author upon reasonable request. Details of the major resources and detailed methods can be found in the Supplemental Material.

Study populations and study design

Two prospective cohorts of patients with first-ever ischemic stroke were examined. The primary outcome measure of both cohorts was a combined vascular end-point composed of myocardial infarction (MI), recurrent stroke and cardiovascular death during 1-year follow-up on a structured interview with the patient or their relatives and, if indicated, with additional information from the hospital or physician.

The inclusion criteria of both studies were: (1) Age 18 years; (2) first ever acute stroke that occurred with stroke onset in the last seven-days and (3) written informed consent by patient or legal guardian prior to study participation. The exclusion criteria were: (1) Prior stroke; (2) patients presenting brain tumor or brain metastasis and (3) participation in an intervention study. Both studies were approved by the respective local research ethics committee. All participants with adjudicated diagnosis of ischemic stroke provided written informed consent and were prospectively enrolled. For evaluation of acute ischemic stroke, diffusion weighted magnetic resonance imaging (Siemens Verio 3.0 Tesla) was performed.

In the first cohort, 78 patients with complete data on primary outcome were enrolled. The relation of the gut microbe-dependent metabolite TMAO with the risk of subsequent (1-year) cardio- and cerebrovascular events (CVE) including myocardial infarction, recurrent stroke and cardiovascular death was examined.

After finding a significant relation between TMAO with the cardiovascular risk in the first cohort, a second larger independent validation cohort including 608 patients from the 'Prospective Cohort with Incident Stroke' study (PROSCIS) with first-ever stroke conducted at the Center for Stroke Research Berlin in Germany (PROSCIS-B: Center for Stroke Research Berlin, Charité University Hospital) was examined with 1-year of follow-up to evaluate if the observed relation persists in an independent non-overlapping patient population. Study protocol and design of the PROSCIS study were described previously[18].

Blood tests

Laboratory workup was performed for total cholesterol, low- and high-density lipoproteins, triglyceride, C-reactive protein, and full blood count. Blood samples were immediately processed and frozen at −80°C for further analyses. Levels of choline and TMAO were quantified by stable isotope dilution liquid chromatography with online tandem mass spectrometry as previously described[19], using a Shimadzu LCMS-8050 CL Triple Quadrupole Mass Spectrometer interfaced with a Nexera LC-30AD CL Ultra High Performance Liquid Chromatograph (UHPLC) system.

Analysis of monocyte subsets in humans

Flow cytometry was used to determine human monocyte subsets as previously described[20]. Blood samples were taken by venipuncture and transferred immediately on ice to the lab and prepared directly without time delay for flow cytometry analysis (antibodies are shown in the major resources table in the Supplemental Material). After pre-selection (side scatter and forward scatter) monocytes were identified as HLA-DR positive and classified according to CD14 and CD16 expression: $CD14^{++}CD16^{-}$ as classical monocytes, $CD14^{+}CD16^{++}$ as non-

classical monocytes and $CD14^{++}CD16^{+}$ as intermediate monocytes. Detailed gating strategy for identification of monocyte subsets is shown in Supplemental Figure I.

Mouse experiments

All animals were bred, raised and housed in the facilities of the "Forschungseinrichtungen für Experimentelle Medizin" (FEM, Charité – University Medicine Berlin, Germany) under specific pathogen-free (SPF) conditions. Adult (10 weeks of age) C57BL/6J mice (Charles River) were placed on normal chow (0.2% choline) or choline supplemented diets (1.3% choline) (Ssniff, Soest, Germany) for 3 weeks prior to monocyte subset analysis. At the end of the 3 weeks period mice were sacrificed for collection of blood and bone marrow (BM). Mouse experiments were approved by the research advisory committee and permitted by LAGeSo (Landesamt für Gesundheit und Soziales Berlin).

Generation of secondary abiotic (gnotobiotic) mice

Secondary abiotic mice were generated through quintuple antibiotic treatment (+ABS) for 6 weeks via the drinking water. The intestinal colonization status of the mice was controlled once a week by highly sensitive cultural analysis of fecal samples as described earlier[21].

Analysis of blood monocyte subsets in mice

Blood samples were collected by cardiac puncture using EDTA-coated syringes. Whole blood (100 μl) or BM cell suspension was incubated with antibodies for 20 minutes at room temperature in the dark (major resources table in the Supplemental Material). After doublet exclusion monocytes were identified as CD45 positive and CD115 positive and further selected by highly positive expression of CD11b, a marker of dendritic cells, monocytes and granulocytes, shown in the CD11b/Ly6C plot. Monocytes were further classified according to Ly6C expression. Detailed gating strategy for identification of monocyte subsets is shown in Supplemental Figure II.

Analysis of bone marrow hematopoietic stem cells and progenitor cells in mice

BM cells were collected from both femur and tibia of mice by flushing the BM with Dulbecco's PBS. Harvested cells were filtered through a 70mm cell strainer and washed with PBS. Erythrocytes from all samples were lysed with red blood cell (RBC) lysis buffer. BM cell suspension was incubated with antibodies for 20 minutes at room temperature in the dark (major resources table in the Supplemental Material). After pre-selection (side scatter and forward scatter) the Lineage negative cells were gated in a histogram and hematopoietic stem cells (HSC) were identified as the C-kit positive and Sca1 positive population and further selected by positive expression of CD135 and CD115 as monocyte and dendritic cell progenitors (MDP).

Statistical Analyses

Database management and statistical analyses were performed with PRISM version 5.0a (GraphPad Software Inc., USA) and SPSS version 23 (IBM SPSS, USA).

Mouse studies—Continuous data were subjected to the Kolmogorov–Smirnov test to determine their distribution and were expressed as mean±standard deviation (SD) or median and range. Comparison of means was performed by independent t-test and Wilcoxon rank-sum test of medians was used if data were not normally distributed. For continuous variables, univariate correlation analyses were performed.

Human studies—For comparison of basic patients' characteristics continuous data were subjected to the Kolmogorov–Smirnov test to determine their distribution and were expressed as mean±standard deviation (SD) or median and range. Comparison of means was performed by independent t-test and Wilcoxon rank-sum test of medians was used if data were not normally distributed. Kaplan–Meier survivor curves were examined to assess the relationship between quartiles of TMAO or choline levels timing of events during follow-up, and the log-rank test for statistical assessment. Cox regression forward selection was used to further characterize the relation of TMAO and choline to outcome. To determine whether TMAO was independently related to outcome, three cox regression models were used: In model 1 the outcome was adjusted for sex and age, in model 2 outcome was adjusted for model 1 and cardiovascular risk factors including hypertension, diabetes, LDL cholesterol and eGFR and in model 3 the outcome was adjusted for model 1 and additionally for stroke severity, stroke etiology (TOAST)[22], cardiovascular risk factors including hypertension, diabetes, LDL cholesterol, history of peripheral or coronary artery disease or myocardial infarction. For additional comparison of the prognostic value of TMAO with regard to CVE or stroke at 1-year, receiver operating characteristic (ROC) curves were generated, and the areas under the curves (AUC) were calculated. To assess the prognostic value of TMAO in patient subgroups Chi-Square and Odds ratio analyses were performed. Additionally, combined ROC curve analyses of the cardiovascular risk factors diabetes, hypertension and dyslipidemia with and without TMAO were performed to assess the additive prognostic value of TMAO over classical cardiovascular risk factors.

Parametric correlation analyses were used to evaluate the correlation between TMAO and intermediate $CD14^{++}CD16^{+}$ monocytes. Multiple linear regression analysis was performed to further assess the correlation between TMAO levels and intermediate monocyte subsets after adjustment for age, sex and the cardiovascular risk factors including diabetes, hypertension, dyslipidemia and smoking. Significance was assumed at a two-sided value of P 0.05.

Results

Patient Characteristics

Demographic and clinical parameters, as well as laboratory test results of all patients in the first study are shown in Table 1, and for the validation study in Table 2. We also compared these parameters between patients with the lowest plasma levels of TMAO (first quartile in the first study n=20; in the validation study n=148) and those with the highest plasma levels of TMAO (fourth quartile in the first study n=20; in the validation study n=148). In the first study, the cohort was composed of 78 sequential consenting patients with adjudicated diagnosis of ischemic stroke. In this cohort, subjects with higher TMAO levels tended to

have higher creatinine levels as compared to subjects with low TMAO levels, while otherwise no differences in baseline clinical or laboratory parameters were observed between patients in the highest quartile versus the lowest quartile of TMAO levels (Table 1). The second larger validation population included 593 patients in the 'Prospective Cohort with Incident Stroke' study (PROSCIS) who had suffered with first-ever stroke. In this cohort, subjects in the highest TMAO quartile tended to be older, had higher creatinine levels, and had lower hemoglobin levels as well as higher leucocyte counts (Table 2) as compared with subjects in the lowest TMAO quartile. Outcomes at 1-year included myocardial infarction in 6 patients, stroke in 21 patients, cardiovascular death in 19 patients, and the composite CVE in 46 patients.

Plasma TMAO Levels in Relation to Subsequent CVE and in the First Study

In the first study cohort (n=78), Kaplan-Meier analyses of TMAO levels, but not its precursor, the trimethylamine-containing nutrient choline, showed a dose dependent relationship with the risk of incident CVE including myocardial infarction, recurrent stroke and cardiovascular death (TMAO, P<0.01, Figure 1A; choline, P=0.37, Figure 1B). The association between TMAO and CVE was further adjusted for age, sex, hypertension, diabetes, LDL-cholesterol and smoking resulting in a significant enhanced risk with an HR 3.5 (95% CI: 1.66 – 7.21; P=0.001). Analysis of the linear trend across quartiles also showed a significant enhanced risk with a Chi^2 of 7.0 (P<0.01).

TMAO Levels and the Risk of Subsequent CVE or Recurrent Stroke in the Validation Study

In the validation study cohort (n=593), Kaplan-Meier plots indicated a graded increased risk of combined CVE including myocardial infarction, recurrent stroke and cardiovascular death (Figure 2A, P=0.01) and also recurrent stroke alone (Figure 2B, P=0.01) with increasing plasma TMAO levels, but not with plasma choline levels (Figure 2C and D, P=0.24 and P=0.29, respectively). The linear trend across quartiles results in a significant enhanced risk of combined CVE with a Chi^2 of 9.8 (p<0.01).

Subjects who experienced incident CVE had higher plasma TMAO levels at presentation than those without an event at 1-year follow-up (no event: 4.91 ± 0.18 μM vs event: 7.28 ± 0.91 μM, P<0.01, Figure 3A). Similarly, subjects who particularly experienced recurrent stroke displayed also higher plasma TMAO levels than those without recurrent stroke at 1-year follow-up (no recurrent stroke: 4.92 ± 0.18 μM vs recurrent stroke: 6.86 ± 1.11 μM, P=0.04, Figure 3A). The dose-dependent relationship between TMAO levels and incident CVE was particularly noticeable in patients in the 4th quartile (6.07–122 μM) of plasma TMAO levels compared to those in the first quartile (<2.7 μM) (HR unadjusted: 5.0; 95% CI: 1.7–14.8; P<0.01; Figure 3B). Moreover, patients in the highest quartile of TMAO levels had an increased risk of CVE over the ensuing 1-year follow-up period after the adjustments for both age and sex (HR model 1: 3.4; 95% CI: 1.2–10.2; P<0.01), and even after further adjustments for cardiovascular risk factors including hypertension, diabetes, LDL cholesterol and eGFR (HR model 2: 3.3 95% CI: 1.2–10.9; P=0.04). In a third model, we adjusted for age and sex and additionally for stroke severity, stroke etiology (TOAST), hypertension, diabetes, LDL cholesterol, Lp(a) and history of peripheral (PAD) or coronary artery disease (CAD) or myocardial infarction (MI) (HR model 2: HR 3.4; 95% CI: 1.1–

10.1; P=0.02). Moreover, patients in the 4th vs. 1st quartile of plasma TMAO levels were at higher risk of recurrent stroke over 1-year follow-up interval (HR 2.7; 95% CI: 1.1–6.1; P<0.01).

ROC Curve Analyses of TMAO and Prognostic Utility in Patient Subgroups

Receiver operating characteristic curve analyses of TMAO further illustrated that TMAO is a considerable indicator of cardiovascular risk with an AUC of 0.66. (Supplemental Figure IIIA). Moreover, combined ROC curve analyses of the cardiovascular risk factors diabetes, hypertension and dyslipidemia with and without TMAO were performed to assess the additive prognostic value of TMAO over classical cardiovascular risk factors. Adding TMAO to classical risk factors including diabetes, hypertension and LDL increased AUC (AUC with TMAO: 0.63; p<0.01vs. AUC without TMAO: 0.57; p=0.09) (Supplemental Figure IIIB). A TMAO value of 4.86 μM yielded the highest sensitivity (63%) and specificity (66%) for the prediction of 1-year cardiovascular risk.

Moreover, the prognostic value of TMAO was assessed in several patient subgroups (Figure 4). A TMAO level > 4.86 μM was associated with an increased cardiovascular risk in patients above 66 years of age, in both males and females, and in patients with hypertension. Moreover, TMAO showed clinical prognostic utility independent of traditional cardiovascular risk factors including within both diabetic and non-diabetic patients, in smokers and non-smokers, in patients with and without dyslipidemia, in patients with normal or impaired renal function, and in patients without history of CAD (Figure 4).

Plasma TMAO Levels in Relation to Monocyte Subsets

Assessment of the proportion of intermediate $CD14^{++}CD16^{+}$ monocytes revealed significant correlation with plasma levels of TMAO (r=0.70, P<0.01) (Figure 5A, B). Additional multiple linear regression analysis was performed to assess the correlation between TMAO levels and intermediate monocyte subsets after adjustment with age, sex and the cardiovascular risk factors diabetes, hypertension, dyslipidemia and smoking. After adjustment, the results still demonstrated significant correlation between intermediate monocyte levels with plasma TMAO concentrations (r=0.38, P<0.01). Further analyses determined the distribution of monocyte subsets in these patients according to TMAO levels as assessed by quartiles (Figure 5C). Classical $CD14^{++}CD16^{-}$ monocytes were not significantly altered between patients with the highest and lowest TMAO levels (Q4: 84.3% vs Q1: 87.8%; P=0.20). Non-Classical $CD14^{+}CD16^{++}$ monocytes tended to be lower in patients with the highest TMAO levels, although this was not statistically significant (Q4: 6.7% vs Q1: 8.6%; P=0.17). Whereas, the proportion of intermediate $CD14^{++}CD16^{+}$ monocytes showed a dose-dependent increase according to TMAO levels with the highest levels being observed in patients with the highest TMAO concentrations (Q4: 9.0% vs Q1: 3.6%; P<0.01).

Impact of the trimethylamine-containing nutrient choline on blood and bone marrow monocyte subset distribution in mice

The levels of $Ly6C^{high}$ monocytes were increased in mice on choline (1.3%) supplemented diet as compared to normal chow (ctr.) (choline: n=6, 9.2±0.5 x 10^{3} per ml vs. ctr.: n=6,

6.5 ± 0.5 x 10^3 per ml; P=0.001). This increase was abolished in mice with depleted gut microbiota (choline + ABS: n=6, 5.4 ± 0.7 x 10^3 per ml; P<0.001 vs. choline) (Figure 6A). Oppositely, the levels of blood Ly6C^{low} monocytes decreased in high choline-fed mice (choline: n=6, 15.0 ± 1.1 x 10^3 per ml vs. ctr.: n=6, 19.2 ± 0.7 x 10^3 per ml, P<0.01). This effect was abolished in gut microbiota-depleted mice (choline +ABS: n=6, 19.3 ± 1.5 x 10^3 per ml; P=0.03 vs. choline) (Figure 6A). To evaluate whether the shift in monocyte subsets occurs at the level of BM exit the levels of Ly6C^{high} and Ly6C^{low} monocytes in the BM were analyzed. Similar to the results in the circulation, the levels of Ly6C^{high} monocytes were increased in the BM in mice on choline-supplemented diet as compared to normal chow (choline: n=6, $9.0.8\pm1.3$ x 10^4; vs ctrl.: n=6, 5.6 ± 0.5 x 10^4 per thighbone; P=0.03) (Figure 6B) which was prevented in mice with depleted gut microbiota (choline + ABS: n=6, 5.4 ± 0.6 x 10^4 per thighbone; P=0.03 vs. choline). Consistently, the levels of Ly6C^{low} monocytes decreased in the BM in choline fed mice (choline: n=6, 3.1 ± 0.2 x 10^3 per thighbone vs ctr.: n=6: 5.5 ± 0.2 x 10^3 per thighbone; P<0.001) but not in gut microbiota depleted mice (choline + ABS: n=6, 4.5 ± 0.3 x 10^3 per thighbone; P<0.01 vs choline.) (Figure 6B).

Impact of the trimethylamine-containing nutrient choline on bone marrow hematopoietic stem and progenitor cells in mice

Analysis of hematopoietic stem and progenitor cells in BM showed no alteration of HSC (choline: n=8, 1.7 ± 0.2% of lineage negative (neg) cells vs ctr.: n=7, 1.1 ± 0.2% of lineage neg cells; P=0.06) (Figure S4A) nor MDP levels (choline: n=8, 1.6 ± 0.1% of lineage neg cells vs ctr.: n=7, 1.2 ± 0.2% of lineage neg cells; P=0.08) (Figure S4B) in choline supplemented mice as compared to normal chow. Accordingly, depletion of the gut microbiota had no significant effects on HSC or on MDP levels in the BM (Supplemental Figure IV).

Discussion

Our study examines for the first time the relationship between the gut microbe-derived metabolite TMAO and recurrent stroke and CVE risk among patients with ischemic stroke in two independent cohorts and the relationship between baseline TMAO with the level of intermediate CD14^{++}CD16^{+} monocytes in patients with stroke. The results indicate the following: (i) elevated TMAO levels are dose-dependently associated with an increased risk of recurrent stroke and subsequent CVE in patients after first-ever stroke; (ii) this relationship holds true even after adjusting for traditional cardiovascular and cerebrovascular risk factors and initial stroke severity; (iii) blood concentrations of TMAO are closely related to the levels of proinflammatory intermediate CD14^{++}CD16^{+} monocytes.

Importantly, the results of the second independent validation cohort demonstrate that the observed clinical association remains robust, indicating the prognostic clinical utility of TMAO for predicting incident adverse event or thrombosis risk replicates in a totally distinct yet clinically relevant cohort.

ROC curve analyses further revealed that TMAO provides added prognostic value to traditional risk factors as a marker of 1-year cardiovascular mortality risk in these patients. At the best cutoff value with the highest sensitivity and specificity, TMAO provided

prognostic information in clinically relevant patient subgroups defined according to age, sex, underlying cardiovascular risk factors, and renal dysfunction.

Notably, we found reduced renal function in patients with the highest TMAO levels. This observation may be partly due to impaired renal elimination of TMAO in these patients. However, it was also previously shown that TMAO likely contributes to the progression of renal disease by increasing tubulointerstitial fibrosis and collagen deposition[23]. Moreover, it was shown that elevated TMAO levels increased the risk of mortality in patients with chronic kidney disease[23]. Subgroup analyses in the present study showed that clinical prognostic utility of elevated TMAO was observed in both subgroups possessing normal versus impaired renal function.

Recently, intestinal microbes and their metabolic products have been identified as potential promoters for the development of cardio-metabolic diseases and atherosclerosis[24]. Microbial composition studies combining clinical phenotypic data with microbial sequencing analysis have identified that an altered gut metagenome is associated, for example, with the development of metabolic disorders such as type 2 diabetes mellitus, obesity, and symptomatic atherosclerotic heart disease[24]. Early metabolomics studies, coupled with mechanistic investigations, have unveiled a specific pathway by which gut microbes contribute to atherosclerosis and cardiovascular disease. This pathway is based on particular dietary nutrients containing a TMA group such as phosphatidylcholine, choline, and carnitine. In the intestine, the TMA containing nutrient is subsequently degraded by a TMA lyase from specific bacterial strains, and, after absorption into the portal circulation, it is further metabolized into TMAO by hepatic flavin monooxygenases (FMOs), in particular FMO3[4, 5, 25–27].

A potential mechanistic link of TMAO to the promotion of atherosclerosis was first demonstrated in animal models, in which TMAO led to increased cholesterol accumulation in macrophages and the formation of foam cells in atherosclerotic lesions by increasing the expression of the scavenger receptors (CD36 and SR-A1) on macrophages[4]. Interestingly, a recent clinical study examining coronary artery anatomy with the SYNTAX score showed a dose-dependent relationship between TMAO levels and enhanced atherosclerotic burden in subjects[28]. Moreover, recent studies suppressing TMAO generation with a small molecule inhibitor of microbial TMA production led to inhibition of atherosclerosis[27], and suppression of FMO3 has been demonstrated to both lead to TMAO reduction and inhibition in atherosclerosis in animal models[29]. Notably, TMAO has been shown to promote vascular inflammation by activating mitogen-activated protein kinase, extracellular signal–related kinase, and nuclear factor-κB signaling cascade in endothelial cells[6]. Furthermore, direct injection of TMAO in rodent models promotes aortic endothelial cell activation and up-regulation of adhesion proteins[6]. Recent studies with cultured endothelial cells indicate that TMAO activates the NLRP3 inflammasome, a multi-enzyme proinflammatory complex thought to be involved in the development of atherosclerosis[30].

While in experimental studies a role of TMAO for inflammatory processes has been shown, our study links increased TMAO values to proinflammatory $CD14^{++}CD16^{+}$ monocytes in humans. The intermediate $CD14^{++}CD16^{+}$ monocytes are considered to promote

inflammatory responses[14], e.g. by secreting high amounts of inflammatory cytokines, such as TNF-α[15]. Moreover, high expression of adhesion molecules, such as CD162/PSGL-1, as well as myeloperoxidase, a major source of reactive oxidant species in innate immune responses[13] has been identified in intermediate monocytes contributing to their pro-thrombotic and pro-atherogenic properties.

Our mouse experiments further support this proposed link and suggest a shift of monocyte subset distribution towards proinflammatory Ly6C^{high} monocytes in the bone marrow without affecting hematopoietic stem cells or monocyte progenitor cells.

Recent lineage-tracing and adoptive transfer studies have supported the concept that Ly6C^{high} monocytes are the precursors to Ly6C^{low} monocytes[31]. Thus, alteration of subset levels reflects a difference in subset conversion. Among molecular pathways to control differentiation of Ly6C^{low} monocytes orphan nuclear receptor mediated signaling, such as the Nr4a1 signaling, has been identified[32]. Further studies are required to dissect the underlying molecular mechanisms by which TMAO regulates monocyte subset conversion and promotes the shift towards increased Ly6C^{high} monocytes.

Despite the observed link between TMAO and its precursor choline and proinflammatory monocytes, future studies will need to clarify whether chronic exposure to TMA-containing nutrients and sustained elevation in TMAO are indeed a causal trigger of (low-grade) inflammatory processes leading to enhanced cardiovascular risk, and whether targeting gut microbiota-dependent TMAO generation in humans can similarly to animal models reduce incident event risks in patients.

The relationship between altered gut microbiota composition/function and different levels of the microbiota-dependent metabolite TMAO has been recently demonstrated. For example, plasma TMAO concentrations were found to be significantly associated with *Prevotella* and *Peptococcaceae*[5]. Additionally, a significant positive correlation between plasma TMAO concentrations with *Clostridiales* and negative correlation with *F. prausnitzii* was identified[33]. Interestingly, microbial transplantation studies have also shown that TMAO production potential and both atherosclerosis[26] and thrombosis[7] and thrombosis susceptibility are transmissible traits, further supporting a role for gut microbiota-generated TMAO in atherogenesis and thrombosis risk. These findings align with our observation that the increase in Ly6C^{high} monocytes could be prevented upon depletion of the gut microbiota suggesting an instrumental role for the gut microbiota for processing inflammation promoting metabolites.

It is tempting to speculate that metagenomics analyses of feces could reveal alterations in the abundance of specific microbes, or more importantly, microbial enzyme pathways, known to be involved in TMA and TMAO formation. Measurement of the downstream functional output of such microbial alterations (i.e. TMAO levels) serves as an integrated sensor of the global changes in microbial community structure and function, with respect to the meta-organismal TMAO pathway. In future investigations, it will be of interest to explore whether demonstration of an altered fecal microbiota serves as an additive predictor beyond TMAO levels of enhanced cardiovascular risk in patients at risk for ischemic stroke. A better

understanding of the role of microbiota-related metabolism in patients with cerebrovascular disease may contribute to the development of novel strategies targeting the gut microbiota (e.g. its enzyme systems) for both primary and secondary prevention in these patients.

Our analysis of incident CVE data in patients with stroke suggests that elevated TMAO levels are linked with an increased risk of future cardiovascular and cerebrovascular thrombotic events. This association is consistent with the recently observed role of TMAO in enhancing platelet hyper-responsiveness and thrombosis potential in vivo[7]. In addition to platelet hyper-responsiveness, the link between microbiota-dependent TMA/TMAO formation and increased levels of proinflammatory monocytes may add to potential pathophysiological mechanisms ultimately cumulating in increased incidence of cardiovascular or cerebrovascular events.

In conclusion, our study suggests a novel link between TMAO and proinflammatory monocytes, as well as with an adverse cardiovascular prognosis in patients after stroke. Our findings contribute to the understanding of a potential role of gut microbiota related mechanisms in patients with cerebrovascular disease progression and encourages further investigation of therapeutic strategies aimed at fostering a reduction in systemic levels of the gut microbial metabolite TMAO.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

Nonstandard Abbreviations and Acronyms

AUC	Area under the curve
BM	Bone marrow
CAD	Coronary artery disease
CD	Cluster of Differentiation
CVE	Cardiovascular events
EGFR	estimated glomerular filtration rate

HR	Hazard Ratio
HSC	hematopoietic stem cells
LDL	Low-density lipoprotein
Lp (a)	Lipoprotein a
Ly6C	Lymphocyte antigen 6C
MDP	Monocyte and dendritic cell progenitor
MI	Myocardial infarction
ROC	Receiver operating characteristic
PAD	Peripheral artery disease
Q	Quartile
TMA	Trimethylamine
TMAO	Trimethylamine *N*-oxide

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Highlights

- Elevated TMAO levels are dose-dependently related with an increased risk of recurrent stroke and subsequent cardiovascular events in patients after first-ever stroke, also after adjustment for traditional cardiovascular and cerebrovascular risk factors and initial stroke severity.

- TMAO adds significant prognostic information in patient subgroups defined by age, sex, cardiovascular risk factors and glomerular filtration rate

- Blood concentrations of TMAO are closely related with the levels of proinflammatory intermediate $CD14^{++}CD16^{+}$ monocytes in patients after ischemic stroke.

- Supplementation of choline, the TMA-containing precursor of TMAO, in mice increases the levels of proinflammatory $Ly6C^{high}$ monocytes in a gut microbiota dependent manner
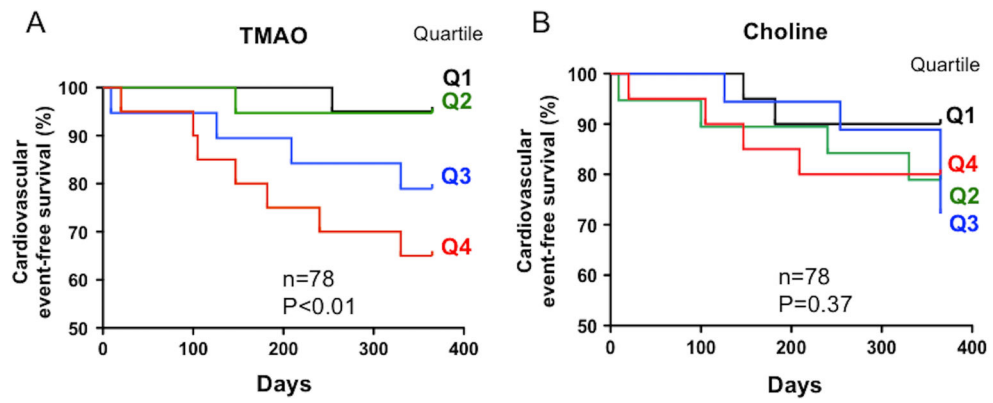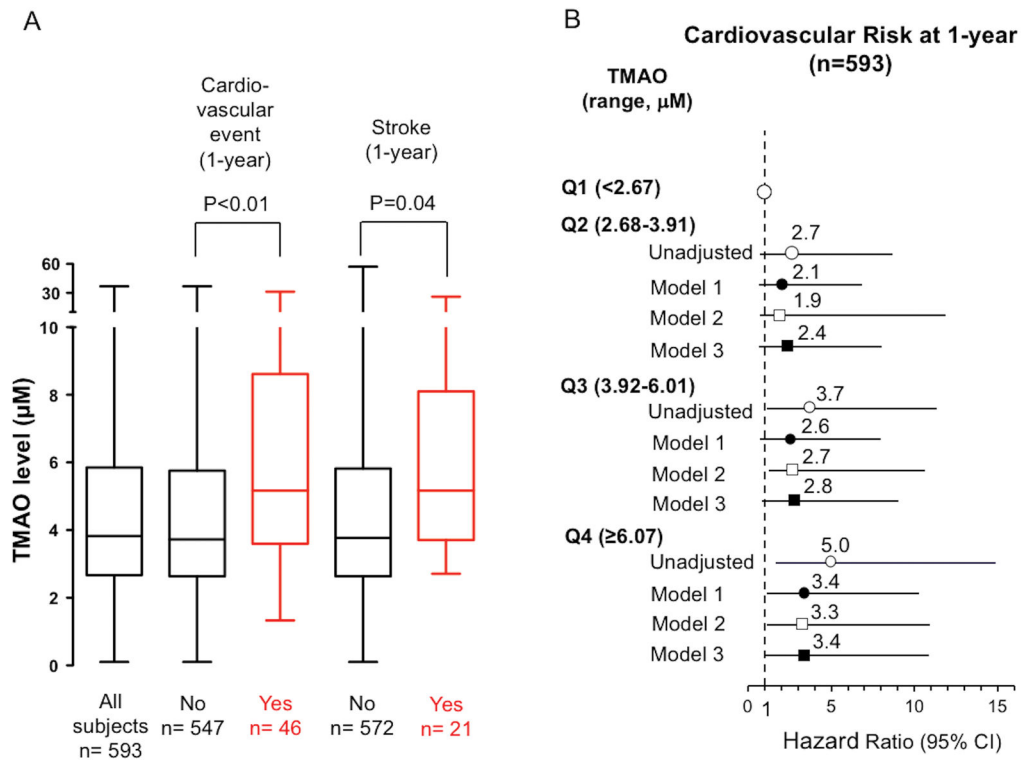
Figure 1. Plasma TMAO levels in relation to subsequent cardiovascular events in patients with ischemic stroke from the first study

In the first cohort, plasma levels of TMAO (A) but not choline (B) dose-dependently trace the risk of incident CVE including myocardial infarction, stroke and death.
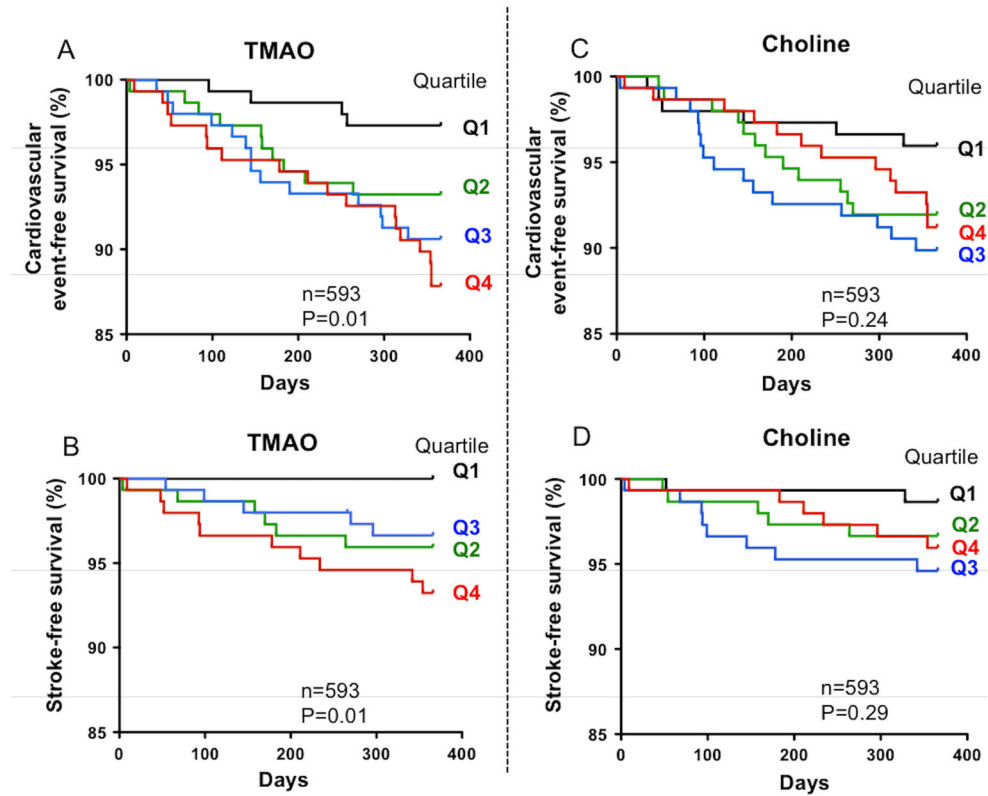
Figure 2. Circulating TMAO levels and risk of incident cardiovascular events or recurrent stroke in the validation study

Kaplan-Meier plots of the validation study confirm that patients in the 4th quartile for plasma TMAO (A, B), but not for plasma choline (C, D), had a significantly increased risk for experiencing a recurrent CVE and recurrent stroke (A and B, respectively).

Figure 3. Relationship between plasma TMAO levels and risk of subsequent cardiovascular events or recurrent stroke

(A) Data from the second lager validation cohort demonstrate higher TMAO levels (Box-Whisker plots) in patients with subsequent CVE or recurrent stroke as compared with patients without events or recurrent stroke, respectively. (B) Illustrates Forest plots of the hazard ratio of 1-year cardiovascular risk according to the quartiles of blood TMAO levels. Bars represent 95% CI. Model 1: adjusted for sex and age. Model 2: adjusted for model 1 plus hypertension, diabetes, LDL cholesterol and eGFR. Model 3: adjusted for model 1 plus stroke severity, stroke etiology (TOAST), hypertension, diabetes, LDL cholesterol, Lp(a), peripheral artery disease, and history of coronary artery disease or myocardial infarction.
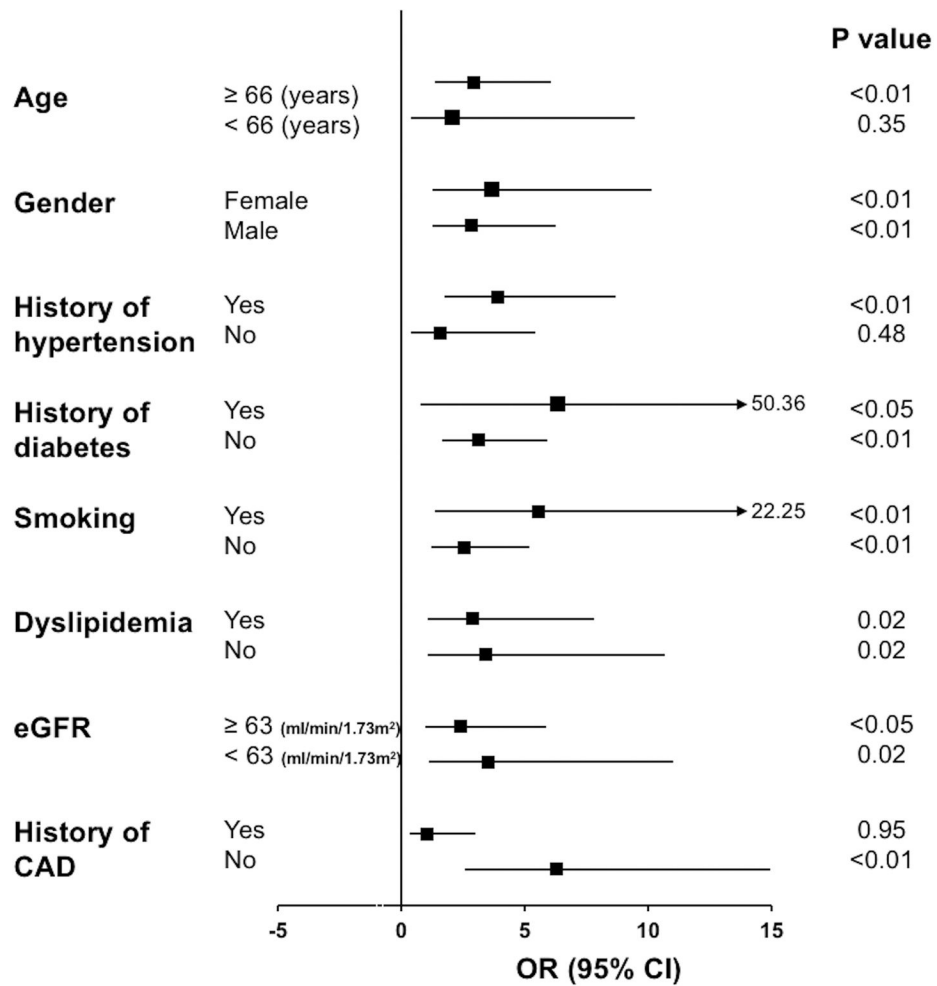
Figure 4. TMAO and cardiovascular risk in patient subgroups
Cardiovascular risk during 1-year follow-up associated with a TMAO level above 4.86 μM
in subgroups of patients from the second validation study. Odds ratio (OD) with 95% CIs
and P values are shown.
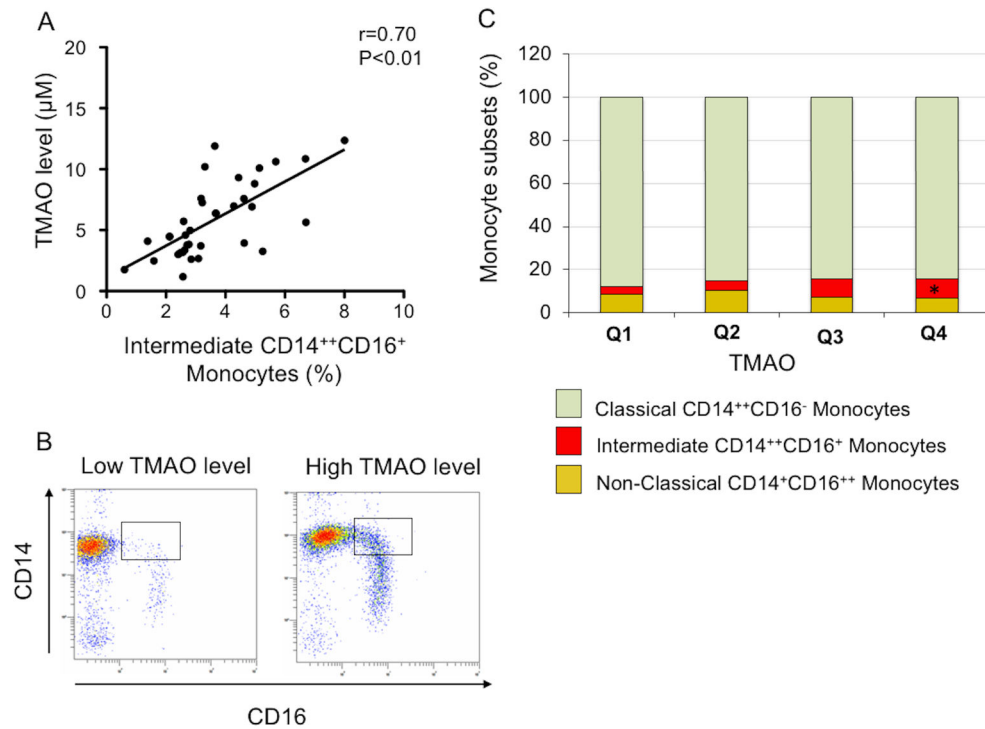
Figure 5. Relation of TMAO concentrations with the level of intermediate CD14++CD16+ monocytes

(A) Graph depicts correlation between TMAO concentrations and the level of intermediate CD14++CD16+ monocytes. (B) shows representative FACS plots of blood monocyte subsets in a patient with low TMAO levels (left plot) and in a patient with high TMAO levels (right plot). (C) Comparison of the monocyte subset distribution between patients with increasing quartiles of TMAO levels (* indicate P<0.05 as compared to CD14++CD16+ monocytes in patients from the first quartiles).
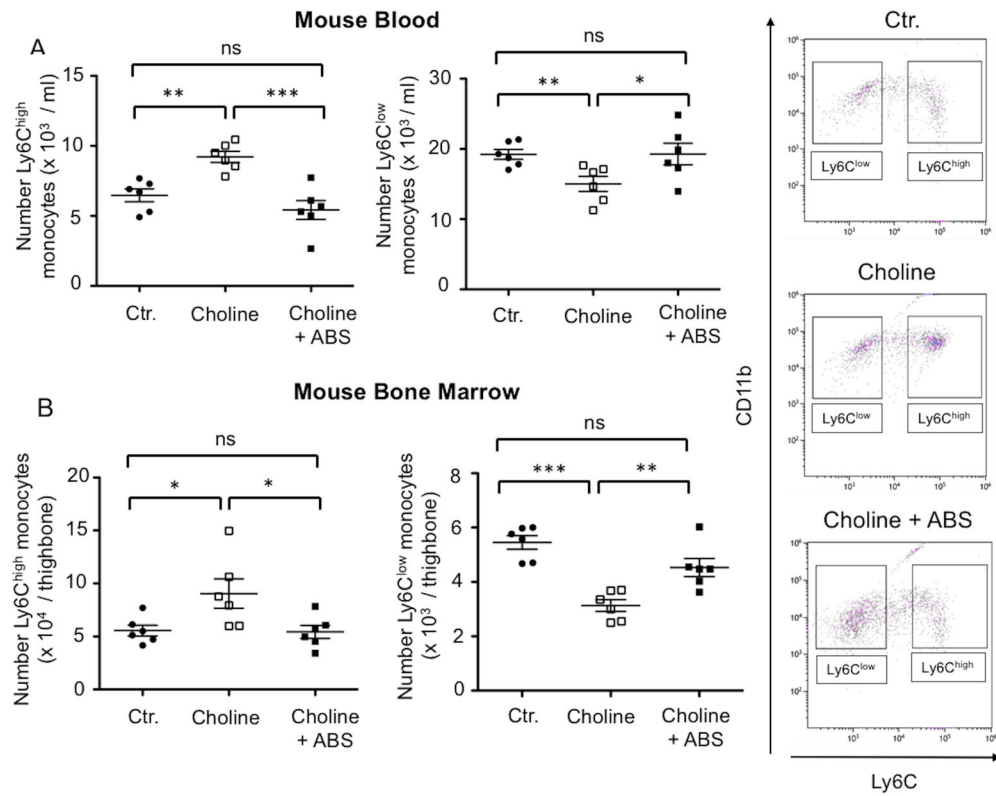
Figure 6. Impact of choline supplemented diet on levels of mouse monocyte subsets in the blood and bone marrow

(A) graphs depict proportions of Ly6C^{high} (left graph) and ly6C^{low} (right graph) blood monocytes in normal chow diet (ctr.) and changes in monocyte subsets in mice on choline supplemented diet with intact gut microbiome (choline) or with depleted gut microbiome (choline + ABS). Corresponding changes in bone marrow monocyte subsets are shown in (B). (C) shows representative FACS plots of blood monocyte subsets in a mouse on normal chow diet (ctr., upper plot), upon choline supplementation with intact gut microbiota (choline, middle plot) or with depleted gut microbiota (choline + ABS, lower plot).

Table 1

Baseline clinical parameters and laboratory test results of the first study

Parameters	All patients (n=78)	Patients in the 1st quartile (n=20)	Patients in the 4st quartile (n=20)	p-value 1st vs 4th quartile
Age (years) (mean±SD)	59 ± 14	59 ± 13	59 ± 11	0.65
Sex (% male)	69	70	75	1.00
Systolic BP (mmHg) (mean±SD)	122 ± 23	128 ± 26	131 ± 21	0.33
Diastolic BP (mmHg) (mean±SD)	66 ± 12	68 ± 13	67 ± 9	0.11
Total Cholesterol (mg/dl) (mean±SD)	202 ± 14	193 ± 53	208 ± 51	0.92
LDL (mg/dl) (mean±SD)	143 ± 39	138 ± 42	144 ± 36	0.53
HDL (mg/dl) (mean±SD)	49 ± 14	46 ± 14	49 ± 11	0.22
Triglyceride (mg/dl) (median, range)	120 (35 –664)	102 (35 –664)	104 (59 –656)	0.93
CRP (mg/l) (mean±SD)	13 ± 29	29 ± 53	6 ± 7	0.09
Creatinine (μmol/l) (median, range)	84 (36 – 706)	81 (52 – 120)	93 (64 – 706)	0.02
Hb (g/dl) (mean±SD)	14 ± 1.4	14.0 ± 1.5	13.9 ± 1.4	0.92
Leukocytes (tsd/μl) (mean±SD)	7.7 ± 2.8	8.3± 4.4	7.8 ± 2.3	0.57
Platelets (tsd/μl) (median, range)	217 (90–445)	214 (104–257)	220 (113–306)	0.66

BMI: body mass index; HR: heart rate; BP: blood pressure; LDL: low-density lipoprotein; HDL: high-density lipoprotein; CRP: C-reactive protein; Hb: hemoglobin;

Table 2

Baseline clinical parameters and laboratory test results of the second independent validation cohort

Parameters	All patients (n=593)	Patients in the 1st quartile (n=148)	Patients in the 4st quartile (n=148)	p-value 1st vs 4th quartile
Age (years) (mean±SD)	67±13	62±14	70±12	0.01
Sex (% male)	61	57	60	0.64
Systolic BP (mmHg) (mean±SD)	139±22	136±22	140±22	0.10
Diastolic BP (mmHg) (mean±SD)	78±14	76±15	76±15	0.80
Total Cholesterol (mg/dl) (mean±SD)	200±48	201±42	198±49	0.50
LDL (mg/dl) (mean±SD)	123±41	123±38	119±40	0.40
HDL (mg/dl) (mean±SD)	51±16	54±18	51±8	0.30
Triglyceride (mg/dl) (median, range)	117 (35–577)	109 (36–553)	122 (36–492)	0.09
CRP (mg/l) (mean±SD)	3.2±10.8	4.7±14.5	3.2±11.9	0.30
Creatinine (μmol/l) (median, range)	80 (31 – 460)	74 (31 – 159)	91 (42 – 460)	<0.01
Hb (g/dl) (mean±SD)	14.2±1.7	14.3±1.6	13.8±1.7	0.02
Leukocytes (tsd/μl) (mean±SD)	8.1±3.5	7.5±2.2	8.3±3.0	0.02
Platelets (tsd/μl) (median, range)	221 (80–526)	213 (87–393)	227 (100–451)	0.13