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ABSTRACT

Background: Trimethylamine *N* oxide (TMAO) has been linked to increased cardiovascular risk. We aimed to determine the prognostic value of TMAO and its dietary precursors, choline and betaine, in heart failure (HF).

Methods and Results: In 112 patients with chronic systolic HF with comprehensive echocardiographic evaluation, we measured plasma TMAO, choline, and betaine by mass spectrometry. Median (interquartile range) TMAO levels, choline, and betaine levels were 5.8 (3.6−12.1) μmol/L, 10.9 (8.4−14.0) μmol/L, and 43.8 (37.1−53.0) μmol/L, respectively, and were correlated with each other (all $P < .0001$ for both). TMAO levels were significantly higher in patients with diabetes mellitus (9.4 [4.9−13.2] vs 4.8 [3.4−9.8] μmol/L; $P = .005$) and in subjects with New York Heart Association functional class III or greater (7.0 [4.7−14.8] vs 4.7 [3.4−11.3] μmol/L; $P = .02$). Elevated TMAO, choline, and betaine levels were each associated with higher plasma N terminal pro−B type natriuretic peptide (NT proBNP) levels and more advanced left ventricular diastolic dysfunction, but not systolic dysfunction or inflammatory and endothelial biomarkers. Higher choline (hazard ratio [HR] 1.64, 95% CI 1.22−2.20; $P = .001$), betaine (HR 1.51, 95% CI 1.10−2.08; $P = .01$), and TMAO (HR 1.48, 95% CI 1.10−1.96; $P = .01$) predicted increased risk for 5 year adverse clinical events (death/transplantation). Only higher TMAO levels predicted incident adverse clinical events independently from age, estimated glomerular filtration rate, mitral E/septal Ea, and NT proBNP levels (HR 1.46, 95% CI 1.03−2.14; $P = .03$).

Conclusion: Elevated plasma TMAO, choline, and betaine levels are each associated with more advanced left ventricular diastolic dysfunction and portend poorer long term adverse clinical outcomes in chronic systolic HF. However, only higher plasma TMAO was associated with poor prognosis after adjustment for cardiorenal indices. (*J Cardiac Fail 2015;21:91−96*)

Key Words: Intestinal microbiota, trimethylamine *N* oxide, diastolic dysfunction, heart failure.

Intestinal microbiota are implicated in the development of metabolic phenotypes such as obesity and insulin resistance.[1] With the use of an unbiased metabolomics approach, our group recently identified 3 metabolites of the dietary lipid phosphatidylcholine choline, betaine and the gut microbiota generated metabolite trimethylamine-*N*-oxide (TMAO) that are associated with atherosclerotic cardiovascular disease.[2] We recently validated those findings in a larger-scale clinical cohort whereby elevated plasma TMAO levels portend greater risk of major adverse cardiac events,[3] and we showed the mechanistic link between TMAO and macrophage activation[2] as well as alterations in cholesterol metabolism and transport.[4] Because choline and betaine are substrates in the formation of TMAO by intestinal microbiota, we have further demonstrated their prognostic values in predicting future major adverse cardiac events to be largely driven by the presentation of elevated TMAO levels.[5]

Heart failure (HF) is a frequent adverse complication of atherosclerotic cardiovascular disease and may present either as myocardial ischemia, vascular dysfunction, and fibrosis leading to progressive diastolic dysfunction or as progressive myocyte damage and cardiac remodeling leading to systolic dysfunction. We recently reported the association between TMAO and long-term mortality risk in a large cohort of patients with a history of chronic HF, independently from renal insufficiency or natriuretic peptide levels.[6] However, the relationship between TMAO and its dietary precursors choline and betaine and myocardial and inflammatory indices, markers of endothelial dysfunction, as well as their relative prognostic values in patients with chronic systolic HF, has not yet been carefully explored. In the present study, our objective was to investigate the relationship between the 3 phosphatidylcholine metabolites TMAO, choline, and betaine and myocardial indices, inflammatory and endothelial biomarkers, and long-term clinical prognosis in subjects with chronic systolic HF.

Materials and Methods

Study Population

This was a single center prospective cohort study approved by the Cleveland Clinic Institutional Review Board, and every subject provided written informed consent. We enrolled 112 ambulatory subjects ≥18 years of age with stable but symptomatic chronic systolic HF (left ventricular [LV] ejection fraction ≤35%;), who underwent comprehensive echocardiographic evaluation as part of a research study at the Cleveland Clinic. Subjects were excluded if they had significant primary valvular abnormalities. Comprehensive transthoracic echocardiographic evaluation of systolic and diastolic myocardial performance was assessed as previously described.[7] The composite end point of adverse clinical events (all cause mortality and cardiac transplantation) was prospectively tracked for 5 years by telephone follow up and medical chart review.

TMAO, Choline, and Betaine Assay

Quantification of TMAO, choline, and betaine was performed with the use of stable isotope dilution liquid chromatography with tandem mass spectrometry (LC/MS/MS), stable isotope dilution assay, and high performance LC with online electrospray ionization (ESI)/MS/MS on an AB Sciex Qtrap 5500 mass spectrometer as previously described.[8] Arginine metabolites (asymmetric dimethylarginine [ADMA], symmetric dimethylarginine [SDMA], L arginine, L ornithine, and L citrulline) were quantified with the use of stable isotope dilution LC/ESI/MS/MS assays on an upgraded ABI 365 triple quadrupole mass spectrometer (Applied Biosystems, Foster City, California) with Ionics EP 10+ redesigned source (Concord, Ontario, Canada) and ESI needle connected to an Aria LX4 series multiplexed high performance LC system with Flux pumps (Cohesive Technologies, Franklin, Massachusetts), as previously described.[7] Global arginine bioavailability ratio (GABR) was calculated as the ratio between the substrates (L arginine) and the products (L ornithine plus L citrulline) of nitric oxide production.[9]

Aminoterminal pro−B type natriuretic peptide (NT proBNP) levels were measured with the use of the Elecsys platform (Roche Diagnostics, Indianapolis, Indiana).[10] Plasma myeloperoxidase (MPO) and high sensitivity C reactive protein (hsCRP) levels were measured by means of CardioMPO assay (Cleveland Heart Labs, Cleveland, Ohio)[11] as well as N Latex Cystatin C CardioPhase assay (Siemens/Dade Behring, Deerfield, Illinois)[12,13] as previously described.

Statistical Analysis

Continuous variables were summarized as mean ± SD if normally distributed and median (interquartile range [IQR]) if non normally distributed. Normality was assessed by means of the Shapiro Wilk *W* test. Spearman rank correlation was used as a nonparametric measure of association between metabolites and dependent variables. Plasma levels of TMAO, choline, and betaine were compared across categoric variables with the use of the Wilcoxon rank sum or Kruskal Wallis test. The optimal receiver operating characteristic (ROC) curve cutoff value for plasma levels of TMAO, choline, and betaine in predicting adverse clinical events was chosen as the value maximizing sensitivity plus specificity. Kaplan Meier survival plots were calculated from baseline to time of adverse event and compared with the use of the log rank test. Cox proportional hazards analysis were used to assess the clinical risks associated with higher TMAO, choline, and/or betaine levels, in which the proportional hazards assumption was verified with log (time) versus log [log (survival)] plots. A 2 sided P value of $<.05$ was considered to be statistically significant. All analyses were performed with the use of JMP 10 Pro (SAS, Cary, North Carolina).

Results

Table 1 illustrates the baseline characteristics of the study population. Overall, the cohort was representative of a stable outpatient cohort of patients with systolic heart failure, with 40 (36%) experiencing at least New York Heart Association (NYHA) functional class III symptoms. Overall, TMAO correlated with choline ($r = 0.40$; $P < .0001$) but not betaine ($r = 0.08$; $P = .43$), and choline correlated with betaine ($r = 0.46$; $P < .0001$).

Table 1. Baseline Characteristics

Variable	Value
Age, y	57 ± 14
Male	84 (75%)
Ischemic etiology	48 (43%)
History of diabetes mellitus	32 (29%)
Echocardiographic indices	
Left ventricular end diastolic volume, indexed (mL/m^2)	111 ± 36
Left ventricular ejection fraction (%)	26 ± 6
Diastolic stage III	44 (40%)
Mitral regurgitation ≥3+	11 (10%)
Right ventricular systolic pressure (mm Hg)	34 ± 13
Right ventricular systolic dysfunction ≥3+	31 (28%)
Left atrial volume index (mL/m^2)	42 ± 15
Mitral E/septal Ea	19 ± 12
Medications	
Angiotensin converting enzyme inhibitor or angiotensin receptor blockers	103 (94%)
Beta blocker	66 (60%)
Spironolactone	31 (30%)
Loop diuretics	87 (78%)
Laboratory data	
Estimated glomerular filtration rate (mL min ^{1} 1.73 m ^{2})	70 ± 26
N Terminal pro−B type natriuretic peptide (pg/mL)	1,473 (543−3,438)
Myeloperoxidase (pg/mL)	312 (263−427)
High sensitivity C reactive protein (mg/L)	3.33 (1.55−6.81)
Cystatin C (mg/dL)	1.23 (1.03−1.66)
Aysmmetric dimethylarginine (μmol/L)	0.43 (0.36−0.55)
Symmetric dimethylarginine (μmol/L)	0.29 (0.23−0.39)
Global arginine bioavailability ratio	0.76 (0.55−1.03)
Choline (μmol/L)	10.9 (8.4−14.0)
Betaine (μmol/L)	43.8 (37.1−53.0)
Trimethylamine *N* oxide (μmol/L)	5.8 (3.6−12.1)

Values are presented as mean ± SD, n (%), or median (interquartile range).

Older patients were more likely to have higher levels of choline, but not of betaine or TMAO. Meanwhile, men had higher choline and betaine levels than women ($P < .001$), yet levels were similar between those with versus without diabetes. As expected, and as previously described,[2,3] those with a history of diabetes mellitus or renal insufficiency had higher levels of TMAO, choline, and betaine compared with those without. Interestingly, there were no significant differences between TMAO, choline, and betaine in those with ischemic versus nonischemic etiologies, nor across MPO or hsCRP levels (Table 2). Also, those receiving loop diuretics had higher TMAO but similar choline and betaine levels compared with those not on loop diuretics ($P < .05$).

The relationship between TMAO, choline, and betaine levels and clinical, laboratory, and echocardiographic parameters are presented in Table 2. Interestingly, there were statistically significant correlations between all the metabolites and multiple echocardiographic indices. Moreover, we observed consistent positive correlations between TMAO and indices of diastolic dysfunction, especially with mitral E/septal Ea and left atrial volume index (Table 2). In contrast, there were no statistically significant correlations noted between TMAO, choline, and betaine in LV ejection fraction or LV dimensions. Figure 1 demonstrates the

Table 2. Univariate Correlations Between Gut Flora−Dependent Phosphatidylcholine Metabolites and Clinical and Echocardiographic Indices

Variable	Choline	Betaine	TMAO
Age	0.26**	0.09	0.12
Left ventricular end diastolic volume, indexed	0.12	0.09	0.06
Left ventricular ejection fraction	0.10	0.19	0.09
Mitral regurgitation	0.20*	0.13	0.05
Right ventricular systolic pressure	0.34**	0.28**	0.14
Right ventricular systolic dysfunction	0.29**	0.34***	0.05
Mitral E/septal Ea	0.33**	0.29*	0.29**
Left atrial volume index	0.45**	0.22*	0.29**
Aminoterminal pro B type natriuretic peptide	0.45**	0.27**	0.26**
Myeloperoxidase	0.03	0.04	0.11
High sensitivity C reactive protein	0.01	0.14	0.19
Cystatin C	0.54****	0.24*	0.40****
Estimated glomerular filtration rate	0.39****	0.13	0.36***
Asymmetric dimethylarginine	0.33***	0.20*	0.11
Symmetric dimethylarginine	0.39****	0.13	0.18
Global arginine bioavailability ratio	0.54****	0.37****	0.23*

TMAO, trimethylamine *N* oxide.
*$P < .05$; **$P < .01$; ***$P < .001$; ****$P < .0001$.

relationship between TMAO, choline, and betaine levels stratified according to a mitral E/septal Ea cutoff at 15. Meanwhile there was strong positive correlation between methylated arginine metabolites and choline and to a lesser extent betaine, and an inverse correlation was observed between both choline and betaine and GABR. In contrast, TMAO did not correlate with any methylated arginine metabolites, and only modestly with GABR. In multivariate linear regression analysis with all variables, choline was independently associated only with cystatin C ($\beta = 0.40$; $P < .0001$) and GABR ($\beta = -0.39$; $P < .0001$), betaine only with GABR ($\beta = -0.31$; $P = .002$) and right ventricular systolic dysfunction class ($\beta = 0.21$; $P = .030$), and TMAO only with NT-proBNP ($\beta = 0.34$; $P = .003$) and cystatin C ($\beta = 0.75$; $P < .0001$).

Table 3 presents the Cox proportional hazard models of TMAO, choline, and betaine for long-term adverse clinical outcomes. There was a total of 40 events over 5 years of follow-up. All 3 measures (choline, betaine, and TMAO) were predictive of the composite end point of 5-year all-cause mortality plus cardiac transplantation. However, only the prognostic value of TMAO remained statistically significant after adjustments for age, estimated glomerular filtration rate (eGFR), mitral E/septal Ea, and NT-proBNP levels (adjusted hazard ratio 1.46, 95% confidence interval 1.03 2.14; $P = .031$). When stratified according to the optimal ROC cutoff value of 6.1 μmol/L for TMAO and median NT-proBNP (1,473 pg/mL) for this cohort, the subset of patients with both elevated TMAO and NT-proBNP demonstrated the greatest risk of future adverse cardiac events (Fig. 2).

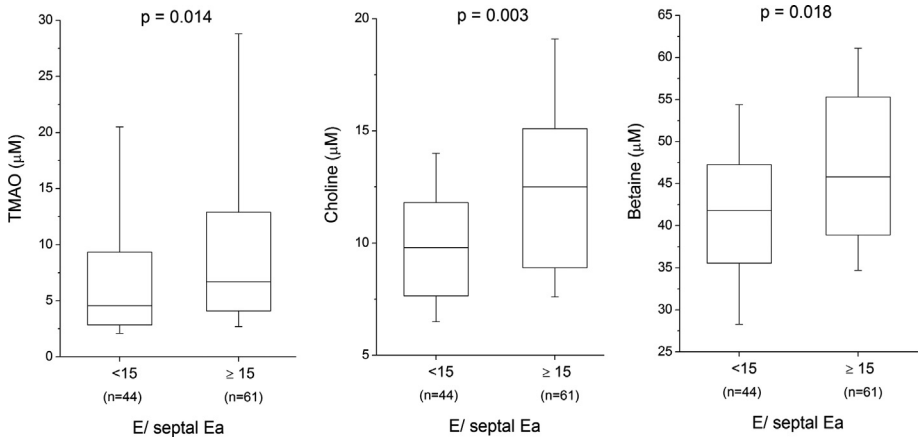

Fig. 1. Relationship between intestinal microflora−dependent phosphatidylcholine metabolites and diastolic dysfunction in chronic systolic heart failure. TMAO, trimethylamine *N* oxide.

Discussion

We recently reported the association between elevated TMAO levels and increased 5-year mortality risk independently from cardiorenal indices in a large cohort of patients with a history of HF.[6] In the present study, we validated our findings in an independent cohort of ambulatory patients with chronic systolic HF, with added insights into the relationship between all 3 phosphatidylcholine metabolites, TMAO, choline, and betaine, and both echocardiographic determinants and renal and inflammatory biomarkers. There are several notable findings. First, we observed a stronger prognostic value in TMAO than in choline and betaine in patients with chronic systolic HF, which was independent from cardiorenal indices. Second, we observed correlations between all 3 metabolites and LV diastolic dysfunction rather than LV systolic dysfunction. Third, the relatively lack of correlations between TMAO, choline, and betaine and several well known inflammatory biomarkers and differential relationships with markers of endothelial dysfunction also suggested an independent pathophysiologic pathway. Of note, the higher level of TMAO found in subjects with renal insufficiency or diabetes mellitus points to an underlying metabolic defect relevant to those disease states rather than a systemic inflammatory response. Nonetheless, the association between elevated TMAO and both HF severity and incident adverse outcomes independent of other cardiorenal indices argues for a potential pathogenic mechanistic link between the gut microbiota pathway generating TMAO and HF development and/or progression. Of note, this was a cohort of ambulatory stable HF patients with LV systolic dysfunction, with an annualized mortality of 7.1% (assuming transplantation as equivalent as death), which was not too different from other reported clinical trials. Taken together, these findings provided validation of the clinical significance of TMAO levels in HF[6] and suggest that more studies are warranted to understand the mechanistic underpinnings of the association.

A mechanistic link between altered intestinal microbiota and myocardial function has previously been suggested to

Table 3. Cox Proportional Hazard Analyses of Adverse Clinical Outcomes

Variable	Hazard Ratio* (95% CI)	*P* Value
Choline		
Unadjusted	1.64 (1.22−2.20)	.001
Adjusted	1.22 (0.82−1.85)	.333
Betaine		
Unadjusted	1.51 (1.10−2.08)	.011
Adjusted	1.33 (0.93−1.89)	.115
Trimethylamine *N* oxide (TMAO)		
Unadjusted	1.48 (1.10−1.96)	.010
Adjusted	1.46 (1.03−2.14)	.031

CI, confidence interval.

Adjusted for age, estimated glomerular filtration rate, mitral E/septal Ea, and N terminal pro−B type natriuretic peptide.

*Hazard ratios per 1 standard deviation (Ln Choline 0.38 μmol/L; Ln Betaine 0.27 μmol/L; Ln TMAO 0.99 μmol/L).

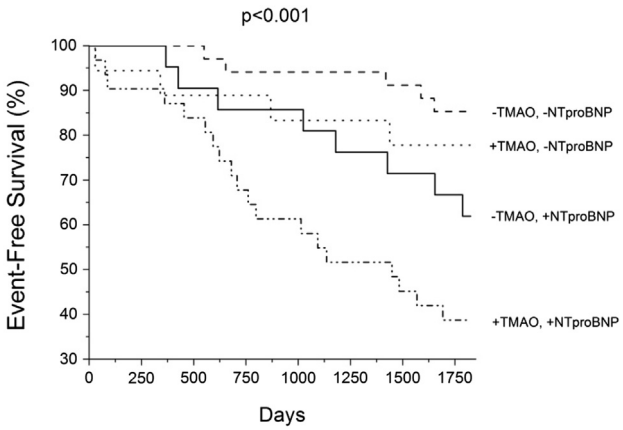

Fig. 2. Kaplan Meier survival curve stratified according to levels of trimethylamine *N* oxide (TMAO) and N terminal pro−B type natriuretic peptide (NT proBNP) levels. /+ TMAO, below versus above 6.1 μmol/L; /+ NT proBNP, below versus above 1,473 pg/mL (median).

result largely in the setting of responses to circulatory insult.[14,15] Dysbiosis (abnormal changes in intestinal microbiota composition) has also been reported in rodent models of myocardial infarction.[11] Interestingly, supplementation with a probiotic was reported to be associated with improved cardioprotection in a surgical model of myocardial infarction in a recent study.[16] The present analysis is the 1st human study to our knowledge to report the relationship between the intestinal microbiota-dependent analyte TMAO and its dietary precursors, choline and betaine, with echocardiographic indices in the setting of chronic systolic HF. Of note, there was no demonstrable relationship between TMAO, choline, or betaine and LV systolic function or degree of cardiac remodeling. Rather, the stronger correlations observed were between all 3 metabolites and LV diastolic functional indices (Table 2). These observations suggest that a greater degree of ''backward failure'' (congestion, with either scarring or ischemia) rather than ''forward failure'' (impaired perfusion) may be associated with the primary metabolic defect driving the observed associations. Consistently with this, associations with renal functional indices were noted for choline and TMAO, but again, the association between TMAO and adverse events among subjects remained even after adjustments for renal function.

Another possibility for the association with elevated TMAO levels among subjects with poorer prognosis may arise because of the greater degree of intestinal congestion from right-sided HF. Indeed, it is tempting to speculate that such congestion and attendant intestinal edema could lead to alterations in intestinal microbiota composition that adversely affects (enhances) TMAO production.[17] Earlier investigations have observed a reduction in active carrier-mediated intestinal transport processes in decompensated HF, leading to epithelial dysfunction possibly as a consequence of intestinal ischemia.[18] As intestinal microbiota shift rapidly from ischemia to reperfusion,[19] it is conceivable that the balance and amount of intestinal commensal versus pathobiontic bacteria may also be affected by venous congestion, thereby leading to altered metabolic milieu and downstream metabolic derangements for the host. Therefore, how HF itself affects intestinal microbial composition and function, and conversely, how intestinal microbiota directly or indirectly affect the HF phenotype and pathophysiology, should be further explored.

The lack of relationship between TMAO and its dietary precursors, choline and betaine, and inflammatory biomarkers is also somewhat unexpected, because earlier studies implied that the lack of intestinal microbiota may be associated with a state of active interleukin-10 mediated inflammatory hyporesponsiveness.[20] That being said, our previous studies in a broad population of cardiac patients also demonstrated prognostic value of TMAO being independent from plasma MPO levels.[3] This is perhaps not too surprising in our cohort of ambulatory chronic systolic HF patients, where organ perfusion remains largely preserved. Therefore, our present findings may imply concepts beyond the traditional gut hypothesis, whereby reduced circulatory efficiency in the HF setting leads to impaired barrier function of the intestinal epithelia and heightened inflammatory responses to endotoxins and peptidoglycans.[21] The dissociation between TMAO and choline/betaine regarding relationships with markers of endothelial dysfunction further suggests that the metabolic defect associated with elevated TMAO may even be independent from traditional measures of vascular dysfunction that have been known to impact diastolic dysfunction.[7,9]

What are the implications of these findings? First, understanding why patients with advancing systolic HF have increased levels of TMAO will be important. Although increased production (from increased dietary sources or microbial/host enzymes) or reduced clearance (because of renal insufficiency) may contribute, there is a need to further determine if the underlying cardiac insufficiency or metabolic defects associated with cardiac dysfunction may contribute to such a phenomenon. The independence of the association between elevated TMAO levels and poor outcomes after adjustments for cardiorenal indices such as eGFR and NT-proBNP argues for additional underlying mechanistic links. Second, neither dietary nor intestinal microbiota influences on HF pathophysiology have been well studied, even though the ''gut hypothesis'' of heart failure has been well established in the literature. Further studies are needed to determine if manipulation of gut microbiota composition or dietary modifications (similar to a renal or diabetic diet) may offer novel therapeutic approaches in the setting of elevated TMAO levels in patients with HF. Indeed, a low-protein diet, which is typical for patients with chronic kidney disease, is anticipated to be low in dietary nutrients such as carnitine, choline, and phosphatidylcholine that can generate TMAO.[4,5] Nutritional and microbiota-associated metabolic factors that can lead to the causation, development, and progression of myocardial dysfunction may therefore provide important therapeutic targets in a disease with grave morbidity and mortality for which treatment has largely focused on hemodynamic augmentation and neurohormonal suppression.

Study Limitation

There are several limitations in this single tertiary referral center experience. Our earlier studies were largely confined to measurements of fasting samples of phosphatidylcholine metabolites, whereas samples in the present study were collected without an overnight fast. Nevertheless, data from our previous studies suggested that the overall plasma TMAO, choline, and betaine levels, although fluctuating shortly after feeding, appear to stay within a confined range.[3] Furthermore, we did not have any information or measurements regarding the dietary habits (especially dietary phosphatidylcholine amounts ingested) of our subjects, nor did we have any quantification of intestinal microbiota composition, intestinal barrier function,

or commensal versus pathobiontic bacterial growth. We also did not have independently adjudicated NYHA func-tional class (the majority of patients were symptomatic in NYHA functional class II III), physical exam findings, or electrocardiographic data at the time of evaluation for the study, and the relatively low number of adverse events (n = 40) may have limited our multivariate analysis.

Conclusion

Elevated plasma TMAO, choline, and betaine levels are each associated with more advanced LV diastolic dysfunction and portend poorer long-term adverse clinical outcomes in chronic systolic HF. However, only higher plasma TMAO levels were associated with poor prognosis after adjustment for cardiorenal indices.

Disclosures

Drs Hazen and Wang are listed as coinventors on pending and issued patents held by the Cleveland Clinic relating to cardiovascular diagnostics and therapeutics. Dr Hazen has been paid as a consultant or speaker for the following companies: Cleveland Heart Lab, Esperion, Lilly, Liposcience, Merck & Co, Pfizer, and Procter & Gamble. Dr Hazen has received research funds from Abbott, Cleveland Heart Lab, Liposcience, Pfizer, Procter & Gamble, and Takeda Pharmaceuticals. Dr Hazen has the right to receive royalty payments for inventions or dis-coveries related to cardiovascular diagnostics or therapeu-tics from the following companies: Cleveland Heart Lab, Esperion, Frantz Biomarkers, Liposcience, and Siemens. Dr Hazen is also partially supported by a gift from the Leonard Krieger endowment and by the Foundaton LeDucq. All other authors report no disclosures.

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