Circulation

ORIGINAL RESEARCH ARTICLE

Reduced Apolipoprotein M and Adverse Outcomes Across the Spectrum of Human Heart Failure

BACKGROUND: Apo (apolipoprotein) M mediates the physical interaction between high-density lipoprotein (HDL) particles and sphingosine-1-phosphate (S1P). Apo M exerts anti-inflammatory and cardioprotective effects in animal models.

METHODS: In a subset of PHFS (Penn Heart Failure Study) participants (n=297), we measured apo M by Enzyme-Linked ImmunoSorbent Assay (ELISA). We also measured total S1P by liquid chromatography–mass spectrometry and isolated HDL particles to test the association between apo M and HDL-associated S1P. We confirmed the relationship between apo M and outcomes using modified aptamer-based apo M measurements among 2170 adults in the PHFS and 2 independent cohorts: the Washington University Heart Failure Registry (n=173) and a subset of TOPCAT (Treatment of Preserved Cardiac Function Heart Failure With an Aldosterone Antagonist Trial; n=218). Last, we examined the relationship between apo M and ≈5000 other proteins (SomaScan assay) to identify biological pathways associated with apo M in heart failure.

RESULTS: In the PHFS, apo M was inversely associated with the risk of death (standardized hazard ratio, 0.56 [95% CI, 0.51–0.61]; $P<0.0001$) and the composite of death/ventricular assist device implantation/heart transplantation (standardized hazard ratio, 0.62 [95% CI, 0.58–0.67]; $P<0.0001$). This relationship was independent of HDL cholesterol or apo AI levels. Apo M remained associated with death (hazard ratio, 0.78 [95% CI, 0.69–0.88]; $P<0.0001$) and the composite of death/ventricular assist device/heart transplantation (hazard ratio, 0.85 [95% CI, 0.76–0.94]; $P=0.001$) in models that adjusted for multiple confounders. This association was present in both heart failure with reduced and preserved ejection fraction and was replicated in the Washington University cohort and a cohort with heart failure with preserved ejection fraction only (TOPCAT). The S1P and apo M content of isolated HDL particles strongly correlated ($R=0.81$, $P<0.0001$). The top canonical pathways associated with apo M were inflammation (negative association), the coagulation system (negative association), and liver X receptor/retinoid X receptor activation (positive association). The relationship with inflammation was validated with multiple inflammatory markers measured with independent assays.

CONCLUSIONS: Reduced circulating apo M is independently associated with adverse outcomes across the spectrum of human heart failure. Further research is needed to assess whether the apo M/S1P axis is a suitable therapeutic target in heart failure.

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Clinical Perspective

What Is New?

- Reduced apo (apolipoprotein) M plasma protein levels are associated with adverse outcomes in heart failure (HF), including HF with reduced ejection fraction and HF with preserved ejection fraction.
- The relationship between reduced apo M and outcomes in HF is particularly pronounced when concentrations of its binding partner, sphingosine-1-phosphate, are also reduced.
- Apo M protein levels are associated with inflammation in human HF.

What Are the Clinical Implications?

- Apo M represents a risk marker in human HF.
- Apo M is associated with inflammation in HF, but its relationship with risk appears to be only partially dependent on this association.
- Further studies are needed to assess whether targeting apo M/sphingosine-1-phosphate can improve outcomes in HF.

Apo (apolipoprotein) M is a lipocalin secreted primarily by the liver and is present in ≈5% of high-density lipoprotein (HDL) and <2% of low-density lipoprotein particles.[1–3] Apo M exerts multiple pleiotropic effects, including anti-inflammatory, antioxidant, and antiatherogenic effects[4,5]; it promotes endothelial protection[6,7] and enhances cell survival.[8] Apo M contains a hydrophobic binding pocket for sphingosine-1-phosphate (S1P), a small signaling sphingolipid. S1P activates multiple G-protein–coupled receptors on cell types, including endothelial cells and cardiomyocytes. Multiple studies suggest that apo M and S1P attenuate ischemic injury, but little is known about the role of apo M or S1P in heart failure (HF) progression.[9–11]

Mechanistically, the apo M/S1P axis inhibits inflammation and attenuates the effect of tumor necrosis factor-α (TNF-α) on gene expression, limiting monocyte adhesion to the endothelium and maintaining endothelial barrier integrity.[12] Apo M also protects animals from nonischemic insults such as lipopolysaccharide-induced death and organ injury through anti-inflammatory and antiapoptotic effects, as well as modulation of the coagulation system.[13] Apo M enhances endothelial nitric oxide production and vasodilation and increases cardiomyocyte survival.[9,14] Despite the strong rationale for a cardioprotective role of apo M, the relevance of apo M in human HF has not been previously investigated.

On the basis of the multiple described protective functions of apo M and S1P in cell and animal models, we hypothesized that reduced levels of apo M are associated with worse outcomes in human HF. Specifically, we tested the hypothesis that reduced circulating apo M is associated with the risk of death, a composite of death/ventricular assist device (VAD) implantation/heart transplantation, and a composite of death/HF-related hospitalization among adults with HF enrolled in PHFS (Penn Heart Failure Study), a large multicenter cohort study with stratified analyses in HF with reduced (HFrEF) and preserved (HFpEF) ejection fraction and subsequent replication in 2 independent cohorts. We also aimed to identify biological pathways associated with apo M using large-scale plasma proteomics.

METHODS

Study Populations

We analyzed primarily data from participants enrolled in the PHFS and replicated our results in 2 independent cohorts: the Washington University Heart Failure Registry and TOPCAT (Treatment of Preserved Cardiac Function Heart Failure With an Aldosterone Antagonist Trial). The data, analytical methods, and study materials are not publicly available for purposes of reproducing the results or replicating the procedures. Such data may be made available to other researchers for collaborative research through the establishment of appropriate data-sharing agreements and regulatory approvals. The parent TOPCAT trial data are available to other researchers through the US National Institutes of Health BioLINCC website.

The Penn Heart Failure Study

The PHFS design has been previously published.[15–18] Briefly, the PHFS was a prospective cohort study of ambulatory patients with chronic HF recruited between 2003 and 2011 at the University of Pennsylvania (Philadelphia), Case Western Reserve University (Cleveland, OH), and the University of Wisconsin (Madison). Patients with a clinical diagnosis of HF as determined by an HF specialist were enrolled. Each participant provided written informed consent. At the time of study entry, standardized questionnaires were administered to participants and their physicians to obtain detailed clinical data. Participants with expected mortality of ≤6 months from a noncardiac condition, mechanical circulatory support, or inability to provide informed consent were excluded. Venous blood samples were obtained at enrollment and stored at –80°C for later analysis. An institutional review board from each participating center approved the protocol.

Washington University Heart Failure Registry

This is a prospective registry of patients with a clinical diagnosis of HF evaluated at Washington University School of Medicine (Barnes Jewish Hospital, St. Louis, MO). As previously described,[19] detailed patient information was prospectively collected, including heart disease onset, pathogenesis, clinical stage, severity, medications, device therapies, comorbidities, demographics, and health status. All patients provided informed consent, and the study was approved by the Washington University Institutional Review Board.

Treatment of Preserved Cardiac Function Heart Failure With an Aldosterone Antagonist Trial

TOPCAT data and samples were obtained from the National Heart, Lung, and Blood Institute of the US National Institutes of Health. TOPCAT was a multicenter, international, randomized,

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double-blinded, placebo-controlled trial of spironolactone that enrolled 3445 adults with HFpEF across 6 countries from 2006 to 2012. The primary goal of the trial was to determine whether spironolactone was associated with a reduction in the composite outcome of cardiovascular mortality, aborted cardiac arrest, or HF hospitalization. The design, general characteristics of the study population, and primary results of the trial have been previously published.[20–22] Because of substantial differences in subject recruitment and study implementation in Russia and Georgia,[21,23,24] we performed measurements only from subjects enrolled in the Americas who had available plasma samples for de novo measurements of apo M levels (n=218).

Apo M Level Determination

Our a priori hypothesis was initially tested in a subset of study participants from the PHFS (n=297) among whom apo M levels were determined by single-plate ELISA with a human apo M antibody, as previously described.[25] Subsequently, we analyzed apo M levels measured with a modified aptamer assay (SomaScan assay), as previously described[26–29] in the PHFS cohort (n=2170), the Washington University Heart Failure Registry (n=173), and TOPCAT (n=218). The SomaScan apo M measurement has been previously validated by mass spectrometry,[26] as detailed in the Data Supplement.

Plasma Proteomics and Pathway Analyses

We used the SomaScan assay version 4, which is a multiplexed, modified aptamer-based binding assay for PHFS and TOPCAT assays. This assay includes 4979 modified aptamer reagents to 4776 unique protein targets. The SomaScan assay uses Slow-Off-Rate Modified Aptamer reagents, which are chemically modified nucleotides, to bind and quantify target proteins in relative fluorescent units directly proportional to the amount of target protein in the sample. We performed knowledge-based pathway analysis to assess the correlates of apo M. First, we assessed the correlation between levels of apo M and all proteins measured in the SomaScan assay after Box-Cox transformation. We adjusted for multiple comparisons on the basis of the principal components underlying the variability of all measured proteins, as previously described.[30–32] Associations between apo M and individual proteins that were significant with an adjusted value of $P<0.01$ were then used to perform pathway analyses with Ingenuity Pathway Analysis software (Qiagen; Hilden, Germany; www.qiagen.com/ingenuity). The full list of proteins and their associations with apo M is provided in the Data Supplement. Proteins were identified according to their UniProt identification annotation. The totality of proteins included in the SomaScan assay was used as the reference set, and both direct and indirect experimentally confirmed relationships from all species were included. The analysis calculates a *P* value (Fisher exact test and right tailed), quantifying the overlap, and a *z* score, quantifying the likelihood and direction (upregulated or downregulated), between the plasma proteomics pattern and known canonical pathways. For Washington University Heart Failure Registry samples, serum samples were analyzed with the SomaScan protein array platform version Plasma_4.2_20161012_1.5k, which included 1306 total analytes, including apo M.

S1P Determination

We measured S1P in a subset of PHFS subjects (n=206). PHFS participants with left ventricular ejection fraction <50% and ischemic cardiomyopathy pathogenesis were matched to participants with nonischemic pathogenesis on the basis of a propensity score that included age, sex, hypercholesterolemia, statin use, and an interaction term between sex and hypercholesterolemia. Matching was performed by means of nearest-neighbor matching, with a caliper width of 0.05 selected for a target sample size of ≈200 using the MatchIt extension package in the R programming environment. Serum samples (10 μL) were diluted with 55 μL Tris-buffered saline (50 mmol/L Tris-HCL, pH 7.5, 0.15 mmol/L NaCl). Precipitation solution (200 μL methanol containing 20 nmol/L internal standard) was added to diluted human serum samples, followed by 30 seconds of vortexing and subsequent centrifugation at 17 000*g* for 2 minutes. Supernatant (5 μL) was injected for liquid chromatography tandem mass spectrometric analysis.[33]

Additional Assays (Inflammatory Biomarkers, Apo AI Measurements, and HDL Particle Isolation)

To confirm the association between apo M and the top canonical pathway identified by pathway analysis (inflammation), we analyzed the relationship between apo M and high-sensitivity C-reactive protein, a well-established marker of inflammation, in the PHFS. We measured high-sensitivity C-reactive protein using standard ARCHITECT immunoassays (Abbott Laboratories, Abbott Park, IL). We also measured various inflammatory biomarkers (TNF, TNF-α receptor I, TNF-α receptor II, interleukin [IL]-1β, IL-6, IL-8, pentraxin 3, and myeloperoxidase) using a Luminex Bead-Based multiplexed assay (Bristol-Myers-Squibb, Ewing Township, NJ).

To discern whether the relationship between apo M and outcomes is independent of its association with HDL cholesterol (HDL-C) or apo AI, we measured apo AI by immunonephelometry among 201 participants, as previously described.[34] HDL-C was measured with the enzymatic colorimetric method.[35]

To assess the correlation between HDL–apo M and HDL-S1P, HDL was isolated from 151 PHFS participants via gradient density ultracentrifugation. Briefly, lipoproteins are separated according to density in sequential ultracentrifugation spins. HDL was isolated by adjusting the density to 1.21 g/mL, as previously described.[36]

Statistical Analysis

Participant characteristics were summarized using mean (SD) for continuous variables with a symmetrical distribution and median (interquartile range) for continuous variables with a skewed distribution. Categorical variables are expressed as counts (percentages).

We stratified the study populations according to tertiles of apo M and compared various clinical characteristics between the strata. We used an ANOVA for symmetrical variables, the Kruskal-Wallis test for skewed variables, and the χ^2 or Fisher exact test, as appropriate, for categorical data. We computed Kaplan-Meier survival curves for tertiles of apo M and compared them with the log-rank test. We assessed the relationship

between apo M and all-cause death and a composite outcome of death, VAD implantation, or heart transplantation. Because VAD implantation and heart transplantation are not part of the standard therapeutic approach to HFpEF, for analyses restricted to participants with HFpEF, we assessed all-cause mortality, as well as the composite outcome of death or HF-related hospitalization, which is increasingly used in HFpEF studies.[37]

We further assessed the relationship between apo M and the risk of outcomes using Cox regression models. To perform unit-independent analyses that can be compared easily between measurement assays and between biomarkers (ie, apo M versus S1P), we express hazard ratios (HRs) per increase of 1 SD (ie, 1-point increase in the *z* score after Box-Cox transformation to improve the normality of data distribution).

We built unadjusted survival models and models that adjusted for confounders, including (1) potential clinical confounders and any characteristics that significantly differed between apo M tertiles and (2) all covariates in adjusted model 2 plus BNP (B-type natriuretic peptide) or NT-proBNP (N-terminal pro-BNP) levels. For analyses in smaller cohorts (ELISA-based apo M measurements in PHFS, stratified analyses in HFpEF versus HFrEF, and analyses in our 2 validation cohorts), we performed unadjusted survival analyses and models adjusted for the following: (1) the MAGGIC (Meta-Analysis Global Group in Chronic Heart Failure) risk score, a single variable that incorporates multiple demographic, clinical, and laboratory parameters,[38] to prevent model overfitting and (2) the MAGGIC risk score plus BNP or NT-proBNP levels. The time of HF diagnosis, one of the components of the MAGGIC risk score, was not available, and no points were given to any subject for this component during the score computation.

To confirm the association between apo M and the top canonical pathway identified by pathway analyses (inflammation), we compared inflammatory biomarkers across tertiles of apo M using an ANOVA. Given the nonnormal distribution of most biomarkers, we compared geometric means (means obtained after log transformation). All values are expressed in the native scale. To assess whether the relationship between apo M and outcomes is dependent on its relationship with inflammation, we built Cox models in which the association with outcomes was assessed with and without adjustment for the inflammatory biomarkers mentioned above to assess the extent to which this adjustment attenuated the relationship between apo M and incident events.

Statistical significance was defined as a 2-tailed value of $P<0.05$. All *P* values presented are 2 tailed. Analyses were performed with the MATLAB statistics and machine learning toolbox (MatLab 2016b, the Mathworks; Natwick, MA) and R Statistical Software version 3.5.2 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

Relationship Between Apo M Measured by ELISA and the Risk of Adverse Outcomes in the PHFS

The general characteristics of PHFS subjects are shown in Table I in the Data Supplement. To test our a priori hypothesis, we measured apo M by ELISA in the PHFS. In this population, mean apo M was 0.92±0.28 µmol/L. During follow-up, 94 deaths occurred, and129 participants reached the composite end point of VAD implantation, heart transplantation, or death. Figure 1 shows Kaplan-Meier survival curves for participants stratified by tertiles of apo M for all-cause death (Figure 1A) and for VAD implantation, heart transplantation, or death (Figure 1B). There was a highly significant difference between the tertiles, with the lowest tertile of apo M (≤0.79 µmol/L) exhibiting the highest risk.

Cox proportional hazard analyses demonstrated that apo M was significantly associated with the risk of death (standardized HR, 0.63 [95% CI, 0.51–0.76]; $P<0.0001$). This association remained significant after adjustment for the MAGGIC risk score (standardized HR, 0.71 [95% CI, 0.56–0.90]; $P=0.0044$). Compared with a base model containing the MAGGIC risk score, the Harrel c index increased from 0.686 to 0.697 for the prediction of death.

As shown in Table II in the Data Supplement, apo M was significantly associated with the risk of VAD implantation, heart transplantation, or death (standardized HR, 0.67 [95% CI, 0.57–0.79]; $P<0.0001$). This association remained significant after adjustment for the MAGGIC risk score (standardized HR, 0.77 [95% CI, 0.63–0.94]; $P=0.0110$). Compared with a base model containing the MAGGIC risk score, the Harrel c index increased from 0.683 to 0.692 for the prediction of VAD implantation, transplantation, and death. Apo M was also associated with the risk of death and the risk of VAD implantation, heart transplantation, or death after further adjustment for BNP.

Prognostic Value of Apo M Versus HDL-C or Apo AI

To assess whether the relationship between apo M and outcomes is independent of its association with HDL-C or apo AI, we built models in which the relationship between apo M and outcomes was assessed with or without adjustment for HDL-C or apo AI (Figures I and II in the Data Supplement). In contrast to apo M, HDL-C was not significantly associated with the risk of death (Figure IA in the Data Supplement) and the risk of VAD implantation, heart transplantation, or death (Figure IB in the Data Supplement). In models that included both apo M and HDL-C, apo M (but not HDL-C) was associated with the risk of death and of VAD implantation, heart transplantation, or death, and its relationship with these outcomes was not attenuated.

In unadjusted analyses, apo AI was significantly associated with the risk of death (Figure IIA in the Data Supplement) or the risk of VAD implantation, heart transplantation, or death. In models that included both apo M and apo AI, apo M was associated with risk of death and the risk of VAD implantation, heart transplantation,

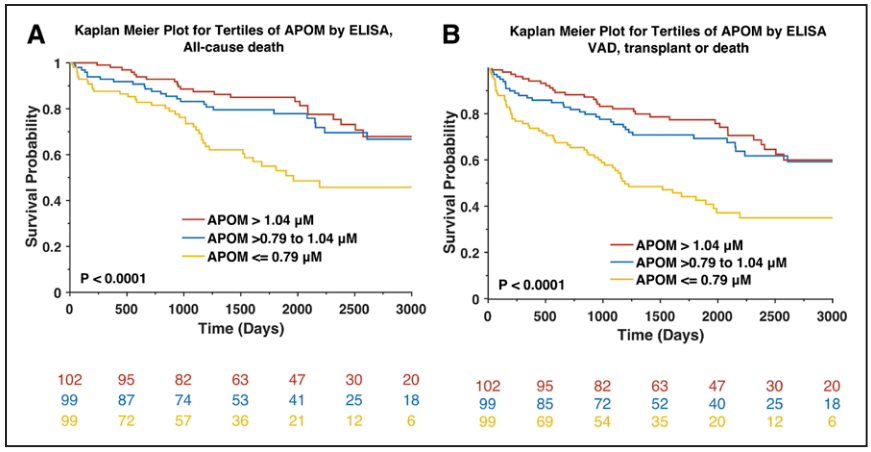

Figure 1. Risk of adverse outcomes among Penn Heart Failure Study participants and tertiles of apo (apolipoprotein) M measured by ELISA.
Kaplan-Meier survival curves for all-cause mortality (**A**) or the composite outcome of death, ventricular assist device (VAD) implantation, or heart transplantation (**B**) are shown. The number of patients at risk at each time point is presented below the graph.

or death, whereas apo AI was not independently associated with these outcomes.

Relationship Between Apo M Measured by a Modified Aptamer Assay and Outcomes in the PHFS

Among subjects with available apo M levels measured by the SomaScan (n=2170), follow-up data were available for 2135 subjects The SomaScan aptamer for apo M was previously validated by mass spectrometry from human serum samples.[29] In the subset of PHFS participants with available ELISA apo M measurements, we found a linear relationship between apo M measured by ELISA and apo M measured by SomaScan, with a Pearson correlation coefficient value of 0.73 (P<0.0001), as shown in Figure III in the Data Supplement. The majority (95%) of apo M is anchored to HDL by its retained signal peptide.[39,40] The correlation between apo M, measured by SomaScan, and HDL-C (r=0.36, P<0.00001) was highly consistent with the previously described association between apo M, measured by ELISA, and HDL-C (r=0.37).[25]

The general characteristics of PHFS subjects stratified by apo M tertiles are shown in Table 1. Lower apo M levels were associated with older age; male sex; higher body mass index; higher serum creatinine; lower blood pressures; ischemic pathogenesis; history of coronary revascularization; diabetes mellitus; history of pacemaker implantation; a lower left ventricular ejection fraction; more advanced New York Heart Association functional class; greater BNP levels; greater use of aspirin, hydralazine and organic nitrates, digoxin, loop diuretics, mineralocorticoid receptor antagonists, and statins; and lower use of lower angiotensin-converting enzyme inhibitors or angiotensin receptor blockers.

During a median follow-up of 5.02 years, 523 participants died and 716 experienced the composite end point of VAD implantation, heart transplantation, or death. Lower apo M levels were significantly associated with an increased risk of death. Kaplan-Meier survival plots for the study population stratified by tertiles of apo M are shown in Figure 2A. Results of unadjusted and adjusted Cox models are shown in Table 2. Each decrease of 1 SD in apo M was associated with nearly a doubling in the mortality risk (standardized HR 0.56 [95% CI, 0.51–0.61]; P<0.0001). In a model that adjusted for age, sex, race, enrollment site, history of percutaneous coronary intervention, coronary artery bypass graft surgery, atrial fibrillation or flutter, pacemaker, biventricular pacer implantation, left ventricular ejection fraction, New York Heart Association class, ischemic versus nonischemic pathogenesis, systolic and diastolic blood pressures, body mass index, history of diabetes mellitus, serum creatinine, and angiotensin-converting enzyme inhibitor/angiotensin receptor blocker, digoxin, hydralazine, loop diuretic, organic nitrate, and statin use, apo M remained significantly associated with the risk of death (standardized HR, 0.73 [95% CI, 0.65–0.81]; P<0.0001). Apo M was also associated with the risk of death after further adjustment for BNP levels (standardized HR, 0.78 [95% CI, 0.69–0.88]; P<0.0001).

Similarly, lower apo M levels were significantly associated with an increased risk of the composite end point of death, VAD implantation, or heart transplantation (standardized HR, 0.62 [95% CI, 0.58–0.67]; P<0.0001; Table 2). Kaplan-Meier survival plots for apo M tertiles are shown in Figure 2B. The association between apo M and the composite end point remained significant after adjustment for multiple potential confounders, clinical risk factors, and BNP (Table 2). Similarly, apo M was also associated with risk of death (standardized HR, 0.80 [95% CI, 0.71–0.91]; P=0.0007) or with the risk of death, VAD implantation, or heart transplantation (standardized HR, 0.88 [95% CI, 0.79–0.99]; P=0.0165) after adjustment for apo AI and apo B.

Interactions With Ischemic Pathogenesis and Key Clinical and Demographic Factors

There was no significant interaction between apo M and ischemic versus nonischemic pathogenesis for

Table 1. General Characteristics of Penn Heart Failure Study Participants, by Tertiles of Apolipoprotein M (n=2170)

Characteristic	Lowest Tertile (n=723)	Middle Tertile (n=724)	Highest Tertile (n=723)	*P* Value
Apolipoprotein M, arbitrary units*	614 (547–665)	804 (761–850)	1027 (962–1134)	…
Age, y*	61.3 (53.2–68.8)	58.3 (48.1–66.1)	53.3 (43.6–62.1)	<0.0001
Male sex, n (%)	525 (72.61)	459 (63.40)	451 (62.38)	<0.0001
Race/ethnicity, n (%)				
White	517 (75.58)	519 (74.78)	544 (77.05)	0.6026
African American	154 (22.51)	157 (22.62)	149 (21.10)	0.7467
Other	13 (1.90)	18 (2.59)	13 (1.84)	0.5551
Body mass index, kg/m^2	30.6 (26.2–36.2)	29.3 (25.2–34.4)	27.5 (24.1–31.1)	<0.0001
Systolic blood pressure, mm Hg	110 (98–125)	114 (100–130)	114 (100–128)	0.0034
Diastolic blood pressure, mm Hg	68 (60–76)	70 (62–78)	70 (62–78)	<0.0001
Ischemic pathogenesis, n (%)	317 (44.27)	204 (28.33)	141 (19.61)	<0.0001
History of percutaneous coronary intervention, n (%)	223 (30.84)	139 (19.20)	106 (14.66)	<0.0001
History of coronary artery bypass graft surgery, n (%)	194 (26.83)	121 (16.71)	77 (10.65)	<0.0001
Current smoking, n (%)	59 (8.16)	64 (8.84)	73 (10.10)	0.4277
Diabetes mellitus, n (%)	317 (43.85)	194 (26.80)	111 (15.35)	<0.0001
Atrial fibrillation or flutter, n (%)	313 (43.29)	261 (36.05)	207 (28.63)	<0.0001
History of pacemaker, n (%)	61 (8.44)	42 (5.80)	34 (4.70)	0.0111
History of implantable cardioverter-defibrillator, n (%)	162 (22.41)	140 (19.34)	154 (21.30)	0.3487
History of biventricular pacer, n (%)	216 (29.88)	173 (23.90)	118 (16.32)	<0.0001
Serum creatinine, mg/dL	1.3 (1–1.8)	1.1 (0.97–1.41)	1.01 (0.9–1.3)	<0.0001
Left ventricular ejection fraction, %	25 (20–40)	30 (20–45)	35 (20–45.8)	0.0003
Ejection fraction category, n (%)				
Reduced ejection fraction	507 (82.84)	534 (81.65)	525 (79.31)	0.2555
Recovered ejection fraction	52 (8.50)	71 (10.86)	80 (12.08)	0.1076
Preserved ejection fraction	53 (8.66)	49 (7.49)	57 (8.61)	0.6888
New York Heart Association class, n (%)				<0.0001
I	69 (9.69)	129 (17.92)	176 (24.38)	
II	343 (48.17)	465 (64.58)	534 (73.96)	
III	365 (51.26)	349 (48.47)	345 (47.78)	
IV	142 (19.94)	164 (22.78)	195 (27.01)	
B-type natriuretic peptide, pg/mL	345 (100–1040)	158 (49–530)	91 (26–283)	<0.0001
Medication use, n (%)				
β-Blocker	649 (89.76)	649 (89.64)	625 (86.45)	0.0790
Aspirin	482 (66.67)	412 (56.91)	340 (47.03)	<0.0001
Angiotensin-converting enzyme inhibitors/ angiotensin receptor blockers	597 (82.57)	626 (86.46)	633 (87.55)	0.0182
Hydralazine	91 (12.59)	67 (9.25)	25 (3.46)	<0.0001
Organic nitrates	157 (21.72)	130 (17.96)	56 (7.75)	<0.0001
Digoxin	294 (40.66)	263 (36.33)	217 (30.01)	0.0001
Loop diuretic	598 (82.71)	515 (71.13)	411 (56.85)	<0.0001
Mineralocorticoid receptor antagonist	264 (36.51)	251 (34.67)	224 (30.98)	0.0777
Statin	454 (62.79)	379 (52.35)	294 (40.66)	<0.0001
Calcium channel blockers	58 (8.02)	80 (11.05)	62 (8.58)	0.1056

*Interquartile range for continuous variables; 95% confidence interval for categorical variables.

death, death or heart failure–related hospitalization, or the composite of death, VAD implantation, or heart transplantation (Table III in the Data Supplement). Accordingly, apo M was associated with these outcomes in both ischemic and nonischemic HF (Table IV in the Data Supplement).

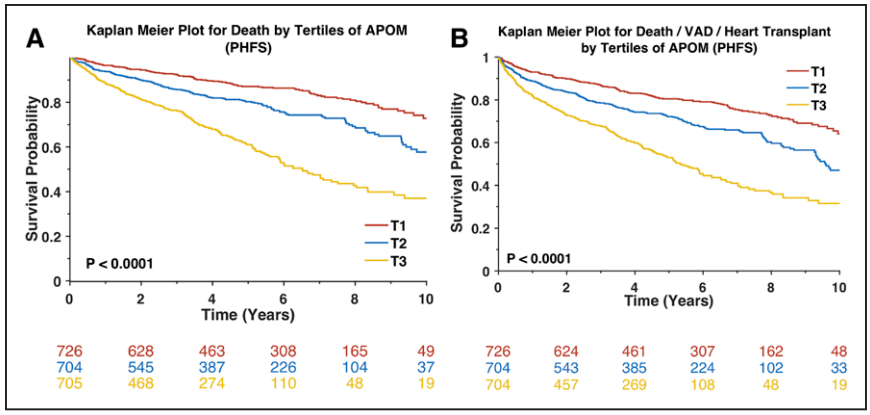

Figure 2. Risk of adverse outcomes among PHFS (Penn Heart Failure Study) participants stratified by tertiles (Ts) of apo (apolipoprotein) M.
Kaplan-Meier survival curves for all-cause mortality (**A**) or the composite outcome of death, ventricular assist device (VAD) implantation, or heart transplantation (**B**) are shown. The number of patients at risk at each time point is presented below the graph.

Although we observed associations between apo M protein levels and age, sex, diabetes mellitus, and renal function, we did not observe any significant interaction between these variables, apo M, and HF outcomes. There was a significant interaction between apo M and African American ethnicity for the outcome of death (*P* for interaction=0.0153) and VAD implantation, heart transplantation, or death (*P* for interaction=0.004). Among African Americans, the HR for death was 0.67 (95% CI, 0.56–0.80; *P*<0.0001), whereas among nonblacks, the HR was 0.53 (95% CI, 0.48–0.59; *P*<0.0001). Similarly, among African Americans, the HR for VAD implantation, heart transplantation, or death was 0.73 (95% CI, 0.62–0.86; *P*=0.0001), whereas among nonblacks, the HR was 0.59 (95% CI, 0.55–0.65).

Analyses Stratified by HFrEF Versus HFpEF

In analyses restricted to subjects with HFrEF (n=1761; Table 3), apo M was associated with death (standardized HR, 0.57 [95% CI, 0.52–0.62]; *P*<0.0001) and the composite end point of death, VAD implantation, or heart transplantation (standardized HR, 0.62 [95% CI, 0.51–0.76]; *P*<0.0001). These associations remained after adjustment for the MAGGIC risk score and adjustment for both the MAGGIC risk score and BNP (Table 3).

In analyses restricted to subjects with HFpEF (n=249), apo M was inversely associated with death. Kaplan-Meier survival plots for this subset of participants stratified by tertiles of apo M are shown in Figure 3A. Each decrease of 1 SD in apo M was associated with a >2-fold increase in mortality risk (standardized HR, 0.44 [95% CI, 0.34–0.57]; *P*<0.0001). Apo M was also associated with the composite end point of death or HF-related hospitalization (standardized HR, 0.62 [95% CI, 0.50–0.75]; *P*<0.0001). Figure 3B shows Kaplan-Meier survival plots for the composite end point of death or HF-related hospitalization among this subset of participants stratified by tertiles of apo M. Among participants with HFpEF, apo M was also associated with these end points independently of the MAGGIC risk score and BNP (Table 3).

Table 2. Hazard Ratios for Apo M Measured by Modified-Aptamer Assay in the Penn Heart Failure Study (n=2135)

Apo M Measured by Modified Aptamer Assay (n=2135)		
Model	**Hazard Ratios**	***P* Value**
All-cause death (n=523)		
Unadjusted	0.56 (0.51–0.61)	<0.0001
Adjusted for clinical factors*	0.73 (0.65–0.81)	<0.0001
Adjusted for clinical factors plus B-type natriuretic peptide	0.78 (0.69–0.88)	<0.0001
Death/ventricular assist device implantation/heart transplantation (n=716)		
Unadjusted	0.62 (0.58–0.67)	<0.0001
Adjusted for clinical factors	0.79 (0.72–0.87)	<0.0001
Adjusted for clinical factors plus B-type natriuretic peptide	0.85 (0.76–0.94)	0.0014

*Clinical factors included in the model were age, sex, race, enrollment site, history of percutaneous coronary intervention, coronary artery bypass graft surgery, atrial fibrillation or flutter, pacemaker, biventricular pacer implantation, left ventricular ejection fraction, New York Heart Association class, ischemic vs nonischemic pathogenesis, systolic and diastolic blood pressures, body mass index, history of diabetes mellitus, serum creatinine, and angiotensin-converting enzyme inhibitor/angiotensin receptor blocker, digoxin, hydralazine, loop diuretic, organic nitrate, and statin use. HR per 1 SD increase in apo M.

Replication in the Washington University Heart Failure Registry

The general characteristics of Washington University Heart Failure Registry participants are shown in Table V in the Data Supplement. During a follow-up of 2 years, 21 deaths occurred, and 29 participants reached the composite outcome of death, left VAD implantation, or heart transplantation. In this cohort, lower apo M levels were significantly associated with an increased risk of death (Table VI in the Data Supplement). Similar to findings in our primary cohort, each decrease of 1 SD in apo M was associated with nearly a doubling in the mortality risk (standardized HR, 0.54 [95% CI, 0.34–0.84]; *P*=0.0064). Apo M was also associated with the composite end point of death, VAD implantation, or heart transplantation (standardized HR, 0.60 [95% CI, 0.41–0.87]; *P*=0.0077). Apo M remained associated with these outcomes after adjustment for the MAGGIC

Table 3. Hazard Ratios for Apolipoprotein M in HFrEF and HFpEF

Model	Standardized Hazard Ratio	95% CI,Lower Bound	95% CI,Upper Bound	*P* Value
Heart failure with reduced ejection fraction (n=1761)				
All-cause death (n=474)				
Unadjusted	0.57	0.52	0.62	<0.0001
Adjusted for the MAGGIC risk score	0.75	0.67	0.83	<0.0001
Adjusted for the MAGGIC risk score plus B-type natriuretic peptide	0.74	0.66	0.82	<0.0001
Death/ventricular assist device implantation/heart transplantation (n=672)				
Unadjusted	0.62	0.51	0.76	<0.0001
Adjusted for the MAGGIC risk score	0.81	0.74	0.89	<0.0001
Adjusted for the MAGGIC risk score plus B-type natriuretic peptide	0.81	0.74	0.90	<0.0001
Heart failure with preserved ejection fraction (n=249)				
All-cause death (n=58)				
Unadjusted	0.44	0.34	0.57	<0.0001
Adjusted for the MAGGIC risk score	0.61	0.43	0.85	0.0035
Adjusted for the MAGGIC risk score plus B-type natriuretic peptide	0.52	0.35	0.77	0.001
Death or heart failure hospitalization (n=110)				
Unadjusted	0.62	0.50	0.75	<0.0001
Adjusted for the MAGGIC risk score	0.68	0.53	0.88	0.0034
Adjusted for the MAGGIC risk score plus B-type natriuretic peptide	0.72	0.55	0.94	0.0162

MAGGIC indicates Meta-Analysis Global Group in Chronic Heart Failure. HR per 1 SD change in apo M.

risk score and after adjustment for the MAGGIC risk score and BNP (Table VI in the Data Supplement).

Replication in the TOPCAT Trial

The general characteristics of TOPCAT trial participants included in this analysis are shown in Table VII in the Data Supplement. During a follow-up of 3.42 years, 48 deaths occurred, and 77 participants reached the composite outcome of death or HF-related hospitalization (Table VI in the Data Supplement). In this cohort, lower apo M levels were significantly associated with an increased risk of death (standardized HR, 0.76 [95% CI, 0.58–0.99]; *P*=0.0419) and an increased risk of the composite end point of death/HF-related hospital admission (standardized HR, 0.65 [95% CI, 0.51–0.82]; *P*=0.0002). Apo M remained associated with these outcomes after adjustment for the MAGGIC risk score and after adjustment for the MAGGIC risk score and BNP (Table VI in the Data Supplement).

Apo M, S1P, and HF Outcomes

Figure VI in the Data Supplement shows the correlation between HDL-associated apo M and S1P among PHFS participants (*R*=0.81, *P*<0.0001). Serum S1P levels were

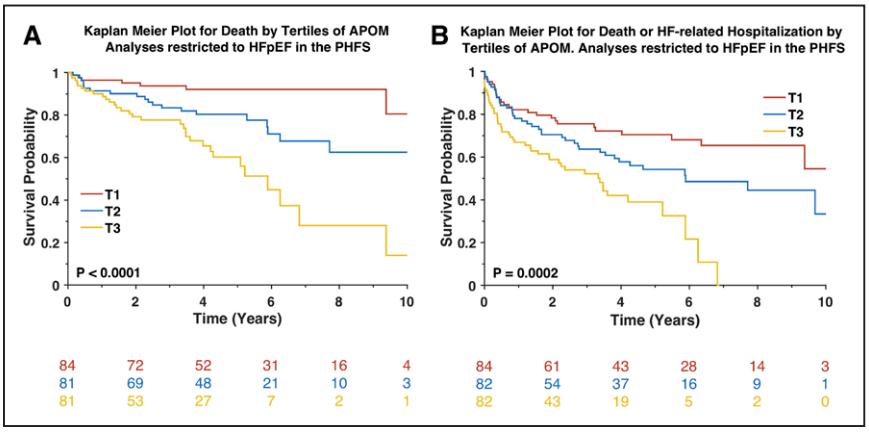

Figure 3. Risk of adverse outcomes among PHFS (Penn Heart Failure Study) participants with heart failure (HF) with preserved ejection fraction (HFpEF) stratified by tertiles (Ts) of apo (apolipoprotein) M. Kaplan-Meier survival curves for all-cause mortality (**A**) or the composite outcome of death or heart failure–related hospitalization (**B**) are shown. The number of patients at risk at each time point is presented below the graph.

associated with death (standardized HR, 0.65 [95% CI, 0.49–0.85]; *P*=0.0021). This association remained after adjustment for the MAGGIC risk score (standardized HR, 0.70 [95% CI, 0.53–0.93]; *P*=0.0129).

There was a weak but significant correlation between S1P and apo M (*R*=0.25, *P*=0.00038). In a model that included both apo M and S1P, both were associated with death (standardized HR for apo M, 0.70 [95% CI, 0.54–0.90], *P*=0.0051; standardized HR for S1P, 0.75 [95% CI, 0.57–0.98], *P*=0.0361). There was a significant interaction between S1P and apo M (*P*=0.0355), indicating that their association with mortality risk was more pronounced at lower levels of apo M and S1P.

Accordingly, among participants with S1P levels below 50th percentile, apo M was associated with death (standardized HR, 0.64 [95% CI, 0.47–0.88]; *P*=0.006), whereas among those ≥50th percentile for S1P, apo M was not significantly associated (standardized HR, 0.71 [95% CI, 0.48–1.07]; *P*=0.10). Similarly, among participants with apo M levels <50th percentile, S1P was significantly associated with death (standardized HR, 0.61 [95% CI, 0.47–0.80]; *P*=0.0004), whereas among those ≥50th percentile for apo M, S1P was not significantly associated with mortality (standardized HR, 0.53 [95% CI, 0.53–1.08]; *P*=0.12).

Pathway Analysis

The top canonical pathways associated with apo M are shown in Figure 4 and listed in Table VIII in the Data Supplement. The results of regression analyses between apo M and all other proteins, used for pathway analysis, can be accessed in the Data Supplement. Apo M protein levels were found to be negatively associated with inflammatory pathways, coagulation pathways, and a number of other biologically plausible pathways, including renin-angiotensin signaling. The top pathways positively associated with apo M plasma protein were the liver X receptor and complement pathways.

We confirmed the association between apo M and the top canonical pathway (inflammation) using inflammatory biomarker levels measured by independent assays. Figure V in the Data Supplement shows a comparison of various inflammatory biomarkers between tertiles of apo M in the PHFS. Lower levels of apo M were associated with higher levels of multiple

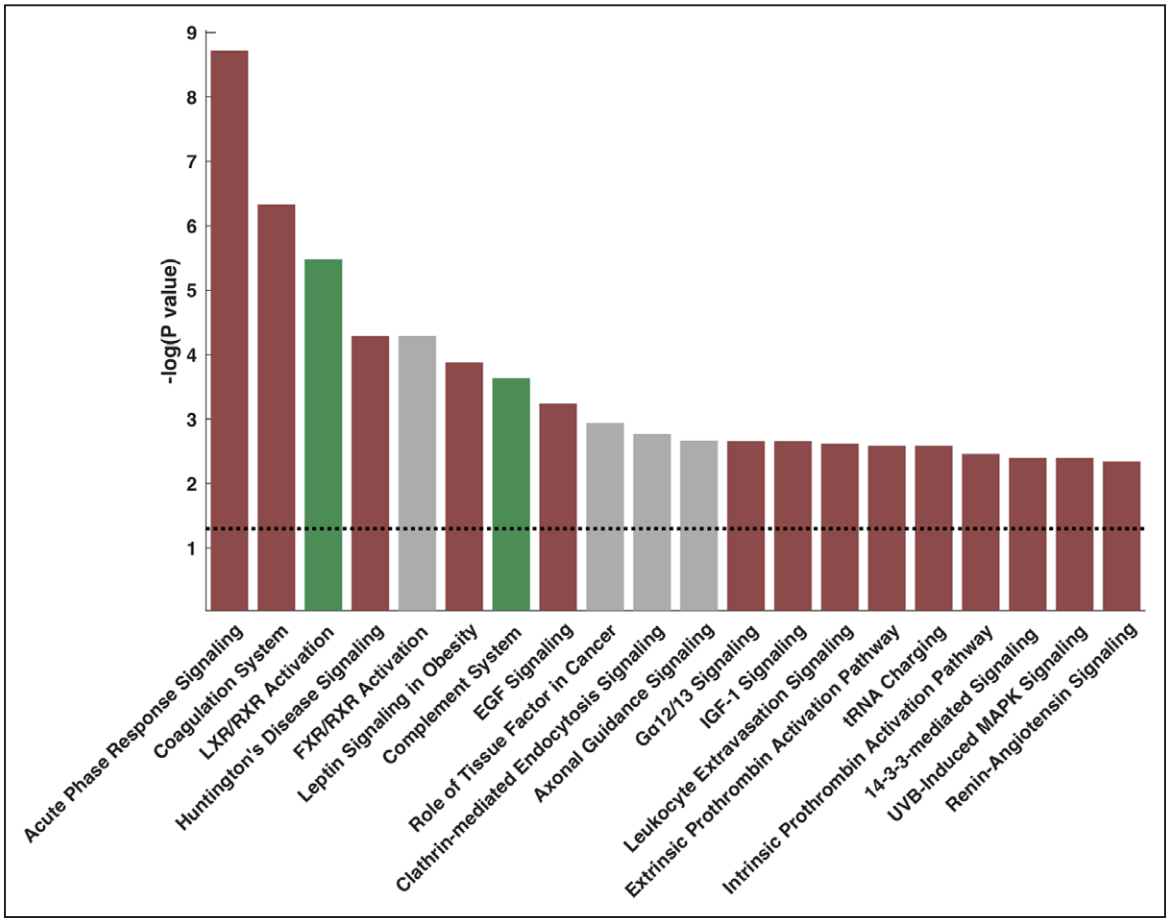

Figure 4. Top 20 canonical pathways associated with apolipoprotein M (apo M).
Red bars indicate negative *z* scores (negative correlations). Green bars indicate positive *z* scores (ie, positive correlations). Gray bars denote pathways in which there is significant overlap with apo M, but the directionality of the relationship is unclear. EGF indicates epidermal growth factor; FXR, farnesoid X receptor; IGF-1, insulin-like growth factor 1; LXR, liver X receptor; MAPK, mitogen-activated protein kinase; RXR, retinoid X receptor; and UVB, ultraviolet B.

inflammatory biomarkers, including TNF-α, TNF-α receptor I, TNF-α receptor II, IL-6, IL-8, pentraxin-3, myeloperoxidase, and high-sensitivity C-reactive protein.

To assess the degree to which the relationship between apo M and outcomes may depend on inflammation, we constructed survival models in which the relationship between apo M and outcomes was compared with versus without adjustment for all the inflammatory biomarkers shown in Figure IV in the Data Supplement. In general, adjustment for these inflammatory biomarkers only partially attenuated the relationship between apo M and outcomes, suggesting that both inflammation and inflammation-independent factors underlie this relationship.

DISCUSSION

We demonstrate that reduced apo M plasma protein levels are associated with the risk of adverse outcomes across the spectrum of human HF by ELISA and SomaScan in the PHFS, a large cohort study performed at 3 academic centers with many years of follow-up and a large number of adjudicated events. In this cohort, we demonstrate that apo M is associated with adverse outcomes in the overall population and in analyses restricted to HFpEF and HFrEF. We validated our findings in 2 independent cohorts, including a mixed HF cohort (Washington University Heart Failure Registry) and an HFpEF-only cohort (TOPCAT). The use of multiple cohorts across multiple centers and different methods to measure apo M provides convincing evidence that reduced levels of circulating apo M protein are independently associated with adverse outcomes (including increased mortality) in both HFpEF and HFrEF. Our findings are novel and provide information that, interpreted in the context of accumulating mechanistic animal data, supports a role for apo M in human HF.

What are the mechanisms by which apo M may promote improved outcomes in human HF? Apo M is a chaperone for S1P, a sphingolipid that activates G-protein–coupled receptors and the phosphoinositide 3-kinase signaling pathway.[14,39] Animal studies suggest that apo M mediates S1P signaling to promote anti-inflammatory effects, survival of cardiomyocytes, and improved endothelial function[10,12,13] (Figure 5). We measured S1P by liquid chromatography tandem mass spectrometry and observed an inverse association between S1P concentration and survival. We also observed a significant interaction between S1P and apo M such that their relationship with outcomes was more pronounced when levels of both were reduced. Apo M may target S1P to endothelial receptors to reduce endothelium-leukocyte adhesion.[12] Alternatively, apo M/S1P may restrain lymphopoiesis via direct effects on inflammatory cells.[40] Further study of the mechanisms by which apo M mediates cardioprotection will be a crucial step forward in both apo M biology and therapeutics targeting this pathway, particularly because S1P receptors are expressed on many cell types and have a multitude of effects. Increasing apo M may bias S1P

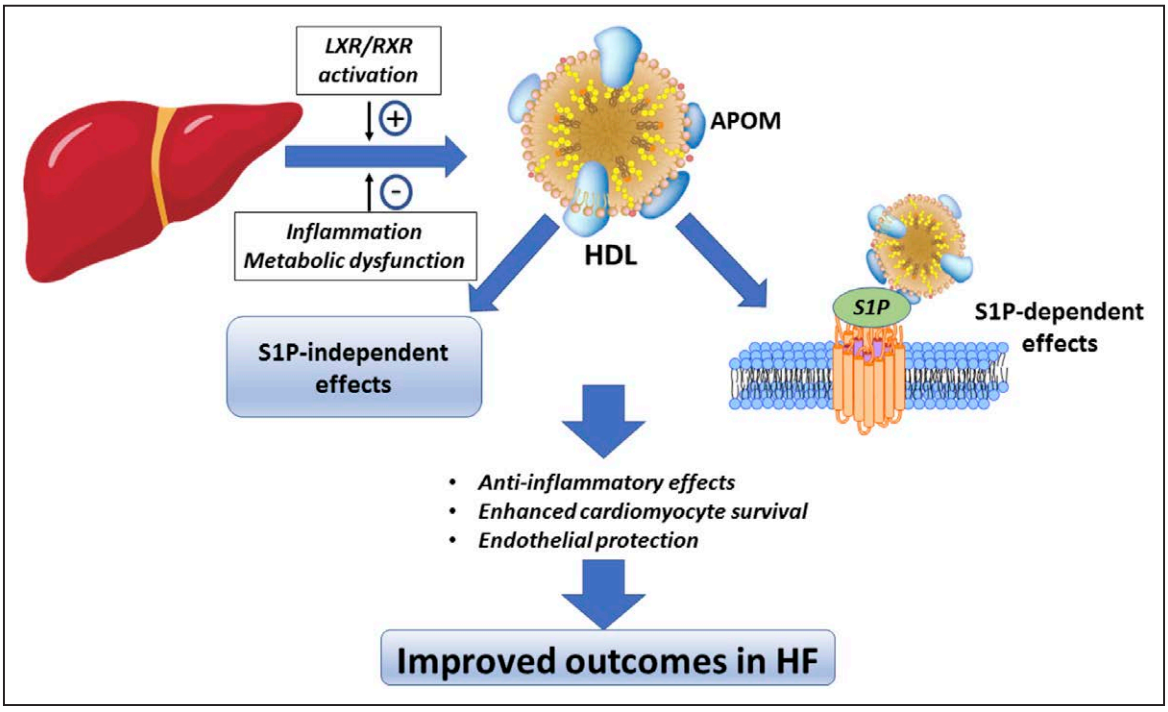

Figure 5. Proposed protective effects of apolipoprotein M (apo M) in heart failure (HF).
Apo M is associated with high-density lipoprotein (HDL) and binds sphingosine-1-phosphate (S1P). S1P signaling enhances cardiomyocyte survival, activates endothelial protective pathways, and is anti-inflammatory. The culmination of these effects may result in improved survival in heart failure. However, whether the relationship between apo M and outcomes is causal remains to be determined. LXR/RXR indicates liver X receptor/retinoid X receptor.

signaling, allowing preferential activation of endothelial, anti-inflammatory, and cardioprotective pathways while avoiding the potential toxicities of indiscriminately increasing S1P. Furthermore, the recent development of apo M peptides highlights the therapeutic potential of directly targeting apo M.[11] The relationship between apo M and death was not fully dependent on the levels of S1P; apo M remained significantly associated with death after adjustment for S1P levels. We found that apo M protein levels are associated with multiple markers of baseline disease status; our pathway analyses suggest a link between apo M and inflammation. Whether the anti-inflammatory effects of apo M are direct or related to the role of apo M in modulating endothelial protection will be the subject of future studies.

Apo M may also link hepatic and lipoprotein metabolism with HF outcomes. Our pathway analyses are consistent with a positive relationship between apo M and the liver X receptor ligand–activated transcription factors, which have been implicated in metabolic homeostasis, inflammation, and hepatic disease but also HF.[41] Although liver X receptor agonism has been suggested to decrease transcription of apo M in vitro, more recent studies suggest a more nuanced interpretation of these initial findings.[42] In theory, liver X receptor agonism could contribute to apo M stability via an effect on apo B–containing lipoproteins, which may decrease apo M clearance.[43,44] In patients with advanced HF, factors including inflammation, metabolic disease, hepatic dysfunction, and reduced lipoprotein levels could contribute to further reductions in apo M secretion or increases in clearance, creating a feed-forward mechanism leading to progressive mortality.

Our findings are relevant to observations linking HDL subsets to adverse outcomes in HF[45] and prior studies demonstrating that reduced circulating lipoproteins are associated with increased mortality risk in patients with HF.[46] There are multiple potential mechanisms by which HDL may play a future role in the treatment of HF. Rodent models support the use of reconstituted HDL[47–49] in HF models but also chimeric apo M peptides that are not HDL associated[11] to prevent cardiac ischemic injury. To account for possible confounding resulting from reductions in lipoprotein levels or apo AI, we adjusted our analyses for HDL-C and apo AI, the major protein constituent of HDL. Although our results support prior findings that reduced levels of apo AI are associated with HF mortality,[50] we observed an independent association between apo M protein levels and all-cause mortality. Mechanistically, apo AI interacts with scavenger receptor BI, the loss of which has been implicated in HF pathogenesis in rodent models.[51] Further basic research must explore the interactions of apo M with apo AI and scavenger receptor class B type I, whether apo M requires apo AI or scavenger receptor class B type I, or whether there might be a synergistic effect of increasing both apo M and apo AI. These questions will need to be tested in preclinical rodent models. Although the possibility remains that apo M is a marker of improved HDL function or improves HDL functionality by an indirect mechanism, increasing apo M may be one mechanism of HDL-mediated cardioprotection.

Our study should be interpreted in the context of its strengths and limitations. Our study was focused on the role of apo M in the prognosis of patients with existing HF; whether apo M is associated with incident HF incidence is a separate question. Second, we did not intend to compare apo M across cohorts; this is a technical limitation as well because the aptamer-specific fluorescence signal depends on the characteristics and the number of other aptamers in the assay, which in turn vary with specific versions of the SomaScan. We cannot rule out that both ELISA and SomaScan measurements of apolipoproteins may be affected by unmeasured biological confounders; nonetheless, the consistent directionality of both assays provides a high degree of confidence in our results. Despite comprehensive adjustments to the extent possible, we cannot rule out residual confounding. Our analyses adjusted for the presence of diabetes mellitus but not for hemoglobin A_{1c} levels (which were not available in these cohorts). Our analyses did not discern the relationship between apo M and cardiovascular versus noncardiovascular death. Because our study is observational in nature, we cannot rule out that apo M and S1P may be inversely associated with increased mortality but not causally related to it, a hypothesis that should be strictly tested with interventions designed to increase apo M.

In addition, our studies were performed in a large number of patients within a well-characterized primary cohort with long-term follow-up and a large number of well-adjudicated events and 2 independent secondary cohorts for validation of our findings for apo M and outcomes. We also convincingly demonstrate that the relationship between apo M and outcomes is present in both HFpEF and HFrEF. After using ELISA to determine that reductions in apo M were associated with worse outcomes in PHFS, we subsequently used SomaScan analyses from 3 separate cohorts at different time points and different institutions. Furthermore, we directly measured S1P to better discern the relationships among apo M, S1P, and mortality and demonstrated that in patients with HF HDL–apo M is associated with HDL-S1P. Last, we performed pathway analysis using a broad proteomic scan, revealing relationships between apo M and specific biological pathways. The mechanistic underpinnings and high biological plausibility, based on animal and preclinical data, increase the relevance and generalizability of our findings. Although our work is an important first step, further insights from human genetics, animal studies, and randomized clinical trials will be required to gain further insights into the

mechanisms by which apo M is associated with mortality in human HF.

CONCLUSIONS

We have identified that reduced circulating apo M protein levels are associated with an increased risk of death across the spectrum of human HF. The apo M/S1P axis merits further exploration as a therapeutic target in patients with HF.

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Supplemental Materials

Data Supplement Figures I–VI
Data Supplement Tables I–VIII
Data Supplement Excel File

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