Version of Record: https://www.sciencedirect.com/science/article/pii/S1874391922003128
Manuscript_cae58745a9d8c7978dba7b9ba773b957

A Proteomic Approach for Investigating the Pleiotropic Effects of Statins in the Atherosclerosis Risk in Communities (ARIC) Study

Authors:
Bruno Bohn[1], Pamela L. Lutsey[1], Weihong Tang[1], James S. Pankow[1], Faye L. Norby[2], Bing Yu[3], Christie M. Ballantyne[3], Eric A. Whitsel[4], Kunihiro Matsushita[5], Ryan T. Demmer[1]

Affiliations:
1. Division of Epidemiology and Community Health, University of Minnesota School of Public Health; 2. Department of Cardiology, Smidt Heart Institute, Cedars-Sinai Health System, Los Angeles, CA; 3. Baylor College of Medicine; 4. Department of Epidemiology, Gillings School of Public Health and Department of Medicine, University of North Carolina - Chapel Hill, NC; 5. Department of Epidemiology, Johns Hopkins Bloomberg School of Public Health.

Corresponding Author:
Ryan Demmer, PhD MPH FAHA
demm0009@umn.edu
300 West Bank Office Building
1300 S 2nd St
Minneapolis, MN, 55454

Funding:
The Atherosclerosis Risk in Communities study has been funded in whole or in part with Federal funds from the National Heart, Lung, and Blood Institute (NHBLI), National Institutes of Health (NIH), Department of Health and Human Services [Contract nos. HHSN268201700001I, HHSN268201700002I, HHSN268201700003I, HHSN268201700004I, HHSN268201700005I]. This work was also supported in part by NIH/NHLBI grants R01 HL134320 (Ballantyne) and K24 HL159246 (Lutsey). SomaLogic Inc. conducted the SomaScan assays in exchange for use of ARIC data.

Acknowledgements
The authors thank the staff and participants of the ARIC study for their important contributions. The Atherosclerosis Risk in Communities study has been funded in whole or in part with Federal funds from the National Heart, Lung, and Blood Institute, National Institutes of Health, Department of Health and Human Services [Contract nos. HHSN268201700001I, HHSN268201700002I, HHSN268201700003I, HHSN268201700004I, HHSN268201700005I]. The authors thank the staff and participants of the ARIC study for their important contributions. SomaLogic Inc. conducted the SomaScan assays in exchange for use of ARIC data. This work was supported in part by NIH/NHLBI grant R01 HL134320.

A Proteomic Approach for Investigating the Pleiotropic Effects of Statins in the Atherosclerosis Risk in Communities (ARIC) Study

Abstract

Background: Statins are prescribed to reduce LDL-c and risk of CVD. Statins have pleiotropic effects, affecting pathophysiological functions beyond LDL-c reduction. We compared the proteome of statin users and nonusers (controls). We hypothesized that statin use is associated with proteins unrelated to lipid metabolism.

Methods: Among 10,902 participants attending ARIC visit 3 (1993-95), plasma concentrations of 4,955 proteins were determined using SOMAlogic's DNA aptamer-based capture array. 379 participants initiated statins within the 2 years prior. Propensity scores (PS) were calculated based on visit 2 (1990-92) LDL-c levels and visit 3 demographic/clinical characteristics. 360 statin users were PS matched to controls. Log2-transformed and standardized protein levels were compared using t-tests, with false discovery rate (FDR) adjustment for multiple comparisons. Analyses were replicated in visit 2.

Results: Covariates were balanced after PS matching, except for higher visit 3 LDL-c levels among controls (125.70 vs 147.65 mg/dL; $p<.0001$). Statin users had 11 enriched and 11 depleted protein levels after FDR adjustment ($q<.05$). Proteins related and unrelated to lipid metabolism differed between groups. Results were largely replicated in visit 2.

Conclusion: Proteins unrelated to lipid metabolism differed by statin use. Pending external validation, exploring their biological functions could elucidate pleiotropic effects of statins.

Introduction

Statins are the first line pharmacotherapy intervention for lowering low-density lipoprotein cholesterol (LDL-c) for the prevention of atherosclerotic cardiovascular disease (ASCVD), with high intensity statin therapy expected to reduce LDL-c by over 50%[1]. In 2012, an estimated 26% of US adults aged 40 and over used statins[2]. In addition to LDL-c reduction, statins have pleiotropic effects spanning many biological pathways and systems. The mechanisms behind the pleiotropic effects of statins are broadly categorized as lipid-dependent (i.e, directly linked to LDL-c synthesis or its removal from circulation) or lipid-independent[3–5], and may vary according to statin type and dosage[4].

Statin use has been hypothesized to directly and indirectly affect a variety of biosynthetic pathways and biological processes, including, cell signaling and functioning, gene expression, and protein synthesis and post-translational modification[4,5]. Through their effects on these many processes, statins have been shown to have a broad impact on human pathophysiology, including in modulation of inflammation and inflammatory cell response, endothelial functioning, nitric oxide (NO) synthesis, and atherosclerotic plaque formation and stability[4,5]. However, no study has looked broadly at the influence of statins on the human circulating proteome. Doing so might help to further elucidate the beneficial effects of statin therapy on ASCVD as well as the effects of the medication on other organ systems, diseases, and biophysiological pathways.

The presented study seeks to investigate differences in protein level expression among statin users versus matched non-users, utilizing the Atherosclerosis Risk in Communities (ARIC) Study SomaScan data. These data provide a resource to enhance understanding the influence of statins on biological pathways more broadly. The objective of this analysis is to characterize differences in human proteome between statin users versus non-users.

Methods

Study Population

ARIC is an ongoing community-based prospective cohort study in the United States[6]. Enrollment began in 1987 in Washington County, Maryland, suburbs of Minneapolis, Minnesota, Jackson, Mississippi, and Forsyth County, North Carolina. Data for the present analysis arise from participants enrolled at visit 1 (baseline: 1987-1989; n = 15,792; ages 45 to 64 years) who returned for visit 2 (1990-1992; n = 14,438) and visit 3 (1993-1995; n = 12,887). All visits included clinical exams and laboratory measurements. Participants were asked to bring all medications and supplements they had taken in the prior 2 weeks to each clinic visit; medication names and dosages were transcribed and coded. Institutional review boards at each individual center approved the study research protocol and all participants provided informed consent.

A diagram summarizing sampling for the present analysis is shown in **Figure 1**. A total of 15,792 participants were enrolled in visit 1. Among these, 104 were excluded due to lack of representativity across race groups and study centers – a standard approach in ARIC data analyses. 14,348 subjects participated in visit 2 and 12,887 subjects participated in visit 3. Our focus was on "new users", defined as individuals who initiated statins between two consecutive visits, in an effort to emulate a clinical trial[7,8]. Therefore, for the primary analytical cohort, we also excluded participants who were on statins at visit 2 or whose visit 2 or visit 3 statin use was unknown (N=570). Additionally, we excluded participants without visit 3 proteomics data (N=1,335) and those missing data on any covariates accounted for in propensity score calculation (N=913), resulting in a final primary cohort of 9,989 participants, with 379 being new statin users.

A replication analyses was conducted utilizing visit 2 proteomics data. Among 14,348 subjects who participated in visit 2, we excluded those who reported statin use at visit 1 or whose statin use at visit 1 or visit 2 (N=318). Additionally, we excluded participants without visit 2 proteomics data (N=2,496) and those missing data on any covariates accounted for in propensity score calculation (N=736), resulting in a final replication cohort of 10,798 participants, with 234 being new statin users.

Proteomics Data

Participant protein levels were determined from fasting blood plasma samples collected on visit 2 and visit 3[9]. Blood samples were centrifuged at room temperature within 10 minutes from collection, aliquoted, and stored at -80°C. Plasma proteins concentrations were quantified utilizing a multiplexed modified DNA-based aptamer technology (SOMAscan assay). Briefly, protein concentrations were converted to matched aptamers, which were then quantified in relative fluorescence units utilizing a DNA microarray technique[9]. Measurements are standardized and normalized utilizing the SOMAscan approach, which includes hybridization control normalization, plate scaling, within-plate median signal normalization, and plate-to-plate calibration through the use SOMAmer reagent calibration samples. Protein

levels were further log-2 transformed to reduce skewness and enhance normality. A total of 4,955 proteins which met QC criterion in visit 2 and visit 3 were included in this analysis.

Statistical Analysis

Statin users were matched 1:1 to non-users (controls) utilizing propensity score (PS) matching with a nearest-neighbor algorithm to minimize confounding by indication, utilizing the R package *MatchIt*. For the primary analyses, PS was determined from sex, race/study center (white MN, white MD, white NC, black NC, black MS), and education level (basic, intermediate, advanced) determined at visit 1, LDL-c levels at visit 2, age, smoking status, body mass index, serum creatinine eGFR, systolic blood pressure, diastolic blood pressure, high density lipoprotein cholesterol (HDL), use of antihypertensive medications, use of non-statin cholesterol lowering/affecting medications, and prevalence of hypertension, diabetes, coronary heart disease, stroke, hypertension, heart failure and myocardial infarction at visit 3. Statin users without a suitable match, defined utilizing a caliper of 0.1 standard deviations of the PS, were excluded from analysis (N = 19).

A similar approach was taken to create a replication matched cohort. PS was determined from sex, race/study center, education level (basic, intermediate, advanced) and LDL-c levels at visit 1, and age, smoking status, body mass index, serum creatinine eGFR, systolic blood pressure, diastolic blood pressure, high density lipoprotein cholesterol (HDL), use of antihypertensive medications, use of non-statin cholesterol lowering/affecting medications, and prevalence of hypertension, diabetes, coronary heart disease, stroke, hypertension, heart failure and myocardial infarction at visit 2. Statin users without a suitable match, defined utilizing a caliper of 0.1 standard deviations of the PS, were excluded from analysis (N = 9).

Statistical analysis was conducted in R (Version 4.0.2). Distribution of covariates between statin users and non-users (controls) were reported and significance of the differences between groups were determined through two-sided t-tests for continuous variables and chi-squared tests for categorical variables. After propensity score matching, simple linear regression models were utilized to compare mean levels of each detected protein between statin users and controls. A false discovery rate (FDR) was utilized to account for multiple comparisons. An analysis of visit 3 protein levels adjusted for visit 2 levels was conducted including those in the primary matched cohort who also had visit 2 proteomics data (N = 642).

Network pathway analysis with Ingenuity Pathway Analysis (IPA; QIAGEN Inc.)[10] was performed to further explore proteins found to be significantly associated with statin use. All proteins found to differ between statin users and controls in the main analysis (visit 3, unadjusted) with a FDR corrected q-value below 0.05 were included in this analysis. The IPA Core Analyses was used to investigate canonical pathways based on these proteins, with a significance threshold of 0.05.

Results

Primary Analysis: Matched Cohort

A total of 9,989 participants were eligible for inclusion in the primary analysis. Of these, 379 (3.79%) participants initiated statin use between visit 2 and visit 3. A description of the cohort prior to matching is shown in **Supplemental Table 1**. After PS matching, 360 statin users were matched to an equal number of nonuser controls. Matched cohort characteristics are summarized in **Table 1**. Overall participants had the mean age of 60.78 (SD=5.53) years, with a small majority of females (55.42%). A majority of participants were white (88.61%). At the time of visit 3, half of participants reported current use of alcohol (51.67%) and only 13.06% identified as current smokers. Over half of the participants suffered from hypertension (51.81%) and 48.06% were on antihypertensive medication. By this time point, prevalence of CHD was 23.75%, stroke was 2.50%, HF was 7.78%, MI was 20.14%, and diabetes was 25.69%. Half of the participants reported use of cholesterol affecting medication (52.92%), and only 5.28% of the participants were on cholesterol reducing medications other than statins. On average, statin users were similar to controls on all measured characteristics, with the exception of LDL-c levels at visit 3. Statin users had significantly lower LDL-c at Visit 3 (125.70 mg/dL) compared to controls (147.65 mg/dL; p-value <.0001).

Primary Analysis: Differences in Visit 3 Protein Levels

In the primary analysis of 360 matched pairs statin users and controls, we identified average levels of 205 proteins to be enriched among statin users and 202 depleted. After FDR adjustment, average levels of 11 proteins remained significantly enriched among statin users, while average levels of 11 proteins were depleted, shown in **Figure 2A**. Notably, cytosolic acetoacetyl-CoA acetyltransferase (ACAT2) and HMG-CoA synthase (HMGCS1), enzymes involved in ketogenesis and upstream of the statins main target in the mevalonate pathway were significantly enriched among statin users. Levels of proprotein convertase subtilisin/kexin type 9 (PCKS9), also involved in cholesterol homeostasis, were also elevated among statin users. Levels of several proteins unrelated to lipid metabolism were found to differ between statin users and controls, with large diversity in protein function, localization, and structure.

Adjusted Analysis: Differences in Visit 3 Protein Levels, adjusted for Visit 2 Protein Levels

Results from the main analyses with an FDR q-value below 0.05 were further investigated with adjustment for their visit 2 levels. Among the 720 participants in the matched primary cohort, 642 (88.67%) had visit 2 proteomics data, being equally distributed between statin users and controls. Results are displayed in **Figure 2B**. Of the 22 proteins found to be significant in the main analyses, all but two (contactin-4 and inositol polyphosphate 5-phosphatase OCRL-1) remained significant (p<.05) after adjustment for their visit 2 levels. The association between all of these proteins and statin use had the same direction in both unadjusted and adjusted analyses, with a difference between linear regression coefficients of less than 25% for 13 of these proteins and a difference of less than 10% for 6 proteins.

Replication Analysis: Matched Cohort

A total of 10,798 participants were eligible for inclusion in the replication analysis, 234 (2.17%) of whom initiated statin use between visit 1 (baseline) and visit 2. **Supplemental Table 2** displays characteristics of this cohort prior to matching. After PS matching, 225 statin users were matched to an equal number of controls. Characteristics of this matched cohort are summarized in **Table 2**. There was small overlap between the primary and replication cohorts, with 20 participants serving as controls in both cohorts and 24 statin users in the primary cohort serving as controls in the replication cohort. Overall, participants characteristics were similar in both cohorts, aside from lower prevalence of most comorbidities and younger mean age. Of note, statin users and controls were similar on all measured characteristics, except fort LDL-c levels at visit 2. Statin users had significantly lower LDL-c at Visit 2 (134.25 mg/dL) compared to controls (159.05 mg/dL; p-value <.0001).

Replication Analysis: Differences in Visit 3 and Visit 2 Protein Levels

Of the 22 proteins that significantly differed between statin users and controls in the main analyses, 14 were also found to significantly differ between statin users and controls in the replication analyses with visit 2 proteomics data. Results are shown in **Figure 2C**. The associations observed between these proteins and statin use had the same direction in both primary and replication analyses. Differences between linear regression coefficients of less than 25% were observed for 9 of these proteins and a difference of less than 10% for 3 proteins.

Results for the Mevalonate Pathway

Differences in average levels of proteins in the mevalonate pathway of LDL-c biosynthesis in primary, adjusted, and replication analyses are depicted in **Figure 3**. No significant differences were observed for the main target of statins, HMG-CoA reductase (HMGCR). The upstream enzymes ACAT2 and HMGCS1 were significantly elevated among statin users in all analyses, remaining significant after FDR adjustment. The other downstream proteins in the pathway mevalonate kinase (MVK), phosphomevalonate kinase (PMVK), diphosphomevalonate decarboxylase (MVD), and isopentenyl-diphosphatase Delta-isomerase 1 (IDI1) were non-significant in all analyses, with the exception of IDI1 at visit 3, which was significantly elevated among statin users ($p<.05$) after adjusting for visit 2 levels.

Ingenuity Pathway Analysis (IPA) Results

IPA was utilized to explore canonical pathways linked to proteins found to be significantly associated with statin use. The 22 proteins found to differ between statin users and controls in the main analysis (visit 3, unadjusted) with a FDR corrected q-value below 0.05 were included. Canonical pathways found to be affected by these proteins are shown in **Figure 4**. In addition to expected pathways associated to cholesterol biosynthesis (e.g. Mevalonate Pathway I) or

other pathways related to lipid metabolism or catabolism (e.g. Ketogenesis, Ketolysis), several additional pathways were identified. Of note, pathways linked to the immune system and inflammation (e.g. Inflammasome Pathway, Phagosome Formation, Complement System) were also found to be differentially abundant in statin users. Lastly, pathways readily linked to human diseases (e.g., Neuroprotective Role of THOP1 in Alzheimer's Disease, Role of Osteoblasts in Rheumatoid Arthritis Signaling Pathway) were also identified although the meaning of these findings is unclear.

Discussion

The present study investigated differential protein level expression among statin users and non-user controls, matched utilizing a propensity score. In the primary matched cohort with 720 participants, we found 22 proteins to significantly differ between the groups after FDR adjustment. 20 of these proteins remained significant when adjusting for visit 2 protein levels in a sub-cohort of 642 participants. Lastly, 14 of these proteins were also found to significantly differ between statin users and controls in a replication matched cohort of 450 participants with visit 2 proteomics data.

The proteins found to differ in these analyses had great variability in functions, structures and localizations, and many of have been previously linked to various non-cardiovascular conditions. Among statin users, we found differential levels of proteins related to the LDL-c biosynthesis, endothelial health, atherosclerosis and inflammation, neurologic function, diabetes, metabolism, and cancer, all of which are indicative of the pleiotropic effect of statins.

Statins and LDL-c Biosynthetic Pathway

LDL-c synthesis occurs through a chain of biochemical reactions taking place primarily in hepatic cells, beginning with the mevalonate pathway[4]. In this pathway, Acetoacetyl-CoA actetyltransferase (cytosolic; ACAT2) and HMG-CoA synthase (cytoplasmic; HMGCS1) catalyze upstream reactions resulting in the formation of 3hydroxy-3-methylglutaryl CoA (HMG-CoA). HMG-CoA is further reduced to mevalonate by the enzyme HMG-CoA reductase in a rate-limiting, irreversible step. Statins primarily act as inhibitors of HMG-CoA reductase (HMGCR) by competitive binding to its active site[4,5]. Limiting this step in the mevalonate pathway reduces the synthesis of various downstream molecules, including LDL-c and isoprenoids[3–5].

In our analyses, statin users had higher levels of the proteins ACAT2 and HMGCS1. These proteins catalyze the first two steps of the mevalonate pathway of LDL-c biosynthesis, prior to the reduction of HMG-CoA by HMGCR. Increased expression of these proteins may be a biological response to statins' inhibition of the mevalonate pathway which is supported by prior animal models[11,12]. The increase in levels of these two proteins or their activity has been previously documented in rat liver models following treatment with lovastatin[11,12]. Meanwhile, no significant differences in levels of HMGCR or proteins downstream from it were observed. The lack of effect on HMGCR is not necessarily surprising as statins inhibit the protein's

activity and not its expression. Future research exploring the lack of effect on other downstream proteins is necessary. Nevertheless, in all analyses, statin-users had significantly lower LDL-c levels, suggesting the matched cohort captured the known, clinically relevant effect of statins.

Statins, Cardiovascular Health, Atherosclerosis, and Inflammation

Statin use has effects on cardiovascular health and systemic inflammation through lipid-dependent and lipid-independent mechanisms. Reduced LDL-c levels affect inflammatory responses and decrease systemic inflammation by mechanisms that include the activation of transmembrane receptors (ex: toll-like receptors) and pro-inflammatory cytokines like interleukin (IL)-1β[3,4]. Further, the impact of statins on inflammation has also been evidenced through lower plasma levels of the high-sensitivity C-reactive protein (hs-CRP), which may occur through lipid-dependent mechanisms or through immunomodulatory functions[3,4]. The reduction of LDL-c bioavailability through the use of statins has direct effects on various biophysiological pathways.

In agreement with prior studies, we observed statin users to have elevated levels of proprotein convertase subtilisin/kexin type 9 (PCSK9). PCSK9 may contribute to atherosclerosis, vascular wall inflammation, and platelet functioning[13]. This protein has also been previously linked to neurological development, neurogenesis, neuronal migration, and apoptosis[14]. PCSK9 has an important role in regulation of LDL-c, mediating its degradation through binding hepatic LDL receptors[15,16]. Gain-of-function mutations in PCSK9 have been linked to familial hypercholesterolemia, while loss-of-function mutations were associated with lower LDL-c levels and decreased risk of cardiovascular disease[15,16]. Additionally, PCSK9 likely reduces the effectiveness of LDL-c lowering via statin use; prior studies have shown that doubling the statin dose only reduces LDL-c by ~6% which is believed to be secondary to increased PCSK9[19]. Therapies that target PCSK9 are used to manage LDL-c levels and reduce the risk of cardiovascular disease[17,18]. The observed increase in PCSK9 levels among statin users has been previously described[19,20]. Therefore, our data support prior suggestions that adjunctive PCSK9 inhibition therapy among statin users represents a logical strategy to enhance statin induced LDL-c reduction[19].

Additionally, statins have been shown to reduce the number of inflammatory cells in plaques by modulating the production and secretion of cytokines, chemokines, and monocytes[3,4]. We found statin use to be associated with proteins involved in inflammatory and innate immune response. Statin users had elevated levels of the killer cell immunoglobulin-like receptor 3DL1 (KIR3DL1), a receptor with critical role in the innate immune response[21,22], suggesting increased immune system activity and inflammation among statin users. KIR3DL1 has not been previously studied in relation to statin use.

We observed a depletion Angiopoietin-related protein 3 (ANGPTL3) among statin users, a trend that has been previously described among patients with hyperlipidemia or familial hypercholesterolemia[23,24]. This protein is mainly expressed in the liver and is likely involved in

regulating LDL-c, HDL-c, and triglycerides, among other biological processes[25–27]. High ANGPTL3 levels have been associated with hyperlipidemia and increased risk of cardiovascular disease, including coronary heart disease and ischemic stroke[23,27,28]. Further, this protein has been previously correlated with elevated plasma glucose, insulin, and HOMA-IR, as well as diabetes risk and liver diseases[25,29]. Regulation of ANGPTL3 levels has been proposed as a novel therapeutic target[26] for reducing coronary heart disease risk.

Lastly, statin users had lower levels of platelet-activating factor acetylhydrolase (PAF-AH), a molecule that inactivates the lipid mediator platelet-activating factor[30]. A reduction of PAF-AH levels and activity due to statins has been described in both *in vivo* and *in vitro* conditions[31–34]. The role of PAF-AH in atherogenesis remains unclear, with both pro- and antiatherogenic activities previously described[35,36]. It has also been hypothesized that PAF-AH is involved in inflammatory responses[36,37].

Other mechanistic pathways connecting statin use to atherosclerosis, inflammation, and cardiovascular health remain to be elucidated. Further investigation is warranted to better understand the pleiotropy of statins in the context of atherosclerosis, inflammation, and cardiovascular disease.

Statins and Other Disease Outcomes

The pleiotropic effects of statins are hypothesized to encompass various others organ systems, diseases, and biophysiological pathways in addition to those described above. A few key examples are outlined below.

Statin users had elevated levels of the procollagen C-proteinase enhancer 1 (PCOLCE), a finding previously reported among asymptomatic HIV patients receiving atorvastatin versus placebo[38]. While the physiological effects of this protein and its role in human pathology remains to be described, PCOLCE has been hypothesized to be associated with liver and heart fibrosis and has been found to be elevated in patients with certain cancers[38–41].

We also observed a significant increase of collagen triple helix repeat-containing protein 1 (CTHRC1). CTHRC1 has a variety of functions, with known or hypothesized roles in collagen matrix deposition, cell migration, and bone formation[42]. This protein also has been linked to the anti-inflammatory process and wound healing through M2 macrophage recruitment among others[42,43]. Higher levels of CTHRC1 has been observed in patients with rheumatoid arthritis[44] and in cardiac fibroblasts following myocardial infarctions, likely due to the proteins role in regulating the scarring process[45]. Notably, elevated CTHRC1 expression has been previously associated to several types of cancers, with this protein having hypothesized roles in tumorigenesis and modulation of tumor microenvironments[46]. Recently, its use as a diagnostic biomarker has been suggested for various cancers and rheumatoid arthritis[44,46].

Other proteins previously associated with higher risk of cancers were identified to be elevated among statin users. Hyaluronidase 1 (HYAL1) is a well-known degrader of hyaluronic acid,

and its elevated expression of HYAL1 has been linked to several types of cancer and metastases. Higher levels of fructose-biphosphate aldolase C (ALDOC) were also observed among statin users. This aldolase is mostly known for its role in the glycolytic pathway in converting fructose 1,6-bisphosphate (F1,6BP) to glyceraldehyde 3-phosphate (G3P) and dihydroxyacetone phosphate (DHAP), but elevated levels have been previously linked to several forms of cancer and metabolic illnesses, including type two diabetes.

Proteins previously associated with neuronal functioning and development and neurological diseases were found to be differentially abundant among statin users vs. controls. Some of these findings are congruent with previous studies linking statin use to neurological disorders, including cognitive decline and neuropathies[47]. The sodium-couple monocarboxylate transporter 1 (SLC5A8) was found to be depleted among statin users, presently. This transporter protein has been previously linked to neuron functioning by facilitating the entry of l-lactate and ketone bodies into neurons[48]. The association between statin use and levels of SLC5A8 has not been previously described and future research may be warranted to confirm the present findings.

In contrast, our findings also suggest statins could have protective effects against neurological disease. Levels of the protease Cathepsin B (CTSB) were depleted among statin users. This protein has been previously linked to rheumatoid arthritis, inflammatory brain disease, brain aging, and several neurological conditions, including Parkinson's and Alzheimer's diseases[44,49,50]. Moreover, CTSB expression has been found to be elevated in various types of cancers and cathepsins have been identified as critical risk factors for cancer progression, suggesting the statin-associated depletion of this protein to be protective against cancer[51,52]. Nevertheless, the association between CTSB and statins remains unclear. *Hurks et al (2010)* found higher levels of CTSB among patients on pravastatin compared to non-users, but a nonsignificant decrease in levels of the protein among simvastatin users ($p > 0.05$)[52]. An inverse relation between CTSB activity and simvastatin concentration *in vitro* has been previously described by *Smith et al (2014)*[53], while higher CTSB activity *in vitro* following treatment with Fluvastatin was observed by *Liao et al (2013)*[54]. Further investigation of this protein may unveil mechanistic links behind these associations, allowing for future precision medicine-oriented approaches to identifying individuals at risk for neurodegenerative outcomes.

<u>Strengths and Limitations</u>

Our present study has some key limitations worth noting. First, we were unable to account for duration of treatment, statin type or dose - characteristics that may differentially influence the proteome. Different doses or statins may induce unique downstream compensatory responses to counteract upstream effects of statin use, leading to alterations in biological pathways that we could not control for. Second, this was an observational study and unmeasured confounding by indication may have been present. To address this, propensity score matching and a new-user design were utilized to minimize this potential[7,8]. Third, genetic and epigenetic variations that could be linked to protein functionality and health outcomes were not

accounted for. Lastly, baseline protein levels pre-statin use were not measured, raising the potential for confounding by pre-statin protein levels. This was partially addressed through adjusting the primary analyses with visit 2 proteomics and through a replication analyses, yielding largely overlapping results. Future studies that can address these limitations will enhance causal inference.

Conclusions

The present study explored the pleiotropic effects of statin use on the human proteome by comparing the proteome of statin users and propensity-matched controls enrolled in the ARIC study. We found that levels of several proteins differed between statin users and controls, many of which have been previously associated with neurological disease, cancers, and atherosclerosis. These findings inform the potential biological mechanisms underlying statin pleiotropy. Target proteomic biomarkers hold promise for precision medicine approaches aiming to both i) identify statin users at risk of rare nonatherosclerotic outcomes; and ii) identify health benefits of statin use independent of LDL-C reduction. Given the importance of statin therapy on reducing atherosclerotic cardiovascular disease event rates and increasing survival, future studies are necessary to replicate these findings and guide decision making to maximize the beneficial effects of statin use.

Figure 1: Selection criteria for the primary and replication matched cohorts. (LTFU = lost to follow up, including death; Visit 1 = ARIC baseline visit (1987-1989); Visit 2 = ARIC second follow up visit (1990-1992); Visit 3 = ARIC third follow up visit (1993-1995); PS = Propensity Score)

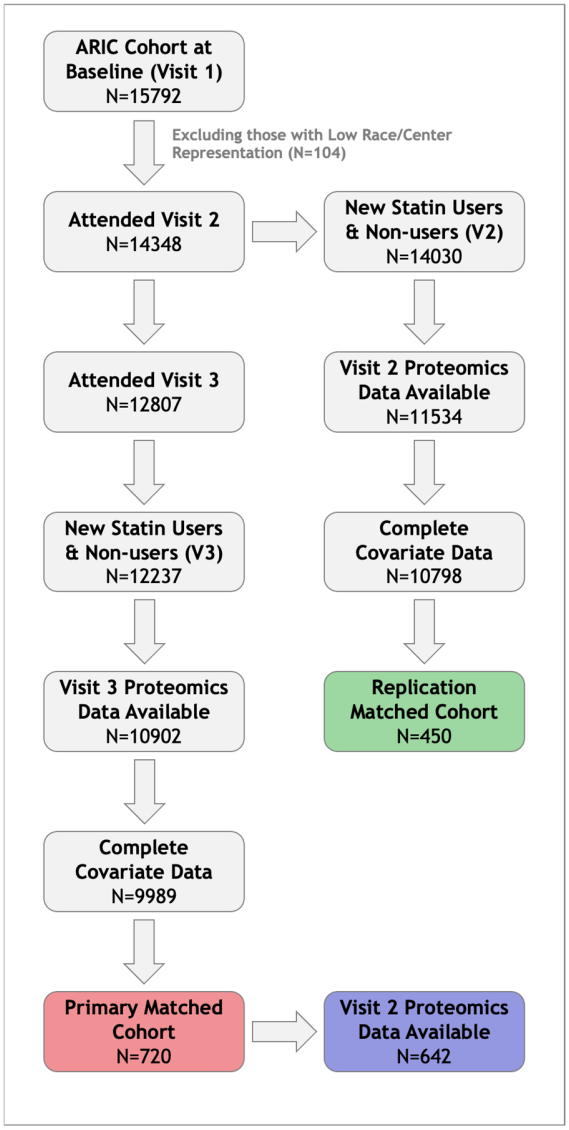
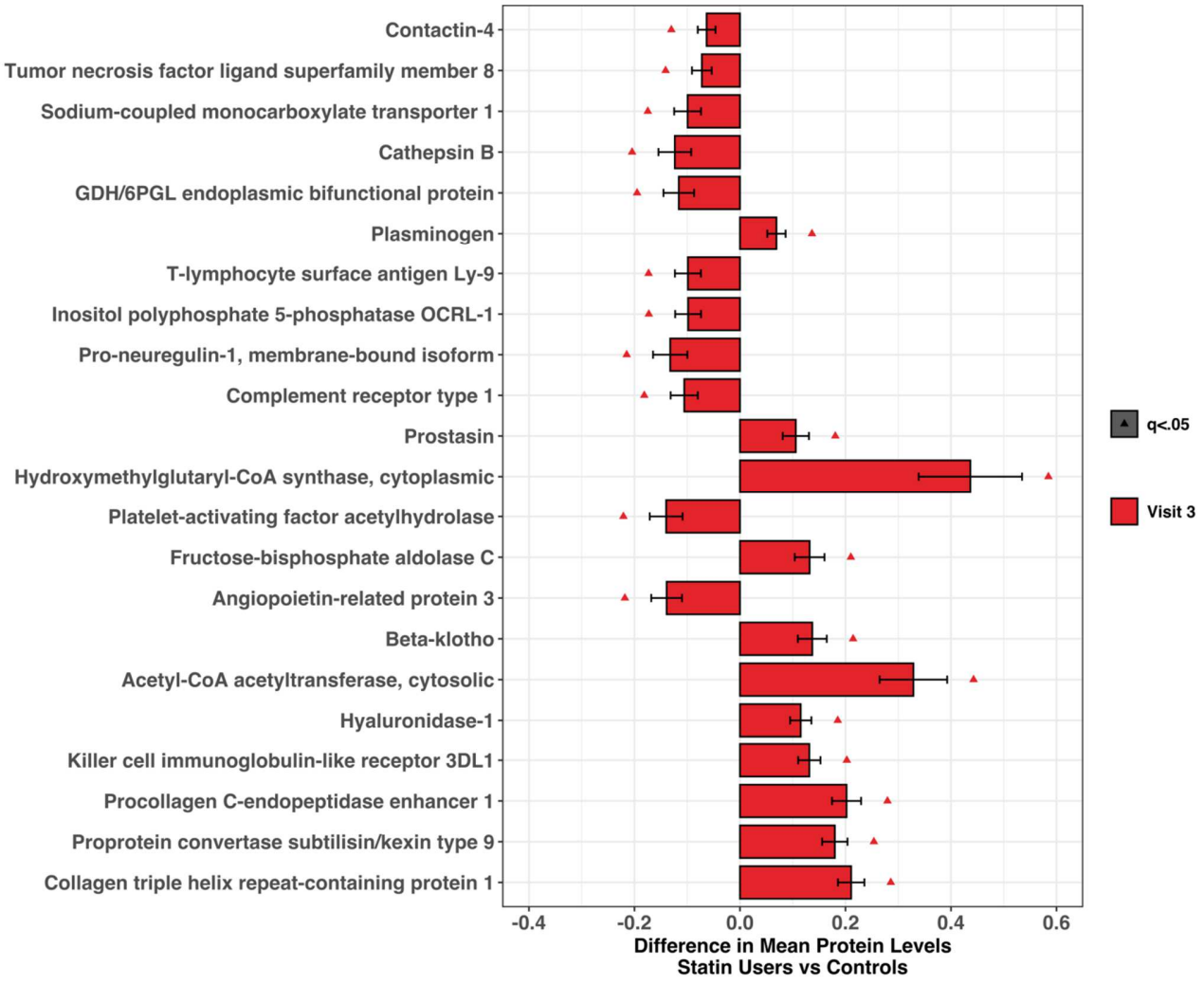
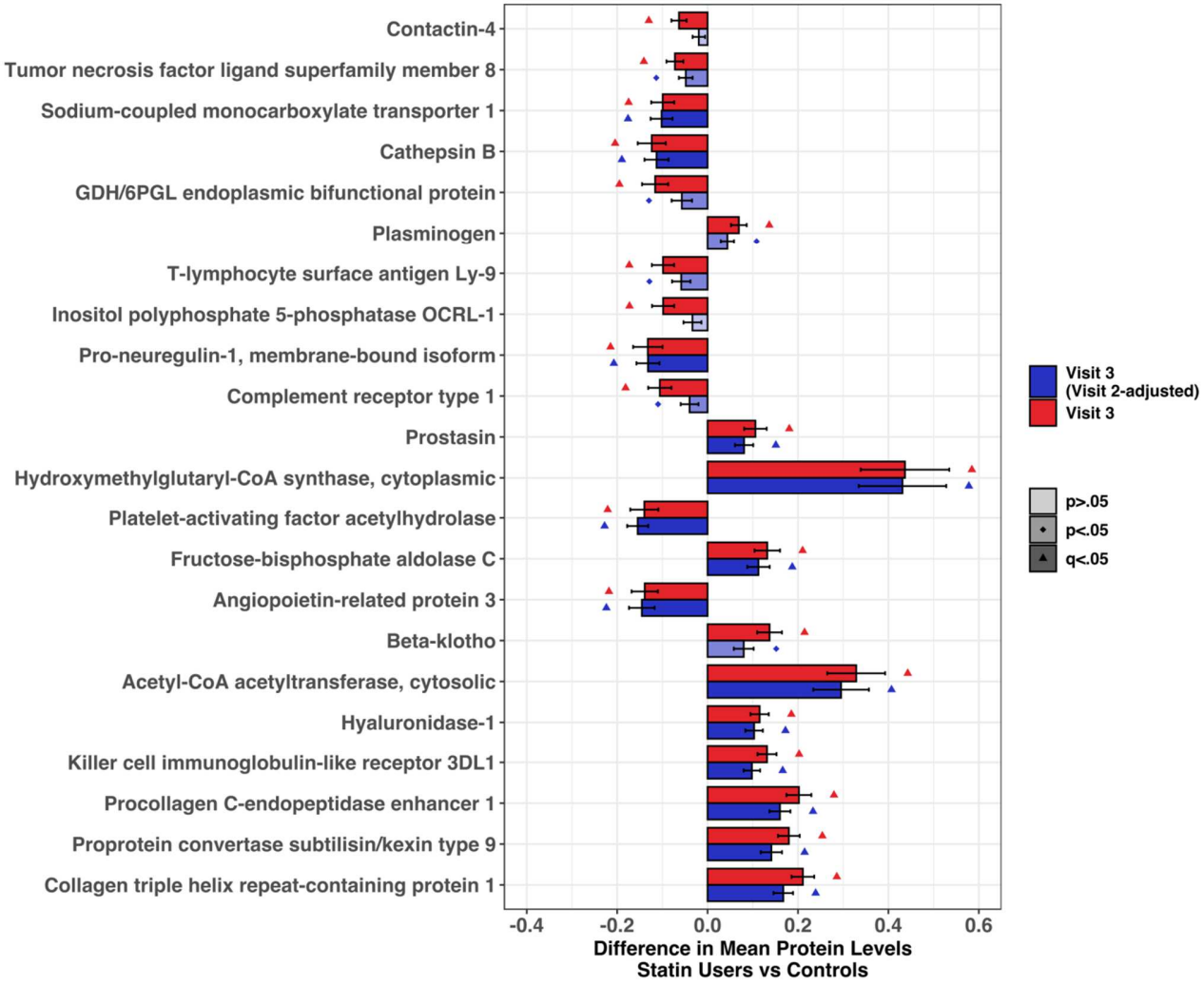


Figure 2: Mean Differences in Protein Levels of Stain Users vs Controls. Proteins shown differed significantly in the Visit 3 Primary Matched Cohort analyses, after false discovery rate adjustment. **A)** Results for Visit 3 proteomics, in the Primary Matched Cohort (N=720); **B)** Results for Visit 3 with and without adjustment for Visit 2 protein levels among N=642 participants with both Visit 2 and Visit 3 proteomics data; and **C)** Results from a Replication Matched Cohort (N=450) using Visit 2 Proteomics. Results from Visit 3 are presented in C for ease of comparison between the Primary and the Replication Matched cohort.

A.



B.


Contactin-4
Tumor necrosis factor ligand superfamily member 8
Sodium-coupled monocarboxylate transporter 1
Cathepsin B
GDH/6PGL endoplasmic bifunctional protein
Plasminogen
T-lymphocyte surface antigen Ly-9
Inositol polyphosphate 5-phosphatase OCRL-1
Pro-neuregulin-1, membrane-bound isoform
Complement receptor type 1
Prostasin
Hydroxymethylglutaryl-CoA synthase, cytoplasmic
Platelet-activating factor acetylhydrolase
Fructose-bisphosphate aldolase C
Angiopoietin-related protein 3
Beta-klotho
Acetyl-CoA acetyltransferase, cytosolic
Hyaluronidase-1
Killer cell immunoglobulin-like receptor 3DL1
Procollagen C-endopeptidase enhancer 1
Proprotein convertase subtilisin/kexin type 9
Collagen triple helix repeat-containing protein 1
-0.4
-0.2
0.0
0.2
0.4
0.6
Difference in Mean Protein Levels
Statin Users vs Controls
Visit 3
(Visit 2-adjusted)
Visit 3
p>.05
p<.05
q<.05

C.

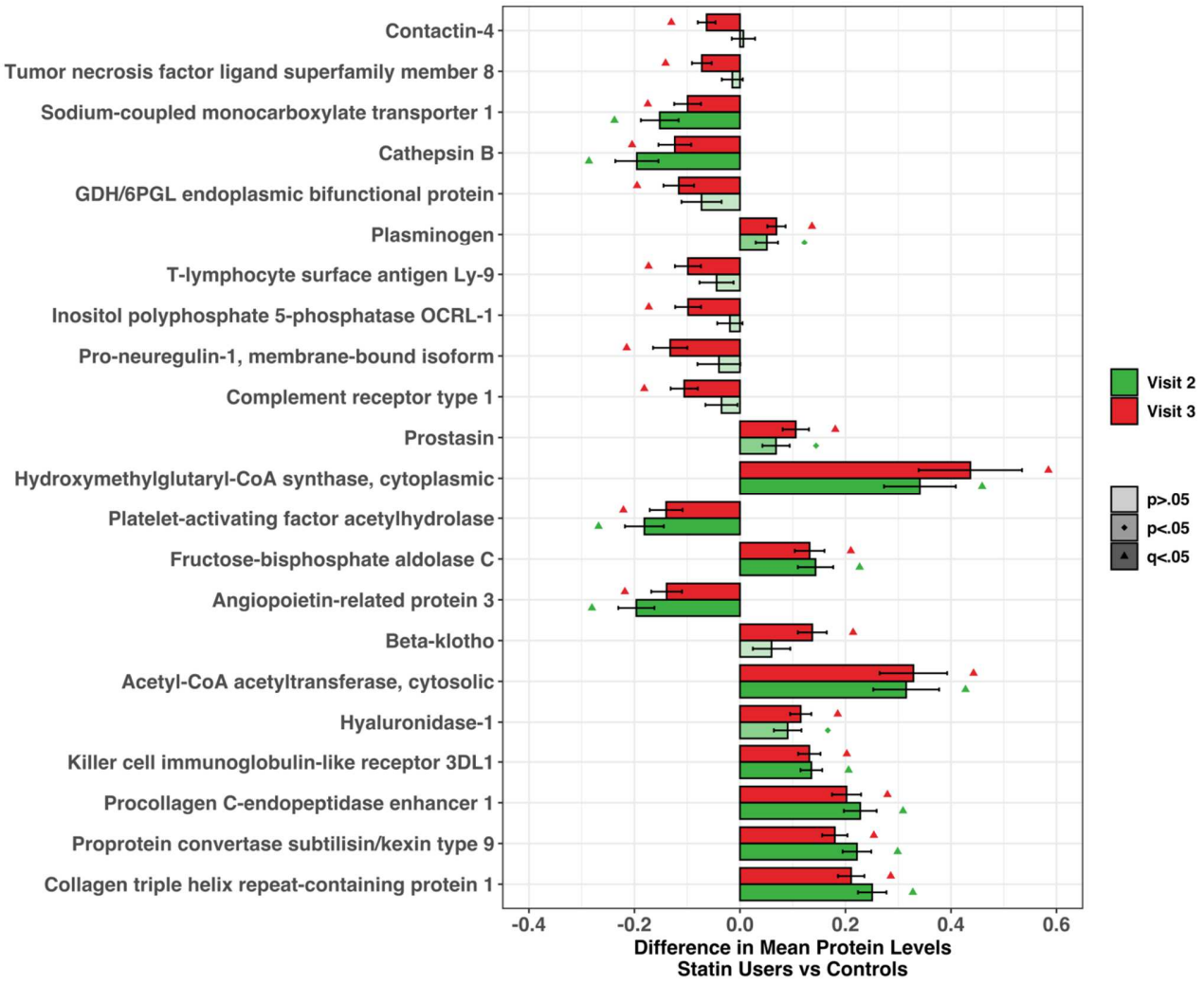

Contactin-4
Tumor necrosis factor ligand superfamily member 8
Sodium-coupled monocarboxylate transporter 1
Cathepsin B
GDH/6PGL endoplasmic bifunctional protein
Plasminogen
T-lymphocyte surface antigen Ly-9
Inositol polyphosphate 5-phosphatase OCRL-1
Pro-neuregulin-1, membrane-bound isoform
Complement receptor type 1
Prostasin
Hydroxymethylglutaryl-CoA synthase, cytoplasmic
Platelet-activating factor acetylhydrolase
Fructose-bisphosphate aldolase C
Angiopoietin-related protein 3
Beta-klotho
Acetyl-CoA acetyltransferase, cytosolic
Hyaluronidase-1
Killer cell immunoglobulin-like receptor 3DL1
Procollagen C-endopeptidase enhancer 1
Proprotein convertase subtilisin/kexin type 9
Collagen triple helix repeat-containing protein 1
-0.4
-0.2
0.0
0.2
0.4
0.6
Difference in Mean Protein Levels
Statin Users vs Controls
Visit 2
Visit 3
p>.05
p<.05
q<.05

Figure 3: Mean Differences in Protein Levels of Proteins in the Mevalonate Pathway of Low-density Lipoprotein Cholesterol of Stain Users vs Controls at Visit 3 (n=720), Visit 3 adjusted for Visit 2 (n=642), and Visit 2 (n=450).

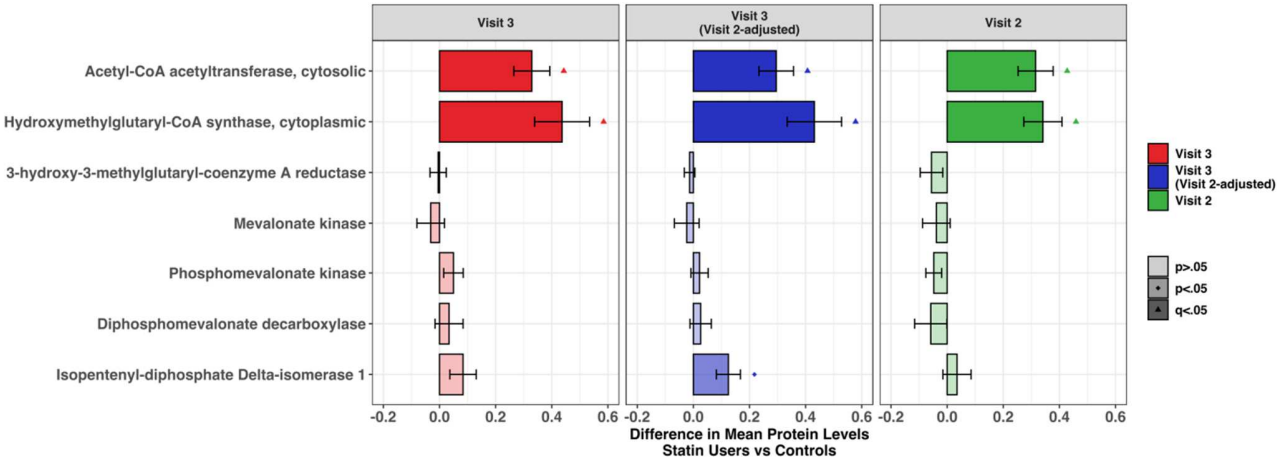
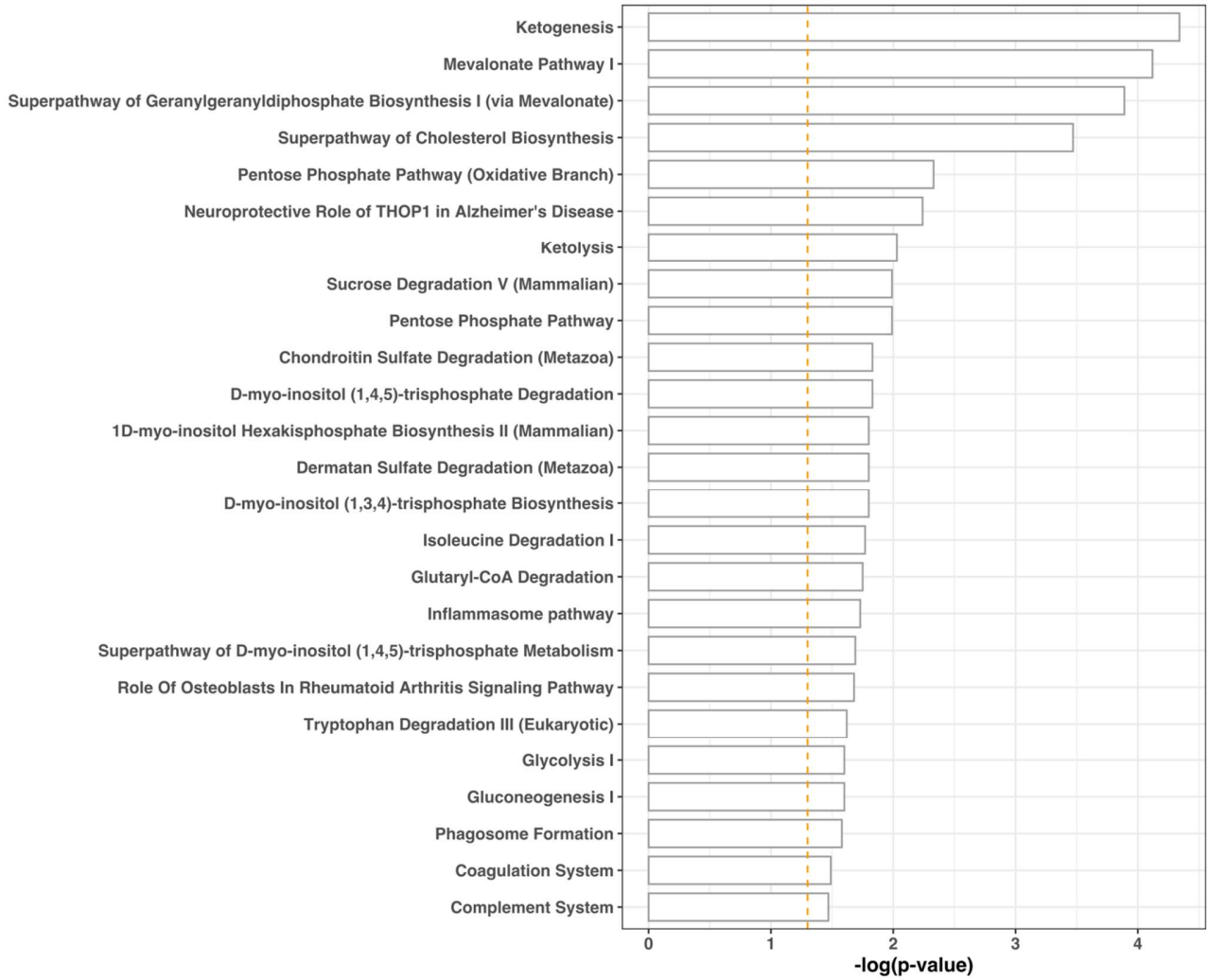


Figure 4: Canonical Pathways Identified with Ingenuity Pathway Analysis of Proteins Differing Significantly (q<0.05) Between Statin Users vs Controls at Visit 3 (N=720). Canonical pathways found to be significantly (p<0.05) altered.



460 **Table 1:** Characteristics of Primary Study Cohort Participants After Matching, Stratified by
461 Statin Use, ARIC, 1993-1995.
462

Demographics and Behavior Variables	**All (N=720)**	**Non-Users (N=360)**	**Statin Users (N=360)**	**p-value**
Age (V3)	60.78 (5.53)	60.61 (5.71)	60.96 (5.34)	0.3849
Sex				0.6528
Male	321 (44.58%)	157 (43.61%)	164 (45.56%)	
Female	399 (55.42%)	203 (56.39%)	196 (54.44%)	
Race/Center				0.8496
White, MN	224 (31.11%)	118 (32.78%)	106 (29.44%)	
White, MD	232 (32.22%)	110 (30.56%)	122 (33.89%)	
White, NC	182 (25.28%)	91 (25.28%)	91 (25.28%)	
Black, NC	11 (1.53%)	5 (1.39%)	6 (1.67%)	
Black, MS	71 (9.86%)	36 (10.00%)	35 (9.72%)	
Education Level				0.4828
Basic	130 (18.06%)	63 (17.50%)	67 (18.61%)	
Intermediate	354 (49.17%)	185 (51.39%)	169 (46.94%)	
Advanced	236 (32.78%)	112 (31.11%)	124 (34.44%)	
BMI				
V2	28.60 (5.15)	28.76 (5.25)	28.45 (5.05)	0.4153
V3	29.14 (5.33)	29.13 (5.48)	29.14 (5.19)	0.9951
Drinking Status (V3)				0.6311
Current	372 (51.67%)	183 (50.83%)	189 (52.50%)	
Former	179 (24.86%)	95 (26.39%)	84 (23.33%)	
Never	169 (23.47%)	82 (22.78%)	87 (24.17%)	
Smoking Status (V3)				0.2088
Current	94 (13.06%)	39 (10.83%)	55 (15.28%)	
Former	326 (45.28%)	167 (46.39%)	159 (44.17%)	
Never	300 (41.67%)	154 (42.78%)	146 (40.56%)	
Clinical Characteristics				
HDL (mg/dL)				
V1[1]	4702 (14.29)	47.45 (14.35)	46.59 (14.24)	0.4217
V2	44.10 (13.19)	44.56 (13.21)	43.64 (13.18)	0.3502
V3	47.31 (15.47)	46.68 (15.22)	47.93 (15.71)	0.2807
LDL (mg/dL)				
V1[1]	164.68 (37.71)	157.30 (38.49)	172.18 (35.42)	<.0001
V2	167.45 (37.91)	166.99 (39.64)	167.90 (36.15)	0.7478
V3[1]	136.86 (34.31)	147.65 (35.08)	125.70 (29.66)	<.0001

sCr-eGFR (mL/min)				
V1[1]	100.70 (12.42)	101.13 (11.69)	100.27 (13.10)	0.3560
V2	94.55 (14.95)	95.25 (13.96)	93.86 (15.88)	0.2126
V3	87.34 (17.03)	88.18 (16.23)	86.49 (17.77)	0.1812
SBP (mmHg)				
V2	122.53 (18.29)	122.37 (18.64)	122.69 (17.96)	0.8181
V3	122.84 (18.51)	122.74 (17.70)	122.94 (19.30)	0.8864
DBP (mmHg)				
V2	72.22 (10.20)	72.34 (10.32)	72.11 (10.09)	0.7591
V3	69.53 (9.90)	69.68 (9.98)	69.39 (9.84)	0.6986
Cardiovascular Disease Prevalence				
Hypertension				
V2	320 (44.44%)	164 (45.56%)	156 (43.33%)	0.5996
V3	373 (51.81%)	190 (52.78%)	183 (50.83%)	0.6545
CHD				
V2[1]	117 (16.27%)	60 (16.67%)	57 (15.88%)	0.8527
V3	171 (23.75%)	85 (23.61%)	86 (23.89%)	>.9999
MI				
V2	110 (15.28%)	59 (16.39%)	51 (14.17%)	0.4684
V3	145 (20.14%)	76 (21.11%)	69 (19.17%)	0.5771
HF				
V2	44 (6.11%)	18 (5.00%)	26 (7.22%)	0.2761
V3	56 (7.78%)	23 (6.39%)	33 (9.17%)	0.2104
Stroke				
V2	12 (1.67%)	6 (1.67%)	6 (1.67%)	>.9999
V3	18 (2.50%)	8 (2.22%)	10 (2.78%)	0.8113
Diabetes				
V2[1]	172 (23.92%)	81 (22.56%)	91 (25.28%)	0.4438
V3	185 (25.69%)	91 (25.28%)	94 (26.11%)	0.8646
Medication Use				
Antihypertensives				
V2	270 (37.50%)	140 (38.89%)	130 (36.11%)	0.4884
V3	346 (48.06%)	178 (49.44%)	168 (46.67%)	0.5020
Cholesterol Affecting				
V2	311 (43.19%)	151 (41.94%)	160 (44.44%)	0.5472
V3	381 (52.92%)	195 (54.17%)	186 (51.67%)	0.5503
Cholesterol Lowering (non-statin)				

V2[1]	94 (13.06%)	11 (3.06%)	83 (23.06%)	>.0001
V3	38 (5.28%)	20 (5.56%)	18 (5.00%)	0.8676
Proteomics Data Availability				
V2	642 (88.67%)	321 (88.67%)	321 (88.67%)	>.9999
V3	720 (100%)	360 (100%)	360 (100%)	-

1. Variables with missing data: HDL (V1) = 4; LDL (V1) = 16; LDL (V3) = 18; sCr-eGFR (V1) = 2; CHD (V2) = 1; Diabetes (V2) = 1;

Abbreviations: V1 = ARIC baseline visit (1987-1989); V2 = ARIC second follow up visit (1990-1992); V3 = ARIC third follow up visit (19931995); BMI = body mass index; HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol; SBP = systolic blood pressure; DBP = diastolic blood pressure; sCr-eGFR = serum creatinine estimated glomerular filtration rate; CHD = coronary heart disease; HF = heart failure; MI = myocardial infarction.

475 **Table 2:** Characteristics of Replication Study Cohort Participants After Matching, Stratified by
476 Statin Use, ARIC, 1993-1995.
477

Demographics and Behavior Variables	**All (N=450)**	**Non-Users (N=225)**	**Statin Users (N=225)**	**p-value**
Age (V2)	58.30 (5.52)	58.60 (5.32)	58.00 (5.72)	0.2567
Sex				0.1312
Male	217 (48.22%)	117 (52.00%)	100 (44.44%)	
Female	233 (51.78%)	108 (48.00%)	125 (55.56%)	
Race/Center				0.9014
White, MN	128 (29.44%)	63 (28.00%)	65 (28.89%)	
White, MD	170 (37.78%)	87 (38.67%)	83 (36.89%)	
White, NC	91 (20.22%)	47 (20.89%)	44 (19.56%)	
Black, NC	6 (1.33%)	2 (0.89%)	4 (1.78%)	
Black, MS	55 (12.22%)	26 (11.56%)	29 (12.89%)	
Education Level				0.4898
Basic	98 (21.78%)	48 (21.33%)	50 (22.22%)	
Intermediate	208 (46.22%)	110 (48.89%)	98 (43.56%)	
Advanced	144 (32.00%)	67 (29.78%)	77 (34.22%)	
BMI (V2)	28.17 (4.93)	28.18 (5.08)	28.16 (4.79)	0.9507
Drinking Status (V2)				0.3955
Current	260 (57.78%)	123 (54.67%)	137 (60.89%)	
Former	106 (23.56%)	56 (24.89%)	50 (22.22%)	
Never	84 (18.67%)	46 (20.44%)	38 (16.89%)	
Smoking Status (V2)				0.8280
Current	86 (19.11%)	41 (18.22%)	45 (20.00%)	
Former	220 (48.89%)	113 (50.22%)	107 (47.56%)	
Never	144 (32.00%)	71 (31.56%)	73 (32.44%)	
Clinical Characteristics				
HDL (mg/dL)				
V1	46.71 (14.10)	47.18 (14.57)	46.23 (13.64)	0.4790
V2	46.71 (14.72)	46.20 (15.30)	47.22 (14.13)	0.4600
LDL (mg/dL)				
V1	181.02 (39.31)	179.48 (40.94)	182.55 (37.65)	0.4091
V2	146.71 (38.19)	159.05 (39.62)	134.25 (32.28)	<.0001
sCr-eGFR (mL/min)				
V1	98.90 (15.08)	99.00 (14.66)	98.60 (15.51)	0.7781
V2	93.28 (16.57)	93 .01 (16.59)	93.55 (16.58)	0.7308
SBP (mmHg) (V2)	121.78 (16.72)	122.61 (15.98)	120.96 (17.43)	0.2935

DBP (mmHg) (V2)	70.89 (9.46)	71.23 (9.24)	70.55 (9.69)	0.4466
Cardiovascular Disease Prevalence at Visit 2				
Hypertension	237 (52.67%)	125 (55.56%)	112 (49.78%)	0.2572
CHD	118 (26.22%)	64 (28.44%)	54 (24.00%)	0.3348
MI	94 (20.89%)	50 (22.22%)	44 (19.56%)	0.5620
HF	36 (8.00%)	18 (8.00%)	18 (8.00%)	>.9999
Stroke	5 (1.11%)	1 (0.44%)	4 (1.78%)	0.3684
Diabetes	83 (18.44%)	42 (18.67%)	41 (18.22%)	>.9999
Medication Use at Visit 2				
Antihypertensives	210 (46.67%)	109 (48.44%)	101 (44.89%)	0.5083
Cholesterol Affecting	226 (50.22%)	118 (52.44%)	108 (48.00%)	0.3961
Cholesterol Lowering (non-statin)	42 (9.33%)	25 (11.11%)	17 (7.56%)	0.2566

Abbreviations: V1 = ARIC baseline visit (1987-1989); V2 = ARIC second follow up visit (1990-1992); V3 = ARIC third follow up visit (19931995); BMI = body mass index; HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol; SBP = systolic blood pressure; DBP = diastolic blood pressure; sCr-eGFR = serum creatinine estimated glomerular filtration rate; CHD = coronary heart disease; HF = heart failure; MI = myocardial infarction.

Supplemental Table 1: Characteristics of Primary Study Cohort Participants Before Matching, Stratified by Statin Use, ARIC, 1993-1995.

Demographics and Behavior Variables	All (N=9989)	Non-Users (N=9610)	Statin Users (N=379)	p-value
Age (V3)	60.07 (5.71)	60.04 (5.73)	60.97 (5.30)	0.0009
Sex				0.7030
Male	4530 (45.35%)	4354 (45.31%)	176 (46.44%)	
Female	5459 (54.65%)	5256 (54.69%)	203 (53.56%)	
Race/Center				0.0001
White, MN	2878 (28.81%)	2764 (28.76%)	114 (30.08%)	
White, MD	2658 (26.61%)	2530 (26.33%)	128 (33.77%)	
White, NC	2430 (24.33%)	2336 (24.31%)	94 (24.80%)	
Black, NC	265 (2.65%)	258 (2.68%)	7 (1.85%)	
Black, MS	1758 (17.60%)	1722 (17.92%)	36 (9.50%)	
Education Level				0.1298
Basic	1936 (19.38%)	1866 (19.42%)	70 (18.47%)	
Intermediate	4252 (42.57%)	4072 (42.37%)	180 (47.49%)	
Advanced	3801 (38.05%)	3672 (38.21%)	129 (34.04%)	
BMI				
V2[1]	2789 (5.29)	27.87 (5.30)	28.45 (4.99)	0.0260
V3	28.44 (5.52)	28.42 (5.54)	29.12 (5.10)	0.0087
Drinking Status (V3)[1]				0.8015
Current	5350 (53.58%)	5148 (53.59%)	202 (53.30%)	
Former	2219 (22.22%)	2130 (22.17%)	89 (23.48%)	
Never	2416 (24.20%)	2328 (24.23%)	88 (23.22%)	
Smoking Status (V3)				0.2220
Current	1758 (17.60%)	1701 (17.70%)	57 (15.04%)	
Former	4151 (41.56%)	3979 (41.40%)	172 (45.38%)	
Never	4080 (40.84%)	3930 (40.89%)	150 (39.58%)	
Clinical Characteristics				
HDL (mg/dL)				
V1[1]	51.92 (16.73)	52.14 (16.79)	46.54 (14.22)	<.0001
V2	49.75 (16.51)	49.99 (16.59)	43.63 (13.11)	<.0001
V3	52.22 (18.10)	52.39 (18.16)	48.05 (15.73)	<.0001
LDL (mg/dL)				
V1[1]	136.64 (37.35)	135.20 (36.64)	173.54 (36.44)	<.0001
V2	133.47 (36.17)	131.95 (35.05)	172.06 (42.34)	<.0001
V3[1]	127.12 (34.00)	127.15 (34.12)	126.41 (30.83)	0.6544

sCr-eGFR (mL/min)				
V1[1]	102.12 (12.10)	102.20 (12.05)	100.12 (13.11)	0.0025
V2[1]	96.81 (13.34)	96.93 (13.22)	93.74 (15.78)	0.0001
V3	89.67 (14.36)	89.80 (14.20)	86.43 (17.64)	0.0003
SBP (mmHg)				
V2	120.87 (18.13)	120.80 (18.14)	122.55 (17.85)	0.0632
V3	124.32 (19.05)	124.38 (19.05)	122.70 (19.02)	0.0912
DBP (mmHg)				
V2	71.93 (10.09)	71.92 (10.09)	72.19 (10.15)	0.6018
V3	71.61 (10.43)	71.71 (10.44)	69.18 (9.78)	<.0001
Cardiovascular Disease Prevalence				
Hypertension				
V2[1]	3349 (33.60%)	3183 (33.20%)	166 (43.80%)	<.0001
V3	4007 (40.11%)	3813 (39.68%)	194 (51.19%)	<.0001
CHD				
V2[1]	485 (4.86%)	420 (4.37%)	65 (17.24%)	<.0001
V3	667 (6.68%)	568 (5.91%)	99 (26.12%)	<.0001
MI				
V2	506 (5.07%)	448 (4.66%)	58 (15.30%)	<.0001
V3	598 (5.99%)	519 (5.40%)	79 (20.84%)	<.0001
HF				
V2	430 (4.30%)	402 (4.18%)	28 (7.39%)	0.0039
V3	502 (5.03%)	467 (4.86%)	35 (9.23%)	0.0002
Stroke				
V2	144 (1.44%)	138 (1.44%)	6 (1.58%)	0.9872
V3	183 (1.83%)	173 (1.80%)	10 (2.64%)	0.3181
Diabetes				
V2[1]	1355 (13.60%)	1258 (13.12%)	97 (25.59%)	<.0001
V3	1486 (14.88%)	1385 (14.41%)	101 (26.65%)	<.0001
Medication Use				
Antihypertensives				
V2[1]	2524 (25.34%)	2386 (24.90%)	138 (36.41%)	<.0001
V3	3047 (30.50%)	2869 (29.85%)	178 (46.97%)	<.0001
Cholesterol Affecting				
V2	2528 (25.31%)	2358 (24.54%)	170 (44.85%)	<.0001
V3	3016 (30.19%)	2815 (29.29%)	201 (53.03%)	<.0001
Cholesterol Lowering (non-statin)				

V2	423 (4.23%)	337 (3.51%)	86 (22.69%)	<.0001
V3	435 (4.35%)	415 (4.32%)	20 (5.28%)	0.4421

1. Variables with missing data: BMI (V2) = 5; Drinking Status (V3) = 4; HDL (V1) = 96; LDL (V1) = 189; LDL (V3) = 116; sCr-eGFR (V1) = 47; sCr-eGFR (V2) = 3; Hypertension (V2) = 23; CHD (V2) = 4; Diabetes (V2) = 25; Antihypertensives (V2) = 27;

Abbreviations: V1 = ARIC baseline visit (1987-1989); V2 = ARIC second follow up visit (1990-1992); V3 = ARIC third follow up visit (19931995); BMI = body mass index; HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol; SBP = systolic blood pressure; DBP = diastolic blood pressure; sCr-eGFR = serum creatinine estimated glomerular filtration rate; CHD = coronary heart disease; HF = heart failure; MI = myocardial infarction.

Supplemental Table 2: Characteristics of Replication Study Cohort Participants Before Matching, Stratified by Statin Use, ARIC, 1993-1995.

Demographics and Behavior Variables	All (N=10798)	Non-Users (N=10564)	Statin Users (N=234)	p-value
Age (V2)	57.09 (5.73)	57.06 (5.73)	58.09 (5.71)	0.0068
Sex				>.9999
Male	4771 (44.18%)	4668 (44.19%)	103 (44.02%)	
Female	6027 (55.82%)	5896 (55.81%)	131 (55.98%)	
Race/Center				0.0003
White, MN	2974 (27.54%)	2908 (27.53%)	66 (28.21%)	
White, MD	2824 (26.15%)	2735 (25.8%)	89 (38.03%)	
White, NC	2602 (24.10%)	2558 (24.21%)	44 (18.80%)	
Black, NC	308 (2.85%)	304 (2.88%)	4 (1.71%)	
Black, MS	2090 (19.36%)	2059 (19.49%)	31 (13.25%)	
Education Level				0.6137
Basic	2279 (21.11%)	225 (21.06%)	54 (23.08%)	
Intermediate	4575 (42.37%)	4474 (42.35%)	101 (43.16%)	
Advanced	3944 (36.53%)	3865 (36.59%)	79 (33.76%)	
BMI (V2)	27.85 (5.30)	27.84 (5.31)	28.24 (4.81)	0.2094
Drinking Status (V2)[1]				0.1784
Current	6183 (57.28%)	6041 (57.20%)	142 (60.68%)	
Former	2230 (20.66%)	2178 (20.62%)	52 (22.22%)	
Never	2382 (22.07%)	2342 (22.18%)	40 (17.09%)	
Smoking Status (V2)				0.0119
Current	2391 (22.14%)	2344 (22.19%)	47 (20.09%)	
Former	4127 (38.22%)	4016 (38.02%)	111 (47.44%)	
Never	4280 (39.64%)	4204 (39.80%)	76 (32.48%)	
Clinical Characteristics				
HDL (mg/dL)				
V1	52.02 (16.80)	52.15 (16.84)	46.24 (13.60)	<.0001
V2	49.97 (16.68)	50.03 (16.73)	47 (13.96)	0.0043
LDL (mg/dL)				
V1	137.63 (38.63)	136.56 (37.85)	185.96 (42.54)	<.0001
V2[1]	133.14 (36.44)	133.07 (36.47)	136.50 (34.69)	0.1393
sCr-eGFR (mL/min)				
V1[1]	102.06 (12.41)	102.13 (12.33)	98.68 (15.40)	0.0008
V2	96.69 (13.79)	96.76 (13.72)	93.66 (16.44)	0.0046
SBP (mmHg) (V2)	121.27 (18.62)	121.27 (18.64)	120.94 (17.54)	0.7775

DBP (mmHg) (V2)	71.91 (10.23)	71.95 (10.24)	70.46 (9.76)	0.0220
Cardiovascular Disease Prevalence at Visit 2				
Hypertension	3796 (35.15%)	3677 (34.81%)	119 (50.85%)	<.0001
CHD	665 (6.16%)	607 (5.75%)	58 (24.79%)	<.0001
MI	662 (6.13%)	616 (5.83%)	46 (19.66%)	<.0001
HF	518 (4.80%)	499 (4.72%)	19 (8.12%)	0.0245
Stroke	205 (1.90%)	201 (1.90%)	4 (1.71%)	>.9999
Diabetes	1558 (14.43%)	1516 (14.35%)	42 (17.95%)	0.1456
Medication Use at Visit 2				
Antihypertensives	2865 (26.53%)	2757 (26.10%)	108 (36.15%)	<.0001
Cholesterol Affecting	2879 (26.66%)	2763 (26.15%)	116 (49.57%)	<.0001
Cholesterol Lowering (non-statin)	445 (4.12%)	426 (4.03%)	19 (8.12%)	0.0032

1. Variables with missing data: Drinking Status (V2) = 3; LDL (V2) = 112; sCr-eGFR (V1) = 7;

Abbreviations: V1 = ARIC baseline visit (1987-1989); V2 = ARIC second follow up visit (1990-1992); BMI = body mass index; HDL-c = high-density lipoprotein cholesterol; LDL-c = low-density lipoprotein cholesterol; SBP = systolic blood pressure; DBP = diastolic blood pressure; sCr-eGFR = serum creatinine estimated glomerular filtration rate; CHD = coronary heart disease; HF = heart failure; MI = myocardial infarction.

1. Grundy, S. M. *et al.* 2018 AHA/ACC/AACVPR/AAPA/ABC/ACPM/ADA/AGS/APhA/ASPC/NLA/PCNA Guideline on the Management of Blood Cholesterol: A Report of the American College of Cardiology/American Heart Association Task Force on Clinical Practice Guidelines. *Journal of the American College of Cardiology.* **73**, e285–e350 (2019).
2. Gu, Q. & Kit, B. K. Prescription Cholesterol-lowering Medication Use in Adults Aged 40 and Over: United States, 2003–2012. 8 (2014).
3. Liberale, L., Carbone, F., Montecucco, F. & Sahebkar, A. Statins reduce vascular inflammation in atherogenesis: A review of underlying molecular mechanisms. *Int J Biochem Cell Biol* **122**, 105735 (2020).
4. Liao, J. K. & Laufs, U. Pleiotropic effects of statins. *Annu Rev Pharmacol Toxicol* **45**, 89–118 (2005).
5. Sirtori, C. R. The pharmacology of statins. *Pharmacol Res* **88**, 3–11 (2014).
6. Wright, J. D. *et al.* The ARIC (Atherosclerosis Risk In Communities) Study: JACC Focus Seminar 3/8. *J Am Coll Cardiol* **77**, 2939–2959 (2021).
7. Ray, W. A. Evaluating medication effects outside of clinical trials: new-user designs. *Am J Epidemiol* **158**, 915–920 (2003).
8. Hernán, M. A. & Robins, J. M. Using Big Data to Emulate a Target Trial When a Randomized Trial Is Not Available. *Am J Epidemiol* **183**, 758–764 (2016).
9. Tin, A. *et al.* Reproducibility and Variability of Protein Analytes Measured Using a Multiplexed Modified Aptamer Assay. *J Appl Lab Med* **4**, 30–39 (2019).
10. Krämer, A., Green, J., Pollard, J. & Tugendreich, S. Causal analysis approaches in Ingenuity Pathway Analysis. *Bioinformatics* **30**, 523–530 (2014).

11. Steiner, S. *et al.* Cholesterol biosynthesis regulation and protein changes in rat liver following treatment with fluvastatin. *Toxicology Letters* **120**, 369–377 (2001).
12. Honda, A. *et al.* Regulation of early cholesterol biosynthesis in rat liver: effects of sterols, bile acids, lovastatin, and BM 15.766 on 3-hydroxy-3-methylglutaryl coenzyme A synthase and acetoacetyl coenzyme A thiolase activities. *Hepatology* **27**, 154–159 (1998).
13. Filippatos, T. D., Christopoulou, E. C. & Elisaf, M. S. Pleiotropic effects of proprotein convertase subtilisin/kexin type 9 inhibitors? *Curr Opin Lipidol* **29**, 333–339 (2018).
14. Mannarino, M. R. *et al.* PCSK9 and neurocognitive function: Should it be still an issue after FOURIER and EBBINGHAUS results? *J Clin Lipidol* **12**, 1123–1132 (2018).
15. Sarkar, S. K. *et al.* A transient amphipathic helix in the prodomain of PCSK9 facilitates binding to low-density lipoprotein particles. *J Biol Chem* **295**, 2285–2298 (2020).
16. Poirier, S. *et al.* The proprotein convertase PCSK9 induces the degradation of low density lipoprotein receptor (LDLR) and its closest family members VLDLR and ApoER2. *J Biol Chem* **283**, 2363–2372 (2008).
17. Amput, P. *et al.* The effects of proprotein convertase subtilisin/kexin type 9 inhibitors on lipid metabolism and cardiovascular function. *Biomed Pharmacother* **109**, 1171–1180 (2019).
18. Giugliano, R. P. *et al.* Stroke Prevention With the PCSK9 (Proprotein Convertase Subtilisin-Kexin Type 9) Inhibitor Evolocumab Added to Statin in High-Risk Patients With Stable Atherosclerosis. *Stroke* **51**, 1546–1554 (2020).
19. Nozue, T. Lipid Lowering Therapy and Circulating PCSK9 Concentration. *J Atheroscler Thromb* **24**, 895–907 (2017).

20. Taylor, B. A. & Thompson, P. D. Statins and Their Effect on PCSK9—Impact and Clinical Relevance. *Curr Atheroscler Rep* **18**, 46 (2016).
21. Vivian, J. P. *et al.* Killer cell immunoglobulin-like receptor 3DL1-mediated recognition of human leukocyte antigen B. *Nature* **479**, 401–405 (2011).
22. O'Connor, G. M. & McVicar, D. The yin-yang of KIR3DL1/S1: molecular mechanisms and cellular function. *Crit Rev Immunol* **33**, 203–218 (2013).
23. Gao, X. *et al.* Angiopoietin-like protein 3 markedly enhanced in the hyperlipidemia related proteinuria. *Lipids in Health and Disease* **18**, 116 (2019).
24. Reeskamp, L. F. *et al.* Statin therapy reduces plasma angiopoietin-like 3 (ANGPTL3) concentrations in hypercholesterolemic patients via reduced liver X receptor (LXR) activation. *Atherosclerosis* **315**, 68–75 (2020).
25. Jiang, S. *et al.* ANGPTL3: a novel biomarker and promising therapeutic target. *J Drug Target* **27**, 876–884 (2019).
26. Wang, X. & Musunuru, K. Angiopoietin-Like 3: From Discovery to Therapeutic Gene Editing. *JACC Basic Transl Sci* **4**, 755–762 (2019).
27. Hussain, A. *et al.* Triglyceride-rich lipoproteins, apolipoprotein C-III, angiopoietin-like protein 3, and cardiovascular events in older adults: Atherosclerosis Risk in Communities (ARIC) study. *Eur J Prev Cardiol* zwaa152 (2021) doi:10.1093/eurjpc/zwaa152.
28. Stitziel, N. O. *et al.* ANGPTL3 Deficiency and Protection Against Coronary Artery Disease. *J Am Coll Cardiol* **69**, 2054–2063 (2017).
29. Christopoulou, E., Elisaf, M. & Filippatos, T. Effects of Angiopoietin-Like 3 on Triglyceride Regulation, Glucose Homeostasis, and Diabetes. *Disease Markers* **2019**, e6578327 (2019).

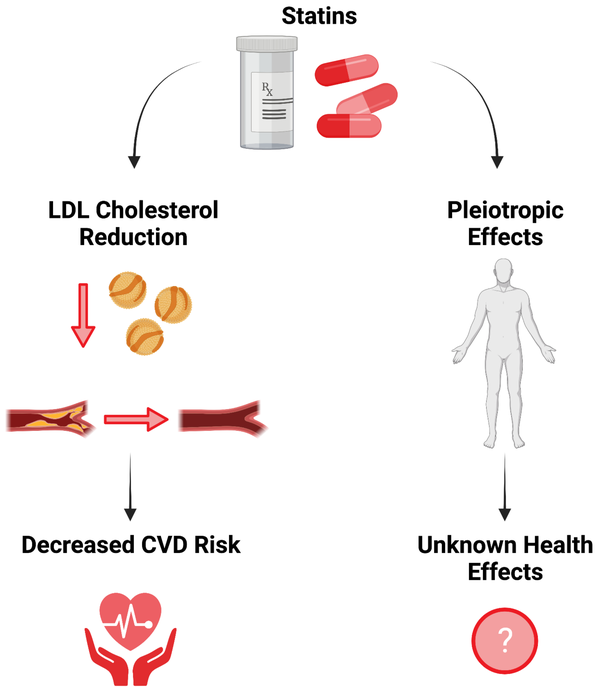
30. Arai, H., Koizumi, H., Aoki, J. & Inoue, K. Platelet-activating factor acetylhydrolase (PAF-AH). *J Biochem* **131**, 635–640 (2002).
31. Tsantila, N. *et al.* In vitro and in vivo effects of statins on platelet-activating factor and its metabolism. *Angiology* **62**, 209–218 (2011).
32. Ryu, S. K. *et al.* Phospholipase A2 enzymes, high-dose atorvastatin, and prediction of ischemic events after acute coronary syndromes. *Circulation* **125**, 757–766 (2012).
33. Stafforini, D. M. & Zimmerman, G. A. Unraveling the PAF-AH/Lp-PLA2 controversy1. *J Lipid Res* **55**, 1811–1814 (2014).
34. Zhang, B. *et al.* Modulating effects of cholesterol feeding and simvastatin treatment on platelet-activating factor acetylhydrolase activity and lysophosphatidylcholine concentration. *Atherosclerosis* **186**, 291–301 (2006).
35. Chen, C.-H. Platelet-activating factor acetylhydrolase: is it good or bad for you? *Curr Opin Lipidol* **15**, 337–341 (2004).
36. Marathe, G. K. *et al.* To hydrolyze or not to hydrolyze: the dilemma of platelet-activating factor acetylhydrolase. *J Lipid Res* **55**, 1847–1854 (2014).
37. deFilippi, C. *et al.* Differential Plasma Protein Regulation and Statin Effects in Human Immunodeficiency Virus (HIV)-Infected and Non-HIV-Infected Patients Utilizing a Proteomics Approach. *J Infect Dis* **222**, 929–939 (2020).
38. DEFILIPPI, C. *et al.* Novel Mediators of Statin Effects on Plaque in HIV: A Proteomics Approach. *AIDS* **32**, 867–876 (2018).
39. Xiang, A. *et al.* PCOLCE Is Potent Prognostic Biomarker and Associates With Immune Infiltration in Gastric Cancer. *Front Mol Biosci* **7**, 544895 (2020).

606 40. Kessler, E. & Hassoun, E. Procollagen C-Proteinase Enhancer 1 (PCPE-1) in Liver
607 Fibrosis. *Methods Mol Biol* **1944**, 189–201 (2019).
608 41. Kessler-Icekson, G., Schlesinger, H., Freimann, S. & Kessler, E. Expression of procollagen
609 C-proteinase enhancer-1 in the remodeling rat heart is stimulated by aldosterone. *Int J*
610 *Biochem Cell Biol* **38**, 358–365 (2006).
611 42. Mei, D., Zhu, Y., Zhang, L. & Wei, W. The Role of CTHRC1 in Regulation of Multiple
612 Signaling and Tumor Progression and Metastasis. *Mediators Inflamm* **2020**, 9578701
613 (2020).
614 43. Qin, S. *et al.* CTHRC1 promotes wound repair by increasing M2 macrophages via
615 regulating the TGF-β and notch pathways. *Biomed Pharmacother* **113**, 108594 (2019).
616 44. Myngbay, A., Manarbek, L., Ludbrook, S. & Kunz, J. The Role of Collagen Triple Helix
617 Repeat-Containing 1 Protein (CTHRC1) in Rheumatoid Arthritis. *Int J Mol Sci* **22**, 2426
618 (2021).
619 45. Ruiz-Villalba, A. *et al.* Single-Cell RNA Sequencing Analysis Reveals a Crucial Role for
620 CTHRC1 (Collagen Triple Helix Repeat Containing 1) Cardiac Fibroblasts After Myocardial
621 Infarction. *Circulation* **142**, 1831–1847 (2020).
622 46. Sial, N. *et al.* CTHRC1 expression is a novel shared diagnostic and prognostic biomarker of
623 survival in six different human cancer subtypes. *Sci Rep* **11**, 19873 (2021).
624 47. Mancini, G. B. J. *et al.* Diagnosis, prevention, and management of statin adverse effects
625 and intolerance: proceedings of a Canadian Working Group Consensus Conference. *Can J*
626 *Cardiol* **27**, 635–662 (2011).

48. Martin, P. M. *et al.* Identity of SMCT1 (SLC5A8) as a neuron-specific Na+-coupled transporter for active uptake of L-lactate and ketone bodies in the brain. *J Neurochem* **98**, 279–288 (2006).
49. Nakanishi, H. Microglial cathepsin B as a key driver of inflammatory brain diseases and brain aging. *Neural Regen Res* **15**, 25–29 (2019).
50. Hu, T. *et al.* Value of serum collagen triple helix repeat containing-1(CTHRC1) and 14-3-3η protein compared to anti-CCP antibodies and anti-MCV antibodies in the diagnosis of rheumatoid arthritis. *Br J Biomed Sci* **78**, 67–71 (2021).
51. Mijanović, O. *et al.* Cathepsin B: A sellsword of cancer progression. *Cancer Lett* **449**, 207–214 (2019).
52. Hurks, R. *et al.* Different effects of commonly prescribed statins on abdominal aortic aneurysm wall biology. *Eur J Vasc Endovasc Surg* **39**, 569–576 (2010).
53. Smith, R. *et al.* Simvastatin inhibits glucose metabolism and legumain activity in human myotubes. *PLoS One* **9**, e85721 (2014).
54. Liao, Y.-H. *et al.* HMG-CoA reductase inhibitors activate caspase-1 in human monocytes depending on ATP release and P2X7 activation. *J Leukoc Biol* **93**, 289–299 (2013).

A Proteomic Approach for Investigating the Pleiotropic Effects of Statins in the Atherosclerosis Risk in Communities (ARIC) Study

Hypothesis: statins have pleiotropic effects that affect levels of proteins in pathways related to and unrelated to lipid metabolism.



ARIC

Proteomics Data
SOMAlogic

Visit 1 (1987-89) | Visit 2 (1990-92) | Visit 3 (1993-95)

Statins

Exclusion of statin users pre-Visit 2

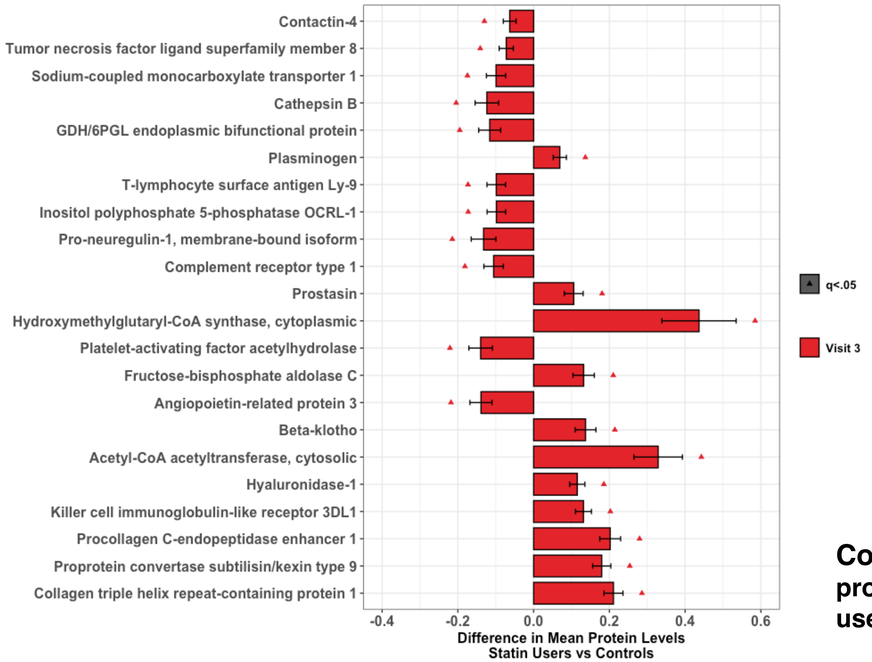
New Statin Users

Nonusers (Controls)

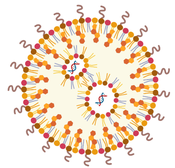
Propensity Score Matching (1:1)

360 Matched Pairs

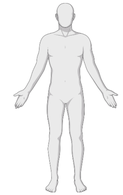
Differences in Protein Levels Between Statin Users and Controls



t-test: $\log_2$(protein levels) ~ statin use (yes or no)
FDR adjustment for multiple comparisons



Proteins Involved in Lipid Metabolism



Proteins Involved in Other Processes (unrelated to lipids)

Conclusions: Levels of several proteins differed between statin users and controls.

Exploring the biological functions of these proteins could elucidate the pleiotropic effects of statins.

[Figures made with BioRender]