Title: Statin-dependent and -independent pathways are associated with major adverse cardiovascular events in people with HIV

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Short Title: Proteomic markers of future MACE in HIV

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ABSTRACT

Background: Statin therapy lowers the risk of major adverse cardiovascular events (MACE) among people with HIV (PWH). Residual risk pathways contributing to excess MACE beyond low-density lipoprotein cholesterol (LDL-C) are not well understood. Our objective was to evaluate the association of statin responsive and other inflammatory and metabolic pathways to MACE in the Randomized Trial to Prevent Vascular Events in HIV (REPRIEVE).

Methods: Cox proportional hazards models were used to assess the relationship between MACE and proteomic measurements at study entry and year 2 adjusting for time-updated statin use and baseline 10-year atherosclerotic cardiovascular disease risk score. We built a machine learning (ML) model to predict MACE using baseline proteins values with significant associations.

Results: In 765 individuals (age: 50.8±5.9 years, 82% males) among 7 proteins changing with statin vs. placebo, angiopoietin-related protein 3 (ANGPTL3) related most strongly to MACE (aHR: 2.31 per 2-fold higher levels; 95%CI: 1.11-4.80; p=0.03), such that lower levels of ANGPTL3 achieved with statin therapy were associated with lower MACE risk. Among 248 proteins not changing in response to statin therapy, 26 were associated with MACE at FDR<0.05. These proteins represented predominantly humoral immune response, leukocyte chemotaxis, and cytokine pathways. Our proteomic ML model achieved a 10-fold cross-validated c-index of 0.74±0.11 to predict MACE, improving on models using traditional risk prediction scores only (c-index: 0.61±0.18).

Conclusions: ANGPTL3, as well as key inflammatory pathways may contribute to residual risk of MACE among PWH, beyond LDL-C.

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Keywords: people with HIV; major adverse cardiovascular events; proteomics; risk prediction; machine learning

ABBREVIATIONS

ANGPTL3	Angiopoietin-related protein 3
ASCVD	Atherosclerotic cardiovascular disease
FDR	False discovery rate
HDL-C	High-density lipoprotein cholesterol
HR	Hazard ratios
IQR	Interquartile ranges
LDL-C	Low-density lipoprotein cholesterol
MACE	Major adverse cardiac events
PWH	People with HIV
STRING	Search tool for the retrieval of interacting genes/proteins

INTRODUCTION

People with HIV (PWH) are known to be at higher risk of major adverse cardiac events (MACE) (1-3). The Randomized Trial to Prevent Vascular Events in HIV (REPRIEVE) demonstrated a 36% reduction in MACE rates among PWH randomized to pitavastatin treatment as compared to placebo over a median of 5.6 years (4, 5). The effects on MACE appeared larger than could be accounted for changes in low-density lipoprotein cholesterol (LDL-C) alone (4). To assess statin effects beyond LDL-C, we previously assessed 255 proteins on 3 Olink platforms (Cardiovascular III, Immuno-oncology and Cardiometabolic panels) within the REPRIEVE mechanistic substudy, showing specific proteins, representing inflammatory and other lipid pathways beyond LDL-C, changed in response to statin therapy. Notably among these proteins, levels of angiopoietin-related protein 3 (ANGPTL3), a protein known to be associated with cardiovascular disease in the general population, decreased with statin therapy (6, 7).

Our objective in this analysis was to further leverage the REPRIEVE mechanistic study to assess the relationship of relevant protein pathways to MACE in PWH. We first evaluated the relationship of statin responsive pathways to incident MACE. Second, we sought to identify inflammatory and immunological markers of residual cardiovascular risk that are associated with MACE in unadjusted and adjusted models accounting for atherosclerotic cardiovascular disease (ASCVD) risk and statin use. Together these data inform the field of relevant mechanistic pathways and potential therapeutic targets beyond LDL-C lowering to prevent MACE in PWH.

RESULTS

Study population

Between 2015 and 2018, 804 participants were randomized in the REPRIEVE mechanistic sub-study. For our current analysis, 39 individuals were excluded due to availability, sampling time and quality of proteomic measurements leading to 765 individuals (379 randomized to pitavastatin and 386 to placebo respectively) being included in our analyses (Figure 1). The average age of our participants was 50.8±5.9 years and 82% (n=631) were males. The individuals were predominantly white non-Hispanic participants or Latino participants. The mean duration of ART was 11.8±6.6 years at baseline. The average 10-year ASCVD risk score of the individuals was 5.0±3.1% and the baseline LDL-C levels were 108.2±29.8 mg/dL and triglyceride levels 133.7 ± 83.2 mg/dL (Table 1).

Over the average follow-up of 5.8±1.9 years in the mechanistic substudy (median: 6.2 years, IQR: 5.5; 7.1 years), 33 participants experienced a MACE event. Participants who experienced MACE were older (53.2 ± 5.4 vs. 50.7 ± 6.0 years respectively), had higher 10-year ASCVD risk scores (6.9 ± 3.6 vs. 5.0 ± 3.1 respectively), were more likely to be current smoker (52% vs. 24% respectively) and had higher high sensitivity C-reactive protein levels (4.5 ± 3.3 vs. 2.8 ± 2.9 mg/L respectively, Table 1). In our analysis population, 20 individuals had a hard MACE (6 cardiovascular deaths, 5 myocardial infarctions, 9 strokes, Supplemental table 1).

Association of statin responsive proteins with MACE

Among a previously identified set of 7 proteins changing in response to pitavastatin treatment (6), 4 proteins showed decreased expression in response to statin therapy and 3 showed increased

expression. In our time-updated Cox models utilizing both baseline and 2-year proteomic measurements, only ANGPTL3 had a significant association with MACE (HR: 2.20 per 2-fold higher protein level; 95%CI: 1.04-4.67; p=0.04 which corresponds to a HR: 1.40; 95%CI: 1.02-1.92 per population standard deviation increase in NPX values), such that lower levels of ANGPTL3 achieved with statin therapy were associated with lower MACE levels. Adjusting our models for 10-year ASCVD risk score and statin use resulted in similar results (HR: 2.31; 95%CI: 1.11-4.80; p=0.03 which corresponds to a HR: 1.43; 95%CI: 1.04-1.96 per population standard deviation increase in NPX values). No other proteins were significantly associated with MACE (Table 2). ANGPTL3 was also significantly associated with hard MACE (HR: 3.27; 95%CI: 1.32-8.13; p=0.01 which corresponds to a HR: 1.66; 95%CI: 1.13-2.45 per population standard deviation increase in NPX values), and this relationship remained consistent in adjusted models accounting for 10-year ASCVD risk score and statin use (HR: 3.46; 95%CI: 1.43-8.53; p=0.005 which corresponds to a HR: 1.70; 95%CI: 1.17-1.48 per population standard deviation increase in NPX values). Supplemental analyses using statin randomization rather than time updated statin use showed similar results (Supplemental Table 2).

In our study population, individuals randomized to statin therapy demonstrated an 11.1% (95%CI: -15.3%; -6.8%, p<0.0001) reduction in ANGPTL3 levels, 15.3 mg/dL (95%CI: -27.4 mg/dL; -3.1 mg/dL; p=0.013) reduction in triglyceride levels and a 27.8 mg/dL (95%CI: -32.5 mg/dL; -23.1 mg/dL; p<0.001) reduction in LDL-C levels from baseline to 2-year follow-up compared to those randomized to placebo. In mediation analysis, 29.8% (95%CI: 11.6%; 52.5%) of the association between statin therapy and triglyceride levels, and 5.9% (95%CI: 1.87%; 10.8%) of the association between statin therapy and LDL-C levels appeared mediated through

the effect of statins on ANGPTL3. We thus further adjusted our analyses relating ANGPTL3 to MACE for time updated LDL-C and triglyceride levels, demonstrating similar results and persistent relationship of ANGPTL3 to MACE (Supplemental table 3).

Inflammatory, immune-oncological and cardiometabolic proteomic markers related to MACE in residual risk analyses.

Among the remaining 248 remaining proteins in the proteomic panels (Supplemental Figure 1), which did not significantly change with statin treatment, in our time-updated Cox models utilizing both baseline and 2-year proteomic measurements, 26 were associated with MACE at an FDR<0.05 after adjusting for statin use and 10-year ASCVD risk scores. Among these, receptor for interleukin-7 (IL7R; HR: 0.29; 95%CI: 0.13; 0.65; p=0.003 which corresponds to a HR: 0.59; 95%CI: 0.42-0.83 per population standard deviation increase in NPX values) and paraoxonase 3 (PON3; HR: 0.45; 95%CI: 0.29; 0.68; p=0.0001 which corresponds to a HR: 0.55; 95%CI: 0.41-0.75 per population standard deviation increase in NPX values) were associated with a lower risk of MACE. All other proteins were associated with an increased the risk of MACE. The effect sizes of the remaining 24 proteins ranged from a hazard ratio of 1.58 to 9.20 per protein doubling (Figure 2, Supplemental table 4). Similar results were seen in supplemental analyses adjusting for statin randomization rather than time-updated statin use (Supplemental Figure 2). Numerically, similar results were also observed for hard MACE (Supplemental table 5). Among the 12 proteins with an FDR<0.05 association with hard MACE, 10 were also identified for MACE. Only EGF-containing fibulin-like extracellular matrix protein 1 (EFEMP1) and Retinoic acid receptor responder protein 2 (RARRES2) were identified as associated with hard MACE but did not reach statistical significance for the MACE outcome. Detailed results,

protein names and abbreviations for proteins identified in these analyses can be found in Table 3. In sensitivity analyses, further adjusting for LDL-C and triglyceride levels, numerically similar results for all proteins (Supplemental figure 3).

Correlation among proteins related to MACE and relation to baseline clinical factors

ANGPTL3 related to multiple proteins, and most significantly to Urokinase plasminogen activator surface receptor (UPAR, ρ=0.54). In contrast, other proteins related to MACE showed only moderate correlation with each other. Proteins related to MACE were only weakly correlated with clinical factors and clinically assessed biomarkers. Detailed correlation heatmap can be found in Supplemental Figure 4 and Supplemental Figure 5.

Protein-protein interaction analysis for non-statin modifiable proteins

For the 28 proteins showing a significant association with MACE or hard MACE at an FDR<0.05 level in residual risk analyses, we created an exploratory protein-protein interaction network to further understand the biological role of these proteins. All proteins except PON3 and Transcobalamin-2 (TCN) were part of a single cluster (average number of connections: 6.21) where Interleukin-6 (IL6) and Interleukin-8 (IL8) were connected to the greatest number of other proteins (n=21 and n=18 respectively, Figure 3). Exploratory gene ontology biological processes enrichment showed an increased representation of leukocyte associated chemotaxis and migration and humoral immune response. Gene ontology molecular function enrichment indicated an increased cytokine and chemokine associated functions in our network. Reactome pathway analysis indicated an increased enrichment of tumor necrosis factor, cytokine and

chemokine pathway functions within our significant proteins. Detailed enrichment results can be found in Figure 3, with lists of proteins corresponding to each term in Supplemental table 6.

Diagnostic power of proteomic markers to predict MACE beyond traditional risk indices

To account for collinearity between the proteomic features, avoid overfitting, and evaluate the additive value of significant proteomic features, we built a machine learning elastic net Cox proportional hazards model using the significant proteomic markers, treatment randomization, and 10-year ASCVD risk score, using stratified 10-fold cross validation. We performed 3 models. Model-1, incorporating statin randomization and 10-year ASCVD risk score, achieved a 10-fold cross-validated c-index of 0.61 (standard deviation: 0.18) for MACE. In contrast, Model-2 incorporating only baseline protein values, demonstrated a cross-validated c-index of 0.74 (standard deviation: 0.12). Model-3, including ASCVD risk, statin randomization and protein values similarly demonstrated a cross-validated c-index of 0.74 (standard deviation: 0.11). Lysosome-associated membrane glycoprotein 3 (LAMP3) had the largest HR among proteins positively associated with MACE, and PON3 had the lowest HR among proteins negatively associated with MACE (Figure 4).

DISCUSSION

In this analysis of the REPRIEVE mechanistic substudy, we identified several novel protein pathways related to MACE in PWH. Among these, *ANGPTL3* is notable, as it inhibits the uptake of triglyceride-rich lipoprotein remnants and was previously identified to decrease in response to statin therapy among PWH in REPRIEVE. We now demonstrate that lower levels of *ANGPTL3* achieved with statin therapy were associated with lower MACE levels. We further identified an additional 26 proteins associated with MACE risk in residual risk analyses accounting for statin use. Enrichment analysis identified an over representation of cytokine and chemokine processes and pathways representing a residual inflammatory risk within this cohort of PWH. A cross-validated machine learning model using the baseline values of these proteins outperformed a model including 10-year ASCVD risk and statin randomization.

ANGPTL3 is an endothelial lipase inhibitor, secreted from the liver, which inhibits lipoprotein lipase, important to overall lipid metabolism, regulation and coronary artery disease (8, 9). By inhibiting lipoprotein lipase, ANGPTL3 interferes with the breakdown and clearance of triglyceride from triglyceride rich particles including VLDL and chylomicrons. Individuals with loss of function variants in ANGPTL3 have lower triglycerides, HDL-C and LDL-C, and reduced cardiovascular disease (10-13). Prior studies have shown that plasma ANGPTL3 levels were 15% lower in statin treated familial hypercholesterolemia patients compared to statin naïve (14). In the ARIC study, ANGPTL3 levels were significantly reduced in a propensity score matched analysis of statin users vs nonusers (15). We now further support these statin effects within PWH, showing in our prior analyses from REPRIEVE, that ANGPTL3 was reduced in individuals randomized to the pitavastatin group by 11% compared to placebo (6). Also, the

effect of statin therapy on circulating ANGPTL3 is supported by evidence from human interventional trials showing a reduction in liver expression of ANGPTL3 in response to statin therapy (16). However, the association between ANGPTL3 and MACE has not yet been previously established.

In REPRIEVE, baseline levels of LDL-C and triglyceride were only modestly increased and often normal. Despite modest baseline LDL-C levels, statin therapy had a major effect to reduce MACE by 36% in REPRIEVE. In the present study, ANGPTL3 appeared to mediate, in part, statin related changes in triglyceride and to a lesser extent LDL-C levels, consistent with the known effects of ANGPTL3 to primarily regulate triglyceride levels. Furthermore, the HR of MACE and hard MACE were increased with higher ANGPTL3 levels, such that lower levels of ANGPTL3 achieved with statin therapy were associated with lower MACE levels. Of note, these relationships remained consistent controlling for 10-year ASCVD risk score, and also in sensitivity analyses controlling for LDL-C and triglyceride, suggesting this may be a direct effect related to ANGPTL3 effects on the vasculature independent of effects on lipid levels.

Given that the effects of ANGPTL3 reduction on LDL-C have been shown in LDL-receptor deficient mice (13), strategies to improve ANGPTL3 may be additive to statin effects. In addition, multiple prior studies have shown insulin resistance in addition to hypertriglyceridemia among PWH, both of which are also known to be regulated by ANGPTL3 (17, 18). Emerging new antisense oligonucleotides, monoclonal antibodies and gene editing solutions to reduce ANGPTL3 levels have shown promise to reduce triglyceride, LDL-C, and, importantly, in coronary artery lesion size (8, 9, 19), suggesting a mechanism whereby directly

modulating ANGPTL3 might affect subsequent MACE. Analyses between people with and without HIV have not shown significant differences in circulating ANGPTL3 levels, suggesting that our results may not be specific to PWH (20). Future studies are now needed to validate of our findings linking ANGPTL3 to MACE and to assess the potential beneficial effects of new therapeutics targeting ANGPTL3 on clinical outcomes in PWH and the general population.

This proteomic analysis informs our understanding of pathways potentially contributing to MACE beyond LDL-C among PWH. These data of almost 300 proteins chosen from key cardiometabolic, immune and inflammatory domains, highlights that many critical pathways may be perturbed in ART treated PWH, independent of ASCVD risk and statin use, contributing to ongoing low-grade inflammation and immune activation and MACE. Prior work has shown that HIV persists in viral reservoirs in T cells and macrophages, despite stable ART, contributing to this phenomenon (21). The current work helps to define the particular pattern of proteins and pathways related to MACE among PWH receiving effective ART

In our exploratory enrichment analysis, biomarkers found to be related to MACE in this study represent key related pathways in cytokine regulation, immune response and chemotaxis. For example, chemotactic factors such as CXCL13 and growth factors driving myelopoiesis such as CSF1 and were tightly interconnected with a central positioning of IL6 and IL8, both cytokines signifying innate, specifically myeloid, immune activation with a known prominent role in atherosclerosis in network analyses. It is of interest to note that in Canakinumab Anti-inflammatory Thrombosis Outcome Study (CANTOS), decreases in IL6 were associated with the observed clinical benefit both in terms of MACE and cancer and it is not surprising that IL6

targeting is now being pursued in atherosclerosis (22). Among PWH the presence of IL6 and also UPAR have been linked to non-AIDS malignancies as well as chronic kidney disease or osteoporosis (23, 24). The strong relationship of ANGTPL3 and UPAR is notable given the central role of UPAR in vascular, fibrotic and coagulation inflammatory pathways and its prior association with non-AIDS events in PWH (25). Future analyses in the main REPRIEVE trial population will relate IL6 and other inflammatory markers to MACE, cancer endpoints, and other comorbidities, to better guide development of novel therapies for different long-term co-morbidities in PWH. It is further important to recognize that modifying systemic factors as well as local biological factors at the plaque may both be important and independent of each other. For example, in prior work we have shown that increased statin-induced expression of collagen forming genes may help to reduce fatty vulnerable plaque (6), though PCOLCE was not significantly associated with a reduction in MACE in the current analysis.

In our exploratory enrichment analysis, we observed that many myeloid-cell associated markers were featured prominently in our network analysis and strongly associated with incident MACE. These data further reinforce the prominent role played by myeloid cells in atherogenesis, including initiation and progression with recruitment of monocytes to the foam cells, release of cytokines by engulfed foam-cell resident macrophages, and potential effects of factors to destabilize rupture-prone plaque and increase thrombosis. The inclusion of many of the non-statin modifiable proteins in enrichment pathways related to myeloid functions of potential impact on atherogenesis, suggests that dysregulation of these pathways in PWH may contribute disproportionately to plaque formation and destabilization and should be targeted for future experimentation and therapeutics beyond statin therapy.

These data further beg the question as to whether clonal hematopoiesis driver mutations may be more common in these patients with this particular inflammatory signature (26). Indeed, recent data from our group and others demonstrated a 2-fold increase in clonal hematopoiesis of indeterminate potential among PWH in the Swiss HIV Cohort vs a matched group of participants in the ARIC cohort (27). These data from the current analysis of REPRIEVE add to our knowledge of a potential role of myeloid dysregulation with respect to MACE in low-moderate risk PWH, on stable ART.

The data in this analysis highlight residual inflammatory pathways that may contribute to MACE, and potentially to other comorbidities in well treated PWH on ART. Although some of the pathways identified in the analysis are statin modifiable, others are not, consistent with data that that trained immunity (more pronounced innate immune responses in previously stimulated cells) persists after statin therapy (28). In contrast, the statin modifiable pathways appear more likely to prevent ASCVD in PWH through effects on plaque stabilization, lipid lowering and improved vascular health, which we now show may relate to statin regulation of ANGPTL3 (6).

Of clinical relevance, we demonstrate in cross validated time dependent Cox elastic net machine learning models that novel proteins identified in this study can increase prediction for MACE. The traditional risk scoring model achieved a modest c-index from 0.61, similar to previously published results in this cohort (29). Adding proteins to the model increased diagnostic performance to a c-index of 0.74. In terms of individual proteins, LAMP3, a lysosome-associated membrane protein that plays a role in various cellular processes, including cell differentiation, autophagy, and immune responses was most significantly related with an HR

well beyond that of ASCVD alone, whereas PON3 was inversely related to a significant degree as a protective pathway. LAMP3 is involved in antigen processing during the immune response and is thought to be linked with the maturation of dendritic cells and potentially a marker of cardiac remodeling though little is known how it relates to atherosclerotic cardiovascular disease (30, 31). External validation of our findings is needed to assess the additive value of proteomic profiling to identify individuals at increased risk of MACE in PWH. Further studies leveraging the larger REPRIEVE cohort will assess these proteins as potentially relevant mechanistic pathways and biomarkers for MACE in PWH.

Our analysis has strengths but some limitations. The number of events is lower than in the overall REPRIEVE trial due to the smaller size of the mechanistic substudy. Due to the limited number of events, our estimates are characterized by a degree of uncertainty, seen in the wide confidence ranges for some of our parameters. Our results are from a population of PWH. Therefore, we cannot evaluate whether our findings are specific to this population or may also apply to the general population. Although our results are highly relevant for primary CVD prevention in those PWH with low-moderate CVD risk, different results might be seen in those with higher CVD risk. Analyses are ongoing to assess key pathways in the overall trial. In addition, the data that we can analyze are limited to the proteins included in the selected panels, and future studies will address other pathways not fully represented in these panels, for example to follow up signals we have seen for statins to reduce Interleukin-1 beta (IL1B) and potentially modulate inflammasome mediated processes (32). Also, the selective coverage of our targeted proteomic approach may not be optimal for enrichment results. Future analyses within the larger REPRIEVE cohort with extended proteomic coverage will provide further validation of our

enrichment results. Furthermore, study specific NPX values of our Olink proteomic analysis preclude the direct translation to other populations. Future analyses using exact protein concentrations will be needed to define normal values and optimal cut-offs for clinical implementation.

These data demonstrate novel pathways beyond LDL that relate to MACE among PWH. Additional interventions beyond statin therapy targeting these pathways may be useful to decrease MACE in PWH by reducing non LDL-related lipid mechanisms and residual systemic inflammation. In particular ANGPTL3, the IL6 axis and related pathways may represent the most obvious targets, but heretofore unrecognized pathways including LAMP3 should also be explored as targets. Relevant protein markers may also be used to improve cardiovascular risk prediction in PWH.

METHODS

Sex as a biological variable

Sex was not considered a biological variable in this study. Our study examined male and female humans, without making any distinction between sexes.

Study design

Detailed study designs of the REPRIEVE randomized clinical trial (NCT02344290) and the US-based nested mechanistic substudy have been published (33, 34). In brief, the REPRIEVE trial enrolled participants with HIV, 40-75 years of age receiving antiretroviral therapy for at least 6 months with low to moderate 10-year ASCVD risk scores defined using the 2013 ACC/AHA Pooled Cohort Equation. Participants with prior statin use within 90-days of enrollment or prior history of ASCVD were excluded (33). At 31 US sites, participants were given the opportunity to co-enroll in the mechanistic substudy and underwent additional blood and imaging analyses (34). Detailed inclusion and exclusion criteria are available in the published study protocols (4, 32). The current analysis is a post hoc analysis of proteomic data, performed to assess the relationship of baseline and time updated inflammatory and immune activation biomarkers to the primary clinical trial endpoint of MACE.

Study population

Overall, 804 participants were randomized in the mechanistic substudy. In our current analyses, we included all participants who had a valid proteomic measurement before the start of randomized treatment. Proteomic measurements were excluded based on routine proteomic

measurement quality control criteria (detailed below). Consort diagram of participant exclusions is shown in Figure 1.

Proteomic analysis

Enrollment and 2-year fasting plasma samples stored at -80ºC were used to measure protein levels. Three commercially available multiplex immunoassays were used: Olink (Thermo Fisher Scientific Inc.) Target 96: Cardiovascular III, Immuno-oncology and Cardiometabolic to measure 275 unique proteins. We excluded participants in whom complete panels were labeled with warnings indicating potential issues with measurement accuracy. We excluded proteins from the analysis if more than 50% of all samples were lower than the lower limit of detection (Supplemental Figure 1) (35-37). Overall, 255 proteins were evaluated. Proteins are expressed in Normalized Protein Expression values which are on $\log_2$ scale.

Lipid measurements

As part of REPRIEVE, fasting blood samples at baseline and 2-years were used to measure LDL-C, high-density lipoprotein cholesterol (HDL-C), total cholesterol and triglyceride levels. Lipids were measured at Quest Diagnostics.

Model building assessing relationship of protein pathway to MACE The goal of the present analysis was to relate the protein pathways to MACE. We first related time updated protein levels to incident MACE in unadjusted analyses. Subsequently, our analyses adjusted for ASCVD risk score and any use of statins during the follow-up period. Individuals underwent regular study evaluations every 4-months according to the REPRIEVE protocol. Changes to

study drug and other medications, including use of non-study statins, were recorded at each visit. Of note, participants stopping randomized treatment or starting non-study statins continued REPRIEVE follow-up unchanged, as previously described (4, 5). Using the recorded medication logs we reconstructed the exact time intervals as to whether participants were either on or off statin therapy. We defined "on statin" time intervals to include use of the randomized statin treatment as per initial treatment assignment to the pitavastatin arm of the trial or receipt of a non-study statin as prescribed by clinical providers. Conversely, time intervals without statin use (i.e., randomized to placebo or holds in randomized pitavastatin) were registered as "off statin" time. We incorporated these time-updated statin use intervals in our statistical models to adjust for the effects of statin treatment on MACE. This method was used as our goal was to gain mechanistic insight, and thus most accurately assess for actual statin use over longitudinal follow up in the trial. In a supplemental analysis, we adjusted for ASCVD risk score and randomization, parallelling the intent to treat design of the trial.

Outcome definitions

The primary endpoint of MACE includes the composite of cardiovascular death, myocardial infarction, hospitalization for unstable angina, coronary, carotid or peripheral arterial revascularization, transient ischemic attack, stroke, and peripheral arterial ischemia and death due to unknown reason. Detailed definitions are provided in the study protocol (33, 34). We further defined hard MACE as: known cardiovascular death, myocardial infarction and stroke.

Statistical analysis

Continuous variables are presented as means and standard deviations or medians and interquartile ranges (IQR), while categorical parameters are shown as counts and percentages. Cox proportional hazard models were used to estimate the association between most recent protein expression and outcomes. Proteomic measurements (baseline and 2 year) were included as time dependent covariate into our models based on the date of the proteomic sampling. Our models were adjusted for baseline 10-year ASCVD risk and time-updated statin use, as previously described. Further analyses were done adding time-dependent LDL-C and triglyceride levels to the model to estimate the protein effect adjusted for current lipid profile. In addition, we performed supplemental analyses adjusting for initial statin randomization, rather than time updated statin use. Hazard ratios (HR) are shown per 2-fold higher levels of the protein value. Analysis of the previously identified seven proteins seen to change with statin therapy used a 5% type 1 error (6). Otherwise, multiple comparisons were adjusted for false discovery rate (FDR) by the Benjamini and Hochberg method using a 5% threshold for statistical significance (38). Survival analysis was done using the survival package (v.3.7-0) in R (39).

In sensitivity analyses, we used the psych (v.2.4.6.26) R package to estimate the proportion of randomized statin effects on triglyceride and LDL-C levels mediated through proteins of interest by calculating delta changes in both protein expression and triglyceride levels defined as difference between 2-year and baseline values. Confidence intervals of the mediated proportion were estimated using the bootstrap method.

Spearman's or point-biserial correlation was used on baseline proteomic values, lipid and inflammatory markers to evaluate association between these factors. Protein-protein interaction

analysis was done using the Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) database. Confidence-based protein interactions using all available interactions sources were analyzed using a medium confidence threshold (confidence score >0.4). Exploratory Gene ontology and Reactome functional enrichments were estimated using the whole genome as a background. P and FDR values of enrichment are not presented due to the possible bias of our limited set of biologically closely related proteins.

To estimate the additive value of baseline proteins to predict MACE and to evaluate the importance of each proteomic feature, we trained three elastic net Cox's proportional hazard's model with 50% lasso and 50% ridge regularization. Model-1: statin randomization + 10-year ASCVD risk score; Model-2: baseline values of proteins associated with MACE; Model-3: Model-1 + Model-2 (including statin randomization, 10-yr ASCVD risk score, and baseline protein level). We estimated cross-validated time dependent inverse probability weighted c-index values of an elastic net Cox model to calculate the diagnostic accuracy of our machine learning model over time. We also plotted the cross-validated average HRs per population standard deviation to predict MACE as feature importance values of each parameter for all three models where at least one of the HRs was different from 1 in one of the models. All machine learning survival analyses were done using the scikit-survival (v.0.23.1) python package (40-42).
All statistical calculations were done in the R (v.4.3.3) and Python (v.3.10.8).

Study approval

The REPRIEVE trial protocol was approved by the institutional review board of the Massachusetts General Brigham and by the ethics committees of each site. All participants

provided written informed consent. Results are reported in compliance with the Consolidated Standards of Reporting Trials. Trial registration: ClinicalTrials.gov Identifier: NCT02344290

Data availability

A Supporting data file containing all values for the data points in graphs and values behind any reported means is available in the supplemental materials. The Olink data have been added as a supplemental file (baseline values: Supplemental dataset 1, follow-up values: Supplemental dataset 2).

AUTHOR CONTRIBUTIONS

MK: performed statistical analysis, interpreted results, wrote the manuscript.
IS: interpreted results, helped in writing the manuscript
MVZ: participated in the conduction of the trial, reviewed manuscript draft
CJF: participated in the conduction of the trial, reviewed manuscript draft
JAA: participated in the conduction of the trial, reviewed manuscript draft
GSB: participated in the conduction of the trial, reviewed manuscript draft
CDM: participated in the conduction of the trial, reviewed manuscript draft
JSC: participated in the conduction of the trial, reviewed manuscript draft
SMC: reviewed manuscript draft
MRD: participated in clinical research coordination, reviewed manuscript draft
ABL: participated in clinical research coordination, reviewed manuscript draft
CD: reviewed manuscript draft
BF: participated in the conduction of the trial, reviewed manuscript draft
SM: collated data, provided statistical support, interpreted results, reviewed manuscript draft
CAS: reviewed manuscript draft
MTU: participated in the conduction of the trial, reviewed manuscript draft
PSD: designed the study, reviewed manuscript draft and provided funding support
HJR: provided statistical supervision, interpreted results, reviewed manuscript draft
SKG: designed the study, interpreted results, wrote the manuscript and provided funding support
All authors participated in the critical review of the final paper and submission.

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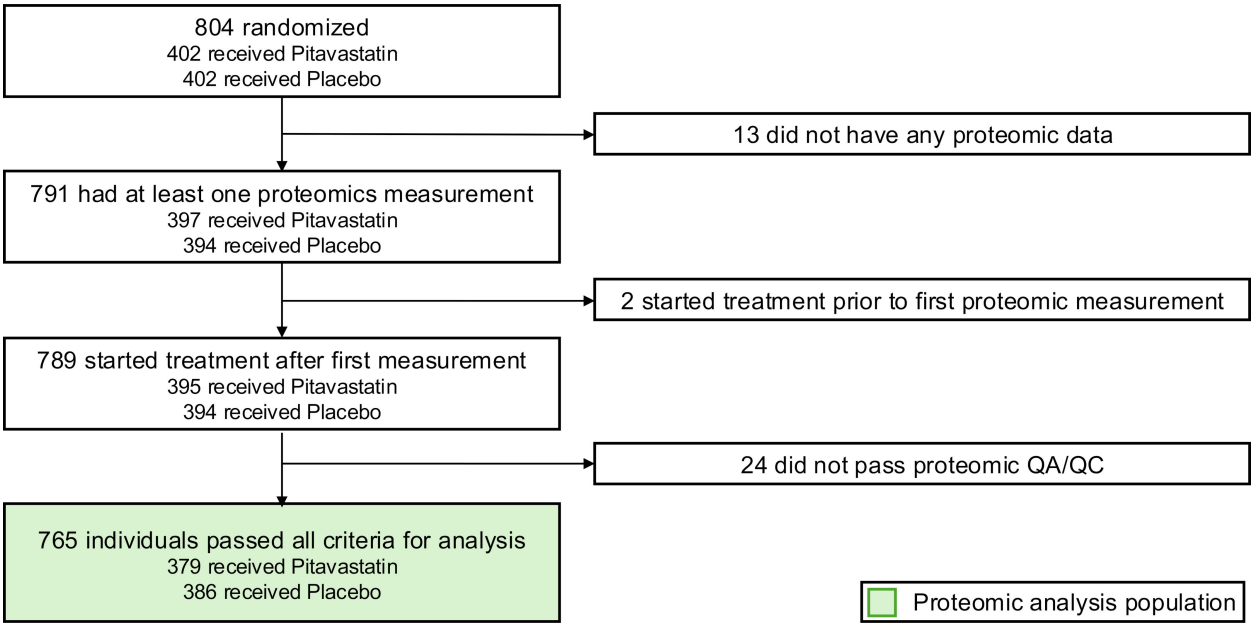
The views expressed in this manuscript are those of the authors and do not necessarily represent the views of the National Heart, Lung, and Blood Institute, the National Institute of Allergy and Infectious Diseases, the National Institutes of Health, or the U.S. Department of Health and Human Services.

FIGURES

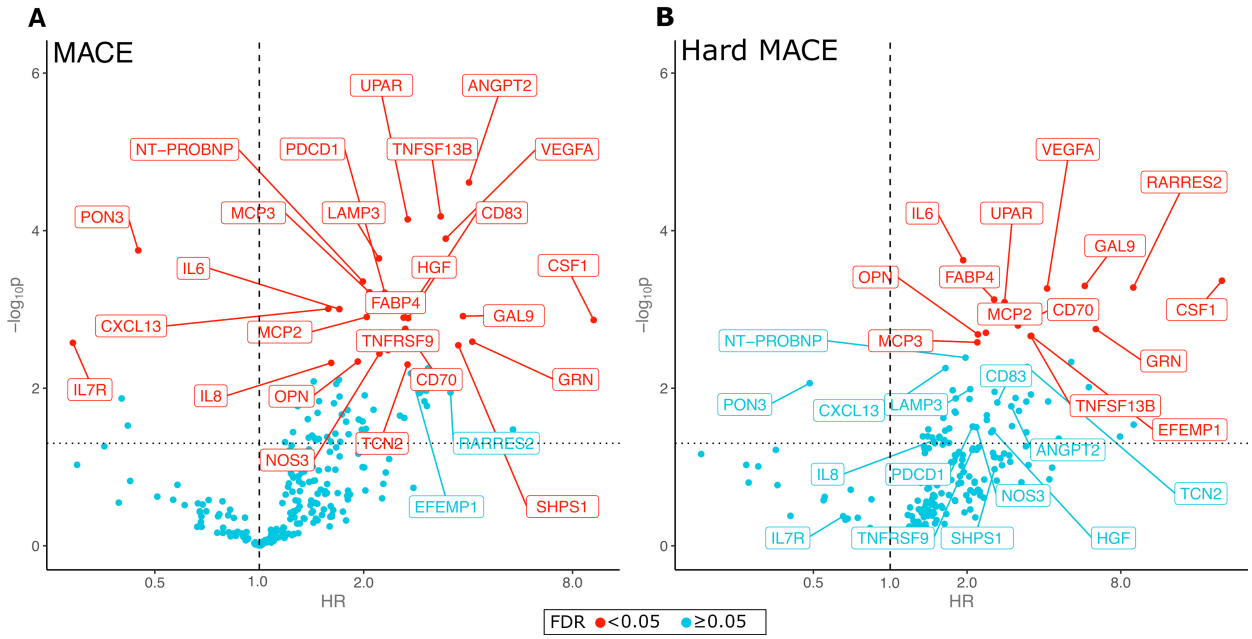
Figure 1. Consort diagram of individuals included in the current analysis.



Overall, 765 individuals were included in our analyses to evaluate the association between proteomic markers and major adverse cardiac events.

Abbreviations: *QA*, quality assurance; *QC*, quality control.

Figure 2. Association between proteomic markers and MACE or hard MACE.

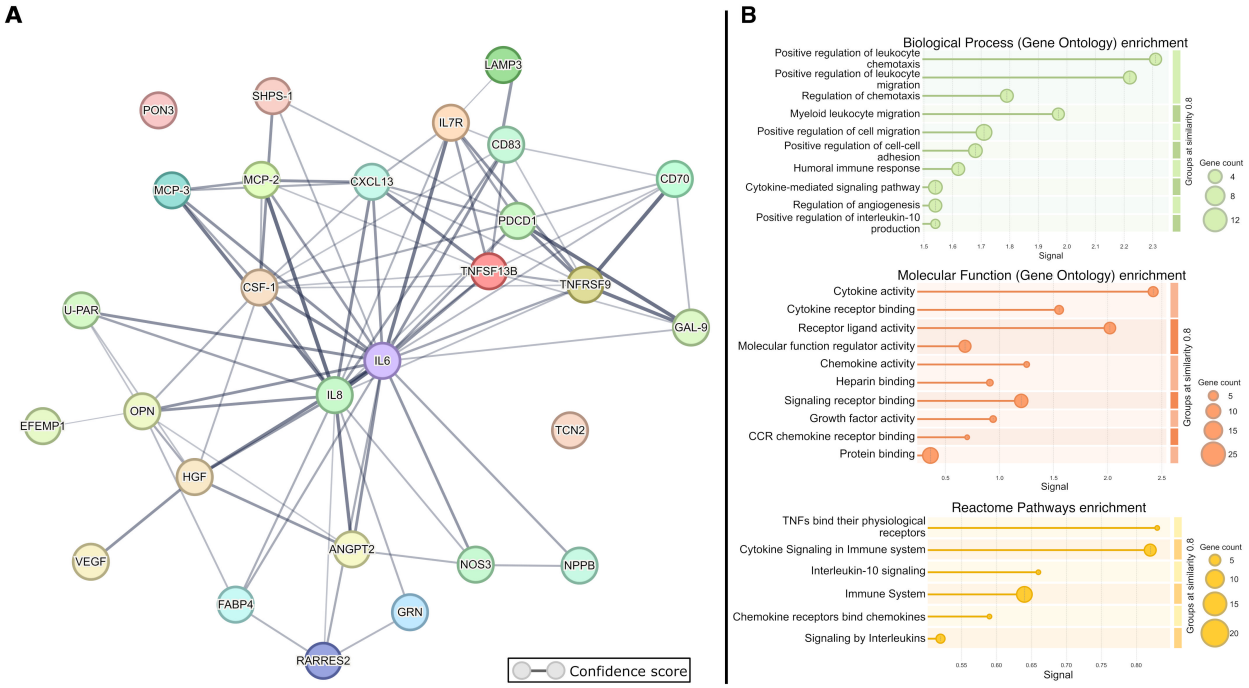


Panel A: Volcano plot of hazard ratios and p values for given proteins and MACE. Hazard ratios were estimated from Cox proportional hazard models using both potential proteomic measurements including statin use as a time-dependent covariate and baseline 10-year baseline ASCVD risk score. Hazard ratios are per protein doubling. Red proteins indicate associations at an FDR<0.05 level. X axis is on a $\log_2$ scale. Dotted line indicates a nominal p value of 0.05. Dashed line indicates a HR of 1.

Panel B: Volcano plot of hazard ratios and p values for given proteins and hard MACE. Hard MACE was defined as: cardiovascular death, myocardial infarction or stroke. Hazard ratios were estimated from Cox proportional hazard models using both potential proteomic measurements including statin use as a time-dependent covariate and baseline 10-year ASCVD risk score. Hazard ratios are per protein doubling. Red proteins indicate associations at an FDR<0.05 level. X axis is on a $\log_2$ scale. Dotted line indicates a nominal p value of 0.05. Dashed line indicates a HR of 1.

Abbreviations: ANGPT2, Angiopoietin-2; CD70, CD70 antigen; CD83, CD83 antigen; CSF1, Processed macrophage colony-stimulating factor 1; CXCL13, C-X-C motif chemokine 13; EFEMP1, EGF-containing fibulin-like extracellular matrix protein 1; FABP4, Fatty acid-binding protein, adipocyte; FDR, False discover rate; GAL9, Galectin-9; GRN, Paragranulin; HGF, Hepatocyte growth factor alpha chain; HR, Hazard ratio; IL6, Interleukin-6; IL7R, Interleukin-7 receptor subunit alpha; IL8, Interleukin-8; LAMP3, Lysosome-associated membrane glycoprotein 3; MACE, Major adverse cardiac event; MCP2, C-C motif chemokine 8; MCP3, C-C motif chemokine 7; NOS3, Nitric oxide synthase, endothelial; NT-PROBNP, NT-proBNP; OPN, Osteopontin; PDCD1, Programmed cell death protein 1; PON3, Serum paraoxonase/lactonase 3; RARRES2, Retinoic acid receptor responder protein 2; SHPS1, Tyrosine-protein phosphatase non-receptor type substrate 1; TCN2, Transcobalamin-2; TNFRSF9, Tumor necrosis factor receptor superfamily member 9; TNFSF13B, Tumor necrosis factor ligand superfamily member 13b, membrane form; UPAR, Urokinase plasminogen activator surface receptor; VEGFA, Vascular endothelial growth factor A

Figure 3. Protein-protein interaction and enrichment analysis among proteins showing a significant association with MACE or hard MACE

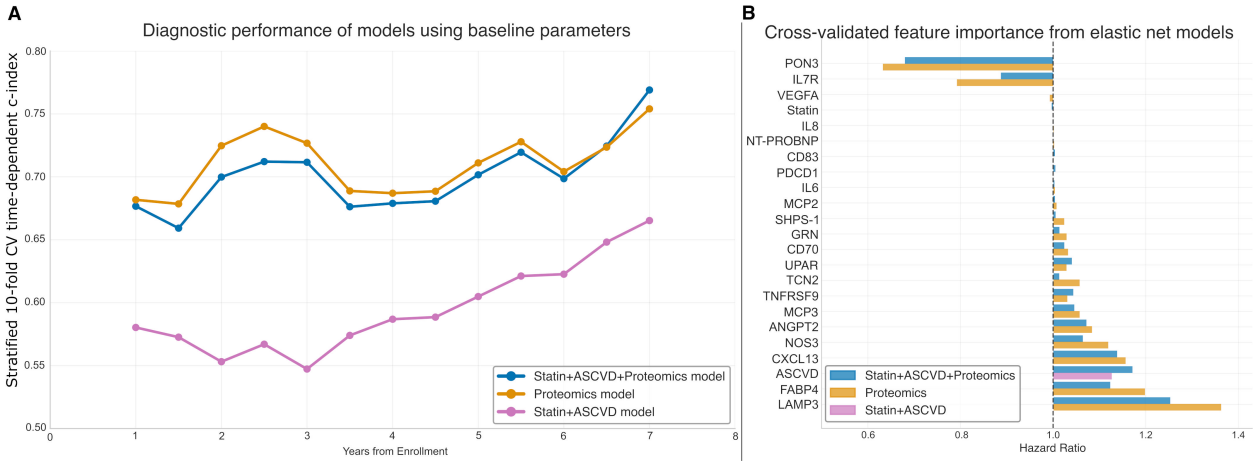


Panel A: Confidence-based protein interaction network using all available interactions sources. The width of the edges between the proteins represents the overall confidence score. The average number of connections between the protein nodes was 6.21, while Interleukin-6 (IL6) and Interleuking-8 (IL8) were connected 21 and 18 proteins respectively.

Panel B: Gene ontology and Reactome functional enrichments analysis. Gene ontology biological processes, molecular function and Reactome pathway enrichment all indicated increased representation of cytokine and chemokine processes and pathway functions within our significant proteins.

Abbreviations: ANGPT2, Angiopoietin-related protein 3; CCR, Chemokine-chemokine receptor; CD70, CD70 antigen; CD83, CD83 antigen; CSF1, Processed macrophage colony-stimulating factor 1; CXCL13, C-X-C motif chemokine 13; EFEMP1, EGF-containing fibulin-like extracellular matrix protein 1; FABP4, Fatty acid-binding protein, adipocyte; FDR, False discover rate; GAL9, Galectin-9; GRN, Paragranulin; HGF, Hepatocyte growth factor alpha chain; IL6, Interleukin-6; IL7R, Interleukin-7 receptor subunit alpha; IL8, Interleukin-8; LAMP3, Lysosome-associated membrane glycoprotein 3; MCP2, C-C motif chemokine 8; MCP3, C-C motif chemokine 7; NOS3, Nitric oxide synthase, endothelial; NT-PROBNP, NT-proBNP; OPN, Osteopontin; PDCD1, Programmed cell death protein 1; PON3, Serum paraoxonase/lactonase 3; RARRES2, Retinoic acid receptor responder protein 2; SHPS1, Tyrosine-protein phosphatase non-receptor type substrate 1; TCN2, Transcobalamin-2; TNF, Tumor necrosis factor; TNFRSF9, Tumor necrosis factor receptor superfamily member 9; TNFSF13B, Tumor necrosis factor ligand superfamily member 13b, membrane form; UPAR, Urokinase plasminogen activator surface receptor; VEGFA, Vascular endothelial growth factor A

Figure 4. Cross-validated performance of significant proteins, unchanged with statin therapy, to predict MACE



Panel A: Stratified 10-fold cross validated time dependent c-index of Cox elastic net machine learning models. Model-1: statin randomization + 10-year ASCVD risk score; Model-2: baseline values of proteins associated with MACE; Model-3: Model-1 + Model-2.

Panel B: Feature importance of the features with a HR different from one at least in one of the models. Bars represent standardized average standardized HR ratios from the 10 fold cross-validation.

Abbreviations: ANGPT2, Angiopoietin-2; ASCVD, Atherosclerotic cardiovascular disease; CD70, CD70 antigen; CV, Cross-validation; CXCL13, C-X-C motif chemokine 13; FABP4, Fatty acid-binding protein, adipocyte; GRN, Paragranulin; IL7R, Interleukin-7 receptor subunit alpha; LAMP3, Lysosome-associated membrane glycoprotein 3; MCP3, C-C motif chemokine 7; NOS3, Nitric oxide synthase, endothelial; PON3, Serum paraoxonase/lactonase 3; TNFRSF9, Tumor necrosis factor receptor superfamily member 9; UPAR, Urokinase plasminogen activator surface receptor

TABLES

Table 1. Patient characteristics of study population stratified by MACE.

Variable	**Overall** N = 765	**No MACE** N = 732	**MACE** N = 33
Demographics			
Age (years)	50.8 ± 5.9	50.7 ± 6.0	53.2 ± 5.4
Male natal sex	631 (82%)	605 (83%)	26 (79%)
Race			
Asian	9 (1.2%)	9 (1.2%)	0 (0%)
Black or African American	281 (37%)	264 (36%)	17 (52%)
White	402 (53%)	388 (53%)	14 (42%)
Other	73 (9.5%)	71 (9.7%)	2 (6.1%)
Ethnicity (n=755)			
Hispanic or Latino	181 (24%)	177 (24%)	4 (12%)
Not Hispanic or Latino	574 (76%)	545 (74%)	29 (88%)
Cardiovascular risk factors			
BMI (kg/m²)	27.4 ± 4.5	27.3 ± 4.4	28.1 ± 5.3
10-year ASCVD risk score (%)	5.0 ± 3.1	5.0 ± 3.1	6.9 ± 3.6
Use of antihypertensive medication	153 (20%)	143 (20%)	10 (30%)
Use of antidiabetic medication	1 (0.1%)	1 (0.1%)	0 (0%)
Family history of premature CVD (n=743)	167 (22%)	158 (22%)	9 (27%)
Smoking status			
Current	192 (25%)	175 (24%)	17 (52%)
Former	233 (31%)	227 (31%)	6 (18%)
Never	338 (44%)	328 (45%)	10 (30%)
Lipids			
Total cholesterol (mg/dL)	185.0 ± 36.0	185.3 ± 36.3	178.4 ± 30.2
LDL-C (mg/dL)	108.2 ± 29.8	108.5 ± 29.9	102.2 ± 25.6
Non-HDL-C (mg/dL)	134.6 ± 35.1	134.8 ± 35.4	129.8 ± 29.6
Triglycerides (mg/dL)	133.7 ± 83.2	133.5 ± 83.5	137.9 ± 76.3
Biomarkers			
High-sensitivity C-reactive protein (mg/L)	2.9 ± 2.9	2.8 ± 2.9	4.5 ± 3.3
Oxidized low-density lipoprotein (U/L)	57.6 ± 21.3	57.5 ± 21.1	59.4 ± 25.1
Lipoprotein-associated phospholipase A2 (ng/mL)	132.3 ± 56.9	132.9 ± 57.3	118.5 ± 45.1
HIV-related health history			
Total ART use (years)	11.8 ± 6.6	11.7 ± 6.6	12.5 ± 6.8
Nadir CD4 (cells/mm³) (n=545)			
<50	167 (22%)	159 (22%)	8 (24%)
50-199	217 (28%)	206 (28%)	11 (33%)
200-349	206 (27%)	198 (27%)	8 (24%)
350+	150 (20%)	144 (20%)	6 (18%)
CD4 count (cells/mm³)	633.4 ± 282.6	634.5 ± 282.3	608.4 ± 293.1
HIV-1 RNA (copies/mL)			
<LLQ	665 (88%)	638 (88%)	27 (84%)
LLQ -< 400	74 (9.8%)	71 (9.8%)	3 (9.4%)
400+	16 (2.1%)	14 (1.9%)	2 (6.3%)
Entry ART regimen class			
NRTI + INSTI	331 (43%)	321 (44%)	10 (30%)
NRTI + NNRTI	198 (26%)	189 (26%)	9 (27%)
NRTI + PI	135 (18%)	127 (17%)	8 (24%)
NRTI-sparing	26 (3.4%)	24 (3.3%)	2 (6.1%)
Other NRTI-containing	75 (9.8%)	71 (9.7%)	4 (12%)

Ethnicity presented per NIH definition for participants in US (including Puerto Rico) and Canada only. 'Other' race includes participants self-identifying as: native or indigenous to the enrollment region; more than one race (with no single race noted as predominant); or of unknown race.

Abbreviations: ART, antiretroviral therapy; ASCVD, atherosclerotic cardiovascular disease; BMI, body mass index; CVD, cardiovascular disease; HDL-C, high-density lipoprotein cholesterol; HIV, human immunodeficiency virus; INSTI, integrase strand transfer inhibitor; LDL-C, low-density lipoprotein cholesterol; LLQ, lower limit of quantification; MACE, major adverse cardiac event; NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitory; PI, protease inhibitor.

Percentages are presented considering the proportion of available data presented in parenthesis for parameters with missing values.

Table 2. Association between proteins changing in relation to statin therapy and MACE and hard MACE.

MACE

Protein	Name	Unadjusted			10-year ASCVD risk score and statin adjusted		
		HR	95% CI	p	aHR	95% CI	p
ANGPTL3	angiopoietin-related protein 3	2.20	1.04; 4.67	0.04	2.31	1.11; 4.80	0.03
MBL2	mannose-binding protein C	0.95	0.74; 1.22	0.67	0.93	0.73; 1.20	0.60
MIC-A/B	MHC class I polypeptide-related sequence A/B	0.95	0.78; 1.16	0.62	0.94	0.77; 1.15	0.56
NRP1	neuropilin-1	1.25	0.25; 6.27	0.78	1.06	0.22; 5.14	0.95
PCOLCE	procollagen C-endopeptidase enhancer 1	1.32	0.62; 2.80	0.47	1.34	0.62; 2.88	0.45
TFPI	tissue factor pathway inhibitor	1.61	0.60; 4.27	0.34	1.54	0.56; 4.22	0.40
TRAIL	TNF-related apoptosis-inducing ligand	1.59	0.53; 4.72	0.41	1.57	0.54; 4.54	0.41

Hard MACE

Protein	Name	Unadjusted			10-year ASCVD risk score and statin adjusted		
		HR	95% CI	p	aHR	95% CI	p
ANGPTL3	angiopoietin-related protein 3	3.27	1.32; 8.13	0.01	3.46	1.43; 8.35	0.005
MBL2	mannose-binding protein C	1.27	0.88; 1.83	0.20	1.25	0.87; 1.80	0.22
MIC-A/B	MHC class I polypeptide-related sequence A/B	1.05	0.79; 1.40	0.74	1.04	0.78; 1.39	0.79
NRP1	neuropilin-1	2.23	0.30; 16.34	0.43	1.82	0.26; 12.9	0.55
PCOLCE	procollagen C-endopeptidase enhancer 1	2.31	0.89; 6.00	0.09	2.42	0.92; 6.39	0.07
TFPI	tissue factor pathway inhibitor	1.73	0.50; 5.99	0.38	1.70	0.47; 6.10	0.42
TRAIL	TNF-related apoptosis-inducing ligand	1.88	0.47; 7.56	0.37	1.87	0.48; 7.26	0.36

Hard MACE was defined as: known cardiovascular death, myocardial infarction or stroke. Estimates are provided per doubling in protein expression from Cox regression models.
Abbreviations: aHR, adjusted hazard ratio; CI, confidence interval; HR: hazard ratio.

Table 3. Proteins not changing in relationship to statin use showing a significant association with MACE and/or hard MACE.

		MACE				hard MACE			
Protein	**Name**	**aHR**	**95%CI**	**p value**	**FDR**	**aHR**	**95%CI**	**p value**	**FDR**
ANGPT2	Angiopoietin-2	4.02	2.11; 7.68	<0.001	0.006	2.98	1.22; 7.30	0.017	0.123
CD70	CD70 antigen	2.63	1.44; 4.83	0.002	0.024	3.16	1.55; 6.47	0.002	0.041
CD83	CD83 antigen	2.69	1.47; 4.90	0.001	0.020	2.63	1.20; 5.73	0.015	0.118
CSF1	Processed macrophage colony-stimulating factor 1	9.20	2.37; 35.81	0.001	0.020	19.92	3.77; 105.39	<0.001	0.027
CXCL13	C-X-C motif chemokine 13	1.58	1.20; 2.08	<0.001	0.020	1.65	1.16; 2.34	0.006	0.077
EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	2.73	1.33; 5.63	0.006	0.057	3.57	1.58; 8.04	0.002	0.041
FABP4	Fatty acid-binding protein, adipocyte	2.10	1.35; 3.24	<0.001	0.020	2.55	1.48; 4.40	<0.001	0.029
GAL9	Galectin-9	3.87	1.70; 8.77	0.001	0.020	5.78	2.15; 15.53	<0.001	0.027
GRN	Paragranulin	4.11	1.64; 10.31	0.003	0.033	6.38	2.00; 20.42	0.002	0.041
HGF	Hepatocyte growth factor alpha chain	2.61	1.46; 4.67	0.001	0.020	2.52	1.07; 5.95	0.034	0.168
IL6	Interleukin-6	1.70	1.24; 2.34	<0.001	0.020	1.93	1.36; 2.74	<0.001	0.027
IL7R	Interleukin-7 receptor subunit alpha	0.29	0.13; 0.65	0.003	0.033	0.65	0.23; 1.84	0.420	0.593
IL8	Interleukin-8	1.61	1.16; 2.25	0.005	0.047	1.56	1.01; 2.41	0.043	0.181
LAMP3	Lysosome-associated membrane glycoprotein 3	2.21	1.45; 3.38	<0.001	0.009	2.06	1.19; 3.57	0.010	0.111
MCP2	C-C motif chemokine 8	2.04	1.32; 3.15	0.001	0.020	2.37	1.37; 4.09	0.002	0.041
MCP3	C-C motif chemokine 7	2.08	1.37; 3.16	<0.001	0.017	2.19	1.32; 3.66	0.003	0.046
NOS3	Nitric oxide synthase, endothelial	2.22	1.30; 3.80	0.004	0.039	2.18	1.07; 4.41	0.031	0.165
NT-PROBNP	NT-proBNP	1.99	1.36; 2.92	<0.001	0.016	1.97	1.24; 3.13	0.004	0.068
OPN	Osteopontin	1.92	1.22; 3.02	0.005	0.047	2.21	1.33; 3.65	0.002	0.045
PDCD1	Programmed cell death protein 1	2.30	1.43; 3.70	<0.001	0.017	2.10	1.07; 4.13	0.031	0.165
PON3	Serum paraoxonase/lactonase 3	0.45	0.29; 0.68	<0.001	0.009	0.48	0.28; 0.83	0.009	0.107
RARRES2	Retinoic acid receptor responder protein 2	3.56	1.33; 9.50	0.011	0.077	8.96	2.59; 30.93	<0.001	0.027
SHPS1	Tyrosine-protein phosphatase non-receptor type substrate 1	3.75	1.57; 8.93	0.003	0.034	2.56	0.82; 8.04	0.107	0.265
TCN2	Transcobalamin-2	2.68	1.35; 5.32	0.005	0.048	3.44	1.44; 8.21	0.005	0.077
TNFRSF9	Tumor necrosis factor receptor superfamily member 9	2.35	1.33; 4.15	0.003	0.037	2.11	0.97; 4.56	0.058	0.204
TNFSF13B	Tumor necrosis factor ligand superfamily member 13b, membrane form	3.33	1.85; 6.02	<0.001	0.006	3.55	1.58; 7.98	0.002	0.041
UPAR	Urokinase plasminogen activator surface receptor	2.68	1.65; 4.36	<0.001	0.006	2.81	1.53; 5.13	<0.001	0.029
VEGFA	Vascular endothelial growth factor A	3.45	1.83; 6.49	<0.001	0.008	4.12	1.85; 9.18	<0.001	0.0267

Hazard ratios are for protein doubling. Cox proportional hazard models are corrected for 10-year atherosclerotic cardiovascular disease risk score and statin use. Hard MACE was defined as: cardiovascular death, myocardial infarction or stroke.
Abbreviations: CI, confidence interval; FDR, false discovery rate; HR, hazard ratio.

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