Associations Between Very Low Concentrations of LDL-Cholesterol, hs-CRP and Health Outcomes in the Reasons for Geographical and Racial Differences in Stroke (REGARDS) Study

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Running Head: *Very low LDL-Cholesterol, hs-CRP and Health Outcomes*

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ABSTRACT:

Introduction: Recent findings have demonstrated the important contribution of inflammation to the risk of cardiovascular disease (CVD) in individuals with optimally managed low density lipoprotein cholesterol (LDL-C). We explored relationships between LDL-C, high sensitivity C-reactive protein (hsCRP) and clinical outcomes in a free-living US population.

Methods: We used data from the REasons for Geographical And Racial Differences in Stroke (REGARDS), and selected individuals at "high risk" for coronary events with a Framingham Coronary Risk Score of ≥10% or atherosclerotic cardiovascular disease (ASCVD) risk ≥7.5% in order to explore relationships between low LDL-C (<70 mg/dl [1.8 mmol/L] in comparison to ≥70 mg/dl [1.8 mmol/L]); hs-CRP <2 compared to ≥2 mg/L and clinical outcomes (all-cause mortality, incident coronary heart disease [CHD] and incident stroke). To assess the association between the LDL-C and hs-CRP categories and each outcome, a series of incremental Cox proportional hazards models were employed on complete cases. To account for missing observations, the most adjusted model was used to interrogate the data using multiple imputation with chained equations (MICE).

Results: In this analysis, 6136 REGARDS high risk participants were included. In the MICE analysis, participants with high LDL-C (≥70 mg/dl) and low hs-CRP (<2 mg/L) had a lower risk of incident stroke (hazard ratio [HR] 0.69, 0.47-0.997) incident CHD (HR 0.71, 0.53-0.95) and CHD death (HR 0.70, 0.50-0.99) than those in the same LDL-C category high hs-CRP (≥2 mg/L). In participants with high hsCRP (≥2 mg/dL), low LDL-C (<70 mg/dL [1.8 mmol/L]) was not associated with additional risk reduction of any investigated outcome, but with the significant increase of all-cause mortality (HR 1.37, 1.07-1.74).

Conclusions: In this high-risk population, we found that low hsCRP (<2mg/L) appeared to be associated with reduced risk of incident stroke, incident CHD and CHD death, whereas low LDL-C (<70 mg/dL) was not associated with protective effects. Thus, our results support other data with respect to the importance of inflammatory processes in the pathogenesis of CVD.

Keywords: Coronary heart disease; LDL-Cholesterol; Mortality; Stroke; hs-CRP.

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ABBREVIATIONS:

ASCVD	Atherosclerotic Cardiovascular Disease
CANTOS	Canakinumab Antiinflammatory Thrombosis Outcome Study
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
CTT	Cholesterol-Treatment Trialists
EAS	European Atheroslerosis Society
ESC	European Society of Cardiology
FOURIER	Further Cardiovascular Outcomes Research With PCSK9 Inhibition in Subjects With Elevated Risk
GFR	Glomerular Filtration Rate
hs-CRP	High sensitivity C-reactive protein
LDL-C	Low density lipoprotein cholesterol
MICE	Multiple imputation with chained equations
REGARDS	Reasons for Geographical and Racial Differences in Stroke

INTRODUCTION

Low density lipoprotein cholesterol (LDL-C) is a causative factor in the development of atherosclerotic cardiovascular disease (ASCVD) [1]. In randomized controlled trials (RCTs), reduction in plasma LDL-C concentration by statins has repeatedly been shown to reduce the mortality and morbidity associated with CVD in a variety of primary [2-4] and secondary [4,5] prevention settings. However, risk is not eliminated when therapeutic LDL-C targets are met [6,7]. Residual risk may result from different variables, including elements of risk owing to LDL-C and risk due to inflammation [8].

Recent therapeutic advances have enabled unpreceedented reductions in plasma LDL-C (and other apolipoprotein B [apo B] containing lipoproteins), thus decreasing he lipid component of residual risk (8). Such approaches have included combination therapy of statins with ezetemibe [9] or other lipid-lowering drugs [10]. Notable success in lipid-lowering has been achieved with the pharmacological attenuation of the action of proprotein convertase subtilisin/kexin type 9 (PCSK9) by monoclonal antibodies such as alirocumab [11,12] and evolucumab [13,14], and by the small interfering RNA, inclisiran [15]. In the Further Cardiovascular Outcomes research with PCSK9 Inhibition in subjects with elevated risk (FOURIER) study, evolocumab decreased LDL-C to a median of 30 mg/dl (0.78 mmol/L) and reduced CVD events by 15% (HR 0.85, 0.79 to 0.92) [16] without evidence of serious adverse effects [17] Importantly, treatment was not associated with cognitive decline [18] or new-onset diabetes [19].

The concept that residual inflammatory risk exists and can be treated has recently been demonstrated in the Canakinumab Antiinflammatory Thrombosis Outcome Study (CANTOS), where investigators showed that treatment with canakinumab (a monoclonal antibody targeting interleukin-1β) reduced the circulating levels of- C-reactive protein (CRP), and significantly reduced recurrent CV events [8].

In contrast to the extensive evidence from clinical trials, the correlation between plasma concentrations of LDL-C and mortality rates in free living populations is less well documented. This is particularly the case in individuals with unusually high or low concentrations of LDL-C, and especially in primary prevention populations of various ethnic origins who have been underrepresented in clinical trials and observational studies. Therefore, we used data from the REasons for Geographical And Racial Differences in Stroke (REGARDS) study to explore relationships between low LDL-C, hsCRP and clinical outcomes (all-cause mortality, incident coronary heart disease [CHD] and incident stroke) with a particular focus on participants with baseline LDL-C <70 mg/dL (1.8 mmol/L) in order to improve our understanding of the associations between lipids, inflammatory markers and the risk of CVD and death in this population. We limited our analysis to participants with high baseline 10-year risk (Framingham-CHD $\geq$10% or ASCVD $\geq$7.5%) to make our study results comparable to populations typically included in lipid-lowering intervention studies.

METHODS

REGARDS study population

The REGARDS longitudinal cohort study recruited 30,239 community-dwelling participants between January 2003 and October 2007. Participants were selected from commercial lists and recruited through a combination of mail and telephone contact. Because of a focus on geographic and racial disparities in stroke mortality, blacks were oversampled (44%), as were residents of the southeastern US Stroke Belt states (56%). The Stroke Belt states were defined as North Carolina, South Carolina, Georgia, Tennessee, Alabama, Mississippi, Arkansas, and Louisiana, with the remaining 44% of the participants being selected from the remaining 40 contiguous US states. Eligibility criteria included non-Hispanic black or white race, aged 45 and older, absence of conditions associated with a life

expectancy of less than 5 years, and not being on a waiting list for a nursing home. Potential participants with diagnosed malignancy at baseline were not eligible to take part in the study. Participation rate was estimated as 33%, a similar value to that seen in other studies. For those agreeing to participate, the telephone interviewers conducted an interview to assess CVD risk factors and medical history. An in-person assessment for direct measurement of risk factors (blood pressure [BP], anthropomorphic characteristics, electrocardiogram) and collection of blood and urine samples was conducted after the interview. Participants were followed by telephone at 6-month intervals to detect suspected cardiovascular events. Details of the study design are provided elsewhere [20].

In this analysis, we included participants with a 2002 Framingham CHD 10-year risk score[21] of ≥10% and, in a separate analysis those with a ASCVD 10-year risk score ≥7.5% who fasted overnight prior to their study visit, had valid measurements of total cholesterol, high density lipoprotein cholesterol (HDL-C) and triglycerides, and with follow-up for incident CHD (as well as other health outcomes).

Laboratory methods

Laboratory assays were conducted as previously described [22]. Samples were centrifuged an average of 97 min after collection and serum or plasma separated and shipped overnight on ice packs to the University of Vermont as previously described [22]. On arrival, samples were centrifuged at 30,000 g at 4^0C and either analyzed (general chemistries) or stored at below -80^0C. hs-CRP was analyzed in batches by particle enhanced immunonephelometry using the BNII nephelometer (N hs-CRP; Dade Behring, Deerfield, IL) with interassay coefficients of variation of 2.1-5.7%. Cholesterol, HDL-C, triglycerides, and glucose were measured by colorimetric reflectance spectrophotometry using the Ortho Vitros Clinical Chemistry System 950IRC instrument (Johnson & Johnson Clinical Diagnostics, New

Brunswick, NJ) [23]. LDL-C was calculated using the Friedewald formula from total cholesterol, HDL-C and triglycerides [24].

Statistical methods

The primary exposure of interest was LDL-C, with particular interest in those individuals with very low LDL-C measurements (<70 mg/dl [1.8 mmol/L]). In addition we explored hsCRP concentration dividing participants into those with concentrations of ≥2mg/L and <2mg/L. The outcomes of interest were all-cause mortality, incident CHD, CHD mortality and incident stroke each at or before December 31, 2013. Incident CHD was defined as either a definite or probable myocardial infarction (MI) or a definite or probable acute CHD death. The participants were contacted every 6 months for CV event information, and medical records were sought for suspected events and adjudicated by physicians. Expanded details of the study follow-up and stroke adjudication are found elsewhere [25]. For all analysis of incident CHD, those participants with a history of heart disease (self-reported MI, coronary artery bypass grafting [CABG], angioplasty or stenting OR evidence of MI *via* ECG [from interview and ECG]) were excluded. Similarly, the analysis of incident stroke excludes those participants with reported stroke at baseline.

Participant age, race (black/white), region of residence and sex were included as demographic variables. Self-reported income level (<$20k, $20k-$35k, $35k-$75k, >$75k) and education level (less than high school, high school graduate, some college, college graduate) were used as measures of socioeconomic status. Alcohol consumption (some/none), physical activity (none/1-3 times per week/4 or more times per week), current smoking were measured through self-reported questionnaires. Diabetes was defined as self-reported glucose-control medication use or fasting glucose ≥126 mg/dL. Body mass index (BMI) (kg/m^2) and systolic blood pressure (SBP) (mmHg) were measured at the in-home

visit. Albumin-to-creatinine ratio (ACR) (≥30 mg/g vs. <30 mg/g), estimated glomerular filtration rate (eGFR) through the CKD-Epi equation [26], hs-CRP (<2mg/L and ≥2 mg/L), HDL-C and triglycerides were measured through specimens. Information regarding the use of statins and other lipid-lowering medications (fibrates or niacin) by participants was obtained from their medication inventory at baseline.

To assess the association between LDL-C, hsCRP and each outcome, Cox proportional hazards models with penalized splines were employed, both unadjusted and adjusted for: demographic factors (age, race, sex), income level, education level, alcohol consumption, physical activity, smoking, BMI, diabetes, eGFR, ACR, hsCRP, statin use, other lipid-lowering medication use, HDL-C and triglycerides. The penalized spline allows the relationship between LDL-C and the log-hazard of each outcome to vary in a non-linear fashion, offering more modeling flexibility. Likelihood ratio tests were used to assess the statistical significance between LDL-C and each outcome.

In an additional analysis, LDL-C categories were defined by fasting LDL-C measurement into the following categories <50 mg/dL (1.3 mmol/L), 50 - <70 mg/dL (1.3 – 1.8 mmol/L) , ≥70 mg/dL (1.8 mmol/L). To assess the association between the LDL-C categories and each outcome, a series of incremental Cox proportional hazards models were employed on complete cases: **Model 1** - adjustment for age, sex, race and region of residence; **Model 2** - additional adjustment for education, income, alcohol use, physical activity, smoking and BMI; **Model 3** - additional adjustment for diabetes, ACR, eGFR, SBP, use of antihypertensive medications, use of lipid-lowering medications, use of beta-blockers and hs-CRP; and **Model 4** - additional adjustment for HDL-C and triglycerides. Model 4 was also used to interrogate the data using multiple imputation with chained equations (MICE) [27-29].

A further analysis was performed as above with the following categories of LDL-C and hsCRP: LDL-C<70, hs-CRP<2; LDL-C<70, hs-CRP≥2; LDL-C≥70, hs-CRP<2; LDL-C≥70,

hs-CRP≥2. The series of incremental Cox proportional hazards models were employed on complete cases and using MICE as above, but the correction for hs-CRP was excluded from model 3. Sensitivity analysis focused on the stratification by statin users. SAS 9.4 (SAS Institute, Inc, Cary, NC) and R [30] were used for all statistical analyses.

RESULTS

Baseline characteristics of participants

Overall, 6136 REGARDS participants with Framingham-CHD 10 year risk scores >10% were eligible for inclusion into this study. Of these, 95% were found to have an LDL-C ≥70 mg/dl, and 5% had an LDL-C <70 mg/dl. Demographic characteristics were broadly similar between the two groups. Compared to the higher LDL-C group, participants in the low LDL-C group were more likely to have a diagnosis of diabetes (67.2% *vs.* 32.6%) and more likely to be on statins (57.1% *vs.* 24.9%) or other lipid-lowering therapy (6.8% *vs.* 3.9%). Additionally, participants in the low LDL-C group were more likely to be black (46.8% *vs.* 41.2%), less likely to consume alcohol (30.8% *vs*. 37.3%), and more likely to smoke tobacco (31.2 *vs.* 25.0%) (**Table 1**).

Association between LDL-C, hs-CRP and all-cause mortality

Over the 7.14-year average follow-up, 1,376 (22.4%) participants suffered a fatal event. We found a significant non-linear relationship between LDL and all-cause mortality, which remained after adjustment for all covariates in all participants, but not in subgroups of statin users and non-users (**Table 2**). The overall tests of association (likelihood ratio tests) indicated a significant association between LDL-C and all-cause mortality in both unadjusted and fully-adjusted models of all participants and subgroups of statin users and non-users (**Table 3**). Inspection of spline plots revealed that LDL measurements between

approximately 70 mg/dl (1.8 mmol/L) and 200 mg/dl (5.2 mmol/L) were protective against all-cause mortality (**Figure 1**) compared to LDL measurements equal to 70 mg/dl (1.8 mmol/L), with levels below 70 mg/dl (1.8 mmol/L) not being associated with decreased risk for mortal events.

When participants with Framingham 10 year risk >10% were categorized according to three LDL-C categories (<50 mg/dL, 50 - < 70 mg/dL, ≥70 mg/dL), LDL-C 50-<70 mg was associated with increased risk of all-cause mortality: HR 1.40 (1.10, 1.78) compared with the referant group of ≥70 mg/dl in a minimally-adjusted model. The effect size was attenuated in adjusted models, but a statistically significant effect was observed when a fully-adjusted MICE was used (**Supplemental Table 1**). Similar patterns were seen in subgroups of participants not taking (**Supplemental Table 1a**) and those taking (**Supplemental Table 1b**) statins, although the hazard ratios for the LDL-C categories did not differ significantly in these subgroups.

In the participants with ASCVD 10-year risk >7.5%, both low LDL-C categories were associated with greater risk for mortality than the referant group. This observation persisted across all models including the fully adjusted MICE model: HR (LDL-C 50- <70 mg/dl) 1.17 (1.04, 1.33); HR (LDL-C <50 mg/dl) 1.32 (1.06, 1.65) (**Supplemental Table 2**). In the subgroup of participants who did not take statins, a similar result was seen across all models: fully adjusted MICE model: HR (LDL-C 50- <70 mg/dl) 1.32 (1.12, 1.56); HR (LDL-C <50 mg/dl) 1.53 (1.13, 2.06) (**Supplemental Table 2a, Figure 4**). In statin-users, low LDL-C was associated with higher mortality in a minimally-adjusted model HR (LDL-C 50- <70 mg/dl) 1.32 (1.12, 1.56); HR (LDL-C <50 mg/dl) 1.53 (1.13, 2.06) this effect was attenuated with progression of adjustment models (**Supplemental Table 2b, Figure 4**).

Categorisation of participants with Framingham 10-year risk >10% into four LDL-C / hs-CRP groups (LDL-C<70 , hs-CRP<2 ; LDL-C<70, hs-CRP≥2; LDL-C≥70, hs-CRP<2;

LDL-C≥70, hs-CRP≥2) revealed that the combination of LDL-C<70 mg/dl and hs-CRP ≥2 was associated with greater risk of mortality than the referent group (LDL-C≥70, hs-CRP≥2) across all complete-case models and MICE: HR 1.37 (1.07, 1.74). Participants with the combination of LDL-C≥70 and hs-CRP<2 were at lowest risk of death in a fully adjusted complete-case model: HR 0.75 (0.67, 0.85), but the difference was not statistically significant in the MICE model (**Table 4**). Similar trends were observed in the subgroup of participants who were not taking statins. In this subgroup LDL-C≥70 and hs-CRP<2 was associated with lower risk across all adjusted models and MICE: HR 0.79 (0.63, 0.98) but the increased risk seen in participants with LDL-C<70 mg/dl and hs-CRP ≥2 in a minimally-adjusted model was attenuated with progression of models (**Supplemental Table 3a**). In participants who were taking statins LDL-C<70 mg/dl and hs-CRP ≥2 was associated with increased risk in a MICE model, but the combination of LDL-C≥70 and hs-CRP<2 was not associated with reduced risk (**Supplemental Table 3b**). Similar results were observed with the group of participants with ASCVD 10-year risk >7.5% in the whole population (**Table 5**), in those not taking statins (**Supplemental Table 4a**) and participants who were taking statins (**Supplemental Table 4b**).

Association between LDL-C, hs-CRP and CHD death

In participants with Framingham 10 year risk >10% the risk of CHD death did not differ significantly between the three LDL-C categories in the whole population (**Supplemental Table 1, Figure 2**) or in subgroups of participants who did not (**Supplemental Table 1a**) or did take statins (**Supplemental Table 1b**).

In the participants with ASCVD 10-year risk >7.5%, LDL-C 50- <70 mg/dl was associated with greater risk for mortality, HR: 1.43 (1.16, 1.75) than the referant group in a minimally adjusted model in the whole population (**Supplemental Table 2**) and in the

subgroup of statin users (**Supplemental Table 2b**). However this relationship was not observed after adjustment for covariables or in the subgroups of statin non-users (**Supplemental Table 2a)**.

Categorisation of participants with Framingham 10-year risk >10% into four LDL-C / hs-CRP groups (LDL-C<70, hs-CRP<2; LDL-C<70, hs-CRP≥2; LDL-C≥70, hs-CRP<2; LDL-C≥70, hs-CRP≥2) revealed that the combination of LDL-C<70 mg/dl and hs-CRP≥2 was associated with greater risk of CHD mortality than the referent group (LDL-C≥70, hs-CRP≥2) across all complete-case models and MICE: HR 1.35 (0.97, 1.88). Participants with the combination of LDL-C≥70 and hs-CRP<2 were at lower risk of death in a fully adjusted complete-case model: 0.67 (0.54, 0.85); this association remained significant in the MICE model: 0.70 (0.50, 0.99) (**Table 4**). Similar trends were observed in the subgroup of participants who were not taking statins. In this subgroup LDL-C≥70 and hs-CRP<2 was associated with lower risk across all adjusted models and MICE: HR 0.62 (0.41, 0.95) but the increased risk seen in participants with LDL-C<70 mg/dl and hs-CRP≥2 in a minimally-adjusted model was attenuated with progression of models (**Supplemental Table 3a**). In participants who were taking statins LDL-C<70 mg/dl and hs-CRP≥2 was associated with increased risk in a MICE model - HR 2.09 (1.09, 3.71), but the combination of LDL-C≥70 and hs-CRP<2 was not associated with significant risk reduction (**Supplemental Table 3b**)

Similar results were observed with the group of participants with ASCVD 10-year risk >7.5% in the whole population (**Table 5**), in those not taking statins (**Supplemental Table 4a, Figure 4**) and participants who were taking statins (**Supplemental Table 4b, Figure 4**).

Association between LDL-C, hs-CRP and incident CHD

Over the 6.91-year average follow-up, 508 (8.3%) participants suffered a coronary event. Inspection of fully-adjusted spline plots indicates an approximate doubling of incident CHD

risk between baseline LDL-C concentrations of 150 mg/dl and 250 mg/dl (**Figure 2**). Nonlinearity was not observed in either unadjusted or adjusted data (**Table 2**) and the overall tests of association (likelihood ratio tests) did not indicate a significant association between LDL-C and incident CHD (**Table 3**).

In Cox proportional hazards models, the two lower categories of LDL were not associated with reduced risk of CHD in participants with high CVD risk as calculated by Framingham (**Supplemental Table 1**, **1a & 1b**) or ACSVD (**Supplemental Tables 2, 2a & 2b**) scores. However, when participants were categorized according to LDL-C and CRP, it was found that risk was the lowest in the group of participants with LDL-C≥70 and CRP<2 in Framingham high risk participants. This effect was statistically significant in the whole study population (**Table 4**) as well as subgroups of those not taking statins (**Supplemental Table 3a, Figure 4**) and statin users (**Supplemental Table 3b, Figure 4**). Similar results were observed in ASCVD high-risk participants (**Table 5, Supplemental Tables 4a & 4b**).

Association between LDL-C, hs-CRP and incident stroke

Over the 8.63-year average follow-up, 352 (5.7%) participants suffered a stroke event. Nonlinearity was not observed in either unadjusted or adjusted data (**Table 2**) and the overall rests of association (likelihood ratio tests) did not indicate a significant association between LDL-C and incident stroke (**Table 3, Figure 3**). No significant differences with respect to stroke were observed across any of the LDL-C categories in Framingham (**Supplemental Table 1, 1a & 1b**) or ASCVD (**Supplemental Tables 2, 2a & 2b**) high-risk participants. When participants were stratified by LDL-C and hs-CRP, stroke risk was lowest in the group with LDL-C≥70 and hs-CRP<2 in both Framingham (**Table 4**) and ASCVD (**Table 5**) populations. Subgroup analysis indicated that this effect was significant in participiants who

did not use statins (**Supplemental Tables 3a & 4a, Figure 4**), but not in those who used statins (**Supplemental Table 3b & 4b, Figure 4**).

DISCUSSION

This study has demonstrated a non-linear association between LDL-C and all-cause mortality in high risk primary prevention individuals with an inverse relationship evident between approximately 70 mg/dl (1.8 mmol/L) and 200 mg/dl (5.2 mmol/L), with higher risk of fatal events below 70 mg/dl (1.8 mmol/L) and for LDL-C levels in this range. Recently, a systematic review was conducted of 19 cohort studies totalling 68,094 participants aged 60 years or older. An inverse association between all-cause mortality and LDL-C was seen in 16 studies representing 92% of the number of participants [31, 32].

We identified 70 mg/dl (1.8 mmol/L) as a convenient reference point for our analyses because American College of Cardiology (ACC)/American Heart Association (AHA) guidelines on lipid reduction suggests 70 mg/dl (1.8 mmol/L) as the lowest value at which lipid-lowering therapy is recommended in individuals without diabetes (although statin therapy should be considered for diabetic individuals with LDL-C below this value, taking into accout patient preferences and comorbidities)[33]. The 2016 European Society of Cardiology (ESC)/European Atherosclerosis Society (EAS) guidelines for the management of dyslipidaemia recommend a target of <70 mg/dL (or >50% reduction in LDL-C) in patients with very high risk of CVD[34]. Thus these very low values of LDL-C are of increasing importance in a primary prevention population. Furthermore, relationships between LDL-C and CV events have been extensively studied for values of LDL-C above 70 mg/dl (1.8 mmol/L). Finally, a large meta-analysis found that 40% of patients treated with high-intensity statin therapy failed to reach a target of 70 mg/dl (1.8 mmol/L) [35].

The absence of a statistically significant relationship between LDL-C and CHD was indeed surprising. Applying the fully-adjusted MICE models to study the relationships between LDL-C categories and all-cause mortality, incident stroke, incident CHD and CHD death, we found that the lower LDL-C categories were not associated with reduced risk of any of the outcomes compared to the reference (≥70 mg/dl [1.8 mmol/L]) group. Using the same MICE models to study participants classified by both hs-CRP and LDL-C we found that in participants with LDL-C ≥70mg/dl (1.8 mmol/L) and with low hsCRP (<2 mg/L) the risk of incident stroke, incident CHD and CHD death was significantly lower than those with higher LDL-C and hsCRP in the Framingham high-risk population. A similar pattern of results was seen in the ASCVD high-risk group. The combination of high hsCRP and low LDL-C was not associated with reduced risk of any outcome in either high risk group, indeed signifincagly higher all-cause mortality was observed. Participants with low values of both hsCRP and LDL-C were not at lower risk for any of the outcomes compared to participants with high hsCRP and high LDL-C.

These data might appear to be at odds with the preponderance of evidence from interventional studies, which strongly suggest that 'lower is better' with respect to LDL-C. In the FOURIER study, evolocumab reduced LDL-C to a median of 30 mg/dl and significantly reduced CVD events (HR 0.85, 95%Cl: 0.79 to 0.92) [16]. The secondary analysis of the same trial revealed that the benefits were observed also at LDL-C levels <20 mg/dL (0.5 mmol/L) [18]. The same results were indeed observed in the recent ODYSSEY OUTCOMES trial with alirocumbab, suggesting however that the higher baseline LDL-C (≥100 mg/dL [2.5 mmol/L]) showed greater risk reduction [36]. A pooled analysis of ODYSSEY studies with alirocumab has demonstrated the feasibility of LDL-C reduction to below 50 mg/dl, which was achieved in one third of the cohort [37]. The investigators found an inverse relationship between LDL-C achieved during treatment and major CV events. The composite end-point

included CHD death, nonfatal myocardial infarction, ischemic stroke, or unstable angina requiring hospitalization [37]. Furthermore, a large meta-analysis of studies employing statins found LDL-C reduction to be associated with reduced CV risk, even as low as 50 mg/dl in the patients for whom this was achievable [35].

However, despite the fact that we limited our analysis to participants with high risk of CVD (calculated using two different methods), our results cannot be directly compared with those of interventional studies for a number of reasons. Firstly, by excluding participants with CHD and stroke at baseline, we studied only a primary prevention population (as opposed to secondary prevention participants in many interventional trials). Based on the data from many available studies, we are aware these are different populations taking into account the risk stratification as well as cardiovascular outcomes observed in hitherto studies [38]. Secondly, the free living population of the REGARDS study is likely to be more hetrogenous than that of interventional studies with numerous inclusion criteria. Thirdly, our follow-up of participants after a single LDL-C-measurement at baseline is not equivalent to controlled studies whereby a LDL-C lowering intervention is employed. LDL-C which is inherently low and LDL-C, which has been lowered by pharmacological agents will not necessarily lead to similar effects on outcomes. Much work has been carried out investigating the pleiotropic effects of statins[4], although less is known about non LDL-mediated effects of newer lipid-lowering drugs. Finally, because of many biochemical roles of cholesterol and concern that low levels of plasma lipids may therefore cause deleterious side-effects beyond the CV system, we chose all-cause mortality as our primary outcome in contrast to the CHD endpoints used in many trials [37].

While the association of lipid levels with stroke risk remains somewhat controversial [39], our results contrast with those obtained from a larger subset of participants in the REGARDS study, which found that baseline concentrations of LDL-C and non-HDL-C baseline levels

were associated with the risk of ischemic stroke [32]. A very large meta-analysis of individual data from 61 prospective studies with 55,000 vascular deaths has also demonstrated only a very weak association between LDL-C and stroke mortality [38]. Perhaps the weaker association of lipid levels with stroke than for coronary disease is a product of the multiple non-atherogentic pathways for stroke, especially as stratifying participants by hs-CRP in addition to LDL-C gave better prediction of stroke risk.

This study focused only on LDL-C, as this measurement was taken at baseline in the REGARDS study. Since then, lipidology has become more sophisticated and a greater appreciation is given to the importance of LDL-C quality, encompassing particle size and number [40]; other atherogenic lipoproteins such as Lp(a) [41]; and combined dyslipidaemia [42]. It is possible that participants with high cholesterol at baseline were later started on statin therapy thus confounding the analysis [31].

The large sample size, long period of follow-up, and rigorous approach to data-collection in the REGARDS study make this cohort an extremely useful tool to explore relationships between biomarkers and risks of disease. Nevertheless, such an approach to research has several limitations. Observational studies such as this are vulnerable to bias by unknown or unmeasured factors and cannot demonstrate causality. By definition, extreme values of any statistic are rare. We found that only 5% of our study population had LDL-C <70 mg/dl (1.8 mmol/L) at baseline; this resulted in small numbers of events within participants with LDL-C <50 mg/dl, yielding relatively wide confidence intervals. This limits the statistical power of our analysis. Due to the limited number of participants that met the inclusion criteria we could not analyse participants with Framigham risk score >20%.

We cannot entirely exclude the possibility of reverse causality, whereby low cholesterol secondary to other disease (e.g. malignancy) is associated with poor prognosis [43]. On this basis, Collins *et al.* have suggested censoring the early period of follow-up in this type of

analysis [4]. However, the potential for reverse causality in our study is reduced by the exclusion of participants with diagnosed malignancy from the REGARDS cohort. In common with all epidemiological studies, which collect participant data at baseline, we cannot be also certain about the interventions and treatments the participants received thereafter. Our results may be confounded by patients with high LDL-C concentrations at baseline initiating lipid-lowering therapy. Similarly, a proportion of those patients taking statins at baseline will have stopped during the follow-up period. Finally, the great improvements in the diagnosis and management of dyslipidaemias over recent years means that the REGARDS population is likely to have received better care with respect to LDL-C (but not necessarily inflammation and hs-CRP) towards the end of the follow-up period, than they did at the start of the study.

CONCLUSIONS

In primary prevention participants from REGARDS study we found a significant non-linear relationship between LDL-C and all-cause mortality, which remained after adjustment for all measured covariates. LDL-C between approximately 70 mg/dl (1.8 mmol/L) and 200 mg/dl (5.2 mmol/L) was protective against all-cause mortality, with levels lower than 70 mg/dl (1.8 mmol/L) not showing any further benefit. We did not find significant associations between LDL-C and incident CHD or incident stroke. Classifying participants by both hsCRP and LDL-C we found that that low hsCRP (<2mg/L) appeared to be associated with reduced risk of incident stroke, incident CHD and CHD death, whereas low LDL-C (<70 mg/dL) was not associated with protective effects. Whilst this is maybe to be expected in a population selected for high cardiovascular risk and low LDL-C, our results support those of the recent CANTOS trial with respect to the importance of inflammatory processes in the pathogenesis of CVD [8].

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CONFLICT OF INTEREST STATEMENT

Peter Penson owns four shares in Astra Zeneca PLC and has received travel/speaker's fees from Amgen; *Maciej Banach:* speakers bureau: Abbott/Mylan, Abbott Vascular, Actavis, Akcea, Amgen, Biofarm, KRKA, MSD, Sanofi-Aventis, Servier and Valeant; consultant to Abbott Vascular, Akcea, Amgen, Daichii Sankyo, Esperion, Lilly, MSD, Resverlogix, Sanofi-Aventis; Grants from Sanofi and Valeant; *Alberico L. Catapano* has received honoraria, lecture fees, or research grants from: Abbot, Aegerion, Amgen, AstraZeneca, Bayer, Eli Lilly, Genzyme, Ionis, Kowa, Mediolanum, Meda, Menarini, Merck, Pfizer, Recordati, Regeneron, Sanofi, SigmaTau. All *other authors* do not declare any conflict of interest related to the results of this study.

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MANUSCRIPT TABLES & FIGURES:

Table 1. Baseline characteristics of participants (N=6136).

	LDL-C < 70 mg/dl/ 1.8 mmol/L	**LDL-C ≥ 70 mg/dl/ 1.8 mmol/L**	**Total**
Overall N (%)	308 (5.0)	5828 (95.0)	6136
Continuous variables, Mean (Std)			
Age (years)	69.2 (8.15)	67.6 (8.65)	67.6 (8.64)
Body Mass Index (kg/m^2)	30.1 (6.07)	29.6 (5.85)	29.6 (5.86)
Estimated GFR (CKD-Epi equation)	78.5 (23.56)	82.9 (19.47)	82.6 (19.72)
HDL cholesterol (mg/L)	42.2 (14.96)	44.6 (12.01)	44.5 (12.18)
Triglycerides (mg/L)	149.0 (93.87)	145.4 (68.11)	145.6 (69.62)
Categorical variables, N (Percent)			
Men	204 (66.2)	3876 (66.5)	4080 (66.5)
Black	144 (46.8)	2401 (41.2)	2545 (41.5)
Education			
Less than High School	56 (18.2)	865 (14.8)	921 (15.0)
High School Graduate	79 (25.7)	1519 (26.1)	1598 (26.0)
Some College	87 (28.3)	1498 (25.7)	1585 (25.8)
College Graduate and More	86 (27.9)	1946 (33.4)	2032 (33.1)
Income			
Less than $20k	59 (19.2)	1081 (18.6)	1140 (18.6)
$20k-$34k	96 (31.2)	1536 (26.4)	1632 (26.6)
$35k-$74k	86 (27.9)	1805 (31.0)	1891 (30.8)
$75k and above	34 (11.0)	765 (13.1)	799 (13.0)

Refused	33 (10.7)	641 (11.0)	674 (11.0)
Alcohol Consumption	95 (30.8)	2174 (37.3)	2269 (37.0)
Physical Activity			
None	135 (43.8)	1991 (34.2)	2126 (34.7)
1-3 times per week	86 (27.9)	2046 (35.1)	2132 (34.8)
4 or more times per week	87 (28.3)	1791 (30.7)	1878 (30.6)
Current Smoking	96 (31.2)	1460 (25.0)	1556 (25.4)
Diabetes	207 (67.2)	1899 (32.6)	2106 (34.3)
Statin Use	176 (57.1)	1451 (24.9)	1627 (26.5)
Other Lipid-lowering Medication Use	21 (6.8)	227 (3.9)	248 (4.0)
Urinary Albumin/Creatinine Ratio > 30 mg/g	73 (23.7)	1142 (19.6)	1215 (19.8)
hs-CRP			
< 1 mg/L	81 (26.3)	1302 (22.3)	1383 (22.5)
1 - < 3 mg/L	95 (30.8)	2006 (34.4)	2101 (34.2)
3+ mg/L	132 (42.9)	2520 (43.2)	2652 (43.2)

Table 2. Tests of nonlinearity of LDL-C Penalized Spline**.**

Outcome	Unadjusted Model			Adjusted Model*		
	All participants	Statin Use	No Statin Use	All participants	Statin Use	No Statin Use
All-Cause Mortality	<0.001	0.003	<0.001	0.002	0.17	0.07
Incident CHD	0.18	0.20	0.97	0.47	0.14	0.01
Incident Stroke	0.81	0.17	0.18	0.94	0.14	0.89

*Includes all adjustment variables in Model 4: age, sex, race, region of residence, education, income, alcohol use, physical activity, smoking, BMI, diabetes, ACR, eGFR, SBP, use of antihypertensive medications, use of lipid-lowering medications, use of beta-blockers, hs-CRP, HDL, and triglycerides.

Table 3: Overall tests of association (Likelihood ratio test) between LDL-C and outcomes.

Outcome	Unadjusted Model			Adjusted Model*		
	All Participants	Statin Use	No Statin Use	All Participants	Statin Use	No Statin Use
All-Cause Mortality	< 0.001	<0.001	<0.001	< 0.001	0.005	<0.001
Incident CHD	0.06	0.07	0.35	0.07	0.048	0.42
Incident Stroke	0.83	0.20	0.48	0.66	0.15	0.26

*Includes all adjustment variables in Model 4: age, sex, race, region of residence, education, income, alcohol use, physical activity, smoking, BMI, diabetes, ACR, eGFR, SBP, use of antihypertensive medications, use of lipid-lowering medications, use of beta-blockers, hs-CRP, HDL, and triglycerides.

Table 4: Hazard ratios (and 95%CI) for each outcome by LDL-C/hs-CRP category among REGARDS participants with Framingham CHD score ≥10%.

		Number of Events /Population	Model 1	Model 2	Model 3	Model 4	Model 4 (MI)
All-cause mortality		1322/5842					
LDL-C/hs-CRP Category	LDL-C<70, hs-CRP<2	31/137	0.81 (0.56, 1.16)	0.77 (0.54, 1.10)	0.70 (0.49, 1.01)	0.72 (0.50, 1.03)	0.85 (0.59, 1.23)
	LDL-C<70, hs-CRP≥2	59/159	1.57 (1.20, 2.04)	1.47 (1.12, 1.91)	1.31 (1.00, 1.71)	1.32 (1.01, 1.73)	1.37 (1.07, 1.74)
	LDL-C≥70, hs-CRP<2	464/2377	0.68 (0.60, 0.76)	0.73 (0.65, 0.82)	0.76 (0.67, 0.86)	0.75 (0.67, 0.85)	0.86 (0.71, 1.03)
	LDL-C≥70,hs-CRP≥2	768/3169	ref	ref	ref	ref	ref
Incident Stroke		285/5458					
LDL-C/hs-CRP Category	LDL-C<70, hs-CRP<2	7/125	0.91 (0.43, 1.94)	0.87 (0.41, 1.86)	0.90 (0.42, 1.95)	0.90 (0.42, 1.94)	0.85 (0.39, 1.84)
	LDL-C<70, hs-CRP≥2	6/136	0.72 (0.32, 1.62)	0.72 (0.32, 1.62)	0.65 (0.29, 1.49)	0.65 (0.28, 1.48)	0.74 (0.36, 1.54)
	LDL-C≥70, hs-CRP<2	102/2239	0.72 (0.56, 0.92)	0.71 (0.55, 0.92)	0.75 (0.58, 0.97)	0.75 (0.58, 0.97)	0.69 (0.47, 0.997)
	LDL-C≥70,hs-CRP≥2	170/2958	ref	ref	ref	ref	ref
Incident CHD		475/5717					
LDL-C/hs-CRP Category	LDL-C<70, hs-CRP<2	9/134	0.65 (0.34, 1.27)	0.67 (0.34, 1.30)	0.61 (0.31, 1.20)	0.62 (0.32, 1.22)	0.55 (0.27, 1.10)
	LDL-C<70, hs-CRP≥2	16/154	1.16 (0.70, 1.92)	1.11 (0.67, 1.84)	0.95 (0.57, 1.59)	0.96 (0.58, 1.61)	1.05 (0.67, 1.64)
	LDL-C≥70, hs-CRP<2	167/2330	0.66 (0.54, 0.80)	0.70 (0.57, 0.86)	0.71 (0.58, 0.87)	0.71 (0.58, 0.87)	0.71 (0.53, 0.95)
	LDL-C≥70,hs-CRP≥2	283/3099	ref	ref	ref	ref	ref
CHD Death		372/5842					
LDL-C/hs-CRP Category	LDL-C<70, hs-CRP<2	7/137	0.62 (0.29, 1.32)	0.58 (0.27, 1.24)	0.52 (0.24, 1.11)	0.51 (0.24, 1.10)	0.58 (0.28, 1.20)
	LDL-C<70, hs-CRP≥2	17/159	1.48 (0.91, 2.43)	1.42 (0.86, 2.33)	1.18 (0.72, 1.96)	1.19 (0.72, 1.97)	1.26 (0.81, 1.97)
	LDL-C≥70, hs-CRP<2	122/2377	0.62 (0.50, 0.78)	0.65 (0.52, 0.82)	0.68 (0.54, 0.86)	0.67 (0.54, 0.85)	0.70 (0.50, 0.99)
	LDL-C≥70,hs-CRP≥2	226/3169	Ref	ref	ref	ref	ref

Model 1: Adjusted for age, sex, race and region of residence
Model 2: Model 1 + education, income, alcohol use, physical activity, smoking and BMI
Model 3: Model 2 + diabetes, ACR, eGFR, SBP, use of antihypertensive medications, use of lipid-lowering medications, use of beta-blockers
Model 4: Model 3 + HDL, triglycerides.

Table 5. Hazard ratios (and 95%CI) for each outcome by LDL-C/hs-CRP category among REGARDS participants with ASCVD risk score ≥7.5%.

		Number of Events /Population	Model 1	Model 2	Model 3	Model 4	Model 4 (MI)
All-cause mortality		3156/14469					
LDL-C/hs-CRP Category	LDL-C<70, hs-CRP<2	162/625	0.91 (0.77, 1.07)	0.95 (0.80, 1.11)	0.91 (0.77, 1.07)	0.90 (0.77, 1.07)	1.08 (0.90, 1.30)
	LDL-C<70, hs-CRP≥2	203/608	1.40 (1.21, 1.62)	1.36 (1.18, 1.58)	1.25 (1.08, 1.45)	1.25 (1.08, 1.45)	1.27 (1.11, 1.46)
	LDL-C≥70, hs-CRP<2	1101/5996	0.65 (0.60, 0.70)	0.72 (0.67, 0.78)	0.76 (0.70, 0.83)	0.76 (0.70, 0.82)	0.91 (0.81, 1.03)
	LDL-C≥70,hs-CRP≥2	1685/7240	ref	ref	ref	ref	ref
Incident Stroke		690/13418					
LDL-C/hs-CRP Category	LDL-C<70, hs-CRP<2	33/564	0.96 (0.67, 1.38)	0.97 (0.68, 1.39)	0.97 (0.67, 1.39)	0.97 (0.67, 1.40)	1.12 (0.76, 1.65)
	LDL-C<70, hs-CRP≥2	29/534	0.92 (0.63, 1.34)	0.90 (0.62, 1.32)	0.86 (0.59, 1.26)	0.86 (0.59, 1.26)	0.83 (0.58, 1.21)
	LDL-C≥70, hs-CRP<2	258/5628	0.77 (0.66, 0.91)	0.79 (0.67, 0.93)	0.83 (0.70, 0.98)	0.83 (0.70, 0.98)	0.92 (0.71, 1.18)
	LDL-C≥70,hs-CRP≥2	370/6692	ref	ref	ref	ref	ref
Incident CHD		710/11117					
LDL-C/hs-CRP Category	LDL-C<70, hs-CRP<2	20/353	0.68 (0.44, 1.07)	0.71 (0.45, 1.12)	0.69 (0.44, 1.09)	0.69 (0.44, 1.10)	0.67 (0.41, 1.09)
	LDL-C<70, hs-CRP≥2	33/372	1.27 (0.89, 1.81)	1.21 (0.85, 1.73)	1.09 (0.76, 1.56)	1.08 (0.76, 1.55)	1.11 (0.80, 1.54)
	LDL-C≥70, hs-CRP<2	254/4743	0.63 (0.54, 0.74)	0.71 (0.60, 0.84)	0.73 (0.62, 0.86)	0.73 (0.62, 0.87)	0.75 (0.59, 0.96)
	LDL-C≥70,hs-CRP≥2	403/5649	ref	ref	ref	ref	ref
CHD Death		1035/14469					
LDL-C/hs-CRP Category	LDL-C<70, hs-CRP<2	49/625	0.82 (0.61, 1.10)	0.85 (0.63, 1.14)	0.77 (0.57, 1.04)	0.77 (0.57, 1.04)	0.75 (0.45, 1.25)
	LDL-C<70, hs-CRP≥2	75/608	1.52 (1.19, 1.93)	1.46 (1.15, 1.86)	1.36 (0.98, 1.60)	1.26 (0.98, 1.61)	1.35 (0.97, 1.88)
	LDL-C≥70, hs-CRP<2	341/5996	0.60 (0.53, 0.69)	0.67 (0.58, 0.77)	0.72 (0.62, 0.82)	0.71 (0.62, 0.82)	0.72 (0.54, 0.96)
	LDL-C≥70,hs-CRP≥2	570/7240	ref	ref	ref	ref	ref

Model 1: Adjusted for age, sex, race and region of residence
Model 2: Model 1 + education, income, alcohol use, physical activity, smoking and BMI
Model 3: Model 2 + diabetes, ACR, eGFR, SBP, use of antihypertensive medications, use of lipid-lowering medications, use of beta-blockers.
Model 4: Model 3 + HDL, triglycerides.

Figure 1: Spline plot of plasma LDL-C-C and mortality rate, normalized to the mortality rate at LDL-C of 70 mg/dl. The left-hand panel shows unadjusted data and the right-hand panel shows data after full adjustment for covariates.

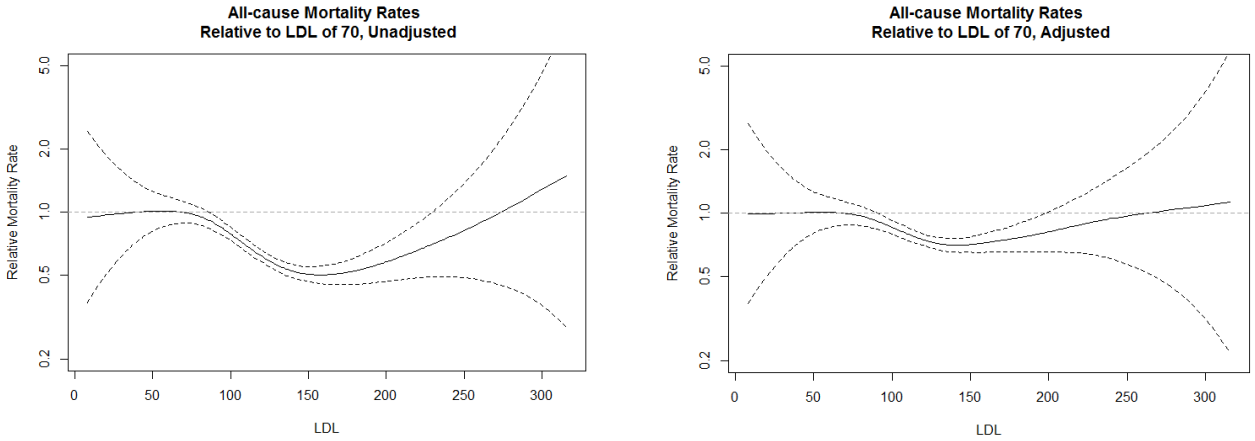


Figure 2: Spline plot of plasma LDL-C and CHD rate, normalized to the CHD rate at LDL-C of 70 mg/dl. The left-hand panel shows unadjusted data and the right-hand panel shows data after full adjustment for covariates..

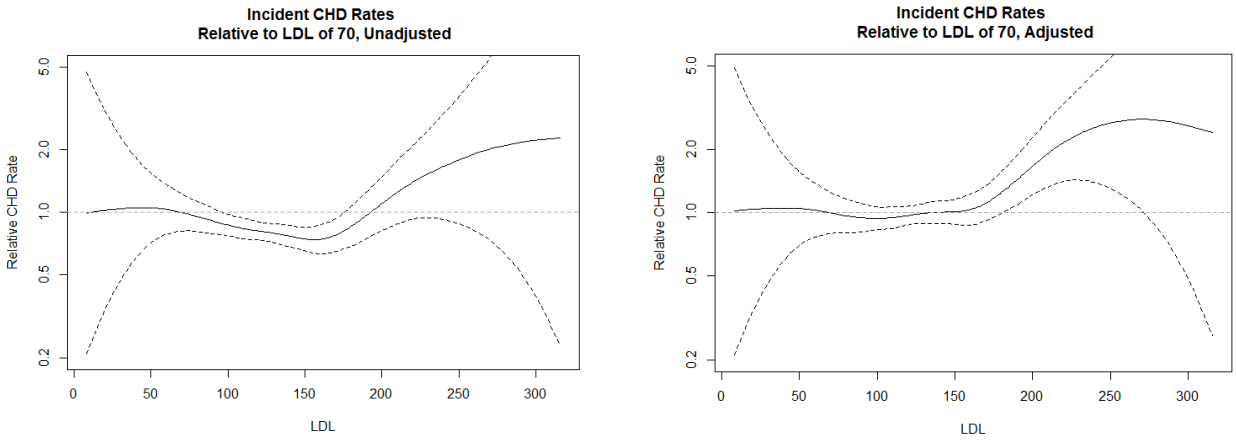


Figure 3: Spline plot of plasma LDL-C and stroke rate, normalized to the stroke rate at LDL-C of 70 mg/dl. The left-hand panel shows unadjusted data and the right-hand panel shows data after full adjustment for covariates.

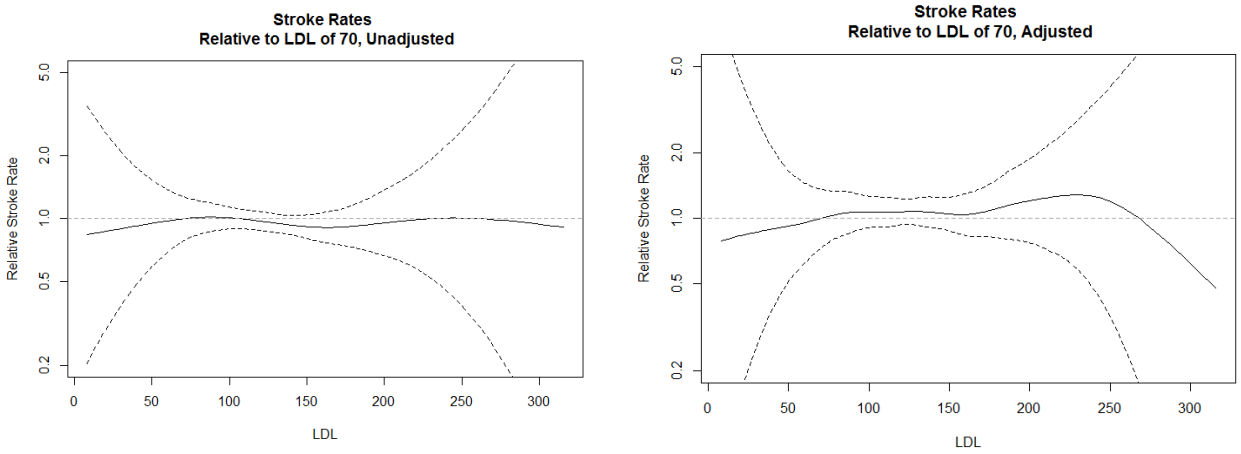
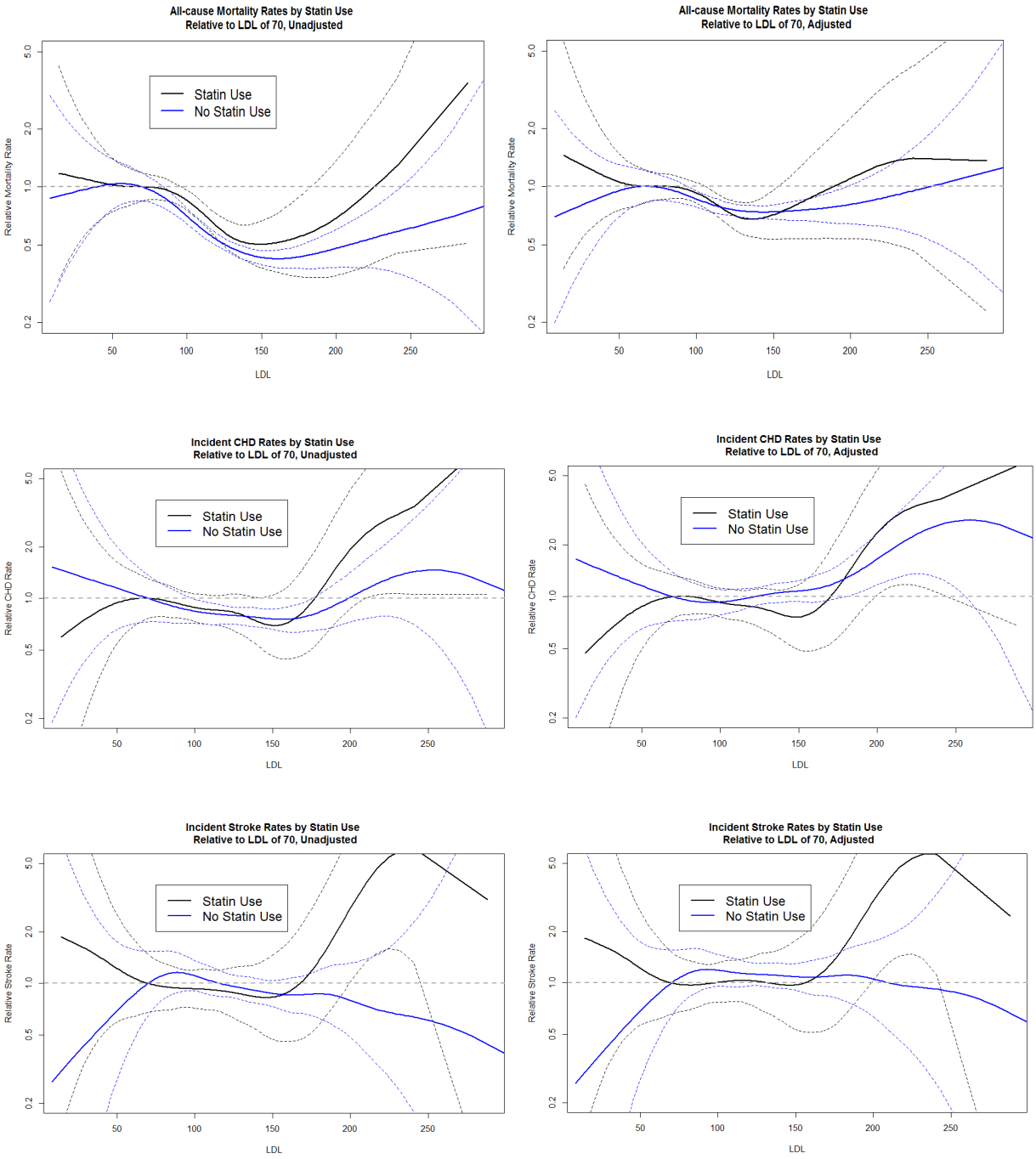


Figure 4: Sensitivity Analysis (by Statin Use).



Summarizing Figure:

Hazard ratios (HRs) for all-cause mortality and cardiovascular outcomes amongst REGARDS participants categorised according to baseline levels of LDL-C and hsCRP. These analyses were performed on participants with Framingham-CHD 10-Year Risk Score of >10% and represent fully-adjusted models with missing data accounted for using Multiple Imputation with Chained Equations.

**** indicates statistically-significant (p<0.05) differences from the referant group (LDL-C ≥ 70 mg/DL, hs-CRP ≥ 2 mg/L).***