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Clinical Correlates, Heritability and Genetic Linkage of Circulating CD40 Ligand in the Framingham Offspring Study

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Abstract

Background—The CD40 receptor and its ligand (CD40L) are known to modulate both inflammation and thrombosis, two processes important for the development and clinical expression of atherosclerosis. Circulating soluble CD40L (sCD40L) concentration predicts cardiovascular risk in selected patient samples. The purpose of this study was to determine the predictors of sCD40L in a large, community-based sample.

Methods and Results—We determined both serum and plasma sCD40L concentration in 3259 participants (54% women) from the Framingham Offspring Study. In multivariable models, advancing age was the only consistent (inverse) correlate of both serum and plasma sCD40L concentration. Overall, the variability explained by clinical covariates was very low for both measurements of sCD40L; with values of only 1.4% and 2.7% for serum and plasma, respectively. We observed that genetic factors accounted for a modest (12% serum; 14% plasma) amount of the adjusted variability in sCD40L.

Conclusions—Circulating sCD40L concentration was poorly associated with known CVD risk factors and were modestly heritable. Determining if either serum or plasma sCD40L are predictive of CVD risk in the community will require longitudinal follow-up.

INTRODUCTION

Inflammation is a cardinal feature of atherosclerosis. Early lesion development is characterized by the adherence of monocytes to the endothelium at lesion-prone arterial sites,(1) a process facilitated by endothelial expression of adhesion molecules (2). Local vascular cell production of inflammatory chemokines facilitates monocyte accumulation and differentiation into

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macrophages that accumulate lipid to become foam cells and initiate the formation of atherosclerotic plaques. Sites of atherosclerotic plaque rupture contain activated macrophages and smooth muscle cells - findings indicative of an ongoing inflammatory response (3). Plaque rupture occurs throughout the course of atherosclerosis and this event typically also produces local thrombus formation that may either contribute to rapid progression of the disease (4), or precipitate acute coronary syndromes (5). Thus, atherosclerosis and its clinical manifestations are influenced by the processes of inflammation and thrombosis.

In this regard, the CD40 receptor, and its ligand (CD40L) is a system of particular interest. This receptor-ligand system was originally identified as an important component of T cell-dependent B-cell differentiation and activation.(6) However, it is now clear this system is expressed in vascular cells(7) and platelet release of preformed CD40L(8) invokes an inflammatory response. In keeping with the known roles of inflammation and thrombosis in atherosclerosis, there are considerable data linking CD40L to atherosclerosis and its clinical manifestations. Blockade of this pathway with CD40L antibody inhibits atherosclerosis,(9) and mice lacking CD40L exhibit impaired lesion progression and inflammatory cell recruitment.(10) These data are consistent with clinical studies indicating that sCD40L concentration predicts cardiovascular disease (CVD) risk in healthy women(11) and clinical outcome in patients with acute coronary syndromes.(12)

We hypothesized that interindividual variability in sCD40L is influenced by both clinical and genetic factors. Hence, we examined the clinical correlates, the heritability and genetic linkage of plasma and serum sCD40L concentration in a large ambulatory cohort, the Framingham Offspring Study.

MATERIALS AND METHODS

Study Sample

The design and selection criteria of the Framingham Offspring Study and the Omni Cohort have been described (13). The current sample was derived from the 3539 Offspring participants attending the seventh examination cycle (1998–2001) and the 405 Omni participants returning for their second examination. All procedures have been approved by the Institutional Review Board of Boston Medical Center and all participants signed informed consent. Clinical definition of diabetes included fasting glucose ≥ 126 mg/dL, or the use of oral hypoglycemic agents or insulin. Information about prior CVD events (coronary heart disease, heart failure, stroke, or intermittent claudication) was obtained via medical history, physical examination at the Heart Study (every 4 to 8 years), and medical records. Records were reviewed by a panel of three experienced investigators as described (14). Participants were excluded if physical examination occurred off-site ($n=206$), serum or plasma was unavailable ($n=65$), or covariate data were missing ($n=16$), leaving 3657 subjects for the current investigation. A separate 8 participant Boston Medical Center sample was recruited to study the characteristics of CD40L in collected blood. This protocol also was approved by the Institutional Review Board at Boston Medical Center and all participants gave written informed consent.

Determination of sCD40L

Serum samples were collected and allowed to clot at room temperature for 30min followed by centrifugation for 22 min at 2500g. Collected plasma was subjected to centrifugation (as described above) within 10 min of collection. All samples were stored at -70°C . For analysis, samples were thawed at room temperature, vortexed vigorously, and sCD40L concentration were determined using a commercially-available ELISA (Bender MedSystems GmbH, Vienna, Austria) according to the manufacturer's instructions. Samples were analyzed in duplicate with an average intra-assay coefficient of variation of $4.69 \pm 4.45\%$ and $4.43 \pm 3.40\%$ for serum and

plasma, respectively. All samples with a coefficient of variation greater than two standard deviations from their respective means were repeated in duplicate and the mean of the repeat duplicate values utilized in this analysis.

Statistical Analysis

The distributions of both serum and plasma sCD40L were highly skewed, so analyses were performed on natural log-transformed data. Age-, sex- and cohort-adjusted linear regression models were constructed to assess the relations of sCD40L to the following individual variables (separate models for serum and plasma concentrations): age, sex, cohort, total/HDL cholesterol ratio, triglycerides, systolic and diastolic blood pressure, body mass index, waist circumference, fasting blood glucose, diabetes mellitus, current smoking (self-reported within the prior year), lipid treatment, hypertension treatment, hormone replacement therapy, aspirin use (at least 3 times per week), prevalent CVD. Sex, age and cohort interactions (Offspring vs. Omni) were also tested for each variable listed above. Stepwise multivariable linear regression models(15) were constructed examining the above variables in relation to serum and plasma sCD40L (dependent variables; separate models for each) with a $p < 0.05$ threshold for inclusion. Analyses were run using SAS version 8.1.(16) Any two-sided p value less than 0.05 was considered to be statistically significant.

The program SOLAR(17) was used to estimate the heritability of sCD40L – that is, the proportion of variability explained by additive genetic factors after accounting for measured covariates in the Offspring sample. CD40L serum and plasma values were first log transformed to remove excess skewness and kurtosis that can cause spurious linkage results,(18) and were adjusted for age and sex in a first model, and subsequently were adjusted for the other clinical covariates without regard to whether the covariates were retained in the stepwise models (i.e. adjusting for age, sex, cohort, smoking, total/HDL cholesterol, triglycerides, systolic and diastolic blood pressure, body mass index, waist, blood glucose, diabetes, medications [lipid lowering, hypertension, hormone replacement, aspirin], prevalent CVD) in a second linear regression model. The proportion of variation resulting from measured covariates was estimated using a regression model with men and women combined. We determined genetic linkage using variance component methods, implemented in the SOLAR software.(17) Linkage methods have been previously described.(19) The Mammalian Genotyping Service conducted the genome scan as described at http://research.marshfieldclinic.org/genetics/Lab_Methods/methods.html in two phases: 1) 1,702 individuals in the largest 330 families were genotyped without regard to clinical characteristics (markers set 8A, average heterozygosity 0.77; sex-averaged mean inter-marker spacing of 8.6 cM); 2) an additional 184 members of the 330 largest pedigrees were genotyped on 382 markers (markers set 13, average heterozygosity 0.76; sex-averaged mean inter-marker spacing of 8.9 cM, 262 markers in common with marker set 8A). A total of 601 markers on the 22 autosomes were included, with an average marker spacing of 5.8cM. Of 1,886 genotyped individuals, 471 were members of the Original cohort and 1415 were Offspring Cohort participants. An additional 634 genotyped individuals without biomarker data were included in the linkage analyses to establish, for each relative pair, the number of alleles shared from common ancestors.

Results

Subject Characteristics and Individual Correlates of sCD40L

The characteristics of the study sample are displayed in Table 1. Since men and women exhibited similar serum concentration of sCD40L, and since we did not observe a significant interaction between sex and the covariates in their relation to sCD40L, we conducted sex-pooled analyses. Age-, sex- and cohort-adjusted associations between the variables in Table 1

and serum sCD40L are presented in Table 2. We found that advancing age, total/HDL cholesterol, and blood glucose were negatively correlated with sCD40L, whereas smoking and the Omni cohort were positively associated with serum sCD40L.

Because controversy exists over the relative value of serum versus plasma determinations of sCD40L, we also measured sCD40L in plasma samples. Of note, the correlation between the serum and plasma sCD40L in this sample was only 0.065. We examined the age- and sex-adjusted correlates of plasma sCD40L; age remained a negative correlate of sCD40L, whereas total/HDL cholesterol and blood glucose did not. Other negative correlates of plasma sCD40L included hypertension treatment, smoking, and the Omni cohort, the latter two contrasting with the positive association for serum sCD40L.

Multivariable correlates of sCD40L

The results of stepwise multivariable linear regression with age, sex and cohort forced into the models are shown in Table 3. Age and total/HDL cholesterol remained significant negative correlates of serum sCD40L, whereas smoking and the Omni cohort remained positive correlates of serum sCD40L after multivariable adjustment. With the exception of age, correlates of plasma sCD40L differed from that of serum. Female sex was positively associated, whereas smoking hypertension treatment and the Omni cohort were negatively related to plasma sCD40L. Overall, the variability explained by clinical covariates was very low for both measurements of sCD40L; the model R^2 for serum and plasma sCD40L was 0.011 and 0.025, respectively.

Heritability of sCD40L concentration

We determined the extent to which circulating sCD40L concentration was explained by genetic factors. The age- and sex-adjusted estimated heritability $\pm$ standard error for sCD40L serum and plasma were 0.13 ± 0.06 and 0.17 ± 0.06 , respectively ($n=1824$). The multivariable adjusted heritability estimates were similar ($n=1818$, 0.12 ± 0.06 , and $0.14 \pm 0.15 \pm 0.06$, serum and plasma). Consistent with a modest degree of heritability, a genome wide scan examining linkage of both plasma and serum sCD40L did not yield any LOD score greater than 1.7 (see supplemental material). Thus, we could find no strong genetic explanation for interindividual variability in either serum or plasma sCD40L concentration in this study sample.

Sample preparation and sCD40L

The two sources of sCD40L had an important influence on the results of multivariable modeling. Previous data in the literature suggest that serum samples contain considerably more sCD40L than plasma, an observation that is in keeping with the liberation of CD40L from platelets during coagulation.(8) As a consequence, we examined the effect of sample acquisition methods on sCD40L in a group of 8 normal volunteers. As shown in the figure, plasma exhibited a relatively low concentration of sCD40L that was stable over a 4 hour time frame if the sample was maintained on ice. Blood samples kept on ice exhibited similar behavior, likely as a consequence of impaired blood clotting at a low temperature. In contrast, Blood samples that were stored at room temperature demonstrated a time-dependent increase in the concentration of sCD40L. These data substantiate the notion that the method of sample acquisition is a critical determinant of sCD40L concentration.

DISCUSSION

We examined the clinical correlates of sCD40L in a large community-based cohort and observed that the results were highly dependent upon whether the samples were determined from serum or plasma. We found that traditional CVD risk factors explained little of the inter-individual variability in sCD40L concentration, regardless of whether it is measured in serum

or plasma. Serum concentration of sCD40L was positively associated with smoking and inversely associated with advancing age. Analysis of plasma sCD40L concentration revealed age and smoking were inversely and female sex positively related to plasma sCD40L. It is uncertain why smoking and cohort appeared to have a paradoxical opposite relation to sCD40L upon whether samples were derived from serum or plasma. Overall, neither plasma nor serum sCD40L concentration appeared to track closely with established CVD or its risk factors.

Conceptually, sCD40L is an attractive target for development as a marker of CVD risk or activity. Most circulating CD40L is derived from a pre-formed pool in the platelet that is released upon platelet activation. This elaboration of CD40L from platelets results in endothelial cell CD40L engagement that evokes both an inflammatory(8) and pro-thrombotic (20) response. Given the well-established roles of inflammation, thrombosis, and endothelial activation(21;22) in the pathogenesis of atherosclerosis, one might speculate that sCD40L concentration provide important information concerning atherosclerotic disease activity. This speculation has been borne out in clinical studies demonstrating sCD40L concentration predict cardiovascular events in both healthy women(11) and patients with acute coronary syndromes. (12;23–25) These observations are consistent with studies in highly selected populations that have linked sCD40L concentration to established CVD risk factors such as hypercholesterolemia(26–28) and diabetes.(29;30) Thus, considerable initial evidence suggested that sCD40L should be a good marker for cardiovascular risk in referral samples.

The data presented here stand in contrast to the aforementioned body of evidence as circulating sCD40L concentration in our sample was not consistently related to either established CVD risk factors (with the exception of age) or prevalent CVD. One potential reason for this apparent discrepancy may relate to the differences in study samples. Many previous findings involved small patient samples with more advanced stages of disease. The present study involved a sample with considerably lower baseline vascular risk leaving open the possibility that sCD40L may only discriminate amongst individuals with widely disparate risk profiles, perhaps those with prevalent platelet activation. This contention is supported by findings from the only other community-based study of sCD40L, The Dallas Heart Study, which also demonstrated a modest relation between plasma sCD40L and established CVD risk factors.(31) In this study, the general lack of association between sCD40L and CVD risk factors is not likely due to lack of power, as this study alone had 95% power to detect a true R-squared of 0.0059 at an alpha level of 0.01. Thus, available data from large community-based cohorts indicate limited association of sCD40L and traditional measures of CVD risk.

Although mutations in the *CD40L* gene, have been described,(32;33) they are extremely rare and characterized by immunodeficiency syndromes. To our knowledge the relation of heritability, genetic linkage and *CD40L* gene single nucleotide polymorphisms to CD40L concentrations have not been reported. Our data suggest that the genetic contribution to variation in sCD40 concentration in ambulatory cohorts is likely to be at most modest.

The source of clinical samples for sCD40L has been a point of contention. Some studies demonstrating increased CV risk as a function of sCD40L have utilized serum samples,(12) whereas others determined sCD40L from plasma.(11;31) These distinctions are not trivial, as platelet activation during sample acquisition may profoundly increase sCD40L concentration up to ten-fold(34;35) as shown in the Figure. As a consequence, it has been argued that plasma is the preferred sample source,(23;31) yet the basis for this argument is anecdotal, since most studies have not directly compared the two measurements. One notable exception is a study by Thom and colleagues(36) that demonstrated results consistent with those reported here, viz., serum concentration was significantly greater than plasma in patients with either CVD or healthy subjects. They concluded that plasma samples processed quickly were the best source of sCD40L derived from patients. Another comprehensive study by Mason and coworkers

(37) examined sCD40L from plasma, serum, and platelet membranes. They observed a weak positive association between plasma and serum sCD40L concentration, but both were negatively correlated with platelet membrane CD40L (37). Mason and coworkers were unable to discriminate between stable and unstable CAD patients using plasma sCD40L concentration, and found that unstable patients had lower serum sCD40L and higher platelet membrane CD40L than stable patients. Overall, the consistency of CD40L was poor in the discrimination of stable versus unstable CAD. The data presented here adds to this body of literature as we analyzed both serum and plasma sCD40L concentration. In our study the serum and plasma measurements were very weakly correlated and had distinct clinical correlates – age being the only consistent (negative) correlate of sCD40L concentration regardless of the sample source. We also found the method of sample acquisition to have a profound effect on sCD40L concentration, particularly the time delay between blood draw and the processing of serum (Figure). The specimen acquisition data are consistent with those of Ahn and colleagues(34) and indicate that serum determinants of sCD40L have the potential for considerable variability, a trait that would hinder its utility as a CVD biomarker.(38)

Our data raise a number of findings that are difficult to explain. For example, we found that age was associated with decreasing sCD40L levels regardless of the sample source, yet age is associated with established CVD. One possible explanation for these findings is decreased sCD40L synthesis, consistent with reports of reduced cognate helper function of CD4 positive T-cells (39). We also found that smoking and cohort were significantly associated with sCD40L, but in opposite directions in serum versus plasma samples. It is likely these two sample sources reflect different pools of sCD40L, with serum indicative of the total blood content (unreleased and released) and plasma reflecting the steady-state pool released from cells. It remains plausible the variables controlling these two pools of sCD40L are distinct and merits further investigation.

How can we reconcile the fact that sCD40L concentration tracks poorly with established CVD risk factors, yet appear to provide prognostic information in selected clinical populations? One possibility put forth by a previous study is that sCD40L in plasma is a predictor of CVD risk only in patient samples characterized by platelet activation, such as those with established CVD or with active coronary disease.(31) The data presented here are consistent with this notion, as our participants were generally healthy and had a high prevalence of medication use. We did not, however, detect any association of aspirin utilization with either serum or plasma sCD40L concentration. Although the results reported here may dampen enthusiasm for the utility of sCD40L as a predictor of CVD risk, there is one favorable interpretation of our data. It is possible that sCD40L provides information that is not captured by traditional risk factor profiling. If true, this contention would explain the results presented here and those of de Lemos and colleagues,(31) and provide some encouragement that sCD40L concentration may add a new dimension to risk stratification. The ultimate test of this hypothesis will require assessment of incident CVD during longitudinal follow-up of studies such as our study, the Dallas Heart Study(31) and other investigations.

Limitations in our study must be acknowledged. The Framingham Study Offspring participants are largely of European descent and middle-aged to elderly; the generalizability of our findings to younger individuals and other ethnicities remains to be established. In addition, the cross-sectional observational nature of our study design results in our inability to draw causal inferences about the relation between CVD and CVD risk factors and serum and plasma sCD40L concentration. Counterbalanced against these limitations, the routine ascertainment of covariate data and sCD40L concentration and the family-based and community-based cohort facilitate multivariable models and genetic analyses.

In summary, we found no consistent association between sCD40L and established CVD risk factors in samples derived from either plasma or serum. These data suggest that the utility of sCD40L in CVD risk prediction in healthy samples will require longitudinal follow-up.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Reference List

1. Ross R. Atherosclerosis - an inflammatory disease. *N Engl J Med* 1999;340:115–126. [PubMed: 9887164]
2. Cybulsky MI, Gimbrone MA Jr. Endothelial expression of a mononuclear leukocyte adhesion molecule during atherogenesis. *Science* 1991;251:788–791. [PubMed: 1990440]
3. van der Wal AC, Becker AE, van der Loos CM, Das PK. Site of intimal rupture or erosion of thrombosed coronary atherosclerotic plaques is characterized by an inflammatory process irrespective of the dominant plaque morphology. *Circulation* 1994;89(1):36–44. [PubMed: 8281670]
4. Waters D, Craven TE, Lesperance J. Prognostic significance of progression of coronary atherosclerosis. *Circulation* 1993;87:1067–1075. [PubMed: 8484829]
5. Davies MJ, Thomas AC. Plaque fissuring: the cause of acute myocardial infarction, sudden ischemic death, and crescendo angina. *Br Heart J* 1985;53:363–373. [PubMed: 3885978]
6. Hollenbaugh D, Ochs HD, Noelle RJ, Ledbetter JA, Aruffo A. The role of CD40 and its ligand in the regulation of the immune response. *Immunol Rev* 1994;138:23–37. [PubMed: 7520883]
7. Karmann K, Hughes CC, Schechner J, Fanslow WC, Pober JS. CD40 on human endothelial cells: inducibility by cytokines and functional regulation of adhesion molecule expression. *Proc Natl Acad Sci U S A* 1995;92(10):4342–4346. [PubMed: 7538666]
8. Henn V, Slupsky JR, Grafe M, Anagnostopoulos I, Forster R, Muller-Berghaus G, et al. CD40 ligand on activated platelets triggers an inflammatory reaction of endothelial cells. *Nature* 1998;391(6667):591–594. [PubMed: 9468137]
9. Mach F, Schonbeck U, Sukhova GK, Atkinson E, Libby P. Reduction of atherosclerosis in mice by inhibition of CD40 signalling. *Nature* 1998;394(6689):200–203. [PubMed: 9671306]
10. Lutgens E, Gorelik L, Daemen MJ, de Muinck ED, Grewal IS, Kotliansky VE, et al. Requirement for CD154 in the progression of atherosclerosis. *Nat Med* 1999;5(11):1313–1316. [PubMed: 10546000]
11. Schonbeck U, Varo N, Libby P, Buring J, Ridker PM. Soluble CD40L and cardiovascular risk in women. *Circulation* 2001;104(19):2266–2268. [PubMed: 11696462]
12. Heeschen C, Dimmeler S, Hamm CW, van den Brand MJ, Boersma E, Zeiher AM, et al. Soluble CD40 ligand in acute coronary syndromes. *N Engl J Med* 2003;348(12):1104–1111. [PubMed: 12646667]
13. Quan SF, Howard BV, Iber C, Kiley JP, Nieto FJ, O'Connor GT, et al. The Sleep Heart Health Study: design, rationale, and methods. *Sleep* 1997;20(12):1077–1085. [PubMed: 9493915]
14. Kannel, WB.; Wolf, PA.; Garrison, RJ., editors. The Framingham Study: An Epidemiological Investigation of Cardiovascular Disease, Section 34. National Heart, Lung, and Blood Institute: Bethesda, MD; 1987. (NIH) 87-2703
15. Kleinbaum, DG.; Kupper, LL.; Muller, KE. Applied regression analysis and other multivariable methods. Second ed. Boston: PWS-Kent; 1988.
16. SAS/STAT User's Guide, Version 8. Cary NC: SAS Institute Inc.; 1999.
17. Almasy L, Blangero J. Multipoint quantitative-trait linkage analysis in general pedigrees. *Am J Hum Genet* 1998;62(5):1198–1211. [PubMed: 9545414]

18. Allison DB, Neale MC, Zannolli R, Schork NJ, Amos CI, Blangero J. Testing the robustness of the likelihood-ratio test in a variance-component quantitative-trait loci-mapping procedure. *Am J Hum Genet* 1999;65(2):531–544. [PubMed: 10417295]
19. Dupuis J, Larson MG, Vasan RS, Massaro JM, Wilson PW, Lipinska I, et al. Genome scan of systemic biomarkers of vascular inflammation in the Framingham Heart Study: Evidence for susceptibility loci on 1q. *Atherosclerosis* 2005;182(2):307–314. [PubMed: 16159603]
20. Slupsky JR, Kalbas M, Willuweit A, Henn V, Kroczeck RA, Muller-Berghaus G. Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD40. *Thromb Haemost* 1998;80(6):1008–1014. [PubMed: 9869175]
21. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. *Circulation* 2002;105(9):1135–1143. [PubMed: 11877368]
22. Vita JA, Keaney JF Jr. Endothelial function: a barometer for cardiovascular risk? *Circulation* 2002;106(6):640–642. [PubMed: 12163419]
23. Varo N, de Lemos JA, Libby P, Morrow DA, Murphy SA, Nuzzo R, et al. Soluble CD40L: risk prediction after acute coronary syndromes. *Circulation* 2003;108(9):1049–1052. [PubMed: 12912804]
24. Kinlay S, Schwartz GG, Olsson AG, Rifai N, Sasiela WJ, Szarek M, et al. Effect of atorvastatin on risk of recurrent cardiovascular events after an acute coronary syndrome associated with high soluble CD40 ligand in the Myocardial Ischemia Reduction with Aggressive Cholesterol Lowering (MIRACL) Study. *Circulation* 2004;110(4):386–391. [PubMed: 15262833]
25. Heeschen C, Dimmeler S, Hamm CW, Fichtlscherer S, Simoons ML, Zeiher AM. Pregnancy-associated plasma protein-A levels in patients with acute coronary syndromes: comparison with markers of systemic inflammation, platelet activation, and myocardial necrosis. *J Am Coll Cardiol* 2005;45(2):229–237. [PubMed: 15653020]
26. Garlachs CD, John S, Schmeisser A, Eskafi S, Stumpf C, Karl M, et al. Upregulation of CD40 and CD40 ligand (CD154) in patients with moderate hypercholesterolemia. *Circulation* 2001;104(20):2395–2400. [PubMed: 11705814]
27. Schonbeck U, Gerdes N, Varo N, Reynolds RS, Horton DB, Bavendiek U, et al. Oxidized low-density lipoprotein augments and 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors limit CD40 and CD40L expression in human vascular cells. *Circulation* 2002;106(23):2888–2893. [PubMed: 12460867]
28. Semb AG, van WS, Ueland T, Smilde T, Waehre T, Tripp MD, et al. Raised serum levels of soluble CD40 ligand in patients with familial hypercholesterolemia: downregulatory effect of statin therapy. *J Am Coll Cardiol* 2003;41(2):275–279. [PubMed: 12535822]
29. Varo N, Vicent D, Libby P, Nuzzo R, Calle-Pascual AL, Bernal MR, et al. Elevated plasma levels of the atherogenic mediator soluble CD40 ligand in diabetic patients: a novel target of thiazolidinediones. *Circulation* 2003;107(21):2664–2669. [PubMed: 12742991]
30. Marx N, Imhof A, Froehlich J, Siam L, Ittner J, Wierse G, et al. Effect of rosiglitazone treatment on soluble CD40L in patients with type 2 diabetes and coronary artery disease. *Circulation* 2003;107(15):1954–1957. [PubMed: 12695287]
31. de Lemos JA, Zirlik A, Schonbeck U, Varo N, Murphy SA, Khera A, et al. Associations between soluble CD40 ligand, atherosclerosis risk factors, and subclinical atherosclerosis: results from the Dallas Heart Study. *Arterioscler Thromb Vasc Biol* 2005;25(10):2192–2196. [PubMed: 16109925]
32. Ferrari S, Giliani S, Insalaco A, Al-Ghonaium A, Soresina AR, Loubser M, et al. Mutations of CD40 gene cause an autosomal recessive form of immunodeficiency with hyper IgM. *Proc Natl Acad Sci U S A* 2001;98(22):12614–12619. [PubMed: 11675497]
33. Winkelstein JA, Marino MC, Ochs H, Fuleihan R, Scholl PR, Geha R, et al. The X-linked hyper-IgM syndrome: clinical and immunologic features of 79 patients. *Medicine (Baltimore)* 2003;82(6):373–384. [PubMed: 14663287]
34. Ahn ER, Lander G, Jy W, Bidot CJ, Jimenez JJ, Horstman LL, et al. Differences of soluble CD40L in sera and plasma: implications on CD40L assay as a marker of thrombotic risk. *Thromb Res* 2004;114(2):143–148. [PubMed: 15306157]

35. Bereczki D, Nagy E, Pal A, Magyar MT, Balla J. Should soluble CD40 ligand be measured from serum or plasma samples? *Arterioscler Thromb Vasc Biol* 2003;23(6):1129–1130. [PubMed: 12807715]
36. Thom J, Gilmore G, Yi Q, Hankey GJ, Eikelboom JW. Measurement of soluble P-selectin and soluble CD40 ligand in serum and plasma. *J Thromb Haemost* 2004;2(11):2067–2069. [PubMed: 15550055]
37. Mason PJ, Chakrabarti S, Albers AA, Rex S, Vitseva O, Varghese S, et al. Plasma, Serum, and Platelet Expression of CD40 Ligand in Adults With Cardiovascular Disease. *Am J Cardiol* 2005;96(10):1365–1369. [PubMed: 16275179]
38. Keaney JF Jr. Circulating biomarkers in acute coronary syndromes: something different or more of the same? *Circulation* 2005;112(6):778–780. [PubMed: 16087806]
39. Eaton SM, Burns EM, Kusser K, Randall TD, Haynes L. Age-related defects in CD4 T cell cognate helper function lead to reductions in humoral responses. *J Exp Med* 2004;200(12):1613–1622. [PubMed: 15611289]

Figure.

Sample preparation and sCD40L concentration. Blood samples from 8 non-Framingham subjects were collected in tubes containing EDTA or no additives. Blood samples were then either kept on ice or at room temperature, as indicated, before centrifugation and preparation of serum or plasma and determination of sCD40L as described in "Methods." Data represent mean $\pm$ SD of 8 determinations.

Table 1

Clinical characteristics of the Offspring and Omni samples.

	Men (n=1682)	Women (n=1975)
Age, years	61±10	60±10
Cohort, % Omni	10%	12%
Total/HDL cholesterol	4.5±1.4	3.7±1.2
Triglycerides, mg/dL	143±99	131±75
Systolic blood pressure, mmHg	128±17	126±20
Diastolic blood pressure, mmHg	76±10	73±10
Body mass index, kg/m ²	28.8±4.6	27.7±5.9
Waist, in	40.6±4.6	38.1±6.2
Fasting glucose, mg/dL	109±31	101±27
Smoking, %	13	13
Diabetes, %	16	11
Lipid lowering treatment, %	24	16
Hypertension treatment, %	36	31
Hormone replacement therapy, %	--	30
Aspirin use, %	39	23
Prevalent CVD, %	17	9
Serum sCD40L (ng/mL)	3.84 ± 3.48	3.97 ± 3.41
Plasma sCD40L (ng/mL)	3.07 ± 4.65	3.44 ± 4.84

All numbers are mean ± SD (continuous), or percent (categorical).

Table 2
Age- sex - and ethnicity-adjusted regression models for serum sCD40L concentration.

Variable, Increment	Serum			Plasma		
	Regression coefficient	SE	P value	Regression coefficient	SE	P value
Age, 10 years	-0.090	0.026	0.0006	-0.080	0.022	0.0002
Sex, female versus male	0.125	0.050	0.012	0.156	0.041	0.0001
Ethnicity, Omni vs Offspring	0.191	0.081	0.019	-0.216	0.067	0.0012
Total/HDL cholesterol, 2 units	-0.098	0.039	0.012	0.013	0.032	0.68
Triglycerides, 100mg/dL	-0.021	0.029	0.46	-0.018	0.023	0.45
Systolic blood pressure, 20 mmHg	0.019	0.029	0.50	0.023	0.023	0.32
Diastolic blood pressure, 10mmHg	0.015	0.026	0.56	0.0057	0.022	0.79
Body mass index, 5 kg/m ²	0.0009	0.023	0.97	-0.024	0.019	0.20
Waist, 5 inch	0.0003	0.023	0.99	-0.023	0.019	0.22
Blood glucose, 25 mg/dL	-0.050	0.022	0.022	-0.022	0.018	0.23
Smoking, present	0.187	0.076	0.014	-0.439	0.062	<0.0001
Diabetes, present	-0.044	0.074	0.55	-0.075	0.061	0.22
Lipid treatment, present	0.022	0.064	0.74	-0.051	0.053	0.34
Hypertension treatment, present	0.018	0.055	0.75	-0.109	0.045	0.016
Hormone treatment, present	0.015	0.074	0.84	-0.077	0.061	0.21
Aspirin use, present	0.0004	0.057	0.99	-0.035	0.047	0.46
CVD, present	0.066	0.079	0.40	-0.052	0.065	0.42

Linear regression coefficients represent the estimated change in sCD40L(ng/mL) per specified number of units (continuous, approximately 1 SD) or presence (dichotomous) of the variable.

Table 3
Multivariable models for serum and plasma sCD40L concentration.

	Serum		Plasma		P value
	Regression coefficients	SE	Regression coefficient	SE	
Cohort, omni vs. offspring					
Age, 10 years	0.207	0.081	-0.241	0.066	0.0003
Sex, female versus male	-0.081	0.027	-0.085	0.023	0.0002
	0.080	0.053	0.150	0.041	0.0002
Total/HDL cholesterol, 2units	-0.102	0.039			
Smoking	0.195	0.076	-0.443	0.062	<0.0001
Hypertension treatment			-0.117	0.045	0.0091
Model R²		0.011		0.025	

Regression coefficients represent the estimated change in sCD40L (ng/mL) per specified number of units (~1 SD) of the variable. All covariates were analyzed simultaneously, selected by a stepwise method as described in the Statistical Analysis section.