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Soluble CD40 ligand and outcome in patients with coronary artery disease undergoing percutaneous coronary intervention

Abstract

Objectives: CD40 ligand (CD40L), a transmembrane glycoprotein belonging to the tumor necrosis factor family and expressed by a variety of cells, is involved in the basic mechanisms of inflammation, atherosclerosis and thrombosis. Some studies suggest that the soluble form of CD40L (sCD40L) is a predictor of major cardiovascular events and mortality in a variety of clinical settings, but data from literature are conflicting.

Methods: We studied consecutive patients with acute (ACS) or chronic (CCS) coronary syndrome who underwent percutaneous coronary artery intervention (PCI). Blood samples for sCD40L dosage were taken at baseline immediately before PCI. We tested the relation between sCD40L and pre-specified outcome measures consisting of new ACS, clinical restenosis and all-cause mortality. We recruited 3,841 patients (mean age 64 ± 11 years, 79% men) with ACS (n=2,383) or CCS (n=1,458).

Results: During a mean follow-up of two years (±0.6 years), 642 patients developed ACS, 409 developed restenosis (≥70% of at least one of the previously treated coronary segments) and 175 died. For each 1-standard deviation increase in sCD40L (0.80 ng/mL), the hazard ratios (HRs) for ACS, restenosis, and mortality were 1.11 (95% confidence interval [CI]: 1.05 to 1.18, p<0.0001), 1.10 (95% CI: 1.02 to 1.19, p=0.010), and 1.00 (95% CI: 0.86 to 1.16, p=0.983), respectively. In multivariable Cox regression models with adjustment for several potential confounders including age, acute or chronic coronary syndrome, multi-vessel disease, stent placement, diabetes, previous coronary events and dyslipidemia, sCD40L remained an independent predictor of ACS and coronary restenosis. There were no interactions between sCD40L and acute or chronic coronary syndrome or stent placement.

Conclusions: Among patients with ACS or CCS who undergo PCI, higher levels of sCD40L predict an increased risk of acute coronary events and coronary restenosis, but not of mortality.

Keywords: angioplasty; atherosclerosis; chronic disease; inflammation; myocardial infarction; soluble CD40 ligand.

Introduction

Inflammation plays an important role in the growth, instability and rupture of the atherosclerotic plaque through different mechanisms including the infiltration of immune defense cells such as activated macrophages and lymphocytes [1–6]. The resulting high levels of cytokines within the plaque may be responsible of progressive lysis of the connective matrix leading to plaque fissuring and disruption [1, 6].

Some transmembrane and circulating glycoproteins contribute to trigger a complex cascade of inflammatory reactions. Monocytes, macrophages, endothelial cells, smooth muscle cells, platelets and dendritic cells express the type I transmembrane protein receptor CD40, a member of the tumor necrosis factor superfamily, whose gene is located on chromosome 20 (q12–q13.2) [4, 7, 8]. CD40 ligand (CD40L) is another transmembrane glycoprotein belonging to the TNF superfamily which is expressed on activated *T* cells, platelets, endothelial cells, smooth muscle cells and macrophages [8]. The contact between CD40L and CD40 triggers conformational changes in the structure of CD40 through a link between intracellular cysteine residues [9], with resulting generation of a dyad

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which triggers inflammatory responses associated with enhanced release of inflammatory cytokines and vascular adhesion molecules [10]. Platelet activation may induce, within a few seconds, a cleavage of membrane-anchored CD40L with generation of the soluble circulating fragment sCD40L, which continues to trigger inflammation and thrombosis [11, 12]. Since more than 95% of sCD40L derives from platelets, it has been assumed that sCD40L reflects the amount of platelet activation [13].

Several studies demonstrated increased circulating levels of sCD40L in patients with acute coronary syndrome (ACS) [6, 14–16] and a few studies suggested that sCD40L may be a predictor of major cardiovascular events and mortality in a variety of clinical settings [13, 17–20]. However, data from literature are conflicting [21–26].

In the present study, we tested the hypothesis that sCD40L is predictive of increased risk of ACS, coronary restenosis and all-cause mortality over a two-year follow-up in a specific population of patients who underwent coronary angioplasty (PCI) in the setting of ACS or chronic coronary syndrome (CCS).

Materials and methods

Our investigation was a satellite analysis of the CK-MB and PCI study [27]. Details of the study have been published [27, 28]. Briefly, CK-MB and PCI study is a prospective, multicentre cohort study that enrolled consecutive patients who underwent PCI, either elective or performed in the context of ACS. We monitored the consecutive enrollment of patients through periodic checking and included in the analysis only those centers which recruited ≥90% of patients who underwent PCI [27]. Thus, entry criteria included either CCS [29] or ACS [30, 31] (non-ST elevation myocardial infarction [NSTEMI], unstable angina [UA], or ST-elevation myocardial infarction [STEMI]).

We excluded patients who refused to provide written informed consent to participate to the study. The study was approved by the local Ethics Committees.

The initial evaluation included a detailed clinical examination, biochemical parameters and 12-lead ECG. The presence of risk factors and comorbidities was defined according to documented medical history. This assessment was performed by any physician during the clinical interview with the patient and by searching through medical records. The selection of medications, devices, degree of revascularization, evaluation of standard laboratory parameters (assessed using standard techniques), and subsequent hospital care were left to the discretion of the treating physician.

The present analysis focused on the prognostic value of pre-procedural serum levels of sCD40L. Blood samples were drawn form a cannulated forearm vein immediately before PCI and collected in citrate-anticoagulated tubes. After centrifugation, plasma was stored at −70 °C and shipped to the core biochemistry laboratory for analysis of biochemical markers. sCD40L levels were measured using an analytically validated automated immunoassay (Roche Diagnostics) according to the manufacturers protocol by staff blinded to clinical end points.

Follow-up

The vital conditions of patients were assessed at 6, 12 and 24 months after hospital discharge by means of hospital visits or phone interviews. Pre-specified endpoints consisted in: (i) hospitalization associated with a CK-MB > 2 times higher than the upper normal value or, in the case of a control ECG, a new onset Q wave with ≥0.04 s duration or ≥1/4 of the amplitude of the corresponding R wave in at least two consecutive leads; (ii) occurrence of spontaneous or effort-induced myocardial ischemia with an angiographically confirmed restenosis >70% of at least one of the previously treated coronary segments, and (iii) all-cause mortality.

Hospital records and other source documents of patients who died were reviewed in conference by the authors of this study.

Data analysis

Analyses were performed using Stata, version 16 (StataCorp LP, College Station, TX, USA) and R version 2.9.2 (R Foundation for Statistical Computing, Vienna, Austria).

We expressed the continuous variables as mean value (±standard deviation [SD]) and the categorical variables as proportions. Unpaired t-test was used to compare continuous variables across groups, and χ^2 test was used for categorical variables.

We evaluated the effect of prognostic factors on endpoints by univariable and multivariable Cox proportional hazards regression models. The hazard ratios (HR) from univariable and multivariable analyses and their corresponding two-sided 95% confidence intervals (CI) were derived from the regression coefficients in the Cox models. For all analyses, the follow-up time was defined as the period between the PCI and the last confirmed follow-up or date of event.

In univariable analyses we tested, in addition to serum levels of sCD40L, the impact of other variables known to influence outcome in this population. These variables were: age (years); sex (male/female); history of hypertension (yes/no); history of type 2 diabetes (yes/no); history of dyslipidaemia (yes/no), current cigarette smoking (yes/no); previous coronary events (yes/no); peripheral arterial disease (yes/no); inclusion in the study for CCS or ACS; coronary multivessel disease (yes/no); stent placement during index PCI (yes vs. no); use of antiplatelet therapy (yes vs. no) and glycoprotein inhibitors (yes vs. no) at the time of PCI. In all cases of PCI, bare metal coronary stents were used.

We defined history of hypertension by the current use of anti-hypertensive drugs or blood pressure values greater than or equal to 140 mmHg systolic or 90 mmHg diastolic. Dyslipidaemia was defined by current use of lipid-lowering drugs or total serum cholesterol >5.2 mmol/L (200 mg/dL). We defined diabetes using the American Diabetes Association criteria of a fasting plasma glucose level of 7.0 mmol/L (126 mg/dL) or higher or current antidiabetic therapy [32]. Peripheral arterial disease was defined by claudicatio intermittens associated with evidence of at least 50% peripheral artery stenosis by duplex scanning or angiography. Multivessel disease was defined by a >50% stenosis in two or more epicardial coronary vessels.

We developed baseline multivariable models that included the covariables which yielded significance in the univariable analyses. Interactions between covariables were also tested, and the Akaike information criterion was used to decide the final model for prediction.

To assess the incremental predictive value for endpoints afforded by sCD40L we calculated the continuous net reclassification

improvement (cNRI), which does not depend on the arbitrary choice of categories, and the integrated discrimination improvement (IDI) using methods suitable for survival data [33, 34].

We developed a nomogram using the results of the validated multivariable models [35]. A nomogram maps the predicted probabilities into points on a scale from 0 to 100 in a user-friendly graphical interface. The total points accumulated by the various covariables correspond to the predicted probability of the outcome for a patient.

Two-sided p-values ≤0.05 were considered statistically significant.

Results

Overall, 3,841 patients were included in the present analysis. Table 1 summarizes the characteristics of the study population. Mean age of patients at hospital admission was 64 ± 11 years. Prevalence of diabetes, hypertension, dyslipidaemia, and current smokers was 19%, 58%, 56%, and 59%, respectively. According to type of coronary syndrome, 2,383 patients were admitted to hospital for ACS (NSTEMI/UA, n=2,027; STEMI, n=356) and 1,458 for CCS with indications for coronary angiography and PCI. Mean levels of sCD40L were 0.59 ng/mL, 0.53 ng/mL, 0.60 ng/mL, and 0.62 ng/mL for CCS, UA, NSTEMI, and STEMI, respectively (p=0.074 for trend).

Participants with ACS at admission were older, had a higher prevalence of diabetes mellitus, dyslipidemia and previous coronary events (all p<0.05, Table 1).

During a mean follow-up period of two years, 642 (17%) patients experienced a new ACS, 409 (11%) patients showed spontaneous or effort-induced myocardial ischemia with an angiographically confirmed restenosis >70% of at least one of the treated coronary segments, and 175 (5%) patients died.

For each 1-SD increase in sCD40L (0.80 ng/mL), the HRs for ACS, clinical restenosis, and mortality were 1.11 (95% CI: 1.05 to 1.18, p<0.0001), 1.10 (95% CI: 1.02 to 1.19, p=0.010), and 1.00 (95% CI: 0.86 to 1.16, p=0.983), respectively. The association between serum levels of sCD40L and the risk of new ACS, as well as clinical restenosis, was significant in patients with ACS or CCS (Figure 1). Remarkably, there were no significant interactions between serum sCD40L and type of coronary syndrome at hospital admission (CCS or ACS) for both outcomes (p=0.654 for ACS; p=0.965 for coronary clinical restenosis; Figure 1).

Figure 2 shows the results of univariable Cox analyses exploring the predictors of new ACS (left panel) and coronary restenosis (right panel) during follow-up.

Among the predictors of new ACS which yielded statistical significance in the univariable analyses, age, multivessel disease, diabetes, previous coronary event, dyslipidemia, type of coronary syndrome at index hospitalization, stent placement, and sCD40L were included in the final multivariable model (Table 2). Use of antiplatelet therapy (HR: 0.91, 95% CI: 0.69 to 1.21, p=0.522) and

Table 1: Main patient characteristics.

Variables	Overall (n=3,841)	Chronic coronary syndrome (n=1,458)	Acute coronary syndrome (n=2,383)	p-Value (chronic vs. acute coronary syndrome)
Age, years	64 ± 11	63 ± 10	64 ± 11	0.002
Male sex, %	79	83	76	<0.0001
Hypertension, %	59	59	59	0.795
Type 2 diabetes, %	19	19	19	0.772
Dyslipidemia, %	56	62	52	<0.0001
Current smoker, %	59	60	58	0.356
Known CAD, %	59	44	56	<0.0001
History of PAD, %	8	9	8	0.153
Multivessel disease, %	41	40	42	0.255
Angiographic EF, %	56 ± 12	57 ± 11	56 ± 12	<0.001
Stent placement, %	81	77	83	<0.0001
Serum creatinine, mg/dL	1.04 ± 0.59	1.02 ± 0.47	1.05 ± 0.66	0.177
Soluble CD40L, ng/mL	0.57 ± 0.80	0.59 ± 0.80	0.57 ± 0.79	0.447
Glycoprotein inhibitors at the time of PCI, %	6	1	9	<0.0001
Antiplatelet therapy at the time of PCI, %				<0.0001
Single therapy, %	47	38	52	
Dual therapy, %	46	59	34	

CAD, coronary artery disease; CD40L, CD40 ligand; EF, ejection fraction; PAD, peripheral artery disease; PCI, percutaneous coronary intervention.
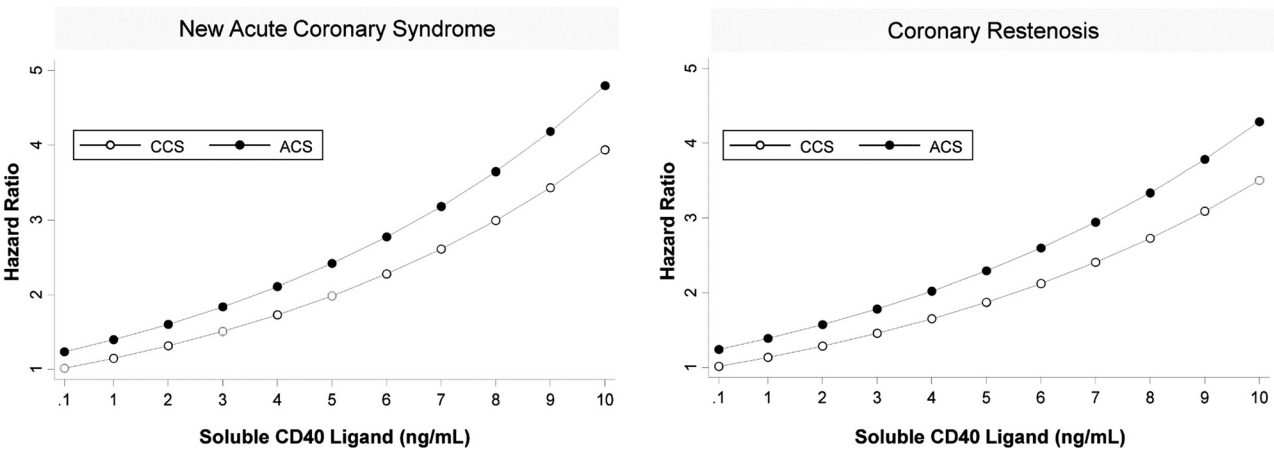
Figure 1: Association between serum levels of sCD40L and the risk of new acute coronary syndrome (left panel) and restenosis (right panel) in both groups of patients with acute or chronic coronary syndromes.

ACS, acute coronary syndrome; CCS, chronic coronary syndrome; sCD40L, soluble CD40 ligand. Interactions with sCD40L: p=0.654 for new acute coronary syndrome; p=0.965 for coronary restenosis.
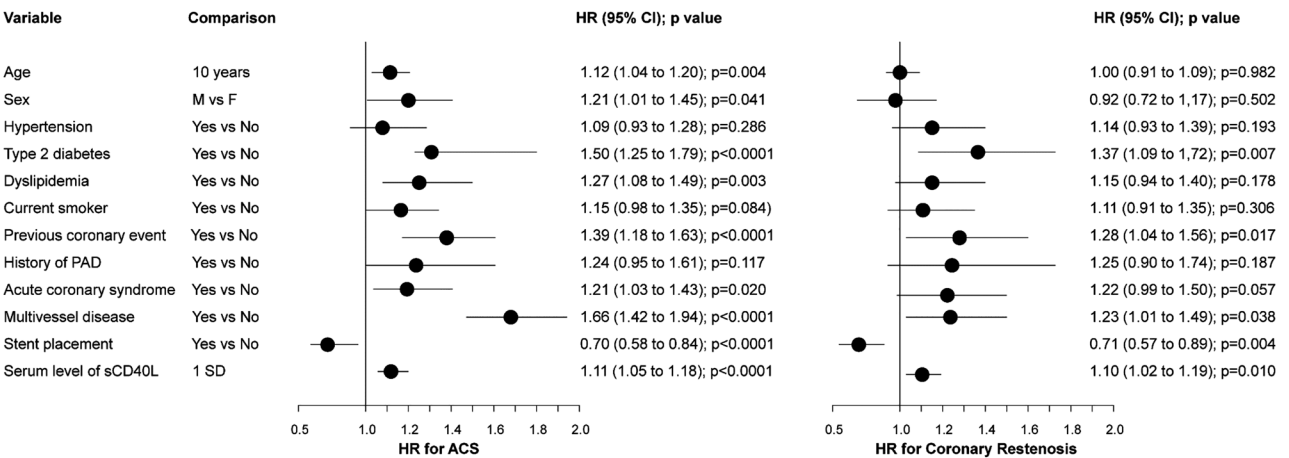

Figure 2: Results of univariable analyses exploring predictors of new acute coronary syndrome (left panel) and restenosis of at least one of the previous treated coronary segments (right panel).

ACS, acute coronary syndrome; CI, confidence interval; HR, hazard ratio; PAD, peripheral artery disease; sCD40L, soluble CD40 ligand.

glycoprotein inhibitors (HR: 1.11, 95% CI: 0.81 to 1.53, p=0.520) prior to PCI did not show any significant associations with new ACS. No significant interactions were observed between covariates (all p>0.05).

For the outcome of clinical restenosis, serum levels of sCD40L, diabetes, and stent placement retained statistical significance to enter in the final prediction multivariable model (Table 3). No significant interactions were noted between these three covariates (all p>0.05). Use of antiplatelet therapy (HR: 0.74, 95% CI: 0.54 to 1.03, p=0.077) and glycoprotein inhibitors (HR: 1.07, 95% CI: 0.71 to 1.60, p=0.754) prior to PCI did not show significant associations with the risk of clinical restenosis during follow-up.

We found a statistically significant improvement in the performance of multivariable models when adding sCD40L as covariate for both outcomes of interest (since cNRI is directly related to the proportion of individuals for which sCD40L improves prediction and IDI to the mean improvement).

For prediction of new ACS, we found a statistically significant cNRI after adding sCD40L to the multivariable model reported above (coefficient=0.1511, standard error [SE]=0.0440, z=3.43, p=0.0006). Separation between events and non-events after the addition of sCD40L resulted in a significant IDI (coefficient=0.0033, SE=0.0010, z=3.46, p=0.0005).

Table 2: Multivariable models exploring the prognostic impact of soluble CD40 ligand on the risk of new acute coronary syndrome.

Variable	Comparison	HR	95% confidence interval	p-Value
Age	10 years	1.08	1.01–1.17	0.047
Multivessel disease	Yes vs. No	1.56	1.33–1.84	<0.0001
Diabetes	Yes vs. No	1.36	1.13–1.64	0.001
Previous coronary event	Yes vs. No	1.22	1.03–1.45	0.023
Dyslipidemia	Yes vs. No	1.29	1.10–1.53	0.002
Acute coronary syndrome	Yes vs. No	1.29	1.09–1.52	0.003
Stent placement	Yes vs. No	0.71	1.05–1.19	<0.0001
sCD40L	1 SD	1.12	1.05–1.19	<0.0001

HR, hazard ratio; sCD40L, soluble CD40 ligand; SD, standard deviation.

Table 3: Multivariable models exploring the prognostic impact of soluble CD40 ligand on the risk of clinical restenosis of at least one of the previously treated coronary segments.

Variables	Comparison	HR	95% CI	p-Value
Diabetes[a]	Yes vs. No	1.33	1.05–1.67	0.018
Stent placement[b]	Yes vs. No	0.71	0.57–0.89	0.004
sCD40L	1 SD	1.11	1.03–1.20	0.008

CI, confidence interval; HR, hazard ratio; sCD40L, soluble CD40 ligand. [a]p for interaction with sCD40L = 0.857; [b]p for interaction with sCD40L = 0.697.

For prediction of restenosis, we found a significant cNRI (coefficient=0.1116, SE=0.0532, z=2.17, p=0.0298) and IDI (coefficient=0.0014, SE=0.0006, z=2.40, p=0.0165) when sCD40L was added to the multivariate predictive model.

Nomograms for the estimation of the risk of new ACS (Figure 3) and clinical restenosis (Figure 4) during follow-up were also generated on the basis of the multivariable Cox regression analyses (Table 2 and 3). As depicted in Figures 3 and 4, each indicator is measured, and the corresponding points are assigned using the row "Score". Thus, the sum is reported on the row "Total Score", and the corresponding probability of the outcome is identified in the row "two-year probability".

For example, a 70-year-old patient (4 points for age) admitted to hospital for ACS (2 points) with history of diabetes (2.5 points) and dyslipidemia (2 points), without previous coronary events (0 point), baseline sCD40L level of 3 (3 points), and treated with PCI (with stent placement [0 points] and evidence of multi-vessel coronary disease [3.5 points]) has a total score equal to 17

points and an estimated probability of new ACS of 40% (Figure 3).

Discussion

In this large prospective study of a well-defined cohort of patients with ACS or CCS undergoing primary or elective PCI, sCD40L showed a significant association with the two-year risk of new ACS and coronary restenosis, but not mortality. Such association was independent of several confounders which yielded significance in the multivariate model (Tables 2 and 3). In addition, sCD40L improved the reclassification of patients at different risk of outcome and the discrimination of patients with and without future events.

Previous studies

Elevated concentrations of sCD40L identify patients with ACS or CCS at increased risk of major complications or death in some studies [13, 17–19, 36, 37], but this association was not confirmed in other studies [20, 22–26, 38].

In a small nested case-control study of apparently healthy women, elevated levels of sCD40L were associated with increased risk of a composite pool of cardiovascular events [19]. In a sub-group analysis of a randomized study comparing abciximab and placebo in 1,088 patients with ACS, elevated concentrations of sCD40L identified patients at higher risk of mortality or nonfatal myocardial infarction in the placebo group, but not in the abciximab group [13]. These data suggests that the inhibition of platelet aggregation induced by abciximab may abolish the potential for sCD40L to identify patients at increased risk of death or reinfarction [13]. In another nested case-control study of 190 patients with ACS, elevated levels of sCD40L were independent predictors of death and reinfarction [37]. In a sub-group analysis of 2,908 patients with unstable angina or non-Q wave myocardial infarction randomized to atorvastatin or placebo, sCD40L >90th centile was associated with increased risk of recurrent coronary events in the placebo group, but not in the atorvastatin group [36]. Thus, atorvastatin also appears to abolish the prognostic value of sCD40L [36]. In a longitudinal study of 499 consecutive patients with STEMI, sCD40L significantly predicted one-year mortality after correction for the significant effect of age and ejection fraction [18].

Other longitudinal studies provided conflicting results. In a small case-control post-hoc analysis of 466 CCS patients randomized to either bezafibrate or placebo,
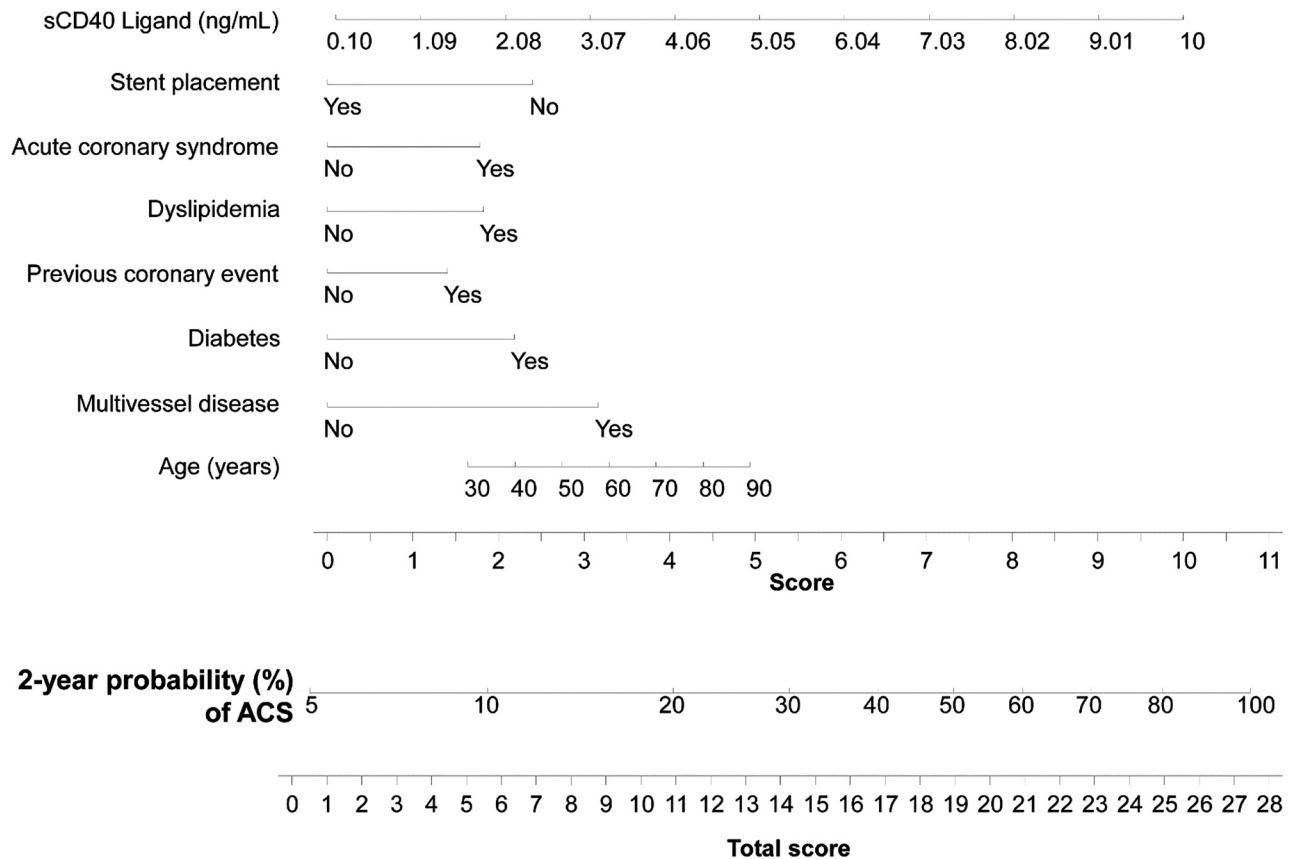
Figure 3: Nomogram for prediction of probability of new acute coronary syndrome at follow-up, based on eight indicator variables. Each indicator is measured, and the corresponding points are assigned using the row "score". The sum is reported on the row "total score," and the corresponding probability of new acute coronary syndrome (ACS) is identified in the row "two-year probability of ACS". ACS, acute coronary syndrome; sCD40, soluble CD40.

sCD40L did not predict an increased risk of coronary events or stroke [38]. In another case-control study of 263 STEMI patients and 262 control patients without coronary artery disease, sCD40L was not associated with the risk of cardiac mortality at two years [25]. In a post-hoc analysis of the TACTICS-TIMI 18 trial, sCD40L determinations were available in 1524 NSTEMI patients treated with aspirin, heparin and tirofiban who had been randomized to an early invasive or conservative strategy [22]. In this population, sCD40L was not associated with non-fatal myocardial infarction or re-hospitalization for ACS at one month [22]. Albeit limited by the relatively small sample and the short duration of follow-up, these data confirms that glycoprotein IIb/IIIa receptor inhibition may abolish the ability of sCD40L to identify patients with ACS at increased risk of major cardiovascular complications [36]. In a study of 303 patients with CCS and cardiac events at one year, 303 patients with CCS and no events at one year, and 303 patients without coronary artery disease and without events, sCD40L did not show any independent association with

cardiac events [24]. In the placebo arm of a randomized study comparing ortofiban and placebo in 2,403 ACS patients, sCD40L did not show any association with the risk of a composite pool of death or myocardial infarction [23]. Similar results were seen in a small study of 77 patients with ACS [20]. Finally, in 97 consecutive patients with unstable angina undergoing coronary angiography, sCD40L was not predictive of a composite pool of death and recurrent ACS at six months [26].

The present study

The present study has the strength of the largest sample size available so far, which allowed a robust assessment of the predictive value of sCD40L in a multivariable model which included several important predictors of recurrent ACS such as age, multivessel disease, diabetes, previous coronary events, dyslipidemia, type of ACS at entry (STEMI vs. NSTEMI) and stent placement. We also found that
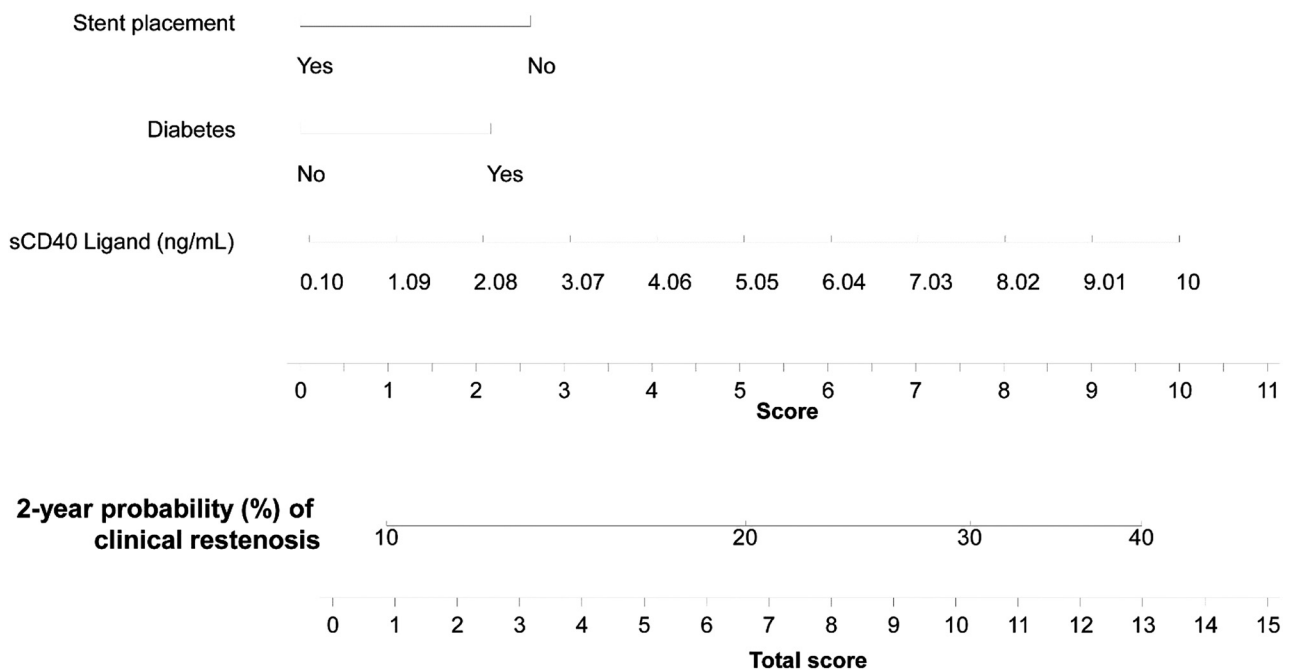
Figure 4: Nomogram for prediction of probability of coronary restenosis at follow-up, based on three indicator variables.
Each indicator is measured, and the corresponding points are assigned using the row "score". The sum is reported on the row "total score," and the corresponding probability of clinical restenosis is identified in the row "two-year probability of clinical restenosis". sCD40, soluble CD40.

sCD40L was a predictor of angiographic clinical restenosis, in line with previous studies [17, 39–41]. Remarkably, higher levels of sCD40L were documented among patients with restenosis after PCI than in those without complication [39], and the same biomarker proved to be a significant predictor of coronary restenosis [40–42].

Several factors might explain the discrepancy between different studies on the ability of sCD40L to identify patients at different cardiovascular risk. Heterogeneity of patient population, different durations of follow-up and type II errors (i.e., false negative) due to small sample sizes may be reasonable sources of variability. It has been suggested [22] that also differences in the technique of analysis including speed of centrifugation, interval between blood drawing and sCD40L determination, and the temperature and assay used for analysis could play a role [43, 44]. Also, plasma should be preferred to serum for sCD40L determination because serum samples yield higher sCD40L values compared to plasma, possibly as a result of release of sCD40L from platelets during clotting [45]. In the present study, we determined sCD40L from plasma and used an analytically validated immunoassay (Roche Diagnostics).

Another factor justifying the discrepancy between different studies is the underlying use of glycoprotein IIb/IIIa receptor inhibitors, which may limit the release of sCD40L from platelets and, hence, its ability to identify

patients at different cardiovascular risk [13, 22] In our study, only 6% of patients were being treated with glycoprotein IIb/IIIa receptor inhibitors at the time of blood drawing just before PCI. The possible lesser inhibition of sCD40L release from platelets may have favored the disclosure of its ability to identify patients at different risk. Because of the observational nature of our study and the potential for selection bias, we cannot make inferences on the relation between drug treatment and outcome. However, neither glycoprotein IIb/IIIa receptor inhibitors nor other antiplatelet drugs showed a significant relation with recurrent ACS or coronary restenosis (data not shown).

The large sample of the present study allowed us to develop nomograms which allow an easy prediction of the two-year probability of developing new ACS or coronary restenosis on the basis of a solid background of factors all of which entered the multivariable models as significant predictors of outcome (Figures 3 and 4).

Limitations of the study

Since the study has been conducted in Caucasian patients, extrapolation of results to different ethnic groups requires caution. Results are applicable to patients with ACS or CCS who require coronary angioplasty, but not necessarily to the general population or to specific cohorts of subjects

with or without risk factors or previous cardiovascular events. Furthermore, administration of drug treatments was not randomized, and subject to selection bias. Angiographic restenosis was locally estimated by researchers who were unaware of sCD40L values. Drug-eluting stents and more powerful P2Y12 platelet receptor antagonists were not available when our study was conducted and these factors could dampen the applicability of our results at the present days.

In other words, clinical applicability of results, including the use of nomogram to predict probability of new ACS and restenosis, should be validated in more recent cohorts. From a pathophysiological standpoint, however, such limitation contributed to a better understanding of the role of the CD40/CD40L system in patients with ACS or CCS through the disclosure of the prognostic value of its soluble form in these patients. Strengths of this study were the large sample size, the central biochemical core-laboratory, the well-defined clinical characteristics of patients, their consecutive recruitment and the two-year follow-up with independent adjudication of clinical events.

Conclusions

In a large sample of consecutive patients with ACS or CCS undergoing coronary angioplasty, sCD40L determined immediately before the procedure showed an independent association with the risk of recurrent ACS and coronary restenosis, but not mortality, at two years. The prognostic value of sCD40L was comparable in patients with ACS and CCS. sCD40L significantly improved the reclassification of patients at different risk of recurrent ACS or coronary restenosis and the discrimination of patients with and without events.

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