Men with Lower HDL Cholesterol Levels have Significant Increment of Soluble CD40 Ligand and High-sensitivity CRP Levels Following the Cessation of Long-term Clopidogrel Therapy

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Aim: The aim of this study was to examine whether the termination of long-term clopidogrel therapy results in a proinflammatory state and whether lipid parameters influence the inflammatory response after stopping the drug.

Methods: A prospective, multicenter study was conducted among 200 patients with implanted coronary stents who received dual antiplatelet therapy for one year, without ischemic or bleeding events. According to the guidelines, clopidogrel was discontinued after one year. In all patients, the high-sensitivity C-reactive protein (hsCRP), soluble CD40 ligand (sCD40L) and lipid [total cholesterol, triglycerides, high-density lipoprotein cholesterol (HDL-C) and low-density lipoprotein cholesterol (LDL-C)] levels were measured twice: on the day of cessation of clopidogrel and 45 days after the termination of clopidogrel treatment.

Results: In men (n = 151), the sCD40L serum levels were significantly higher 45 days after the discontinuation of clopidogrel (p = 0.007), while the hsCRP levels were not significantly different (p = 0.407). Furthermore, when analyzed across the HDL-C quartiles, the hsCRP and sCD40L values were found to be associated with the levels of HDL-C after the discontinuation of clopidogrel in men. In addition, the men in the first HDL-C quartile exhibited the most pronounced increase in the sCD40L levels (p = 0.001) and had significantly higher hsCRP levels (p = 0.001) compared to the subjects in the other quartiles. Other lipid parameters did not show any associations with the sCD40L or hsCRP levels.

Conclusions: The discontinuation of clopidogrel is associated with higher increments in the sCD40L level, and a pronounced proinflammatory response is associated with a lower HDL-C concentration.


Key words: Clopidogrel cessation, Inflammatory markers, HDL-C, Men

Introduction

The beneficial clinical effects of the application of dual antiplatelet therapy in patients with acute coronary syndrome and those undergoing coronary artery stenting are well known. According to current recommendations, combined therapy with aspirin and clopidogrel should be administered for at least one year in these patients. However, recent research has shown that the discontinuation of long-term clopidogrel therapy is often followed by an increase in the rate of adverse cardiovascular events, i.e. the rebound phe-
nomenon\(^4-6\)). Clopidogrel is a drug with pleiotropic effects\(^7\). In addition to its proven antiaggregating and antithrombotic actions, clopidogrel also exerts anti-inflammatory effects\(^8\). In this regard, the potential rebound of the platelet activity can lead to the development of a prothrombotic and/or proinflammatory state following the cessation of clopidogrel\(^8-11\).

It remains unknown whether certain factors and/or patient characteristics influence the rebound phenomenon. The aim of this study was therefore to examine whether the cessation of long-term clopidogrel therapy is followed by an increase in the levels of specific inflammatory biomarkers, which may explain the observed changes in the platelet activity. Considering the frequent associations between inflammation, dyslipidemia and an increased platelet activity, we also investigated the relationships between lipid parameters and inflammatory markers after clopidogrel cessation.

Materials and Methods

Study Design

This study was an open, multicenter, prospective trial that included 200 patients who underwent intracoronary stent implantation at the Clinical Centre of Serbia and Military Medical Academy. All patients received dual antiplatelet therapy (aspirin 100 mg/day + clopidogrel 75 mg/day) one year after stent placement. Patients were excluded from the study if they met any of the following criteria: ischemic events during the one-year period of clopidogrel therapy, less than 20 or more than 80 years of age, unable to give informed consent, selected hematological abnormalities (hemoglobin < 100 g/L, platelet count < 100 × 10\(^9\) cells/L or > 600 × 10\(^9\) cells/L, neutrophil count < 1.5 × 10\(^9\)/L), liver failure or renal failure. In addition, individuals taking anticoagulants, non-steroidal anti-inflammatory drugs or glucocorticoids were not enrolled. Informed consent was obtained from each patient for participation in this study. After providing consent, patients who met the entry criteria discontinued clopidogrel, while all previous therapy was continued, including aspirin.

The study protocol was approved by the Ethics Committee of the Medical Faculty in Belgrade and conducted according to the Declaration of Helsinki.

Blood Sampling and Analysis

Blood samples for examinations of the expression levels of inflammatory biomarkers were obtained twice:
• on the day of discontinuation of clopidogrel
  one year after coronary stent implantation
• forty-five days after the termination of clopido-
  grel treatment

The measured inflammatory biomarkers included the high-sensitivity C-reactive protein (hsCRP) and soluble CD40 ligand (sCD40L) levels.

Samples of venous blood were collected into vacutainer tubes (Becton-Dickinson GmbH, Heidelberg, Germany), which were then left for 30 minutes to allow the clotting process to occur.

The hsCRP levels were determined on the day of blood collection. The analysis was performed using serum obtained via centrifugation at 3,000 g for 10 minutes at room temperature. The samples were automatically diluted 1:20 in accordance with the corresponding protocol of the manufacturer. The diluted samples were used within four hours, according to the manufacturer's instructions.

Quantitative measurements of the hsCRP levels were acquired using the Behring BN II Nephelometer device, reagents and calibrators supplied by Dade Behring, Marburg GmbH, Germany.

The results were automatically evaluated in comparison with standard known concentrations and are presented in units of mg/L. The limit of detection of the test was 0.175 mg/L, and the upper limit of the reference value was 3.00 mg/L. The coefficient of variation was 7.6% registered in 10 repetitions of a sample containing a CRP level of 0.41 mg/L.

Blood samples for the analysis of the sCD40L levels, performed during the clotting process within 30 minutes at room temperature, were centrifuged at 1,800 g for 10 minutes in order to obtain serum. The serum samples were then aliquoted and stored at −70°C.

The serum levels of human sCD40L were determined using a quantitative sandwich enzyme immunoassay (Human sCD40L Platinum ELISA, Bender Medsystems). All analyses were performed in duplicate, and the assays were performed according to the manufacturer's instructions. The intra- and inter-assay coefficients of variation for sCD40L were 7.0% and 9.6%, respectively, with a sensitivity of 0.06 ng/mL (according to the manufacturer).

In detail, the samples, standards and controls were mixed with HRP-conjugated sCD40L antibodies in the dilution plate (dilution 1:5) and then added to each well of precoated plates. Following incubation and washing, the substrate TMB (3,3′,5,5′-Tetramethylbenzidine) was added and the reaction was terminated by the addition of acid. The absorbance at 450 nm was measured using an automated microplate reader (Sunrise, TECAN, UK). The sCD40L concentrations were calculated based on a standard curve created by plotting the mean absorbance values of the standards against those of the concentrations using a
five-parameter curve fit.

All lipid parameters were measured in fasting blood samples using commercial tests on the Dimension clinical chemistry system (Siemens, Dimension RxL Max). The total cholesterol levels were measured according to an oxidase assay, the HDL-C levels were assessed using a polyethylene glycol test and the triglyceride levels were determined using a lipase test.

### Statistical Analysis

The study group consisted of 151 men and 49 women. Regarding the comparison of the hsCRP and sCD40L levels according to the quartiles of the lipid parameters 45 days after the cessation of clopidogrel, a separate analysis was performed for men and women due to the small number of patients in the female subgroup. The results for men are presented in Table 1 and Fig. 1-3. The results for women are presented in the two supplemental tables. The entire cohort of patients was divided into four groups according to the quartiles of the HDL-C level measured 45 days after the cessation of clopidogrel. Categorical variables were compared between the four groups using the Chi-square test and age was compared according to a one-way ANOVA. The Wilcoxon rank test was used for comparisons of the hsCRP and sCD40L levels obtained baseline and day 45. The high-sensitivity CRP and sCD40L values and the changes in the hsCRP and sCD40L levels from baseline to day 45 were compared between the first HDL-C quartile and the rest of the three quartiles using the Mann-Whitney test for each quartile separately, and the global differences between the quartiles were tested using the Kruskal-Wallis test for independent samples. All tests were two-sided, and statistical significance was defined as a p value of 0.05. The statistical program IBM SPSS Statistics 20.0 was used for all calculations.

### Results

Two hundred patients with intracoronary stents who discontinued clopidogrel treatment (as a part of dual antiplatelet therapy) after one year were enrolled in this study. Second measurements of the laboratory parameters on day 45 after the cessation of clopidogrel were not obtained in two patients, one of whom had a fatal ischemic stroke and the other developed unstable angina and is currently scheduled for repeat coronary
angiography. The baseline characteristics and lipid parameters of all study participants and the male sub-group across the quartiles of HDL-C after clopidogrel cessation are provided in Table 1. The data for women are presented in the supplemental tables.

In the men, there were no statistically significant differences in the hsCRP levels at baseline or after clopidogrel discontinuation [1.2 (0.70-2.3) vs. 1.2 (0.67-2.3), \( p = 0.407 \); Fig. 1A]. However, the sCD40L levels were significantly lower while the patients were on dual anti-platelet therapy compared with those observed 45 days after the discontinuation of clopidogrel [12.9 (10.05-15.5) vs. 13.39 (10.94-16.13), \( p = 0.007 \); Fig. 1B]. Meanwhile, the concentrations of HDL-C (Fig. 1C) were not significantly different between baseline and 45 days after clopidogrel cessation (1.04 \( \pm \) 0.24 vs. 1.5 \( \pm \) 0.25, \( p = 0.239 \)). In women, there were no significant differences between the baseline and later measurements of the hsCRP, sCD40L and HDL-C levels (Supplemental Tables).

The patients in the first quartile of HDL-C had the highest levels of hsCRP versus the other three quartiles (\( p = 0.017 \), \( p = 0.001 \), \( p = 0.001 \); I vs. II, I vs. III, I vs. IV HDL-C quartile respectively; Fig. 2A). In contrast, the sCD40L levels were similar among the HDL-C quartiles (Fig. 2B).

The delta hsCRP and delta sCD40L values (the differences between the levels of these parameters at baseline and on day 45) are shown in Fig. 3, panels A and B. The delta sCD40L values decreased across the quartiles of HDL-C (\( p = 0.010 \), \( p < 0.001 \) and \( p = 0.003 \); I vs. II, I vs. III, I vs. IV HDL-C quartile, respectively). The \( p \) values for the differences in the delta hsCRP values between the first and other three quartiles of HDL-C were \( p_1 = 0.063 \), \( p_2 = 0.022 \) and \( p_3 = 0.095 \), respectively.

Neither the hsCRP nor sCD40L levels changed significantly across the HDL-C quartiles in women (Supplemental Table 1).

**Discussion**

The main findings of the present study carried out among a relatively large cohort of patients indicate that the abrupt cessation of clopidogrel after one year of treatment in men is followed by: a) a significant increase in the serum concentration of sCD40L, b) no significant changes in the release of hsCRP, c) a significant increment in the serum concentration of sCD40L and elevation of the concentration of hsCRP in patients in the lowest HDL-C quartile compared to that observed in the other quartiles.

Inflammation plays an important role in the
pathogenesis and progression of atherosclerotic disease, both in the process of plaque rupture and activation of the coagulation cascade, which facilitates the development of thrombosis. Platelets, in addition to their well-known role in homeostasis, influence the process of thrombus formation as well as the inflammation that follows vascular injury. Therefore, inhibiting the platelet function may have an effect on certain inflammatory markers, primarily those associated with activated platelets. Clopidogrel, as an antithrombotic drug, exhibits proven inhibitory effects on platelet aggregation as well as additional (pleiotropic) actions, primarily an anti-inflammatory activity, which may modify the degree of vascular inflammation. Since there is increasing evidence that treatment with clopidogrel results in a reduction in the levels of sCD40L, P-selectin and CRP and inhibits platelet-leukocyte aggregate formation, it is an acceptable hypothesis that the cessation of clopidogrel induces an increase in biomarkers of vascular inflammation due to the loss of the inhibitory effect of this drug.

The high-sensitivity CRP level has significant predictive value in both the primary and secondary prevention of cardiovascular disease and is an accepted independent marker of adverse coronary events. In our group of patients, there were no significant differences in the mean values of the hsCRP levels following the cessation of clopidogrel compared to that observed under clopidogrel therapy, consistent with the findings of the DECADES and CESSATION studies. On the other hand, Angiolillo et al. documented significant increases in the CRP levels following the cessation of clopidogrel.
values, although it should be noted that their study was conducted among patients with diabetes mellitus, which should raise concern, since diabetes mellitus itself is a risk factor for both the development and progression of atherosclerosis\(^\text{22}\). Therefore, the increase in proinflammatory markers observed in that study must be viewed in light of the overall health status of the patients, not simply as a consequence of the discontinuation of clopidogrel.

Regarding the sCD40L level, there is increasing evidence to suggest that the CD40-CD40L pathway plays an important role in the progression of atherosclerotic disease and destabilization of atherosclerotic plaque. Soluble CD40L has autocrine, paracrine and endocrine activities, which stimulate the activation and aggregation of platelets and platelet-leukocyte conjugation, potentially resulting in atherothrombosis\(^\text{23}\). Soluble CD40L is a marker of platelet activation, and the majority of sCD40L in the blood is derived from activated platelets\(^\text{23, 24}\). It has also been demonstrated that a higher degree of platelet aggregation prior to angioplasty is associated with a greater increase in sCD40L release into the circulation after the procedure\(^\text{25}\).

In this study, we observed a statistically significant increase in the sCD40L levels following the cessation of clopidogrel in relation to the values obtained under treatment with clopidogrel. Although the increase in sCD40L registered in the CESSATION study was not statistically significant, that seen in the DECADES study, similar to our results, was found to be significant at four weeks after the withdrawal of clopidogrel\(^\text{19, 20}\).

Analyzing the results of the present study, the question arises as to whether the increase in specific inflammatory markers is caused by the withdrawal of clopidogrel and the loss of its protective effects on the platelet function or rather a true pro-inflammatory rebound effect. The significant increase in the sCD40L levels with similar CRP levels after the discontinuation of clopidogrel noted in this study appears to favor an increase in platelet activation more so than an increase in proinflammatory conditions, pointing perhaps to a wider (more systemic) role of hsCRP and narrower (more platelet) role of sCD40L, particularly in the context of the administration of clopidogrel.

Accumulating evidence indicates the presence of a complex interplay between dyslipidemia, platelet reactivity and thrombogenic potential. Against this background, we investigated the relationships between lipids parameters and proinflammatory markers after the discontinuation of clopidogrel and found some interesting results. Namely, an inverse correlation was observed between the changes in the sCD40L and HDL-C levels, i.e., patients with lower HDL-C levels displayed higher increments in the sCD40L levels in the serum, which implies the existence of a higher degree of platelet reactivation in patients with low HDL-C levels following clopidogrel cessation. On the other hand, the patients with the lowest HDL-C levels had higher hsCRP levels after the cessation of clopidogrel. This observation provides a connection between inflammation, platelet activation and the lipid status and is a unique finding of this study. With respect to other lipid parameters (total cholesterol, LDL-C, triglycerides), no associations with increments in inflammatory markers were noted.

The cardioprotective role of HDL is clear and well documented and may be attributed to its actions in the process of reverse cholesterol transport as well as its antioxidant, anti-inflammatory and antithrombotic effects\(^\text{26-29}\), wherein the plasma HDL level is inversely related to the incidence of cardiovascular disease. Reverse cholesterol transport is characterized by the transfer of HDL particles from extrahepatic tissues to the liver for additional metabolism and excretion\(^\text{30, 31}\), thus reducing the total cholesterol level and consequent risk of cardiovascular disease. One of the potential mechanisms by which HDL participates in reducing the platelet activity involves a direct effect on platelets. Scavenger receptor B type 1 (SR-BI), a major HDL receptor, among other things, is expressed on platelets and megakaryocytes, whereas its expression is positively correlated with the serum HDL level and inversely correlated with the cholesterol ester content in platelets in addition to their ability to aggregate\(^\text{31}\). In accordance with these findings, we observed lower values of HDL in our patients with higher levels of inflammatory markers released from activated platelets after clopidogrel cessation.

In addition, analyzing the data in Table 1, there was a statistically significant difference in the number of patients with previous myocardial infarction based on the HDL-C quartile, i.e., the lowest number was recorded in the highest HDL-C quartile, thus indirectly reaffirming the protective role of HDL-C against the development of thrombosis.

**Conclusion**

Activated platelets play key roles in the pathogenesis of inflammation, providing a link between inflammation, atherosclerosis and thrombosis. Following the cessation of clopidogrel, its protective effects are lost, which potentially increases the risk of adverse cardiovascular outcomes. It is therefore necessary to
conduct further studies of larger numbers of patients in order to provide additional protection after clopidogrel cessation, particularly in patients with certain risk factors, such as a low HDL cholesterol level. In accordance with our results, various factors may affect the increase in HDL-C, resulting in positive effects, which should be examined in appropriate clinical studies. Furthermore, it is unknown whether similar effects occur with new antiplatelet drugs (ticagrelor, prasugrel), an issue that must also be investigated. Finally, as previously mentioned, given the small number of women in our study, we are unable to draw firm conclusions regarding the correlations between inflammatory and lipid parameters after clopidogrel cessation in this population, which should also be a subject of future research.

Acknowledgment

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Conflicts of Interest

None.

References


15) Lindahl B, Toss H, Siegbahn A, Venge P, Wallentin L: Markers of myocardial damage and inflammation in rela-
Macaya C: Clopidogrel withdrawal is associated with pro-inflammatory and prothrombotic effects in patients with diabetes and coronary artery disease. Diabetes, 2006; 55: 780-784


Supplemental Table 1.
Baseline characteristics, lipid status and serum hsCRP and sCD40L levels 45 days after clopidogrel cessation in women according to the HDL-C quartile

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Quartiles of HDL-C – 45 days after stopping clopidogrel in women</th>
<th>( n = 48 )²</th>
<th>( p )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Parameter</td>
<td>I ( n = 12 )</td>
<td>II ( n = 12 )</td>
<td>III ( n = 12 )</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59 ± 10</td>
<td>60 ± 12</td>
<td>56 ± 9</td>
</tr>
<tr>
<td>Active smokers, n (%)</td>
<td>4 (33.3)</td>
<td>1 (8.3)</td>
<td>8 (66.6)</td>
</tr>
<tr>
<td>Arterial hypertension, n (%)</td>
<td>9 (75.0)</td>
<td>10 (83.5)</td>
<td>9 (75.0)</td>
</tr>
<tr>
<td>Diabetes mellitus, n (%)</td>
<td>3 (25.0)</td>
<td>4 (33.3)</td>
<td>1 (8.3)</td>
</tr>
<tr>
<td>Hypercholesterolemia, n (%)</td>
<td>10 (83.3)</td>
<td>10 (83.3)</td>
<td>8 (66.7)</td>
</tr>
<tr>
<td>Family history of CAD, n (%)</td>
<td>12 (100.0)</td>
<td>12 (100.0)</td>
<td>10 (83.3)</td>
</tr>
<tr>
<td>BMI, kg/m²</td>
<td>28.2 ± 2.7</td>
<td>26.9 ± 2.6</td>
<td>24.4 ± 2.5</td>
</tr>
<tr>
<td>Obesity, n (%)</td>
<td>11 (91.7)</td>
<td>7 (58.3)</td>
<td>4 (33.3)</td>
</tr>
<tr>
<td>Previous MI, n (%)</td>
<td>9 (75.0)</td>
<td>10 (83.3)</td>
<td>7 (58.3)</td>
</tr>
<tr>
<td>DES, n (%)</td>
<td>5 (41.7)</td>
<td>3 (25.0)</td>
<td>5 (41.7)</td>
</tr>
<tr>
<td>Laboratory Parameters at 45 day</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LDL-C, mmol/L</td>
<td>2.4 ± 0.8</td>
<td>2.6 ± 0.7</td>
<td>3.1 ± 1.3</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>4.5 ± 0.9</td>
<td>5.0 ± 1.4</td>
<td>5.2 ± 1.7</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>2.0 ± 0.7</td>
<td>2.1 ± 1.2</td>
<td>1.7 ± 0.7</td>
</tr>
<tr>
<td>hsCRP, mmol/L</td>
<td>2.2 (1.0-3.7)</td>
<td>1.3 (0.5-5.4)</td>
<td>0.8 (0.4-3.8)</td>
</tr>
<tr>
<td>sCD40L, mmol/L</td>
<td>11.6 (9.7-14.8)</td>
<td>14.2 (11.7-15.8)</td>
<td>13.0 (11.0-19.4)</td>
</tr>
<tr>
<td>Comedication</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta-blocker, n (%)</td>
<td>10 (83.3)</td>
<td>8 (66.7)</td>
<td>11 (91.7)</td>
</tr>
<tr>
<td>ACE inhibitors, n (%)</td>
<td>10 (83.3)</td>
<td>7 (58.3)</td>
<td>8 (66.7)</td>
</tr>
<tr>
<td>Statins, n (%)</td>
<td>10 (83.3)</td>
<td>9 (75.0)</td>
<td>10 (83.3)</td>
</tr>
<tr>
<td>Calcium blockers, n (%)</td>
<td>1 (8.3)</td>
<td>2 (16.7)</td>
<td>7 (58.3)</td>
</tr>
</tbody>
</table>

1 Quartiles of HDL-C at 45 days for women: I – less than 1.066 mmol/L, II – 1.066-1.225 mmol/L, III – 1.226-1.435 and IV – above 1.435 mmol/L.
2 In one woman, data for the HDL-C level on day 45 were missing (the patient underwent repeated coronary angiography for unstable angina)

Supplemental Table 2.
High-sensitivity CRP, sCD40L and HDL-C levels in women at baseline and 45 days after clopidogrel cessation

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Baseline concentration</th>
<th>Concentration at 45th day</th>
<th>( p )</th>
</tr>
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<tr>
<td>hsCRP, mg/L (IQR)</td>
<td>1.70 (0.80-3.40)</td>
<td>1.25 (0.82-3.67)</td>
<td>0.976</td>
</tr>
<tr>
<td>sCD40L ng/mL (IQR)</td>
<td>13.05 (11.03-15.15)</td>
<td>12.71 (10.63-15.98)</td>
<td>0.273</td>
</tr>
<tr>
<td>HDL-C, mmol/L ± SD</td>
<td>1.20 ± 0.26</td>
<td>1.24 ± 0.28</td>
<td>0.057</td>
</tr>
</tbody>
</table>