Low C1-Inhibitor Levels Predict Early Restenosis After Eversion Carotid Endarterectomy


Objective—Homozygotes for the normal (A) allele of mannose-binding lectin (MBL2) gene have higher risks to develop an early restenosis after eversion carotid endarterectomy (CEA). Activation of the lectin pathway is regulated by C1-inhibitor (C1-INH). The objective of the present study was to determine the predictive value of C1-INH in restenosis after CEA.

Methods and Results—C1-INH and MBL-associated serine protease-2 (MASP-2) were determined in samples serially taken from 64 patients with CEA, who were followed-up with carotid duplex scan (CDS) examinations for 14 months. MBL2 genotypes were also determined. Patients with >50% restenosis had lower C1-INH levels at 6 weeks (P=0.0052) and at 4 days (P=0.0277) postsurgery. C1-INH levels at 6 weeks correlated inversely with the CDS values at 14 months (r =-0.3415, P=0.0058), but only in MBL2 A/A homozygotes (r =-0.5044, P=0.0015). Patients with low C1-INH levels (C1-INH <115%) had higher CDS values already at 7 months postsurgery. Patients with MBL2 A/A and low C1-INH levels at 6 weeks postsurgery had 13.97 (95% CI:1.95 to 100.21, P=0.0087) times higher risk to develop an early restenosis. Differences in the MASP-2 concentration were not associated with restenosis.

Conclusions—Determining C1-INH levels at 6 weeks postsurgery—together with genotyping of MBL2—might be a useful marker in the identification of patients with high risk for early carotid restenosis. (Arterioscler Thromb Vasc Biol. 2007;27:000-000.)

Key Words: carotid arteries ■ genetics ■ inflammation ■ restenosis ■ risk factors

Carotid endarterectomy (CEA) is a routine surgical intervention to prevent stroke in patients with severe symptomatic or asymptomatic carotid atherosclerosis.¹,² The long-term outcome of CEA is highly affected by the development of recurrent carotid stenosis; either as a consequence of myointimal hyperplasia (early restenosis, which develops within 24 months after CEA) or of the renewal of atherosclerosis (late restenosis). The incidence of an early restenosis within 24 months after CEA is approximately 13% in the first 2 years. It seems that traditional risk factors of atherosclerosis are unable to predict early restenosis,³,⁴ although studies are controversial.⁵,⁶ This might reflect that the pathomechanism of an early restenosis—which is a consequence of an inflammatory response to the vessel wall leading to myointimal hyperplasia of smooth muscle cells—differs from those of primary atherosclerosis.

It seems that the complement system plays an important role in the inflammatory response in early restenosis after eversion CEA. Recently in a prospective study, we demonstrated that homozygous carriers of the wild-type (A) alleles of the mannose-binding lectin (MBL) gene (MBL2) have significantly higher risk to develop early restenosis after eversion CEA compared with carriers of the defect (B, C, or D) alleles of MBL2.⁹ Moreover, we have also demonstrated that elevated levels of complement C3 (C3), but not other noncomplement acute-phase reactants (APRs)—such as C-reactive protein (CRP)—are associated with and might directly contribute to the development of early restenosis, depending on MBL2 genotype.¹⁰ Thus the lectin pathway has a dual role in carotid artery disease: on the one hand an intact lectin pathway seems to be protective against primary atherosclerosis,¹¹ whereas it plays a key role in the development of early restenosis after CEA.⁹,¹⁰

Activation of the lectin pathway occurs when MBL (or ficolins) bind their ligands, through MBL associated serine proteases (MASPs, mainly through MASP-2), which leads...
to activation of the terminal complement cascade by C4 and C2.13 C1-inhibitor (C1-INH) is the main inhibitor of lectin pathway activation, but also inhibits classical pathway of complement and the coagulation, fibrinolysis, and kinin-kallikrein systems also.14 Only scarce literature data are available on the association of C1-INH with atherosclerotic vascular diseases.15 The objective of the present study was to determine whether an association of C1-INH levels with early restenosis after eversion type CEA exists, based on our previous findings with MASP-2 to early restenosis was also considered.9,10,16

Study Design

Patients included in the present study represent a subgroup of a prospective study of 123 consecutive patients, in which MBL2 genotypes,9 changes in CRP16 and C310 levels were previously published. The reason for including less patients in the present study was the limited availability of the serum samples, otherwise the study setup was the same.9,10,16

Methods

Patients

In this prospective study, we included 64 Hungarian patients (21 female and 43 male, aged between 43 and 79 years), with a median age of 65.5 (56.0–73.0) years. Patients included in the present study represent a subgroup of a prospective study of 123 consecutive patients, in which MBL2 genotypes,9 changes in CRP16 and C310 levels were previously published. The reason for including less patients in the present study was the limited availability of the serum samples, otherwise the study setup was the same.9,10,16

Briefly, all patients underwent eversion type CEA: the surgical technique has been described earlier.17 The indication for CEA was in accordance with the American Heart Association guidelines.18 Blood samples were collected at 4 different time intervals; preoperatively and 4 days, 6 weeks, and 14 months postsurgery. Serum was immediately separated and stored at −80°C until laboratory use, whereas EDTA-anticoagulated blood samples were obtained for DNA preparation preoperatively.

CDS examinations (ATL Ultramark 9 HDI system) were performed preoperatively and at 6 weeks, 7 months, and 14 months postsurgery by an experienced radiologist. The common, internal, and external carotid arteries on both sides were examined in the standard fashion, spectral measurements were taken with a Doppler angle between 55° and 60°. The diagnostic criteria for internal carotid artery stenosis and restenosis were based on peak systolic velocities and end diastolic velocities as well as internal carotid artery/common carotid artery ratios. Patients had medical check-up and CDS examinations at 6 weeks (median 5.7 [4.6 to 8.0]), 7 months (median 6.8, [6.2 to 7.9]) and at 14 months (median 13.8 [12.3 to 19.0]) postsurgery. Early restenosis was defined as >50% stenosis of the internal carotid artery at 14 months postsurgery (which reflects the pathomechanism). Early restenosis developed in 10/64 (15.63%) patients during the follow-up, which is comparable to data published in the literature,4 in 3/64 (4.7%) cases clinically significant (>70%) restenosis developed (that indicated a second surgery/intervention).

Statistical Analysis

Statistical analysis was performed with SPSS for Windows 13.0.1 (SPSS Inc) and Prism for Windows 4.02 (GraphPad Software)

| Table 1. Baseline Characteristics of the Patients With or Without Restenosis During the Follow-Up |

<table>
<thead>
<tr>
<th>Patients With Restenosis (n=10)</th>
<th>Patients Without Restenosis (n=54)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, years</td>
<td>65.5 (56.0–73.0)</td>
<td>67.5 (58.0–73.0)</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>7/3 (70.0%/30.0%)</td>
<td>36/18 (66.7%/33.3%)</td>
</tr>
<tr>
<td>Smoking status, ever/never</td>
<td>4/6 (40.0%/60.0%)</td>
<td>24/30 (44.4%/55.6%)</td>
</tr>
<tr>
<td>Diabetes mellitus, yes/no</td>
<td>3/7 (42.9%/57.1%)</td>
<td>11/43 (20.4%/79.6%)</td>
</tr>
<tr>
<td>Hypertension, yes/no</td>
<td>10/0 (100.0%/0.0%)</td>
<td>46/8 (85.2%/14.8%)</td>
</tr>
<tr>
<td>CAD, yes/no</td>
<td>6/4 (60.0%/40.0%)</td>
<td>30/24 (55.6%/44.4%)</td>
</tr>
<tr>
<td>History of TIA/stroke, yes/no</td>
<td>7/3 (70.0%/30.0%)</td>
<td>41/13 (75.9%/24.1%)</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>25.08 (24.79–28.60)</td>
<td>25.18 (22.84–27.86)</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.94 (6.67–7.84)</td>
<td>6.15 (5.54–6.94)</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.75 (1.30–2.35)</td>
<td>1.90 (1.33–2.41)</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>4.60 (1.18–21.56)</td>
<td>6.45 (3.05–13.75)</td>
</tr>
</tbody>
</table>

*Mann–Whitney nonparametric test, †Fisher exact test. Values presented as absolute numbers (percentages) or medians (interquartile ranges). Restenosis was defined as >50% stenosis of the operated artery at the end of the study period. CAD indicates coronary artery disease; TIA, transient ischemic attack; CRP, C-reactive protein; C1-INH, C1-inhibitor.
statistical software products. As many of the variables had non-
Gaussian distributions we used nonparametric tests throughout the
analysis. We used the Mann–Whitney U test to compare 2 indepen-
dent groups, the Fisher exact test to compare categorical variables,
the Friedman test for variance analysis (using Dunn’s post hoc test
comparisons between 2 repeated measures), Spearman rho to calcu-
late correlations, and 2-way ANOVA with Bonferroni posttests to
detect interactions. Multiple logistic regression and receiver operator
characteristics (ROC) analyses were also performed. All statistical
analyses were performed 2-tailed and P<0.05 was considered as
significant. Values presented in the text are medians (interquartile
ranges), unless otherwise stated.

Results

Changes in C1-INH Levels During the Follow-Up

Concentrations of C1-INH were determined in the patients at
4 different time intervals; preoperatively, as well as 4 days, 6
weeks, and 14 months postsurgery (Figure 1A). C1-INH
levels significantly increased at 4 days postsurgery compared
with the preoperative values (156.0 [131.0 to 172.5]% versus
115.0 [95.5 to 131.0]%, P<0.01 Dunn’s post hoc test after
Friedman’s test). During the follow-up, we observed a sharp
decrease in C1-INH levels at 6 weeks compared with the 4
days postsurgery values (P<0.01) and C1-INH levels re-
mained lower at 14 months as compared with the 4 days
postsurgery levels (P<0.01).

Next, patients were divided into 2 subgroups according to
the MBL2 carrier state. Thirty-seven patients were homozy-
gous for the MBL2 normal (A) allele (MBL A/A), whereas 27
patients carried at least 1 variant (B, C, or D) MBL2 allele
(MBL A/O or O/O). We found no association between MBL2
genotypes and in changes in C1-INH levels during the
follow-up (data not shown).

Changes in MASP-2 Concentrations According to
Different MBL2 Genotypes During the Follow-Up

Concentrations of MASP-2 were also determined in the
samples. The increase in MASP-2 levels at 4 days postsur-
gery compared with the baseline values did not reach statis-
tical significance, however an increasing trend could be
observed. Considering all patients, MASP-2 levels were
lower at 6 weeks (P<0.01) and at 14 months (P<0.01)
compared with the 4 days postsurgery results, respectively
(Figure 1B). However, the decrease in MASP-2 levels at 6
weeks compared with 4 days postsurgery was only significant
(P<0.01) in patients with the MBL A/A genotype, where
MASP-2 even decreased below baseline values (P<0.01). In
patients with MBL A/O or O/O MASP-2 levels were not
decreased at 6 weeks compared with 4 days postsurgery
levels.

Association of C1-INH and MASP-2 Levels to
Early Restenosis

Concentrations of C1-INH and MASP-2 were compared in
patients who developed (n=10) and who did not develop
(n=54) early restenosis at 14 months postsurgery. Patients
with early restenosis had lower C1-INH levels in blood
samples taken at 6 weeks (P=0.0052) and also at 4 days

Table 2. Association of C1-INH and MASP-2 Levels to Early Restenosis at Different Time Intervals

<table>
<thead>
<tr>
<th>Time of Sampling</th>
<th>C1-INH, % of NHS</th>
<th>MASP-2, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Restenosis, Yes*</td>
<td>Restenosis, No*</td>
</tr>
<tr>
<td>Pre-OP</td>
<td>102.5 (87.0–115.0)</td>
<td>115.0 (97.0–131.0)</td>
</tr>
<tr>
<td>4 days</td>
<td>134.0 (123.75–153.5)</td>
<td>158.0 (135.5–177.0)</td>
</tr>
<tr>
<td>6 weeks</td>
<td>112.5 (105.75–120.25)</td>
<td>131.0 (115.5–144.5)</td>
</tr>
<tr>
<td>14 months</td>
<td>135.5 (122.0–147.25)</td>
<td>124.0 (108.0–146.5)</td>
</tr>
</tbody>
</table>

*Restenosis was defined as >50% reduction in diameter during the CDS examination at 14 months. †Mann–Whitney nonparametric test. Values
presented as absolute numbers or medians (interquartile ranges). C1-INH indicates C1-Inhibitor; NHP, normal human plasma of healthy blood donors;
pre-OP, preoperatively.
levels determined at 6 weeks postsurgery was included in the preoperative (0.6907) C1-INH measurements. Thus C1-INH (0.7796), followed by the 4 days postsurgery (0.7204) and the curve for the ROC analysis was the highest at 6 weeks value in the prediction of early restenosis. The area under the blood sampling time of C1-INH (pre-operative, 4 days, or 6 weeks postsurgery) has the best specificity and sensitivity degree of restenosis correlation of C1-INH concentrations with the

Next, a ROC analysis was performed to determine which C1-INH concentrations at 14 months postsurgery and restenosis.

Considering MASP-2 concentrations, no significant associations with early restenosis were detected (Table 2).

Correlation of C1-INH Concentrations With the Degree of Restenosis

Next, a ROC analysis was performed to determine which blood sampling time of C1-INH (pre-operative, 4 days, or 6 weeks postsurgery) has the best specificity and sensitivity value in the prediction of early restenosis. The area under the curve for the ROC analysis was the highest at 6 weeks (0.7796), followed by the 4 days postsurgery (0.7204) and the preoperative (0.6907) C1-INH measurements. Thus C1-INH levels determined at 6 weeks postsurgery was included in the further analysis.

We found a strong significant inverse correlation between C1-INH concentrations at 6 weeks and the degree of restenosis at 14 months ($r = -0.3415$, $P = 0.0058$). Correlations were also determined in the patients, grouped by $MBL2$ genotypes. The strong inverse correlation between serum C1-INH levels at 6 weeks and the degree of restenosis at 14 months postsurgery was only present in patients with $MBL A/A$ ($r = -0.5044$, $P = 0.0015$), but not in patients with $MBL A/O$ or $O/O$ genotypes ($r = -0.1418$, $P = 0.4805$).

Predictive Value of Low C1-INH Levels at 6 Weeks in the Development of an Early Restenosis

To assess the predictive value of C1-INH levels on the development of restenosis during the follow-up, patients were categorized according to low versus normal/high C1-INH concentrations measured at 6 weeks postsurgery. Low C1-INH levels were defined according to a ROC analysis with the best specificity (0.741) and sensitivity (0.700) values and were set as C1-INH < 115.0%, which equaled to the lowest tertile of the whole group. As presented on Figure 2 CDS values progressively increased in the group of low C1-INH patients whereas those with high C1-INH were characterized with a less pronounced increase. Two-way ANOVA with Bonferroni posttests were performed. Patients with low C1-INH levels had significantly higher CDS values at 7 months ($P < 0.01$) and later at 14 months ($P < 0.05$) postsurgery. Low C1-INH levels are associated with high CDS values during the follow-up period at high statistical significance ($P < 0.0001$ for 2-way ANOVA).

Next, multiple logistic regression analysis was performed to calculate the odds ratio of low C1-INH levels at 6 weeks postsurgery for the prediction of an early restenosis (CDS > 50% at 14 months postsurgery; Table 3). In the model we adjusted for age and sex, as no traditional risk factors or CRP concentrations determined at 6 weeks showed association with restenosis (data not shown). In all patients we found that those patients with low C1-INH levels at 6 weeks postsurgery have 6.93 (95% CI: 1.55 to 31.02, $P = 0.0113$) times higher risk to develop an early restenosis in 14 months, compared with the patients with C1-INH levels in the 2 higher tertiles, independent of age and sex (Table 3).

The predictive value of low C1-INH levels was significant and much more pronounced in patients with the $MBL2$ A/A genotype (Table 3), the odds ratio for the development of an early restenosis was 13.97 (95% CI: 1.95 to 100.21, $P = 0.0087$) in patients with low C1-INH levels. On the contrary, in patients with MBL A/O or O/O, low C1-INH levels did not have significant predictive value ($P = 0.3791$).

Discussion

The main novel finding of the present report is that low C1-INH levels, determined at 6 weeks postsurgery, are able to predict early restenosis after eversion CEA, depending on

![Figure 2. Dynamic changes of CDS values during the follow-up period in groups of patients stratified according to 6 week C1-INH levels. Patients with low C1-INH showed sharp increase and had higher CDS values during the follow-up. Means and 95% confidence intervals are presented, 2-way ANOVA with Bonferroni posttests: *$P < 0.05$, **$P < 0.01$.](image-url)

Table 3. Predictive Value of Low C1-INH Levels at 6 Weeks for the Development of an Early Restenosis Adjusted for Age and Sex

<table>
<thead>
<tr>
<th>C1-INH low/normal* patients with $MBL2$ A/A (n=37)</th>
<th>B</th>
<th>SE</th>
<th>P</th>
<th>Odds Ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C1-INH low/normal* patients with $MBL2$ A/O or O/O (n=27)</td>
<td>2.6369</td>
<td>1.0053</td>
<td>0.0087</td>
<td>13.9698 (1.9475–100.2056)</td>
</tr>
<tr>
<td>C1-INH low/normal*</td>
<td>1.3996</td>
<td>1.5912</td>
<td>0.3791</td>
<td>4.0535 (0.1792–91.6848)</td>
</tr>
</tbody>
</table>

*Low C1-INH levels were defined according to a ROC analysis, with the best specificity (0.741) and sensitivity (0.700) values and was set as C1-INH < 115.5%, which equaled to the lowest tertile of the patient group. Multiple logistic regression analysis, age is a continuous variable, sex is a categorical variable in the model. C1-INH indicates C1-inhibitor.
MBL2 genotypes. Patients with C1-INH levels at the lowest tertile had almost 7-fold higher risk to develop early restenosis compared with patients with C1-INH levels in the 2 higher tertiles, adjusted for age and sex. The risk is even higher in subjects with the MBL2 A/A genotype: patients with low C1-INH concentrations have nearly 14-times higher risk for early restenosis. Patients with low C1-INH levels had significantly higher CDS values already at 7 months postsurgery compared with the patients with normal/high C1-INH. MASP-2 levels did not have significant predictive value in early restenosis and showed no associations with CDS values during the follow-up. We found that C1-INH levels are increased at 4 days postsurgery compared with the preoperative values. As C1-INH is a well known positive APR, this observation might confirm our previous finding, that a strong acute-phase reaction follows CEA at 4 days postsurgery, which can be detected by sensitive inflammatory markers, such as CRP. At 6 weeks postsurgery—with the abatement of the acute-phase reaction—levels of C1-INH returned to baseline. During the 14 months of follow-up, however, we observed an increasing trend of C1-INH levels in patients who develop restenosis—similar to our previous observation with C3. Thus it seems that the regulation of complement proteins differs from noncomplement APRs, and there might be an upregulation in patients who develop restenosis after CEA.

C1-INH is not only a potent inhibitor of the lectin pathway, but also inhibits classical pathway of complement and proteases of 3 other plasma enzyme cascades (coagulation, fibrinolysis, and kinin-kallikrein). It is a serine-protease inhibitor, which forms inactive complexes with its target proteinases (C1r, C1s, coagulation factors XIa, and XIIa, and plasma kallikrein). The primary sources of C1-INH synthesis are hepatocytes, however secondary extrahepatic sites of production has also been described (monocytes, fibroblasts, endothelial cells, megakaryocytes, microglial cells, neurons, placenta). Several patients who developed significant early restenosis at the end of the follow-up, had slightly lower serum C1-INH levels already prior CEA (at the margin of statistical significance). During the follow-up this observation has become significant and low levels of C1-INH were able to predict restenosis in the patient group. Thus we assume that initially low C1-INH levels are not able to regulate complement activation sufficiently in patients. This assumption is confirmed by the observation that low C1-INH has significant predictive value only in patients with the MBL2 A/A genotype. Thus we conclude—as we reported earlier—that an intact lectin pathway might be required for restenosis, and insufficient regulation by low C1-INH levels contributes to the uncontrolled complement activation (Figure 3 shows the pathophysiology). However, we cannot rule out the possibility that dysregulation of the other plasma enzyme systems—because of the decreased C1-INH levels—also contributes to the process.

Complement activation plays pathophysiologic roles during ischemia/reperfusion injury and the involvement of the

![Figure 3](http://journals.ahajournals.org/doi/10.1161/01.ATVP.87.10.1725)
lactin pathway is also suggested.25,26 During evasion CEA tissue hypoxia occurs for the time of cross-clamping (approx. 20 minutes) of the carotid artery, which may result in complement activation. During surgery, the atherosclerotic plaque is removed together with the intima, exposing a new surface in the vessel wall, which is mainly composed by smooth muscle cells, inflammatory cells, and extracellular matrix proteins. We assume that the initiation of the lectin pathway occurs by binding of MBL to the endothelial cells or intermedier filaments,27 and it is not sufficiently controlled by low C1-INH levels attributable to low hepatic synthesis or lack of local production of neointimal cells. This leads to activation and proliferation of smooth muscle cells28 and macrophages, which might lead to the increased production of cytokines and growth factors,29 which finally leads to myointimal hyperplasia. Prior studies conducted on patients with acute myocardial infarction have shown that treatment with intravenous C1-INH may reduce myocardial injury.30,31 Based on our findings it can be assumed that supplementation with C1-INH during the postoperative period might be beneficial in the prevention of restenosis in patients with CEA.

It seems that monitoring complement proteins—especially C3 and C1-INH—has a rationale in the follow-up of CEA. Previously we have shown that high C3 levels are associated with the degree of restenosis determined by CDS after CEA.10 High C3 levels might have a direct role in the hyperproliferation of vascular smooth muscle cells28 and indicate the chronic complement dependent inflammation as a special marker, and not as a common acute phase reactant.10 However C3 was not able to predict early restenosis in these patients. In contrast to the increasing evidence on C3 as a marker, and not as a common acute phase reactant.10 How-ever C3 was not able to predict early restenosis in these patients. In contrast to the increasing evidence on C3 as a useful risk factor in coronary32–34 or carotid10 artery disease, scarce data are available on the association of C1-INH with vascular diseases. Kostner et al found that patients with unstable angina pectoris tended to have higher C1-INH levels compared with patients with stable angina pectoris.33 In the present study we reported that low C1-INH levels are associated with poorer outcome after evasion CEA. Thus the relation of C1-INH levels with different vascular diseases remains to be further examined.

In conclusion, we have shown that low C1-INH levels are associated with and are able to predict early restenosis after CEA. These results are one step toward understanding restenosis development after evasion CEA, however further studies are required to confirm the role of C1-INH as a marker in the identification of patients with high risk for early recurrent carotid stenosis. Moreover it would be interesting and important to study the role of C1-INH in patients with carotid artery stenting, which are currently under way in our Departments.

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Disclosures
None.

References


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Polyclonal antibody directed against recombinant CCP1-CCP-2-SP fragment of human MASP-2 was produced in rabbits. ELISA plates (high binding microplates from Greiner Bio-One) were coated with 100 µl 2.5 µg/ml antibodies in 0.1 M NaHCO₃, pH 9.6, and incubated overnight at 4 ºC. Plates were washed after each step three times with 100 µl/well wash buffer (PBS, 0.05% Tween-20 from Sigma-Aldrich). Incubation in each case was sustained for 1 hour at 37 ºC. Wells were blocked with 1 % BSA (Fluka&Riedel) in PBS. Human serum samples were diluted 1/10 (v/v) in VBS buffer containing 0.05% Tween-20, 0.1% gelatin (Reanal Ltd., Hungary), 1 M NaCl, 10 mM EDTA, pH 7.5. The third layer of our sandwich ELISA was digoxigenin labeled rabbit IgG anti human MASP-2 diluted 1/500 (v/v) in detection buffer (PBS, 1 % BSA, 0.05 % Tween-20). 100 µl HRP-conjugated sheep anti-digoxigenin antibody (Fab fragments, from Boehringer Mannheim) diluted 1/4000 (v/v) in detection buffer was used as second antibody. Development was carried out with ABTS (2,2’-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid from Sigma-Aldrich) at a concentration of 2.5 mg/ml in 0.1 M citrate/Na₂ HPO₄ buffer, pH 4.2, in the presence of 0.01% H₂O₂. The absorbance at 415 nm was recorded after 15-30 min incubation at room temperature. The ELISA was standardized using negative controls and dilutions of highly purified recombinant human MASP-2 CCP1-CCP2-SP fragment.