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 evasion 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;27:2756-2762.)

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 hyperplasia3 (early restenosis, which develops within 24 months after CEA) or of the renewal of atherosclerosis3 (late restenosis). The incidence of an early restenosis after CEA is approximately 13% in the first 2 years. It seems that traditional risk factors of atherosclerosis are unable to predict early restenosis,5,6 although studies are controversial.7,8 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 cells3—differs from those of primary atherosclerosis.

It seems that the complement system plays an important role in the inflammatory response in early restenoses after 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 evasion 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 (MSPs, mainly through MASP-212), which leads

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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.

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 were performed by polymerase chain reaction (PCR) using sequence specific priming (PCR-SSP), as described previously.17

Study Design
Patients included in the present study represent a subgroup of a prospective study of 123 consecutive patients, in which MBL2 genotypes 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.

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

<table>
<thead>
<tr>
<th></th>
<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>
<td>0.9410</td>
</tr>
<tr>
<td>Sex, male/female</td>
<td>7/3 (70.0%/30.0%)</td>
<td>36/18 (66.7%/33.3%)</td>
<td>1.0000†</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>
<td>1.0000†</td>
</tr>
<tr>
<td>Diabetes mellitus, yes/no</td>
<td>3/7 (42.9%/70.0%)</td>
<td>11/43 (26.4%/79.6%)</td>
<td>0.6775†</td>
</tr>
<tr>
<td>Hypertension, yes/no</td>
<td>10/0 (100.0%/0.0%)</td>
<td>46/6 (85.2%/14.8%)</td>
<td>0.3373†</td>
</tr>
<tr>
<td>CAD, yes/no</td>
<td>6/4 (60.0%/40.0%)</td>
<td>30/24 (55.6%/44.4%)</td>
<td>1.0000†</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>
<td>0.7011†</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>
<td>0.4484</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>
<td>0.0214</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.75 (1.30–2.35)</td>
<td>1.90 (1.33–2.41)</td>
<td>0.7463</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>4.60 (1.18–21.56)</td>
<td>6.45 (3.05–13.75)</td>
<td>0.3271</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.

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, and 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 opened 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,14 in 3/64 (4.7%) cases clinically significant (>70%) restenosis developed (that indicated a second surgery/intervention). Baseline characteristics of the patients who developed or did not develop early restenosis are shown in Table 1.

Laboratory Analysis

Serum concentrations of C1-INH were measured by radial immunodiffusion method,19 using goat antisera against human C1-inhibitor (Quidel) for detection. C1-INH concentrations are expressed as percentages of the standard calibrator, which was pooled human serum of healthy blood donors. Total genomic DNA was extracted from white blood cells using the method of Miller.20 Determination of the alleles of the MBL2 gene were performed by polymerase chain reaction (PCR) using sequence specific priming (PCR-SSP), as described previously.21 MASP-2 levels were measured by enzyme linked immunosorbent assay (ELISA) method as described in supplemental Table I (available online at http://atvb.ahajournals.org).

Statistical Analysis

Statistical analysis was performed with SPSS for Windows 13.0.1 (SPSS Inc) and Prism for Windows 4.02 (GraphPad Software)
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 independent 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 calculate 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 remained 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 homozygous 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 postsurgery compared with the baseline values did not reach statistical 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>
<thead>
<tr>
<th>Time of Sampling</th>
<th>C1-INH, % of NHS</th>
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<tbody>
<tr>
<td></td>
<td>Restenosis, Yes*</td>
</tr>
<tr>
<td></td>
<td>(n=10)</td>
</tr>
<tr>
<td>Pre-OP</td>
<td>102.5 (87.0–115.0)</td>
</tr>
<tr>
<td>4 days</td>
<td>134.0 (123.75–153.5)</td>
</tr>
<tr>
<td>6 weeks</td>
<td>112.5 (105.75–120.25)</td>
</tr>
<tr>
<td>14 months</td>
<td>135.5 (122.0–147.25)</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Time of Sampling</th>
<th>MASP-2, mg/L</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Restenosis, Yes*</td>
</tr>
<tr>
<td></td>
<td>(n=10)</td>
</tr>
<tr>
<td>Pre-OP</td>
<td>0.37 (0.18–0.63)</td>
</tr>
<tr>
<td>4 days</td>
<td>0.73 (0.34–1.05)</td>
</tr>
<tr>
<td>6 weeks</td>
<td>0.37 (0.24–0.49)</td>
</tr>
<tr>
<td>14 months</td>
<td>0.37 (0.26–0.89)</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; NHS, normal human serum of healthy blood donors; pre-OP, preoperatively.
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 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

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**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></th>
<th>B</th>
<th>SE</th>
<th>P</th>
<th>Odds Ratio (95% Confidence Interval)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients (n=64) C1-INH low/normal*</td>
<td>1.9363</td>
<td>0.7646</td>
<td>0.0113</td>
<td>6.9330 (1.5493–31.0248)</td>
</tr>
<tr>
<td>C1-INH low/normal* patients with MBL2 A/A (n=37)</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* Patients with MBL2 A/O or O/O (n=27)</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.
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 developed 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 lectin pathway is also suggested. During eversion 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, 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 cells and macrophages, which might lead to the increased production of cytokines and growth factors, 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. 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. High C3 levels might have a direct role in the hyperproliferation of vascular smooth muscle cells and indicate the chronic complement dependent inflammation as a special marker, and not as a common acute phase reactant. However 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 coronary or carotid 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. In the present study we reported that low C1-INH levels are associated with poorer outcome after 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 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.

Sources of Funding
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Disclosures
None.

References


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Table I

<table>
<thead>
<tr>
<th>Description of the MASP-2 enzyme linked immunosorbent (ELISA) assay</th>
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<tbody>
<tr>
<td>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 °C. 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 °C. Wells were blocked with 1 % BSA (Fluka&amp;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.</td>
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</table>