Reduced ADAMTS13 activity is associated with thrombotic risk in systemic lupus erythematosus

S Martin-Rodriguez1, JC Reverter3, D Tassies1, G Espinosa2, M Heras1, M Pino1, G Escolar1 and M Diaz-Ricart1

1Hemotherapy-Hemostasis Department; 2Systemic Autoimmune Diseases Department; and 3Cardiology Department. Institut Clinic del Torax. Institut d Investigacions Biomèdiques August Pi i Sunyer, Hospital Clínic, Universitat de Barcelona, Barcelona, Spain

Background: Severe deficiency of ADAMTS13 activity leads to von Willebrand factor (VWF) ultralarge multimers with high affinity for platelets, causing thrombotic thrombocytopenic purpura. Other pathological conditions with moderate ADAMTS13 activity exhibit a thrombotic risk. We examined the ADAMTS13 activity in systemic lupus erythematosus (SLE) and its value as a thrombotic biomarker.

Methods: ADAMTS13 activity, VWF antigen and multimeric structure, and vascular cell adhesion molecule 1 (VCAM-1) were measured in plasma samples from 50 SLE patients and 50 healthy donors. Disease activity (systemic lupus erythematosus disease activity index; SLEDAI) and organ damage (systemic lupus international collaborating clinics) scores, thrombotic events, antiphospholipid syndrome (APS) and antiphospholipid antibodies (aPLs) were registered.

Results: SLE patients showed decreased ADAMTS13 activity and high VWF levels compared with controls (66/27% vs. 101/26%, P < 0.01, and 325/151% vs. 81/14%, P < 0.001). VCAM-1 levels were higher in SLE patients (P < 0.05). Considering three groups of SLE patients depending on ADAMTS13 activity (>60%, 60–40% and <40%), comparative analysis showed significant association between ADAMTS13 activity and SLEDAI (P < 0.05), presence of aPLs (P < 0.001), APS (P < 0.01) and thrombotic events (P < 0.01). Reduced ADAMTS13 activity together with increased VWF levels were especially notable in patients with active disease and with aPLs. Conclusion: ADAMTS13 activity, in combination with other laboratory parameters, could constitute a potential prognostic biomarker of thrombotic risk in SLE. Lupus (2015) 0, 1–7.

Key words: Systemic lupus erythematosus; ADAMTS13 activity; SLEDAI

Introduction

Von Willebrand factor (VWF) is an adhesive, multimeric glycoprotein synthesized by endothelial cells1 and megakaryocytes,2 which plays an essential role in hemostasis.3 After vascular damage, VWF mediates platelet adhesion by binding to exposed subendothelial collagen. VWF also carries and stabilizes factor VIII in blood circulation.4

The normal hemostatic function of VWF depends on its multimeric size. This is physiologically regulated by the metalloproteinase ADAMTS13. ADAMTS13 (a disintegrin and metalloproteinase with a thrombospondin type 1 motif, member 13) is a protease synthesized by human stellate hepatic cells and vascular endothelial cells, which cleaves specifically ultralarge VWF multimers at the VWF-A2 domain, generating shorter globular multimers in the normal circulation.5,6 A lack of proteolytic activity results in the presence of unusual hyperadhesive large VWF strings on the endothelial cell surface, which are more likely to bind to platelets than normal globular VWF, leading to platelet aggregation and thrombus formation in the microvasculature.7,8

ADAMTS13 severe deficiency (<5% of ADAMTS13 activity) is considered to be the cause of thrombotic thrombocytopenic purpura (TTP), a syndrome characterized by thrombocytopenia, hemolytic anemia and thrombotic events in microvasculature, which results in organ damage, of which renal impairment and
neurological symptoms are the most common clinical features. ADAMTS13 activity deficiency is caused by mutations in the ADAMTS13 gene (Upshaw–Schulman syndrome) or with the development of inactivating autoantibodies against the protease (acquired TTP). In addition, there are studies demonstrating that ADAMTS13 activity could also be reduced in other pathological conditions such as sepsis, cirrhosis, malignancy, autoimmune disorders, multisystemic inflammatory states and in other conditions such as organ transplantation or associated to the administration of specific drugs. Moreover, it has been described that a mild to moderate deficiency of ADAMTS13 has clinical significance, contributing to increase the risk of thrombosis in diseases that share in common the presence of systemic inflammation.

Systemic lupus erythematosus (SLE) is an autoimmune disease characterized by multisystemic chronic inflammation and the presence of immune complexes associated with organ damage and systemic clinical manifestations involving the skin, joints, kidneys, nervous system and blood, among other elements. SLE patients have an increased risk of cardiovascular events, resulting from both atherosclerosis and thromboembolic events. Thrombosis is one of the life-threatening complications in SLE, with an approximate prevalence of 10% in the SLE population. These thrombotic events in SLE have similar clinical features to those found in TTP, and could be ascribed to endothelial damage caused by the inflammatory condition present in SLE.

This study was designed to evaluate the potential relation between low to moderate levels of ADAMTS13, endothelial damage and the thrombotic risk present in patients with SLE.

Patients and methods

Experimental design

A total of 50 consecutive SLE patients from the systemic autoimmune outpatient clinic were included in this study. Healthy individuals without autoimmune disease, thrombotic risk or a history of thrombosis were selected as controls. The two groups were matched for age (range 16–58 years in patients, range 20–50 years in controls) and gender (4% men and 96% women in patients, 10% men and 90% women in controls).

SLE clinical manifestations were evaluated according to the American Rheumatism Association glossary committee, and activity of the disease and SLE-associated organ damage were scored using the systemic lupus erythematosus disease activity index (SLEDAI) and systemic lupus international collaborating clinics (SLICC), respectively. Other laboratory parameters related to SLE were also registered (Table 1). Sixty per cent of the SLE patients were on treatment with prednisone, and 25% were on treatment with immunosuppressants (cyclophosphamide, azathioprine and methotrexate). Other medications used were non-steroidal anti-inflammatory agents, hydroxychloroquine and antplatelet agents. Four out of 50 SLE patients included had thrombotic episodes: ischemic stroke (one), deep venous thrombosis (one) and deep venous thrombosis and pulmonary embolism (two). Three of these patients received oral anticoagulation.

Informed consent was obtained from all patients and donors for blood utilization. The study was approved by the ethical committee of the hospital clinic and was carried out according to the principles of the Declaration of Helsinki.

To carry out the present study, ADAMTS13 activity, VWF and VCAM-1 levels were measured in plasma samples from SLE patients and the

### Table 1 Clinical characteristics of the patients with SLE included

<table>
<thead>
<tr>
<th>Age</th>
<th>32 (16–58)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (female (%)/male (%))</td>
<td>96%/4%</td>
</tr>
<tr>
<td>Antiphospholipid antibodies (%)</td>
<td>28%</td>
</tr>
<tr>
<td>Lupus anticoagulant (LA)</td>
<td>16%</td>
</tr>
<tr>
<td>Anticardiolipin antibodies (aCL)</td>
<td>20%</td>
</tr>
<tr>
<td>Anticardioliqin antibodies IgG/IgM/IgG-IgM</td>
<td>8%/8%/4%</td>
</tr>
<tr>
<td>Anti-β2-glycoprotein-1 antibodies (anti-β2GPI)</td>
<td>12%</td>
</tr>
<tr>
<td>Anti-β2GPI IgG/IgM/IgG-IgM</td>
<td>4%/6%/2%</td>
</tr>
<tr>
<td>SLE clinical manifestations (%)</td>
<td></td>
</tr>
<tr>
<td>Arthritis</td>
<td>98%</td>
</tr>
<tr>
<td>Skin involvement</td>
<td>78%</td>
</tr>
<tr>
<td>Glomerulonephritis</td>
<td>54%</td>
</tr>
<tr>
<td>Class V/IV/III/II</td>
<td>8%/36%/6%/4%</td>
</tr>
<tr>
<td>Nephrotic syndrome</td>
<td>40%</td>
</tr>
<tr>
<td>Renal failure</td>
<td>8%</td>
</tr>
<tr>
<td>Hematological involvement</td>
<td>42%</td>
</tr>
<tr>
<td>Hemolytic anemia/thrombocytopenia</td>
<td>22%/34%</td>
</tr>
<tr>
<td>Serositis</td>
<td>28%</td>
</tr>
<tr>
<td>Neurological involvement</td>
<td>16%</td>
</tr>
<tr>
<td>Antiphospholipid syndrome</td>
<td>16%</td>
</tr>
<tr>
<td>SLEDAI mean ± SD (range)</td>
<td>6.64 ± 4 (2–21)</td>
</tr>
<tr>
<td>SLICC mean ± SD (range)</td>
<td>2.66 ± 1 (1–6)</td>
</tr>
</tbody>
</table>

Controls were matched for age (range 20–50 years) and gender (90% women and 10% men).

SLE: systemic lupus erythematosus; SLEDAI: systemic lupus erythematosus activity index; SLICC: systemic lupus international collaborating clinics.
controls. Results were correlated with clinical parameters of the disease in patients with SLE.

Sample collection and processing
Nine parts of venous blood from SLE patients were collected into one part of 3.8% sodium citrate. Platelet-poor plasma (PPP) was obtained by centrifugation at 2500 rpm for 20 minutes and PPP samples were aliquoted and immediately stored at −80°C until testing. Plasma samples from 50 healthy donors were used as controls.

Tests using plasma samples were performed in random order and analysis of results was performed on a blinded basis.

ADAMTS13 activity assay
Fluorescence resonance energy transfer (FRET) methodology was performed using a synthetic 73 amino acid VWF peptide as fluorescence-quenching substrate.27 Plasma samples were diluted 1:25 in 5 mM Tris HCl, 25 mM calcium chloride (pH 6), 0.005% Tween-20, 100 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, hydrochloride PEFABLOC SC (Roche, Mannheim Germany) dilution buffer.

One hundred microlitres of calibration test and control samples were added into a 96-well white plate (Sterilin Ltd., Newport, UK) and incubated at 37°C for 10 minutes. After this, 100 μl of the FRET-VWF 73 substrate solution were added to each well and fluorescence was measured by a fluorescence microplate reader (Fluoskan Ascent FL; Thermolab Systems, Waltham, MA, USA) (λex = 340 nm, λem = 450 nm at 37°C) every 5 minutes up to 1 hour. The normal range for ADAMTS13 activity was calculated based on measurements on 50 different healthy donors, being 101 ± 8%.

Measurement of VWF antigen and VCAM-1 levels
Measurement of VWF antigen levels was performed by an enzyme-linked immunoabsorbent assay (ELISA), using a commercial sandwich ELISA (DG-EIA vWF; Grifols, Barcelona, Spain). Sample concentration was optimized because VWF antigen exceeded the highest point of the standard curve. Plasma levels of soluble VCAM-1 were measured by using the human VCAM-1 ELISA kit (Millipore Corporation, Billerica, MA, USA) according to the manufacturer’s instructions. Absorbance was immediately read at 450 nm by MultiSkan Ascent (Thermo Electron Corporation, Finland). Normal ranges are 50–160% for VWF (according to the manufacturer’s protocol) and 400–650 ng/ml for VCAM-1 (according to the laboratory standards).

Evaluation of the VWF multimer structure by agarose gels
Analysis of VWF multimers was carried out by sodium dodecyl sulphate-agarose discontinuous gel electrophoresis followed by protein transfer to nitrocellulose membranes by western blotting. Blots were probed using horseradish peroxidase (HRP) conjugated rabbit anti-VWF; visualisation of VWF multimers was achieved using a commercially available enhanced chemiluminescence kit for detecting HRP labelled antibodies on western blots.28

Anti-ADAMTS13 antibodies assay
The TECHNOZYM ADAMTS13 INH ELISA kit (Technoclone, Vienna, Austria) was used for the detection of human IgG autoantibodies against ADAMTS13 in those patients whose ADAMTS13 activity was lower than 60%. The ELISA test was performed according to the manufacturer’s protocols. Measurement was performed by ELISA reader MultiSkan Ascent (Thermo Electron Corporation, Finland) at 450 nm (IgG ADAMTS13 antibodies positive if above 15 U/mL).

Statistical analysis
Quantitative results were expressed as mean ± standard deviation and percentages. Statistical analysis was performed using the Student’s t test and linear regression analysis for parametric data and chi-squared (χ²) for non-parametric data. Results were considered statistically significant when P < 0.05. Statistically significant levels indicated in the text refer to the t test, except where indicated.

Results

ADAMTS13 activity levels
ADAMTS13 activity values were significantly reduced in patients with SLE (66 ± 27% vs. 101 ± 8% in healthy individuals, P < 0.01). In SLE patients, ADAMTS13 activity was below 60% (38 ± 14%, n = 20) in 40% of the SLE patients. ADAMTS13 activity was between 60% and 40% (50 ± 6, n = 9) in 18% of SLE patients, whereas 22% showed ADAMTS13 activity below
40% (29 ± 11%, n = 11). No patient could be identified as having a severe deficiency of ADAMTS13 activity (<5%), 13% being the lowest value in the group of SLE patients studied (Figure 1(a)).

**VWF antigen levels and multimeric structure**

VWF levels were significantly elevated in all SLE patients included in our study (325 ± 151% vs. 81 ± 14% in healthy individuals, P < 0.001) (Figure 1(b)).

Although moderate, there was a statistically significant inverse correlation (Pearson correlation, r = −0.3) between levels of VWF and activity levels of ADAMTS13 (P < 0.05).

No abnormalities were observed in the multimeric patterns of VWF in the patients’ plasma when compared with those observed in control samples (see Figure 1(c)).

**Evaluation of endothelial activation through soluble adhesion molecule VCAM-1 analysis**

Levels of soluble VCAM-1 were found to be notably and significantly higher in almost all the plasma samples from patients with SLE (range of 564–4991 ng/ml vs. 502–694 ng/ml in healthy donors, P < 0.05). Comparing subgroups of patients with ADAMTS13 activity greater than 60% and less than 60%, we found that those who had protein activity values less than 60% presented VCAM-1 levels significantly higher than those with ADAMTS13 activity levels greater than 60% (mean ± SEM of 2319 ± 293 vs. 1561 ± 223 ng/ml, respectively, P < 0.05). However, no significant correlation was found between ADAMTS13 activity levels and VCAM-1 levels.

**Relationship between ADAMTS13 activity levels and indicators of disease activity (SLEDAI) and organ damage (SLICC)**

The population of SLE patients included in the present study showed low to medium SLEDAI (6.64 ± 4) and SLICC (2.6 ± 1) scores. The SLEDAI score was significantly higher in SLE patients with ADAMTS13 activity levels less than 60% (8.6 ± 5) than in patients with ADAMTS13 activity greater than 60% levels (5.3 ± 3) (P < 0.01).

The presence of active disease, considered when the SLEDAI score was greater than 6, was significantly associated with low levels of ADAMTS13 activity (P < 0.05, χ² test), from the comparative analysis among three groups of SLE patients established depending on ADAMTS13 activity (>60%, between 60–40% and <40%).

In relation to SLE patients with SLEDAI scores greater than 6 (n = 22), 59% had ADAMTS13 activity levels less than 60% and 36% had ADAMTS13 activity levels less than 40% (Table 2).

Statistical analysis showed no correlation between SLICC and the metalloprotease levels.

**IgG anti-ADAMTS13 autoantibodies**

IgG anti-ADAMTS13 autoantibodies were determined by ELISA only in those plasma samples from SLE patients with ADAMTS13 activity levels below 60%. The presence of IgG against ADAMST13 was positive only in three SLE plasma samples (15%) being of 63 U/ml in two samples and 70 U/ml in the other. These samples showed ADAMTS13 activity levels of 38%, 40% and 51%. Additional evaluation of ADAMTS13 activity in mixtures of patients plasma and control plasma in a 1:1 proportion gave values of 70%, 53% and 81%, respectively. Therefore, only one sample out of 50 showed significant inhibitory action on ADAMTS13 activity (around 50% of inhibition).

**ADAMTS13 activity and other disease parameters**

Fourteen SLE patients were positive for one, two or three of the antiphospholipid antibodies (aPLs)
LS, LA, aCL and aβ2GPI. Twelve of these patients (85%) exhibited reduced ADAMTS13 activity, and seven of them showed values less than 40%.

Of the eight patients diagnosed with APS SLE, seven (88%) had ADAMTS13 activity levels less than 60%, being less than 40% in 50% of them.

Four out of the 50 SLE patients included in the study had a thrombotic episode (which stands for 8% of the patients with SLE). All four patients who experienced thrombotic events were found to present ADAMTS13 activity levels less than 60%.

Interestingly, three out of four of these patients presented ADAMTS13 activity levels less than 60%, being less than 40% in 50% of them. Levels of ADAMTS13 activity in these patients were 46%, 37%, 32% and 17%.

An additional comparison of the distribution (χ² test) of the number of SLE patients with the presence of aPLs, APS SLE and thrombotic events among the three groups of SLE patients (established depending on ADAMTS13 activity >60%, between 60–40% and <40%), demonstrated a statistically significant association between low levels of ADAMTS13 activity and the presence of aPLs (P < 0.001), APS SLE (P < 0.01) and thrombotic events (P < 0.01).

Discussion

SLE is an autoimmune disease in which the main pathological features are systemic inflammation together with elevated levels of proinflammatory cytokines, the presence of autoantibodies and the deposition of immune complexes in target tissues. One of the main causes of morbidity and mortality among SLE patients is the development of thrombotic events, which are caused by microvasculature occlusion similar to that occurring in TTP. While the presence of autoantibodies neutralizing ADAMTS13 is known to be the main cause of microthrombosis in acquired TTP, the pathogenic mechanisms of thrombosis in SLE still remain unknown. The hypothesis of our present study was that ADAMTS13 activity could be reduced in SLE and this deficiency could have an impact on the clinical outcome of the disease.

ADAMTS13 activity in the patients included in our study was found to be mildly to moderately deficient, although never as severe as in TTP. These results were associated with very high levels of plasma VWF, with a moderate inverse correlation. VWF is a marker of endothelial damage and these high values, together with the increased levels of VCAM-1, another marker of endothelial harm, could be indicating the presence of endothelial activation and damage in association with SLE.

Endothelial injury is a common pathological condition in different diseases in which inflammation is present, such as obesity, diabetes and chronic renal failure, and even in association with treatments such as hematopoietic stem cell transplantation. In these pathological entities, decreased levels of ADAMTS13 have been reported. In autoimmune disorders, inflammation and the presence of immune complexes can produce not only endothelial activation, with the expression of adhesion molecules that induce a pro-adhesive and thrombogenic endothelium, but also endothelial dysfunction. The cause of ADAMTS13 deficiency in SLE is still unclear although different hypotheses could be proposed. Considering that ADAMTS13 is synthesized in the liver, it has been suggested that a lack of synthesis of metalloproteinase may be due to liver damage, potentially ascribed to the SLE condition and/or to the treatment with immunosuppressant agents, although low levels of ADAMTS13 activity were observed in both treated and untreated SLE patients. Turner et al. demonstrated that ADAMTS13 is also synthesized by endothelial cells. These authors detected the presence of both ADAMTS13

Table 2 Clinical and laboratory parameters of the SLE patients included and correlation with the ADAMTS13 activity levels (% with respect to the total number of SLE patients)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>ADAMTS13 activity</th>
<th>ADAMTS13 activity</th>
<th>ADAMTS13 activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>All</td>
<td>&gt;60%</td>
<td>60–40%</td>
</tr>
<tr>
<td>SLEDAI ≥6 (%)*</td>
<td>22 (44%)</td>
<td>9 (18%)</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>APS SLE**</td>
<td>8 (16%)</td>
<td>1 (2%)</td>
<td>4 (8%)</td>
</tr>
<tr>
<td>Antiphospholipid antibodies***</td>
<td>14 (28%)</td>
<td>2 (4%)</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>Lupus anticoagulant antibodies</td>
<td>10 (20%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>4 (8%)</td>
<td>1 (2%)</td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>4 (8%)</td>
<td></td>
<td>2 (4%)</td>
</tr>
<tr>
<td>IgG, IgM</td>
<td>2 (4%)</td>
<td></td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Anti-β2GPI</td>
<td>6 (12%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>IgG</td>
<td>2 (4%)</td>
<td>1 (2%)</td>
<td></td>
</tr>
<tr>
<td>IgM</td>
<td>3 (6%)</td>
<td></td>
<td>2 (4%)</td>
</tr>
<tr>
<td>IgG, IgM</td>
<td>1 (2%)</td>
<td></td>
<td>1 (2%)</td>
</tr>
<tr>
<td>Thrombotic events*</td>
<td>4 (8%)</td>
<td></td>
<td>1 (2%)</td>
</tr>
</tbody>
</table>

Levels of statistical significance reached by using χ² test for comparison between levels of ADAMTS13 activity and clinical parameters in the three groups of SLE patients established depending on ADAMTS13 activity levels, as indicated: *P < 0.05; **P < 0.01; ***P < 0.001.

SLE: systemic lupus erythematosus; SLEDAI: systemic lupus erythematosus activity index; APS: antiphospholipid syndrome.
messenger RNA and antigen in cultured human endothelial cells from venous and arterial locations, functionally active even under static conditions. Therefore, considering that the endothelium may be an important source of ADAMTS13 because of the high number of endothelial cells along the microvasculature, the potential activation and damage due to the inflammatory state in SLE, as derived from the high levels of VWF and VCAM-1, could be causing ADAMTS13 activity deficiency. Moreover, reduced ADAMTS13 activity levels may result from an increased consumption due to the high amounts of its physiological substrate VWF.

Development of autoantibodies against ADAMTS13 could be another putative mechanism. In our SLE patients, the presence of anti-ADAMTS13 IgG was rare and there was not a strong correlation with the corresponding ADAMTS13 activity levels. However, other subclasses of antibodies, such as IgM anti-ADAMTS13, or the development of non-neutralizing antibodies that induce modifications in the protein clearance may be also be considered as possible mechanisms, but no conclusion can be drawn at the moment.

Our SLE cohort included patients with both APS secondary to SLE and different types of aPL without a diagnosis of APS. aPLs belong to a heterogeneous group of antibodies against different plasma proteins that bind phospholipids. The presence of aPLs, characteristic of APS, is associated with the development of a prothrombotic state. APS was first described in SLE, although it may appear as a primary disease, but aPLs may also be found in SLE without APS. It is interesting to point out that most of the patients with detectable aPLs showed levels of ADAMTS13 activity below 60%. Other in-vitro studies suggest that the presence of aPLs promote endothelial activation through the expression of adhesion molecules, such as VCAM-1. Taking all these findings together, the high levels of VWF and VCAM-1 described and the reduced levels of the VWF cleaving protease would lead to the development of a prothrombotic state increasing the thrombotic risk present in SLE patients.

In our study, ADAMTS13 activity was specially reduced in patients with active disease. The pathogenic role of ADAMTS13 in unexpected thrombotic events occurring in acute episodes of chronic inflammatory diseases is the subject of current investigation. These thrombotic events have been associated with decreased ADAMTS13 activity levels in ischemic stroke, and in other clinical situations such as sepsis or bone marrow transplantation.

In conclusion, our present findings show that SLE is a pathological condition with mild to moderate ADAMTS13 activity deficiency and high levels of VWF and VCAM-1, potentially related to endothelial damage. ADAMTS13 activity in SLE is specially reduced in patients with active disease and in those with aPLs. Further studies are needed to clarify the pathogenesis of this deficiency and its role in the thrombotic complications observed in SLE. Since the size of the study population is relatively small, we cannot state that lower levels of ADAMTS13 are predictive of thrombotic risk. Nevertheless, our results are powerful enough to detect significant differences in the thrombotic risk in subgroups of SLE patients with lower levels of ADAMTS13. Further studies in larger groups of patients will be required to explore whether levels of ADAMTS13 and VWF could be combined with other parameters to generate a score or even an algorithm that could be more predictive of the thrombotic risk in SLE patients.

Funding

This work has been partially supported by Ministerio de Economía y Competitividad (SAF2011-28214, Fondos FEDER), and Health Institute Carlos III (ISCIII): Fondo de Investigación Sanitaria (FIS PI13/00517) and Cardiovascular Research Network (RIC) (RD12/0042/0016) together with the European Regional Development Funds (FEDER).

Conflict of interest statement

The authors declare no conflicts of interest.
References


