

Increase of coagulation potential in chronic spontaneous urticaria

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Abstract

Background: The pathogenesis of chronic spontaneous urticaria (CU) has recently been conceived to be associated with thrombin generation through the extrinsic coagulation pathway. However, little is known about the components of the intrinsic coagulation pathway potentially involved.

Methods: To investigate the whole process of coagulation, both classical coagulation assays and a global coagulation test, the intrinsic coagulation pathway-dependent activated partial thromboplastin time (APTT) clot waveform analysis, were performed using plasma of 36 patients with CU who had various severities.

Results: Classical coagulation assays revealed that levels of fibrinogen, D-dimer, and fibrin and fibrinogen degradation products (FDP), and positive rates of soluble fibrin monomer complex (SFMC) were significantly elevated in patients with CU, whereas the elevation of prothrombin fragment 1 + 2 was not statistically significant. On the other hand, all parameters of a global coagulation test, APTT clot waveform analysis, evidently showed a hypercoagulable pattern and were significantly correlated to disease severity of CU.

Conclusions: CU is characterized by elevated blood coagulation potential with involvement of the intrinsic coagulation factors, which may contribute *in vivo* to the generation of fibrin even by small amounts of thrombin.

Chronic spontaneous urticaria (CU) is a common skin disorder characterized by spontaneously appearing wheals and pruritus anywhere on the body for 6 weeks or longer (1). It severely impairs the quality of life of patients (2). In patients with urticaria, degranulation of cutaneous mast cells by a variety of reasons results in the release of histamine, which induces the activation of sensory nerves, vasodilatation, and extravasation of plasma from microvessels into the dermis. The administration of anti-histamines effectively suppresses symptoms of many patients with CU. Although IgG antibody to IgE and/or those to the high affinity IgE receptor plays a role in the mechanism for activation of cutaneous mast cells and blood basophils in CU (3, 4), the detailed mechanism of mast cell stimulation in CU remains to be fully elucidated and a substantial population of patients with CU is not sufficiently controlled with anti-histamines alone. On the other hand, beneficial effects of anticoagulants such as

heparin and warfarin have been reported in some patients with intractable urticaria (5–7), but the pharmacological mechanisms by which anticoagulants exert their effects in urticaria has long been unexplained.

In recent years, a series of reports by Asero and his colleagues has shed light on the involvement of coagulation in the pathogenesis of CU. They described enhanced thrombin generation through the observation of high levels of prothrombin fragments 1 + 2 (PF1 + 2) in patients with CU (8). They argued that thrombin generation is accelerated by the increase of tissue factor (TF) expressed in dermal tissues via the activation of extrinsic coagulation (9). They advocated that the accelerated generation of thrombin would then activate mast cells and increase endothelial permeability (8). However, Kaplan and Greaves recently challenged this scenario on several points: (i) lack of evidence for thrombin-dependent activation of human mast cells, (ii) immediate

inactivation of thrombin by plasma inhibitors, (iii) possible presence of yet to be defined plasma factor other than thrombin that leads to an increase in endothelial permeability (10). In this context, the extrinsic coagulation pathway evoked by activated factor VII (FVIIa)-TF binding is known to be promptly attenuated due to a large amount of tissue factor pathway inhibitor (TFPI) in tissues (11). Therefore, the coactivation of the intrinsic pathway coagulation factors, especially factors VIII and IX, should be requisite to overcome the inhibition by TFPI and complete coagulation by full thrombin generation (i.e. thrombin burst).

To elucidate a more detailed condition of coagulation in CU, including the involvement of the intrinsic coagulation pathway, it is fundamental to assess the whole process of coagulation. However, conventional assays for blood coagulation measure only a part of the coagulation process, such as the initiation time of clot formation, and do not necessarily reflect the overall coagulation process. Both prothrombin time (PT) and activated partial thromboplastin time (APTT) are useful to obtain information for only an early stage of clot formation. On the other hand, classical thrombin assays, such as prothrombin fragment 1 + 2 (PF1 + 2), estimate the amount of thrombin that has already been generated before blood collection. However, recently developed clot waveform analysis assesses the whole process of coagulation beyond the initiation time of clot formation through continuously charting the changes in light transmittance on standard coagulation assays such as APTT (12, 13). Data are mathematically processed to derive several unique parameters, such as to reflect hemorrhagic and thrombophilic conditions perceptively. In this study, we evaluated hemostatic profiles of plasma in patients with CU using APTT clot waveform analysis as well as classical coagulation assays in order to elucidate the precise involvement of coagulation in CU.

Methods

Patients and plasma samples

We recruited 15 normal healthy individuals and 36 patients with CU of various severities. Mean $\pm$ SD age of healthy individuals was 37.4 ± 14.0 years (range 27–80) and that of CU patients was 47.0 ± 18.0 years (range 9–76). No significant age differences were seen between these groups. All patients were diagnosed at Department of Dermatology, Hiroshima University Hospital on the basis of the appearance of spontaneous wheals anywhere on the body with or without angioedema for more than 6 weeks (1). All patients with CU whose severity was properly assessed and described in the medical record at the time of blood collection were divided into three groups using a clinical urticaria-severity score (1–3: mild, moderate, and severe, respectively) as described elsewhere (14, 15). According to this score, 10 patients were evaluated as mild, 19 as moderate, and 7 as severe.

Blood was drawn into evacuated anticoagulant tubes [nine volumes of blood to one volume of 3.8% (w/v) trisodium citrate solution]. After centrifugation for 15 min at 1500 g,

the platelet-poor plasma was stored at -80°C and thawed at 37°C immediately prior to performing the assays described.

The study protocol conformed to the ethical guidelines of the Declaration of Helsinki, was approved by the Nara Medical University Hospital Ethical Committee and the Institutional Review Board at the Hiroshima University Hospital, and all subjects gave their informed consent before participation.

Classical coagulation assays

Among coagulation factors, fibrinogen and factors VII, VIII, and X clotting activities (FVII:C, FVIII:C, and FX:C) were measured by a one-step clotting assay on a MDA-II Haemostasis System (bioMérieux, Lyon, France) using the respective factor-deficient plasma (Sysmex, Kobe, Japan), Thrombocheck Fib-L (Sysmex), Thrombocheck PT Plus (Sysmex), and Thrombocheck APTT-SLA (Sysmex). A standard curve was prepared using Verify Reference Plasma (bioMérieux) in serial doubling dilutions (1:10–1:1280) in 0.05 M imidazole saline buffer containing 0.05% sodium azide (pH 7.3). The Verify Reference Plasma is calibrated by the manufacturer against an International FVIII Standard (IRP-SSC1 or 2). Each test sample was diluted to 1:10 in imidazole saline buffer. Plasma von Willebrand factor antigen (VWF:Ag) level was determined by ELISA using double-antibody sandwich techniques (Dako, Glostrup, Denmark).

Plasma FDP and D-dimer were measured by the latex agglutination method using Nanopia P-FDP (Daiichi Pure Chemicals, Tokyo, Japan), COBAS INTEGRA 700 (Roche Diagnostics, Tokyo, Japan), respectively. The plasma PF1 + 2 levels were measured using an enzyme immunoassay (Enzygnost PF1 + 2 micro, Dade Behring, Germany) according to the manufacturer's instructions. Plasma soluble fibrin monomer complex (SFMC) was determined by the fibrin monomer-coated erythrocyte aggregation method using the FM test (Roche Diagnostics), which gave qualitative results.

APTT clot waveform analysis

APTT clot waveform analysis of the optical data obtained from the modified APTT assay was performed on the MDA-II Haemostasis System as described previously (16). As with an APTT assay, the analysis is performed by adding to plasma a surface activator, phospholipids, and calcium ions to activate the intrinsic clotting system. Intrinsic coagulation potential of plasma is reflected in clot waveform. The obtained data were processed subsequently by export research tools (WIT/WET, bioMérieux, Fig. 1). The first derivative of the transmittance (dT/dt) reflects the coagulation velocity at each time point along the waveform plot of changes in light transmission, which in turn reflects the conversion of fibrinogen to a fibrin clot. Point 'a' marks the beginning of the recording by the instrument which occurs 8 s after the addition of CaCl_2 . The minimum value of the first derivative (Min1), defining the maximum velocity of change in light transmission achieved (point 'c'), was calcu-

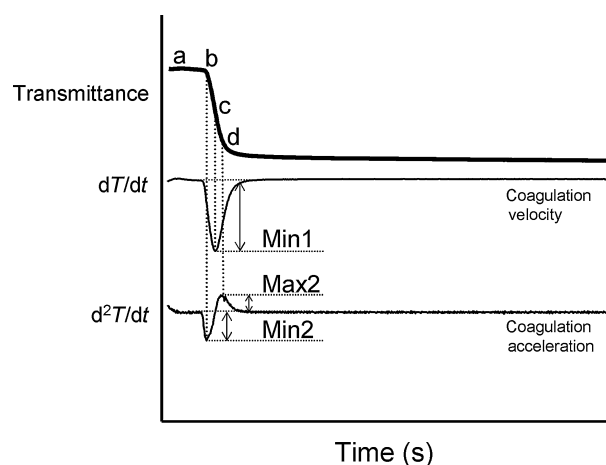


Figure 1 APTT clot waveform of normal plasma. The upper trace shows the recording of the changes in light transmittance (T) observed over time (t) during the performance of a normal diluted APTT. The middle trace shows the first derivative (dT/dt : Min1) derived from these transmittance data. The lower trace shows the second derivative (d^2T/dt^2 : Min2).

lated as an indicator of the maximum velocity of coagulation. The second derivative of the transmittance data (d^2T/dt^2) reflects the acceleration of the reaction at any given time point. The minimum value of the second derivative (Min2), measured at point 'b', and the maximal value of the second derivative (Max2) at point 'd' were also calculated as an index of the maximum acceleration of the reaction achieved (16). Reduction in Clot Time and increase in |Min1|, Max2, and |Min2| shows a hypercoagulable state, whereas prolonged Clot Time and decreased |Min1|, Max2, and |Min2| indicate a hypocoagulable state or bleeding tendency.

Statistical analysis

Data are presented as mean $\pm$ SD. Statistical analysis of differences among two groups was determined by the Mann–Whitney test. The chi-square test or Fisher's exact test was used to determine differences with respect to SFMC test. Correlations were calculated according to Spearman rank correlation coefficient. The level of significance was set at $P < 0.05$. All analyses were done using the GRAPHPAD PRISM (GraphPad Software, San Diego, CA, USA) and YSTAT 2002 software package (Igaku Tosho Shuppan, Tokyo, Japan).

Results

Hemostatic profiles in the CU patients

The results of classical coagulation assays; coagulation factors and coagulation activation markers, are shown in Fig. 2. Among coagulation factors, a significantly elevated level was observed in fibrinogen in the CU patient group ($P < 0.05$) (Fig. 2A). Among markers of activated coagulations, the levels of D-dimer and FDP and the positive rate of SFMC were higher in the CU patient group than in the control

group ($P < 0.05$). The levels of PF1 + 2 levels in the CU group were also higher than those in control groups, but the difference was not statistically significant (Fig. 2B). On the other hand, all parameters in clot waveform analysis showed the hypercoagulable pattern in the CU patient group: significantly reduced Clot Time and elevated |Min1|, Max2, and |Min2|: $P < 0.05$, $P < 0.01$, $P < 0.001$, $P < 0.001$, respectively (Fig. 2C).

When patients were divided according to clinical severities of CU, they were significantly correlated to levels of D-dimer ($P < 0.05$) and PF1 + 2 ($P < 0.01$), and positive rates of SFMC ($P < 0.05$), but not to levels of FDP and coagulation factors (Fig. 3A and B). On the other hand, significant correlations were observed between disease severities and all parameters in clot waveform analysis, Clot Time, |Min1|, Max2, and |Min2| ($P < 0.01$) (Fig. 3C).

Discussion

In this study, we have demonstrated that increased coagulation potential is strongly associated with symptoms of CU by performing APTT clot waveform analysis in addition to assays for the levels of coagulation factors and markers of activated coagulations.

We first evaluated hemostatic profiles using classical coagulation assays. Among coagulation factors in patients with CU, the level of fibrinogen was higher than that of healthy controls (Fig. 2A). However, no correlation was observed between the levels of any coagulation factors and disease severities of CU (Fig. 3A). Coagulation activation markers in CU, D-dimer, FDP, PF1 + 2, and positive rate of SFMC tend to be elevated and correlated with disease severities in patients with CU. However, the increase of PF1 + 2 in patients with CU was not significant as compared with healthy controls, and levels of FDP were not significantly correlated with disease severities of CU (Figs 2B and 3B).

On the other hand, all parameters in APTT clot waveform analysis showed significantly hypercoagulable patterns in patients with CU (Fig. 2C), and significantly correlated to disease severities of CU (Fig. 3C). It is known that the level of PF1 + 2, the level of D-dimer, and the positive rate of SFMC reflect the amount of thrombin actually generated, and the level of FDP reflects both the amount of actually generated thrombin and fibrinolytic activity. However, we do not have clear data to explain the discrepancy between the levels of actually activated coagulation markers and those of coagulability represented by global coagulation test parameters. Taking into account that any clinically thrombotic complications are not usually observed in association with CU, there may be an urticaria intrinsic mechanism that prevents the burst of thrombin formation, while it drives coagulation to produce a small amount of thrombin.

Asero et al. (9) concluded that no activation of FXII was evident in CU. We also found that FXII activity measured by a one-step clotting assay was not elevated in CU patients recruited in this study (data not shown). These observations appear to suggest that the intrinsic coagulation pathway is not involved in CU, since FXII is essential as an initiator of

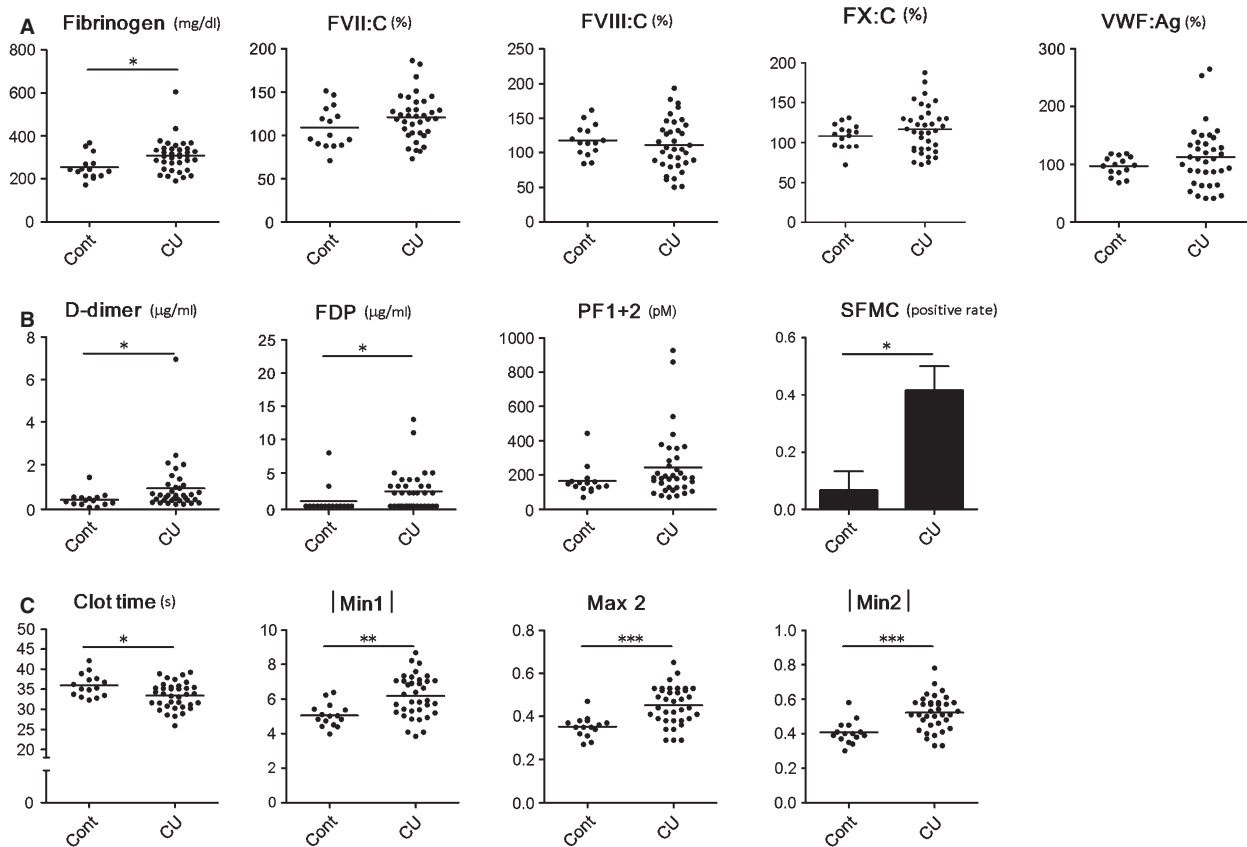


Figure 2 Hemostatic profiles in patients with CU. (A) Coagulation factors in healthy controls (Cont) and patients with CU (CU). (B) Coagulation activation markers in healthy controls (Cont) and patients with CU (CU). (C) Parameters of APTT clot waveform

analysis in healthy controls (Cont) and patients with CU (CU). Bars indicate means. * indicates $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$ (compared to healthy controls).

the intrinsic pathway in the model of classical coagulation cascade (contact system model). However, according to the recently proposed 'cell based model' of thrombin generation (17), the intrinsic pathway can be directly activated by a small amount of thrombin via factor XI (FXI) on an activated platelet membrane without the activation of FXII *in vivo* (18, 19). Moreover, thrombin generated by the extrinsic coagulation pathway should be promptly inactivated by TFPI without the activation of the intrinsic pathway. Furthermore, APTT clot waveform analysis applied in this study is sensitive to intrinsic factors VIII and IX, which are essential to overcome TFPI-mediated inhibition of the extrinsic pathway (19). Although we did not measure individual activities of all intrinsic factors, the hypercoagulable pattern in APTT clot waveform analysis suggests a high potential of the intrinsic coagulation cascade. Thus, it is feasible that the state of hypercoagulability based on both intrinsic and extrinsic activation may play an important role in the pathogenesis of CU.

The relation of mast cell activation and the state of hypercoagulability is a matter of discussion. Asero et al. (8) argued that thrombin activates skin mast cells via the activation of protease-activated receptor (PAR)-1 based on their

observations of positive autologous plasma skin test in patients with CU. However, there has not been enough evidence to confirm thrombin-dependent activation of mast cells (10). In addition, thrombin formation is reduced by subcutaneously-injected histamine, which is released from activated mast cells *in vivo* (20). Moreover, heparin proteoglycans released from mast cells strongly inhibit interaction of platelets with collagen (21), an important physiological agonist for platelets. Therefore, mast cell activation should result in the inhibition of full platelet activation that is requisite for thrombin burst. In fact, the amount of thrombin generation observed in patients with CU in this study was small.

In the hypercoagulable state, the activation of mast cells might be triggered or augmented by coagulation factors other than thrombin through the activation of PAR-2 rather than PAR-1. Recently, Wang et al. has mentioned that FVIIa may cause mast cell degranulation via PAR-2 activation in patients with CU (22). PAR-2 is cleaved by proteases with trypsin-like specificity but not by thrombin that cleaves PAR-1 and PAR-4. Moreover, as PAR-2 can be activated by tryptase (23), once mast cells are activated, mast cells may be further activated by tryptase secreted by activated mast cells.

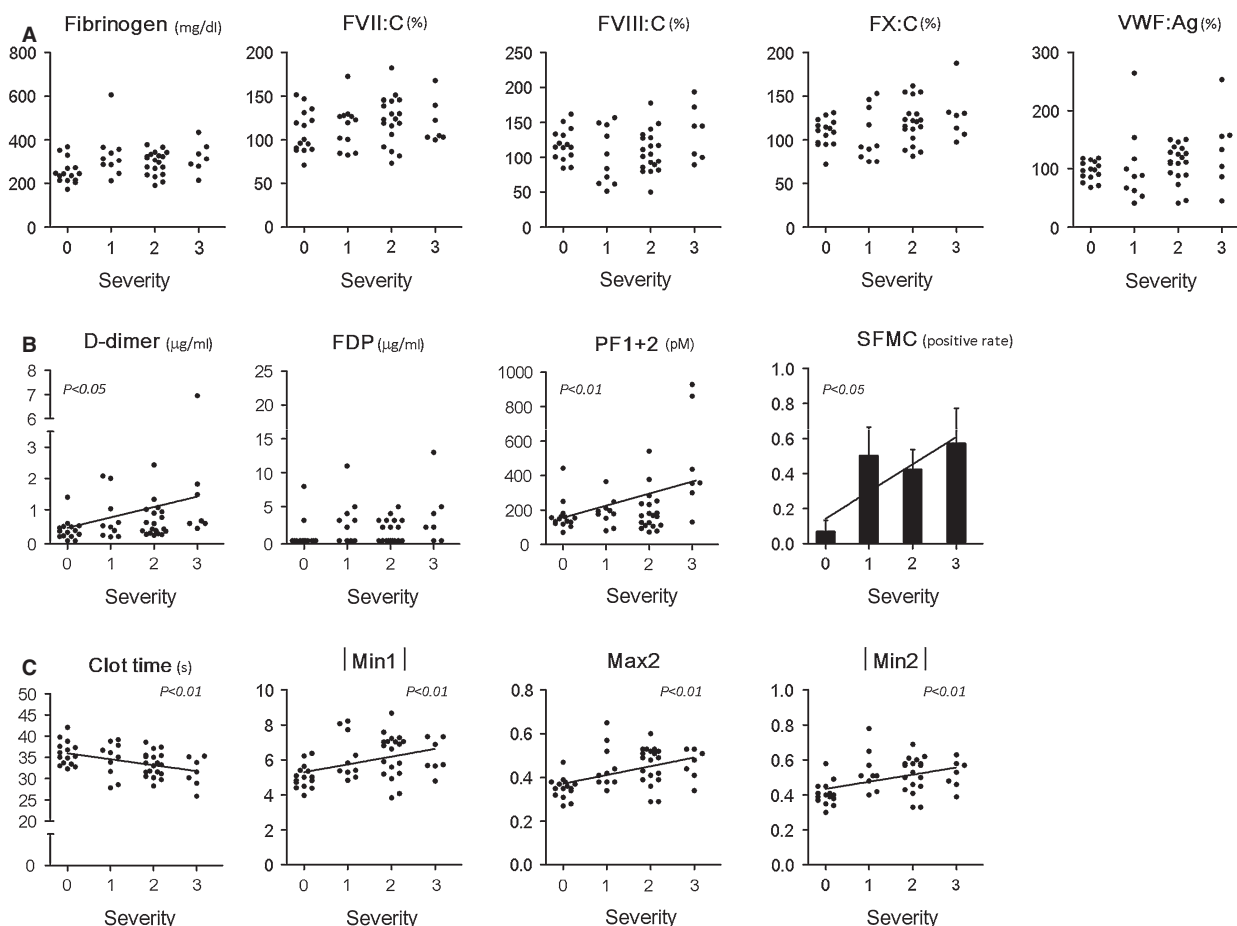


Figure 3 Correlation with severities in patients with CU. (A) Correlation between severities and coagulation factors. (B) Correlation between severities and coagulation activation markers. (C) Correlation between severities and parameters of APTT clot waveform analysis. Statistical significance is shown in each figure.

tion between severities and parameters of APTT clot waveform analysis. Statistical significance is shown in each figure.

It is noteworthy that the complex of FVIIa, activated FX (FXa), and TF may work more efficiently as a PAR-2 agonist than FVIIa only (24–26). Since extrinsic TF-FVIIa complex is promptly attenuated by TFPI, FXa generated with help of intrinsic coagulation factors VIII and IX should be crucial for PAR-2 activation. Thus, a hypercoagulable state with low level of thrombin generation observed in CU may potentially evoke or augment mast cell activation through PAR-2 cleavage by activated coagulation factors, and prevents a progress of excessive thrombin generation. The efficacy of several anti-coagulants, including nafamostat mesilate (27), heparin (6, 7) and warfarin (28, 29), reported in a few cases of CU support this possibility. We are currently studying if FVIIa actually induces histamine release from human skin mast cells *in vitro*. Further studies about longer and detailed time courses of hemostatic activities in patients with CU, in relation to wheal formation, disease activities, and mast cell activation should reveal their precise roles in the pathogenesis of urticaria.

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Authorship

T.T. and Y.S. designed the overall project, performed research, and wrote the manuscript; J.K. acquired data; S.T. and K.Y. acquired and interpreted data and drafted the manuscript; M.H. designed the overall project and wrote the manuscript; A.Y. and M.S. made critical revisions of the manuscript for important intellectual content.

Conflicts of interest

The authors declare no competing financial interests.

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