

## Original article

## Severe chronic urticaria is associated with elevated plasma levels of D-dimer

**Background:** Patients with chronic urticaria (CU) frequently show signs of thrombin generation as a result of the activation of the extrinsic pathway of coagulation and signs of fibrinolysis as shown by slightly increased mean D-dimer plasma levels. Here, we studied patients with severe CU to see whether the activation of coagulation and fibrinolysis parallels the severity of the disease.

**Methods:** Eight consecutive patients with severe exacerbations of CU and 13 with slight CU were studied. Plasma prothrombin fragment  $F_{1+2}$  as well as D-dimer were measured by ELISA. Serum histamine-releasing activity was assessed by basophil histamine release assay. Seventy-four normal subjects were used as controls.

**Results:** In patients with severe CU, median levels of both D-dimer (11.20 nmol/l) and  $F_{1+2}$  (592 pmol/l) largely exceeded those found in patients with slight CU [D-dimer: 2.66 nmol/l ( $P = 0.001$ ) and  $F_{1+2}$ : 228 pmol/l ( $P = 0.003$ )] and in normal subjects [D-dimer: 1.41 nmol/l ( $P = 0.0001$ ) and  $F_{1+2}$ : 159 pmol/l ( $P = 0.0001$ )]. Sera from 25% of patients with severe CU and 31% of those with slight CU, but from none of normal subjects, showed *in vitro* histamine-releasing activity. D-dimer and  $F_{1+2}$  levels were significantly correlated each other ( $r = 0.64$ ,  $P = 0.002$ ) and with CU severity score ( $r = 0.80$ – $0.90$ ,  $P = 0.0001$ ), but no correlation was observed between serum histamine-releasing activity and coagulation parameters or severity score.

**Conclusions:** Severe exacerbations of CU are associated with a strong activation of coagulation cascade and fibrinolysis. Whether this activation is the cause of CU or acts as an amplification system is still a matter of debate.

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Chronic ordinary urticaria (CU) has long been considered as a 'mysterious' disorder and has been often ascribed to anxiety or intolerance to foods, food dyes and food additives. It is now clear that in a substantial proportion of cases CU is an autoimmune disorder caused by histamine-releasing autoantibodies directed against the alpha subunit of the high affinity IgE receptor (anti-FcεRI) or against IgE (anti-IgE) (1, 2). These autoantibodies can be demonstrated *in vivo* by intradermal injection of autologous serum (ASST), which is commonly used in clinical settings, and *in vitro* by a functional assay employing basophils from normal donors (as a satisfactory immunoassay for anti FcεRI and anti-IgE antibodies is not available) (3, 4). The pathomechanism remains elusive in those cases of CU in which ASST is negative and no histamine-releasing autoantibodies can be demonstrated. Indirect evidence of the possible involvement of the coagulation cascade in CU came from the observation that the proportion of skin test-positive patients rises substantially if autologous plasma is

injected instead of autologous serum (about 80% vs 50%). Elevated plasma levels of prothrombin fragment  $F_{1+2}$ , suggesting thrombin generation were detected in CU patients (5), and recent findings showed that this is the result of the activation of the tissue factor pathway of coagulation cascade (6). In animal models thrombin has been shown to increase vascular permeability (7, 8) both directly, acting on endothelial cells (9), and indirectly, inducing release of proinflammatory mediators by mast cells (8, 10, 11), and to generate C5a in the absence of C3, thus bypassing the whole first part of the complement cascade (12). Furthermore, in outpatients with slight to moderate CU, mean D-dimer plasma levels were significantly higher than in a group of age- and sex-matched normal controls, although only in very few cases D-dimer levels exceeded the normal range (6). In this study, D-dimer and  $F_{1+2}$  plasma levels were measured in a selected population of patients with particularly severe CU to see if the activation of the coagulation cascade is related to the severity of the disease.

## Methods

### Patients

Eight consecutive adults (M/F 3/5; mean age  $\pm$  SD  $47 \pm 22$ ) admitted to the Dermatology Clinic or to the Allergy Unit of the Ospedale Maggiore Policlinico, University of Milan, Italy, because of severe exacerbations of CU were studied. CU was diagnosed on the basis of a history of continuous or recurrent hives with or without angioedema for more than 6 weeks (9). Physical urticarias and other possible causes of urticaria (food and drug allergy, bacterial infections and parasitoses) were excluded on the basis of clinical history and appropriate investigations (i.e., physical tests, skin prick tests and/or specific IgE determinations, search for bacterial foci and parasites). Urticarial activity was estimated according to the number of wheals present at the time when blood samples from our patients were collected as previously described (10): 1–10 small ( $<3$  cm in diameter) wheals grade 1 (slight); 10–50 small wheals or 1–10 large wheals grade 2 (moderate);  $>50$  small wheals or  $>10$  large wheals grade 3 (severe); virtually covered with wheals grade 4 (very severe). Based on this scale of disease activity, eight patients were considered as having a severe disease (two of them were almost completely covered with wheals) and 13 as having a slight to moderate disease when they were first visited and when blood samples for *in vitro* studies were drawn. At the time of blood collection the patients were not assuming any medication. Sodium citrate-anticoagulated plasmas and sera from patients and from 74 normal subjects were stored into plastic cones at  $-20^{\circ}\text{C}$  until *in vitro* assays were carried out. The study protocol conformed to the ethical guidelines of the Declaration of Helsinki, and all subjects gave their informed consent before participation.

### Prothrombin fragment<sub>1+2</sub> measurement

Prothrombin fragment  $\text{F}_{1+2}$  was measured by a sandwich ELISA (Enzygnost  $\text{F}_{1+2}$ ; Behring Diagnostics GmbH, Frankfurt, Germany) (13, 14) according to manufacturer's instructions. Intra-assay and inter-assay CVs were 5% and 8% respectively.

### D-dimer measurement

D-dimer levels were measured by ELISA (Zymutest D-dimer; Hyphen BioMed, Neuville-sur-Oise, France) according to manufacturer's instructions. The intra- and inter-assay coefficients of variation were 10% and 15% respectively.

### Basophil histamine release assay

A leucocyte suspension from a normal blood donor was prepared by dextran sedimentation of peripheral venous blood, anticoagulated with 0.01 M EDTA, and mixed with 6% dextran in saline solution (Plander 70, Fresenius Kabi Italia, Isola della Scala, Verona, Italy) and 30 mM dextrose (Sigma Chemicals, St Louis, MO, USA). The cells were allowed to settle for 60–90 min at room temperature. Then leucocyte-rich plasma was aspirated and centrifuged at 300 *g* for 15 min at  $4^{\circ}\text{C}$ , and the cell button was washed twice in Tyrode's buffer, pH 7.4, containing (mM) 140 NaCl, 5.5 dextrose, 2.7 KCl, 0.36  $\text{NaH}_2\text{PO}_4$ , and 12  $\text{NaHCO}_3$ . Leucocytes (with about  $7 \times 10^4$  basophils) were resuspended in 100  $\mu\text{l}$  volume of Tyrode's buffer with 1.8 mM  $\text{CaCl}_2$  and 0.5 mM  $\text{MgCl}_2$ , and incubated with 100  $\mu\text{l}$  of the serum under examination, making a final volume of 200  $\mu\text{l}$ . After incubation for 40 min at  $37^{\circ}\text{C}$ , the reaction was stopped by addition of 800  $\mu\text{l}$  of ice-cold buffer solution and centrifugation at 1000 *g* for 10 min at  $4^{\circ}\text{C}$ . After centri-

fugation, the supernatants were aspirated, mixed with an equal volume of 6%  $\text{HClO}_4$  and centrifuged at 1000 *g* for 10 min at  $4^{\circ}\text{C}$ . Histamine concentration in the supernatants was measured by an automated fluorimetric method, according to Ruff et al. (17). Spontaneous histamine release was evaluated by measuring histamine concentration in the supernatant of unstimulated cells, incubated for 40 min at  $37^{\circ}\text{C}$ . Total histamine content was obtained by adding 100  $\mu\text{l}$  of 6%  $\text{HClO}_4$  to 100  $\mu\text{l}$  of cell suspension. Net histamine release was calculated as percentage of total histamine content, after subtraction of spontaneous release. A 5% release cut-off value was used. Sera were tested with leucocyte suspension from one normal donor whose basophils were previously shown to release 30% of total histamine content on challenge with an optimal dose of goat polyclonal anti-human IgE (10  $\mu\text{g}/\text{ml}$ ; Sigma Chemicals).

### Statistical analysis

Data are reported as median and interquartile range (25th and 75th percentiles). Differences between groups were evaluated by Mann–Whitney nonparametric test. Correlation was evaluated by Spearman's rank test. Significance level was set at  $P < 0.05$ .

## Results

Plasma D-dimer levels were significantly higher in patients with severe CU [median and interquartile range 11.20 nmol/l (5.68–86.13 nmol/l)] than in patients with mild disease [2.66 nmol/l (1.21–5.00 nmol/l)] and in normal subjects [1.41 nmol/l (0.96–1.62 nmol/l)] ( $P = 0.001$  and  $P = 0.0001$  by comparison with positive and negative controls respectively) (Fig. 1). Similarly, in patients with severe disease,  $\text{F}_{1+2}$  plasma levels [592 pmol/l (530–1744 pmol/l)] largely exceeded those found both in patients with slight urticaria [228 pmol/l (180–420 pmol/l);  $P = 0.003$ ] and in normal subjects [159 pmol/l (123–196 pmol/l);  $P = 0.0001$ ] (Fig. 2). The correlation between the severity score of single patients and individual plasma levels of D-dimer and  $\text{F}_{1+2}$  is shown in Fig. 3.

In one patient we had the opportunity to measure plasma levels of D-dimer and  $\text{F}_{1+2}$  during an episode of severe exacerbation of the disease and at remission after 1 week of therapy with steroids, antihistamines and low molecular weight heparin. At remission we observed a dramatic reduction of plasma levels of D-dimer (from 110.18 nmol/l to 0.64 nmol/l) and  $\text{F}_{1+2}$  (from 603 pmol/l to 160 pmol/l). Another patient who showed very high plasma levels of D-dimer (575.45 nmol/l) and  $\text{F}_{1+2}$  (1998.61 pmol/l) was checked again after 1 year during disease remission obtained with steroids and antihistamines, and also in this patient, we found a dramatic drop in plasma levels of both D-dimer (2.06 nmol/l) and  $\text{F}_{1+2}$  (116.78 pmol/l) (Fig. 4).

Sera from two of eight (25%) patients with severe CU and from four of 13 (31%) patients with mild CU induced histamine release  $>5\%$  vs 0/20 (0%) sera from normal subjects ( $P = 0.0002$  and  $P < 0.0001$  respectively) (Fig. 5). The proportion of sera inducing histamine

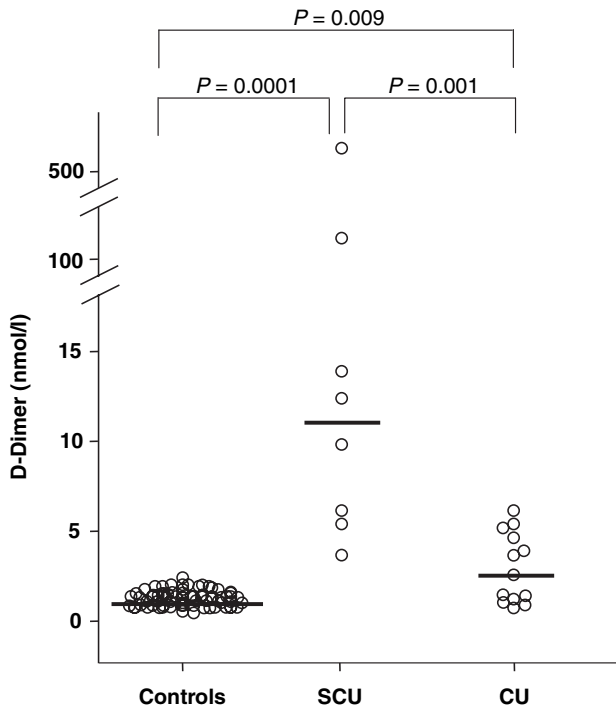


Figure 1. Plasma levels of D-dimer in normal subjects, patients with severe exacerbations of chronic urticaria (SCU) and mild chronic urticaria (CU). Results are expressed as nmol/l, and horizontal lines represent the median values. D-dimer plasma levels were increased in patients with chronic urticaria, particularly in those with severe disease exacerbations.

release was similar in patients with severe or mild CU ( $P = \text{NS}$ ). In the 21 patients with CU, D-dimer and  $F_{1+2}$  levels were significantly correlated ( $r = 0.64$ ,  $P = 0.002$ ), whereas coagulation parameters were not correlated with serum histamine-releasing activity [neither D-dimer ( $r = 0.054$ ;  $P = 0.815$ ) nor  $F_{1+2}$  ( $r = 0.087$ ;  $P = 0.707$ )].

## Discussion

In previous studies we observed an activation of coagulation cascade via the tissue factor pathway in most outpatients with mild to moderate CU (5, 6). We interpreted the light increase of plasma D-dimer levels found as the result of a minimal activation of fibrinolysis (6). In this study, we found significantly elevated fragment  $F_{1+2}$  and D-dimer plasma levels, which are well-known markers of coagulation and fibrinolysis activation, in a selected group of patients with severe exacerbation of CU, showing that in this subset the coagulation cascade may be activated, up to the production of fibrin as a result of the generation of thrombin. As experimental data have demonstrated the vasopermeability effect of thrombin (7, 8), its generation should be considered relevant to the pathophysiology of the disease. This is

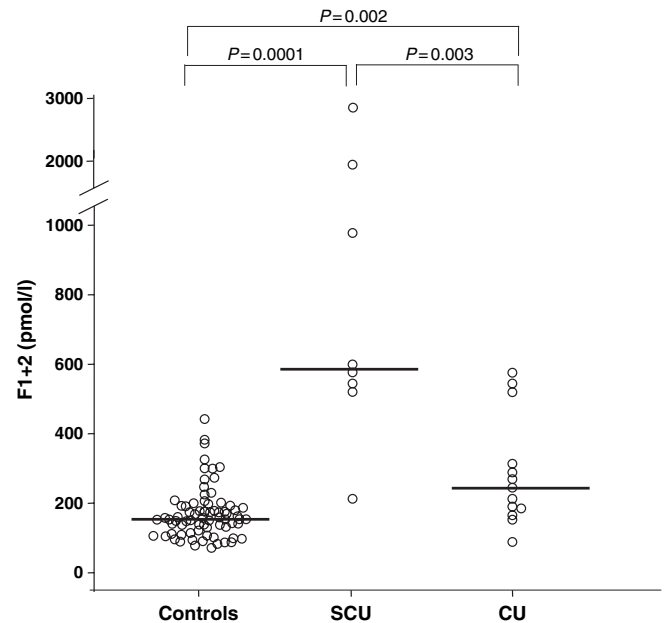


Figure 2. Plasma levels of prothrombin fragment  $F_{1+2}$  in normal subjects, patients with severe exacerbations of chronic urticaria (SCU) and mild chronic urticaria (CU). Results are expressed as pmol/l and horizontal lines represent the median values.  $F_{1+2}$  plasma levels were increased in patients with chronic urticaria, particularly in those with severe disease exacerbations.

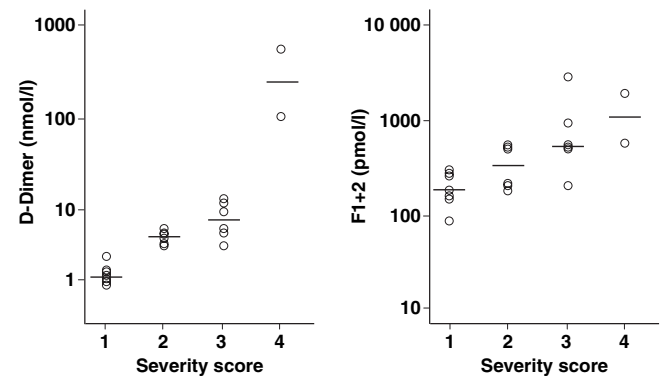


Figure 3. Plasma levels of D-dimer and prothrombin fragment  $F_{1+2}$  in patients with chronic urticaria (CU) divided on the basis of their severity score. Horizontal lines represent the median values.

further supported by the dramatic drop of D-dimer and  $F_{1+2}$  plasma levels which were extremely high during an acute phase of severe CU and perfectly normal after remission (Fig. 4).

Although direct evidence is still missing, thrombin could be involved in CU not only because it increases vascular permeability but also because it can induce mast cell degranulation, a phenomenon that is crucial to the pathophysiology of CU (7, 8, 10). Whether this

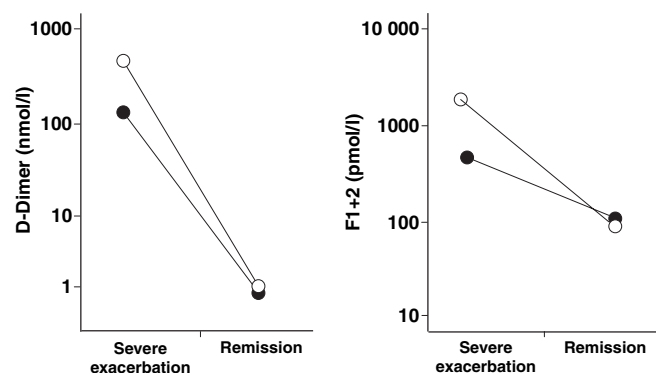


**Figure 4.** D-Dimer and  $F_{1+2}$  levels in plasma samples taken from two patients during severe exacerbation of chronic urticaria and at remission. In one patient, the remission blood sampling was performed after 1 week of therapy with steroids, antihistamines and low molecular weight heparin (full circles). In the other patient, remission blood sampling was performed 1 year later, when the patient was not assuming any therapy (empty circles).

phenomenon is the cause of the disease or acts simply as an amplification system is still to be defined. It is conceivable that autoantibodies and activation of the coagulation cascade act synergistically in some patients. It is notable that, although serum histamine-releasing activity was significantly higher in CU patients than in normal subjects, only two of eight (25%) patients with severe CU and four of 13 (31%) patients with mild CU had evidence of circulating histamine releasing factors as shown by the basophil assay. While these proportions are in line with those found in our previous studies (16), they further suggest that mechanisms of histamine release other than autoantibodies may be operating in CU. The fact that both  $F_{1+2}$  and D-dimer plasma levels were increased in patients with severe CU, and to a lesser extent in patients with mild CU, but were not correlated with histamine-releasing activity of patients' sera suggests that activation of the coagulation cascade and serum

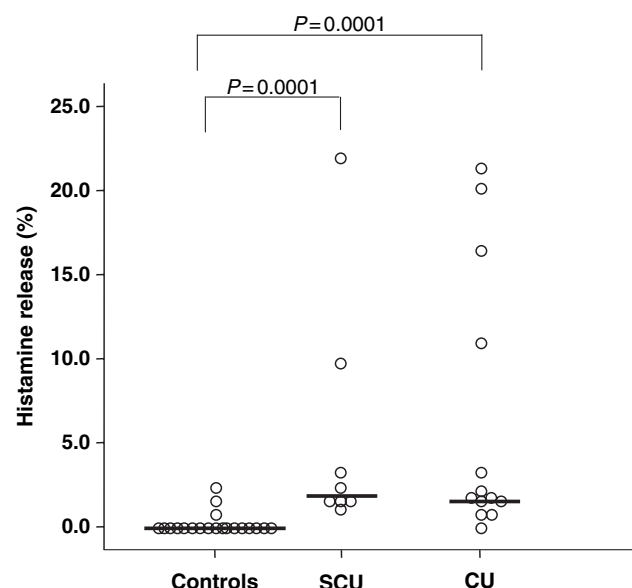


**Figure 5.** Histamine-releasing activity in sera from normal subjects, patients with severe exacerbations of chronic urticaria (SCU) and mild chronic urticaria (CU). Results are expressed as net per cent histamine release from basophils of a normal donor, and horizontal lines represent the median values. Histamine-releasing activity was significantly higher in patients with chronic urticaria than in normal subjects.

histamine-releasing autoantibodies might both separately contribute to the pathomechanism of CU. The relative weight of coagulation factors and that of histamine-releasing autoantibodies seem to vary from one patient to another suggesting that multiple mechanisms are involved in CU.

Although our study considered a relatively small number of patients, our data also provide the rationale for the evaluation of anticoagulant therapy in large populations of CU patients as to date the efficacy of heparin and warfarin in the treatment of CU has been assessed only in scattered reports (17, 18).

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