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Blood coagulation and fibrinolysis in patients with Cushing's syndrome: Increased plasminogen activator inhibitor-1, decreased tissue factor pathway inhibitor, and unchanged thrombin-activatable fibrinolysis inhibitor levels

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ABSTRACT. ***Background and objectives:*** **Cushing's syndrome (CS) is associated with an increased cardiovascular mortality and morbidity. Chronic endogenous and exogenous hypercortisolism frequently induce a hypercoagulable and thrombotic condition. Little is known about hemostatic features of patients with CS. To our knowledge, plasma tissue factor pathway inhibitor (TFPI) and thrombin-activatable fibrinolysis inhibitor (TAFI) levels in these patients have not been investigated. Therefore, the main purpose of this study was to evaluate the markers of endogenous coagulation/fibrinolysis, including TFPI and TAFI, and to investigate the relationships between cortisol and these hemostatic parameters and serum lipid profile in patients with CS.** ***Design and methods:*** **Twenty-four patients with CS and 24 age-matched healthy controls were included in the study. Prothrombin time (PT), activated partial thromboplastin time (aPTT), fibrinogen, factors V, VII, VIII, IX, and X activities, von Willebrand factor (vWF), antithrombin III (AT III), protein C, protein S, tissue plasminogen activator (t-PA), tissue plasminogen activator inhibitor-1 (PAI-1), TFPI and TAFI, as well as common lipid variables, were measured. The relationships between serum cortisol and these hemostatic parameters were examined.** ***Results:*** **Compared with the control subjects, platelet count, PT, fibrinogen, AT-III and PAI-1 were significantly increased in patients with CS ($p<0.05$, $p<0.0001$, $p<0.01$, $p<0.05$, and $p<0.0001$, respectively), whereas aPTT and TFPI levels were significantly decreased ($p<0.0001$ and $p<0.01$, respectively). Plasma TAFI Ag levels did not significantly change in patients with CS compared with the controls. In patients with CS, we showed a negative correlation between serum cortisol: 08:00 h and aPTT (r:–0.469, $p<0.05$). Serum cortisol: 24:00 h was positively correlated with PAI-1 Ag levels (r: 0.479, $p<0.05$).** ***Interpretation and conclusions:*** **In conclusion, we found some important differences in the hemostatic parameters between the patients with CS and healthy controls. Increased platelet count, fibrinogen, PAI-1, and decreased TFPI levels in these patients represent a potential hypercoagulable and hypofibrinolytic state, which might augment the risk for atherosclerotic and atherothrombotic complications. This condition may contribute to the excess of mortality due to cardiovascular disease seen in patients with CS.**

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INTRODUCTION

Patients with Cushing's syndrome (CS) present an increase in cardiovascular disease (CVD), such as coronary artery disease, congestive heart failure, myocardial infarction, and endothelial dysfunction, which contribute to a 4-times higher mortality rate compared to an age- and sex-matched population (1-4). This increased cardiovascular risk is closely related with systemic arterial hypertension, impaired glucose tolerance or diabetes, central obesity, hyperlipidemia, and hypercoagulability (5, 6). Hypercoagulability and thromboembolic complications have occurred in patients with endogenous and exogenous hypercortisolism (7-10).

High levels of circulating cortisol increase plasma clotting factors and impair fibrinolytic capacity (decreased fibrinolytic activity) (7, 9-13). Increased coagulation factors, including factors II, V, VII, VIII, IX, X, XI, XII, XIII, von-Willebrand factor (vWF), and plasminogen activator inhibitor-1 (PAI-1) are reported in patients with CS (9, 11, 12, 14, 15). It has been suggested that, because of these alterations in the coagulation and fibrinolysis, patients with CS have a tendency toward thrombogenesis (10, 16, 17).

The thrombin-activatable fibrinolysis inhibitor (TAFI), an enzyme that may act as a link between coagulation and fibrinolysis, inhibits fibrinolysis by removing carboxyterminal residues from partially degraded fibrin, thus decreasing plasminogen binding on the surface of fibrin (18, 19). Increased TAFI levels have been associated with several thrombotic conditions such as venous thromboembolism (20, 21) and ischemic stroke (22, 23). Tissue factor pathway inhibitor (TFPI) is secreted by the endothelium and stored in platelets (24). TFPI binds directly and inhibits the earliest steps in extrinsic pathway activation by binding factor Xa (which is involved in the activation of prothrombin to thrombin) and tissue factor (TF)/factor VIIa complexes in an inactive quaternary complex (25). Low plasma TFPI levels have been reported in patients with ischemic stroke (26), thrombotic thrombocytopenic purpura (27), and in women taking combined oral contraceptives (28).

Although several studies indicate that coagulation and the fibrinolytic system are disturbed in patients with CS,

Key-words: Cushing's syndrome, Hemostasis, thrombin-activatable fibrinolysis inhibitor, tissue factor pathway inhibitor.

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the levels of plasma TAFI antigen and TFPI have not been investigated in patients with CS. Therefore, in a case-control study, we determined the profile of a wide range of coagulation and fibrinolytic parameters including TAFI and TFPI and lipid profile in patients with active CS. We also investigated the relationships between serum and urine cortisol levels and hemostatic parameters in these patients. A hypercoagulable state might increase the risk for thromboembolic complications and predispose to an increased prevalence of vascular disease.

MATERIALS AND METHODS

Patients and study design

The study was performed at Karadeniz Technical University Medical Faculty, Department of Internal Medicine. We prospectively evaluated 24 untreated patients with CS (19 women and 5 men; mean age, 41.2±16.8 yr). The diagnosis of CS was based on the clinical signs and symptoms (centripedal obesity, hypertension, hirsutism, moon face, purple stria, Buffalo hump, muscle weakness, diabetes or glucose intolerance, osteoporosis) and hormonal data. The diagnosis of hypercortisolism was based on increased daily urinary free cortisol (UFC) excretion, rise in serum cortisol concentrations at 08:00 h, with absence of physiological circadian rhythm or failure of cortisol to suppress after a standard 2-day low-dose (2 mg/day) dexamethasone suppression test (LDDST) (cut-off value of 1.8 µg/dl). The midnight (24:00 h) serum cortisol levels were measured for all patients. The cut-off value was chosen as 7.5 µg/dl. All patients had levels above cut-off value. ACTH levels, standard two-day, high dose (8 mg/day) DST (HDDST), CRH stimulation test, and appropriate imaging [pituitary magnetic resonance imaging (MRI), adrenal MRI/computed tomography (CT) scan] were used for the differential diagnosis of CS. Cushing's disease (CD) was diagnosed in the presence of normal/elevated ACTH levels, suppression >50% of serum cortisol levels after HDDST and/or ACTH response >35% to the CRH test. Adrenal CS was diagnosed in the presence of suppressed ACTH levels, lack of suppression of serum cortisol levels after HDDST and/or lack of response of ACTH to the CRH stimulation test. All patients with CD underwent surgical selective resection of the ACTH-secreting pituitary adenomas by the transsphenoidal approach; immunohistochemistry confirmed the diagnosis in all patients. All adrenal adenomas were surgically removed. The diagnosis was additionally confirmed by histological examination. In one of the patients with adrenal CS, radiological characteristics of the mass (size, infiltration, enhancement, dishomogenity) with hepatic metastases suggested malignancy. Etiology of CS was pituitary-dependent CS (CD) (6 macro-and 7 microadenomas) in 13 cases, cortisol-secreting adrenal adenomas in 10 cases, and cortisol-secreting adrenal carcinoma in 1 case. No thrombotic complication was observed during follow-up period in patients with CS.

Patients did not receive medical treatment (e.g. estrogen therapy) and did not have any known disease (e.g. thyroid dysfunction, coronary heart disease, collagen disease, liver cirrhosis, atrial fibrillation, or renal disease) that might affect blood coagulation or fibrinolysis, and lipid profile at the time of the study. At diagnosis, risk factors for coagulation and thromboembolism, including known cancer, pregnancy, known thrombophilia, recent childbirth, and use of oral contraceptives were excluded from patient group. Also, no medication known to influence the serum lipid concentration was administered. Twenty-four healthy age-and-sex-matched subjects (18 women and 6 men, mean age 43.0±10.8 yr) were used as controls. Their biochemical values were within normal ranges. None of the controls were taking any drugs affecting the levels of serum cortisol and hemostatic parameters and lipid levels. All participants including patients and control subjects were non-smokers, and there was no minor illness such as viral infections or family history of clotting disorders in patients and controls.

Laboratory analysis

Blood was collected in the morning between 08:00-09:00 h after an overnight fast to avoid the differences of diurnal variation, especially for hormonal and hemostatic parameters. Serum cortisol levels were measured by automated chemiluminescence method (Dpci Immulate). The determination of 24-h excretion of UFC was assayed by chemiluminescence method (Dpci Immulate). Serum ACTH levels were measured by automated immunoradiometric assay (Immulite 2000 DPC, Diagnostic Product Corporation, 5210 Pacific Concourse, Los Angeles, USA). Normal ranges of biochemical parameters were 6.2-19.4 µg/dl for serum cortisol, 20-90 µg/day for 24-h UFC levels, 9-52 pg/ml for serum ACTH.

Serum total cholesterol (TC) was measured using a cholesterol oxidase enzymatic method; triglycerides (TG), by a glycerol oxidase enzymatic method; HDL cholesterol (HDL-C), by a cholesterol oxidase enzymatic method in supernatant after precipitation with phosphotungstic acid-MgCl2. These routine analyses were carried out by autoanalyzer (Technicon AXON). LDL cholesterol (LDL-C) was calculated by Friedewald's formula. Fasting blood glucose (FBG) was measured using an enzymatic (glucose oxidase) colorimetric method. All determinations were performed with an autoanalyzer (Roche, Modular, Switzerland). Reagents were supplied by the same manufacturer.

For coagulation and fibrinolysis, a venous blood sample (9 vol) was collected into Vacutainer tubes (Becton Dickinson, Mountain View, CA) containing 0.129 mol/l trisodium citrate (1 vol). Apolipoproteins AI and B were determined by an immunonephelometric assay method (Dode Behring, Marburg, GmBH Emil-Von-Behring-Str 76 35041 Marburg, Germany). Normal ranges are 125-215 mg/dl for apo AI, and 55-125 mg/dl for apo B.

Platelet-poor plasma was obtained by centrifugation 3500×*g* at 10 C for 20 min. Platelet count, mean platelet volume (MPV), fibrinogen, antithrombin III (AT III), factors V, VII, VIII, IX, and X measurements were performed immediately. Aliquots of plasma were transferred into plastic tubes without delay and frozen at –80 C until assays for determination of von-Willebrand factor (vWF), protein C, protein S, tissue plasminogen activator (t-PA), and plasminogen activator inhibitor-1 (PAI-1). Platelet count and MPV was measured with automatic cell counter (Coulter Micro Diff II). Fibrinogen was determined using a nephelometric assay by commercial kits for fibrinogen (Cat No.OSCA 09, Dade Behring Marburg GmbH, Germany). D-Dimer measurement was performed using a NycoCard assay by commercial kits for D-Dimer. Factors V, VII, VIII, IX, and X activities were measured with coagulometer (Diagnostica Stago) using commercial kits of Diagnostica Stago. AT III assay was performed with spectrophotometric method (Behring turbitimer, Turbiquant, AT III, Dade Behring). Normal ranges are 200-400 mg/dl for fibrinogen, <0.3 ng/ml for D-Dimer, 50-150% for factors V, FVII, FVIII, FIX and FX. Protein C and Protein S activity assays were per-

formed with enzyme-linked immunosorbent assay (ELISA) method using commercial kits of Biopool International. vWF activity was determined by ELISA method using commercial kits of Imtec Immundiagnostica Gmbh. T-PA, PAI-1, TAFI antigens (Ag), and TFPI assays were performed with ELISA using commercial kits of American Diagnostica. According to our hematology laboratory, normal ranges are 22-39 mg/dL for AT III, 70-150% for vWF, 72-160% for protein C activity, 60-150 % for protein S activity, 1-20 ng/ml for t-PA Ag, 20-44 ng/ml for PAI-1 Ag, 40-250% for TAFI, and 75-120 ng/ml for TFPI Ag.

Statistical analysis

Statistical analyses were performed by Student's t test for normal distribution data and Mann Whitney U test for not normal distribution data. In patient group, correlations among biochemical parameters and cortisol and coagulation and lipid profile were carried out using Pearson (normal distribution data) and Spearman (not normal distribution data) correlation analysis. Results are cited as mean±SD, $p<0.05$ was accepted significantly.

RESULTS

Table 1 summarizes the clinical characteristics and laboratory parameters in patients with CS and control subjects. As expected, BMI, systolic and diastolic blood presuure (SBP, DBP, respectively), the levels of fasting blood glucose (FBG), TC, LDL-C, TG, apo B, serum cortisol and UFC were significantly higher in the patient group than those in controls.

Compared with the control subjects, platelet count, prothrombin time (PT), fibrinogen, AT-III, and PAI-1 were significantly increased in patients with CS ($p<0.05$, $p<0.0001$, $p<0.01$, $p<0.05$, and $p<0.0001$, respectively), whereas activated partial thromboplastin time (aPTT) and TFPI levels were significantly decreased ($p<0.0001$ and $p<0.01$, respectively). Plasma TAFI Ag levels did not significantly change in patients with CS compared with the controls. The other coagulation/fibrinolysis parameters in patients with CS were not different from the controls.

Table 2 shows the number of out of normal ranges or cut-off values of controls and patients with Cushing's syndrome. Compared with the control subjects, fibrinogen, AT-III, and PAI-1 were significantly increased in patients with CS ($p<0.05$, $p<0.05$, and $p<0.001$, respectively).

In patients with CS in multiple regression model, hypertension was significantly associated with the hemostatic parameters. The risk of impairment of hemostatic parameters was significantly greater in patients with hypertension (p: 0.016, odds ratio: 12.653). We did not find an association between BMI and hemostatic variables.

In patients with CS, serum cortisol: 24:00 h was positively correlated with PAI-1 Ag levels (r: 0.479, $p<0.05$) (Fig. 1). We did not find any significant correlation between serum and urine cortisol and the other hemostatic parameters that we measured.

Table 1 - *Clinical and biological parameters of controls and patients with Cushing's syndrome.*

	Controls	CS	*p*
No. of subjects	24	24	–
Age (yr)	43.0±10.8	41.2±16.8	ns
BMI (kg/m^2)	26.3±3.3	30.9±5.9	0.002
SBP (mmHg)	127.8±10.3	147±32.8	0.013
DBP (mmHg)	81±6.7	95±18.3	0.002
FBG (mg/dl) (no.=70-110)	87.5 ± 8.3	125.8 ±47.1	0.001
Potassium (mg/dl) (no.=3.5-5.0)	4.2±0.8	4.3±0.5	ns
Cortisol: 08.00h (µg/dl)	15.7±1.6	26.6±12.1	0.0001
Cortisol: 24.00h (µg/dl)	2.3±2.0	22.7±9.6	0.0001
UFC (µg/d) (no.=20-90)	50.9±14.9	385.5±383	0.001
TC (mg/dl)	157.3±30.1	230.1±42.5	0.0001
Triglycerides (mg/dl)	95.7±39.8	165±114.7	0.012
LDL-C (mg/dl)	95.4±32.1	149.8±39.6	0.0001
HDL-C (mg/dl)	46.8±11	53±14	ns
Apo AI (mg/dl)	146.2±19.5	142.7±32.2	ns
Apo B (mg/dl)	88.4±22.1	117.6±29.1	0.004
Platelet count (per µl)	249.6±47.2	293.4±75.6	0.021
MPV (fl)	7.9±0.6	11.2±17.1	ns
PT (sec)	11.8±1.2	13.1±0.6	0.0001
aPTT (sec)	30.2±3.0	25.8±2.0	0.0001
Fibrinogen (mg/dl)	249.7±13.8	352.5±117.0	0.004
D-Dimer (ng/ml)	0.251±0.15	0.31±0.21	ns
F V (%)	85±28.1	92.7±50	ns
F VII (%)	96.3±22.6	111±51.3	ns
F VIII (%)	120±20.1	145.9±50.1	ns
F IX (%)	119.7±24.1	141.9±53.5	ns
F X (%)	97.1±18.7	95.3±37.4	ns
AT III Ag (mg/dl)	24.3±2.2	29.9±6.4	0.022
Protein C (%)	120.9±35.19	134.4±22.7	ns
Protein S (%)	119.8±25.9	107.7±19.6	ns
vWF activity (%)	126.9±34.8	132.8±22.2	ns
t-PA Ag (ng/ml)	13.9±2.5	14.5±5.8	ns
PAI-1 Ag (ng/ml)	29.5±8.0	61.6±33.8	0.0001
TFPI Ag (ng/ml)	95.3±30.0	70.5±13.4	0.008
TAFI Ag (%)	148.6±24.3	140.3±4.6	ns

ns: $p>0.05$; CS: Cushing's syndrome; BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; FBG: fasting blood glucose; UFC: urinary free cortisol; TC: total cholesterol; LDL-C: LDL cholesterol; HDL-C: HDL cholesterol; Apo: apolipoprotein; MPV: mean platelet volume; PT: prothrombin time; aPTT: activated partial thromboplastin time; F: factor; AT III: antithrombin III; Ag: antigen; vWF: von Willebrand Factor; t-PA: tissue plasminogen activator; PAI-1: plasminogen activator inhibitor-1; TFPI: tissue factor pathway inhibitor; TAFI: thrombin activatable fibrinolysis inhibitor.

DISCUSSION

It is known that either chronic glucocorticoid administration or endogenous hypercortisolism frequently induce a hypercoagulable condition (6). Increased cortisol levels stimulate the synthesis of several clotting factors. Thromboembolic events (e.g. pulmonary embolism and deep venous thrombosis) are 4 times more frequent in CS than in the general population and contribute to this increase in mortality (16). Also, about 10% of patients with CS present serious thromboembolic complications especially after surgery or after inferior petrosal sinus sampling (7, 29). Therefore, patients with active CS should be treated as having a prothrombotic disorder, and antithrombotic prophylaxis should be considered (30).

There are few reports regarding the effects of cortisol on platelet count and function in the literature (31). Cortisol

Table 2 - *The number of out of normal ranges or cut-off values of controls and patients with Cushing's syndrome (χ^2 test).*

	Controls	CS	*p*
No. of subjects	24	24	–
Fibrinogen*	0	5	0.025
D-Dimer	9	8	ns
F V	2	3	ns
F VII	1	2	ns
F VIII	1	4	ns
F IX	3	5	ns
F X	0	1	ns
AT III Ag*	0	6	0.022
Protein C	2	2	ns
Protein S	3	0	ns
vWF activity	6	9	ns
t-PA Ag	0	0	ns
PAI-1 Ag*	0	11	0.0006
TFPI Ag **	7	14	ns
TAFI Ag	0	0	ns

*No. of above normal ranges; **no. of under normal ranges. F: factor; AT III: antithrombin III; Ag: antigen; vWF: von Willebrand Factor; t-PA: tissue plasminogen activator; PAI-1: plasminogen activator inhibitor-1; TFPI: tissue factor pathway inhibitor; TAFI: thrombin activatable fibrinolysis inhibitor.

increases platelet production directly. Increased and/or hyperactive platelets are one of the main findings in hypercoagulable states and arterial thrombosis (32). In the present study, platelet count was significantly increased in patients with CS compared to controls. This increase may lead to a tendency to thrombosis and coagulation in patients with CS.

In our study, the mean PT values were lengthened and the mean aPTT values were shortened in patients with CS. These results are consistent with the findings of literature (7, 9, 11, 33). Interestingly, aPTT values showed an inverse correlation with serum cortisol 08:00 h levels, suggesting the use of aPTT as a convenient routine hemostatic variable.

Several prospective studies have consistently shown that a direct, independent, and statistically significant association exists between fibrinogen levels and the subsequent incidence of heart disease (34). Moreover, among individuals who had increased serum LDL-C and increased fibrinogen levels, there was a 6.1-fold increase in coronary risk (35). In the literature, increased fibrinogen levels have been reported in patients and canine with CS (6, 7, 16). In the present study, we found a significant increase in fibrinogen levels in patients with CS. This increase may be a tendency to atherothrombosis and coagulation in these patients.

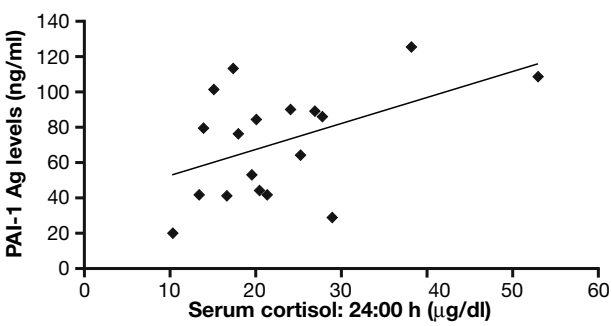

Fig. 1 - *Correlation between serum cortisol: 24:00 h and plasminogen activator inhibitor-1 antigens (PAI-1 Ag) levels in patients with Cushing's syndrome (r: 0.479, p<0.05).*

AT III is the most important anticoagulant molecule in mammalian systems (36). It controls the activity of thrombin and inhibits activated FVII (in the presence of heparin), activated FX, activated FIX, and activated FXII (36, 37). The critical role that AT III plays in controlling coagulation is reflected in the strong correlation between AT III deficiency states and thrombosis (38). Despite some limited studies in the literature, the relationship between CS and AT-III is still controversial. Jacoby et al. reported that AT-III is significantly lower in canine with CS (16), but others have reported it to be increased (39) or unchanged (11). In the present study, interestingly, we found a significant increase in AT III levels in patients with CS. The increase in AT-III may be a protective mechanism and/or compensatory response *vs* hypercoagulable state seen in CS.

It has been shown that PAI-1, the main inhibitor of the fibrinolytic system, and fibrinogen are both associated with coronary heart disease (40). At present, there are no compelling epidemiological studies that define PAI-1 as a clear risk factor for arterial thrombotic complications (35). In the present study, we found a significant increase in PAI-1 levels in patients with CS. This result is consistent with the some studies in the literature (7, 33). In other studies, there was no significant difference in PAI-1 levels between patients with CS and control subjects (6, 8). Moreover, in the present study, PAI-1 was positively correlated with serum cortisol: 24:00 h levels. It is reported that PAI-1 is often elevated and may cause thromboembolic events by lowering fibrinolytic activity in patients with CS (15). In our study, increased PAI-1 levels in patients with CS may be tendency to thrombosis which is crucial in cardiovascular events.

TFPI regulates FX activation. Low TFPI is a risk factor for a first venous thrombosis, recurrent venous thromboembolism, and stroke (26, 41, 42). To our knowledge, plasma TFPI Ag levels have not been investigated in patients with CS. In the present study, we found a significant decrease in TFPI levels in patients with CS. Decreased TFPI levels in patients with CS may be tendency to thrombosis and coagulation in these patients. However, we did not find an association between cortisol levels and TFPI levels in the correlation analysis. Also, there was no significant difference in the number of out of normal ranges or cut-off values between controls and patients with CS for TFPI.

TAFI, also known as procarboxypeptidase B, is a plasma zymogen that potently inhibits fibrinolysis (43, 44). It protects the fibrin clots from breakdown by removing C-terminal lysine residues from partially degraded fibrin which are necessary for t-PA-mediated plasmin regeneration (44). Increased activation of TAFI might exacerbate a prothrombotic disposition. Increased plasma TAFI Ag levels were associated with a mild risk for venous thrombosis

(45). One study reported that patients with a recent myocardial infarction presented lower TAFI Ag values and that increased TAFI levels were actually protective against myocardial infarction (46). On the other hand, high TAFI levels were reported to be associated with an increased risk of first ischemic stroke (22). To our knowledge, this is the first study to evaluate TAFI Ag levels in patients with CS. In the present study, TAFI Ag levels did not change in our patients with CS.

Limitations

Hypertension and BMI may influence the coagulation state. Hypertension was significantly associated with the hemostatic parameters. However, we did not find an association between BMI and hemostatic variables.

In conclusion, we found some important differences in the hemostatic parameters between patients with CS and healthy controls. Increased platelet count, fibrinogen, PAI-1, and decreased TFPI levels in these patients represent a potential hypercoagulable and hypofibrinolytic state, which might augment the risk for atherosclerotic and atherothrombotic complications. This condition may contribute to the excess mortality due to CVD seen in patients with CS. However, our study comprised a small number of patients with CS. A larger number of patients should be included in a prospective study to explain the association between CS and TAFI.

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