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Asthma is associated with enhanced thrombin formation and impaired fibrinolysis

Running Head: Hypercoagulable state in asthma

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Abstract

Background: There is evidence that altered blood coagulation and fibrinolysis are involved in the pathogenesis of asthma. Increased thromboembolic risk has been reported in asthmatics.

Objective: To investigate whether enhanced thrombin generation and impaired fibrinolysis occur in asthmatics.

Methods: Plasma thrombin generation profile together with a computational assessment of thrombin dynamics and fibrinolytic capacity expressed as clot lysis time (CLT) were determined in 164 consecutive patients with stable asthma and 72 controls matched for age, sex, weight and smoking.

Results: Asthma patients had 20.2% increased endogenous thrombin potential (ETP), 41.4% higher peak thrombin concentration, 61% higher maximal prothrombin conversion rate, 15.5% faster rate of thrombin formation (all, $p < 0.0001$) and 10% lower thrombin decay capacity ($p = 0.0004$) compared with controls. Asthmatics had also 14.4% longer CLT ($p = 0.001$) associated with 21.3% higher plasminogen activator inhibitor-1 (PAI-1) ($p < 0.0001$), and 13% higher plasma α_2 -macroglobulin ($p = 0.0002$). Using ETP and CLT above 75th percentile of the control values as the cut-off levels, we found increased risks of enhanced thrombin generation and hypofibrinolysis in asthmatics, also after correction for potential confounders. ETP and CLT were associated inversely with forced expiratory volume in 1 second/vital capacity (FEV₁/VC) index, after adjustment for age and body mass index. Non-allergic asthma ($n = 70$, 42.6 %) was characterized by 17.5% longer CLT ($p = 0.02$), which

positively associated with PAI-1. Thrombin generation profile was not affected by allergy.

Conclusion and Clinical Relevance: Asthma is associated with enhanced thrombin generation and impaired fibrinolysis, which might contribute to thromboembolic events in this disease.

Key words: asthma, thrombin generation, clot lysis time, thrombosis

Introduction

Asthma is a chronic, inflammatory disease of the airways, characterized by reversible airflow obstruction and bronchial hyper-responsiveness [1]. Inflammatory diseases are associated with a hypercoagulable state [2, 3, 4]. There is growing evidence for heightened activation of blood coagulation in the airways of asthmatic subjects and pro-coagulant plasma protein leakage (including factors [F] VII, X and thrombin) into the bronchoalveolar space [5]. Thrombin locally transforms fibrinogen to fibrin, which is necessary for normal airway epithelial repair [6]. In severe asthma, however, fibrin production was substantially increased, with a large deposition in the alveoli and distal airways in fatal cases [7]. Tissue factor (TF), the main initiator of blood coagulation *in vivo*, becomes exposed on the surface of some mononuclear, epithelial (including alveolar epithelium), and endothelial cells on stimulation by bacteria and/or proinflammatory cytokines [8]. Patients with severe asthma demonstrated increased soluble TF in induced sputum as compared to moderate asthma or healthy subjects [9]. Asero et al. showed higher plasma prothrombin fragments 1+2 and D-dimer in asthma subjects, suggesting marked activation of blood coagulation [10]. Animal studies yielded further evidence supporting the concept of involvement of coagulation proteins in the pathogenesis of asthma. Genetically modified FVII^{tTA/tTA} mice, with a very low expression of FVII, demonstrated a diminished influx of eosinophils into the airways, together with

decreased inflammatory cytokines production and reduced hyper-responsiveness after allergen challenge [11]. Moreover, exposure of wild mice to aerolized fibrinogen, followed by thrombin, enhanced airway inflammation and hyper-responsiveness [7]. Fibrinolysis mediated by plasmin is favoured in normal human lungs [12]. Over-expression of plasminogen activator inhibitor-1 (PAI-1), which is a key regulator of fibrinolysis, has been reported in the airways of asthmatics [13]. Platelets are also involved in asthma pathology. Increased circulating platelet-leukocytes aggregates and higher plasma concentrations of platelet factor 4 (PF4) have been observed in asthmatics [14, 15]. Clinical relevance of altered hemostatic balance in asthma remains unclear. Majoor et al. [16] reported a hazard ratio (HR) of 3.33 (95% Confidence Interval [CI]: 1.16-9.93) of pulmonary embolism (PE) in severe asthma. Increased risks of PE (HR 3.38 [95%CI: 1.70-6.74]) and stroke (HR 1.37 [95%CI: 1.27-1.48]) were also demonstrated in large cohorts of asthmatics from Taiwan [17, 18]. Higher rates of stroke and coronary heart disease (CHD), defined as definite or probable myocardial infarction (MI) or death, were reported in women with adult-onset asthma (HR 2.08 [95%CI: 1.3-3.32] and HR 1.78 [95%CI: 1.18–2.67], respectively) [19].

Given the available data concerning the potential links between asthma and thromboembolic events, we sought to evaluate thrombin generation and fibrinolysis measured in the plasma of asthmatic patients.

Materials and Methods

Patients and Controls

We studied 164 white adult patients with clinically stable asthma who were recruited at the outpatient clinic in Cracow, Poland, from October 2012 to April 2013. The diagnosis of asthma was established based on a history of recurrent respiratory symptoms (wheeze,

shortness of breath, chest tightness and cough) together with currently and/or historically documented post bronchodilator increase in forced expiratory volume in 1 second (FEV₁) of at least 12% and 200 ml from the baseline. Atopic status was confirmed by a positive skin prick testing for at least one inhaled allergen (Allergopharma, Reinbeck, Germany). Allergic asthma was diagnosed if the relevance of allergen exposure and its relation to symptoms was confirmed by the patient's history [1]. All asthma medications, with the exception of omalizumab, were permitted, including oral corticosteroids at a daily dose equivalent to ≤ 10 mg of prednisolone, unless the doses were unchanged during the preceding 3 months. Asthma patients could not be exacerbated during the preceding 6 months. Severity of asthma was categorized according to the Global Initiative for Asthma (GINA) guidelines [1]. Intermittent asthma was defined as treated only with inhaled short-acting β_2 -agonists on demand and not receiving inhaled or systemic corticosteroids. "Mild" asthma was defined as a mild persistent disease treated with a low daily dose of inhaled corticosteroids (ICS) (< 250 μ g of fluticasone propionate [FP] [dry powder inhaler] or equivalent). "Moderate" asthma was defined as a mild persistent disease treated with low (combined with long-acting β_2 -agonists) or medium doses of ICS (250-500 μ g of FP or equivalent). "Severe" asthma was defined as a severe persistent disease treated with a high daily dose of ICS (> 500 μ g of FP or equivalent) [1]. Spirometry was performed using a Jaeger, Master Screen spirometer in all enrolled subjects according to the American Thoracic Society standards. If a basal spirometry showed bronchial obstruction, the bronchial reversibility test with 400 μ g of albuterol was performed and parameters of the repeated spirometry were analyzed.

Control subjects (n=72) matched for sex, age, body mass index (BMI), and smoking habits were enrolled from the hospital personnel and their relatives. They had normal spirometry and no history of allergic diseases or bronchial obstructions in the past.

The exclusion criteria for both groups were: age above 70 years, any acute illness, congestive heart failure, atrial fibrillation, previous venous thromboembolism, MI or stroke, cancer, hyper- or hypothyroidism, current anticoagulant therapy, liver injury, and chronic kidney disease (stage 3 or more). Subjects were eligible if they had arterial hypertension, diabetes mellitus (DM), hypercholesterolemia or stable CHD. DM was defined as the use of insulin or oral hypoglycemic agents or fasting serum glucose >7.0 mmol/l. Arterial hypertension was defined based on a history of hypertension (blood pressure $>140/90$ mmHg) or preadmission antihypertensive treatment. Hypercholesterolemia was defined as previously diagnosed and treated with statins, or serum total cholesterol >5.2 mmol/l. Liver injury was diagnosed when serum alanine aminotransferase was elevated (at least 3 times above upper limit of the reference range). CHD was defined as documented coronary stenosis or stable angina.

Ex-smokers were included as non-smokers if they had stopped smoking at least 6 months previously.

The study was approved by the Ethics Committee of the Jagiellonian University. All subjects gave written informed consent to participate in the study.

Laboratory investigations

Fasting blood samples were drawn from the antecubital vein using minimal stasis between 8:00 and 11:00 A.M. Lipid profile, glucose, creatinine with estimated glomerular filtration rate, alanine aminotransferase, blood cell and platelet count were assayed by routine laboratory techniques. Fibrinogen was determined with the Clauss method. High-sensitivity C-reactive protein (hsCRP) and immunoglobulin E (IgE) were measured by latex nephelometry (Siemens, Marburg, Germany). Blood samples were drawn into tubes containing 0.109 mol/l sodium citrate (vol/vol, 9:1), centrifuged $2,000 \times g$ for 20 minutes, at room temperature. The supernatant was frozen in aliquots and stored at -70°C until analysis.

Plasminogen and antiplasmin activities were measured by chromogenic assays (STA Stachrom plasminogen and STA Stachrom α_2 -antiplasmin, Diagnostica Stago, Asnieres, France). Prothrombin and antithrombin activities were assessed by an ACL TOP 500 CTS analyzer with appropriate reagents (Instrumentation Laboratory, Bedford, MA, USA). Commercially available immunoenzymatic assays were used to measure PAI-1 antigen (American Diagnostica, Stamford, CT, USA) and platelet activation markers: PF4 and P-selectin (R&D Systems, Minneapolis, MN, USA). Measurement of plasma α_2 -macroglobulin [20], the Calibrated Automated Thrombogram (CAT) [21, 22], computational assessment of thrombin generation kinetics [20], and Clot Lysis Time (CLT) [23] were determined using the previously described methods. A detailed methodology is provided in the **online supplement**. A typical course of thrombin generation with its key parameters are shown in Fig. 1. The maximum concentration of thrombin formed during the recording time is described as the “thrombin peak” and the area under the curve represents the “endogenous thrombin potential” (ETP). “Lag-time” is the time from the start of analysis until thrombin starts to generate and “time to thrombin peak” is the time from the start of thrombin generation until the maximum thrombin value is achieved. In this assay elevated values of ETP and thrombin peak concentrations, together with shorter time to thrombin peak levels reflect an enhanced and faster activation of blood coagulation and is typical of a prothrombotic/hypercoagulable state in vivo [20- 22]. Longer CLT that is a well-established test assessing a global efficiency of endogenous enzymatic fibrin clot degradation being crucial for the vessel patency, indicating impaired fibrinolysis, which is associated with an increased risk of thrombotic events [23]. Increased thrombin formation leading to increased fibrin formation accompanied by its slower digestion predisposes to vascular thrombosis.

Statistical Analysis

The assumption of normality was verified with the Shapiro-Wilk test. All continuous variables were non-normally distributed and presented as medians with 95% confidence intervals (CI), according to the STROBE Statement [24]. Data was shown as medians with interquartile ranges, and maximum and minimum values. Continuous variables were compared by the Mann-Whitney U-test, Kruskal-Wallis and multiple repetition tests, as appropriate, whilst categorical variables by an χ^2 test. To test the relationship between continuous variables a univariate linear regression model was used. Because IgE had a skewed distribution, a 10 log-transformed was entered for a better fit of the model. Other variables were calculated in the original scale. Independent determinants of CLT and ETP were established in a multiple linear regression model, built by a forward stepwise selection procedure, verified by F Snedecore's statistics, with $F > 1$. The R^2 was used as a measure of the variance. Log-linear analysis and unconditional multivariate logistic regression were performed to make adjustments for categorical variables (age [cut-off value of 50 years], sex, BMI [$< 25 \text{ kg/m}^2$ and $\geq 25 \text{ kg/m}^2$], hypertension, DM, CHD, smoking, and medications, i.e. statins, aspirin, antihypertensives, glucocorticosteroids, montelukast, theophyllin, and oral estrogens). To calculate odds ratios (ORs) with 95% CIs, ETPs and CLTs were divided using the value of 75th percentile in controls as a cut-off point. To study determinants of the highest ETPs and the longest CLTs, the two variables were grouped into quartiles based on the distribution in the control subjects. The ORs with 95% CIs were calculated for clinical and laboratory variables determined for asthmatics in the 4th quartile, in reference to the values in the 1st and 2nd quartiles for ETP or 1st quartile for CLT. The choice of the reference group for ETP resulted from a very low number of asthmatics with ETPs in the first quartile ($n=2$). P-values < 0.05 were considered statistically significant. Analysis was performed with the STATISTICA 10.0 software package (StatSoft, Inc, Tulsa, OK, USA).

The present study was powered to have a 80% chance of detecting a 15% difference in CLT using a p-value of 0.05, based on the published values of this parameter [25]. In order to demonstrate such a difference or greater in CLT, at least 36 patients were required in each group.

Results

Patient characteristics

Asthma patients and control subjects were well matched for demographic variables and cardiovascular risk factors (Table 1). Allergic asthma was diagnosed in 94 patients (57.3%) who were younger, more often male, with a lower prevalence of hypertension compared with non-allergic asthmatics (Table 1). All asthmatics were on theophyllin and all but one on montelukast received inhaled corticosteroids. Ten patients on montelukast (25%) and 13 on theophyllin (50%) were also treated with oral corticosteroids.

Routine laboratory tests

Compared with the control subjects, asthmatics had a slightly lower fasting glucose, lower platelet count, and higher total cholesterol (Table 2). Asthmatics had 7.1% higher fibrinogen and 46% higher hsCRP. As expected, serum IgE levels were 75.2% higher in allergic asthma. Unexpectedly, we observed 13% higher α_2 -macroglobulin and 5.6% lower antithrombin in asthmatics (Table 2). α_2 -macroglobulin was positively associated with prothrombin ($\beta=0.24$ [95% CI: 0.12-0.35]) only in subjects with allergic asthma. Among platelet activation markers, PF4 was 51% higher in asthma, whilst P-selectin was similar to the values in the control group. There were no differences between allergic and non-allergic asthma regarding these laboratory variables (Table 2).

Thrombin generation and thrombin dynamics

Asthmatics had a significantly altered thrombin generation profile compared with the controls (Table 2, Fig. 2). Asthma was associated with 20.2% increased ETP, 41.4% higher thrombin peak, a 15.5% faster rate of thrombin formation (time to peak) and 11% shorter lag-time (Table 2). Asthma patients were also characterized by a 6.7% higher amount of converted prothrombin, 61% higher maximal prothrombin conversion rate, and 10% lower thrombin inactivation capacity (thrombin decay capacity) compared with healthy controls (Table 2). These differences in parameters of thrombin formation suggest a prothrombotic state in asthma. There were no differences between allergic and non-allergic asthma in thrombin generation and thrombin dynamic variables (Table 2).

ETP associated inversely but weakly with forced expiratory volume in 1 second/vital capacity (FEV_1/VC) index ($\beta=-0.11$ [95% CI:-0.19 to -0.03]), also after correction for age and BMI ($\beta=-0.09$ [95% CI:-0.17 to -0.004]). ETP was higher in persistent mild, moderate or severe stages of asthma (Fig. 2). Differences in ETP associated with asthma severity remained significant after adjustment for BMI, age, and sex ($\beta=0.24$ [95% CI: 0.16-0.32]). In asthmatic patients, ETP associates with BMI ($\beta=0.32$ [95% CI: 0.22-0.42]), blood platelet count ($\beta=0.21$ [95% CI: 0.13-0.29]), white blood cells (WBC) ($\beta=0.21$ [95% CI: 0.13-0.29]), total cholesterol ($\beta=0.23$ [95% CI: 0.16-0.3]), fibrinogen ($\beta=0.27$ [95% CI: 0.2-0.34]), prothrombin ($\beta=0.29$ [95% CI: 0.22-0.37]), and hsCRP ($\beta=0.4$ [95% CI: 0.33-0.47]). All of these associations remained significant after correction for age and BMI in a univariate linear regression model (data not shown).

A multiple regression model showed prothrombin as a major independent determinant of ETP variability in controls. Prothrombin was also important in asthma, but its potency was weakened by the effect of other variables such as hsCRP, total cholesterol, plasminogen,

platelet count, and BMI, all of which explained 41% of the ETP variability. Figure 3 presents ORs for clinical and laboratory variables that determined the ETP values in the 4th quartile in reference to those in the 1st and 2nd quartiles. Clinical determinants of the highest ETPs in this analysis were: older age (50 years or more), female sex, BMI ≥ 25 kg/m², hypertension, and FEV₁/VC <0.7. ORs of ETPs in the 4th quartile were significant for the 4th quartiles of all analyzed laboratory variables except antithrombin. Notably, elevated ETP was observed in women with asthma younger than 50 years (n=40) compared with men (n=23) at the same age (1536.1 [1489.6-1582.6], vs. 1405.4 [1347.5-1463.3] nmol/l thrombin x min, p=0.04). Moreover, asthmatics treated with montelukast had higher ETP compared with those not receiving this medication (1574.4 [1528.6-1622.1] vs. 1500 [1471.9-1528.1] nmol/l thrombin x min, p=0.03), also after adjustment for asthma severity, bronchial obstruction, age, sex, BMI, and co-morbidities (β =0.12 [95% CI: 0.05-0.19]). Women on oral contraceptives or hormone replacement therapy (n=19) had increased ETP than the remaining female asthmatics (1614.5 [1549.3-1679.8] vs. 1508.3 [1479.9-1536.7] nmol/l thrombin x min, p=0.02). The use of antihypertensive medications, statins, aspirin, or oral glucocorticosteroids, as well as smoking habits showed no associations with ETP.

Fibrinolysis

Impaired fibrinolysis was observed in asthmatics, as reflected by 14.4% longer CLT, accompanied with 4.2% higher plasminogen and 21.3% raised PAI-1, but unaltered antiplasmin (Table 2). PAI-1 was higher in asthmatics also after an adjustment for age, BMI, and sex (β =0.22 [95% CI: 0.16-0.28]). Longer CLT was demonstrated in allergic and non-allergic asthma after correction for potential confounders (β =0.19 [95% CI: 0.12-0.26], and β =0.32 [95% CI: 0.24-0.4], respectively), with higher values of CLT in non-allergic patients (β =0.1 [95% CI: 0.02-0.18]). As shown in Table 3, low fibrinolytic capacity, defined as CLT

above the 75th percentile of the control values, was found more frequently in asthmatics.

Compared with the control group, longer CLT was demonstrated in persistent mild, moderate, and severe asthma (Fig. 4). A weak association between CLT and asthma severity was also documented after adjustment for potential confounders ($\beta=0.08$ [95% CI: 0.01-0.15]). CLT associated positively, but weakly, with asthma duration ($\beta=0.09$ [95% CI: 0.01 - 0.17]), but after adjustment for confounders this association was non-significant ($\beta=0.05$ [95% CI: -0.02-0.12]). We observed a positive associations of CLT with age in asthma ($\beta=0.19$ [95% CI: 0.12-0.27]), BMI ($\beta=0.2$ [95% CI: 0.13-0.28]), ETP ($\beta=0.24$ [95% CI: 0.17-0.32]), and an inverse with FEV₁/VC index ($\beta=0.13$ [95% CI: -0.21 to -0.05]), also after adjustment for age and BMI ($\beta=0.09$ [95% CI: -0.17 to -0.008]).

To demonstrate the independent effect of continuous variables on CLT, a multiple regression model was used. CLT was independently associated with PAI-1, antipiasmin, WBC, ETP, and P-selectin in the control group. This model explained 62% of the CLT variability. In asthmatics, the most significant determinants of CLT were PAI-1, ETP, total cholesterol, and hsCRP, with a negative contribution of P-selectin, IgE, and WBC. However, this model explained only 28% of the CLT variability.

Figure 5 presents ORs for CLTs in the 4th quartile as compared with those in the 1st quartile of CLT. Except hypertension, clinical predictors of CLTs in the 4th quartile were the same as of ETPs. Among laboratory parameters total cholesterol, glucose, prothrombin, hsCRP, ETP, plasminogen, and PF4 were found to be predictors of the longest CLT values in asthma. The use of antihypertensive medications, statins, aspirin, oral glucocorticosteroids, or oral estrogens, showed no association with CLT. The same was true for smoking. CLT was longer in asthmatics treated with montelukast (109 [96.6-121.4] vs. 93.2 [87.9-121.4] min, $p=0.04$) also after adjustment for asthma severity, bronchial obstruction, age, sex, BMI, and co-morbidities ($\beta=0.21$ [95% CI: 0.13-0.29]).

Discussion

The present study shows that asthma is characterized by an unfavourably altered thrombin generation profile, including higher prothrombin conversion, faster rate of thrombin formation, and slower thrombin inactivation, which are accompanied by significantly impaired fibrinolysis largely driven by elevated PAI-1. Our findings provide evidence for a hypercoagulable state in persistent clinically stable asthmatics, which could explain a rising number of reports on the increased risk of thromboembolic events in asthma patients [16-19]. Very recently, Sneeboer et al. [26] has published a similar study also performed in asthmatics that documented enhanced thrombin generation together with higher levels of PAI-1, D-dimer, von Willebrand factor, and plasmin- α_2 -antiplasmin complexes in peripheral blood of these subjects. Our report confirms those observations with a similar magnitude of differences and expands their findings by showing impaired global efficiency of fibrinolysis and increased platelet activation.

Thrombin generation

Thrombin generation determined in the CAT assay is a reproducible and reliable automated tool for assessment of overall thrombotic-haemostatic functions of plasma in an integrated manner [20-22]. This approach allowed us to document a prothrombotic state in persistent asthmatics, with the pattern of thrombin formation similar to that reported by Sneeboer et al. [26] in asthma and Undas et al. [27] in chronic obstructive pulmonary disease, which is an established risk factor for thromboembolic events [2]. As expected, the key determinant of thrombin formation in asthmatics and controls was prothrombin. Its activity was, however, similar in both groups. Thus, we employed a computational analysis of thrombin generation curves to investigate the balance of pro- and anticoagulant mechanisms during thrombin generation [20]. Interestingly, this equilibrium was extensively disturbed by increased

prothrombin conversion and reduced thrombin inactivation in asthma patients. Increased prothrombin conversion in patients can be attributed to higher plasma prothrombin levels to some extent. However, the maximum rate of the prothrombinase complex was higher in asthma patients than controls, and this parameter is dependent on other processes including FV and FX activation, and the TF-induced coagulation pathway as a whole. Increased prothrombin conversion leads to higher thrombin generation in asthma patients, and this effect is augmented by the attenuation of thrombin inactivation capacity. The reduction of the thrombin inactivation rate is probably due to lower antithrombin activity, and it is partially counteracted by the increase of α_2 -macroglobulin levels.

Other laboratory variables that independently determined ETP in asthma, including fibrinogen, hsCRP, plasminogen, total cholesterol, and α_2 -macroglobulin that were higher in asthmatics, indicate a complex regulation of increased thrombin formation in this disease. One of the strongest associations, however, was observed for hsCRP. It might be speculated that a prothrombotic state in asthma is at least in part driven by an increased biological activity of proinflammatory cytokines, namely IL-1/IL-6/TNF α , and is not particularly unique to this disease.

Another novel finding of our study was increased α_2 -macroglobulin levels in blood of clinically stable asthma patients. This protein acts as a general serpin scavenger and inhibits active enzymes, including plasmin and thrombin, as well as kallikrein [28]. Circulating levels of α_2 -macroglobulin could be increased in response to activated blood coagulation, contributing to the attenuation of a prothrombotic state [29]. On the other hand, higher levels of α_2 -macroglobulin can display a procoagulant activity due to the formation of complexes with activated protein C. This might lead to a loss of its anticoagulant activity and enhanced thrombin generation [30]. A recent pilot study showing attenuation of neutrophil migration

and protein release in allergic asthmatics treated with recombinant human activated protein C might indicate that suppressed thrombin formation could indeed be beneficial in this disease [31]. Moreover, anticoagulant therapy is not used in asthma treatment nowadays, although the beneficial effect of heparin on airways inflammation and asthma symptoms is well known [5]. It might be speculated that favourable actions of diverse anticoagulant agents on airway inflammation in asthma might be attributable in part to suppression of excessively activated thrombin formation and elimination of harmful thrombin-mediated cellular effects.

Fibrinolysis

Clot lysis time measured using a plasma-based assay has been successfully used to determine the efficiency of fibrinolysis in various disease states including subjects at risk of thrombosis [25, 32, 33, 34]. PAI-1 was the main determinant of CLT, followed by plasminogen [33]. We demonstrated that in asthmatics prolonged CLT is also largely determined by elevated plasma PAI-1. However, the most important determinant of longer CLT among asthmatics was higher ETP, which confirms that more compact fibrin clot networks typically formed in the presence of higher thrombin amounts are lysed slowly in asthma, similarly to patients at risk of venous thromboembolic diseases [34]. Longer euglobulin lysis time, a simple global lysis test, was previously demonstrated in 41 recently diagnosed and untreated symptomatic asthma patients [35]. Heightened PAI-1 was reported in asthmatic airways [36] and blood samples from these subjects [26, 37]. Moreover, recent studies have suggested that PAI-1 may promote the development of asthma by regulating airway inflammation, remodeling and hyperresponsiveness [36]. A role of other fibrinolysis inhibitors, particularly thrombin activatable fibrinolysis inhibitor (TAFI), in prolongation of CLT remains to be established. There is ongoing debate as to whether atopic asthma and non-atopic asthma are two immunopathologically distinct entities or if both are driven by similar mechanisms [38]. There is evidence supporting the concept that IgE-mediated mechanisms play a major role in

asthma pathogenesis. Notably, the synthesis of IgE may take place in the bronchial mucosal epithelium of patients with both types of asthma [39]. Our results seem consistent with this hypothesis, suggesting that inflammation is critical for promotion of the prothrombotic state in asthma, particularly in terms of enhanced thrombin formation.

Platelet activation

Analysis of blood platelet count, as well as plasma levels of PF4 and P-selectin showed that asthma subjects had a slightly lower platelet count (all values within a reference range), and similar P-selectin, but higher PF4 levels as compared to the controls. There have been a number of reports on platelet activation in allergic diseases and asthma [14, 15]. Our study provided additional evidence for the role of increased platelet activity in the pathogenesis of asthma, suggesting the contribution of a platelet specific proteins, namely PF4, in this disease. Furthermore, a slightly decreased platelet count in asthmatics reported by us might suggest continuous recruitment of peripheral blood platelets to the lungs with their further consumption as a result of chronic activation in the inflammation site [15].

Study limitations

The study group was relatively small. However, the study was adequately powered to detect intergroup differences in thrombin generation and fibrinolysis variables. Subgroup analysis in particular should be interpreted with caution. We determined each variable at a single time point and therefore we cannot exclude changes of the variables studied over time. The assessment of medication-induced alterations to blood coagulation and fibrinolysis was beyond the scope of our study. Interestingly, we observed that treatment with systemic corticosteroids had no effect on the studied parameters, whilst montelukast was associated with increased ETP and CLT not related to asthma severity and other potential confounders, including age, BMI or co-morbidities, which deserves further studies. We did not determine other potential modulators of coagulation and fibrinolysis, e.g. genetic polymorphisms. Given

prothrombotic abnormalities in first-degree relatives of patients with venous thrombosis [40], we cannot exclude that a prothrombotic state observed in asthma is to some extent genetically determined [41]. A potential genetic factor involved in impaired fibrinolysis could be a common deletion/insertion polymorphism (4G/5G) at a promoter region of the PAI-1 gene that largely determines the PAI-1 expression [36]. Statistical associations reported here may not necessarily indicate cause-effect relationships. Finally, clinical relevance of prothrombotic alterations in asthmatics in terms of thromboembolic risk and their molecular mechanisms remains to be established.

Conclusion and clinical relevance

Our study demonstrates enhanced thrombin generation and attenuated fibrinolysis in patients with asthma. Since haemostasis is closely regulated in vivo, intergroup differences by 10-20% are commonly reported in various patient groups at risk of thromboembolic arterial and venous events, in which the same tests were successfully used [25, 32, 41]. To our knowledge, larger differences in thrombin or fibrinolysis variables in subjects without clinically overt acute thrombotic episodes are unlikely. This study increases our knowledge of thrombotic mechanisms in asthma and could have practical implications.

In summary, clinicians treating patients with symptomatic asthma should be aware of a prothrombotic state, especially in those who are at risk of venous thromboembolism. Further larger studies are needed to elucidate a prognostic role of a prothrombotic state in asthmatics in terms of thromboembolism during follow-up.

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All authors declare no conflict of interests.

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Legends to figures

Figure 1. Representative thrombin generation curves in the Calibrated Automated Thrombogram for a healthy control (blue curve) and for patients with persistent mild (green curve) or severe asthma (red curve).

In this assay after a period in which no observable thrombin is formed, the thrombin concentration steeply goes up, raises to a peak, and then goes down again. The maximum concentration of thrombin formed during the time of registration is described as the "thrombin peak" (nmol/l) and the area under the curve represents the "endogenous thrombin potential" (ETP, nmol/l x min). "Lag-time" is the time from start of analysis until thrombin started to generate, and the "time to thrombin peak" represents the time from start of thrombin generation until the maximum thrombin value is achieved.

Figure 2. Distribution of Endogenous Thrombin Potentials in controls and patients with intermittent, persistent mild, moderate and severe asthma.

Data is presented as median, interquartile range, and maximum and minimum values.

Numbers on the graph represent p-values in comparison to control. Statistically significant differences are marked with an asterisk.

ETP - Endogenous Thrombin Potential

Figure 3. Associations of clinical and laboratory variables with the highest values of Endogenous Thrombin Potential.

Endogenous thrombin potentials (ETPs) were grouped into quartiles based on the distribution in the control subjects. The odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for asthma subjects with ETPs in the fourth quartile, referred to those in the first and second quartiles. The value of the 75th percentile in controls as cut-off point was used for laboratory variables.

BMI - body mass index

FEV₁/VC - forced expiratory volume in 1 second/vital capacity

hsCRP - high-sensitivity C-reactive protein.

IgE - Immunoglobulin E

PAI-1- Plasminogen activator inhibitor-1

Figure 4. Distribution of Clot Lysis Times in controls and patients with intermittent, persistent mild, moderate and severe asthma.

Data is presented as median, interquartile range, and maximum and minimum values.

Numbers on the graph represent p-values in comparison to control. Statistically significant differences are marked with an asterisk.

CLT - Clot Lysis Time

Figure 5. Associations of clinical and laboratory variables with the longest values of Clot Lysis Time.

Clot lysis times (CLTs) were grouped into quartiles based on the distribution in the control subjects. The odds ratios (ORs) with 95% confidence intervals (CIs) were calculated for

asthma subjects with CLTs in the fourth quartile referred to those in the first quartile. The value of the 75th percentile in controls as cut-off point was used for laboratory variables.

ETP - Endogenous Thrombin Potential, for other abbreviations see the description of Figure

3.

Tables

Table 1. Demographic and clinical characteristics of the subjects studied.

	Control n=72	Asthma n=164	p-value control vs. asthma	Allergic asthma n=94	p-value control vs. allergic asthma	Non-allergic asthma n=70	p-value control vs. non-allergic asthma	p-value allergic vs. non-allergic asthma
Clinical data								
Age, years	50.5 (48.4-52.6)	53.5 (52.1-54.9)	0.34	49.5 (47.6-51.4)	0.64	56.5 (54.4-58.6)	0.049*	0.009*
Male gender, n (%)	18 (25)	42 (25.6)	0.92	31 (33.0)	0.26	11 (15.7)	0.24	0.01*
Body mass index, kg/m ²	25.6 (25-26.1)	26.6 (26-27.2)	0.15	25.9 (25-26.8)	0.58	27.4 (26.8-28.1)	0.04*	0.09
Current smoking, n (%)	14 (19.4)	19 (11.6)	0.16	12 (12.8)	0.34	7 (10)	0.21	0.84
Number of pack-years	16 (12-20.1)	12 (9.9-14.1)	0.14	13 (10.8-15.2)	0.1	12 (7.7-16.3)	0.5	0.5
Former smoking, n (%)	5 (6.9)	17 (10.4)	0.56	11 (11.7)	0.44	6 (6.6)	0.96	0.7
Number of pack-years	15 (10.4-19.6)	10 (8.2-11.8)	0.43	9 (6.6-11.4)	0.41	10 (7.2-12.8)	0.6	0.75
Arterial hypertension, n (%)	19 (26.4)	57 (34.7)	0.21	26 (27.7)	0.30	31 (44.3)	0.001*	0.02*
Diabetes mellitus, n (%)	2 (2.8)	13 (7.9)	0.14	5 (5.3)	0.42	9 (12.9)	0.03*	0.09
Hypercholesterolemia, n (%)	36 (50)	88 (53.6)	0.6	51 (54.3)	0.59	37 (52.9)	0.73	0.86
Coronary heart disease, n (%)	3 (4.2)	16 (9.7)	0.14	6 (6.38)	0.53	7 (10)	0.17	0.40

Medications used:								
Antihypertensives, n (%)	19 (26.4)	55 (33.5)	0.27	24 (25.5)	0.9	31 (44.3)	0.03*	0.01*
Statins, n (%)	12 (16.7)	19 (11.6)	0.29	8 (8.5)	0.11	11 (15.7)	0.88	0.15
Aspirin, n (%)	3 (4.2)	8 (4.8)	0.81	3 (3.2)	0.74	5 (7.1)	0.44	0.25
Oral estrogens, n (%)	0	19 (11.6)	0.003*	11 (11.7)	0.003*	8 (11.4)	0.003*	0.96
Asthma duration, years		12.5 (11.2-13.8)		15 (13.3-16.7)		10 (8.1-11.5)		0.15
Asthma severity (GINA)								
intermittent, n (%)		20 (12.2)		13 (13.8)		7 (10)		
persistent mild, n (%)		36 (21.9)		19 (20.2)		17 (24.3)		
persistent moderate, n (%)		45 (27.4)		24 (25.5)		21 (30)		
persistent severe, n (%)		63 (38.4)		38 (40.4)		25 (35.7)		0.73
Asthma treatment:								
Inhaled corticosteroids, n (%)		144 (87.8)		81 (86.2)		63 (90)		0.46
Long-acting β_2 -agonists, n (%)		124 (75.6)		72 (76.5)		52 (74.3)		0.73
Montelukast, n (%)		40 (24.4)		23 (24.5)		17 (24.3)		0.98
Theophyllin, n (%)		26 (15.9)		13 (13.8)		13 (18.6)		0.41
Oral corticosteroids, n (%)		34 (20.7)		21 (22.3)		13 (18.6)		0.56
Spirometry values								
FEV ₁ , % of predicted value	96 (94.3-97.7)	89 (86.43-91.57)	0.01*	90 (86.04-93.96)	0.06	89 (86.17-91.83)	0.006*	0.45

FEV ₁ /VC	0.89 (0.87-0.90)	0.76 (0.75-0.77)	<0.0001*	0.77 (0.76-0.78)	<0.0001*	0.76 (0.75-0.77)	<0.0001*	0.71
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Categorical variables are presented as numbers (percentages), continuous variables as median with 95% confidence interval.

Abbreviations: FEV₁ - Forced expiratory volume in 1 second, FEV₁/VC - Forced expiratory volume in 1 second/vital capacity, GINA - Global

Initiative for Asthma.

* - statistically significant, n – number

Table 2. Laboratory variables.

	Control n=72	Asthma n=164	p-value control vs. asthma	Allergic asthma n=94	p-value control vs. allergic asthma	Non-allergic asthma n=70	p-value control vs. non-allergic asthma	p-value allergic vs. non-allergic asthma
Laboratory tests								
Blood platelets, 10 ³ /μl	236 (228.3-243.7)	214.5 (209.4-219.6)	0.002*	213 (205.8-220.2)	0.07	215 (207.9-222.1)	0.02*	0.57
Red blood cells, 10 ⁶ /μl	4.74 (4.7-4.8)	4.78 (4.7-4.85)	0.64	4.74 (4.68-4.79)	0.56	4.8 (4.65-4.95)	0.85	0.78
White blood cells, 10 ³ /μl	6.4 (6.2-6.6)	6.48 (5.72-7.24)	0.25	6.65 (5.35-7.95)	0.04*	6.14 (5.86-6.42)	0.77	0.03*
Total cholesterol, mmol/l	4.81 (4.65-4.96)	5.1 (5.01-5.19)	0.009*	5.15 (5.04-5.26)	0.02*	5 (4.85-5.15)	0.24	0.42
Triglycerides, mmol/l	1.15 (1.04-1.26)	1.2 (1.14-1.26)	0.88	1.2 (1.13-1.27)	0.88	1.14 (1.04-1.24)	0.91	0.94
Glucose, mmol/l	5.1 (5.02-5.18)	4.9 (4.82-4.98)	0.009*	4.85 (4.78-4.92)	0.003*	5 (4.84-5.16)	0.84	0.1
Fibrinogen, g/l	3.36 (3.3-3.4)	3.6 (3.52-3.68)	0.001*	3.5 (3.4-3.6)	0.03*	3.65 (3.52-3.78)	0.004*	0.39
Prothrombin, %	103 (101.03-104.97)	108 (106.76-109.24)	0.13	107 (105.45-108.55)	0.21	109 (106.96-111.04)	0.16	0.58
hsCRP, mg/l	0.87 (0.6-1.13)	1.27 (1.05-1.49)	0.03*	1.21 (0.94-1.47)	0.34	1.85 (1.47-2.23)	0.04*	0.67
Immunoglobulin E, IU/ml	1.5 (1.45-1.54)	1.92 (1.87-1.98)	0.003*	2.26 (2.19-2.32)	<0.0001*	1.29 (1.23-1.34)	0.23	<0.0001*

α_2 -macroglobulin, nmol/l	3.29 (3.17-3.40)	3.72 (3.62-3.82)	0.0002*	3.78 (3.65-3.91)	<0.0001*	3.54 (3.39-3.68)	0.02*	0.22
Antithrombin, %	107 (105-109)	101 (99.78-102.2)	0.008*	101 (99.4-102.6)	0.02*	101.5 (99.6-103.4)	0.02*	0.89
Thrombin generation profile in Calibrated Automated Thrombogram								
Lag-time, min	2.62 (2.53-2.70)	2.33 (2.28-2.38)	0.0002*	2 (1.94-2.06)	<0.0001*	2.33 (2.25-2.41)	0.03*	0.06
Thrombin peak, nmol/l	200.5 (192-208.9)	283.6 (277.4-289.8)	<0.0001*	286.0 (278.2-293.8)	<0.0001*	281.24 (271.1-291.4)	<0.0001*	0.30
Time to thrombin peak, min	5.92 (5.69-6.14)	5 (4.89-5.11)	<0.0001*	5 (4.87-5.13)	<0.0001*	5.33 (5.14-5.52)	0.004*	0.10
Endogenous thrombin potential, nmol/l thrombin x min	1255 (1222.94-1287.1)	1508.3 (1483.8-1532.6)	<0.0001*	1516.3 (1484.8-1544.8)	<0.0001*	1497.4 (1458.1-1536.6)	0.0001*	0.81
Computational assessment of thrombin dynamics								
Total amount of prothrombin converted, nmol/l	848.1 (816.7-879.5)	904.7 (887-922.4)	0.03*	915.2 (895.3-935.1)	0.01*	883.1 (850.2-915.9)	0.27	0.24
Maximal prothrombin conversion rate, nmol/l x min	167.6 (150.2-184.9)	269.8 (258.9-280.8)	<0.0001*	275.8 (263-288.6)	<0.0001*	256.6 (236.9-276.2)	0.0002*	0.34
Thrombin-antithrombin complex formation, nmol/l	807 (775.9-837.6)	843.9 (826.9-861)	0.1	860.8 (841.9-879.6)	0.04*	825.8 (793.8-857.8)	0.48	0.28
Thrombin- α_2 -macroglobulin complex formation, nmol/l	44.5 (41.4-47.5)	47.2 (45.6-48.9)	0.6	46.2 (44.1-48.3)	0.96	49.1 (46.4-51.7)	0.29	0.15
Thrombin decay capacity, min ⁻¹	0.7 (0.69-0.73)	0.63 (0.62-0.64)	0.0004*	0.63 (0.62-0.64)	0.002*	0.62 (0.61-0.63)	0.001*	0.46

Fibrinolysis								
Clot lysis time, min	83.2 (80.5-85.87)	95.2 (90.1-100.3)	0.001*	90.75 (84.5-97)	0.24	106.6 (98.4-114.8)	<0.0001*	0.02*
Plasminogen, %	102.4 (101.1-103.7)	106.7 (104.97-108.53)	0.02*	105 (102.39-107.61)	0.08	107.5 (105.24-109.76)	0.01*	0.79
Antiplasmin, %	100.3 (98.61-101.89)	103 (101.4-104.6)	0.09	104 (101.8-106.2)	0.02*	102 (99.69-104.31)	0.12	0.42
Plasminogen activator inhibitor-1, ng/ml	24.3 (23.27-25.33)	29.48 (27.91-31.06)	<0.0001*	28.73 (26.69-30.78)	0.0009*	29.72 (27.25-32.19)	0.0007*	0.80
Platelet activation markers								
P-selectin, ng/ml	23.5 (21.85-25.15)	23.94 (22.58-25.3)	0.62	23.94 (22.3-25.58)	0.82	22.08 (19.77-24.39)	0.50	0.72
Platelet factor 4, ng/ml	97 (94.28-99.72)	146.65 (144.56-148.76)	<0.0001*	148.29 (145.51-151.06)	<0.0001*	144.96 (141.71-148.2)	<0.0001*	0.62

Categorical variables are presented as numbers (percentages), continuous variables as median with 95% confidence interval.

Abbreviations: hsCRP - high-sensitivity C-reactive protein.

* - statistically significant, n – number

Table 3. Odds Ratios (ORs) with 95% confidence intervals (95% CIs) for asthmatics of having Endogenous Thrombin Potential and Clot Lysis Time above 75th percentile of controls values with subjects below this cut-off point as a reference group, with adjustment for age, sex and body mass index.

	Endogenous Thrombin Potential >1454.15 nmol/l thrombin x min (75 th percentile of the control values)		Clot Lysis Time > 99.0 min (75 th percentile of the control values)	
Control, numbers	19		17	
Asthma, numbers	91		75	
OR (95%CI)	4.03 (2.30-7.05)		2.24 (1.4-3.59)	
Allergic asthma, numbers	54		35	
OR (95%CI)	4.33 (2.20-8.53)		1.73 (1.03-2.92)	
Non-allergic asthma, numbers	37		40	
OR (95%CI)	3.69 (1.83-7.41)		3.19 (1.83-5.56)	
	<50 years	≥50 years	<50years	≥50years
Control, numbers	10	9	10	7

Asthma, numbers	34	57	22	53
OR (95%CI)	2.64 (1.28-5.42)	2.28 (1.26-4.13)	1.2 (0.61-2.34)	3.78 (1.87-7.62)
	Men	Women	Men	Women
Control, numbers	4	15	2	15
Asthma, numbers	17	75	13	62
OR (95%CI)	2.32 (0.85-6.29)	2.05 (1.26-3.35)	2.89 (0.85-9.84)	2.16 (1.28-3.66)
	Body mass index <25	Body mass index ≥25	Body mass index <25	Body mass index ≥25
Control, numbers	5	14	9	8
Asthma, numbers	18	73	16	59
OR (95%CI)	2.68 (0.99-7.23)	4.68 (2.3-9.56)	0.93 (0.42-2.08)	3.5 (1.86-6.57)





