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ORIGINAL ARTICLE

Enhanced thrombin and plasmin generation profiles in alpha-2-antiplasmin–deficient patients: Data from the Rare Bleeding disorders in the Netherlands study

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Abstract

Background: α2-Antiplasmin (A2AP) deficiency is a rare and often unidentified disorder characterized by increased fibrinolysis and subsequent bleeding. Global hemostasis assays may increase insight into the altered coagulation and fibrinolysis in these patients.

Objectives: To explore thrombin and plasmin generation profiles in A2AP-deficient patients, corresponding A2AP activity levels and associated bleeding phenotypes.

Methods: The Nijmegen hemostasis assay was used to assess thrombin and plasmin generation in 23 A2AP-deficient patients (median age, 50 years; 70% women) from the cross-sectional Rare Bleeding disorders in the Netherlands study. Analyzed parameters included thrombin peak height, thrombin potential, fibrin lysis time, plasmin peak height, plasmin velocity index, and plasmin potential. These parameters were expressed as percentages of a reference obtained from 37 healthy controls (median age, 46 years; 57% women). The Nijmegen hemostasis assay data were correlated with A2AP activity

levels and International Society on Thrombosis and Hemostasis Bleeding Assessment Tool scores using Pearson correlation coefficients.

Results: Patients' A2AP activity levels ranged from 23% to 83% (reference range, 89%-122%). Plasmin generation increased, as evidenced by significantly shorter fibrin lysis times (73%; $P < .001$) and higher plasmin peak heights (203%; $P < .001$), plasmin velocity indices (302%; $P < .001$) and plasmin potentials (154%; $P < .001$) in A2AP-deficient patients than those in healthy controls. Moreover, significantly higher thrombin potentials (146%; $P < .001$) and thrombin peak heights (132%; $P < .001$) were observed. Enhanced plasmin generation parameters showed statistically significant correlations with lower A2AP activity levels and higher International Society on Thrombosis and Hemostasis Bleeding Assessment Tool scores.

Conclusion: A2AP-deficient patients exhibited augmented plasmin generation profiles that correlated with A2AP activity level and bleeding phenotype. Interestingly, increased thrombin generation profiles were also found in these patients.

KEYWORDS
α2-antiplasmin, blood coagulation disorders, fibrinolytic defect, hemostasis, plasmin, thrombin

Essentials

- α2-Antiplasmin (A2AP) deficiency is a rare bleeding disorder due to accelerated clot dissolution.
- The cross-sectional Rare Bleeding disorders in the Netherlands study gathered data from a unique group of 23 A2AP-deficient patients.
- Severe A2AP deficiency leads to higher plasmin generation and more bleeding than mild deficiency.
- A2AP-deficient patients showed enhanced thrombin generation.

1 | INTRODUCTION

α2-Antiplasmin (A2AP) is a serine protease inhibitor that plays a pivotal role in regulating fibrinolysis, the physiologic process responsible for the dissolution of fibrin clots [1]. A2AP is primarily synthesized in the liver and functions by irreversibly inhibiting plasmin, the key enzyme responsible for fibrin degradation [2,3]. The fine-tuned balance between clot formation and dissolution, orchestrated in part by A2AP, is crucial for maintaining hemostatic equilibrium.

A2AP deficiency is a very rare congenital bleeding disorder with an undetermined prevalence. The literature describes approximately 120 cases, of which 14 homozygous for A2AP deficiency [4,5]. The disease is characterized by reduced levels of this antifibrinolytic inhibitor, leading to enhanced fibrinolysis, and subsequent increased susceptibility to bleeding with potentially severe hemorrhagic complications [1,4,6]. Biallelic (ie, homozygous or compound heterozygous) affected patients commonly exhibit moderate to severe bleeding problems with onset in early childhood. Posttraumatic, postdental extraction, and postsurgical bleeding are most frequently reported [4]. In addition, spontaneous bleeding may also occur, including more severe manifestations such as intramedullary hematomas, hemarthrosis, and kidney bleeding [7–11]. In contrast, monoallelic (ie, heterozygous) affected individuals may remain asymptomatic, although a minority of these patients experience pronounced bleeding after invasive dental procedures, surgery, or trauma later in life [4–6]. Gastrointestinal, retroperitoneal and umbilical cord bleeding, menorrhagia, and obstetric complications have also been described in monoallelic affected patients [12–18]. These data demonstrate that mild A2AP deficiencies may also have clinical significance and underscore the importance of further research and awareness regarding this disorder.

Patients with A2AP deficiency exhibit normal screening tests for primary and secondary hemostasis, including platelet function, prothrombin time, and activated partial thromboplastin time [1,13]. As a consequence, A2AP deficiency goes undetected with routine hemostatic assessment. Moreover, evaluation for this deficiency is only available in specialized hemostatic laboratories. This underlines the possibility of underdiagnosis, raising concerns about the prevalence and clinical impact of A2AP deficiency within the broader population [4,13,19]. In the absence of an accurate diagnosis, addressing and preventing bleeding complications becomes challenging, as individualized therapy cannot be employed [4]. The relative scarcity of reported cases and limited research dedicated to A2AP further contributes to the challenge of recognizing and understanding all molecular, physiologic and clinical aspects of this disorder, impeding progress in the development of effective diagnostic and therapeutic strategies.

The Rare Bleeding disorders in the Netherlands (RBiN) study is a multicenter study conducted from 2017 to 2019 in the Netherlands, analyzing the clinical and laboratory phenotype of patients with rare bleeding disorders (RBDs). This study also invited patients with congenital fibrinolytic disorders to participate, including A2AP deficiency. The aim of this RBiN substudy was to unravel the underlying hemostatic balance in A2AP-deficient patients by measuring thrombin generation (TG) and plasmin generation (PG) profiles and analyze the relation between these profiles, A2AP activity levels, bleeding phenotype, and genotype.

2 | MATERIALS AND METHODS

2.1 | The RBiN study

This study is an additional analysis within the nationwide cross-sectional RBiN study, which encompassed patients with rare coagulation factor deficiencies and fibrinolytic disorders from all 6 Dutch Hemophilia Treatment Centers. Inclusion criteria comprised individuals diagnosed with an RBD subsequent to referral to a Hemophilia Treatment Center due to a bleeding diathesis, a family history of RBD, or abnormal values in prior screening laboratory tests. Patients aged 1 year or older were eligible, and enrollment occurred between October 1, 2017, and November 30, 2019. The specific criteria for patient inclusion and study design have been previously documented [20]. For this study, we focused on the 23 A2AP-deficient patients included in the RBiN study (Table). These patients exhibited A2AP activity levels below 89% (lower limit of normal). The study received approval from the Medical Ethical Committee of Arnhem-Nijmegen, and written informed consent was obtained from all patients or their parents in the case of minors.

2.2 | Clinical phenotype, laboratory results, and genotype

The International Society on Thrombosis and Haemostasis Bleeding Assessment Tool (ISTH-BAT) was uniformly used to determine the clinical bleeding phenotype in each participant at study inclusion [20]. The ISTH-BAT was consistently conducted by a single investigator during the study visit. Moreover, blood samples were collected at the Hemophilia Treatment Centers during the study visit for subsequent laboratory analyses. The samples were collected in citrate tubes, centrifuged at 4200*g* for 15 minutes, and snap frozen onsite at −80 °C immediately after collection. To ensure consistency, all laboratory analyses were performed in the hematology laboratory of the Radboudumc. A2AP activity was measured using a chromogenic assay of antiplasmin (STA-Stachrom Antiplasmin; Stago). The reference range for A2AP activity was 89% to 122%. Furthermore, genotype sequencing using targeted whole exome sequencing was executed with a panel of 156 genes involved in hemostasis and thrombosis disorders. In this article, only class 3 (variant of unknown significance), 4 (likely pathogenic), and 5 (clearly pathogenic) genetic variants in the *A2AP* gene were evaluated. Variant classification was performed using the American College of Medical Genetic and Genomics and the Association for Molecular Pathology system.

TABLE Characteristics of studied α2-antiplasmin (A2AP)–deficient patients.

Characteristics of A2AP deficient patients		
Characteristics		**Value**
Age (y)		50 (31-69)
Adult women (%)		13/23 (57)
Adult men (%)		6/23 (26)
Children (%)		4/23 (17)
ISTH-BAT score[a]		7 (4-13)
Bleeding severity grade (%)	0	11
	1	21
	2	58
	3	11
A2AP (%)		68 (59-73)
PT (s)		14 (14-15)
aPTT (s)		30 (28-31)
Fibrinogen (mg/L)		3320 (2905-3683)
Biallelic genetic variants in *A2AP* (%)		3/23 (13)
Monoallelic genetic variants in *A2AP* (%)		15/23 (65)
Unknown mutation status (%)		5/23 (22)

All patients were of the Caucasian race. For sex, bleeding grade and genetic status, percentages are shown. Other characteristics are expressed as medians with IQR.

aPTT, activated partial thromboplastin time; ISTH-BAT, International Society for Thrombosis and Hemostasis bleeding assessment tool; PT, prothrombin time.

[a]Of the 23 patients, 19 completed the interview to determine their ISTH-BAT score.

2.3 | TG and PG assay

TG and PG were measured using the Nijmegen hemostasis assay (NHA) [21]. Briefly, citrated plasma was mixed with a low tissue factor concentration (estimated at 0.28 pM), recombinant tissue-plasminogen activator (around 190 IU/mL), cephalin (as a source of phospholipids), and a thrombin-specific and plasmin-specific fluorescent substrate. After starting the reaction with calcium (16.7 mM), fluorescence was measured alternately for thrombin at excitation and emission wavelengths of 355 and 460 nm, respectively, and for PG at 485 and 520 nm, respectively. Calibration curves were prepared with human α-thrombin and human plasmin. In each run, a sample of normal pooled plasma, consisting of pooled plasma from 60 healthy donors, was included in the measurements to serve as a control. Several parameters were calculated from the first derivative of the fluorescence signal to describe the proteolytic activity of thrombin

and plasmin. For analyses, we selected the TG parameters lag time, time to peak, peak height, and thrombin potential, and the PG parameters fibrin lysis time, plasmin peak height, plasmin potential, and plasmin velocity index (Supplementary Figure S1). As shown in the PG curve, the measured fluorescence remains at a high level after reaching the plasmin peak. This is due to the plasmin-α2-macroglobulin complex, which retains the ability to cleave the plasmin substrate in the NHA. Manual evaluation of the data extracted from TG and PG curves preceded the analysis of NHA results. Normalization was carried out using the average TG and PG parameter values of a cohort of 37 healthy individuals (mean and median age, 46 years; 57% women). Blood samples from these voluntary donors were obtained from the Dutch blood bank and met all national donation requirements. The blood group distribution in this cohort was consistent with that of the general population. Individuals with a bleeding disorder and individuals using oral contraceptives or other coagulation-interfering medication were excluded from the healthy control cohort.

2.4 | Statistical analysis

Continuous variables were presented as medians with IQR, while categorical variables were reported as counts and percentages. Any missing data are indicated within the tables or in the accompanying legends and did not result in exclusion of patients from the analyses. To select the appropriate statistical tests for further analysis, the data were tested for normality using the Shapiro–Wilk test. The results indicated that A2AP activity values were not normally distributed, while the ISTH-BAT scores and TG and PG values were. A Mann–Whitney *U* test was used to compare the PG and TG parameter values of A2AP-deficient patients and healthy controls. Spearman correlation analysis was employed to assess associations between the A2AP activity levels and the PG and TG parameter values. Additionally, correlations were investigated between the ISTH-BAT scores and both the baseline A2AP activity levels (using Spearman method) and the PG parameter values (using Pearson method). The strength of correlations was classified as absent (0-0.19), weak (0.20-0.39), moderate (0.40-0.59), strong (0.60-0.79), or very strong (0.80-1.00). Visual representation and statistical analysis were conducted using R (version 4.2.2; www.R-project.org). A P value of <.05 was considered statistically significant.

3 | RESULTS

3.1 | TG and PG profiles

In total, 23 patients with an A2AP deficiency were included. The median age was 50 years, with the majority being female (Table). The median A2AP activity level was 68% (IQR, 59%-73%), and the median ISTH-BAT score was 7 (IQR, 4-13). Examples of TG and PG curves of 2 A2AP deficient patients are shown in Figure 1. PG profiles of the patients were normalized, and the subsequent PG parameters fibrin lysis time, plasmin peak height, plasmin velocity index, and plasmin potential are shown in Figure 2A. A2AP-deficient patients exhibited a significantly shorter median fibrin lysis time (73%; $P < .0001$) and higher median plasmin peak height (203%; $P < .0001$) compared with healthy individuals. This resulted in a substantially elevated median plasmin velocity index (302%; $P < .0001$) in A2AP-deficiency patients. Additionally, the median plasmin potential was significantly higher in A2AP-deficient patients (154%; $P < .0001$) than in healthy individuals. Figure 2B shows the normalized TG parameter values of A2AP-deficient patients. No significant differences exist between A2AP-deficient patients and controls for lag time and thrombin peak time. However, the median thrombin peak height (132%; $P < .001$) and thrombin potential (146%; $P < .0001$) were significantly higher in A2AP-deficient patients than those in healthy individuals.

3.2 | Correlation analyses

3.2.1 | Plasmin generation

Subsequently, correlation analyses were performed between A2AP activity level and PG parameters to assess the association between the severity of the A2AP deficiencies and underlying mechanism in the PG. A moderate positive correlation existed between A2AP activity level and fibrin lysis time ($R = .58$, $P = .012$), while a moderate negative correlation was observed between A2AP activity level and plasmin peak height ($R = -.51$, $P = .03$), and between A2AP levels and plasmin velocity index ($R = -.57$, $P = .013$) (Figure 3A–C). The A2AP activity level showed no significant correlation with the plasmin potential ($R = .34$, $P = .16$) (Figure 3D). Figure 4 illustrates individual PG curves for 4 patients with A2AP activity levels of 23%, 36%, 65%, and 83%, along with the median PG curve of the group of healthy individuals (measured A2AP activity value of 100%). The curves highlight the correlations depicted in Figure 3, indicating that a more severe A2AP deficiency leads to a shorter fibrin lysis time, higher plasmin peak height, and higher plasmin velocity index as a reflection of enhanced fibrinolysis.

3.2.2 | Thrombin generation

In continuation of the PG parameter analysis, the correlations between TG parameters and A2AP activity levels were investigated, as shown in Figure 5. No correlation existed between A2AP activity level and lag time, thrombin peak time, and thrombin peak height ($R = .09$, $P = .72$; $R = .14$, $P = .58$; and $R = -.18$, $P = .46$, respectively) (Figure 5A–C). Interestingly, A2AP activity level exhibited a moderate negative correlation with thrombin potential ($R = -.52$, $P = .03$) (Figure 5D).

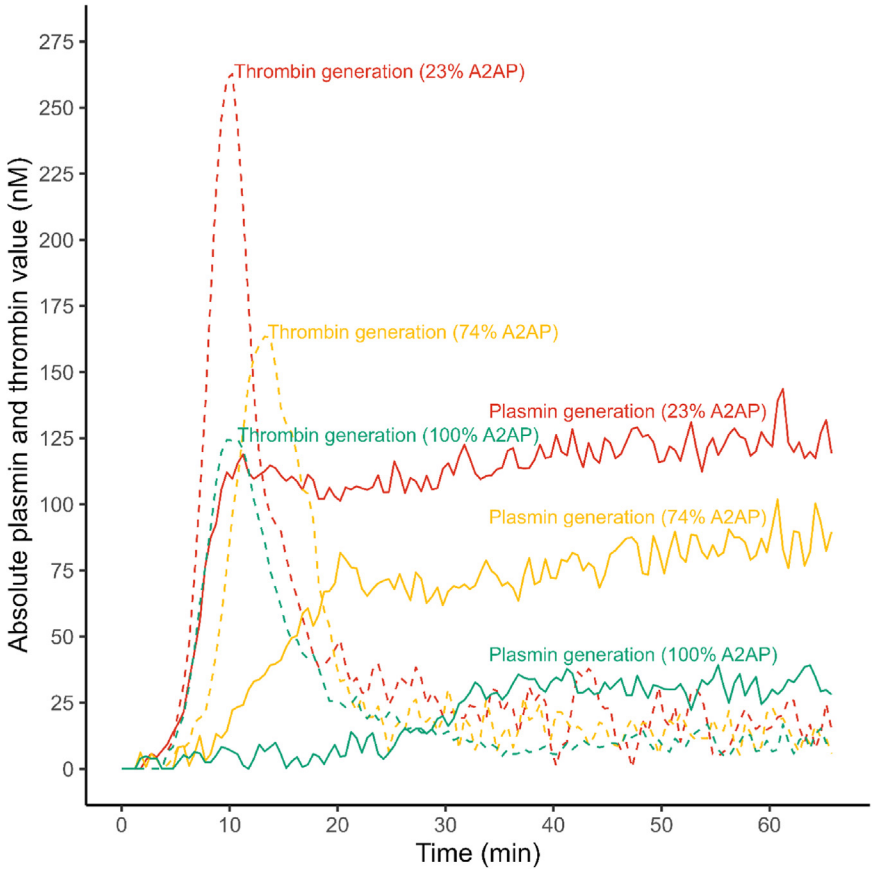

FIGURE 1 Examples of thrombin and plasmin generation assay curves from 2 patients with different A2AP activity levels (23% and 74%, as shown next to the curves) and a healthy individual (100%) to illustrate the relationship between thrombin and plasmin generation curves. A2AP, α2-antiplasmin.

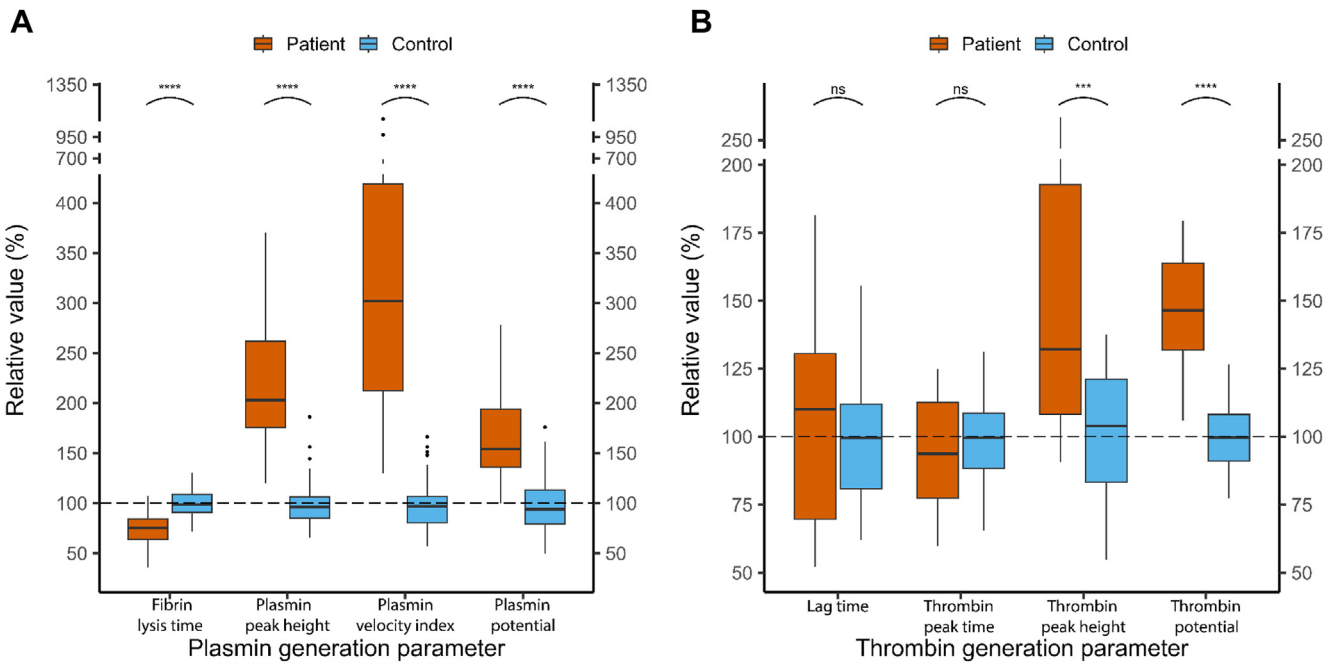

FIGURE 2 Relative plasmin (A) and thrombin (B) generation assay parameter values of alpha-2-antiplasmin deficient patients and controls. The dashed line corresponds to the average value of the controls (100%). ns, not significant; ***$P < .001$, ****$P < .0001$.

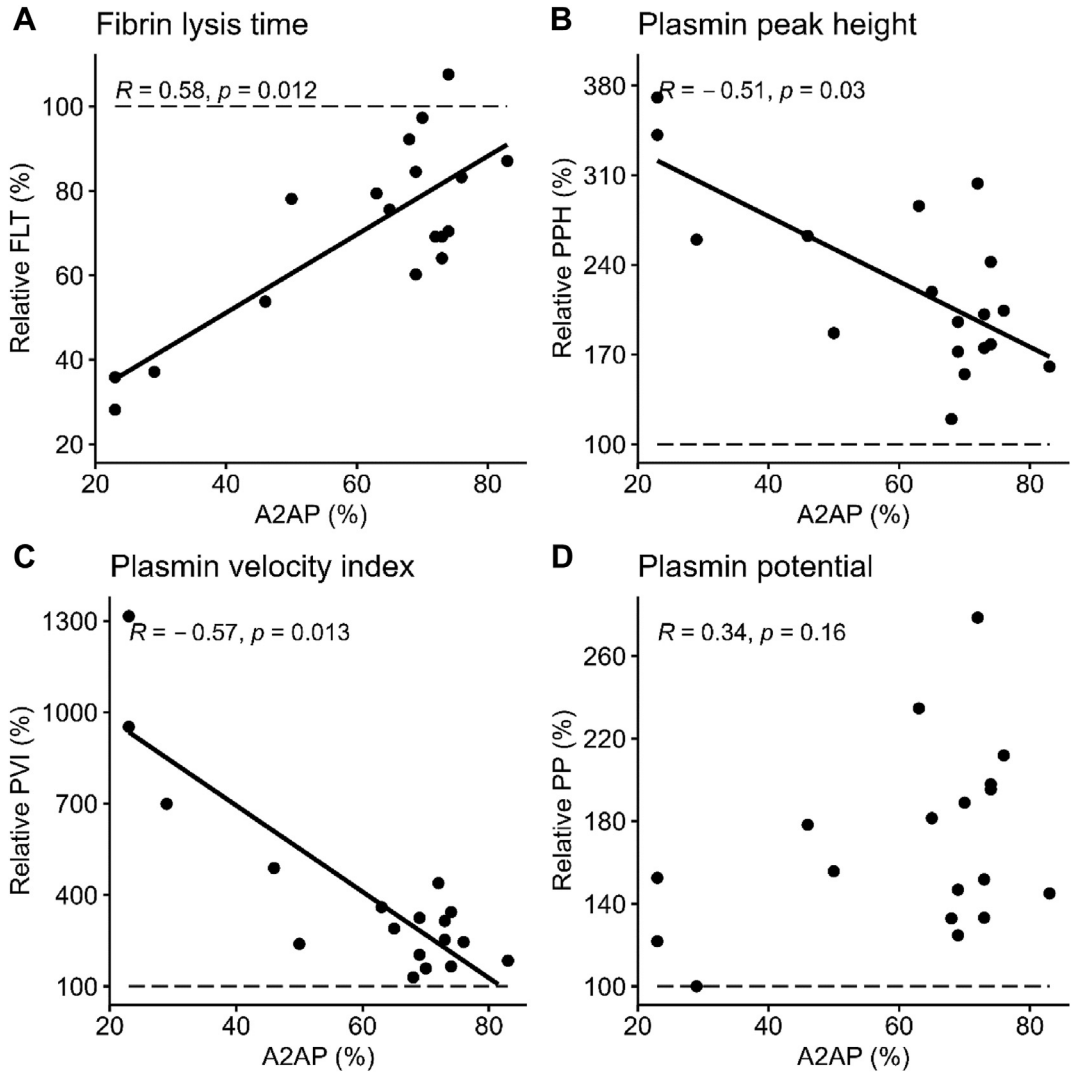

FIGURE 3 Correlation between plasmin generation assay parameter values and A2AP activity level. A2AP, α2-antiplasmin; FLT, fibrin lysis time; PP, plasmin potential; PPH, plasmin peak height; PVI, plasmin velocity index.

3.2.3 | Bleeding phenotype

A subsequent analysis focused on the bleeding tendency in relation to A2AP activity levels and PG parameter values. No statistically significant correlation was found between the A2AP activity level and the ISTH-BAT score of the A2AP-deficient patients ($R = -.35$, $P = .14$) (Supplementary Figure S2). Figure 6 displays the correlations between ISTH-BAT scores and various PG parameter values. ISTH-BAT scores exhibit a strong negative correlation with fibrin lysis time ($R = -.67$, $P = .003$) (Figure 6A) and strong positive correlations with plasmin peak height ($R = .60$, $P = .008$) (Figure 6B) and plasmin velocity index values ($R = .68$, $P = .002$) (Figure 6C).

3.3 | Genetic analysis

Finally, results of genetic analysis were available for 17 of the 23 A2AP-deficient patients, revealing 5 different genetic variants in the *A2AP* gene (Supplementary Table S1). The biallelic affected patients were either homozygous for the c.1185_1187dup variant or compound heterozygous for the c.528_530del and c.116delC variants, and they exhibited a severe laboratory and clinical phenotype. Monoallelic affected individuals possessed the c.528_530del, c.165+1G>A, or c.801G>A variant and displayed a milder laboratory and clinical phenotype. The variation in laboratory values and ISTH-BAT scores among family members and among unrelated patients was comparable. Consequently, no clustering of family members was observed in the various correlation plots.

4 | DISCUSSION

In this study, clearly enhanced PG profiles were observed in 23 A2AP-deficient patients from the Dutch RBiN population. These augmented PG profiles were characterized by shorter fibrin lysis times and higher plasmin peak heights, plasmin velocity indices, and plasmin potentials.

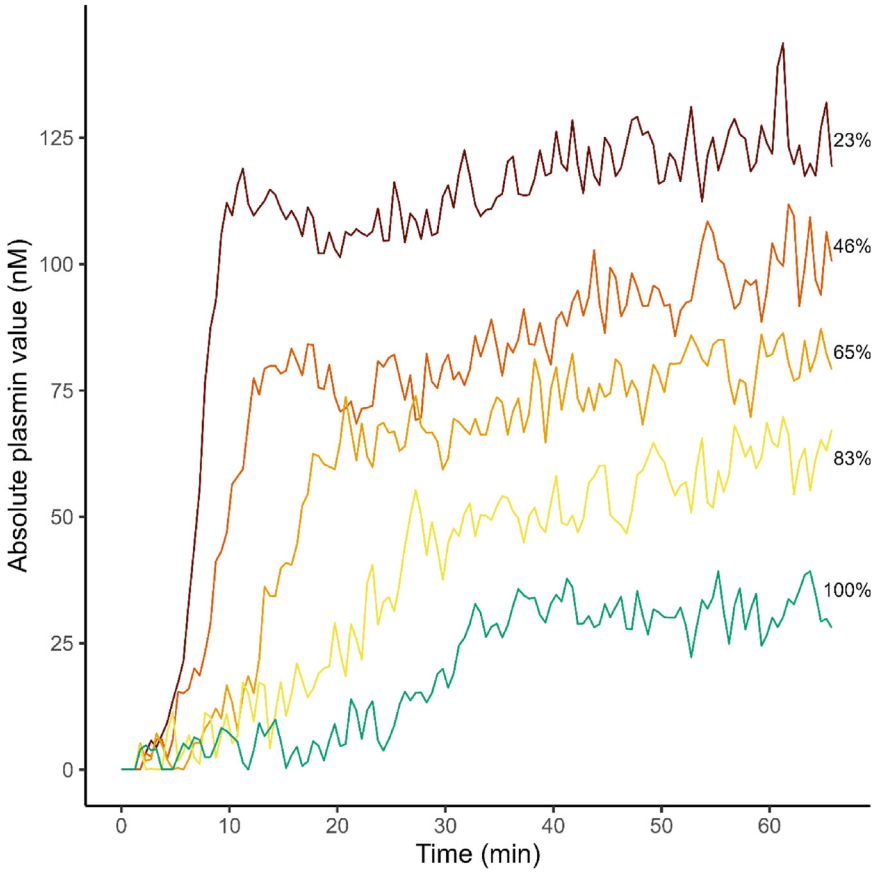

FIGURE 4 Examples of plasmin generation assay curves from patients with different A2AP activity levels (23%, 46%, 65%, and 83%, as shown next to the curves) and from a healthy control subject (A2AP activity level of 100%). A2AP, α2-antiplasmin.

Interestingly, TG profiles increased as well, with higher thrombin peak heights and thrombin potentials. These changes in PG and TG parameters (except for plasmin potential) were more pronounced in patients with a more severe A2AP deficiency and correlated with a more severe bleeding phenotype, as indicated by a higher ISTH-BAT score.

4.1 | Regulation of fibrinolysis

Fibrinolysis is a crucial and intricate process involving the binding of plasminogen to fibrin, followed by its conversion into plasmin through its natural activators tissue-type plasminogen activator and urokinase [22]. Kallikrein, factor (F)XIa, and FXIIa have also been identified as activators of plasminogen *in vitro* [23,24]. Subsequently, plasmin facilitates the dissolution of fibrin, making it a central enzyme in fibrinolysis [1]. To prevent excessive fibrinolysis leading to bleeding, regulatory proteins are involved in inhibiting the formation or action of plasmin, such as the nonspecific antiprotease α2-macroglobulin (A2M). This protein can inhibit various proteases, although this inhibition is not directed at the active site of the protease [25]. In this way, A2M can inhibit plasmin and thrombin, as well as other hemostatic proteins, such as FXa, activated protein C, tissue plasminogen activator, and urokinase. However, the net effect of A2M on the regulation of coagulation and fibrinolysis remains unclear, both in healthy individuals and in patients with bleeding disorders such as A2AP deficiency [26]. Other, more specific inhibitors of fibrinolysis include plasminogen activator inhibitor 1, thrombin activatable fibrinolysis inhibitor, and A2AP, with A2AP likely playing the principal role in fibrinolysis regulation [27,28]. A2AP serves as a direct inhibitor of plasmin by forming a stable inactive complex (A2AP-plasmin) with this protein. Additionally, A2AP can establish a strong binding with plasminogen, preventing the adsorption of plasminogen to fibrin, thereby inhibiting its activation [29,30]. Furthermore, A2AP indirectly blocks the activity of the tissue plasminogen activators and urokinase by competing for the identical binding site on the plasminogen molecule, resulting in reduced conversion of plasminogen to plasmin [1,30]. The pivotal role of A2AP in fibrinolysis inhibition underscores its potential as a therapeutic target, with ongoing research exploring an anti-A2AP antibody for deep vein thrombosis treatment (BAY3018250, Sirius trial).

4.2 | PG in A2AP-deficient patients

In our study, strong correlations of A2AP activity levels and several PG parameters were found, pointing to a dominant role of A2AP to inhibit plasmin. The hyperfibrinolytic state in A2AP deficiency was associated with a reduced fibrin lysis time and increased values for plasmin peak height, plasmin velocity index, and plasmin potential.

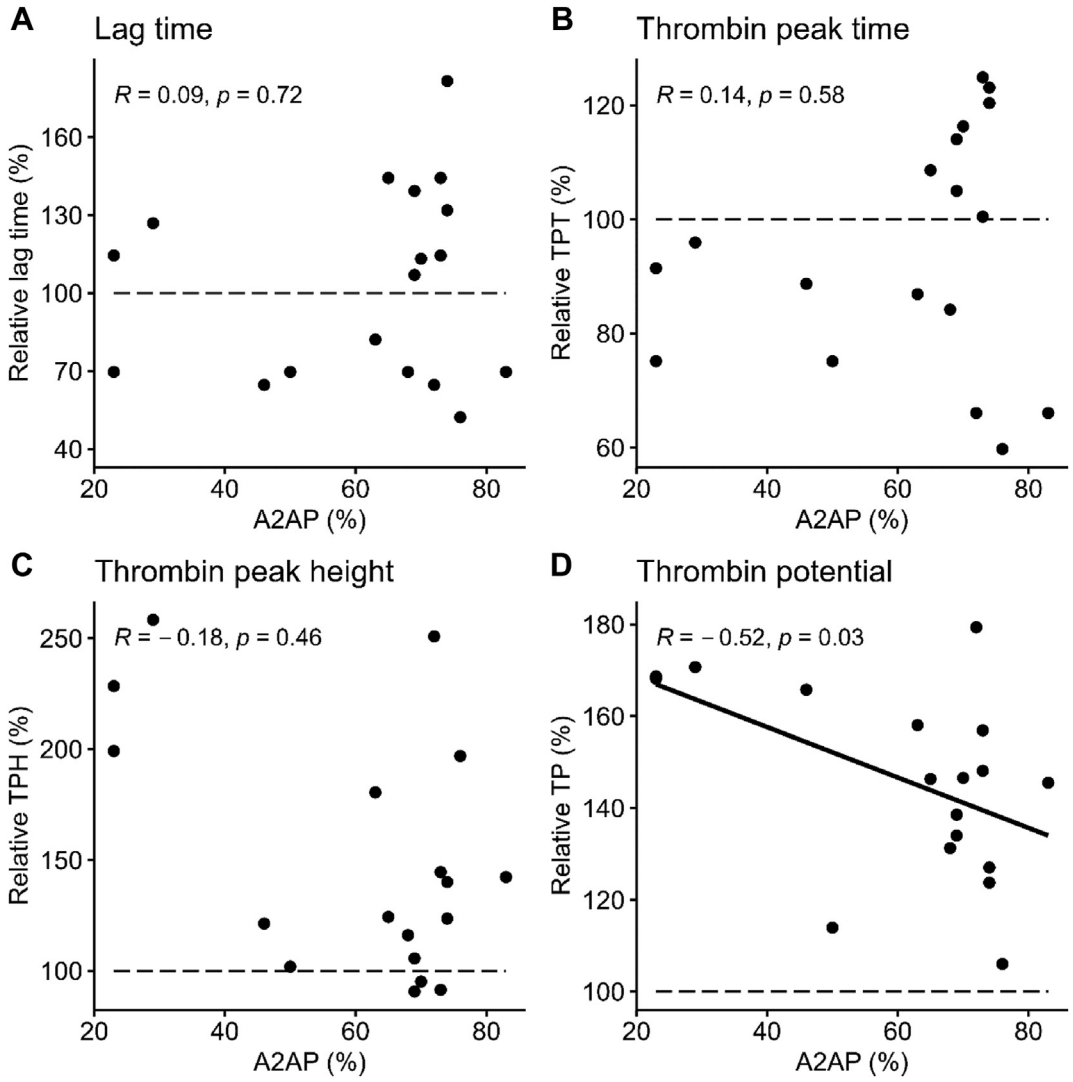

FIGURE 5 Correlation between thrombin generation assay parameter values and A2AP activity level. A2AP, α2-antiplasmin; LT, lag time; TP, thrombin potential; TPH, thrombin peak height; TTP, time to thrombin peak.

These effects were accentuated in more severe A2AP deficiency cases, except for the plasmin potential. Although a more severe deficiency resulted in a higher plasmin peak height, the concurrent further reduction in fibrin lysis time counteracts an increase in the overall area under the curve representing plasmin potential. This phenomenon is illustrated in Figure 4, showing patients with different A2AP activity levels. For this reason, the parameter plasmin potential seems less suitable as an indicator of hyperfibrinolysis. Therefore, we propose using fibrin lysis time, plasmin peak height, and a combination of these parameters (represented by the plasmin velocity index) as reliable measures for (hyper)fibrinolysis.

Because of the pivotal role of A2AP in fibrinolysis regulation, A2AP deficiency leads to a state of hyperfibrinolysis once fibrin is generated. The extent of hyperfibrinolysis can be inferred from the PG curve. Limited research has been conducted on PG in patients with a fibrinolytic disorder. Saes et al. [31] measured PG parameter values in 10 patients with a plasminogen activator inhibitor 1 deficiency. In these patients, hyperfibrinolysis was reflected by an increased plasmin peak height and plasmin potential compared with those of healthy individuals [31]. PG measurements in A2AP-deficient patients have not been conducted previously, although experiments have been performed on plasma samples immunodepleted of A2AP [32]. In the study by Simpson et al. [32], elevated plasmin peak height (116%), plasmin potential (150%), and plasmin velocity index (304%) were observed, which aligns with our findings. However, due to the study setup, no correlation analysis could be performed between the level of enhanced PG and the bleeding phenotype.

4.3 | TG in A2AP-deficient patients

As discussed, A2AP-deficient patients also exhibited significantly elevated thrombin potentials compared with healthy controls, with thrombin potential correlating with the severity of A2AP deficiency.

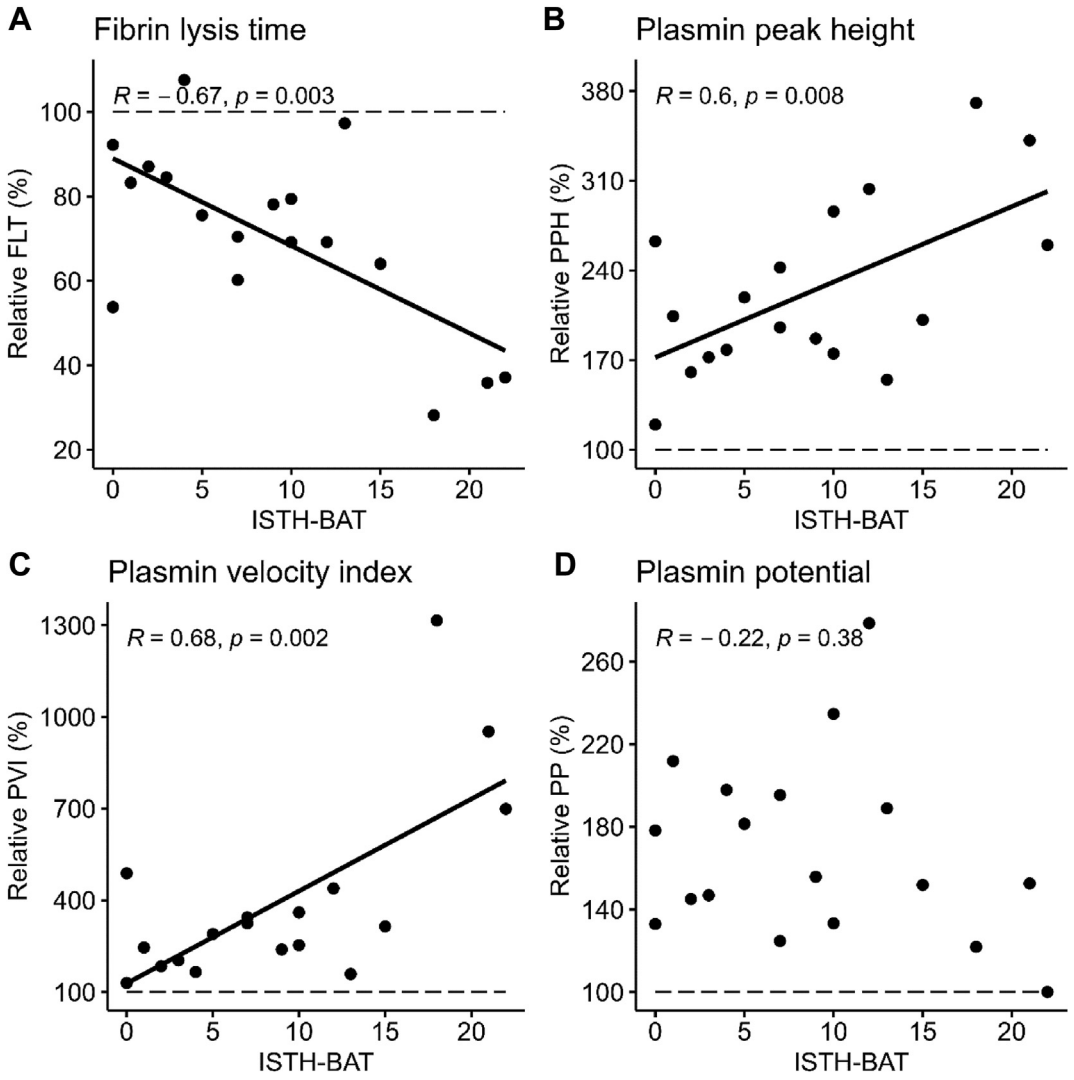

FIGURE 6 Correlation between plasmin generation assay parameter values and ISTH-BAT score. ISTH-BAT, International Society for Thrombosis and Hemostasis bleeding assessment tool; FLT, fibrin lysis time; PP, plasmin potential; PPH, plasmin peak height; PVI, plasmin velocity index.

A straightforward explanation for this effect might be off-target cross-reactivity of plasmin to the thrombin substrate used in the NHA. However, a thorough validation study for the NHA did not show any cross-reactivity of plasmin to the thrombin substrate used in the NHA [21]. While plasmin is most commonly known for its role in fibrinolysis through the cleavage of cross-linked fibrin, as a relatively nonspecific protease, it is also involved in the activation and degradation of other plasma proteins. Therefore, it is conceivable that higher plasmin levels in A2AP deficiency may lead to increased net activation of coagulation factors, resulting in enhanced TG. Previous research has demonstrated that plasmin is capable of activating coagulation FV and FVII *in vitro* [33,34]. Moreover, in other studies it was found that low concentrations of plasmin could activate FVIII:C and the VIII/von Willebrand factor complex in the presence of platelets. Plasmin is also able to activate platelets, although the effect of plasmin on FVIII:C and FVIII/von Willebrand factor was shown to be independent of the platelet secretion reaction [35,36].

In addition to these laboratory-based studies, several clinical studies support our results. A study involving trauma patients with hyperfibrinolysis noted elevated TG profiles compared with those of control subjects [37]. Furthermore, other studies have documented the occurrence of the thrombolytic paradox, as administration of fibrinolytic agents to achieve thrombolysis in patients with acute myocardial infarction induces an augmented procoagulant response [38,39]. In the absence of heparin treatment, this effect may contribute to adverse outcomes, such as early reocclusion, prolonged duration until reperfusion, or failure to achieve reperfusion [40]. Ewald and Eisenberg [41] demonstrated that plasmin-medicated activation of the intrinsic pathway appears to account at least in part for this procoagulant effect of fibrinolytic drugs.

4.4 | Bleeding phenotype

Our study also revealed the correlation between enhanced PG and a more severe bleeding phenotype, expressed as the ISTH-BAT score. The most frequently observed bleeding symptoms in our study included bleeding after major trauma or surgery, bleeding after tooth extraction and menorrhagia, in line with existing literature on bleeding events in A2AP deficiency [4,5]. These manifestations were evident in both biallelic and monoallelic patients, with biallelic patients (representing a severe A2AP deficiency) describing more severe symptoms. Additionally, biallelic patients reported occurrence of muscle bleeding, hemarthrosis, hematomas, and oral bleeding, whereas these bleeding manifestations were almost absent in the monoallelic patients. In comparison with bleeding scores of other RBDs included in the RBiN study, the median ISTH-BAT score was slightly lower for A2AP deficiency (7 vs 10) [20].

This study clearly demonstrates an association between the bleeding phenotype of A2AP-deficient patients and the degree of PG, exhibiting a well-defined distribution. The heterogenicity of the patients' bleeding tendency in relation to the A2AP activity level can be explained by different genetic variations or variability in proteoforms unrelated to A2AP. These effects are not reflected in the A2AP activity level but will affect PG. This highlights the practical value of incorporating a PG assay in assessing the bleeding risk of patients with A2AP deficiency.

4.5 | Limitations

A potential limitation of our study is the relatively small study group of A2AP-deficient patients, with the majority of included patients possessing a mild A2AP deficiency based on a monoallelic genetic variant. However, given the extremely low prevalence of the disorder, we consider this group to be a reasonable representation of A2AP-deficient patients. Additionally, the distribution of A2AP activity levels in the investigated population aligns with the epidemiology of A2AP-deficient patients [4]. A second potential limitation is that some of the included patients belong to families with relatives who were also included in the study. Therefore, an analysis was conducted to assess the impact of familial relationships on the results, revealing no correlation between familial ties and A2AP activity levels, TG, PG, and bleeding phenotype. Nevertheless, the influence of familial relationships on the results cannot be entirely ruled out. Third, the levels of free and complexed α2-macroglobulin were not measured, and consequently, the influence of this nonspecific inhibitor could not be assessed in our study. However, it remains unclear what these measurements would imply for the *in vivo* situation, as complexed α2-macroglobulin is cleared from circulation by the LRP-1 receptor [42,43]. Lastly, in order to provide any recommendations on the bleeding risk based on individual TG and PG profiles, a prospective clinical study should be conducted in the future for patients with these very RBDs, even focusing on international collaborations.

5 | CONCLUSION

In conclusion, this is the first study describing the enhanced patterns of PG and TG in patients with an A2AP deficiency and the strong correlation with the bleeding phenotype. These observations apply not only to the presumably very rare biallelic patients but also to the more common monoallelic A2AP-deficient patients with milder deficiencies. Consequently, currently, a potential distinct group of patients with a mild A2AP deficiency who exhibit a clear bleeding phenotype are not recognized as such. Therefore, increased awareness and efforts to diagnose mild forms of A2AP deficiency are warranted in patients with an unexplained bleeding tendency in order to increase our knowledge of and provide appropriate care for this RBD.

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AUTHOR CONTRIBUTIONS

M.H.C., P.L.d.E., I.C.K., K.M., L.N., N.v.E., R.E.G.S., W.L.v.H., and S.E.M.S. are members of the steering committee that designed the RBiN study and performed data collection. They are delegates of all Dutch Hemophilia Treatment Centers. N.M.A.B. is the head of the Department of Hematology at Radboud University Medical Center and head of the RBiN project management team. B.H. performed statistical analyses, interpreted data, and wrote the manuscript. S.R.R., W.L.v.H and S.E.M.S. interpreted data. All authors critically revised and approved the final version of the manuscript.

RELATIONSHIP DISCLOSURES

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SUPPLEMENTARY MATERIAL

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