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RESEARCH ARTICLE

# Generation and characterization of monoclonal antibodies against the N-terminus of alpha-2-antiplasmin

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## Abstract

Around 70% of circulating alpha-2-antiplasmin ( $\alpha$ 2AP), the main natural plasmin inhibitor, is N-terminally cleaved between residues Pro12 and Asn13 by antiplasmin-cleaving enzyme. This converts native Met- $\alpha$ 2AP into the more potent fibrinolysis inhibitor Asn- $\alpha$ 2AP. The Arg6Trp (R6W) polymorphism affects the N-terminal cleavage rate of Met- $\alpha$ 2AP in a purified system, with ~8-fold faster conversion of Met(R6)- $\alpha$ 2AP than Met(W6)- $\alpha$ 2AP. To date, assays to determine N-terminally intact Met- $\alpha$ 2AP in plasma have been limited to an ELISA that only measures Met(R6)- $\alpha$ 2AP. The aim of this study was to generate and characterize monoclonal antibodies (mAbs) against Met(R6)- $\alpha$ 2AP, Met(W6)- $\alpha$ 2AP and all  $\alpha$ 2AP forms (total- $\alpha$ 2AP) in order to develop specific Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP ELISAs. Recombinant Met(R6)- $\alpha$ 2AP, Met(W6)- $\alpha$ 2AP and Asn- $\alpha$ 2AP were expressed in *Drosophila* S2 cells. Using hybridoma technology, a panel of 25 mAbs was generated against a mixture of recombinant Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP. All mAbs were evaluated for their specific reactivity using the three recombinant  $\alpha$ 2APs in one-site non-competitive ELISAs. Three mAbs were selected to develop sandwich-type ELISAs. MA-AP37E2 and MA-AP34C4 were selected for their specific reactivity against Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP, respectively, and used for coating. MA-AP15D7 was selected for its reactivity against total- $\alpha$ 2AP and used for detection. With the novel ELISAs we determined Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP levels in plasma samples and we showed that Met(R6)- $\alpha$ 2AP was converted faster into Asn- $\alpha$ 2AP than Met(W6)- $\alpha$ 2AP in a plasma milieu. In conclusion, we developed two specific ELISAs for Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP, respectively, in plasma. This will enable us to determine N-terminal heterogeneity of  $\alpha$ 2AP in plasma samples.

decision to publish, or preparation of the manuscript.

**Competing interests:** The authors have declared that no competing interests exist.

## Introduction

Alpha-2-antiplasmin ( $\alpha$ 2AP), a member of the serine protease inhibitor (SERPIN) superfamily, is the main natural inhibitor of the fibrinolytic enzyme plasmin [1–3]. Native  $\alpha$ 2AP is secreted as an approximately 67-kDa single-chain glycoprotein of 464 amino acids, containing 11–14% carbohydrate, with a methionine (Met) as its N-terminus (Met- $\alpha$ 2AP) [4].  $\alpha$ 2AP is a heterogeneous protein possessing unique N- and C-terminal ends of which the N-terminus is involved in  $\alpha$ 2AP incorporation into a clot and the C-terminus in the initial interaction of  $\alpha$ 2AP with plasmin(ogen), as we recently extensively reviewed [5].

In the circulation  $\alpha$ 2AP undergoes both N- and C-terminal modifications, which significantly alter its inhibitory activity. In approximately 65% of circulating  $\alpha$ 2AP the C-terminus is intact so it can bind to plasmin(ogen) (plasminogen-binding form of  $\alpha$ 2AP, PB- $\alpha$ 2AP), whereas the remainder (~35%) has lost its ability to bind plasminogen (non-plasminogen-binding form of  $\alpha$ 2AP, NPB- $\alpha$ 2AP) [6–9]. Approximately 70% of circulating  $\alpha$ 2AP is N-terminally cleaved by antiplasmin-cleaving enzyme (APCE) between residues Pro12 and Asn13, converting native Met- $\alpha$ 2AP into the more potent fibrinolysis inhibitor Asn- $\alpha$ 2AP [10]. APCE is also known as a soluble, circulating derivative of fibroblast activation protein [11]. A glutamine residue in the N-terminus of  $\alpha$ 2AP (Gln14) serves as a substrate for activated Factor XIII (FXIIIa) leading to crosslinking and incorporation of  $\alpha$ 2AP into a fibrin clot [12]. This crosslinking reaction is more efficient when the N-terminus is removed, since Asn- $\alpha$ 2AP has been shown to crosslink 13 times more quickly into fibrin than Met- $\alpha$ 2AP [10]. Crosslinking of  $\alpha$ 2AP into fibrin clots results in resistance to fibrinolysis, especially when clots retract [13].

A genetic variation in the *SERPINF2* gene, the gene coding for human  $\alpha$ 2AP, has been described previously [14–16]. Christiansen *et al.* showed that the Arg to Trp polymorphism of codon 6 (R6W) in the N-terminus of  $\alpha$ 2AP is functionally significant, as it affects the conversion of Met- $\alpha$ 2AP to Asn- $\alpha$ 2AP by APCE, and thereby the rate of  $\alpha$ 2AP incorporation into fibrin [17]. They showed in a purified system that Met(R6)- $\alpha$ 2AP was cleaved approximately 8 times more quickly by APCE than Met(W6)- $\alpha$ 2AP, relating the polymorphism to the percentage of Met- $\alpha$ 2AP in plasma, with the highest percentage Met- $\alpha$ 2AP in W6 homozygote individuals. Methods for the measurement of N-terminal variation of  $\alpha$ 2AP will be useful in order to understand the role of  $\alpha$ 2AP N-terminal variation in crosslinking and thrombotic disease.

To date, assays to specifically determine N-terminally intact Met- $\alpha$ 2AP in plasma have been limited to an ELISA that can only measure Met(R6)- $\alpha$ 2AP, as we previously described [18]. The polyclonal antibody used in this ELISA lacked reactivity against Met(W6)- $\alpha$ 2AP, meaning that only R6 homozygote individuals could be analyzed. With an allele frequency of 81% for the R allele, on average 66% of study samples can be measured with this ELISA, resulting in loss of power. Therefore, in the current study we generated and characterized monoclonal antibodies (mAbs) with selective reactivity against Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP, to improve the assessment of N-terminal variation of  $\alpha$ 2AP.

## Materials and methods

### Plasma samples

$\alpha$ 2AP-depleted plasma was obtained from Affinity Biologicals (Ancaster, Ontario, Canada).

Normal pooled plasma was prepared from citrated apheresis plasma (Sanquin blood bank, Rotterdam, The Netherlands) from 5 healthy donors. Pooled plasma from R6 homozygote individuals (R6 pooled plasma) or W6 homozygote individuals (W6 pooled plasma) was prepared by pooling 100  $\mu$ l plasma samples from 18 and 17 previously genotyped control subjects of the ATTAC study population, respectively [19]. Genotyping of the  $\alpha$ 2AP polymorphism

R6W (rs2070863) was performed by TaqMan assay [18]. Both plasma pools had a similar  $\alpha$ 2AP activity in a chromogenic substrate assay.

### Production of recombinant $\alpha$ 2AP

Generation of recombinant Met(R6)- $\alpha$ 2AP and recombinant Asn- $\alpha$ 2AP was performed as described previously [20]. The wildtype construct which results in recombinant Met(R6)- $\alpha$ 2AP was used as a template and exposed to a mutagenesis procedure for the production of recombinant Met(W6)- $\alpha$ 2AP.

### Generation of mAbs and screening of hybridomas

mAbs against  $\alpha$ 2AP were produced in-house as described by Galfré and Milstein [21]. A mixture of recombinant Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP was used as antigen. The immunization, cell fusion and screening procedure have been performed as described previously [22]. Briefly, BALB/c mice were immunized by subcutaneous injection of 10  $\mu$ g  $\alpha$ 2AP in complete Freund's adjuvant, followed two weeks later by intraperitoneal injection of 10  $\mu$ g  $\alpha$ 2AP in incomplete Freund's adjuvant. After an interval of at least six weeks, the mice were boosted intraperitoneally with 10  $\mu$ g  $\alpha$ 2AP in saline on days four and two before the cell fusion. Spleen cells were isolated and fused with myeloma cells using polyethylene glycol. After selection, the supernatants were screened for specific antibody production with one-site non-competitive ELISAs using microtiter plates coated with recombinant Met(R6)- $\alpha$ 2AP, Met(W6)- $\alpha$ 2AP or Asn- $\alpha$ 2AP. The bound immunoglobulins were detected with horseradish peroxidase (HRP)-conjugated rabbit antimouse IgG. Positive clones were cultivated and the IgG fraction of the mAbs was purified from the culture supernatants by affinity chromatography using a ProSep-VA Ultra column (Merck Millipore, Darmstadt, Germany). HRP-conjugated mAbs were produced as described by Nakane and Kawaoi [23]. Determination of antibody isotype was carried out using the IsoStrip Mouse Monoclonal Antibody Isotyping kit (Roche Diagnostics, Vilvoorde, Belgium).

All mouse experiments were carried out after prospective approval of the KU Leuven Ethical Committee for Animal Experimentation (ECD) (Approval Number: P/055/2015). Anesthesia of the mice was performed using sevoflurane.

### Characterization of selected mAbs directed against Met- $\alpha$ 2AP by one-site non-competitive ELISA

Purified mAbs were tested for their specific reactivities with  $\alpha$ 2AP by one-site non-competitive ELISA. 96-well microtiter plates (Nunc A/S, Roskilde, Denmark) were coated with 2  $\mu$ g/ml (200  $\mu$ l per well in PBS containing 137 mM NaCl, 2.7 mM KCL, 6.5 mM Na<sub>2</sub>HPO<sub>4</sub> and 1 mM KH<sub>2</sub>PO<sub>4</sub> (pH 7.4)) of recombinant Met(R6)- $\alpha$ 2AP, Met(W6)- $\alpha$ 2AP or Asn- $\alpha$ 2AP for 24 hours at 4°C. Non-specific sites were blocked with 200  $\mu$ l 1% bovine serum albumin (Sigma, Steinheim, Germany) in PBS and incubated for two hours at RT. The plates were washed four times with 200  $\mu$ l 0.002% Tween-80 in PBS (PBST). Subsequently, 180  $\mu$ l of serial dilutions (100–0.0001  $\mu$ g/ml) of purified mAbs in PBST containing 0.1% BSA were added to the wells and incubated for two hours at RT. After four rounds of washing with 200  $\mu$ l PBST, HRP-conjugated polyclonal rabbit anti-mouse IgG (2 mg/ml) (Sigma) was added at an optimized dilution of 1:10000 and incubated for two hours at RT. Following four washes with 200  $\mu$ l PBST, the enzymatic activity was determined using o-phenylenediamine (Acros Organics, Geel, Belgium) in a solution containing 0.1 M sodium citrate and 0.2 M di-sodium phosphate and H<sub>2</sub>O<sub>2</sub> (Merck, Darmstadt, Germany) and the reaction was stopped after 45 minutes by adding

50  $\mu$ l of 4 M  $H_2SO_4$ . The absorbance was read at 492 nm on a ELx808 Absorbance Microplate Reader (BioTek Instruments, Winooski, VT).

Relative cross-reactivities of the selected mAbs with Met(W6)- $\alpha$ 2AP or Met(R6)- $\alpha$ 2AP were calculated by dividing the mAb concentrations at which half-maximal mAb binding occurred and by multiplying this ratio by 100.

### Reactivity of monoclonal antibodies on Western blot

To investigate the reactivities of the mAbs by Western blot, recombinant Met(R6)- $\alpha$ 2AP, Met(W6)- $\alpha$ 2AP or Asn- $\alpha$ 2AP in TBS were boiled for five to ten minutes at 95°C in the presence of XT Sample Buffer and XT reducing agent (BioRad, Richmond, CA) and resolved on a 10% SDS-polyacrylamide gel (300 ng/lane). After electrophoresis the proteins were transferred to a nitrocellulose membrane using a PowerPac™ HC power supply (BioRad) with transfer buffer (25 mM Tris, 192 mM glycine (pH 8.3) and 20% methanol) at 100V constant voltage for one hour. After protein transfer, non-specific sites on the nitrocellulose membrane were blocked with 5% milk in PBS, pH 7.4 followed by three washings in PBS with 0.1% Tween-20. Post-blocking, the blots were incubated with selected purified mAbs (800 ng/ml) in PBS with 0.1% Tween-20 for 18 hours under constant motion at 4°C. After three washes with PBS with 0.1% Tween-20, IRDye® 800CW donkey anti-mouse secondary antibodies (1 mg/ml) (Lincoln Nebraska, USA) were added at an optimized dilution of 1:10000 and incubated for one hour at RT. Following three washes with PBS, the blots were scanned in the 800 nm channel of an Odyssey® Imaging System (Lincoln Nebraska, USA).

### Development of Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP sandwich-type ELISAs

Three selected mAbs (MA-AP37E2 for Met(R6)- $\alpha$ 2AP, MA-AP34C4 for Met(W6)- $\alpha$ 2AP and MA-AP15D7 for all  $\alpha$ 2AP variants (total- $\alpha$ 2AP)) were used to develop sandwich-type ELISAs. MA-AP15D7 was HRP-conjugated to function as detection antibody. 96-well microtiter plates (Nunc A/S, Roskilde, Denmark) were coated with 2  $\mu$ g/ml of MA-AP37E2 or MA-AP34C4 (110  $\mu$ l per well in 0.05 M carbonate buffer (pH 9.6)) overnight at 4°C. Non-specific sites were blocked with 150  $\mu$ l 1% BSA in PBST for one hour at RT and plates were washed once with PBST. Subsequently, serial dilutions (1000–15.6 ng/ml) of recombinant Met(R6)- $\alpha$ 2AP, Met(W6)- $\alpha$ 2AP or Asn- $\alpha$ 2AP with 0.1% BSA in PBST were added. Plates were incubated for two hours at RT followed by four rounds of washing with PBST. Next, MA-AP15D7-HRP (2 mg/ml) was added and incubated for one hour at RT. For the Met(R6)- $\alpha$ 2AP ELISA MA-AP15D7-HRP was diluted 8000 times and for the Met(W6)- $\alpha$ 2AP ELISA MA-AP15D7-HRP was diluted 2000 times. Following four washes with PBST, enzyme activity was determined using 3,3',5,5'-tetramethylbenzidine (Sigma) as substrate, and the reaction was stopped after ten minutes by adding 100  $\mu$ l per well of 1 M  $H_2SO_4$ . The absorbance was read at 450 nm on a Victor<sup>3</sup> 1420 multilabel plate counter (Perkin Elmer, Waltham, MA, USA).

For the analysis of native Met- $\alpha$ 2AP in plasma, serial dilutions of plasma samples (normal pooled plasma, R6 pooled plasma, W6 pooled plasma and  $\alpha$ 2AP-depleted plasma) were added. The assays were performed using plasma dilutions of 1:40–1:2560 for the Met(R6)- $\alpha$ 2AP ELISA and plasma dilutions of 1:5–1:320 for the Met(W6)- $\alpha$ 2AP ELISA. The responses of the different plasma samples in the ELISAs are expressed as the mean result of triplicate measurements.

For the calculation of Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP concentrations in plasma samples we took the following into account: (1) normal pooled plasma of healthy individuals contains 70  $\mu$ g/ml  $\alpha$ 2AP [5], which is an average value (normal range 45–100  $\mu$ g/ml), and which refers to total  $\alpha$ 2AP, including both Met- $\alpha$ 2AP and Asn- $\alpha$ 2AP, (2) our ELISAs only detect Met- $\alpha$ 2AP (3), W6 pooled plasma contains 56.4% Met- $\alpha$ 2AP and R6 pooled plasma contains

23.6% Met- $\alpha$ 2AP [17]. This implied that R6 pooled plasma contains 16.5  $\mu$ g/ml Met(R6)- $\alpha$ 2AP and W6 pooled plasma contains 39.5  $\mu$ g/ml Met(W6)- $\alpha$ 2AP. Standard curves of R6 pooled plasma and W6 pooled plasma were used for calibration in order to determine Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP concentrations in plasma samples. Standard curves were fitted by means of a non-linear regression model using Graphpad Prism (version 5.01).

### Variation of Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP

Variation of Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP in R6 homozygote individuals ( $n = 18$ ) and W6 homozygote individuals ( $n = 17$ ) was determined by the Met(R6)- $\alpha$ 2AP ELISA and Met(W6)- $\alpha$ 2AP ELISA, respectively. The assays were performed using plasma dilutions of 1:600 for the Met(R6)- $\alpha$ 2AP ELISA and plasma dilutions of 1:20 for the Met(W6)- $\alpha$ 2AP ELISA. Normal pooled plasma was used to determine the inter- and intra-assay coefficients of variation (CV) of the ELISAs.

### Conversion of Met- $\alpha$ 2AP into Asn- $\alpha$ 2AP during incubation of normal pooled plasma

The conversion of Met- $\alpha$ 2AP into Asn- $\alpha$ 2AP by APCE was assessed by incubating normal pooled plasma at 29°C for 0, 6, 24, 30, 48, 54 and 72 hours followed by analysis in the Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP ELISAs.

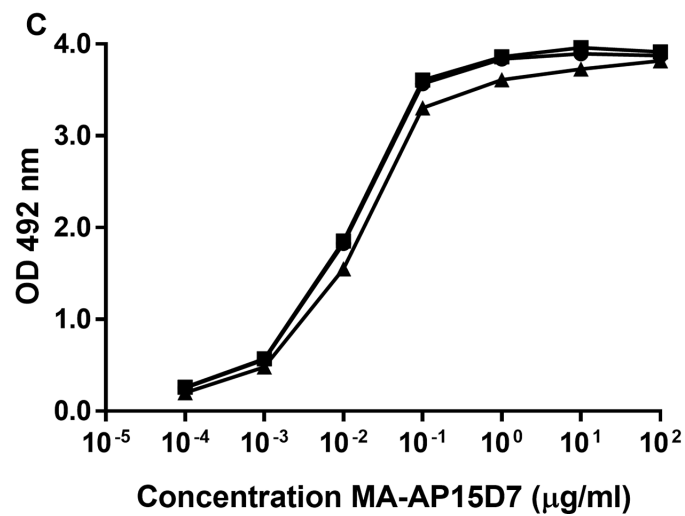
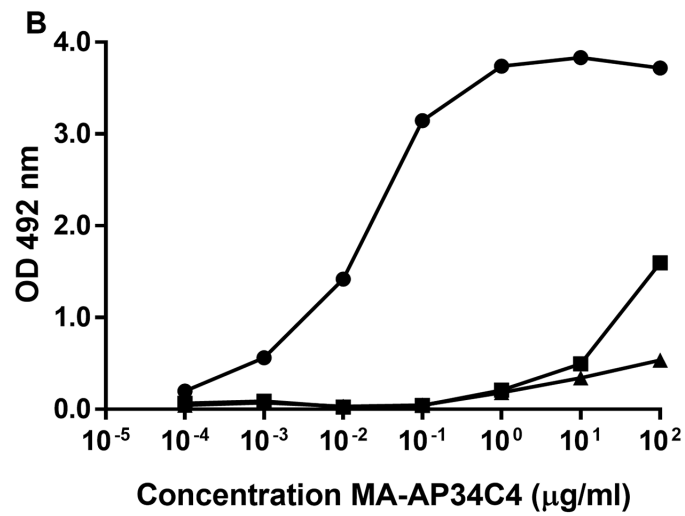
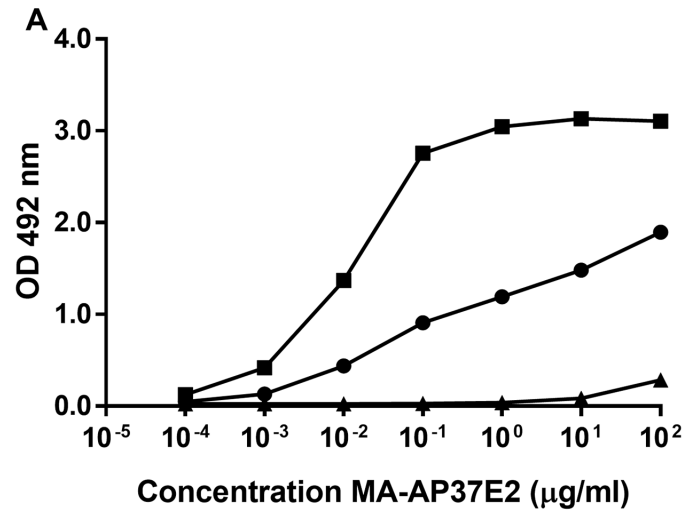
## Results

### Characterization of monoclonal antibodies

In total a panel of 25 mAbs against  $\alpha$ 2AP was generated of which 8 mAbs were specific for Met- $\alpha$ 2AP. For the development of novel Met- $\alpha$ 2AP ELISAs we selected three mAbs. MA-AP37E2 and MA-AP34C4 were selected for their relatively specific reactivity against Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP respectively (Fig 1A and 1B). MA-AP15D7 was selected for its reactivity against total- $\alpha$ 2AP as it reacted equally with Met(R6)- $\alpha$ 2AP, Met(W6)- $\alpha$ 2AP and Asn- $\alpha$ 2AP (Fig 1C). Isotyping of the three selected mAbs revealed that MA-AP37E2 and MA-AP34C4 are IgG1 antibodies with kappa light chains and that MA-AP15D7 is an IgG2b antibody with kappa light chains.

MA-AP37E2 showed 0.1% cross-reactivity with Met(W6)- $\alpha$ 2AP and MA-AP34C4 showed 0.01% cross-reactivity with Met(R6)- $\alpha$ 2AP (Fig 1A and 1B). MA-AP37E2 showed a small degree of cross-reactivity (estimated to be less than 0.0001%) with Asn- $\alpha$ 2AP when MA-AP37E2 was applied at a high concentration (100  $\mu$ g/ml), while MA-AP34C4 showed some cross-reactivity with Asn- $\alpha$ 2AP when it was applied at a concentration from 1  $\mu$ g/ml to 100  $\mu$ g/ml. The relative cross-reactivities of both mAbs with Asn- $\alpha$ 2AP could not be determined reliably, since half-maximal binding was not reached. Since the mAb concentrations at which cross-reactivity with Asn- $\alpha$ 2AP occurred were high, these responses are considered negligible in our assays (see below).

In addition to the ELISA analyses, we analyzed the reactivity of the selected mAbs by Western blot. Results showed reactivities of MA-AP37E2, MA-AP34C4 and MA-AP15D7 with protein bands with molecular weights of ~53-kDa, representing recombinant Met(R6)- $\alpha$ 2AP, recombinant Met(W6)- $\alpha$ 2AP and recombinant Asn- $\alpha$ 2AP respectively (Fig 2A, 2B and 2C; S1 Fig). In this approach considerable cross-reactivity was observed. Besides response to Met(R6)- $\alpha$ 2AP, MA-AP37E2 showed a weak response to Met(W6)- $\alpha$ 2AP (Fig 2A). And MA-AP34C4 showed a similar response to both Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP (Fig 2B). Importantly,



**Fig 1. Reactivity of (A) MA-AP37E2, (B) MA-AP34C4 and (C) MA-AP15D7 with recombinant  $\alpha$ 2AP by one-site non-competitive ELISA.** 96-well microtiter plates were coated with recombinant Met(R6)- $\alpha$ 2AP (■), Met(W6)- $\alpha$ 2AP (●) or Asn- $\alpha$ 2AP (▲) (2  $\mu$ g/ml) and serial dilutions (100–0.0001  $\mu$ g/ml) of MA-AP37E2, MA-AP34C4 or MA-15D7 were added to determine their reactivity. One representative experiment is shown out of three similar experiments. Concentration mAb is shown on a log scale.

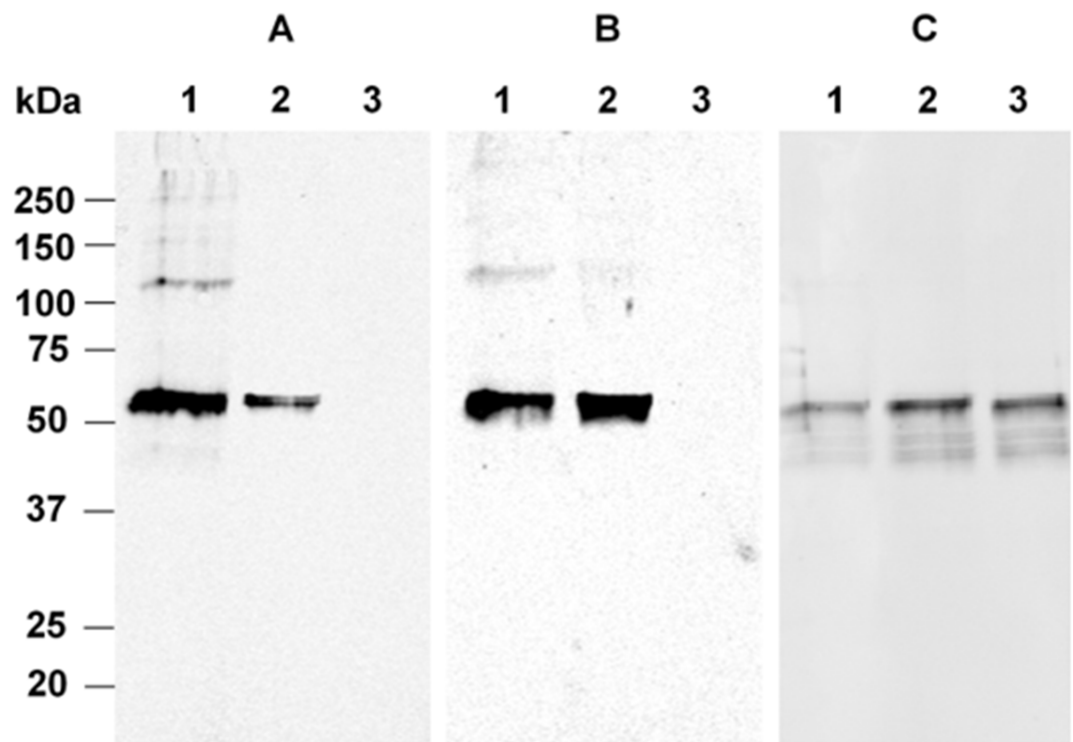
<https://doi.org/10.1371/journal.pone.0196911.g001>

MA-AP37E2 and MA-AP34C4 did not show any reactivity against recombinant Asn- $\alpha$ 2AP (Fig 2A and 2B), indicating their selective reactivity for N-terminally intact Met- $\alpha$ 2AP.

### Development of Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP sandwich-type ELISAs

For the development of sandwich-type ELISAs, MA-AP37E2 (used in the Met(R6)- $\alpha$ 2AP ELISA) and MA-AP34C4 (used in the Met(W6)- $\alpha$ 2AP ELISA) were applied as coating antibodies and HRP-conjugated MA-AP15D7 as detection antibody. Recombinant Met(R6)- $\alpha$ 2AP showed high reactivity in the Met(R6)- $\alpha$ 2AP ELISA, whereas virtually no reactivity was observed for recombinant Met(W6)- $\alpha$ 2AP and recombinant Asn- $\alpha$ 2AP up to a concentration of 1000 ng/ml (Fig 3A). Recombinant Met(W6)- $\alpha$ 2AP showed high reactivity in the Met(W6)- $\alpha$ 2AP ELISA, whereas virtually no reactivity was displayed for recombinant Met(R6)- $\alpha$ 2AP and recombinant Asn- $\alpha$ 2AP up to a concentration of 1000 ng/ml (Fig 3B). These results demonstrate the specificities of the Met(R6)- $\alpha$ 2AP ELISA and Met(W6)- $\alpha$ 2AP ELISA for Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP, respectively.

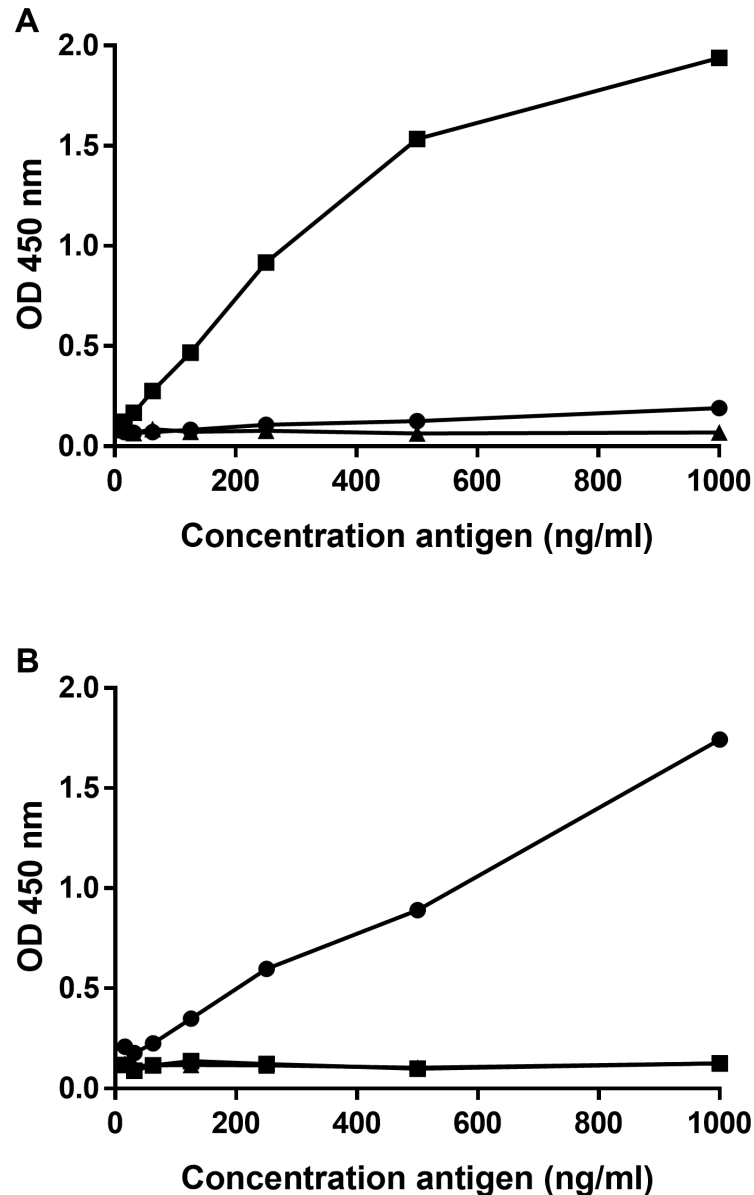
Applying plasma to the novel ELISAs revealed a high reactivity for R6 pooled plasma in the Met(R6)- $\alpha$ 2AP ELISA, but no reactivity for W6 pooled plasma. Additionally, W6 pooled plasma showed high reactivity in the Met(W6)- $\alpha$ 2AP ELISA, but no significant response was displayed for R6 pooled plasma (Fig 4A and 4B). A matrix effect possibly explains why the



**Fig 2. Reactivity of (A) MA-AP37E2, (B) MA-AP34C4 and (C) MA-AP15D7 with recombinant  $\alpha$ 2AP on Western blot.** Lane 1: Met(R6)- $\alpha$ 2AP (300 ng), lane 2: Met(W6)- $\alpha$ 2AP (300 ng), lane 3: Asn- $\alpha$ 2AP (300 ng). The migration distances of molecular weight marker proteins are indicated on the left.

<https://doi.org/10.1371/journal.pone.0196911.g002>





**Fig 3. Reactivity of recombinant  $\alpha$ 2AP in the (A) Met(R6)- $\alpha$ 2AP ELISA and (B) Met(W6)- $\alpha$ 2AP ELISA.** 96-well microtiter plates were coated with MA-AP37E2 or MA-AP34C4 (2  $\mu$ g/ml) and serial dilutions (1000–15.6 ng/ml) of recombinant Met(R6)- $\alpha$ 2AP (■), Met(W6)- $\alpha$ 2AP (●) or Asn- $\alpha$ 2AP (▲) were added. HRP-conjugated MA-AP15D7 was used as a detection antibody. Representative experiments out of three similar experiments are shown.

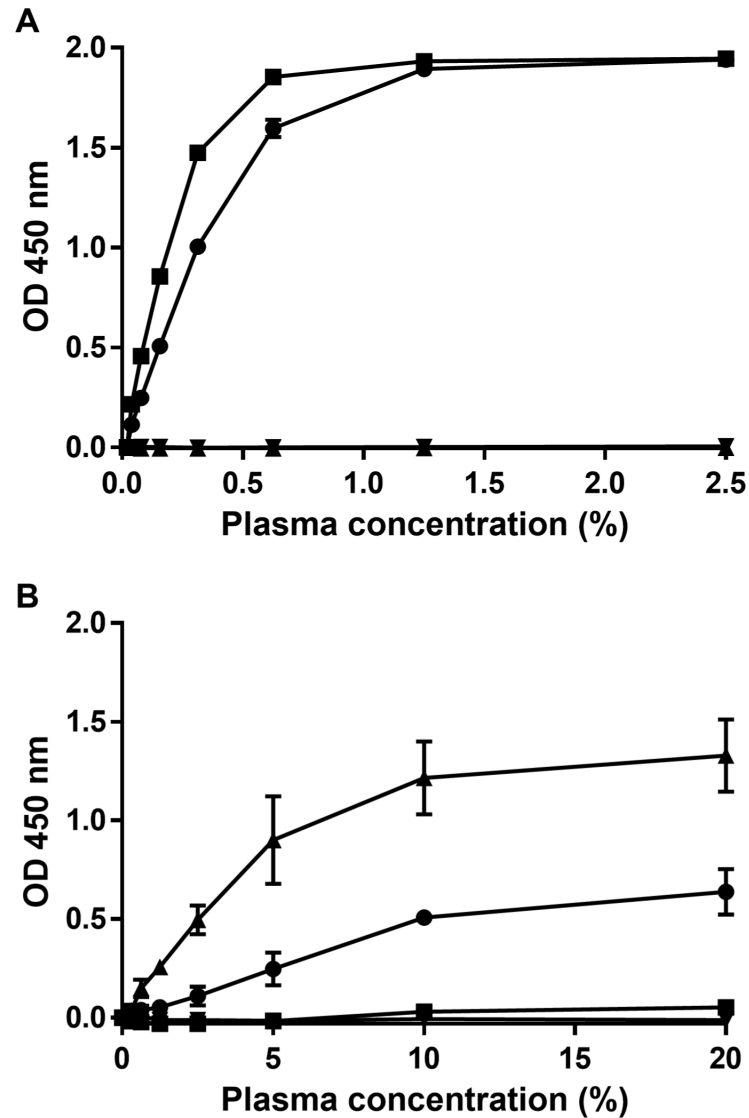
<https://doi.org/10.1371/journal.pone.0196911.g003>

responses of the least diluted plasma samples did not further increase in Fig 4B. No reactivity was observed for  $\alpha$ 2AP-depleted plasma in both ELISAs.

Using the Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP ELISAs, we quantified native Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP in normal pooled plasma. We found that normal pooled plasma used in the current study contains 6.4  $\mu$ g/ml Met(R6)- $\alpha$ 2AP and 6.9  $\mu$ g/ml Met(W6)- $\alpha$ 2AP.

### Variation of Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP

Plasma samples of R6 and W6 homozygote individuals were analyzed in the Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP ELISA, respectively (Fig 5). Met(R6)- $\alpha$ 2AP levels in R6 homozygote



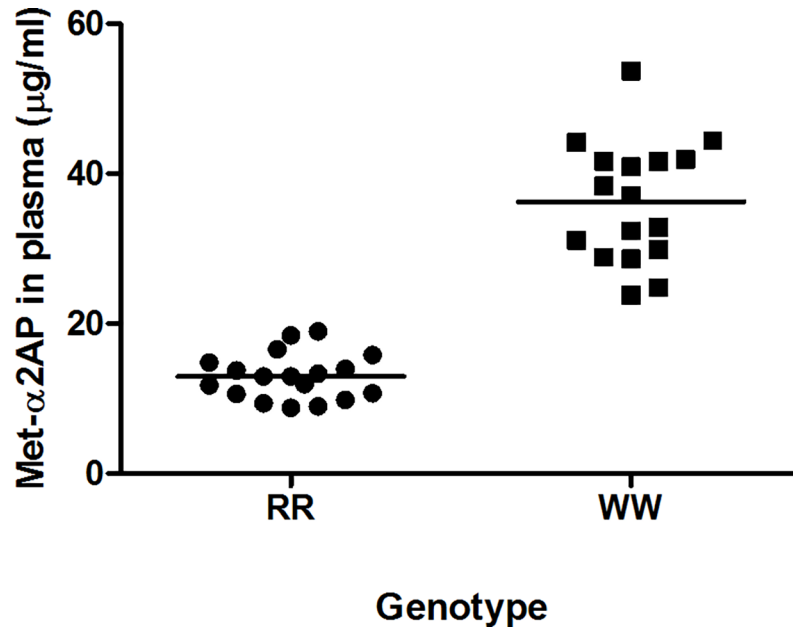
**Fig 4. Reactivity of plasma  $\alpha$ 2AP in the (A) Met(R6)- $\alpha$ 2AP ELISA and (B) Met(W6)- $\alpha$ 2AP ELISA.** 96-well microtiter plates were coated with MA-AP37E2 or MA-AP34C4 (2  $\mu$ g/ml) and varying plasma concentrations (0–2.5% for MA-AP37E2 and 0–20% for MA-AP34C4) of R6 pooled plasma (■), W6 pooled plasma (▲), normal pooled plasma (●) or  $\alpha$ 2AP-depleted plasma (▼) were tested. Representative experiments out of three similar experiments are shown. Standard deviations of assays performed in triplicate are shown as error bars.

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individuals ranged from 9 to 19  $\mu$ g/ml with a mean  $\pm$  sd of  $13 \pm 3$   $\mu$ g/ml (CV = 24%). Met (W6)- $\alpha$ 2AP levels in W6 homozygote individuals ranged from 24 to 54  $\mu$ g/ml with a mean  $\pm$  sd of  $36 \pm 8$   $\mu$ g/ml (CV = 22%). Inter- and intra-assay CV for the Met(R6)- $\alpha$ 2AP ELISA were 9.4% and 2.7%, respectively. Inter- and intra-assay CV for the Met(W6)- $\alpha$ 2AP ELISA were 16.9% and 4.9%, respectively.

### Conversion of Met- $\alpha$ 2AP to Asn- $\alpha$ 2AP during incubation of normal pooled plasma

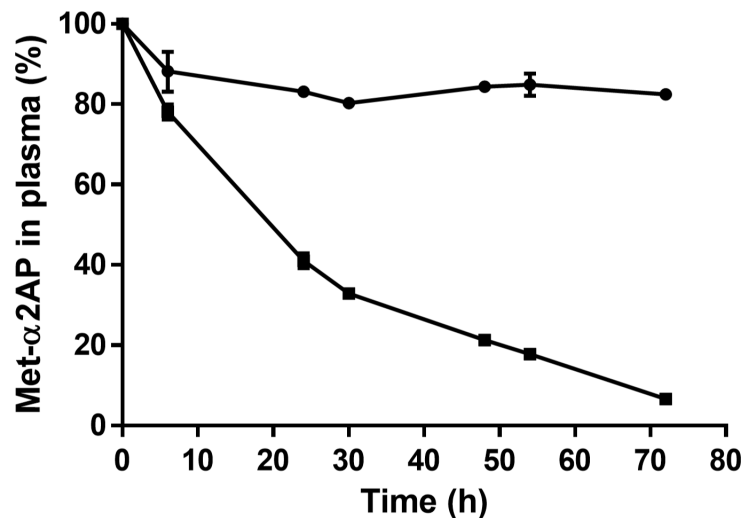
Christiansen et al. showed in a purified system that APCE cleaves Met(R6)- $\alpha$ 2AP approximately 8 times more quickly than Met(W6)- $\alpha$ 2AP [17]. To study this cleavage in a plasma



**Fig 5. Variation of Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP in R6 and W6 homozygote individuals.** Concentrations of Met(R6)- $\alpha$ 2AP (●) as determined by the Met(R6)- $\alpha$ 2AP ELISA and concentrations of Met(W6)- $\alpha$ 2AP (■) as determined by the Met(W6)- $\alpha$ 2AP ELISA. Met(R6)- $\alpha$ 2AP levels in 18 R6 homozygote individuals (RR) ranged from 9 to 19  $\mu$ g/ml with a mean  $\pm$  sd of  $13 \pm 3$   $\mu$ g/ml (CV = 24%). Met(W6)- $\alpha$ 2AP levels in 17 W6 homozygote individuals (WW) ranged from 24 to 54  $\mu$ g/ml with a mean  $\pm$  sd of  $36 \pm 8$   $\mu$ g/ml (CV = 22%). Bars represent the mean Met- $\alpha$ 2AP level.

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milieu, we evaluated the conversion of Met- $\alpha$ 2AP into Asn- $\alpha$ 2AP in normal pooled plasma using the new ELISAs. Over a course of 72 hours, Met(R6)- $\alpha$ 2AP was almost completely converted into Asn- $\alpha$ 2AP, whereas only approximately 20% of Met(W6)- $\alpha$ 2AP was converted (Fig 6). The ELISA results were not sufficiently accurate for a kinetic analysis, however they



**Fig 6. Evaluation of Met- $\alpha$ 2AP to Asn- $\alpha$ 2AP conversion in normal pooled plasma incubated at 29°C for 72 hours.** Percentage of Met(R6)- $\alpha$ 2AP (■) and Met(W6)- $\alpha$ 2AP (●) as determined by the novel Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP sandwich-type ELISAs. The variability of assays performed in duplicate is shown with error bars.

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confirm that Met(R6)- $\alpha$ 2AP is converted substantially more quickly into Asn- $\alpha$ 2AP by APCE than Met(W6)- $\alpha$ 2AP. Furthermore, they show that APCE is active in plasma incubated at 29°C for at least 72 hours.

## Discussion

Current assays to determine N-terminal variation of  $\alpha$ 2AP in plasma are limited to an ELISA that can only measure Met(R6)- $\alpha$ 2AP [18]. Here we describe the development of Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP specific ELISAs to overcome this limitation. For this, we generated and characterized novel mAbs against Met(R6)- $\alpha$ 2AP (MA-AP37E2) and Met(W6)- $\alpha$ 2AP (MA-AP34C4). Additionally, we generated a mAb against total- $\alpha$ 2AP (MA-AP15D7) that we used for detection in our ELISAs.

Previously, Christiansen et al. showed that R6 and W6 homozygous individuals have on average 23.6% Met(R6)- $\alpha$ 2AP and 56.4% Met(W6)- $\alpha$ 2AP, respectively [17]. Based on these percentages, we calculated that R6 homozygous individuals have 16.5  $\mu$ g/ml Met(R6)- $\alpha$ 2AP and that W6 homozygous individuals have 39.5  $\mu$ g/ml Met(W6)- $\alpha$ 2AP, when taking in account that plasma contains 70  $\mu$ g/ml  $\alpha$ 2AP. Using our ELISAs we found comparable values, since we determined a mean level of 13  $\mu$ g/ml Met(R6)- $\alpha$ 2AP in R6 homozygote individuals and a mean level of 36  $\mu$ g/ml Met(W6)- $\alpha$ 2AP in W6 homozygote individuals. The Met(R6)- $\alpha$ 2AP levels in R6 homozygote individuals were lower compared to Met(W6)- $\alpha$ 2AP levels in W6 homozygote individuals. This difference could be explained by 8-fold faster cleavage rate of Met(R6)- $\alpha$ 2AP, by APCE, as compared with cleavage of Met(W6)- $\alpha$ 2AP [17]. The data of the separate individuals showed that the biological variation in Met(R6)- $\alpha$ 2AP (CV = 24%) and Met(W6)- $\alpha$ 2AP (CV = 22%) is similar.

Earlier studies have generated and characterized mAbs reacting with distinct parts of the  $\alpha$ 2AP protein. A mAb against  $\alpha$ 2AP's C-terminus has been shown to be useful for the assessment of C-terminal cleavage [24], whereas mAbs against the internal region of  $\alpha$ 2AP were used for the determination of total- $\alpha$ 2AP [25]. Additionally, mAbs that inhibit  $\alpha$ 2AP activity have been described [26]. A recent study demonstrated that inactivation of  $\alpha$ 2AP by a mAb results in the dissolution of experimental pulmonary emboli in mice [27]. In this study a similar result was shown after the treatment of pulmonary emboli with recombinant r-tPA. However, in contrast to the r-tPA treatment, the mAb-directed inactivation of  $\alpha$ 2AP did not show side-effects such as fibrinogen degradation or enhanced experimental bleeding. The authors of the study suggested that modulation of the activity of  $\alpha$ 2AP by the mAb might have unique therapeutic value in pulmonary embolism. To date, no mAbs have been described as being against the  $\alpha$ 2AP N-terminus. One of the reasons is that the original preparations of purified  $\alpha$ 2AP contained only Asn- $\alpha$ 2AP [28]. Newer preparations revealed also Met- $\alpha$ 2AP [4], while the R6W SNP was only recognized in 2007 [17].

In a previous study on the assessment of  $\alpha$ 2AP N-terminal variation in plasma, the Met- $\alpha$ 2AP ELISA used a polyclonal antibody raised against the first twelve N-terminal amino acids of Met-(R6)- $\alpha$ 2AP [18]. This polyclonal antibody only recognized Met(R6)- $\alpha$ 2AP and not Met(W6)- $\alpha$ 2AP. The current study confirms that the immunological properties of the Met(W6)- $\alpha$ 2AP N-terminus differ from those of the Met(R6)- $\alpha$ 2AP N-terminus. It is interesting to note that none of the 8 generated Met- $\alpha$ 2AP specific antibodies reacted equally well with Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP (results not shown). The limitation of specificity of the polyclonal antibody for only Met(R6)- $\alpha$ 2AP is solved by our ELISAs as we can now measure both Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP.

We used the newly developed ELISAs to show that endogenous Met(R6)- $\alpha$ 2AP present in plasma is converted more quickly into Asn- $\alpha$ 2AP by APCE compared to Met(W6)- $\alpha$ 2AP. This

confirms and extends a previous study performed with purified Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP by Christiansen et al., who showed that Met(R6)- $\alpha$ 2AP is a better substrate for APCE than Met(W6)- $\alpha$ 2AP, since Met(R6)- $\alpha$ 2AP was cleaved ~8 times more quickly to Asn- $\alpha$ 2AP [17]. Possibly, the difference in the reactivity of APCE to either Met- $\alpha$ 2AP variant is affected by the same differences in conformational properties between Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP N-termini that cause their antigenic properties. Moreover, Met(R6)- $\alpha$ 2AP decreased over the course of a 72-hour plasma incubation, indicating that APCE remains active during that time period.

The selective reactivities of MA-AP37E2 and MA-AP34C4 in ELISA were not observed in the Western blot experiments. On the blots we found considerable cross-reactivity from both mAbs. The discrepancy between the ELISA- and Western blot results may partially be explained by our definition of cross-reactivity in ELISA (for instance, the ratio of mAb concentrations at which half-maximal mAb binding occurred in Fig 1) and partially by the conformational status of both Met- $\alpha$ 2AP variants, which are different in the ELISA and Western blotting procedures. Incomplete renaturation of Met- $\alpha$ 2AP during Western blotting, resulting in a more linear protein, might result in cross-reactivity of MA-AP37E2 with Met(W6)- $\alpha$ 2AP and of MA-AP34C4 with Met(R6)- $\alpha$ 2AP. This incomplete renaturation should not be present in ELISA, since the native conformation of the proteins is retained. Since Met(R6)- $\alpha$ 2AP only differs from Met(W6)- $\alpha$ 2AP by one amino acid residue at position 6 (R6W) on a stretch of 12 amino acids, the recognition of a linear amino acid sequence of both proteins by MA-AP37E2 and MA-AP34C4 will be partially similar. However, since a tryptophan is a more complex amino acid than an arginine, this substitution may have consequences for the 3D protein structure of the proteins [17]. To date, there is no 3D protein structure available for the entire N-terminus of  $\alpha$ 2AP [29].

To summarize, we have described the development of two novel ELISAs for Met(R6)- $\alpha$ 2AP and Met(W6)- $\alpha$ 2AP in plasma. This will enable us to improve the evaluation of the fibrinolytic potential in plasma samples in various disease states.

## Supporting information

**S1 Fig. Original Western blot result showing the reactivity of (A) MA-AP37E2, (B) MA-AP34C4 and (C) MA-AP15D7 with recombinant  $\alpha$ 2AP.** Lane 1: Met(R6)- $\alpha$ 2AP (300 ng), lane 2: Met(W6)- $\alpha$ 2AP (300 ng), lane 3: Asn- $\alpha$ 2AP (300 ng).  $\alpha$ 2AP protein bands are visualized in green. The molecular weight marker proteins are visualized in red. (TIF)

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**Writing – review & editing:** Frank W. G. Leebeek, Paul J. Declerck, Dingeman C. Rijken, Shirley Uitte de Willige.

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