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Effects of (Pre-)analytical Variables on Activated Protein C Resistance Determined Via a Thrombin Generation-based Assay

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Keywords

APC Resistance, Factor V_{Leiden}

Summary

The normalized activated protein C sensitivity ratio (nAPC-sr) determined with an assay that quantifies the effect of APC on thrombin formation initiated via the extrinsic coagulation pathway identifies hereditary and acquired defects of the protein C system We investigated the influence of assay conditions (analytical variables) and plasma handling (pre-analytical variables) on nAPC sr obtained with this APC resistance test The effect of the analytical variables ($CaCl_2$, phospholipid and APC concentrations and the concentration and source of tissue factor) was determined in pooled normal plasma Inhibition of thrombin formation by APC was dependent on the APC concentration and was also affected by the tissue factor, Ca^{2+} and phospholipid concentrations Thus, strict standardization of reactant concentrations is required to obtain reproducible nAPC sr Three different tissue factor preparations were compared by determining nAPCsr in plasma samples obtained from 90 healthy individuals nAPC sr were similar for all three tissue factor preparations although, compared with the non-commercially available tissue factor used in earlier studies, values determined with commercial tissue factor preparations showed larger variation Pre-analytical variables, investigated in plasma of nine volunteers (3 normal individuals and 6 individuals with an APC-resistant phenotype) were concentration of anticoagulant (3 2% vs 3 8% trisodiumcitrate), time before processing of blood (0, 4 and 24 h), centrifugation speed, storage temperature of plasma (–20° C vs –80° C) and sample thawing Multiple linear regression analysis showed that only the citrate concentration affected the nAPC-sr, which was higher in samples collected in 3 2% trisodiumcitrate than in samples collected in 3 8% trisodiumcitrate

Introduction

The identification of the A1691G mutation in the factor V gene (1 4), resulting in a mutated factor V molecule (factor V_{Leiden}, factor V_{R506Q}), has led to a higher number of patients with an identifiable prothrombotic disorder Arg_{506} represents a predominant target for proteolytic inactivation of factor Va by activated protein C (APC) and as a result of the Arg_{506}-Gln mutation factor Va_{Leiden} is more resistant to APC (1, 5 7) Moreover, it appears that the mutation in factor V_{Leiden} also results in a substantial loss of the cofactor activity of factor V in APC dependent down regulation of factor VIII(a) (8, 9) APC resistance, which is found in 20% of patients with venous thrombosis (10, 11), also occurs in individuals without factor V_{Leiden} and recently it was shown that APC resistance in the absence of the factor V_{Leiden} mutation is a risk factor for venous thrombosis (12 14) APC resistance without factor V_{Leiden} can be attributed to other hereditary defects of the protein C pathway or to acquired coagulation abnormalities occurring during oral contraceptive (OC) use or pregnancy (15 18)

In 1997 we developed an assay (17, 19) in which the anticoagulant effect of APC in plasma is quantified by measuring the effect of APC on thrombin generation initiated via the extrinsic coagulation pathway This assay not only allows detection of the factor V_{Leiden} mutation, but is also sensitive for acquired APC resistance occurring during OC use (17, 20, 21) In contrast, the classical aPTT based APC resistance test is much less affected by OC use (20)

Since APC sensitivity ratios (APCsr) determined with the thrombin generation-based assay change in parallel with thrombotic risks reported in epidemiological studies (22), this assay may gain interest of other research or coagulation laboratories In this paper we report how the quantification of APC resistance with the thrombin generation based test is affected by analytical variables i e the APC, Ca^{++} ions and phospholipid concentrations, and the source and concentration of tissue factor and by pre-analytical variables i e blood collection and handling and storage of plasma

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Materials and Methods

Materials

Tris (Tris (hydroxymethyl amino methane), Hepes (N-(2 Hydroxyethyl) piperazine N' (2 ethanesulfonic acid), NaCl, EDTA (Ethylenedinitrilo tetraacetic acid), BSA (bovine serum albumin) and ovalbumin were purchased from Sigma, St Louis, USA The chromogenic substrates D-Phe (pipecolyl) Arg pNA (S2238) and L-pyroGlu-Pro Arg-pNA (S2366) were supplied by Chromogenix, Molndal, Sweden Ancrod, obtained from the WHO International Laboratory for Biological Standards (NIBSC, Hertfordshire, England) was dissolved in distilled water (50 U/ml) and stored at –20° C

1,2 Dioleoyl-*sn* glycero-3 phosphoserine (DOPS), 1 2 dioleoyl-*sn* glycero 3 phosphocholine (DOPC) and 1 2 dioleoyl *sn* glycero 3 phosphoethanolamine

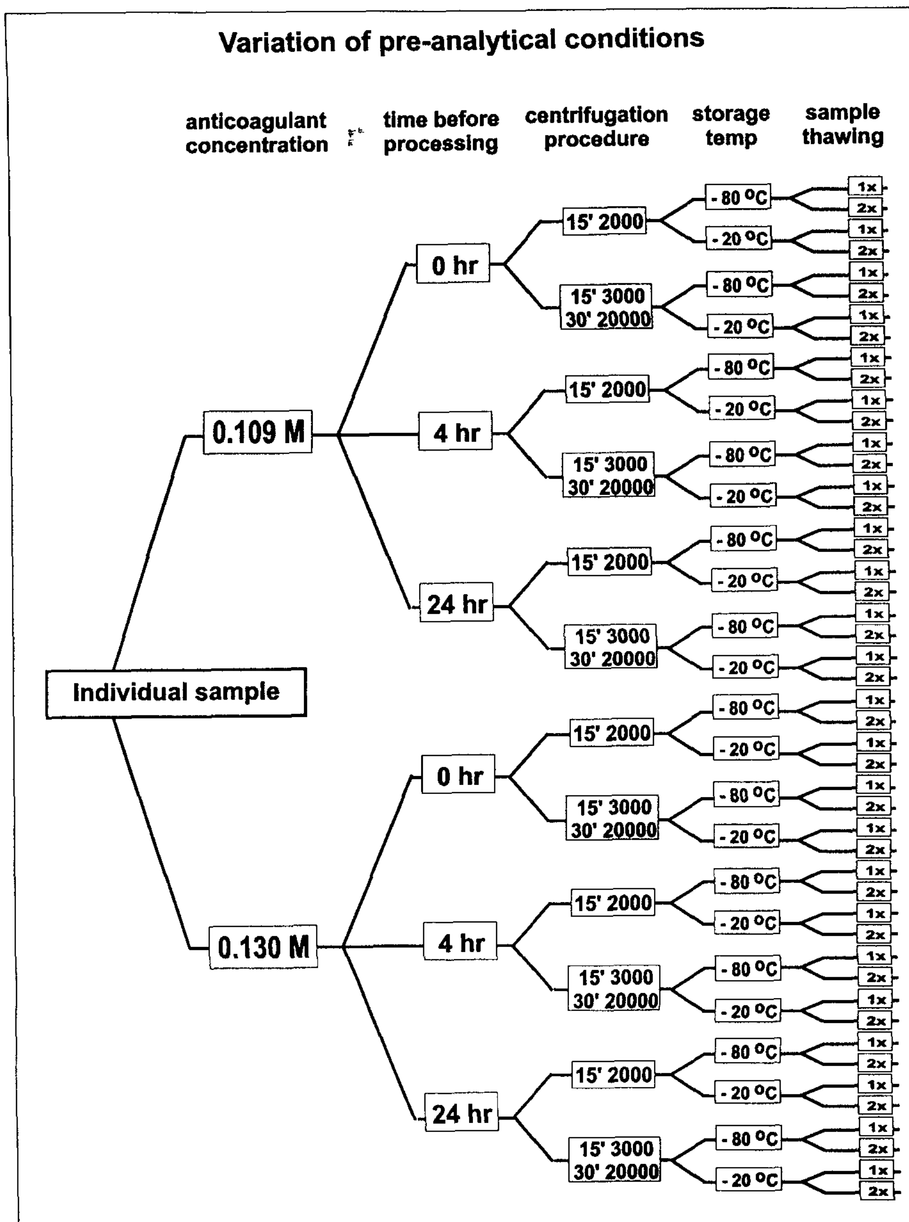

Fig 1 Schematic diagram of different procedures of plasma preparation and storage

(DOPE) were obtained from Avanti Polar Lipids, Alabaster, Alabama, USA Small unilamellar phospholipid vesicles composed of DOPS/DOPE/DOPC (20/20/60, M/M/M) were prepared by mixing appropriate quantities of phospholipid dissolved in $CHCl_3/CH_3OH$ (9/1 v/v) in a glass tube The phospholipids were dried under a mild flow of N_2 and stored at –20° C Before use the dried phospholipids were brought to room temperature, suspended in 25 mM Hepes (pH 7 5) 175 mM NaCl by rigorous vortexing for at least 1 min and subsequently sonicated for 10 min at 4° C with a MSE Soniprep 150 ultrasonic disintegrator set at 7 5 μm peak to peak amplitude Phospholipid concentrations were determined by phosphate analysis (23)

Purified human APC was from Enzyme Research Laboratories (ERL) and supplied by Kordia Laboratory Supplies, Leiden, The Netherlands APC was diluted in 25 mM Hepes (pH 7 5 at 37° C), 175 mM NaCl, 5 mg/ml BSA and frozen in small portions at –80° C APC concentrations were determined with S2366 using kinetic parameters reported by Sala et al (24)

Recomboplastin S without additives was a kind gift of Dr H Pelzer (Dade, USA) Relipidated recombinant tissue factor Dade Innovin® was purchased from Behring RecombiPlasTin© (Ortho) was from Instrumentation Laboratories Tissue factor preparations were reconstituted according to the instructions of the manufacturer In standard coagulation assays tissue factor is added in excess and can be used for several days when kept at 2–8° C following reconstitution In the thrombin generation based APC resistance assay tissue factor is used in high dilutions (see below and Results) and to improve day to day reproducibility reconstituted tissue factor preparations were stored in small aliquots at –80° C Tissue factor concentrations were determined with an antigen assay (American Diagnostics)

Plasma Preparation for Investigating the Effect of Analytical Variables on the nAPCsr

Blood was obtained by clean venipuncture in the forearm, without pressure and discarding the first 2 ml of blood Nine parts of blood were collected in one part of 0 13 M (3 8%) trisodium citrate (pH 7 8) and the blood was centrifuged for 15 min at 3000 × g at room temperature, followed by centrifugation for 30 min at 20,000 × g at 4° C (17, 20) The cell free plasma was stored at –80° C until analysis A pooled normal plasma was prepared from plasma of healthy volunteers not on medication, not using OC and non pregnant (21 females and 44 males, mean age 35 years) The individuals who donated blood for pooled normal plasma were not screened for factor V_{Leiden} or other hereditary coagulation abnormalities

Plasma Preparation for Investigating the Effect of Pre analytical Variables on the nAPCsr

Blood was collected from nine volunteers three factor V_{Leiden} negative individuals (2 men, 47 and 55 years, 1 woman, 51 yr), three heterozygous factor V_{Leiden} carriers (1 man, 30 yr, 1 woman, 41 yr and a 32 yr old woman who was 1 month pregnant) and three women (<30 years of age) using a monophasic oral contraceptive (OC) preparation containing 30 µg ethinyl estradiol and 150 µg desogestrel The presence of the factor V_{Leiden} mutation was established by DNA analysis (25)

The handling of the blood samples and the preparation and handling of plasma samples from the volunteers is schematically presented in Fig 1 Nine parts of blood were collected in one part of 3 2% (0 109 M) or 3 8% (0 130 M) trisodium citrate and divided into three equal portions, one part of which was immediately further processed and the others were left 4 or 24 h at room temperature before processing Platelet poor plasma was obtained by centrifuging either 15 min at 2000 × g at room temperature or 15 min at 3000 × g at room temperature followed by 30 min at 20,000 × g at 4° C Samples were frozen in small aliquots and stored at either –20° C or –80° C until analysis For each variable one aliquot was thawed once and refrozen again Taken together this resulted in 432 samples (9 individuals × 48 combinations) available for analysis The nAPCsr of all 48 samples belonging to an individual were determined in the same session The nAPC sr in six samples was not determined because the plasma was clotted upon thawing and 27 samples were left out of analysis because in these measurements residual thrombin formation determined in the presence of APC in normal plasma fell outside the range 6-16% (see also Results section)

APC resistance Assay and Determination of the nAPCsr

Plasmas were thawed and defibrinated with Ancrod (1 U/ml final concentration) for 10 min at 37° C after which the clot was removed with a plastic spatula (Sarstedt) Defibrinated plasma was used within 3 h after defibrination The APC resistance assay was routinely performed as described before (17, 20) Briefly, 80 µl defibrinated plasma was incubated at 37° C and thrombin formation was initiated with 45 µl starting solution (prewarmed at 37° C) containing tissue factor, $CaCl_2$, phospholipid vesicles with or without APC in 25 mM Hepes (pH 7 5 at 37° C), 175 mM NaCl, 5 mg/ml BSA

This resulted in final concentrations of 0 4 ng/ml tissue factor 16 mM added $CaCl_2$, 15 µM phospholipid vesicles (DOPS/DOPC/DOPE, 20/60/20, M/M/M) and if present, 5 nM APC Since commercial tissue factor preparations, after reconstitution according the manufacturers instructions, usually contain between 300 and 400 ng/ml tissue factor the final tissue factor concentration in the assay mixtures corresponds to an approximate 900-fold final dilution of reconstituted tissue factor preparations It should be noted that in earlier publications (17, 19, 20) the tissue factor concentration was abusively given as 0 1 ng/ml When quantified with an antigen assay (American Diagnostics) the tissue factor concentration used in the previous studies was 0 4 ng/ml

After 20 min an aliquot from the plasma mixture was diluted 50-fold in a 50 mM Tris buffer (pH 7 5 at 37° C) containing 175 mM NaCl, 20 mM EDTA and 0 5 mg/ml ovalbumin The amount of Alpha$_2$-Macroglobulin thrombin (α_2M-IIa) complex present in the diluted plasma mixture, which is a measure for the amount of thrombin generated (19, 26), was quantified by adding an appropriate aliquot from the diluted plasma (100 µl without APC, 200 µl with APC) to the well of a microtiterplate containing buffer with S2238 The well finally contained 250 µl 50 mM Tris (pH 7 5 at 37° C), 175 mM NaCl, 0 5 mg/ml ovalbumin, 20 mM EDTA and 235 µM S2238 The rate of change in absorbance was determined at 405 minus 492 nm at 37° C in a 340 ATTC microtiterplate reader (SLT Labinstruments, Salzburg, Austria) set in the kinetic mode The amidolytic activity was corrected by blank values measured in plasma samples (80 µl) to which 45 µl 25 mM Hepes (pH 7 5 at 37° C), 175 mM NaCl, 5 mg/ml BSA with or without APC was added and which were subjected to the same incubation and dilution procedure as described above

In routine nAPC sr determinations 21 subject plasmas together with 3 samples of pooled normal plasma (first, middle and last sample) were determined in duplicate to yield a total of 96 amidolytic measurements on a single microtiterplate (48 with and 48 without APC) From the α_2M-IIa amidolytic activities (± APC) thus obtained in subject plasma and in pooled normal plasma determined in the same microtiterplate the nAPC sr was calculated as

$$\text{nAPC-sr} = (\alpha 2\text{M-IIa}_{+APC}/\alpha 2\text{M-IIa}_{APC})_{\text{plasma sample}}/(\alpha 2\text{M-IIa}_{+APC}/\alpha 2\text{M IIa}_{APC})_{\text{pooled normal plasma}}$$

Statistics

The influence of variation of pre-analytical variables on the nAPCsr of the plasma samples of the nine individual volunteers (see also above) was assessed in a multiple linear regression model with nAPC-sr as dependent variable using indicator variables for the nine individuals In the comparison of different tissue factor preparations differences of means and 95% confidence intervals were calculated in the standard fashion

Results

Effects of Tissue Factor, $CaCl_2$ and Phospholipid Concentrations on Thrombin Generation in the Absence and Presence of APC

For quantification of the effect of APC on thrombin formation it is not necessary to measure complete time courses of thrombin generation Thrombin generation curves typically reach a residual level of amidolytic activity that can be attributed to the α_2M IIa complex (27, 28) The α_2M IIa level remains constant in time and is a direct indicator for the amount of thrombin generated in plasmas with the same α_2M levels (19, 26) Since this is particularly the case when the same plasma is tested with and without an effector of thrombin generation, the effect of reactants e g APC on thrombin generation can simply be quantified on the basis of single amidolytic assays of the end levels of α_2M-IIa present in plasma in which thrombin was generated in the absence and presence of APC

Fig 2 shows the results of titration experiments in which the concentration of one of the reactants (tissue factor, $CaCl_2$ or phospholipid) was varied while the others were kept constant at concentrations at which the routine assay is performed (see below) In the absence of APC (open symbols) optimal thrombin formation was observed at 0 4 ng/ml tissue factor (Fig 2A), 16 mM added $CaCl_2$ (Fig 2B) and 2 5 µM phospholipid vesicles in addition to the lipid present in the relipidated tissue factor preparation (Fig 2C)

In the presence of 5 nM APC thrombin formation was inhibited at all reaction conditions tested (Figs 2 A-C, closed symbols) However, with 5 nM APC thrombin formation increased at increasing tissue factor and $CaCl_2$ concentrations This indicates that the efficacy by which APC inhibited thrombin formation gradually decreased at higher tissue factor (Fig 2A) and $CaCl_2$ concentrations (Fig 2B) In the presence of APC, thrombin formation progressively decreased when the amount of phospholipid in the assay was increased, indicating that the ability of APC to inhibit thrombin formation was enhanced at higher phospholipid concentrations (Fig 2C, closed symbols)

Based on these data routine assay conditions (15 µM phospholipid, 0 4 ng/ml tissue factor and 16 mM $CaCl_2$) were chosen such that thrombin formation in the absence of APC was insensitive to small variations in concentrations of phospholipid, tissue factor or $CaCl_2$ (see also Discussion)

Effect of APC on Thrombin Formation in Normal Plasma and in Factor V_{Leiden} Plasma

The experiment presented in Fig 3 shows the effect of increasing APC concentrations on thrombin generation in pooled normal plasma

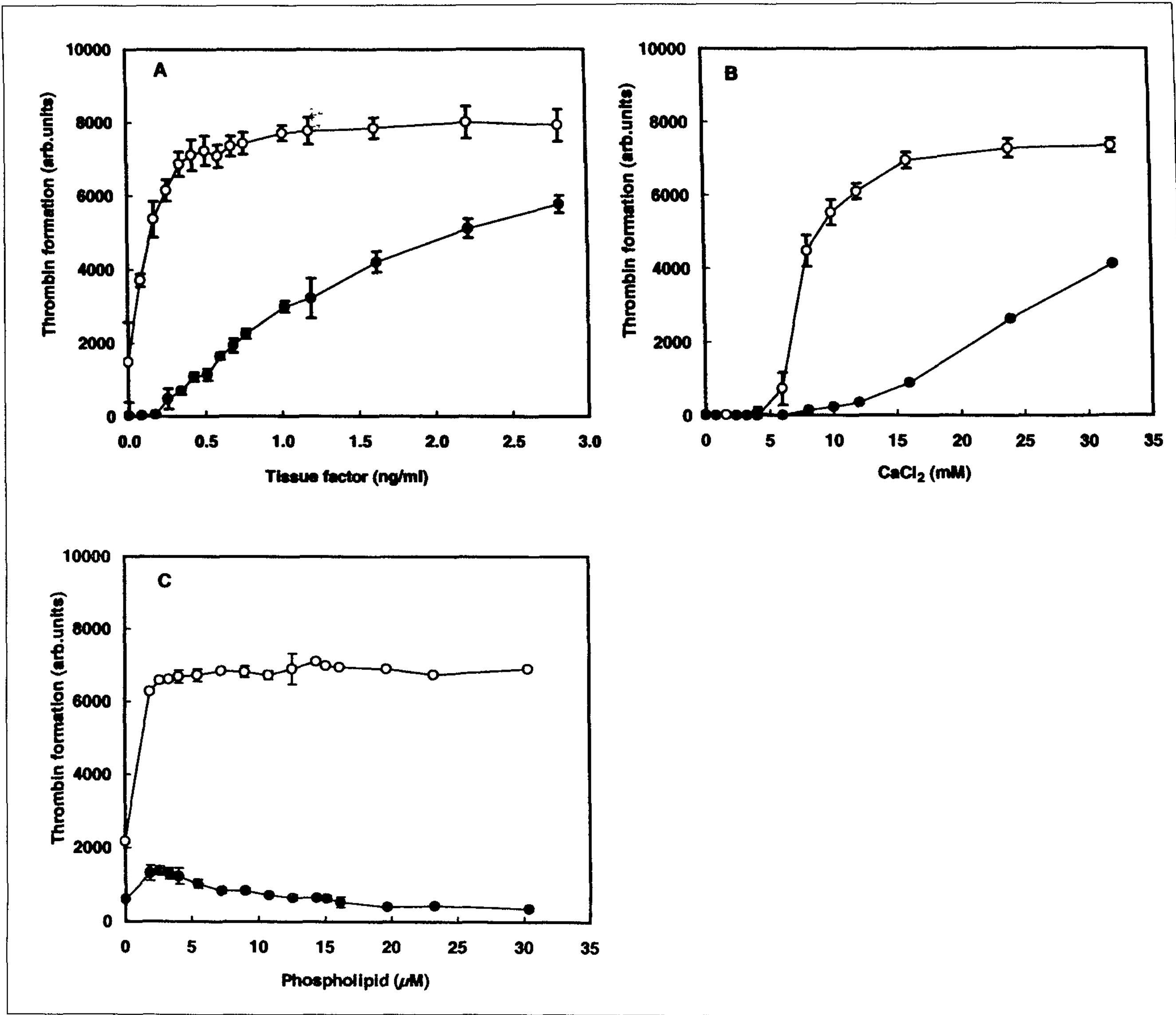

Fig. 2 Effects of varying amounts of tissue factor, Ca^{2+} ions and phospholipid on thrombin generation in pooled normal plasma. Thrombin formation was initiated in defibrinated pooled normal plasma with a mixture containing varying amounts of: A) tissue factor (DADE® Innovin®), B) Ca^{2+}-ions or C) phospholipid vesicles while keeping the others constant at 0.4 ng/ml tissue factor, 16 mM Ca^{2+} ions and 15 µM phospholipid either without (○) or with (●) 5 nM APC (final concentrations in the assay mixture). Thrombin formation is expressed in arbitrary units (final levels of α_2M-IIa complex present in plasma after 20 min). Y-error bars indicate the 95% confidence interval (n = 4). Some error bars are not visible since their size was less than that of the markers indicating the data points

and in plasmas from a normal male volunteer and from a male heterozygous carrier of factor V_{Leiden}. In all plasmas residual thrombin formation (expressed as percentage of thrombin formation determined in the absence of APC) gradually decreased at increasing APC concentrations. However, thrombin formation in the plasma from the heterozygous carrier of factor V_{Leiden} was less sensitive to APC than that in plasma from the wildtype volunteer which at all APC concentrations tested showed a sensitivity towards APC similar to that of pooled normal plasma.

Quantification of the Effect of APC on Thrombin Generation

In APC resistance tests the sensitivity of a particular plasma for APC is commonly determined on the basis of an APC sensitivity ratio (APCsr) which is defined as the ratio of the test result with and without APC. In the thrombin generation-based APC resistance test:

$APCsr = (\alpha 2M\text{-}IIa_{+APC}/\alpha 2M\text{-}IIa_{-APC})$

The nAPCsr was introduced (25) to minimize day-to-day variation and is calculated by dividing the APCsr of a plasma sample by the APCsr of pooled normal plasma determined in the same experiment i.e.

$nAPC\text{-}sr = APCsr_{plasma\ sample}/APCsr_{pooled\ normal\ plasma}$

During the development of the thrombin generation-based APC resistance test the concentration of APC added to plasma was chosen such that residual thrombin generation in the presence of APC was 10% (19). However, due to small variations in reaction conditions, residual thrombin formation in normal pooled plasma may deviate from 10%. This has different effects on the APCsr and the nAPCsr of a particular plasma sample. Fig. 4 compares the APCsr and the nAPC-sr of plasma from the wild type volunteer (Fig. 4A) and from the heterozygous carrier of factor V_{Leiden} (Fig. 4B) when residual thrombin formation in pooled normal plasma varied between 1% and 18% (data from Fig. 3). The APCsr of the wildtype volunteer showed a 20-fold increase at

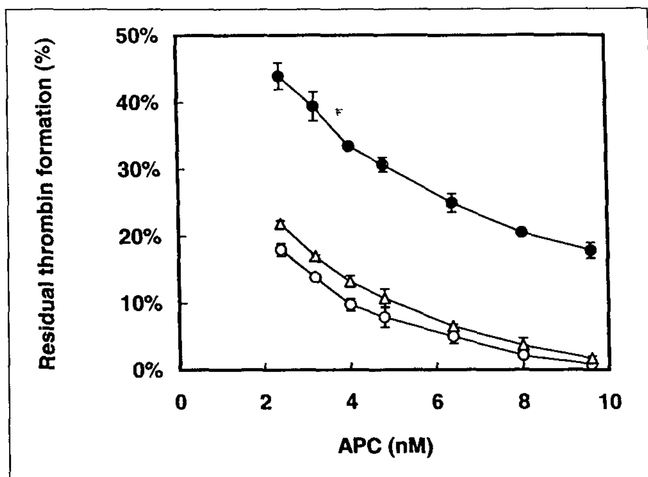

Fig 3 Effects of varying amounts of APC on residual thrombin formation in pooled normal plasma plasma from a healthy man and factor V_{Leiden} plasma Thrombin formation was initiated in defibrinated plasma at 0 4 ng/ml tissue factor (Innovin) 16 mM Ca^{++} ions and 15 μM phospholipid vesicles and amounts of APC as indicated in the figure Thrombin formation in the presence of APC was expressed as percentage of thrombin formation determined in the absence of APC for (○) pooled normal plasma (△) plasma of a male volunteer, (●) plasma of a heterozygous carrier of factor V_{Leiden} Y-error bars indicate the 95% confidence interval (n = 4)

increasing levels of residual thrombin formation in pooled normal plasma, while the nAPC-sr remained virtually constant when residual thrombin formation in the pooled normal plasma varied between 5% and 18% (Fig 4A) In the plasma from the factor V_{Leiden} carrier an opposite trend was observed The nAPC sr of factor V_{Leiden} plasma strongly depended on the percentage of residual thrombin formation in pooled normal plasma When this was less than 5% the nAPC-sr of the factor V_{Leiden} plasma became very high with large error margins (Fig 4B) The APCsr (not normalized) of factor V_{Leiden} plasma was much less affected under these conditions At levels of residual thrombin formation in normal plasma above 5% the APCsr and the nAPCsr showed a similar but opposite variation

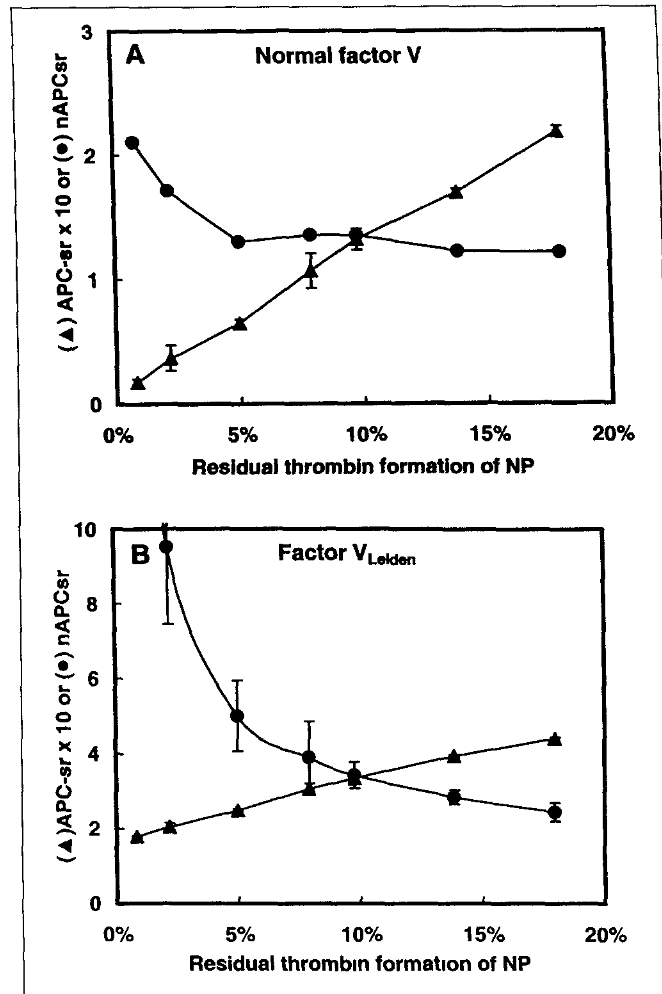

Fig 4 Comparison of APCsr and nAPC sr APC sr (▲) and nAPC sr (●) determined in plasma from the healthy volunteer (A) and the carrier of factor V_{Leiden} (B) were calculated from the data presented in Fig 3 as described in the Results section The APC sr (× 10 to enable direct comparison with the nAPC sr) and the nAPC sr are plotted as function of residual thrombin formation in pooled normal plasma Y error bars indicate the 95% confidence interval (n = 4)

Table 1 Between assay variation of the thrombin generation based APC resistance test

	(n)APCsr (mean)	Between assay variation (%CV)
Healthy individual without factor V_{Leiden}		
APCsr (x 10)	1 80	23 1%
nAPCsr	1 79	14 8%
Factor V_{Leiden} plasma		
APCsr (x 10)	2 75	17 5%
nAPCsr	2 76	12 4%

The APCsr (x 10 to enable direct comparison with the nAPCsr) and nAPCsr were determined on 18 different days in pooled normal plasma, plasma from an individual with mild phenotypic APC resistance and plasma from a heterozygous carrier of factor V_{Leiden} For futher details see text and Materials and Methods

Tissue factor	subjects	n	nAPC-sr (mean)	5-95% CI
Innovin	all	90	1.19	1.14-1.24
	men	52	0.95	0.91-1.01
	women	38	1.51	1.43-1.59
RecombiPlasTin	all	90	1.20	1.15-1.24
	men	52	1.02	0.97-1.07
	women	38	1.44	1.38-1.50
Recomboplastin S	all	90	1.23	1.19-1.24
	men	52	1.05	1.02-1.08
	women	38	1.46	1.42-1.52

With all tissue factor preparations a significant difference between the nAPCsr of men and women was observed (p <0.001). For further details see text and Materials and Methods.

Table 2 Effect of tissue factor preparations on the nAPC-sr

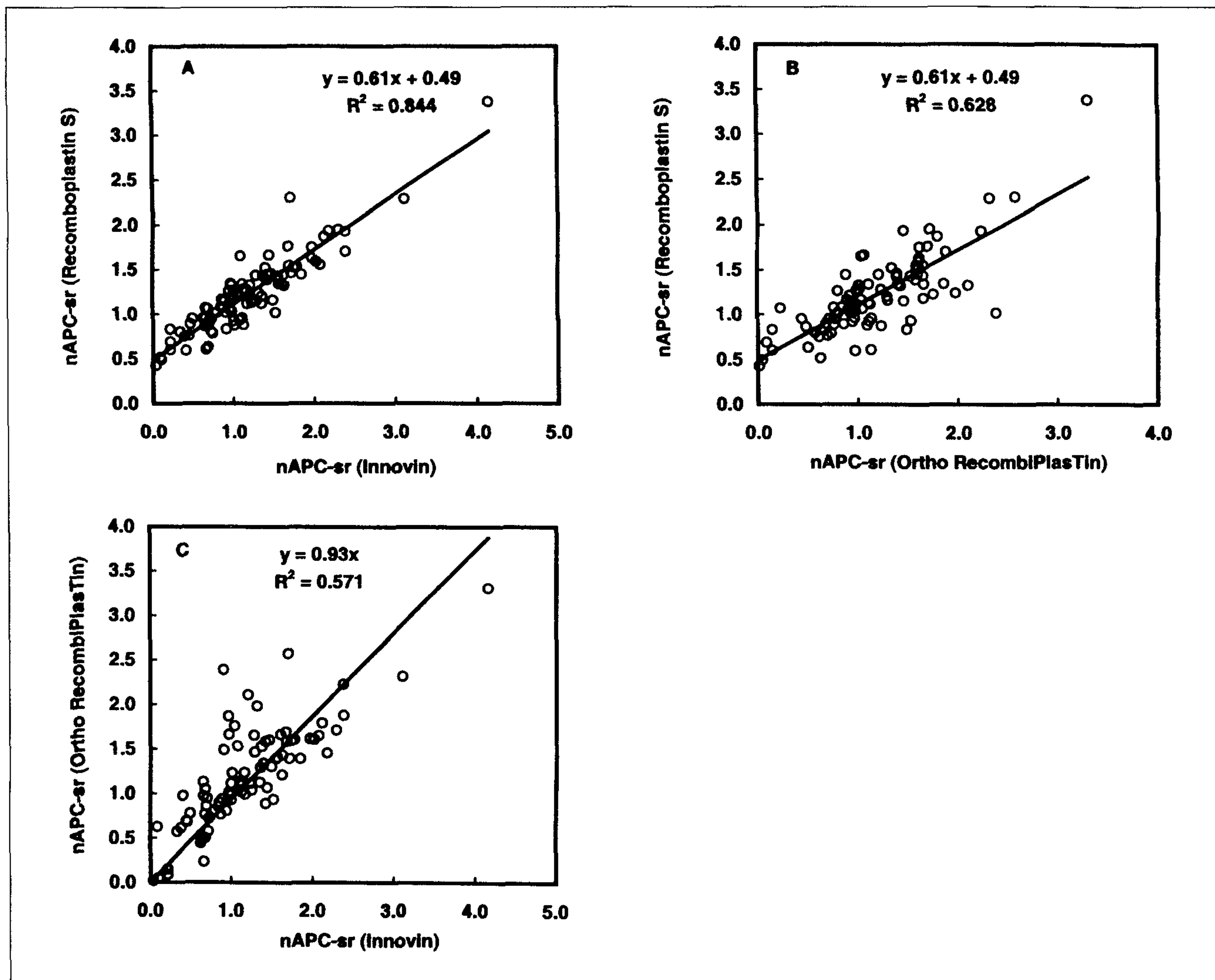

Fig 5 Correlations between nAPC-sr determined with different tissue factor preparations Correlation plot of the nAPC-sr determined with (A) Innovin and Recomboplastin S, (B) RecombiPlasTin and Recomboplastin S and (C) Innovin and RecombiPlasTin

Table 3 Effects of pre-analytical variables on the nAPCsr

Anticoagulant		3 2 % citrate		3 8 % citrate*	
Genotype	n	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI
GG	3	1 77	1 22-2 32	0 89	0 73-1 04
AG	2	3 22	1 81-4 62	2 52	0 96-4 07
GG+O	3	4 47	3 58-5 37	3 99	3 33-4 64
AG+pregnancy	1	7 33		6 97	

Centrifugation		15 min 2000xg RT		15 min 3000xg at RT 30 min 20 000xg at 4°C*	
Genotype	n	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI
GG	3	1 34	1 15-1 53	1 32	0 80-1 84
AG	2	2 80	1 27-4 33	3 00	1 63-4 36
GG+OC	3	4 27	3 53-5 02	4 19	3 35-5 02
AG+pregnancy	1	7 21		7 10	

Hours at RT		0 hrs*		4 hrs		24 hrs	
Genotype	n	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI
GG	3	1 20	0 73-1 67	1 31	1 20-1 41	1 48	1 00-1 97
AG	2	2 92	1 37-4 47	2 91	1 67-4 15	2 82	1 23-4 41
GG+OC	3	4 15	3 09-5 20	4 07	3 55-4 58	4 47	3 67-5 26
AG+pregnancy	1	7 14		7 16		7 15	

Storage temperature plasma		-20°C		80°C*	
Genotype	n	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI
GG	3	1 32	0 99-1 66	1 34	0 96-1 72
AG	2	2 69	1 56-3 82	2 92	1 44-4 41
GG+OC	3	4 41	3 54-5 28	4 06	3 33-4 79
AG+pregnancy	1	7 23		7 08	

Thawing		1 x*		2 x	
Genotype	n	nAPC-sr	5-95% CI	nAPC-sr	5-95% CI
GG	3	1 33	0 92-1 74	1 33	1 03-1 63
AG	2	2 90	1 47-4 33	2 88	1 38-4 38
GG+OC	3	4 14	3 53-4 74	4 33	3 34-5 31
AG+pregnancy	1	7 34		6 97	

Mean nAPC-sr values and 95% C I were calculated for individuals with a similar genotype/phenotype GG = normal genotype, AG = factor V_{Leiden} heterozygous genotype, OC = oral contraceptives * refers to the routinely used plasma preparation procedure

Table 1 summarizes the between assay variation of the (n)APCsr obtained by determining the effect of 5 nM APC on thrombin formation in pooled normal plasma, plasma from a healthy volunteer with an intermediate APCsr and a factor V_{Leiden} plasma on 18 different days The average residual thrombin formation in the normal plasma was 10 1% (range 6 5 13 8%) with a coefficient of variation (%CV i e SD/mean × 100%) of 20 8% The %CV of the (n)APCsr determined in plasma from the healthy normal volunteer were 14 8% (nAPCsr) and 23 1% (APCsr) Also in the case of factor V_{Leiden} plasma, the %CV of the nAPCsr (12 4%) was lower than that of the APCsr (17 5%)

Comparison of Different Tissue Factor Preparations

In earlier studies concerning the thrombin generation-based APC resistance test (17, 19 21) a tissue factor preparation was used that is not commercially available To compare this preparation with commercial tissue factor preparations, we determined the nAPC-sr of 90 plasmas from normal healthy individuals (52 men, 38 women) using our earlier preparation (Recomboplastin S) and two commercially available tissue factors (Dade® Innovin® and Ortho RecombiPlasTin®) at 0 4 ng/ml tissue factor, 16 mM $CaCl_2$ and 15 μM added phospholi pid vesicles The results obtained are summarized in Table 2 APC concentrations needed to attain 10% residual thrombin formation in pooled normal plasma were 4 8 nM APC for Innovin®, 5 2 nM APC for RecombiPlasTin® and 5 0 nM APC for our original tissue factor preparation (Recomboplastin S), which hints at small differences between the preparations Average nAPC-sr obtained were, however, not statistically different for all tissue factor preparations Plasma from women was more resistant to APC than plasma from men (Table 2) An excellent correlation was observed between the original non commercial recomboplastin S and Innovin® (r = 0 92, Fig 5A) and a somewhat lower correlation with RecombiPlasTin® (r = 0 79, Fig 5B) However, due to a relative lack of low nAPC-sr values in the determination with the original recomboplastin S preparation, regression lines did not cross the origin The two commercially available tissue factor preparations showed a good correlation (r = 0 71) with a regression line which passes through the origin and which has a slope of 0 94 indicating that nAPC-sr values showed similar individual variation with these preparations (Fig 5C)

Effect of Plasma Handling on the nAPC sr of Nine Individual Plasmas

To obtain information on the influence of plasma handling (i e pre-analytical variables) on the nAPC-sr, plasmas from nine individual

Variables	β	95% CI
3.2% citrate vs 3.8% citrate	0.739	0.647 - 0.831
4 hrs delay vs immediate processing	-0.016	-0.130 - 0.096
24 hrs delay vs immediate processing	0.149	0.037 - 0.261
single vs double centrifugation	0.089	-0.003 - 0.181
storage at −20°C vs −80°C	-0.023	-0.117 - 0.071
thawing twice vs once	-0.035	-0.127 - 0.057

nAPC-sr were obtained in plasmas of nine individuals processed by 48 different methods and analyzed in a multiple regression model. The nine individuals were included as indicator variables and the effect of a change in preanalytical variable was assessed against the routine method of plasma preparation (3 8% citrate as anticoagulant, immediate processing of the blood sample, double centrifugation, storage at -80°C and no extra thawing)

Table 4 Regression model for the thrombin generation-based nAPC-sr

volunteers with a large variation in the nAPC-sr were collected and subjected to different treatments as described under Materials and Methods (cf Fig 1) The pooled normal plasma used for normalization of the nAPCsr was collected according to the standard procedure (nine parts of blood in one part 3 8% citrate) and processed as described in Materials and Methods

The results are summarized in Table 3 Under all circumstances the plasma sample from the heterozygous carrier of factor V_{Leiden}, who was 1 month pregnant, had the highest nAPC-sr The nAPC sr determined for the heterozygous factor V_{Leiden} carriers showed values comparable to the women who did not carry the factor V_{Leiden} mutation but who were using oral contraceptives Values close to normal plasma were observed for the non-factor V_{Leiden} men and for the non-factor V_{Leiden} woman who was not using oral contraceptives (Table 3) These nAPC-sr are in agreement with earlier reported values (17, 20)

The data obtained for all possible combinations of plasma processing were analyzed in a multiple regression model which included the nine individuals as indicator variables (Table 4) Thereby, adjustment for individual differences (factor V_{Leiden} carriers, OC users and pregnant woman) was achieved The model showed that the citrate concentration and the 24 h wait at room temperature before the blood was processed were variables that clearly influenced the nAPC-sr measurement (Table 4) The largest effect was observed with the anticoagulant concentration Samples collected on 3 2% citrate showed an average increase of 0 74 of the nAPCsr compared to plasma collected on 3 8% citrate The difference between the nAPCsr of plasma collected on 3 2% and 3 8% citrate became less in samples with higher nAPC-sr (Table 3) The other pre-analytical variations i e centrifugation procedure, 4 h wait at room temperature before processing of the blood sample, temperature during 3-month storage as well as additional sample thawing did not notably influence the nAPC-sr (Table 4)

Discussion

The data reported here concern the effects of variations in plasma handling and assay conditions on nAPC-sr values determined with the thrombin generation-based APC resistance test described in earlier publications (17, 19, 20) It has been reported that the measurement of the nAPC-sr with this assay, apart from allowing detection of the factor V_{Leiden} mutation, is very sensitive for conditions of acquired APC-resistance such as it occurs during the use of oral contraceptives (17, 20, 21) and pregnancy (29-31) Part of this sensitivity is likely due to the decision to use an APC concentration which diminishes thrombin formation in normal plasma by 90% This results in a large window for the nAPC-sr (values between 1 and 10) of APC resistant samples However, in order to obtain results that are comparable from study to study and between different laboratories the assay conditions need to be standardized The assay conditions were chosen such that thrombin formation in the absence of APC is insensitive to small variations in the phospholipid, $CaCl_2$ or tissue factor concentrations The amount of phospholipid present in the assay mixture (15 μM) is higher than the concentration required for optimal thrombin formation in the absence of APC The use of a high phospholipid concentration makes the assay insensitive to small amounts of phospholipid present in plasma and also limits the amount of APC required to inhibit thrombin formation However, measurements obtained in the presence of APC and thus, the nAPC-sr remain sensitive to variations in concentrations of reactants (cf Figs 2-4) and therefore, standardization is essential in order to minimize within- and between laboratory variation

It is not surprising that the ability of APC to inhibit thrombin formation is decreased at higher tissue factor and $CaCl_2$ concentrations and increases when the amount of phospholipid present in the assay mixture is increased At high tissue factor concentrations excessive amounts of factor Xa and factor Va will be formed, which will result in a condition at which APC is incapable to effectively inactivate factor Va and inhibit thrombin generation The observation that in model systems containing purified proteins APC-catalyzed factor Va inactivation has a sharp Ca^{2+} optimum (3 mM) and is inhibited at high Ca^{2+} concentrations (32), can explain the increase of residual thrombin formation at increasing amounts of Ca^{2+} The fact that APC has a rather low affinity for negatively charged phospholipids (33) can account for the increased effectivity by which APC down-regulates thrombin formation at higher phospholipid concentrations

In order to obtain reproducible nAPC-sr, it is necessary to ensure that the residual thrombin formation determined in the presence of APC in normal plasma is close to 10% (Fig 4) A good day-to-day reproducibility is then achieved, which can be further improved by normalizing against the APC-sr obtained in pooled normal plasma in

the same run When the level of residual thrombin formation in pooled normal plasma is kept within a narrow range (6 16%) the coefficients of variation of the nAPC sr (SD/mean × 100%) were 14 8% and 12 4% for plasma of a healthy donor and a heterozygous factor V_{Leiden} carrier, respectively (Table 1)

When residual thrombin formation in pooled normal plasma is below 6%, normalization i e dividing the percentage of residual thrombin formation in the plasma sample by that determined in pooled normal plasma leads to systematic overestimation of nAPC-sr in APC resistant samples and the inter- and intra-assay variability becomes too large (Fig 4) The extent of overestimation of the APCsr is then reduced by omitting the normalization procedure There is a second reason why normalization may affect the actual value of the nAPCsr determined for an individual plasma sample The presence of as low as 2 5% factor V_{Leiden} carrier plasma already affects the APCsr of the pooled normal plasma (34) Thus, normalization against a normal plasma in which users of oral contraceptives or carriers of factor V_{Leiden} are included in the subjects who donate blood will result in lower nAPC-sr for individual plasma samples than nAPCsr normalized against a pooled normal plasma from which users of oral contraceptives and carriers of factor V_{Leiden} are excluded In order to allow comparison of nAPC-sr values obtained in different studies it is recommended to use pooled normal plasma containing samples from a large number of volunteers (to minimize variations in the percentage of carriers of factor V_{Leiden}) and to exclude women who use oral contraceptives

nAPCsr obtained with the thrombin generation-based APC resistance test are hardly affected by the handling of plasma The major variable to control appears to be the concentration of anticoagulant (Tables 3, 4) This is not surprising considering the $CaCl_2$ dependence of the measurement in the presence of APC (cf Fig 2) and the fact that the normal plasma pool used for normalization was collected at 3 8% An influence of anticoagulant concentration has also been reported for coagulation tests (e g INR) that are based on measurement on the clotting of plasma initiated with tissue factor (35) Therefore, it is preferable to normalize APCsr against a pooled normal plasma that is collected on the same concentration of anticoagulant The linear regression analysis further indicated a small influence of the storage time of blood before plasma is prepared (Table 4) Other variables appeared to have little or no influence

We feel that the data presented in this paper will allow other laboratories to implement the thrombin generation based APC resistance measurement but it will be clear that strict attention has to be given to standardization not only of assay conditions but also of the way plasmas and normal pools are prepared in order to pave the way for a larger inter laboratory study in the future

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