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A COMPARATIVE STUDY USING THROMBIN GENERATION AND THREE DIFFERENT INR METHODS IN PATIENTS ON VITAMIN K ANTAGONIST TREATMENT

Running title: INR monitoring in patients on VKA treatment

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ABSTRACT

Introduction: Vitamin K antagonist (VKA) treatment requires routine monitoring using the international normalized ratio (INR). However, different INR assays may vary in their results. The aim of this study was to assess the agreement of three different INR methods, compared with thrombin generation, in patients on VKA treatment.

Methods: Sixty patients attending the Anticoagulation Clinic at Mater Dei Hospital (Msida, Malta) for VKA monitoring between August-September 2015 were enrolled. The INR was tested using a point-of-care (POC) device (CoaguChek XS Plus, Roche Diagnostics) for both capillary and venous blood samples, a photo-optical (Sysmex CS-2100i/CA-1500, Siemens) and a mechanical clot detection system (Thrombolyzer XRC, Behnk Elektronik). All assays used human recombinant thromboplastin as reagent. Thrombin generation was performed using the Calibrated Automated Thrombogram.

Results: There was a negative curvilinear correlation between the Endogenous Thrombin Potential and different INR assays (r ≤ -0.75) and a strong positive linear correlation between the CoaguChek XS Plus on capillary samples and the other INR methodologies (r ≥ 0.96).

Conclusion: All different INR assays showed good correlation with the thrombin generation potential. The POC INR showed one of the highest correlation coefficients with thrombin generation, confirming the POC devices as an accurate, valid alternative to laboratory INR in VKA patients.

Keywords: Accuracy, Warfarin, International Normalized Ratio, Point-of-Care Systems, Thrombin Generation

INTRODUCTION

Vitamin K antagonists (VKAs) have a narrow therapeutic window, several food and drug interactions and a variable anticoagulant response, which explain the need for periodical anticoagulation monitoring and dose adjustment (1). Since VKAs inhibit the synthesis of vitamin K-dependent coagulation factors (factors II, VII, IX and X), they are monitored using laboratory tests that assess the extrinsic pathway of the coagulation cascade. The prothrombin time (PT) measures the time to clot formation of citrated plasma, after recalcification and addition of thromboplastin to trigger coagulation, and is usually expressed as international normalized ratio (INR). The WHO recommended method for PT testing in relation to VKA therapy is the manual tilt-tube technique (2), but currently most PT determinations are performed using automated coagulation analysers, such as photo-optical or electro-mechanical coagulometers. Furthermore, in the last two decades several portable coagulometers, also known as point-of-care (POC) devices, have been developed for the self-care of patients prescribed with VKAs (3). More recently, we saw the advent of global coagulation assays, such as thrombin generation, which may have the potential to better evaluate all phases of coagulation (4).

Several studies indicated an excellent correlation between photo-optical and electro-mechanical coagulation analysers (5, 6), while the comparison between POC and laboratory or manual INRs showed a certain variability in the results, with potential clinical disagreement and differences in VKA dosing (7-10). However, it is not known which test actually correlates better with the overall blood coagulation potential, since these three INR methods have never been compared simultaneously with global coagulation assays.

The aim of this study was to assess the agreement of three different INR assays, compared with thrombin generation, in patients on VKA treatment.

MATERIAL AND METHODS

Study population

Consecutive adult patients attending the Anticoagulation Clinic at Mater Dei Hospital (in Msida, Malta) for warfarin monitoring were screened. We included 30 patients deemed eligible for POC monitoring according to the local protocol (target INR $\leq$ 3.0 and at least 3 consecutive INRs within the therapeutic range, absence of antiphospholipid syndrome, liver disease, severe renal failure, active cancer, or dual antiplatelet therapy) and 30 random patients, in order to cover a broader range of INR values. Patients were recruited during the months of August and September 2015.

The study was reviewed and approved by the University of Malta Research and Ethics Committee and written informed consent was obtained from all patients before inclusion.

Sample collection and tests performed

Laboratory INR

From each patient, one venous blood sample was collected using a 10 mL syringe and a 21G needle, in order to fill in 3 coagulation tubes, each containing 2 mL of whole blood and sodium citrate 0.109M/3.2% (Vacuette, Greiner Bio-One). One tube was processed according to the standard system at Mater Dei Hospital at the time of this study. This tube was centrifuged for 10 minutes at 2500 g and plasma was analysed using a photo-optical clot detection system (Sysmex CS-2100i or CA-1500, Siemens Healthcare

Diagnostics) and human recombinant thromboplastin (Dade Innovin Reagent, Siemens Healthcare Diagnostics). We had previously tested with both Sysmex analysers 33 samples with various INRs, ranging from 0.9 to 4.45, and found no statistical difference in the PT and the INR between the two analysers (data not shown).

The two remaining tubes underwent double centrifugation (2500 g for 10 min twice) with plasma separation, in order to obtain platelet poor plasma (PPP) within a 2 hour time frame from phlebotomy. They were stored in 300 µL aliquots at -80° C. It has previously been demonstrated that freezing plasma does not affect INR testing (11).

Afterwards, one scientist tested the INR on thawed PPP using a mechanical clot detection system (Thrombolyzer XRC, Behnk Elektronik) and the same human recombinant thromboplastin (Dade Innovin Reagent, Siemens Healthcare Diagnostics).

The INR is calculated dividing the patient's PT by the mean of the PTs of adult normal subjects, to the power of the thromboplastin's International Sensitivity Index (ISI) (12), according to the following formula:

$$INR = \left(\frac{\text{patient PT}}{\text{mean normal PT}} \right)^{ISI}$$

INR calibration was performed locally, on each analyser, using a calibrator kit (PT-Multi Calibrator, Siemens) composed of five lyophilized calibration plasmas.

Point-of-care testing

All 60 patients were tested using the CoaguChek XS Plus (Roche Diagnostics) coagulometer. Quality control (QC) analysis for POC was performed at the beginning of

each testing day. One researcher performed all the tests. Both capillary and venous blood samples were tested with the CoaguChek XS Plus. Capillary blood samples were obtained by finger prick and applied on a test strip within 10 seconds. Non-citrated venous blood samples were obtained from the syringe used to draw the venous blood, after filling the coagulation tubes and after discharging few blood drops. The same CoaguChek XS Plus coagulometer was used throughout study. Two lots of test strips were used during the study (233 430-11 and 202 053-11). As per manufacturer instructions, the CoaguChek XS Plus system utilizes human recombinant thromboplastin with ISI=1.0 (13).

Thrombin generation

Frozen aliquots were shipped to the Coagulation Laboratory at the Royal Hallamshire Hospital (in Sheffield, United Kingdom) in dry-ice. Thrombin generation was performed using the Calibrated Automated Thrombogram (CAT), according to the method described by Hemker et al (14).

Prior to this analysis, samples were thawed in a water bath at 37° C for 5 minutes. Afterwards, 80 µL of PPP were added to 20 µL of tissue factor trigger at a concentration of 5pM (PPP-reagent, Thrombinoscope BV, Maastricht, the Netherlands) in a 96-well plate. All samples were tested in duplicate and one calibrator (Thrombin Calibrator, activity 580 nM) well was run in parallel. Three QC plasma samples were tested in each run.

The reaction was initiated after automated dispensing of 20 µL of fluorogenic substrate (FluCa-kit, Thrombinoscope BV, Maastricht, the Netherlands). The fluorescence intensity was measured for 1 hour using a Fluoroskan Ascent fluorimeter (Thermo

Electron Corporation), after the samples were incubated for 10 min at 37ºC. Using a dedicated software (Thrombinoscope BV, Maastricht, the Netherlands, version 3.4.0.154), the following parameters were calculated: lag time (LT), peak thrombin concentration (Peak), time to peak thrombin (ttPeak), endogenous thrombin potential (ETP) and velocity index.

Statistical analysis

We collected information regarding demographic characteristics of the population, past medical history, details of the warfarin treatment and concomitant medications.

Continuous variables were expressed as mean with standard deviation (SD) or median with interquartile range (IQR); categorical variables were expressed as counts and percentages. Continuous variables were compared using the Student's t-test or the Mann-Whitney U test; categorical variables were compared using the Chi square or Fisher's exact tests, as appropriate. The correlation between different laboratory tests was evaluated using the non-parametric Spearman's rank correlation test, according to data distribution, and the correlation coefficients (r) were calculated. The mean INRs obtained with different methodologies were compared using one-way repeated measures ANOVA with Bonferroni's post-hoc correction.

The statistical agreement between different INR methodologies was evaluated creating Bland-Altman plots (or difference plots) with the mean of the two measurements on the *x*-axis and the difference between the two values on the *y*-axis (15). The estimated mean bias is the mean difference between the two values and the 95% limits of agreement are computed as mean bias $\pm$ 1.96 SD (15).

In order to evaluate the clinical agreement and to estimate the percentage of INR values which might have resulted in a different clinical management, the INR values were categorized as above, within or below the INR therapeutic range (2.0-3.0 for patients with atrial fibrillation, venous thromboembolism and aortic valve replacement; and 2.5-3.5 for patients with mitral valve replacement).

Data analysis was performed using the statistical software STATA SE 12 (StataCorp LP, College Station, TX, USA). Two-tailed p values less than 0.05 were considered statistically significant.

RESULTS

Study population

Sixty patients were enrolled in this study. Mean (SD) age was 68.5 (11.5) years and 26 (43.3%) were males. The most common indications for warfarin treatment were atrial fibrillation (63.3%) and venous thromboembolism (26.7%), followed by mechanical heart valve replacement (8.4%). The majority of patients (73.3%) were on oral anticoagulant treatment for more than a year. The current median (IQR) dose of warfarin was 4 (3-5) mg. Comorbidities and concomitant medications in our population are summarized in Table 1. None of these patients had known antiphospholipid syndrome.

Different INR methodologies

Using the standard laboratory instrumentation in our Coagulation Laboratory (the Sysmex CS-2100i/CA-1500), mean (± SD) INR was 2.46 (± 0.75), with a range from 1.37 to 4.92. Mean and median INR values measured with the other methodologies

were slightly higher and are summarized in Table 2. Mean INR obtained using the Sysmex CS-2100i/CA-1500 analysers was significantly different from the CoaguChek XS Plus on capillary and venous samples and from the Thrombolyzer XRC results (p values < 0.001).

Thrombin generation

The intra-assay coefficient of variation (CV) of thrombin generation was 4.3%. The inter-assay CV was 13.7% for the normal QC and 6.8% for the warfarin QC.

Thrombin generation results are summarized in Table 3. Patients with VTE had a slightly lower lag time and time to peak compared to patients with atrial fibrillation (AF), although this was not statistically significant (p=0.08 and p=0.06, respectively). This result was not explained by other variables that were comparable in the two groups (e.g. median INR 2.35 in AF patients *vs*. 2.3 in VTE patients, p=0.99; median age 69.5 *vs*. 67 years, p=0.54; warfarin treatment duration more than 1 year 71.7% *vs*. 73.3%, p=1.00; median TTR in the previous 3 months 67.8% *vs*. 67.0%, p=0.77, respectively). There was no difference in the other parameters of the thrombin generation curve, as reported in Table 3.

Correlation between thrombin generation and different INR methodologies

There was a negative curvilinear correlation between the ETP and the INR measured with the Sysmex CS-2100i/CA-1500 (r = -0.75, p<0.001), the CoaguChek XS Plus on capillary (r = -0.80, p<0.001) and venous blood (r = -0.78, p<0.001), and the Thrombolyzer XRC (r = -0.78, p<0.001), as shown in Figure 1.

Comparison between INRs

A strong positive linear correlation was found between the CoaguChek XS Plus, tested on capillary samples, and the other INR methodologies, showing Spearman's r coefficients above 0.95 (Table 4 and Figure 2). The CoaguChek XS Plus tended to overestimate the INR by a mean of approximately 0.3 INR units, compared to the Sysmex CS-2100i/CA-1500. The agreement, represented by the Bland-Altman or difference plots, is reported in Figure 3.

From a clinical perspective, the INR values within the same clinical category, compared to the CoaguChek XS Plus on capillary samples, were 93.3% for the CoaguChek XS Plus on venous samples; 78.3% for the Sysmex CS-2100i/CA-1500; and 93.2% for the Thrombolyzer XRC. However, the disagreement between the two methods would never lead to antagonistic behaviour (such as dose increase *vs*. dose reduction or viceversa).

DISCUSSION

To the best of our knowledge, this is the first time that three different INR assays (namely the CoaguChek XS Plus, the Sysmex CS-2100i/CA-1500 and the Thrombolyzer XRC) have been simultaneously compared with the thrombin generation assay. All the INR assays used human recombinant thromboplastin as reagent, therefore the difference in results was mainly due to the different analysers.

We found a negative curvilinear relationship between the ETP measured by the CAT and the INR values, with Spearman's coefficients ranging between -0.80 and -0.75. A similar negative correlation was already reported by Gatt *et al.* in comparison with the Sysmex CA-1500 (16). In our study thrombin generation showed a better correlation

11

with the CoaguChek XS Plus and the Thrombolyzer XRC, than with the Sysmex CS-2100i/CA-1500.

We also found a strong positive linear correlation between the CoaguChek XS Plus, tested on capillary samples, and the other INR methodologies, with all Spearman's coefficients above 0.95. The correlation was almost perfect for the CoaguChek XS Plus tested on capillary samples *vs.* venous samples with a mean (± SD) bias of 0.002 (± 0.11) INR units, suggesting that this pre-analytical variable does not interfere with the INR values, if the test is correctly performed. Similar results were obtained by Plesch *et al.* who found a mean bias of less than ± 0.02 INR units between the capillary and venous sample, albeit using a different device, the CoaguChek XS (17). In our study, the correlation was also very strong when the CoaguChek XS Plus was compared with the photo-optical (Sysmex CS-2100i/CA-1500) and the mechanical clot detection methods (Thrombolyzer XRC). Previous studies, that compared the CoaguChek XS Plus with photo-optical (Sysmex analysers) or mechanical clot detection methods (STAGO analysers), found correlation coefficients approximately 0.95-0.96 (8, 18, 19); however, the CoaguChek XS Plus had never been compared before with different laboratory techniques simultaneously.

Although the statistical agreement was very good, clinical disagreement between the CoaguChek XS Plus on capillary samples and the other INR assays ranged from 6.7% to 21.7% of patients, resulting in possibly different, but never antagonistic, warfarin management. A previously published study reported clinical disagreement in 26-29% of cases, but the management differed only by minor interventions (9). Furthermore, considering that VKA patients managed with a POC device should be monitored in this way for a certain period of time, without continuously switching between POC and

laboratory INR, this small difference is unlikely to negatively interfere with the clinical management of VKA patients.

Our findings have important implications in the international literature. Despite the recent discovery of the novel direct oral anticoagulants, VKAs will remain the treatment of choice for several categories of patients, such as those with valvular AF, mechanical heart valves or with severe renal insufficiency. Portable coagulometers, compared to traditional laboratory INR, are less invasive and can provide immediate results. Furthermore, POC can allow a more practical INR monitoring, since they can be used in different settings outside the hospital and they can also allow patient self-testing and self-management. Portable coagulometers therefore represent an alternative to standard laboratory INR and our results can provide reassurance on the accuracy of the CoaguChek XS Plus device.

The strengths of our study include the simultaneous comparison of thrombin generation measured by the CAT with three different INR assays, all using the same thromboplastin in order to reduce possible variability due to this analytical variable. Furthermore, we decided to reduce variability by asking a single investigator to perform all the POC tests. However, there are also some limitations that need to be acknowledged. First, the small number of patients, although similar to previous studies (17, 18), resulted in a small number of INRs above 4.0, thus precluding the possibility of a sensitivity analysis in this patient subgroup. Second, despite the potential to better assess all phases of coagulation, thrombin generation is not yet considered a validated test for monitoring anticoagulation. However, we chose to compare different INR methodologies with thrombin generation because the latter is known to show more variation in VKA patients and has the potential to better identify small differences in

test accuracy, than simply comparing different INR methodologies among each other. Third, all POC measurements were performed by a trained scientist, and our results might not generalizable, for example, to all patients performing INR self-testing.

In conclusion, our study showed that the relationship between INR results and thrombin generation does not differ depending on the assay used for INR measurement. Despite not being generally considered as the 'gold standard', the POC INR showed one of the highest correlation coefficients with thrombin generation, therefore confirming the POC devices as an accurate and valid alternative to laboratory INR in VKA patients.

Conflict of interest

The authors have no relevant conflicts of interest to declare in relation to this study.

Author contributions

N.Riva and A.Gatt contributed to the conception and design of the study, analysis and interpretation of data and drafted the article. K.Vella, S.Meli, K.Hickey, D.Zammit and C.Calamatta contributed to acquisition, analysis and interpretation of data. M.Makris, S.Kitchen and W.Ageno contributed to interpretation of data and critical revision of the manuscript. All authors provided final approval of the manuscript.

LEGEND TO FIGURES AND TABLES

Table 1.   Baseline characteristics of the population

Table 2.   Summary of INR measurements using different methodologies

Table 3.   Results of thrombin generation test in the overall population and in the comparison between patients with venous thromboembolism and atrial fibrillation

Table 4.   Agreement of the CoaguChek XS Plus on capillary blood samples, with the other INR methodologies

Figure 1.   Correlation between the Endogenous Thrombin Potential (ETP) and the INR, measured with the Sysmex CS-2100i/CA-1500 (a), the Thrombolyzer XRC (b), the CoaguChek XS Plus on capillary samples (c) and on venous samples (d)

Figure 2.   Correlation between the INR measured with the CoaguChek XS Plus on capillary samples and the CoaguChek XS Plus on venous samples (a), the Sysmex CS-2100i/CA-1500 (b), and the Thrombolyzer XRC (c)

Figure 3.   Bland Altman plots representing the difference between the CoaguChek XS Plus on capillary samples and the Sysmex CS-2100i/CA-1500

Table 1. Baseline characteristics of the population

	N. of patients = 60
Age (years), mean (SD)	68.5 (11.5)
Male sex, n (%)	26 (43.3%)
Indication for anticoagulant treatment:	
• Atrial fibrillation, n (%)	38 (63.3%)
• Venous thromboembolism, n (%)	16 (26.7%)
• Aortic valve replacement, n (%)	4 (6.7%)
• Mitral valve replacement, n (%)	1 (1.7%)
• Cerebrovascular accident, n (%)	1 (1.7%)
Duration of the anticoagulant treatment:	
• ≤ 3 months, n (%)	6 (10.0%)
• 3-6 months, n (%)	6 (10.0%)
• 6-12 months, n (%)	4 (6.7%)
• > 1 year, n (%)	44 (73.3%)
Current warfarin dose (mg), median (IQR)	4 (3-5)
Comorbidities:	
- Hypertension, n (%)	49 (81.7%)
- Diabetes mellitus, n (%)	22 (36.7%)
- Dyslipidemia, n (%)	32 (53.3%)
- Coronary artery disease, n (%)	18 (30.0%)
- Hypothyroidism, n (%)	8 (13.3%)
- Previous stroke, n (%)	3 (5.0%)
- Chronic obstructive pulmonary disease, n (%)	5 (8.3%)
- Malignancy, n (%)	8 (13.3%)
- Smokers: current, n (%) / previous, n (%)	5 (8.3%) / 13 (21.7%)
- Obesity, n (%)	29 (48.3%)
Concomitant medications:	
- Antiplatelet*, n (%)	5 (8.3%)
- Steroids, n (%)	1 (1.7%)
- Statins, n (%)	35 (58.3%)
- ACE-inhibitors or ARBs, n (%)	42 (70.0%)
- Diuretics, n (%)	34 (56.7%)
- Beta-blockers, n (%)	22 (36.7%)
- Calcium channel blockers, n (%)	11 (18.3%)
- Digoxin, n (%)	14 (23.3%)
- Levothyroxine, n (%)	8 (13.3%)
- Proton pump inhibitors, n (%)	10 (16.7%)
- Metformin, n (%)	17 (28.3%)

Legend: ACE = angiotensin-converting-enzyme; ARB = angiotensin II receptor blockers; IQR = interquartile range; SD = standard deviation

* Antiplatelet therapy refers to aspirin or clopidogrel, none of the patients was receiving dual antiplatelet therapy.

Table 2. Summary of INR measurements using different methodologies

Instrument (n of tests)	Mean INR (SD)	Median INR (IQR)	INR range
Sysmex CS-2100i/CA-1500 (60)	2.46 (0.75)	2.31 (1.95-2.74)	1.37-4.92
CoaguChek XS Plus (capillary blood) (60)	2.74 (0.86)	2.6 (2.2-3.1)	1.4-5.8
CoaguChek XS Plus (venous blood) (60)	2.74 (0.82)	2.6 (2.2-3.0)	1.4-5.7
Thrombolyzer XRC (59*)	2.71 (0.85)	2.52 (2.14-2.97)	1.34-5.33

Legend: INR = international normalized ratio; IQR = interquartile range; SD = standard deviation

*Thrombolyzer results were available for 59 patients, since one patient had a difficult blood sampling and only a limited amount of plasma was available.

Table 3. Results of thrombin generation test in the overall population and in the comparison between patients with venous thromboembolism and atrial fibrillation

Overall population *			
Parameter	*Mean (SD)*	*Median (IQR)*	*Range*
Lag time (min)	6.35 (1.99)	6 (5.17-7.17)	3.47-13
Peak thrombin concentration (nM)	101.66 (44.51)	91.49 (72.51-121.2)	29.99-269.37
Time to peak (min)	9.23 (2.15)	8.83 (7.67-10.17)	5.97-16
Endogenous thrombin potential (nM/min)	596.75 (265.26)	547.5 (419-722.5)	186.5-1835
Velocity index (nM/min)	36.53 (17.76)	31.34 (25.22-47.91)	8.59-85.19

Comparison between patients with atrial fibrillation and venous thromboembolism **			
Parameter	*AF patients (n = 38)*	*VTE patients (n = 15)*	*p value*
Lag time (min)	6.33 (5.33-7.67)	5.17 (4.8-6.33)	0.08
Peak thrombin concentration (nM)	83.82 (69.05-121.2)	97.57 (77.37-135.39)	0.43
Time to peak (min)	9.33 (8-10.65)	7.83 (7.67-9)	0.06
Endogenous thrombin potential (nM/min)	508.5 (398-722.5)	547.5 (465-803)	0.40
Velocity index (nM/min)	30.6 (24.02-42.72)	38.45 (25.45-47.98)	0.44

* Thrombin generation results were available for 59 patients, since the thrombin generation curve was not computable in one patient with VTE
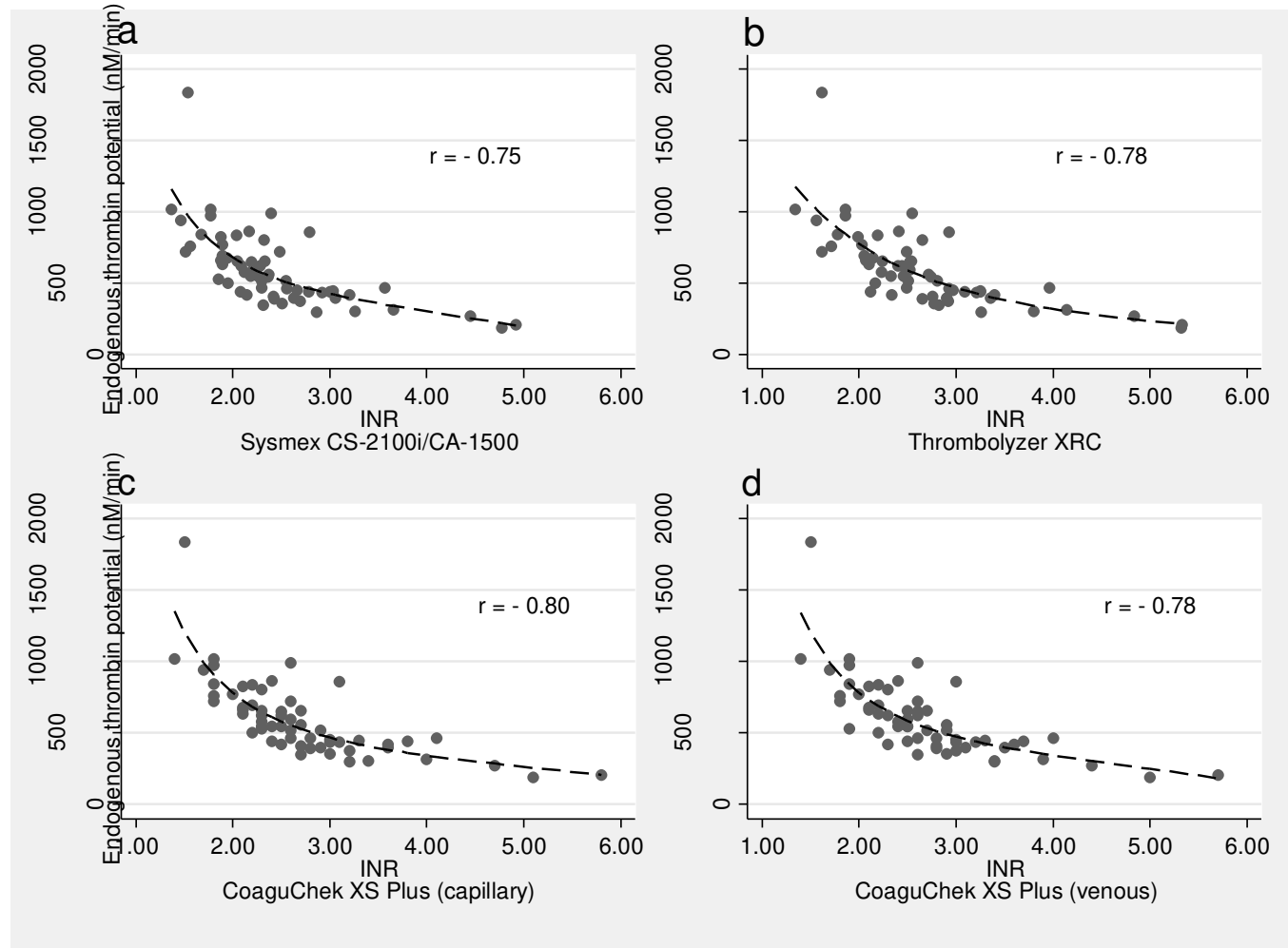
** All parameters are reported as median (IQR)

Legend: AF = atrial fibrillation; IQR = interquartile range; SD = standard deviation; VTE = venous thromboembolism

Table 4. Agreement of the CoaguChek XS Plus on capillary blood samples, with the other INR methodologies

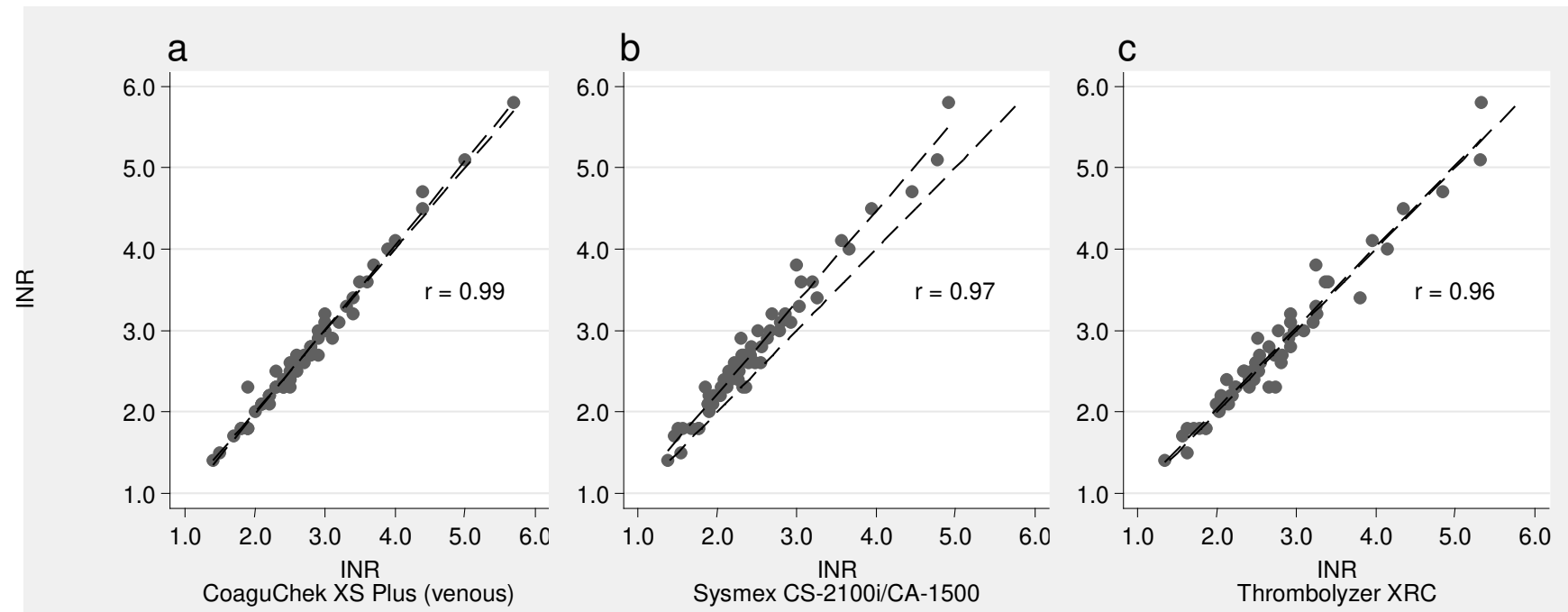| *Comparison*	*Spearman's correlation coefficient r (p value)*	*INR difference, mean (± SD)*	*Magnitude of absolute difference, n (%)*		
			< 0.5	**0.5-1.0**	**> 1.0**
CoaguChek XS Plus (capillary blood) *vs.* CoaguChek XS Plus (venous blood)	0.9856 (< 0.001)	0.002 (0.11)	60 (100%)	0	0
CoaguChek XS Plus (capillary blood) *vs.* Sysmex CS-2100i/CA-1500	0.9699 (< 0.001)	0.28 (0.18)	53 (88.3%)	7 (11.7%)	0
CoaguChek XS Plus (capillary blood) *vs.* Thrombolyzer XRC	0.9646 (< 0.001)	0.04 (0.18)	58 (98.3%)	1 (1.7%)	0

Legend: INR = international normalized ratio; SD = standard deviation

Figure 1. Correlation between the Endogenous Thrombin Potential (ETP) and the INR, measured with the Sysmex CS-2100i/CA-1500 (a), the Thrombolyzer XRC (b), the CoaguChek XS Plus on capillary samples (c) and on venous samples (d)

Figure 2. Correlation between the INR measured with the CoaguChek XS Plus on capillary samples and the CoaguChek XS Plus on venous samples (a), the Sysmex CS-2100i/CA-1500 (b), and the Thrombolyzer XRC (c)
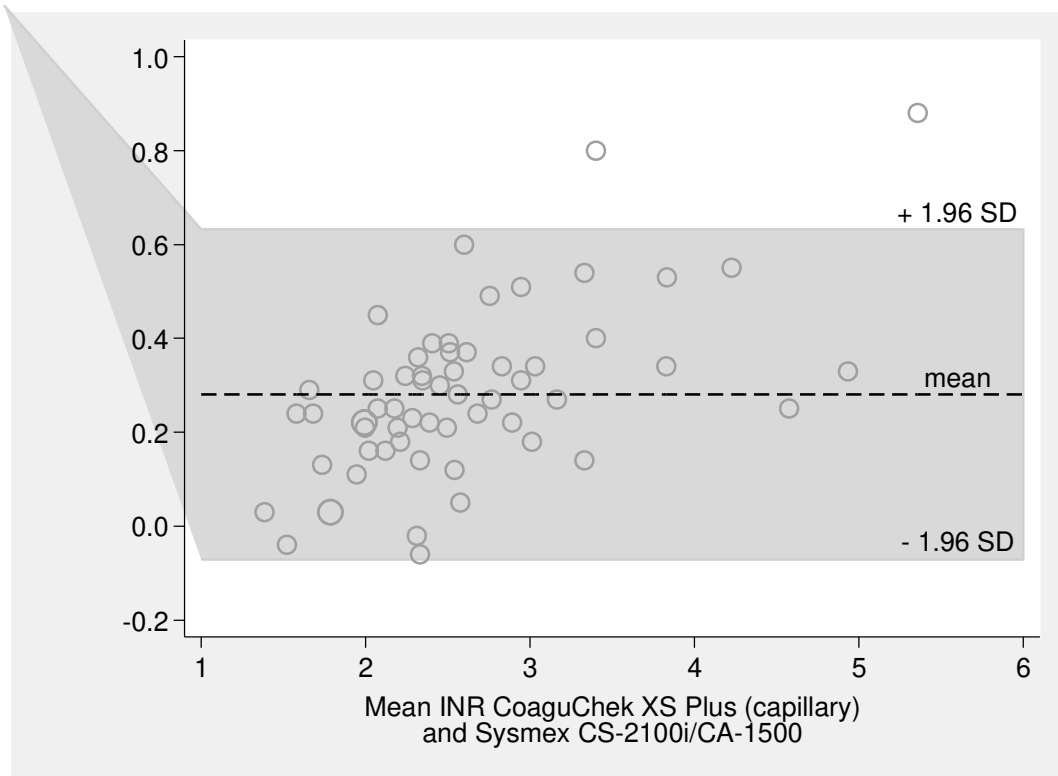
The dashed line represents the perfect correlation, while the continuous line is the actual correlation between the two different INR methodologies

Figure 3. Bland Altman plots representing the difference between the CoaguChek XS Plus on capillary samples and the Sysmex CS-2100i/CA-1500

The dashed line represents the mean difference, while the grey area defines the 95% limits of agreement

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