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Commutability Assessment of Processed Human Plasma Samples for Normetanephrine and Metanephrine Measurements Based on the Candidate Reference Measurement Procedure

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Background: To identify candidate external quality assessment (EQA) materials for normetanephrine and metanephrine measurements, we assessed the commutability of eight processed human plasma samples. The agreement between routine assays and the candidate reference measurement procedure (cRMP) was also evaluated.

Methods: Fifty-three clinical samples and eight processed plasma samples were prepared. The processed samples included pooled and individual plasma samples spiked with pure normetanephrine and metanephrine and non-spiked pooled and individual plasma samples. The clinical and processed samples were subjected to four routine isotope dilution tandem mass spectrometry assays and cRMP. Commutability was assessed based on two approaches recommended by the CLSI and International Federation of Clinical Chemistry (IFCC). Passing–Bablok regression and Bland–Altman analysis were used to evaluate the agreement between the routine assays and cRMP.

Results: The commutability results of the CLSI approach were better than those of the IFCC approach. For the CLSI approach, spiked individual plasma samples and spiked high-concentration pooled plasma samples were commutable for all routine assays for both analytes. The non-spiked pooled plasma sample was commutable for two out of four routine assays for metanephrine and three out of four routine assays for normetanephrine. The agreement between the routine assays and the cRMP was satisfactory, except for one routine assay showing significant bias.

Conclusions: High-concentration spiked pooled plasma samples and spiked individual plasma samples are candidate EQA materials for normetanephrine and metanephrine measurements.

Key Words: Tandem mass spectrometry, Commutability, Normetanephrine, Metanephrine

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INTRODUCTION

Pheochromocytomas and paragangliomas (PPGLs) are rare endocrine tumors associated with the over-secretion of catecholamine, metanephrine, and normetanephrine [1]. The Endocrine Society Guideline recommends plasma free or urinary fractionated normetanephrine and metanephrine as preferred biomarkers for the diagnosis of PPGLs [2]. Normetanephrine and metanephrine measurements are also useful in judging surgical quality and monitoring tumor metastasis and recurrence [3]. Notably, the sensitivity and specificity of plasma free normetanephrine and metanephrine assays are better than those of urinary fractionated normetanephrine and metanephrine assays [4–6].

External quality assessment (EQA) is widely used to monitor the performance of clinical laboratories [7]. Commutable EQA materials that behave like patient samples can promote the credibility of the role of EQA in evaluating the performance of routine assays and harmonization status [7, 8]. However, for practical reasons (such as stability, analyte concentrations, cost), EQA materials sometimes are processed samples for which commutability is unknown [8–10]. Without commutability information, between-assay variation observed in EQA is hard to improve. Therefore, repeat assays may be required, especially when patients are transferred between hospitals. Considering the significant role normetanephrine and metanephrine play in diagnosing PPGLs, it is urgent to identify the commutability of existing EQA materials and explore suitable EQA materials for normetanephrine and metanephrine measurements.

Recently, we developed a candidate reference measurement procedure (cRMP) for normetanephrine and metanephrine [11]. In 2019, the Chinese National Center for Clinical Laboratories (NCCL) launched an EQA program for normetanephrine and metanephrine measurements in China. Forty-six laboratories participated in the program, and 39 of them used isotope dilution tandem mass spectrometry (ID-LC-MS/MS). We selected the four most commonly used routine ID-LC-MS/MS assays and the cRMP developed in our laboratory as a comparative assay to assess the commutability of eight processed human plasma samples for normetanephrine and metanephrine measurements. To the best of our knowledge, no studies on the commutability of processed human plasma samples for normetanephrine and metanephrine measurements have been published. The two most widely used commutability assessment approaches, i.e., the linear regression approach recommended by the CLSI (EP30-A) [12] and the difference in bias approach

recommended by the International Federation of Clinical Chemistry (IFCC), were applied [13–15]. The agreement between the routine assays and the cRMP was evaluated by comparing the results of 53 clinical samples determined using the routine assays and the cRMP.

MATERIALS AND METHODS

Clinical samples

More than 200 individual lithium-heparin-anticoagulated plasma samples with normetanephrine and metanephrine concentrations of 5–1,600 pg/mL and 20–1,800 pg/mL (normetanephrine: 0.03–8.73 nmol/L, 1 nmol/L = 183.20 pg/mL; metanephrine: 0.10–9.13 nmol/L, 1 nmol/L = 197.23 pg/mL), respectively, were collected at Beijing Hospital (Beijing, China) between October 2020 and May 2021. All samples were from patients undergoing normetanephrine and metanephrine assays and were stored at –80°C. Samples with hemolysis, icterus, or lipemia were considered deviant and were excluded. This retrospective study was approved by the Ethics Committee of Beijing Hospital, with exemption from informed consent (approval No. 2016BJYYEC-121-03). Samples were pooled and aliquoted for measurement because of limited sample volumes. For pooling, the samples were sorted based on their normetanephrine and metanephrine concentrations and the relative differences in the concentrations of the two analytes. No more than three samples with similar normetanephrine and metanephrine concentrations were thawed and pooled to achieve sample volumes of at least 4 mL. Thus, 53 pooled samples were prepared and split into five aliquots. After preparation, the samples were stored at –80°C until analysis.

Processed human plasma samples

A set of eight processed human plasma samples, including five EQA samples (202013, 202014, 202113, 202114, 202115) obtained from the 2021 Chinese National EQA program for normetanephrine and metanephrine and three processed human plasma samples (Plasma 1, Plasma 2, Plasma 3) were used in this study. The national EQA samples from 2020 (202013, 202014) and 2021 (202113, 202114, 202115) were prepared by pooling fresh leftover patient plasma samples collected from the clinical laboratory at Beijing Hospital. All pooled plasma samples were assayed to ensure they were not reactive for anti-HIV antibodies, anti-hepatitis C virus antibodies, and hepatitis B virus surface antigen and were stored at –80°C. Before EQA sample preparation, all plasma was thawed, pooled on a magnetic stirrer (Vario-

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mag Compact, Thermo Fisher Scientific, Waltham, MA, USA), and filtered sequentially through 0.45-μm and 0.22-μm membranes using a vacuum pump (Thermo Scientific™ RAP, Thermo Fisher Scientific, Waltham, MA, USA). Except for sample 202113, all EQA samples were spiked with pure normetanephrine and metanephrine (normetanephrine: Sigma Chemical Company, St. Louis, MO, USA, Lot No.: N7127, purity: ≥98%; metanephrine: Aladdin Biochemical Technology, Shanghai, China, Lot No.: R134561, purity: ≥98%) at physiological and pathological concentrations (Table 1). Eventually, the processed pooled plasma was aliquoted into 1.5-mL vials and stored at –80°C until analysis. To ensure homogeneity, all processed pooled plasma samples were thoroughly mixed throughout the aliquoting process using a magnetic stirrer.

Plasma 1, Plasma 2, and Plasma 3 were individual plasma samples collected from Beijing Hospital. Plasma 2 and Plasma 3 were spiked with pure substances at pathological concentrations, whereas Plasma 1 was not. All samples, including the 53 clinical and eight processed samples, were distributed to participating laboratories on dry ice and were stored at –80°C until analysis. The cRMP and routine assays were used to measure all clinical and processed samples two times in a single run. The eight processed plasma samples were randomly inter-spersed among the clinical samples during the measurements. Each laboratory was requested to return two values for each clinical sample and processed sample.

Analytical methods

The four routine assays used in this study are the most commonly used routine assays for plasma normetanephrine and metanephrine in China. The assays are denoted as A, B, C, and D. Detailed information on the instruments, calibrators, internal standard materials, and sample preparation procedures was provided by the participating laboratories (Supplemental Data Table S1).

The cRMP used in this study was developed in our laboratory and comprises a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) and a API6500 tandem mass spectrometer (AB Sciex, Framingham, MA, USA) [11]. Protein precipitates prepared using methanol and cation solid-phase extraction (SPE) (Waters Corporation) were used to isolate normetanephrine and metanephrine from the plasma matrix. The spike recovery rates of the cRMP were 98.5%–101.9% for normetanephrine and 98.3%–101.7% for metanephrine; the within-run imprecision was 1.10%–1.34% for normetanephrine and 0.79%–1.36% for metanephrine; and the total imprecision was

Table 1. Commutability assessment results from the CLSI and IFCC approaches

Sample	Analyte (pg/mL)	CLSI EP30-A				IFCC			
		A	B	C	D	A	B	C	D
Plasma 1	Metanephrine (25.9)*	C	C	C	C	C	C	C	C
	Normetanephrine (68.9)*	C	C	C	–	C	I	C	I
Plasma 2	Metanephrine (268.0)*	C	C	C	C	C	C	I	C
	Normetanephrine (287.8)*	C	C	C	C	C	C	C	C
Plasma 3	Metanephrine (533.8)*	C	C	C	C	C	I	C	C
	Normetanephrine (449.4)*	C	C	C	C	C	I	C	C
202113	Metanephrine (37.1)*	+	C	+	C	C	C	I	I
	Normetanephrine (73.1)*	C	C	C	–	C	C	I	NC
202013	Metanephrine (1,344.9)*	C	C	C	C	C	I	C	C
	Normetanephrine (1,137.2)*	C	C	C	C	C	I	C	C
202014	Metanephrine (634.8)*	C	C	C	C	C	I	I	C
	Normetanephrine (579.9)*	C	C	C	C	C	I	C	C
202114	Metanephrine (206.1)*	+	C	C	C	C	I	I	C
	Normetanephrine (299.3)*	C	C	C	–	C	I	C	C
202115	Metanephrine (388.3)*	+	C	+	C	C	C	I	C
	Normetanephrine (620.1)*	C	C	C	C	C	C	C	C

*Refers to the mean of cRMP results. A, B, C, and D refer to the four most commonly used routine normetanephrine and metanephrine assays in China.
Abbreviations: C, commutable; IFCC, International Federation of Clinical Chemistry; +, positive matrix effect; –, negative matrix effect; NC, non-commutable; I, inconclusive results; cRMP, candidate reference measurement procedure.

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1.53%–1.87% for normetanephrine and 1.15%–1.64% for metanephrine.

Statistical analysis

The means of the two repeat values of the clinical and processed samples were used to assess the commutability of the processed samples. Log_{10}-transformed and ln-transformed concentrations were used to obtain the consistent scatter of the difference plot over the concentrations in the CLSI and IFCC approaches, respectively. The two approaches used to assess the commutability of the processed samples are described in detail below.

To evaluate the agreement between the cRMP and four routine assays, the means of the two repeat values of the 53 clinical samples were used in Passing–Bablok (PB) regression and Bland–Altman (BA) analysis, and Spearman's analysis was used to calculate the correlation coefficients between the methods. P <0.05 was considered statistically significant. The cRMP was considered as the standard. The Chinese NCCL sets ±30% of the target values as the total allowable error (TEa) limits for normetanephrine and metanephrine measurements. We used one third of the TEa (10%) to assess the intra-run imprecision of the routine assays and half of the TEa (15%) to assess the bias of the routine assays. The two repeat values of each clinical sample were used to calculate the intra-run imprecision (intra-run %CV). Outliers were screened by visual inspection. The generalized extreme studentized deviate technique was used for reinspection according to the CLSI EP 09-A3 guidelines [16]. All calculations were conducted using Microsoft Excel 2016 and MedCalc statistical software 18.11.6-64-bit (MedCalc Software, Ostend, Belgium).

CLSI approach based on linear regression analysis

As described in the CLSI EP30-A (formerly, C53-A) guidelines, the commutability of processed samples can be identified by regression analysis. The linearity was visually inspected using ordinary linear regression. The 95% prediction interval of Deming regression was calculated to assess the commutability of the processed samples. When the results of the processed samples were within or exceeding the 95% prediction interval of Deming regression, they were considered commutable and non-commutable, respectively.

IFCC approach based on difference in bias analysis

The IFCC recommends assessing the difference in bias and its uncertainty to determine the commutability of processed sam-

ples [13–15]. Briefly, the uncertainty of bias of the routine assays was calculated according to equations 1, 2, and 3.

$$d_{RM} = B_{RM} - B_{CS} \qquad (1)$$

$$u(d_{RM}) = \sqrt{\frac{(S_x^2 + S_y^2)}{p} + \frac{S_B^2}{n}} \qquad (2)$$

$$U(d_{RM}) = k \times u(d_{RM}) \qquad (3)$$

where B_{RM} is the mean bias of the processed samples, and B_{CS} is the mean bias of the clinical samples. S_x and S_Y represent the standard deviation of the processed sample results derived from the cRMP and routine assays, respectively, p is the number of repeat assays (p=2), S_B is the standard deviation of the biases of the results of the clinical samples as measured by routine assays, n is the number of clinical samples, and k is the expansion factor (k=2). U (d_{RM}) is the expanded uncertainty of d_{RM}. The commutability evaluation limit C is usually fixed and based on the medical requirements of the assay. In this study, half of the TEa as recommended by the Chinese NCCL was set as the commutability evaluation limit C (15%). When $d_{RM} \pm U (d_{RM})$ was within, exceeding, or overlapping 0±C, the processed samples were considered commutable, non-commutable, and inconclusive, respectively.

RESULTS

Commutability assessment based on the CLSI approach (EP30-A guidelines)

For normetanephrine, two out of 53 A assay results and one out of 53 C assay results were identified as outliers and thus excluded. For metanephrine, two, one, and two out of 53 A, B, and C assay results, respectively, were identified as outliers and thus excluded. Since the routine assays presented a nearly constant %CV (the differences between the routine assays and the cRMP increased in proportion to the concentrations of normetanephrine and metanephrine), according to the CLSI approach, log_{10} (concentration) was used for the commutability assessment. The commutability of the eight processed samples is summarized in Table 1 and Fig. 1. Plasma 2 and Plasma 3 (spiked individual plasma samples) were commutable for all routine assays for both analytes. Sample 202113 (non-spiked pooled patient sample) showed a positive matrix effect for metanephrine measurement by assays A and C and a negative matrix effect for normetanephrine measurement by assay D. Plasma 1 (non-spiked individual plasma sample) showed a negative matrix effect for normetanephrine measurement by as-
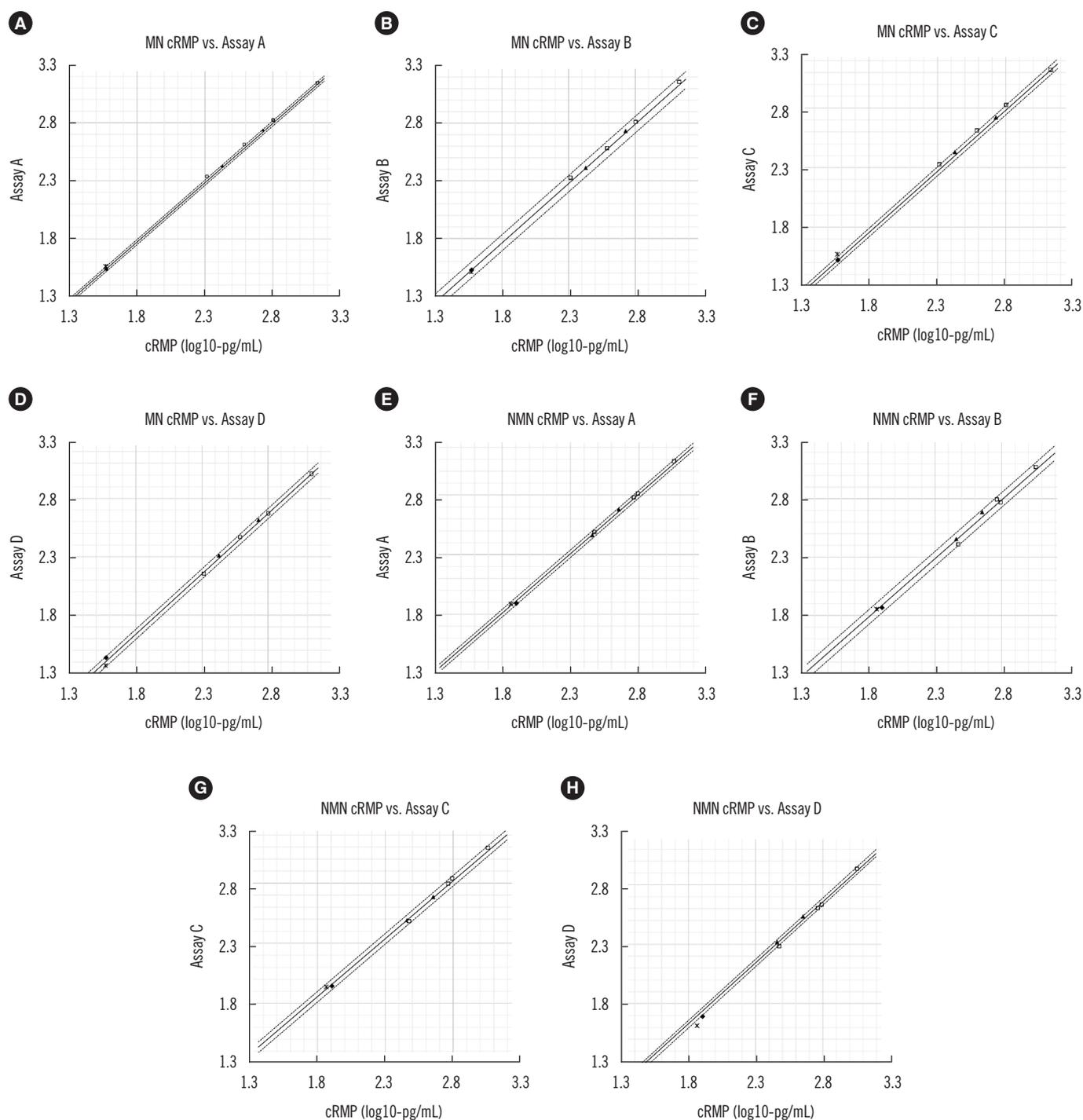
Fig. 1. Commutability of the processed samples based on the CLSI approach. The black solid lines are the regression lines, and the black dashed lines are the two-tailed 95% prediction lines. The X-axis indicates the log10-transformed results of the cRMP, and the Y-axis indicates the log10-transformed results of routine assays. Panels A–D present the commutability assessment results for metanephrine. Panels E–H present the commutability results for normetanephrine. The EQA materials (202013, 202014, 202114, and 202115), spiked individual plasma (Plasma 2 and Plasma 3), non-spiked individual plasma (Plasma 1), and non-spiked EQA (202113) samples are shown as squares, black triangles, black diamonds, and stars, respectively.
Abbreviations: NMN, normetanephrine; MN, metanephrine; cRMP, candidate reference measurement procedure.
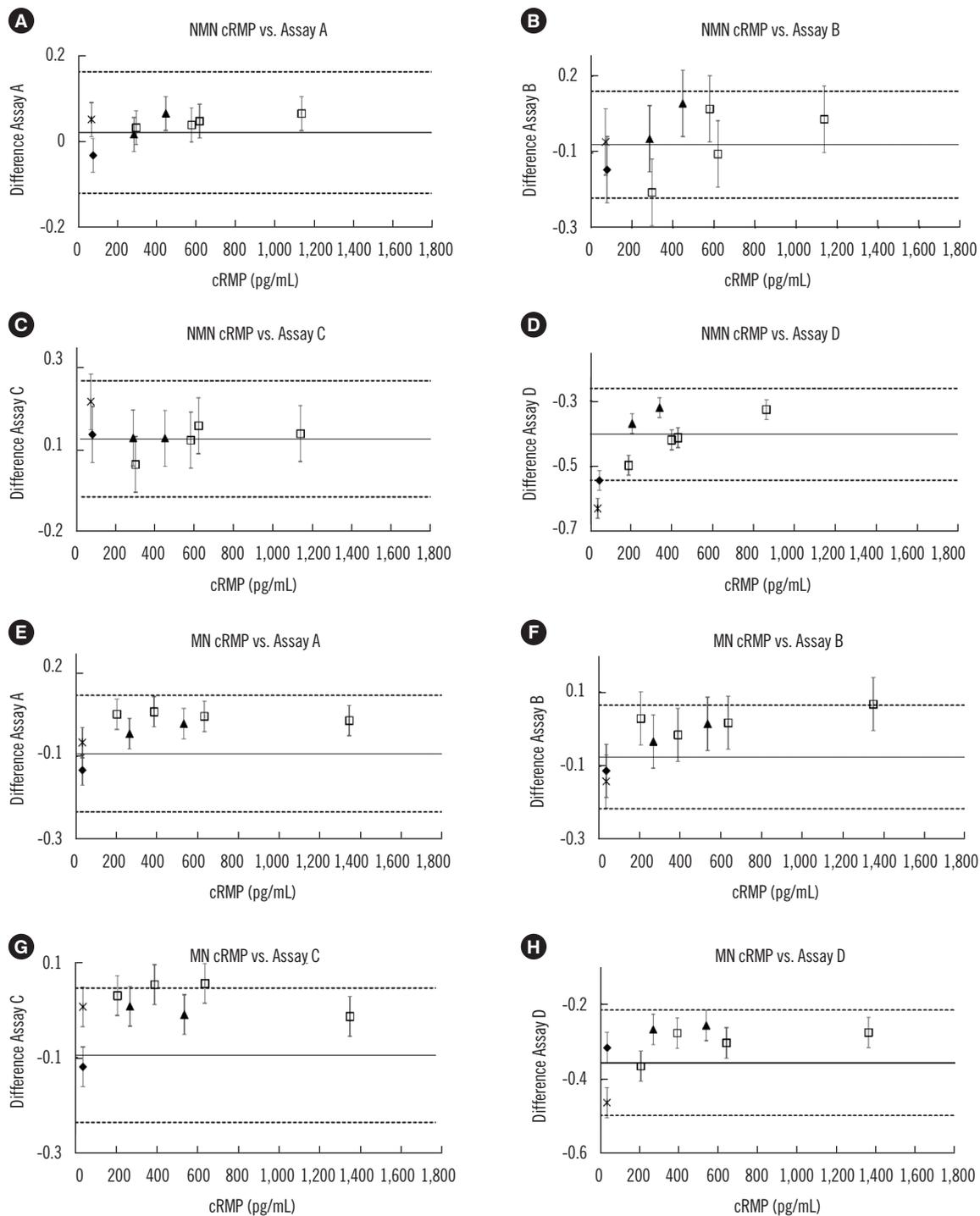
Fig. 2. Commutability results based on the IFCC approach. The mean bias of clinical samples is presented by a solid black line, and the assessment limits are shown by dashed lines. Panels A–D present the commutability assessment results for normetanephrine. Panels E–H present the commutability results for metanephrine. The EQA samples (202013, 202014, 202114, and 202115), spiked individual plasma (Plasma 2 and Plasma 3), non-spiked individual plasma (Plasma 1), and non-spiked EQA (202113) samples are shown as squares, black triangles, black diamonds, and stars, respectively. The X-axis indicates the concentration defined by the cRMP.

Abbreviations: NMN, normetanephrine; MN, metanephrine; cRMP, candidate reference measurement procedure; IFCC, International Federation of Clinical Chemistry.

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say D (Table 1 and Fig. 1).

EQA samples 202114 and 202115 were non-commutable in some clinical assays and the matrix effects were all positive for metanephrine measurement and all negative for normetanephrine measurement (Table 1 and Fig. 1). However, 202013 and 202014 were commutable for all routine assays for both analytes. Notably, all processed samples were commutable in assay B (Table 1 and Fig. 1).

Commutability assessment based on the IFCC approach

The SD of clinical samples increased with increasing normetanephrine and metanephrine concentrations (Supplemental Data Figure S1). However, plots of the clinical sample concentrations and (ln [routine assay results]–ln [cRMP results]) revealed approximately constant scatter widths (Supplemental Data Figure S2). Therefore, according to the IFCC guidelines, ln (concentration) was used for statistical analysis. The criterion (15%) was converted to an absolute number (± 0.140) using Equation (4):

Relative bias (%)$\approx 100(e$ ln $bias$-1) (4)

The commutabilities of nearly half of the processed samples for assays B and C were inconclusive (Table 1, Fig. 2). Notably, all processed samples were commutable for assay A. Except for Plasma 1 (non-spiked individual sample) for normetanephrine measurement and EQA sample 202113 (non-spiked pooled plasma) for both analytes, all processed samples were commutable for assay D (Table 1).

Agreement among the cRMP and four routine assays

The concentrations of normetanephrine and metanephrine in the 53 clinical samples were 17–1,502 pg/mL and 22–1,571 pg/mL, respectively. Scatter plots of the results of the cRMP and each routine assay for the 53 samples are presented in Supplemental Data Fig. S3. The results of PB regression and BA analysis are presented in Table 2 and Fig. 3. Strong correlations among the cRMP and routine assay results were observed, with Spearman's correlations coefficients (R) of 0.992–0.997. The PB regression slopes were 0.72–1.03 (Table 2, Fig. 3). The slopes for assay D were 0.74 and 0.72 for metanephrine and normetanephrine, respectively, indicating that this assay showed significant biases for metanephrine and normetanephrine measurements (Table 2). BA analysis showed that the mean biases of all routine assays except D met the bias limit of $\pm 15\%$ (assay D bias was 29.45% for normetanephrine and 29.62% for metanephrine) (Table 2).

DISCUSSION

To explore possible commutable materials for normetanephrine and metanephrine measurements, we assessed the commutability of existing EQA samples and other processed materials using two different statistical approaches.

According to the CLSI approach (EP30-A guidelines), the spiked individual plasma samples (Plasma 2 and Plasma 3) and spiked high-concentration pooled plasma samples (EQA samples 202013 and 202014) were commutable for both analytes for all routine assays; therefore, they are suitable EQA materials for normetanephrine and metanephrine measurements. However, the low-concentration pooled plasma samples (EQA samples 202114 and 202113) were non-commutable for some routine assays for both analytes. One possible explanation is that the worse precision of the routine assays at low concentrations may have affected the statistical calculation in the CLSI approach. Fortunately, high-concentration normetanephrine and metanephrine are more clinically significant in diagnosing PPGLs. Normally, the non-spiked individual plasma (Plasma 1) should have been commutable for all routine assays. However, it was non-commutable for normetanephrine in assay D. One possible explanation is that the concentrations of normetanephrine and metanephrine in Plasma 1 were close to the limits of quantification of assay D, and thus, the results of assay D for Plasma 1 may have been insufficiently accurate.

The results of commutability assessment based on the CLSI approach were significantly discrepant from those based on the IFCC approach, especially for assays B and C (Table 1, Figs. 1, 2). One possible explanation is that our measurement protocol based on the EP30-A guidelines does not consider the position effect. This may have affected the calculation and interpretation of the final results in the IFCC approach. Another possible explanation is that the criterion ($Inbias \pm 0.140$) of the IFCC guidelines is too strict for normetanephrine and metanephrine measurements. According to the relevant statistical principles of both approaches, the commutability assessment results based on the IFCC approach were closely associated with assay precision. Assays with poor precision would have larger U (d_{RM}) and thus, $d_{RM} \pm U$ (d_{RM}) easily exceeded or overlapped the fixed criterion, yielding non-commutable or inconclusive results. However, in the CLSI approach, the width of the 95% prediction interval of the Deming regression depends on the residual error (imprecision) of the assays [17]. Methods with poor precision would have wider prediction intervals; therefore, the results of the processed samples were more likely to fall within the wide
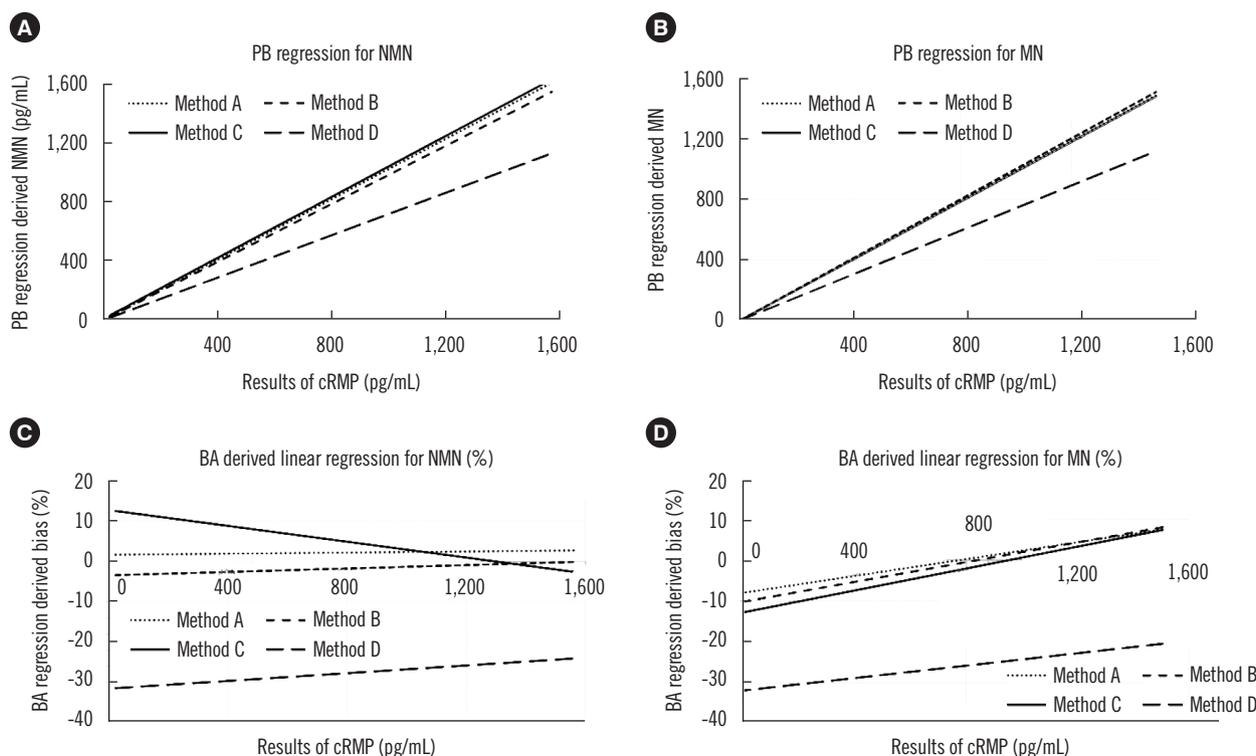
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Fig. 3. PB regression and BA analysis of the results of the four routine ID-LC-MS/MS assays and the cRMP. (A, B) PB regression lines for each routine assay relative to the cRMP. (C, D) regression lines of biases of each assay derived from BA analysis.
Abbreviations: PB, Passing–Bablok; BA, Bland–Altman; NMN, normetanephrine; MN, metanephrine; cRMP, candidate reference measurement procedure.

Table 2. PB regression and BA analysis for normetanephrine and metanephrine measurements of the 53 clinical samples

PB and BA analysis	PB regression (Y = a´ results of cRMP+b)				Regression of biases derived from BA analysis (Y = a´ results of cRMP+b)			
	R	P	Slope (95%CI)	Intercept (95%CI)	Mean bias	Slope	Intercept	SD (±1.96 SD)
Normetanephrine								
A vs. cRMP	0.997	<0.0001	1.02 (1.01, 1.05)	−0.95 (−4.74, 1.59)	1.89	0.0007	1.5739	4.35 (−6.64, 10.41)
B vs. cRMP	0.993	<0.0001	0.99 (0.96, 1.02)	−2.42 (−6.23, 0.91)	−2.58	0.0021	−3.4934	7.49 (−17.27, 12.12)
C vs. cRMP	0.992	<0.0001	1.03 (1.01, 1.05)	7.06 (1.61, 13.10)	8.22	−0.0098	12.665	8.80 (−9.03, 25.47)
D vs. cRMP	0.996	<0.0001	0.72 (0.70, 0.75)	−1.74 (−9.39, 0.37)	−29.45	0.0048	−31.615	6.76 (−42.71, −16.23)
Metanephrine								
A vs. cRMP	0.996	<0.0001	1.00 (0.96, 1.02)	−2.27 (−5.15, 0.75)	−4.81	0.0103	−8.0056	6.20 (−16.94, 7.34)
B vs. cRMP	0.993	<0.0001	0.99 (0.96, 1.02)	−2.65 (−4.96, 0.95)	−6.62	0.0121	−10.164	8.48 (−23.23, 10.00)
C vs. cRMP	0.995	<0.0001	0.98 (0.96, 1.01)	−3.78 (−5.38, 2.60)	−8.65	0.0134	−12.974	7.72 (−23.79, 6.48)
D vs. cRMP	0.997	<0.0001	0.74 (0.73, 0.76)	−1.62 (−2.46, 0.77)	−29.62	0.0076	−31.885	5.38 (−40.17, −19.07)

Abbreviations: PB, Passing–Bablok; BA, Bland–Altman; A, assay A; B, assay B; C, assay C; D, assay D; cRMP, candidate reference measurement procedure.

Table 3. Mean intra-run imprecision (intra-run %CV) of the routine assays

Mean intra-run %CV	Assay A	Assay B	Assay C	Assay D
Metanephrine	1.86	8.27	4.53	3.35
Normetanephrine	2.00	7.00	4.60	0.55

The intra-run %CV was calculated from the two repeat values for the 53 clinical samples measured by the four routine assays.

interval and to be considered commutable.

The intra-run %CV of assays B and C was substantially larger than that of assays A and D (Table 3). Therefore, for assays B and C, processed samples were more likely to be judged commutable based on the CLSI approach, whereas non-commutable and inconclusive results were more likely to be observed when the IFCC approach was used. This may explain the signifi-

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cant discrepancy between the results for assays B and C when different methods were used to assess commutability. However, it bears mentioning that, despite the imprecision of assays B and C being poorer than that of assays A and D, the intra-run imprecision of all routine assays met the imprecision limit of 10% (Table 3). Therefore, the impact of imprecision should not be exaggerated. We believe that the criterion (*Inbias* ± 0.140) used in this study in the IFCC approach was too strict, and the commutability assessment results based on the CLSI approach may more accurately reflect the commutability of the processed samples.

The normetanephrine and metanephrine concentrations in the 53 clinical plasma samples measured by the four routine assays and cRMP were compared using PB and BA analyses. Similar to previous findings [18], we observed good correlations between the cRMP and routine assays. Notably, we previously demonstrated that using patient-derived plasma samples assigned by the cRMP to recalibrate routine assays greatly decreased the large biases of the routine assays [11]. Therefore, the inconsistency among the cRMP and clinical assays may be mainly due to the data transfer and differences in the traceability systems of the different routine assays.

This study had some limitations. First, because of limited clinical sample volumes, each sample was measured only twice; however, according to the EP30-A guidelines, clinical and processed samples should be analyzed thrice. Second, although 3-methoxytyramine is also useful in screening PPGLs and metastatic diseases, it was not investigated in our study.

In conclusion, spiked pooled as well as individual plasma samples are potential commutable materials for normetanephrine and metanephrine measurements and can be used as EQA materials or quality control materials for routine assays. We observed a good agreement between the results of the cRMP and most routine ID-LC-MS/MS assays.

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AUTHOR CONTRIBUTIONS

Deng Y wrote the manuscript. Liu Q conducted the experiments and analyzed the data. Liu Z and Zhao H participated in the discussion of the study results. Zhou W and Zhang C conceived the study.

CONFLICTS OF INTEREST

No potential conflicts of interest relevant to this article are reported.

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Supplemental Data Table S1. Details of the four routine ID-LC-MS/MS assays and the cRMP

| Method	A	B	C	D	cRMP
MS Instrument	AB Sciex 5500 (AB Sciex, Framingham, MA, USA)	Thermo TSQ Endura (Thermo Fisher Scientific, Waltham, MA, USA)	AB Sciex 6500 (AB Sciex)	Waters Xevo TQ-S (Waters Corporation, Milford, MA, USA)	AB Sciex 6500 (AB Sciex)
LC- Instrument	Shimadzu LC-20ADXR (Shimadzu, Kyoto, Japan)	Thermo U3000 (Thermo Fisher Scientific)	Waters/Eixon (Waters Corporation)	Acquity UPLC (Waters Corporation)	Waters Acquity UPLC FI-I class system (Waters Corporation)
Standard materials	MN: Dr. Ehrenstorfer GmbH (Augsburg, Germany) NMN: Sigma (Sigma-Aldrich St. Louis, MO, USA)	National institutes for food and drug control in China	MN: Aladdin (Shanghai, China) NMN: Toronto Research Chemicals (Toronto, Canada)	Sigma (Sigma-Aldrich)	Cerilliant (Sigma-Aldrich)
Internal Standard	MN-D3 (Medical Isotopes, Pelham, NH, USA) NMN-D3 CDN	Sigma (Sigma-Aldrich)	Sigma (Sigma-Aldrich)	Sigma (Sigma-Aldrich)	Cambridge Isotope Laboratories, Andover, MA, USA
QC materials	In-house-prep	In-house-prep	In-house-prep	In-house-prep	In-house-prep
Solvent of working solution	Plasma MNs: buffer	Water	50% Methanol (Thermo Fisher Scientific)/water	Water (Millipore, Billerica, MA, USA)	1 mg/mL Ascorbic acid aqueous (Sigma Chemical Company, St. Louis, MO, USA) solution
Samples	Plasma and urine	Plasma and urine	Plasma	Plasma and Urine	Plasma
Linear range for plasma (pg/mL)	MN:15–2,500 NMN:15–2,600	MN:10–5,000 NMN:10–5,000	MN:15–1,500 NMN:15–1,500	10(2)–5,000	N/A
Sample volume required (µL)	Plasma MNs: 200	400	200	200	20–400
Sample preparation	SPE	SPE	SPE	SPE	SPE (WCX, Waters Corporation. Milford, MA, USA)
Redissolving solvent before MS detection	Plasma MNs: 100 µL 0.2% FA/Water	Plasma: 100 µL Acetonitrile (Thermo Fisher Scientific)/FA/water	5 mM Ammonium acetate/40% acetonitrile/water	40 µL Water	0.1% FA aqueous solution
Injection volume of MS measurement (µL)	Plasma: 30	Plasma: 10	Plasma: 20	Plasma: 10	Plasma: 20
Chromatographic column	PFPP (Shimadzu)	XBridge amide	Waters BEH C18 (Waters Corporation,)	Waters HSS PFP	Shim-pack Velox PFPP column (1.8 µm, 100 mm×2.1 mm, Shimadzu)
Mobile phase	Plasma MNs: 0.2% FA/water, 0.2% FA/acetonitrile (Thermo Fisher Scientific Waltham)	FA/water, FA/acetonitrile (Thermo Fisher Scientific)	5 mM Ammonium acetate (Sigma Chemical Company)/water, 5 mM ammonium acetate/95% acetonitrile/water	0.1% FA/water, acetonitrile (Thermo Fisher Scientific)	0.1% FA and 2-mmol/L ammonium formate solution and methanol
Total %CV					
NMN	0.88%–4.47%	2.84%–7.23%	2.60%–4.23%	7.58%–9.64%	1.15%–1.64%
MN	1.80%–7.75%	4.89%–6.58 %	2.93%–6.94%	4.50%–8.59%	1.53%–1.87%
Accuracy activities	Spike-and-recovery assay, American CAP	Spike-and-recovery assay	Spike-and-recovery assay	Spike-and-recovery assay	Spike-and-recovery: MN: 98.3%–101.7%; NMN: 98.5%–101.9%

Abbreviations: SPE, solid-phase extraction; NMN, normetanephrine; MN, metanephrine; MS, mass spectrometry; LC, liquid chromatography; cRMP, candidate reference measurement procedure; FA, formic acid.
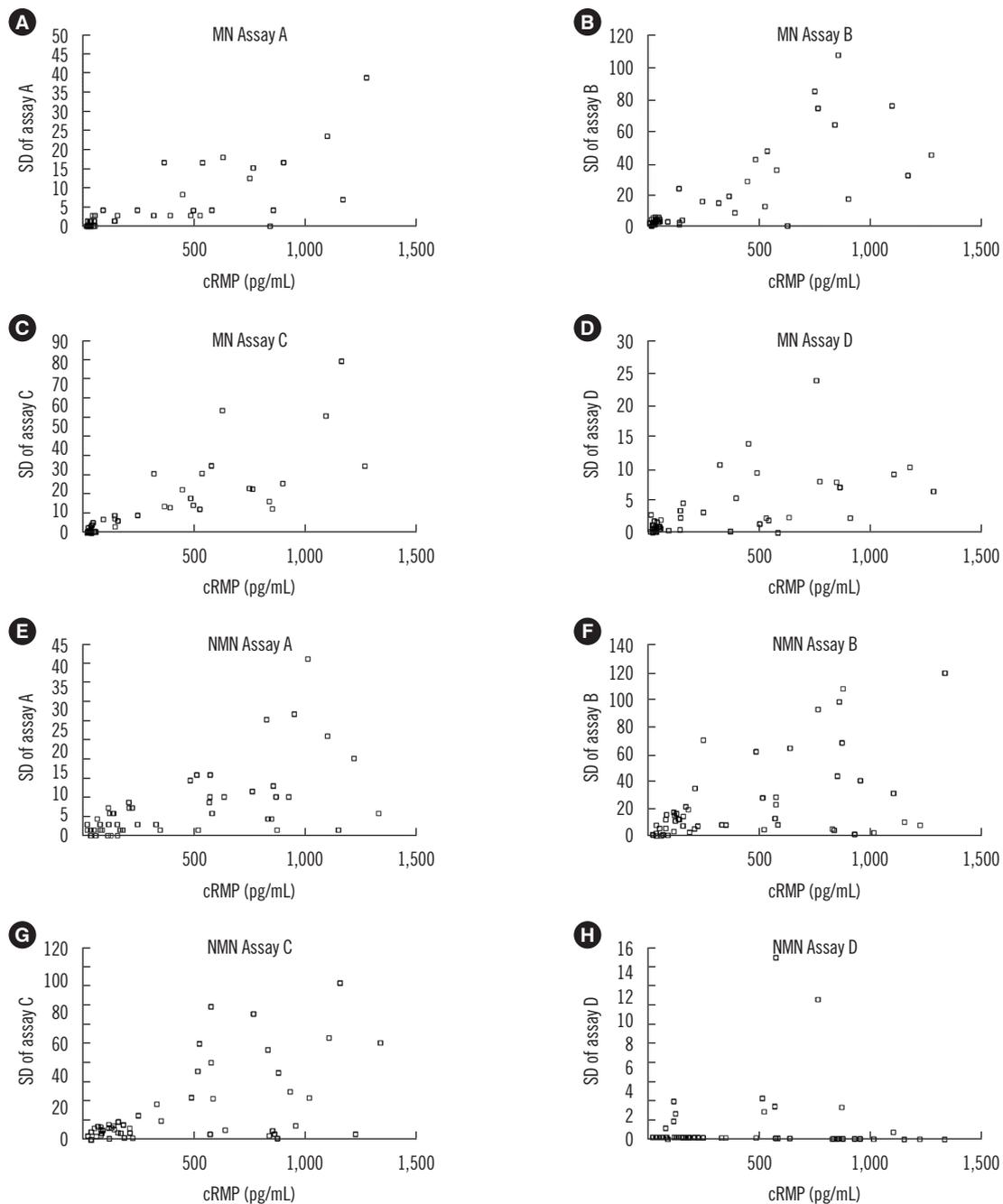
Supplemental Data Fig. S1. The standard deviation (SD) scatter plots of the 53 clinical samples for the four routine assays. The X-axis indicates the concentration defined by the cRMP. Panels A–D are SD scatter plots for metanephrine; panels E–H are SD scatter plots for normetanephrine.
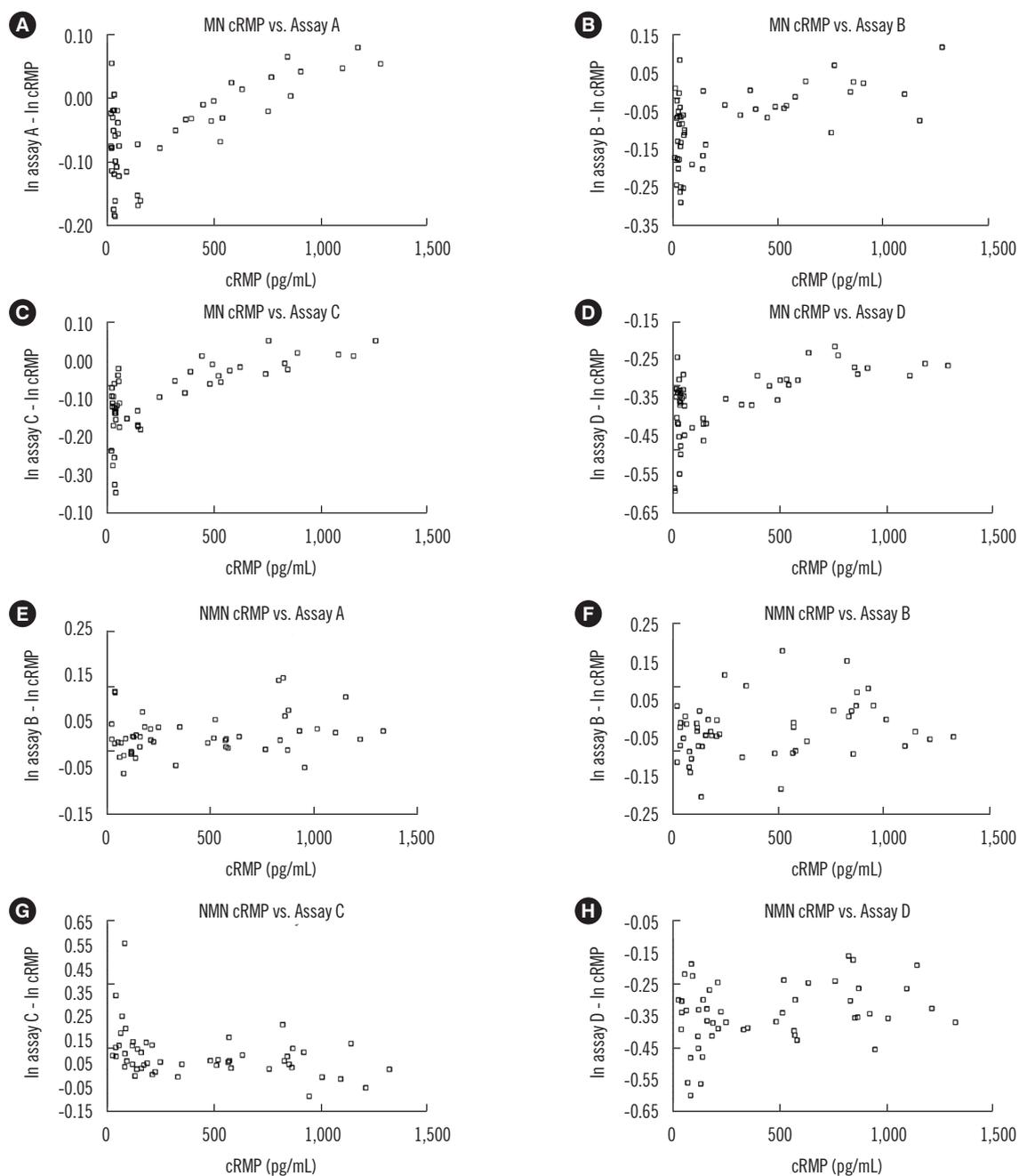
Supplemental Data Fig. S2. Bias (ln [routine assay results] – ln [cRMP results]) plots for the four routine assays. The X-axis indicates the concentration defined by the cRMP. Panels A–D show the results for metanephrine; panels E–H show the results for normetanephrine.

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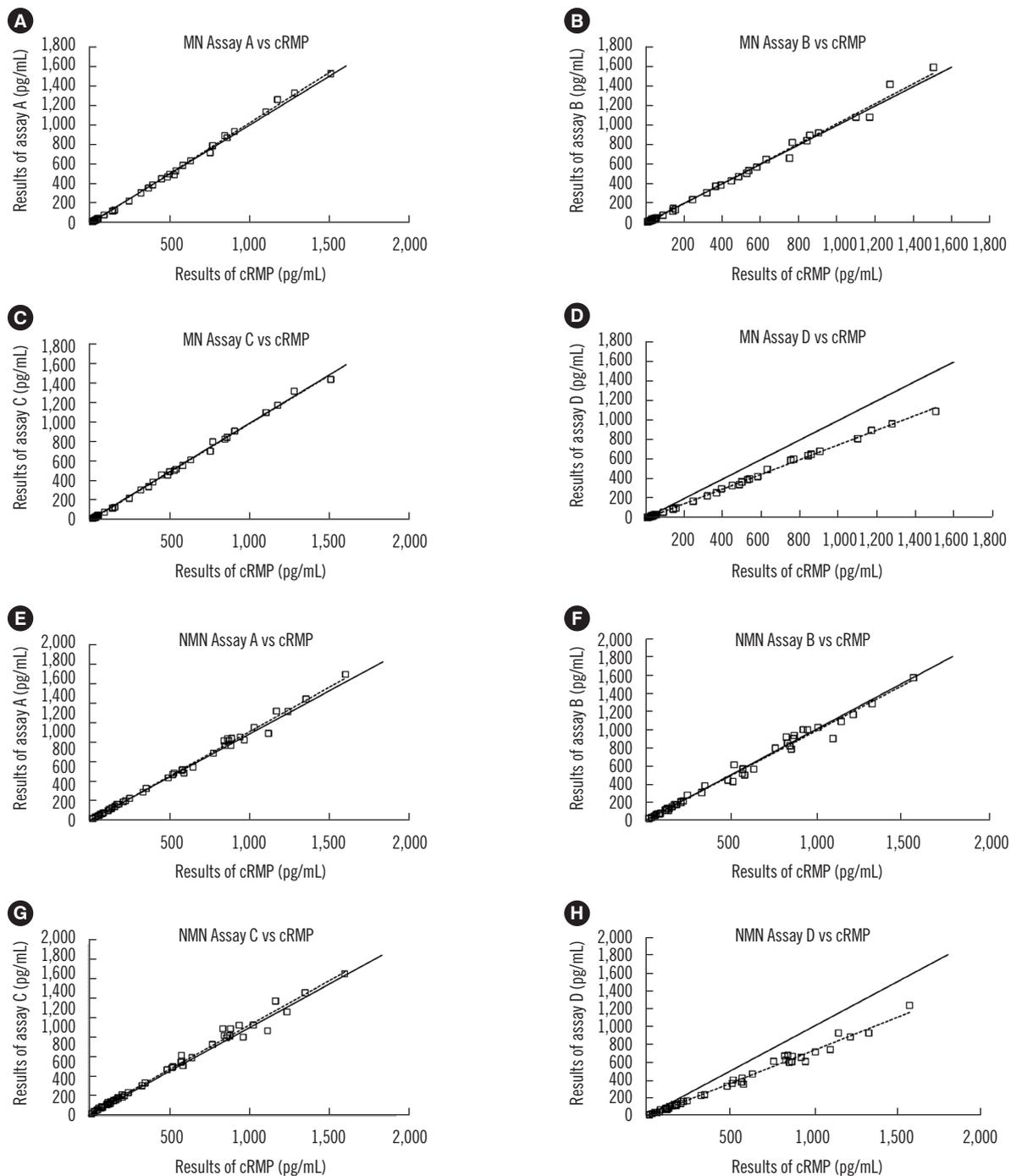
Supplemental Data Fig. S3. Scatter plots of the 53 patient samples for the cRMP versus routine assays. The black dashed lines are the ordinary linear regression lines, and the black solid lines are the lines of Y = X. The X-axis shows the results of the cRMP assay, and the Y-axis shows the results of the routine assays. Panels A–D show scatter plots for metanephrine (MN), and panels E–H show scatter plots for normetanephrine (NMN).