Second Round Robin for plasma hepcidin methods: First steps toward harmonization

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Measurements of the iron regulatory hormone hepcidin by various methodologies and laboratories are not harmonized. As a result different numeric results are obtained for the same clinical sample. We investigated whether better agreement between plasma hepcidin methods can be achieved by harmonization. Native plasma pools (n = 11) of a variety of hepcidin concentrations and blank plasma spiked with three different quantities of synthetic hepcidin-25 purchased from two different commercial sources (n = 6), were distributed in duplicate among 21 methods worldwide. We assessed commutability by comparing results from synthetic hepcidin with those from native samples in various method couples by Bland-Altman plots. Methods differed substantially in absolute values and reproducibility. For the majority of methods we found that samples with synthetic hepcidin-25 were noncommutable with the native samples. In an attempt to harmonize by using native hepcidin results, we selected two methods that showed good mutual agreement of native results and calculated consensus values as the medians for the 11 duplicate native samples obtained by these two methods. Finally, we constructed algorithms enabling the laboratories to calculate the hepcidin consensus (HEPCON) value using their own native hepcidin results. We found that the use of these algorithms substantially reduced the between-method CV. Until commutable materials are defined, hepcidin harmonization can be achieved by exploiting specific algorithms, allowing each lab to report their native hepcidin concentrations in HEPCON values. This study represents the first step toward harmonization of plasma hepcidin methods and facilitates aggregation of hepcidin data from different research investigations. Am. J. Hematol. 00:000–000, 2012. © 2012 Wiley Periodicals, Inc.

Introduction

Hepcidin plays a central role in iron metabolism [1,2], and could become a target of treatment and useful biomarker for the diagnosis and monitoring of iron disorders (reviewed in [1,3]). Hepcidin is downregulated in diseases that demand increased iron concentrations (i.e., increased erythropoietic activity, iron deficiency, and hypoxia) and upregulated in inflammation and infection [1,2,4,5]. Hepcidin causes degradation and internalization of the cellular iron exporter ferroportin [6], leading to iron retention in the cell and less iron available in the circulation for red blood cell synthesis.

Quantitative hepcidin methods have been developed on Mass Spectrometry (MS) [7–19] and Immunochemical (IC) platforms [19–25]. In our previous send out of samples for the comparison of hepcidin methods, the so called first Round Robin (RR1) for hepcidin methods [26], we found low within-sample variance of the measurements of the methods but large differences between absolute hepcidin levels measured by the various methods. This precludes comparability between the data obtained by methods and hinders the use of plasma hepcidin method in medical practice.

To address this variation in hepcidin outcomes between methods we aimed to harmonize the various available hepcidin methods by sending out plasma samples in a second Round Robin (RR2). Harmonization for analytes such as hepcidin, for which higher order materials and methods are not (yet) available, is becoming an accepted process [27]. In this RR2, we (i) tested two commercially available synthetic hepcidin-25 preparations to assess their suitability as material for harmonization, and (ii) determined method specific regression of native samples with consensus values and assessed the suitability of these algorithms for harmonization purposes. We anticipated this study to further contribute to harmonization of hepcidin methods, which should allow the definition of universal reference values and clinical decision limits for plasma hepcidin values.

Materials and Methods

Sample collection and participants. A prospective blinded measurement design was used to assess concordance in plasma hepcidin analysis. Eleven MS, 9 IC methods and one ligand binding method (later included amongst the IC methods) of in total 16 laboratories participated in the analysis, with a total of 21 methods [8–15,17–25,28]. The study was coordinated by the Department of Laboratory Medicine, Laboratory of Genetic, Endocrine & Metabolic Disorders of the Radboud University Nijmegen Medical Centre. The study was designed to compare hepcidin levels in 11 biological samples as well as in six samples composed of blank plasma spiked with different concentrations of two different materials for harmonization, i.e. synthetic human hepcidin-25 purchased from two different companies.

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Conflict of Interest: DWS and HT steer the hepcidinanalysis.com service for hepcidin measurements in body fluids.
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TABLE I. Characteristics of Methods Used for Plasma Hepcidin Measurements

Platform	Method	Hepcidin extraction		Standard	Purchased from	Reference
MS	HPLC-MS/MS	Reversed Phase	Internal	Synthetic Hepcidin-25 [13C6,15N1]Ile6, [13C6,15N1]Ile8	Bachem, in house made	[10]
MS	LC-MS/MS	WCX	Internal	Mouse synthetic Hepcidin 1	Peptide Int.	–
MS	LC-MS/MS	WCX magnetic nanoparticles	Internal	Synthetic Hepcidin-25 [13C6]Phe9, [15N,13C2]Gly20	Peptide Int., in house made	[11]
MS	LC-MS/MS	Reversed Phase	Internal	Synthetic Hepcidin-25 [13C9,15N1]Phe4	In house made	[14]
MS	LC-MS/MS	Reversed Phase	Internal	Synthetic Hepcidin-25 [13C8,15N3]	Peptide Int.	[9]
MS	LC-MS/MS	Protein ppt & SPE	Internal	Calcitonin gene-related peptide	Peptide Int.	[8]
MS	MALDI-TOF MS	None	Internal	Synthetic Hepcidin-25 [15N4,13C6]Arg16	Sigma-Genosys	[17]
MS	MALDI-TOF MS	WCX	Internal	Synthetic Hepcidin-24	Peptide Int.	[13,19]
MS	Q-TOF LC/MS	HBD and ACNppt	External	Synthetic Hepcidin-24[15N2,13C6]Lys24	Sigma AQUA	[18]
MS	SELDI-TOF MS	IMAC	External	Synthetic Hepcidin-25	Peptide Int.	[15]
MS	SELDI-TOF MS	IMAC	Internal	Synthetic Hepcidin-25[15N1,13C9]Phe9	AltaBioscience	[12]
IC	Competitive ELISA	None	External	Synthetic Hepcidin-25	Peptide Int.	[19]
IC	Competitive ELISA	None	External	Synthetic Hepcidin-25	Bachem	[20]
IC	Competitive ELISA	None	External	Synthetic Hepcidin-25	Bachem	*
IC	Competitive ELISA	None	External	Modified Hepcidin-25 fragment	DRG	[28]
IC	Competitive ELISA	None	External	Recombinant Hepcidin-25	In house made	[22]
IC	Competitive RIA	None	External	Synthetic Hepcidin-25	Bachem	[23]
IC	Competitive RIA	None	External	Synthetic Hepcidin-25	Peptide Int.	[24]
IC	Sandwich ELISA	None	External	Synthetic Hepcidin-25	Peptide Int.	[25]
IC	Sandwich ELISA	None	External	Recombinant Hepcidin-25	In house made	–
IC	HBD Method	None	Internal	^{125}I-Hepcidin-25	In house made	[21]

MS, mass spectrometry based; IC, immunochemical based; HPLC-MS/MS, high performance liquid chromatography; MS/MS, tandem MS; LC, liquid chromatography; MALDI, matrix assisted laser desorption/ionization; TOF, time of flight; Q, quadrupole; SELDI, surface enhanced laser desorption/ionization; ELISA, enzyme linked immunosorbent assay; HBD, hepcidin binding domain; WCX, weak cation exchange; ppt, protein precipitation; SPE, solid phase extraction; ACN, acetonitrile; IMAC, immobilized metal affinity chromatography.

Bachem standard: Bachem LTD, St. Helens, UK; Peptide Int., Peptide Int. Inc. Louisville, KY, USA; Sigma-Genosys, Sigma-Genosys, Woodlands, TX, USA; AltaBioscience standard, University of Birmingham, Birmingham, UK; DRG standard: DRG Instruments GmbH, Marburg, Germany.

* Bachem EIA kit (Cat. No S-1337) purchased from Bachem in August 2010. Note that the methods are randomly numbered compared to Tables II–IV.

Specimens. Ten heparin plasma pools were composed from hospitalized patient sample remnants (March 2010), so as to cover a wide variation in hepcidin levels, one plasma sample was composed from a iron-depleted patient with juvenile hemochromatosis (blank plasma) [29].

Six additional plasma samples were composed from the blank plasma and spiked to three end concentrations, that is to 5, 12.5, and 20 nmol/L with hepcidin-25 from Peptide International (Louisville, KY; lot No 580714, date 2008-7-23) and to 3.4, 8.6 and 13.7 nmol/L with hepcidin-25 from Bachem LTD (St. Helens, UK; Lot number 3005438; date 2009-6-29).

These difference in additions of the Bachem and Peptide International peptides can be attributed to our initial assumption that the values provided by both companies are nett amounts determined by amino-acid analysis of the synthetic human hepcidin-25. In a later stage of the study, however, we discovered that we added less Bachem peptide to the blank plasma than originally assumed. This because Bachem provided the gross amount of hepcidin on the vial that consisted of 68.6% hepcidin-25 (as assessed by amino-acid analysis; the remaining being salts and water) and for which we did not correct for in the preparation of the samples. According to manufacturers protocol both peptides had disulfide bonds between Cys_1- Cys_8, Cys_2-Cys_7, Cys_3-Cys_6, and Cys_4-Cys_5 [30]. Purity as assessed by HPLC was $>$98% for Peptide International hepcidin ($\leq$1% with the methionine analog and $\leq$1% with other impurities) and $>$95% for Bachem hepcidin. Both companies did not determine the nature of the impurities, but in the case of peptides with multiple disulphide bridges as with hepcidin, they may be misfolded peptide.

Each sample, of the total of 17 samples, was split into two aliquots and coded (blind) for each participating laboratory and stored at -80^{o}C. Three weeks after collection and storage, the samples were shipped on dry ice to all participants, and measured within 4 months of receipt. All laboratories performed single measurements on the 34 samples (17 duplicate samples). All native samples underwent one freeze-thaw cycle before analysis (except for two methods for which the samples underwent 2 freeze thaw cycles). Note that a previous study showed that plasma samples are not susceptible to loss of hepcidin by 2 freeze-thaw steps [7].

Hepcidin methods. Characteristics of the methods used for the plasma hepcidin measurements of this study are schematically and in random order presented in Table I. MS-based methods that are available for quantitative hepcidin measurement and participated in this round robin are based on MALDI, surface enhanced laser desorption/ionization (SELDI), or electrospray ionization (ESI) for generation of ions, combined with TOF or quadrupole analyzers for MS principles. Hepcidin enrichment was achieved by WCX chromatography, liquid chromatography, reversed phase extraction, solid phase extraction or protein precipitation. Internal and external standards that were used were synthetic hepcidin analogues, stable isotope labeled hepcidin-25 and hepcidin-24, and recombinant hepcidin-25 (Table I).

Immunochemical (IC) based methods that are available for quantification of hepcidin and participated in this round robin are either competitive or sandwich immunochemical methods (with biotinylated hepcidin-25 or ^{125}I-hepcidin-25 as tracer for quantification) and a hepcidin ligand binding method (Table I). Internal and external standards that were used were synthetic hepcidin-25, recombinant hepcidin-25, modified hepcidin-25 fragments and ^{125}I-hepcidin-25 (Table I). Most methods have been published previously [8–10,12–14,16–25,28], although one has only been described for urine samples [15].

Statistical methods. Analytical characteristics of the methods. At first we studied the repeatability of the methods for plasma (native samples). Therefore, we partitioned the total variance of each hepcidin method into the following components: (i) the between-sample variance (population variance) and (ii) the within-sample variance (analytical variance or repeatability). Note that the repeatability is the measure of the within-sample variance under identical conditions. The higher the within-sample variance, the lower the probability that samples with only small differences can be distinguished. A linear mixed model was used to estimate these variance components of each method separately. The dependent variable was hepcidin and the independent random variables were native sample (11 levels) and duplicate measurement (2 aliquots). Because we were interested in the analytical variation on a single measurement of each method, we omitted the last term from the final model and did not present the variation due to the two aliquots separately.

The standard deviation (SD, absolute error) and the percentage variance relative to the total variance of both the within- and the between-(native) sample are presented for each method separately. The relative variance of each component is presented to indicate the percentage explained variance of that component. The Spearman rank correlation between each combination of two methods was calculated using all 22 paired hepcidin values (11 native samples, two aliquots).

"Well performing" methods. For the use of this article we selected methods with a within-sample variance of $<$10% and a spearman correlation with all other methods $>$0.90.

Commutability. To assess whether the synthetic hepcidin-25 peptides of Peptide International and Bachem mimic native serum samples in their analytical behavior (commutability), according to the Clinical and Laboratory Standards Institute (CLSI) C53-A protocol [31] we determined whether the synthetic samples followed the same trend as the native samples for each couple of "well performing" methods, separately. For each couple of "well performing" methods the regression of the difference

TABLE II. The Mean and Standard Deviation (SD) of the Duplicates of the Levels of Hepcidin (nmol/L) by Sample by Method

Method / Sample		MS(1) Mean (SD)	MS(2) Mean (SD)	MS(3) Mean (SD)	MS(4) Mean (SD)	MS(5) Mean (SD)	MS(6) Mean (SD)	MS(7) Mean (SD)	MS(8) Mean (SD)	MS(9) Mean (SD)	MS(10) Mean (SD)	MS(11) Mean (SD)
Native												
1		0.0 (0.0)	0.0 (0.0)	0.0 (0.0)	3.2 (0.3)	0.0 (0.0)	0.5 (0.1)	0.0 (0.0)	1.5 (0.2)	0.0 (0.0)	0.0 (0.0)	0.00 (0.00)
2		2.6 (0.1)	5.6 (1.8)	6.2 (0.1)	11.7 (1.5)	4.1 (0.5)	9.4 (1.6)	13.6 (5.7)	8.5 (0.7)	4.4 (0.1)	2.5 (0.6)	0.00 (0.00)
3		3.8 (0.1)	4.7 (0.5)	9.2 (0.2)	15.1 (2.0)	5.0 (0.1)	10.7 (0.4)	29.9 (5.1)	11.8 (4.0)	5.8 (0.0)	2.4 (0.8)	0.01 (0.01)
4		5.1 (0.1)	7.2 (1.0)	11.6 (0.9)	18.6 (0.4)	6.9 (1.3)	16.9 (3.7)	37.6 (5.6)	12.6 (1.0)	7.8 (0.0)	6.0 (2.2)	0.01 (0.00)
5		8.8 (0.1)	12.6 (0.5)	21.7 (1.9)	31.6 (0.1)	12.4 (1.2)	25.3 (1.0)	44.7 (5.9)	24.6 (0.6)	15.3 (0.5)	6.8 (1.0)	0.28 (0.37)
6		10.4 (0.1)	14.9 (1.8)	28.3 (1.0)	38.2 (0.2)	18.4 (1.3)	32.2 (3.3)	75.1 (7.6)	28.9 (0.2)	18.3 (0.9)	13.2 (9.0)	0.01 (0.02)
7		11.5 (0.0)	14.9 (0.3)	30.6 (0.8)	42.7 (1.1)	24.5 (0.1)	33.3 (1.2)	72.7 (3.1)	36.3 (4.6)	18.9 (1.2)	13.7 (6.8)	0.02 (0.01)
8		10.9 (0.2)	17.4 (2.3)	30.7 (2.3)	43.5 (1.5)	21.4 (0.8)	34.7 (2.9)	52.9 (2.7)	43.0 (0.7)	19.6 (0.5)	10.9 (1.0)	0.01 (0.01)
9		12.4 (0.0)	20.4 (2.5)	38.4 (0.5)	47.1 (2.1)	31.9 (1.4)	38.5 (0.4)	73.7 (0.3)	39.2 (2.5)	24.6 (0.1)	24.0 (2.8)	0.02 (0.02)
10		14.3 (0.1)	22.6 (1.5)	41.1 (0.2)	54.2 (1.2)	33.0 (1.6)	48.7 (5.2)	81.9 (0.5)	46.1 (0.1)	26.1 (1.0)	11.7 (1.5)	0.02 (0.01)
11		20.0 (0.4)	29.8 (2.5)	62.4 (0.5)	81.2 (0.5)	48.7 (1.9)	64.7 (0.0)	71.5 (0.8)	72.8 (13.1)	33.0 (9.6)	26.3 (2.8)	0.08 (0.08)
Synthetic												
PI	5 nM	4.1 (0.3)	4.1 (0.3)	8.1 (0.4)	13.7 (1.2)	5.0 (0.7)	11.5 (0.2)	35.0 (3.6)	8.4 (2.0)	6.0 (0.1)	4.0 (1.1)	0.01 (0.01)
	12.5 nM	10.4 (0.2)	11.7 (0.3)	20.5 (1.3)	29.6 (1.5)	11.5 (0.5)	25.1 (0.4)	66.6 (16.1)	24.4 (1.1)	15.3 (0.1)	15.3 (9.9)	0.01 (0.01)
	20 nM	16.6 (0.3)	18.8 (0.8)	35.0 (0.6)	45.8 (1.0)	18.9 (0.8)	38.2 (0.8)	86.0 (3.6)	41.5 (2.0)	25.3 (0.4)	13.6 (5.1)	0.20 (0.24)
B	3.4 nM	2.6 (0.4)	3.2 (0.0)	5.4 (0.0)	8.2 (0.6)	3.0 (0.2)	8.0 (0.1)	20.8 (5.9)	5.4 (0.2)	3.7 (0.0)	1.7 (0.4)	0.00 (0.00)
	8.6 nM	5.0 (0.1)	6.3 (1.3)	10.7 (0.2)	17.5 (0.7)	6.3 (0.5)	13.6 (0.1)	44.2 (10.1)	11.1 (1.9)	7.6 (0.0)	5.0 (0.1)	0.01 (0.01)
	13.7 nM	7.6 (0.3)	9.1 (0.8)	17.8 (0.9)	22.6 (0.3)	9.0 (0.1)	21.5 (0.4)	61.0 (15.9)	20.1 (1.5)	11.8 (0.0)	14.5 (6.9)	0.01 (0.01)

Method / Sample		IC(1) Mean (SD)	IC(2) Mean (SD)	IC(3) Mean (SD)	IC(4) Mean (SD)	IC(5) Mean (SD)	IC(6) Mean (SD)	IC(7) Mean (SD)	IC(8) Mean (SD)	IC(9) Mean (SD)	IC(10) Mean (SD)
Native											
1		0.0 (0.0)	0.2 (0.0)	0.0 (0.0)	0.0 (0.0)	0.4 (0.1)	4.3 (1.8)	44.4 (4.0)	2.3 (1.8)	6.2 (0.0)	8.8 (2.0)
2		1.5 (0.6)	3.2 (0.2)	3.5 (0.0)	5.5 (0.8)	7.1 (0.2)	20.8 (1.6)	40.1 (17.6)	18.5 (1.3)	5.8 (0.9)	10.8 (1.8)
3		5.2 (0.1)	5.2 (0.5)	4.9 (0.1)	8.1 (0.5)	12.7 (0.2)	26.5 (4.3)	26.5 (9.5)	29.2 (1.8)	6.4 (1.3)	13.5 (4.2)
4		7.7 (0.4)	7.8 (0.6)	6.1 (0.1)	10.9 (0.7)	19.1 (4.1)	45.8 (1.0)	25.5 (1.4)	45.9 (10.1)	3.8 (0.4)	18.5 (5.8)
5		16.1 (0.5)	13.0 (0.6)	8.7 (0.6)	21.4 (1.8)	32.7 (0.1)	77.0 (7.8)	31.0 (0.2)	94.7 (21.8)	3.4 (0.0)	52.6 (35.4)
6		24.1 (0.4)	21.3 (1.9)	11.6 (0.9)	31.8 (0.4)	61.7 (2.1)	98.2 (7.8)	31.0 (7.0)	83.0 (13.4)	4.5 (0.4)	23.8 (8.2)
7		25.4 (0.4)	23.1 (1.0)	12.4 (1.1)	34.2 (2.0)	52.0 (8.3)	115.0 (30.0)	44.3 (8.4)	73.7 (12.9)	3.4 (0.4)	20.1 (6.3)
8		30.9 (0.0)	26.1 (1.2)	14.2 (0.8)	36.0 (2.4)	48.4 (8.0)	122.5 (4.4)	46.1 (9.7)	74.2 (4.6)	4.9 (2.5)	64.5 (66.6)
9		29.1 (1.4)	25.0 (0.7)	14.8 (0.8)	37.1 (1.4)	62.8 (1.1)	117.3 (13.2)	55.2 (6.4)	71.2 (24.1)	5.1 (0.9)	77.4 (68.6)
10		34.0 (1.3)	23.8 (0.6)	18.1 (0.4)	42.3 (2.5)	45.0 (6.9)	118.0 (18.2)	106.7 (1.5)	62.7 (12.7)	5.9 (0.1)	65.3 (72.4)
11		50.6 (2.5)	43.9 (1.9)	27.1 (0.0)	56.6 (2.7)	89.1 (0.2)	155.2 (1.0)	43.6 (19.7)	84.6 (16.7)	5.8 (0.0)	35.2 (1.6)
Synthetic											
PI	5 nM	3.3 (0.1)	3.7 (0.3)	4.7 (0.3)	5.2 (0.4)	7.4 (0.4)	20.3 (2.2)	30.4 (2.4)	23.3 (0.5)	7.1 (0.3)	8.8 (1.0)
	12.5 nM	7.4 (0.4)	7.4 (1.3)	10.1 (0.0)	12.5 (0.1)	24.1 (2.9)	50.8 (0.1)	35.8 (3.0)	77.1 (9.6)	6.0 (0.3)	22.9 (9.1)
	20 nM	16.7 (1.1)	17.5 (1.2)	17.0 (1.3)	22.2 (1.6)	32.8 (1.5)	76.8 (2.4)	33.0 (5.6)	141.6 (2.0)	6.0 (0.5)	26.1 (16.4)
B	3.4 nM	2.1 (0.1)	2.1 (0.3)	3.0 (0.1)	3.2 (0.3)	6.0 (1.1)	12.5 (0.2)	33.3 (7.3)	12.0 (0.8)	7.3 (0.1)	10.7 (4.9)
	8.6 nM	4.2 (0.8)	4.7 (0.4)	5.5 (0.1)	6.8 (0.4)	10.8 (2.7)	25.9 (2.7)	44.2 (14.5)	35.9 (7.1)	6.6 (0.9)	8.8 (1.0)
	13.7 nM	7.0 (1.3)	7.8 (0.1)	8.1 (0.1)	10.7 (0.6)	16.9 (1.3)	48.8 (10.4)	29.1 (13.2)	63.8 (10.1)	6.1 (0.1)	16.1 (2.3)

MS, Mass spectrometry based; IC, Immunochemical based; nM, nmol/L; PI, Peptide International and Bachem samples are synthetic hepcidin peptides purchased from Peptide Int. (Louisville, KY, USA). B, Bachem LTD (St. Helens, UK), respectively, which are spiked into blank plasma samples. According the inserts of the companies, level assignment of synthetic peptides is achieved by amino acid sequence analysis and purity is checked by HPLC. Native samples are obtained in the Radboud University Nijmegen Medical Center, by pooling plasma samples from hospitalized patients. All native samples underwent one freeze-thaw cycle before analysis (except for MS5 and IC3 for which the samples underwent 2 freeze thaw cycles). *Note that the methods are randomly numbered within the type of platform compared to Table* I.

against average was calculated (Bland-Altman plots [32]) with the 95% confidence band for single measurements, using native samples only. Subsequently, the commutability of the synthetic sample was assessed by the calculation of the relative differences of its value to the regression line in the Bland-Altman plots (normalized SD). Synthetic samples were defined to be not commutable when the normalized SD was > 2, in other words when the measurement was outside the 95% confidence band.

Notably, assessing the commutability of materials in assays which were not "well performing" is not indicated, as the confidence intervals of the method comparison with native samples is too wide to be able to make an appropriate interpretation.

Harmonization/standardization. Theoretically, a qualification of a reference method is that it is measured with optimal precision. In the absence of traceability to higher order materials and reference methods, precision is therefore the first requirement for alternatives of reference methods for their hepcidin values to be used as consensus values for harmonization purposes. In fact, all methods with equal hepcidin values of the same sample will contribute to a more optimal precision of their consensus value. The consensus of each sample is the mean value of the hepcidin values of those methods that showed good mutually agreement. This mutual agreement of two methods is evaluated regarding the agreement of the hepcidin values of that couple with the $x=y$ line in the scatter plot. An essential requirement of methods to be selected to determine the consensus value, is their very good mutual agreement to increase precision and control the noise of the consensus value.

A method specific regression line (algorithm) was constructed from the duplicates of the reported native hepcidin results, against the preliminary hepcidin consensus version 1 (HEPCON1) values. This method specific regression line can be used to calculate the consensus value of its own measured result. In addition the appropriate confidence interval is calculated using the delta-method. The performance of the algorithms was studied by the improvement of the between-method CV of the HEPCON1 compared to the between-method CV of the observed hepcidin values.

Results

Analytical characteristics of the methods

Table I presents the 21 participating methods (11 MS and 10 IC) from in total 16 laboratories. The various assays differ widely in numerous aspects of the methodology used, such as extraction procedures and the (internal and external) standards. Mean hepcidin levels with SD measured by the 21 hepcidin methods are shown in random order in Table II. We observed that absolute hepcidin values differed widely between samples and the various methods. For example, native samples 1 and 11 were measured between 0.0–44.4 nmol/L and 0.08–155.2 nmol/L, respectively. Table III shows the absolute within- and between-sample SDs, and the within- and between-sample variance relative to the total variance for each method. The within- and between-sample SDs differed widely between the assays. The contribution of the within-sample variance to the total variance is low for 9 MS and 6 IC methods (0.1–7.9%) and high for 2 MS and 4 IC methods (17.4–100.0%).

TABLE III. The Total, Between- and Within-Sample Standard Deviation (SD) and the Contribution of the Between- and Within-Sample Variance to the Total Variance of the Hepcidin Measurements of Each Method, Using a Linear Mixed Model on the Native Samples

Method	Total SD	Between-sample SD	Within-sample SD	Variance (%)	
				Between-sample	Within-sample
MS(1)	5.8	5.8	0.2	99.9	0.1
MS(2)	8.9	8.8	1.6	96.8	3.2
MS(3)	18.3	18.2	0.9	99.7	0.3
MS(4)	22.4	22.4	1.2	99.7	0.3
MS(5)	15.0	15.0	1.1	99.4	0.6
MS(6)	18.8	18.6	2.4	98.3	1.7
MS(7)	27.7	27.4	4.3	97.6	2.4
MS(8)	21.0	20.6	4.4	95.6	4.4
MS(9)	10.5	10.1	2.9	92.1	7.9
MS(10)	8.9	8.1	3.8	82.3	**17.7**
MS(11)	0.1	0.0	0.1	1.1	**98.9**
IC(1)	15.8	15.8	1.0	99.6	0.4
IC(2)	13.0	13.0	1.0	99.4	0.6
IC(3)	7.6	7.6	0.6	99.4	0.6
IC(4)	17.9	17.8	1.7	99.1	0.9
IC(5)	27.6	27.3	4.3	97.6	2.5
IC(6)	50.8	49.4	12.0	94.4	5.6
IC(7)	23.5	21.4	9.8	82.6	**17.4**
IC(8)	31.5	28.5	13.4	81.9	**18.1**
IC(9)	1.3	0.9	1.0	44.8	**55.2**
IC(10)	36.7	0.0	36.7	0.0	**100.0**

SD, absolute error; Between-sample, segment due to variation between samples; Within-sample, segment due to repeated measurements; MS, Mass Spectrometry based; IC, Immunochemical based. Data are expressed in nmol/L. The within-sample variance $>$ 10 % of the total variance are indicated in bold.

Note that the methods are randomly numbered within the type of platform compared with Table I.

Selection of methods for further analysis

We excluded 6 methods based on the criteria of a within-sample variance of $>$10% of the total variance of this method with these samples. Of the remaining 15 methods we determined the Spearman rank correlations of each combination of two methods (Supporting Information Table I). Based on the results, we excluded two additional method (MS7 and IC5) that despite having a significant correlation with the other methods, did not reach the required coefficient threshold $>$0.90. This restricted our selection to 13 "well performing" methods.

Commutability of synthetic hepcidin

Figure 1 shows a selection of 4 out of the 91 possible Bland-Altman plots, illustrating 4 different patterns of analytical behavior of synthetic relative to native samples in the comparison of "well performing" method pairs. The figure top panels represent two examples for which the synthetic samples are not commutable to native samples, i.e., results obtained for the synthetic samples and native samples are substantially different. The left and top right panels of Figure 1 represent examples with large differences between two methods. Figure 1 also shows that expected values of the pure hepcidin standards do not match measured values, e.g. in each of the panels the highest Bachem standard (13.7 nmol/L) is lower than the middle Peptide International standard (12.5 nmol/L).

Figure 2 shows the relative difference (in normalized SD) of the value of the synthetic sample to the regression line in the Bland-Altman plots, that ranges from less than two for the samples with the lowest concentration of Peptide International or Bachem synthetic hepcidin peptides, to over 9 for the samples with the highest concentration of synthetic hepcidin peptide from Peptide International. For the majority of the method couples in the Bland-Altman plots the synthetic hepcidin samples were situated outside the 95% confidence interval (CI) of the regression line of the native samples, i.e. $n = 76$ and $n = 73$ from the total of $n = 91$ for Peptide International and Bachem, respectively. Normalized SDs increase for both synthetic peptides with increasing concentrations. Note that the Bachem synthetic samples tend to have lower normalized SDs compared to the Peptide International synthetic samples.

Harmonization by native samples

Two MS methods (6 and 8) had acceptable agreement between the $x=y$ line in their mutual scatter diagram (Supporting Information Figure 1). For all other couples this agreement is far worse. These two methods were therefore selected to calculate hepcidin consensus (HEPCON1) values.

Algorithms designed on the HEPCON1 consensus values could be used to recalculate a single method specific hepcidin value to the HEPCON1 value and vice versa (Table IV and Supporting Information Table II). For example, methods MS1 and IC6 measure 8.8 nmol/L and 77.0 nmol/L, respectively, for a specific sample. Using the algorithm this results in the respective HEPCON1 values of 28.2 nmol/L and 26.9 nmol/L. These algorithms to calculate method specific hepcidin values from the HEPCON1 succeed to explain between 89 and 98% of the variance (R-square) for the methods with a within sample variance $<$ 10% of the total variance and with a correlation with the other methods $>$ 0.90. Note that these algorithms may be less reliable in the low and high hepcidin concentration range.

To validate the performance of the algorithms, we compared the between-method CV using the observed values and the between-method CV using the HEPCON1 values for each aliquot separately. The results are presented in the Supporting Information Table III. We found that the performance of the algorithms was extremely good for aliquots with higher hepcidin values (mean $>$ 10 nmol/L), i.e., a decrease of 84–90% of the intermethod CV if the HEPCON1 was used compared to the between-method CV of the observed hepcidin values. This decrease is less, but still 55–82% for the aliquots with mean hepcidin between 5 and 10 nmol/L. For aliquots with mean hepcidin values below 5 nmol/L, the algorithm provided no additional improvement and should not be used. Note that in the latter case most methods measured zero hepcidin concentration in the aliquot.

Discussion

We confirmed the observations of the hepcidin RR1 that hepcidin values differ widely between plasma hepcidin methods [26]. Moreover, we observed large differences in SD, but the contribution of the within-sample variation to the total variation appeared to be similar for the majority of

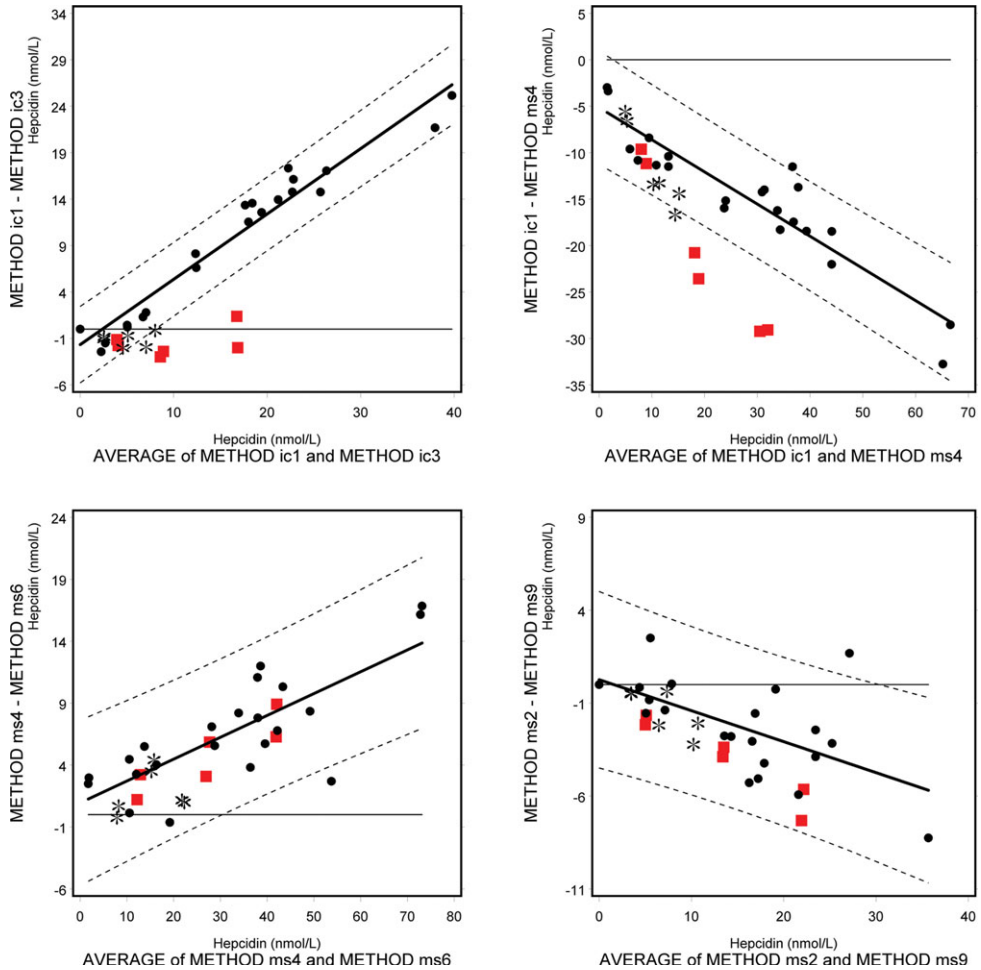

Figure 1. Four examples of 91 Bland-Altman plots for the comparison of hepcidin measurements (in nmol/L) of two methods with the regression lines (thick solid line) using the native samples (closed circles) only. The dashed lines indicate the 95% confidence interval. The horizontal line at 0 refers to no difference between methods. The red squares indicate the synthetic hepcidin of Peptide International and the stars indicate the synthetic hepcidin of Bachem. The left and top right panels are three examples with large differences between the two methods. The top panels are two examples for which the samples with synthetic hepcidin are not commutable. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the methods. Importantly, we found samples with synthetic hepcidin not to be commutable, thus not suitable for harmonization purposes. As an alternative, we used native plasma samples for harmonization, and constructed algorithms that allow to compute the values obtained by the methods into international hepcidin consensus (HEPCON1) values and vice versa. We found the performance of these algorithms to be very well, meaning that they substantially improved the between-method CV.

The observations of widely differing hepcidin values between methods corroborate those obtained in our first round robin for hepcidin methods [26]. In fact, hepcidin assay results are not traceable to reference materials and/or reference measurement procedures (RMP) because neither reference materials nor a RMP for hepcidin exists. This observation was the main reason to set up the harmonization study. These differences between assays might be attributed to differences in the values that laboratories and companies assign to the internal and external standards used by the different methods, to impurities in these standards or to loss of the standard during storage, e.g. by aggregation [30,33] or differential sticking of the synthetic hepcidins to the tubes. Hence, studies on the optimal handling and storage conditions of synthetic hepcidin standards are warranted to allow the formulation of recommendations on this point. Moreover accurate value assignment of the

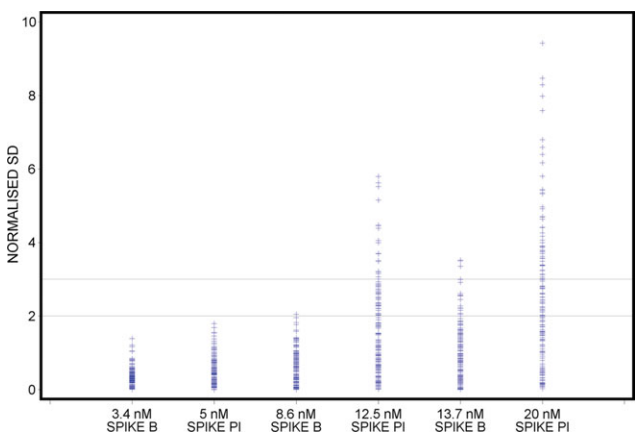

Figure 2. The normalized SD of the samples with synthetic hepcidin relative to the regression line in the 91 Bland-Altman plots. Spikes were considered not commutable when the normalized SD > 2. The synthetic samples consist of hepcidin purchased from the Peptide International (Spike PI) and hepcidin purchased from Bachem (Spike B), each spiked in three different concentrations to the native blank plasma. Note that the *X*-axis is not of linear order. nM, nmol/L. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

synthetic peptides could also contribute to bringing the values of the various methods together. This could be achieved by techniques of peptide quantification, such as

TABLE IV. The Algorithm to Calculate the Estimated Hepcidin Value (in nmol/L) from the HEPCON1 Value (nmol/L), Using a Linear Regression Model on the Native Samples Only

Method								SEM	R-square
IC1	Y	=	−2.67	+	0.79	×	HEPCON1	0.39	0.98
IC2	Y	=	−1.23	+	0.64	×	HEPCON1	0.45	0.96
IC3	Y	=	−0.11	+	0.38	×	HEPCON1	0.19	0.98
IC4	Y	=	−0.15	+	0.89	×	HEPCON1	0.54	0.97
IC5**	Y	=	1.75	+	1.29	×	HEPCON1	1.85	0.85
IC6	Y	=	11.37	+	2.42	×	HEPCON1	2.94	0.89
MS1	Y	=	0.62	+	0.29	×	HEPCON1	0.16	0.97
MS2	Y	=	0.87	+	0.44	×	HEPCON1	0.35	0.95
MS3	Y	=	−1.17	+	0.91	×	HEPCON1	0.44	0.98
MS4	Y	=	2.41	+	1.13	×	HEPCON1	0.46	0.99
MS5	Y	=	−2.92	+	0.74	×	HEPCON1	0.52	0.96
MS6	Y	=	1.19	+	0.94	×	HEPCON1	0.45	0.98
MS7**	Y	=	16.43	+	1.17	×	HEPCON1	2.66	0.69
MS8	Y	=	−1.19	+	1.06	×	HEPCON1	0.45	0.98
MS9	Y	=	1.25	+	0.50	×	HEPCON1	0.60	0.89
MS10*	Y	=	−0.36	+	0.38	×	HEPCON1	0.84	0.70

MS, Mass Spectrometry based; IC, Immunochemical based. HEPCON1, average of MS6 and MS8; SEM, standard error of the mean.

** correlation with other methods $\leq$0.90; * Within- sample variance 17.7% of total variance.

All other methods have a within-sample variance$<$ 10% of total variance and a correlations$>$ 0.90 with other methods. The HEPCON1 hepcidin value can be calculated from the method specific value by the reverse of the regression formula (Supporting Information Table II). Note that the methods are randomly numbered within the type of platform compared to Table I.

amino-acid analysis, which however also have their analytical uncertainties. Alternatively, stable isotopes can be send out from reference laboratories to calibrate the standards.

We found the majority of methods to have a relatively low contribution ($<$10%) of the within-sample variance to the total variance, implying that these method have a good ability to distinguish samples of different hepcidin concentrations. Methods and their corresponding laboratories that showed a relatively large within-sample variance (>10%) or a relatively low correlation with the other methods were encouraged to improve the precision and accuracy of their method.

We found that the expected values of the pure standards of Bachem to be lower than those of Peptide International. This difference suggests an issue with value assignment of at least one of these materials, and will affect the possible use of these materials as higher order reference materials. This might also explain the tendency of the lower normalized SD's of the Bachem samples compared to the Peptide International samples. We moreover found the between-method behavior of both synthetic samples to be different from that of pooled native patient plasma for the large majority of the method couples. Even in the low concentration range, samples with synthetic hepcidin from both Bachem and Peptide International behaved differently from native samples. This difference in between -method behavior between synthetic and native hepcidin increases with increasing hepcidin concentrations. Thus, the lower concentrations of Bachem peptide did not result in false judgment of the commutability. These observations imply that the samples with synthetic hepcidin are not commutable to the native samples. These artificial samples are therefore not suitable for measurement of between-method level variability, precluding the exploitation of these samples as materials for harmonization purposes, i.e. for equalizing the levels of hepcidin of the different methods [34]. This non-commutability of these samples with synthetic hepcidin can be caused by a matrix alteration, by a non-native (synthetic) analyte, or by the inadequate analytical specificity of some methods for the analyte of interest [27,35–37]. More specifically, in the case of hepcidin-25: i) the structure of the native hepcidin might be different from synthetic hepcidin, e.g. because of thermodynamic processes that differ for the *in vitro* and *in vivo* production of hepcidin [33,38], ii) *in vitro* addition of a synthetic (or native hepcidin) peptide to plasma might result in different binding to plasma proteins, such as α-2 macroglobulin or albumin [39] than the *in vivo* secreted hepcidin, and iii) the non-native samples only comprise (synthetic) hepcidin-25, whereas the native samples might also comprise the smaller hepcidin isoforms hepcidin-22 and 20; these hepcidin isoforms might interfere in some of the immunochemical methods due to nonspecificity of the hepcidin antibody [1]. Thus even when we could succeed to make a synthetic or recombinant hepcidin that has a structure identical to native hepcidin-25, and spike it to plasma, it is unclear if it behaves similar to the hepcidin secreted in vivo.

Since samples with synthetic hepcidin appeared not be useful as reference materials for harmonization, we used pools of native patient samples and constructed algorithms which allow computing hepcidin consensus (HEPCON1) values for the results obtained by the various methods and vice versa. In the absence of an IFCC validated reference method [40] and/or alternative reference materials, we selected two methods for the definition of consensus values, that showed a good precision and correlation with the majority of other methods as criteria, and which gave hepcidin results for the native samples that are (almost) equal. Any of the methods could have passed to be used as a consensus because of low within-sample variations. However, to increase the precision and control the noise of the consensus value, we averaged those methods that produced "identical" results. Whether this selection results in consensus values which reflect true values is unknown. Furthermore, the selected two methods are not necessarily the most precise methods. Therefore, the HEPCON1 values obtained by these two methods should be seen as preliminary, interim, and provisional rather than that these values are more correct than others. Nevertheless, they allow us to harmonize the many different assays by use of a range of patient samples (a concept that is becoming an accepted process for analytes when higher order materials or methods are not (yet) available [27]) by generating algorithms by which hepcidin consensus values can be obtained and can be compared worldwide.

We used the data of the first round robin [26] to validate the algorithms and observed a decrease of 55–64% in the between-method CV if the HEPCON1 was used compared to the between-method CV of the observed values. This is a considerable decrease, regarding some changes in methodology in time.

The better the precision of the hepcidin method the better the precision of the calculated HEPCON1 values. Moreover, reduction of the between-method variation in hepcidin results, using the HEPCON1 values, is blunted by variation in the individual hepcidin results of each method.

Currently, reference intervals and decision limits are method dependent. Therefore, for each (carefully validated) hepcidin methodology these intervals and limits should be determined separately. Here, we show that in the absence of a reference method and calibrator, harmonization of the methods is amended by transforming the original hepcidin results by various methods to HEPCON1 values. This harmonization by HEPCON1 values may contribute to i) confirmation of generally accepted and usable reference intervals and decision limits, ii) application of consistent clinical decision limits for medical care and best practice guidelines, and iii) pooling and comparison of data from various studies to facilitate medical research and research translation.

Since the proposed algorithms are susceptible to changes caused by adaptations to the methodologies, the "hepcidin community" should further work on the development of commutable calibrators and reference methods. Meanwhile, we intend to regularly update the algorithms by the organization of biannual round robins.

In conclusion, until commutable materials for harmonization are defined, harmonization can be achieved by exploiting specific algorithms allowing each lab to report in HEP-CON1 values. Importantly, this study represents the first step toward harmonization of plasma hepcidin methods and facilitates aggregation of hepcidin data from different research investigations and development of appropriate clinical practice guidelines.

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