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Evaluation of the necessity and the feasibility of the standardization of procalcitonin measurements: activities of IFCC WG-PCT with involvement of all stakeholders

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Keywords: procalcitonin, standardization, harmonization, traceability, reference measurement system

Abbreviations

International system (SI)

External quality assessment (EQA)

In vitro diagnostic device (IVD)

Reference measurement procedure (RMP)

Isotope dilution-liquid chromatography tandem mass spectrometry (ID-LC-MS/MS)

Analytical performance specifications (APS)

Isotope dilution mass spectrometry (IDMS)

Stable-isotope-labelled (SIL)

High-resolution mass spectrometry (HRMS)

Reference measurement system (RMS)

Abstract

Procalcitonin (PCT) is an important biomarker for sepsis diagnosis and management. To date, there is no higher-order reference measurement procedure (RMP) and certified reference material to achieve global standardization of results and results traceability to the SI units.

Although efforts have been made to harmonize PCT results, a number of correlation studies and external quality assessment (EQA) schemes show conflicting results regarding results comparability and to date, equivalence of PCT results across the assays remains questionable in absence of studies relying on commutable EQA materials. In this context, the IFCC initiated activities to fill these gaps through the creation of the working group on standardization of PCT assays that gathers experts from National Metrology Institutes, calibration laboratories, clinicians, biologists, EQA providers and assay manufacturers. Among the activities, a higher order RMP and commutable reference materials are under development to build a robust reference measurement system (RMS). A commutability study is being organized to identify EQA materials that are fit for purpose to reliably estimate the current comparability of PCT results. This work will make it possible to evaluate the necessity and the feasibility for establishing and maintaining a new RMS for PCT assays, if deemed necessary.

Introduction

Clinical laboratory tests play an important role across the healthcare continuum to diagnose and monitor patients and to guide treatment decisions. A study based on 72196 patients reported that 56% of patients from the emergency department and 98% of inpatients had at least one laboratory test ordered for diagnosis purpose during their care pathway [1]. Among clinical diagnostic technologies, immunoassay are the most widely used technique in routine laboratory [2]. In response to the high demand for testing, numerous immunoassays have been developed and commercialized. However, the co-existence of in vitro diagnostic medical devices marketed by different vendors may introduce some variability of the results between laboratories equipped with different analyzers. Depending on the purpose of the laboratory test prescription and the use of the test result in clinical management, such a variability could potentially lead to diagnostic errors, inappropriate treatment, and a misleading longitudinal patient monitoring [3]. This variability may have a crucial impact when an inpatient is moving from one unit to another, where different clinical laboratories with potentially different measurement procedures take over the patient follow-up, or when a biomarker has to be monitored for long period where analysis in different laboratories is inevitable. Moreover, from a research perspective as well as clinical decision, the lack of results comparability may impact conclusions and the subsequent definition of clinical practice guidelines and public healthcare strategies based on biomarkers measured values [3]. Thus, not only the precision and accuracy of quantitative methods are fundamental prerequisites for a proper diagnosis and appropriate therapeutic monitoring, but also the comparability and the reliability of the results from different routine assays.

A privileged mean to ensure accuracy and comparability of the results is to establish metrological traceability of results to the International System (SI) of units [3–5]. By definition, metrological traceability is the property of a measurement result whereby the result can be related to a reference through a documented unbroken chain of calibrations, each contributing to the measurement uncertainty [6]. For each measurement procedure used in clinical laboratory for a specific measurand, values associated with the calibrators should be traceable to the available higher-order reference measurement procedure (RMP) and/or reference materials, as requested by ISO 17511:2020 and the European regulation 2017/746 for in vitro diagnostic devices (IVD) that will come into full force on 26th May 2022 to replace the 98/79/EC Directive. Although ISO 17511:2020 includes alternative procedures to establish metrological traceability of results (e.g. harmonization protocols based on ISO 21151:2020), the ideal mean to establish metrological traceability of results to the SI Units is to rely on a reference measurement system consisting in higher-order RMP and materials (Figure 1) [7]. In this case, a primary RMP represents the highest metrological order and is used for the value assignment of a primary reference material. This primary reference material is in turn used to calibrate the secondary RMP that is used to assign certified values to secondary reference materials. Assay manufacturers then use these materials to calibrate routine assays and maintain an unbroken chain of calibrations between the primary RMP and the routine assay used to measure patient samples. In that context, standardization of measurement procedures thus ensures metrological traceability to the SI units. However, primary and/or secondary reference materials and procedures are not always available. In such a situation, a common reference measurement system that does not allow establishing results traceability to the SI units could still be agreed as a reference by convention with the objective to obtain equivalent results across the different commercially available assays [3]. This is the concept of harmonization.

To be used in a calibration hierarchy of one or more end-user measurement procedures and successfully lead to harmonized results, it is crucial that the secondary reference material is commutable for the concerned assays, i.e. that it behaves the same way as patient samples [8–10]. While non-commutability of secondary calibrators breaks the traceability chain and results in the inability to achieve results harmonization [3,9], commutable reference materials are also needed to evaluate accuracy and comparability of routine assays [11–13]. Additionally, commutable reference materials are needed to conduct appropriate post-market vigilance to ensure that standardization has been achieved. Overall, establishing and maintaining a reference system leading to a consistent harmonization level appears demanding and only a limited number of success stories can be found in the field of clinical chemistry [14,15].

To improve global standardization and harmonization of laboratory tests, a wide range of activities are conducted by the Scientific Division of the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC). Within this article, we describe the objectives and activities of the working group on standardization of procalcitonin (PCT) that was created in 2018.

1. Procalcitonin and its current measurement procedures

In the last 20 years, it has been shown that PCT, a 116 amino acids protein, is useful for both diagnosis and antibiotic stewardship in sepsis and lower respiratory tract infections [16–23], two global threats and health priorities [24–27]. Indeed, sepsis affected 48.9 million people, leading more than 11 million deaths worldwide [28], whereas antimicrobial resistance caused approximately 700,000 deaths every year. Without appropriate actions to address this issue, the number of annual deaths caused by antimicrobial resistance could reach 10 million by 2050 [26]. PCT measurement has already supported the clinical community for timely decision to start or terminate antibiotic treatment [29,30] supporting cost-effective patient management [31]. Changes in PCT have been clearly associated with disease outcome in ICU patients [32]. Moreover, elevation of PCT concentration has recently been reported in severe forms of coronavirus disease COVID-19 presenting bacterial coinfection [33,34]. Longitudinal PCT measurement is recommended by IFCC Task Force on COVID-19 to predict its severe form [35]. PCT measurement in COVID-19 patients has been associated with disease severity and mortality [36] but whether can serve as predictor of secondary bacterial infections is still unclear [37], possibly due to variations in PCT measurements that introduce limitations in meta-analyses.

In non-infectious conditions, PCT is cleaved in different products (NProCT, Calcitonin-GKKR-Katacalcin, Calcitonin and Katacalcin). Therefore PCT concentration remains low in the bloodstream (≤ 0.05 ng/mL) (Figure 2) [38]. In infectious conditions, PCT is produced in thyroid C-cells and in various tissues and organs such as liver, lung, pancreas and is directly released into the blood [39]. Therefore, an increase up to 1100 fold of PCT concentration from the basal concentration was observed [39,40]. Moreover, in this condition, it was shown that PCT exists in three different isoforms in the bloodstream (intact PCT (1-116), PCT (2-116) in which the first amino acid is cleaved, PCT (3-116) in which the first two amino acids are cleaved). Among these forms, PCT (3-116) has been reported as the predominant form, but little is known about the relevance of the different forms [41,42].

Interestingly, PCT concentration remains low in the case of viral infections and localized bacterial infections [44,45]. Therefore, PCT concentration has been used to establish clinical guidelines for sepsis stratification and monitoring of antimicrobial therapy [16,17,46]. An example of decision-making process based on PCT concentration is presented in Figure 3.

The use of PCT tests has been gaining widespread in the clinical laboratory settings [48,49]. In consequence, multiple immunoassays measuring PCT concentration in human serum or plasma have been developed to meet the important demand of PCT testing and developments are continuing with new assays commercialized recently (e.g. Architect Brahms PCT assay in 2017, B-R-A-H-M-S PCT direct in 2018, Access PCT assay in 2019, Diasys PETIA PCT assay in 2020) (Table 1). To meet the need for short turnaround times, an increasing number of point-of-care tests for PCT have been developed for a rapid clinical decision and treatment initiation. As underlined previously, the PCT measurement results could vary from one immunoassay to another depending on different factors including detection method, antibody specificity, reagents, instrument platforms or calibrators. Commercially available immunoassays for PCT employ different mechanisms of detection such as chemiluminescence, electroluminescence, time-resolved fluorescence, or immune-turbidimetry whose sensitivity could be different. Moreover, not all immunoassays use the same antibodies. As different antibodies could recognize different epitopes of PCT, this could result in differences in assay specificity and selectivity. Currently, most PCT immunoassays use a pair of antibodies. However, assays like the Diazyme assay employ a single precipitating antibody, and assays like Advia Centaur Brahms, Lumipulse G Brahms and Atellica Brahms assays use a third antibody for the detection. In addition, most immunoassays employ monoclonal antibodies that recognize a single epitope of PCT with high specificity, while the BRAHMS Kryptor assay and Diasys PETIA assay are the only immunoassays using polyclonal antibodies that can potentially recognize multiple epitopes. There is generally limited information available on the PCT isoform(s) being actually measured or on cross reactivity, e.g. with PCT fragments. Liaison Brahms and Lumipulse Brahms assays reported the use of two monoclonal antibodies targeting two epitopes situated in calcitonin and katalcalcin segments (Table 1). Furthermore, Brahms PCT LIA, the first commercial kit employed two monoclonal antibodies interacting with two different binding sites: the epitope residues 96-106, part of the katalcalcine segment, and residues 70-76, part of the calcitonin segment [50]. However, these assays are not predominantly used in clinical practice.

Another source of variability across PCT assays lies in the use of different reagents such as biotin, streptavidin, ruthenium, acridinium, as described in Table 1. As demonstrated for other biomarkers, the use of different reagents may have a significant effect on measurement reliability [51–55]. As a consequence, these technical differences could potentially contribute to a lack of comparability across results provided by different immunoassays.

Table 1. Characteristics of the current commercially available immunoassays for PCT

Immunoassay (Company)	Date of first market authorization	Market authorization	Predicate Device	Instrument	Assay principle	Antibodies and reagents	Sample volume Sample matrix	Measuring range (ng/mL)
BRAHMS PCT LIA (BRAHMS Diagnostica)	2005	FDA 510(k) - CE marked	Endotoxin Activity Assay	Luminometer	Immunoluminometric assay - non automated	Two mouse anti-PCT mAb	20 µL Se, P	0.3-500
BRAHMS PCT Sensitive Kryptor (BRAHMS diagnostica)	2009	FDA 510(k) – CE marked	BRAHMS PCT LIA	Kryptor Test System	Time-resolved amplified cryptate emission (TRACE) immunoassay	Sheep anti-PCT pAb cryptate labelled Mouse anti-PCT mAb XL665 labelled	50 µL Se, H, E	0.02-50
Architect BRAHMS PCT (Abbott)	2017	FDA 510(k) – CE marked	BRAHMS PCT Sensitive Kryptor	Architect i2000SR Analyzer	Chemiluminescent microparticle immunoassay (CMIA)	Rat anti-PCT mAb microparticles coated Mouse anti- PCT mAb acridinium-labelled conjugate	100 µL Se, L, K2	0.02-100
Vidas BRAHMS PCT (Biomérieux)	2009	FDA 510(k) – CE marked	BRAHMS PCT LIA	Vidas	Enzyme-linked fluorescent immunoassay (ELFA)	Mouse anti-PCT mAb microwells coated Mouse anti- PCT mAb alkaline phosphatase-labelled	200 µL Se, L	0.05-200
Liaison BRAHMS PCT II Gen (Diasorin)	2015	FDA 510(k) – CE marked	BRAHMS PCT Vidas	Liaison analyzer	Chemiluminescence Immunoassay (CLIA)	Mouse anti katacalcin PCT mAb magnetic particle coated. Mouse anti calcitonin PCT mAb isoluminol labelled	225 µL Se, L	0.02-100
Lumipulse G BRAHMS PCT (Fujirebio)	2016	FDA 510(k) – CE marked	BRAHMS PCT Sensitive Kryptor	Lumipulse G system	Chemiluminescence Enzyme Immunoassay	Mouse anti PCT mAb Mouse anti calcitonin PCT mAb coated particles Mouse anti katacalcin PCT mAb alkaline phosphatase labelled	60 µL Se, N, L, K2	0.02-100
Elecsys BRAHMS PCT (Roche Diagnostics)	2009	FDA 510(k) – CE marked	BRAHMS PCT Sensitive Kryptor	Cobas e 411 analyzer	Electrochemiluminescence immunoassay (ECLIA)	Streptavidin-coated microparticles Mouse anti-PCT mAb biotinylated Mouse anti-PCT mAb ruthenium labelled	30 µL Se, K2, K3, L	0.02-100
Atellica IM BRAHMS PCT (Siemens healthcare)	2018	FDA 510(k) – CE marked	BRAHMS PCT Sensitive Kryptor	Atellica IM Analyzer	CLIA	Mouse anti-fluorescein mAb paramagnetic particles coated Mouse anti-PCT mAb acridinium ester labelled Mouse anti-PCT mAb fluorescein labelled	100 µL Se, E, L, N	0.04-50

Alinity I BRAHMS PCT (Abbott)	n/a	FDA 510(k) – CE marked	BRAHMS PCT Sensitive Kryptor	Alinity i analyzer	Chemiluminescent microparticle immunoassay (CMIA)	Rat anti-PCT mAb microparticles coated Mouse anti- PCT mAb acridinium- labelled conjugate	100 µL Se, L, K2	0.02-100
Advia Centuar BRAHMS PCT (Siemens)	2011	CE marked	BRAHMS PCT Sensitive Kryptor	Advia centaur XP and CP analyzers	CLIA	Mouse anti-fluorescein mAb paramagnetic particles coated Mouse anti-PCT mAb acridinium ester labelled Mouse anti-PCT mAb fluorescein labelled	100 µL Se, E, H	0.02-75
Vitros BRAHMS PCT (Ortho Clinical Diagnostics)	2020	FDA 510(k) - CE marked	BRAHMS PCT Sensitive Kryptor	VITROS 3600	Luminescence immunoassay	Streptavidin-coated wells Anti-PCT mAb biotin labelled Anti-PCT mAb horseradish peroxidase labelled	30 µL Se, E, L	0.03-100
Access PCT (Beckman Coulter)	2019	FDA 510(k) – CE marked	BRAHMS PCT Vidas	UniCel DXI or Access 2	CLIA	Anti-PCT mAb paramagnetic particles coated (does not use biotin- streptavidin particle) Anti-PCT mAb alkaline phosphatase conjugate	35 µL Se, L, E	0.01-100
Diazyme PCT (Diazyme Laboratories)	2017	FDA 510(k) – CE marked	BRAHMS PCT Vidas	Olympus Beckman AU400 chemistry analyzer	Latex-enhance immunoturbidimetric assay	Anti-human PCT mAb latex particles coated	15 µL Se, E, L	0.2-52
Diasys PCT FS (Diasys Diagnostic GmbH)	2020	CE marked	BRAHMS PCT Sensitive Kryptor	Cobas c501 chemistry analyzer	Latex-enhance immunoturbidimetric assay	Anti-human PCT pAb latex particles coated	10 µL Se, L	0.2-50
Maglumi PCT (Shenzhen)	n/a	CE marked	n/a	Analyzer Maglumi	CLIA	Mouse anti-PCT mAb nano magnetic microbeads coated Mouse anti-PCT mAb ABEI labelled	40 µL Se, E, Ewb	0.13-100

mAB : monoclonal antibody ; pAb : polyclonal antibody ;

Se : serum ; P : plasma ; H : heparin plasma ; E : EDTA plasma ; L : lithium heparin plasma ; K2 : K2EDTA plasma ; N : sodium heparin plasma ; K3 : K3EDTA plasma ; Ewb : EDTA whole blood

1 To assess the comparability of PCT measurement results, a number of correlation studies
2 have been conducted but these have shown conflicting results. Indeed, some studies showed
3 significant differences between PCT concentration obtained from different immunoassays,
4 whereas other studies reported the opposite [56–59]. In addition, the evaluation of different
5 external quality assessment (EQA) schemes results has shown a substantial discrepancy
6 across the different EQA schemes: while some highlighted quite good agreement between the
7 different assays, other EQA schemes showed the contrary [60]. Although substantial variations
8 in the PCT concentration measured by different immunoassays were reported in several
9 correlation studies and EQA schemes, the current clinical decision for diagnosis of sepsis and
10 therapy monitoring is based on common clinical cutoff values regardless of the immunoassay
11 employed for PCT measurements. Even if a good clinical concordance at clinical cutoffs has
12 been reported in the different correlation studies [60], several studies came up to the
13 conclusion that it would be preferable to compare results obtained from the same
14 immunoassay when interpreting serial evaluation of PCT concentration for a given patient [61–
15 63].

16 The current situation regarding the comparability of PCT measurement results may be
17 explained either by differences in assays selectivity or a calibration issue caused by the lack
18 of higher-order reference materials for PCT measurement and thus a lack of traceability to the
19 SI Units. It should be noted that manufacturers offering a PCT assay belonging to the Brahms-
20 like family participate in a harmonization program through the use of the Brahms Kryptor assay
21 as surrogate reference method in absence of any higher order RMP and certified reference
22 materials. However, not all assays commercially available take part in this harmonization effort,
23 thus there is currently no commonly agreed reference material for all available PCT
24 immunoassays at the international level. Therefore, the need for a new reference system
25 based on higher order reference materials and/or a higher-order reference method to achieve
26 global standardization/harmonization of PCT results worldwide has been pointed out in a
27 number of studies [56–59,64,65].

28 **2. IFCC working group on standardization of PCT assays (IFCC WG-PCT)**

29 To address the question of PCT measurements reliability and comparability and more
30 specifically to evaluate the need and the feasibility for standardization or harmonization, a
31 dedicated IFCC working group was created in 2018. This working group gathers clinicians,
32 biologists, in vitro diagnostic manufacturers, EQA providers and metrology institutes. The
33 participation of all concerned stakeholders is of utmost importance for the successful
34 implementation of a reference measurement system. Within this working group, the efforts
35 focused on five terms of reference (Figure 4): the development of a reference measurement
36 system (reference materials and procedures), the evaluation of the variability of results
37 provided by the different commercially available PCT assays, the evaluation of the need and
38 the feasibility for standardization of PCT assays, and the implementation of standardization of
39 PCT assays if deemed needed and feasible.

40

41

- 42 **▪ The first term of reference** consists in the development and validation of a RMP for
43 PCT absolute quantification by stable isotope dilution-liquid chromatography-tandem
44 mass spectrometry (ID-LC-MS/MS) in order to establish metrological traceability of
45 measurement results to the SI Units. The standardization of PCT tests could only be
46 implemented if the following prerequisites are met to achieve results comparability
47 independent of time, location and measurement procedures [3]. First, a proper

48 definition of the measurand should be established, i.e. clearly define what entity is
49 intended to be measured. Then, the selectivity of the different assays should be close
50 enough, i.e. measure the same entity. Finally, a reference measurement system
51 enabling to establish results traceability to the SI units should be established. This
52 includes availability of primary reference materials, commutable secondary reference
53 materials and a higher-order RMP meeting analytical performance specifications (APS)
54 that are fit for purpose to meet the medical need (e.g. sufficiently small measurement
55 uncertainties, high precision, high selectivity and high accuracy).
56

57 **Definition of the clinically relevant measurand:** , Measurand is defined by the
58 International Vocabulary of Metrology as a quantity intended to be measured [6].
59 Defining the measurand comes down to respond to the question “What is being
60 measured?”. At first sight the answer may seem simple, however that may be difficult
61 to answer according to the complexity of analyte, particularly in case of proteins [8].
62 Indeed, proteins could be subjected to heterogeneity and present as a mixture of
63 different molecular species in biological fluids such as it has been shown for human
64 growth hormone [66], human cardiac Troponin I [8,67], brain natriuretic peptide (BNP)
65 and N-terminal fragment of the BNP [68,69]. Thus, for an accurate quantitative analysis
66 and implementation of a reference measurement system, the isoforms of interest
67 should be clearly defined as it has been performed for HbA1c [70]. In the case of PCT,
68 there is currently no assay that detects the whole form of PCT, particularly the N-
69 terminal side of the sequence to discriminate the three different PCT isoforms.
70 Therefore, the most practical solution has been to define the measurand as total serum
71 PCT, including PCT (1-116), PCT (2-116) and PCT (3-116) isoforms.
72

73 **Selection and development of candidate reference measurement system**

74
75 Isotope Dilution Mass spectrometry (IDMS) is widely applied for protein absolute
76 quantification [66,71–79]. Thanks to its high selectivity and the use of stable-isotope-
77 labelled (SIL) compounds as internal standards, IDMS allows accurate quantification
78 of the measurand with low measurement uncertainties and is recognized as a primary
79 reference method by the Consultative Committee for Amount of Substance of BIPM
80 (Bureau International des Poids et Mesures). In a number of Reference Measurement
81 Systems, IDMS successfully serves as higher-order reference method and a number
82 of IDMS-based RMPs are listed as such in JCTLM database [80]. Still, validation of an
83 IDMS-based RMP remains a challenge for different reasons, including the need to
84 extensively characterize purity of primary calibrators, to obtain stable-isotope-labelled
85 (SIL) compounds which behavior is close enough to the endogenous compound to
86 make them suitable for use as internal standards, and to reach sufficiently low limit of
87 quantification with sufficiently small measurement uncertainties while achieving high
88 accuracy and high precision.
89

90 In a healthy state or in the absence of infection, plasma or serum concentration of PCT
91 is below 0.05 ng/mL. Due to ion suppression phenomena occurring in mass
92 spectrometry-based analysis, the sample preparation is critical for PCT absolute
93 quantification in a complex serum matrix by mass spectrometry. For sample
94 preparation, an antibody-free approach based on the physicochemical properties of
95 PCT was preferred in contrast to immuno-affinity approaches, which are currently
96 employed in commercially available immunoassays. After optimized sample
97 purification, PCT is then digested into peptides with trypsin. Peptides are then
98 quantified using high-resolution mass spectrometry in order to reduce interferences.

99 The ID-LC MS/MS analysis consists in the separation, the fragmentation and the
100 detection of PCT-related peptides generated by the digestion of the protein. The
101 different strategies for targeted quantification of proteins by ID-LC MS/MS are
102 described in Figure 5. As the selected bottom-up strategy provides an indirect detection
103 of PCT through the quantification of peptides derived from tryptic digestion, it is crucial
104 to select PCT-related peptides that are unique representatives of PCT protein
105 (proteotypic peptides). Two PCT-related peptides, located in two different segments of
106 PCT amino acid sequence, were selected to improve the specificity of the quantitative
107 method by discriminating the different PCT cleavage forms such as NProCT,
108 Calcitonin-GKKR-Katacalcin, Calcitonin and Katacalcin.

109 PCT concentration is obtained through the measurement of the concentration of the
110 PCT-related proteotypic peptides calculated from a calibration curve that is established
111 by measuring calibrators that are prepared by mixing known amounts of the labeled
112 and non-labelled primary calibrators. Depending on the calibration strategy, the
113 measured concentration of peptides might be negatively biased due to material loss
114 during the different step of the sample preparation, incomplete protein extraction,
115 incomplete proteolysis by trypsin, peptides loss by adsorption) [81]. The two commonly
116 used calibration quantification strategies include peptide-based calibration or protein-
117 based calibration, in which calibrators are prepared with synthetic PCT-related peptides
118 or PCT recombinant protein, respectively (Figure 5). The protein-based calibration
119 approach presents one main advantage: as the protein-based internal standard
120 undergoes the same sample processing as the endogenous protein, incomplete protein
121 extraction, incomplete proteolysis by trypsin and any material loss during sample
122 preparation steps occurring after spiking of the labeled protein are all compensated and
123 do not affect method's accuracy. However, the characterization of protein-based
124 primary calibrator is more challenging than for peptide-based calibrators as protein-
125 based impurities are expected to present, impurity identification and impurities
126 quantification is harder than for peptides. In addition, protein-based calibrators are
127 expensive to produce and can be subject to batch to batch variations. In order to
128 evaluate the most appropriate approach to ensure high accuracy and precision, these
129 two approaches have been employed and compared for the development of a
130 candidate higher-order RMP. Both peptide- and protein-based calibrators were
131 obtained from different providers. The peptides and proteins PCT standards produced
132 from the selected providers were then SI-traceably quantified by the primary RMP
133 composed of amino acid reference method using ID-LC MS and correction of the
134 contribution from impurities containing amino acids (Figure 1). The homogeneity and
135 stability of these materials still need to be evaluated before certification of the primary
136 reference material can be finalized.

137
138 **The second term of reference** consists in documenting and understanding the
139 variability of measurement results provided by different commercially available PCT
140 assays. The state of the art of PCT measurement shows conflicting results regarding
141 the comparability of PCT measurement results [60]. Moreover, in almost all correlation
142 studies available in the literature and all EQA programs, no information on the
143 commutability of the study samples is available when the studies are performed using
144 samples other than fresh single donations. However, this is a crucial aspect to
145 accurately document the need for PCT standardization/harmonization. In this respect,
146 agreement and correlation between the different PCT assays will be evaluated through
147 the organization of an interlaboratory comparison involving most popular PCT assays
148 and relying on samples of proven commutability. Thus, the two main tasks of second
149 term of reference consist in the production of candidate EQA materials and the

150 organization of a commutability study. This will result in the identification of appropriate
151 EQA materials to properly evaluate the comparability of the different PCT assays and
152 evaluate the need for further improving results consistency through calibration
153 standardization.

154 In a recent review based on data provided from 10 EQA providers of PCT assays, the
155 use of spiked materials was suspected to cause the inability to properly evaluate the
156 comparability between measurement results obtained from the different commercially
157 available PCT assays [60]. Surprisingly, the suitability of EQA materials consisting in
158 pools of fresh unspiked human serum was not evidenced, possibly due to ethical issues
159 and limitations in material stability and challenges to get sample collection, shipment
160 and testing conducted in a sufficiently low period of time [60]. The fact that no data is
161 provided regarding commutability of the materials and the measurement protocol of
162 laboratory participants represents a major limitation to determine what lessons can be
163 learned from such studies. In fact, various modifications/alterations of functional nature
164 could occur during the process to produce commutable candidate material such as
165 lyophilization, freezing, pooling, which could induce matrix effects for all or most of
166 assays. As commutability of a reference material cannot be predicted, it has to be
167 demonstrated against a set of authentic clinical specimens. Different EQA materials
168 will therefore be evaluated to identify the most suitable way to consistently obtain
169 commutable materials. Candidate EQA materials will consist in different matrices,
170 including frozen or lyophilized pools of human serum, artificial matrices, spiked or not
171 with recombinant PCT from different sources. A number of EQA providers already
172 agreed sharing their materials for inclusion in the commutability study. As
173 recommended in the IFCC latest recommendation, commutability of the candidate EQA
174 materials will be evaluated against a set of clinical specimens which PCT concentration
175 brackets that of the EQA materials. Ideally, clinical specimens should consist in fresh
176 unadulterated single donations (i.e. not pooled, not frozen, not spiked with any
177 exogenous substance) because these are commutable by definition. However,
178 maintaining materials stability from sample collection to testing is an important issue, it
179 is challenging to collect within one or two days a set of 30-50 fresh single donations
180 covering the entire concentration range, to ship the materials to all participating
181 laboratories and to get samples tested with a sufficiently short turnaround time to
182 maintain samples stability. It is therefore anticipated that clinical specimens will consist
183 in frozen single donations. In such a case, the absence of freeze-thaw effect should be
184 demonstrated beforehand. As a commutability assessment of a candidate reference
185 material should ideally include all commercially available assays, another challenge
186 lies in the need to collect a sufficiently large volume of each single donation to allow
187 conducting measurements with the different assays with appropriate replication
188 number. Although a commutability assessment should include as many PCT
189 measurement procedures as possible, involved assay should target the same
190 measurand and have adequate APS. The measurement results obtained will be
191 analyzed using the difference in bias approach [82]. An important prerequisite will be
192 to select appropriate acceptance criteria for commutability, ie. to decide what level of
193 non-commutability is acceptable, which is the current topic of the IFCC Working Group
194 on Commutability in Metrological Traceability (WG-CMT). It is expected that results of
195 the commutability will allow identifying the manufacturing process leading to the highest
196 commutability level. An EQA scheme relying on commutable material(s) will allow to
197 accurately document the current state of the art of harmonization of PCT results and
198 consequently to determine whether standardization is needed or not to improve the
199 comparability of the results provided from the different PCT assays used in routine
200 clinical practice.

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- **The third term of reference** consists in evaluating the need for standardization of PCT assays through a common calibration with commutable calibrators value assigned with the ID-LC HRMS RMP. The consultation of the International Consortium for Harmonization of Clinical Laboratory Results (ICHCLR) has already revealed that standardization/harmonization of PCT assays is considered as a high priority due to the growing utilization of PCT and to the implementation and integration in clinical guidelines and antimicrobial stewardship programs [83]. Moreover, a high medical impact of this standardization was pointed out by the ICHCLR. The strength of the IFCC working group on PCT assays, that gathers almost all IVD manufacturers marketing PCT assays, is particularly relevant for the objective of this term of reference. Indeed, the consultation of assay manufacturers was initiated with the creation of the working group and reveals that the need and demand for standardization on PCT assays differ across assay manufacturers. For some commercially available PCT assays (BRAHMS licensed assays group), a harmonization system is already in place through the use of the Kryptor method as a consensus reference method. Thus, for this group of methods, the recalibration of PCT assays is not perceived as a priority. On the contrary, for PCT assays other than BRAHMS PCT assays, there is currently no common calibrators nor reference system. Thus, standardization or harmonization is needed for these assays. Although views differ, all IFCC WG-PCT members agreed that commutable EQA materials are needed to accurately estimate between-method agreement (second term of reference) and thus assess the need for standardization of PCT.

- **The fourth term of reference** consists in evaluating the feasibility for standardization of PCT assays through common calibration with commutable calibrators, value assigned with the ID-LC HRMS RMP. This activity is highly dependent on the activities of the first and second terms of reference. Indeed, if a RMP is not available or does not meet appropriate APS, standardization will not be feasible. When validated, it will be necessary to evaluate if the candidate reference method by ID-LC MS/MS is robust enough at PCT concentration of clinical interest and if it enables to properly establish results traceability to the SI Units with sufficiently low measurement uncertainty. In addition, a sufficiently strong correlation between the different commercially available PCT assays and the candidate reference methods by ID-LC HRMS has to be demonstrated. Finally, the availability of the commutable calibrators has to be ensured. The feasibility of the standardization of PCT assays will thus be assessed if all these criteria are met.

The fifth term of reference consists in implementing the standardization of PCT assays at the global scale, if it appears desirable and feasible. If all the conditions for feasibility are met and if discrepant results between the different PCT assays are still observed from the EQA scheme relying on commutable EQA materials, standardization of PCT assays could be done either through panels of patient or commutable secondary calibrators value assigned with the ID-LC HRMS RMP. As recalibration of commercially available assays for PCT would require important efforts from IVD manufacturers, there should be strong evidence that standardization is necessary and feasible. EQA schemes will be needed to assess the accuracy and comparability of PCT measurement and evaluate the impact of the recalibration. Subsequently, it would be necessary to assess if the current clinical decision used in clinical practice need to be reconsidered or not.

251 **Conclusion**

252 Due to the growing threat of antimicrobial resistance and high mortality associated with sepsis,
253 having accurate and comparable PCT results appears of high importance. Still, in absence of
254 neither any higher order reference material nor any accuracy-based program relying on EQA
255 materials of proven commutability, the current state of the art regarding comparability of PCT
256 results across the different commercially available assays remains to be properly documented.
257 In this context, the IFCC Working Group on standardization of PCT assays has initiated
258 activities to fill this gap. A higher order RMP and commutable reference material are under
259 development to evaluate the necessity and the feasibility for establishing and maintaining a
260 new reference measurement system for PCT. As cooperation from all stakeholders is of major
261 importance, the working group gathers experts from National Metrology Institutes, calibration
262 laboratories, clinicians, biologists, EQA providers and assay manufacturers.

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Figure captions

Figure 1. Example of a metrological traceability chain for procalcitonin (PCT) measurement (ISO 17511-2020) relying on the development of a reference measurement system consisting of a hierarchy of different reference materials and reference measurement procedures.

Figure 2. Amino acid sequence of procalcitonin (PCT) and the different forms of PCT derived products which can be detected at different concentrations in the bloodstream of healthy subjects (figure adapted from Maruna et al 2000 and Becker et al 2004 [38,43])

Figure 3. PCT clinical cut-off values for patient stratification (figure adapted from Meisner 2014 [47])

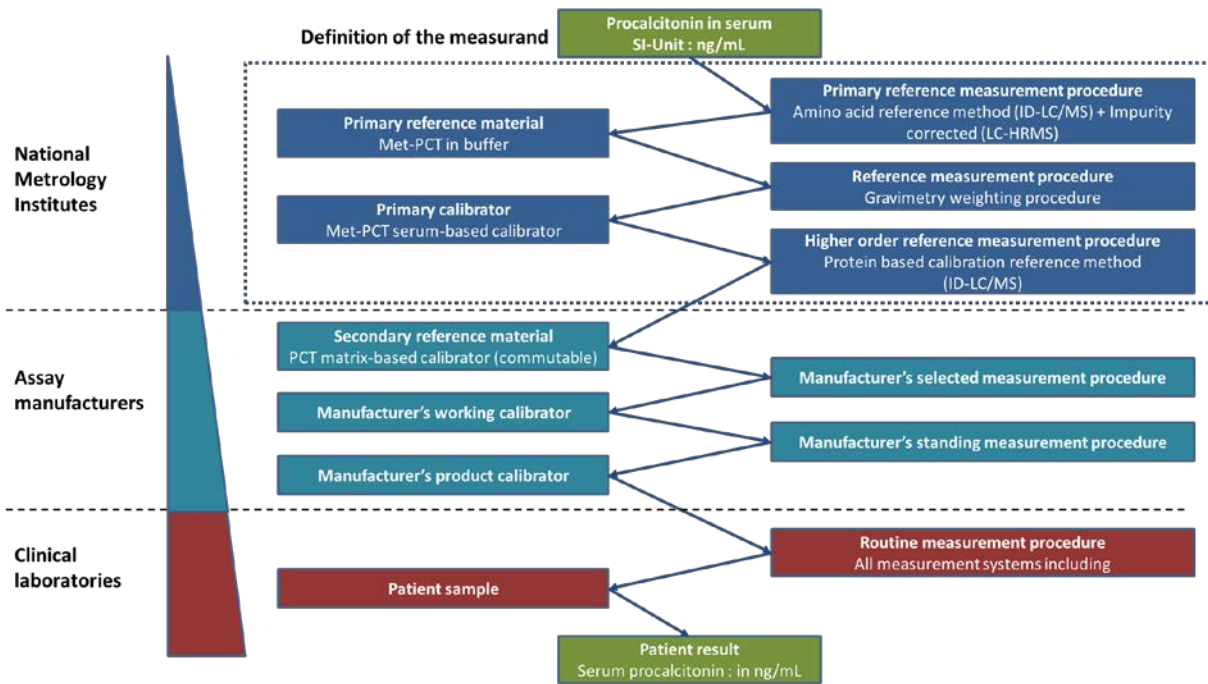
Figure 4. Terms of Reference of IFCC working group on Standardization of Procalcitonin (PCT) assays (IFCC WG-PCT)

Figure 5. Different calibration strategies for procalcitonin (PCT) targeted quantification by ID-LC MS/MS.

Analyte and internal standard (IS) could be spiked at different steps of the sample preparation process: 1 – before protein purification step; 2 – after protein purification step and before protein digestion step; 3 – after digestion step and before LC-MS/MS analysis. Depending on when analyte and IS are added to calibrators or patient samples, results accuracy could be influenced by various random variabilities during the whole quantification process such as sample handling, inefficient protein purification/digestion, protein/peptides adsorption, LC injection volume or MS ion suppression/enhancement.

Peptide-based calibration: calibrator produced by the addition of peptide analyte and SIL-peptide internal IS after protein digestion step (3)

Protein-based calibration: calibrators produced by the addition of protein analyte and SIL-protein IS before (2) or after (3) protein purification step.



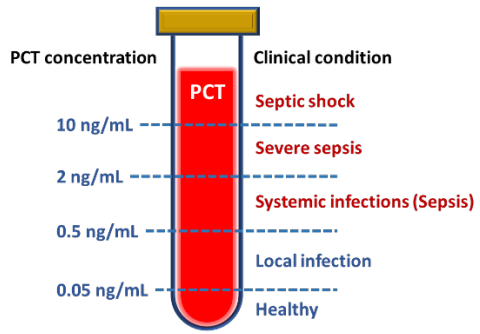
PCT amino acid sequence (NProCT-KR-Mature Calcitonin-GKKR-Katacalcin)

H₂N-APFRSALESS PADPATLSED EARLLAALV QDYVQMKASE LEQEQEREGS

SLDSPRSKRC GNLSTCMLGT YTQDFNKFHT FPQTAIGVGA PGKKRDMSSD

LERDHRPHVS MPQNaN-COOH

					<u>Concentration</u>
NProCT	-KR-	Mature Calcitonin	-G-KKR-	Katacalcin	0.19 fmol/mL
NProCT					4.15 fmol/mL
		Mature Calcitonin	-G-KKR-	Katacalcin	0.17 fmol/mL
				Katacalcin	3.17 fmol/mL
		Mature Calcitonin			1.84 fmol/mL



Standardization of Procalcitonin assays (IFCC WG-PCT)

