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Diagnosis of Sepsis by Use of Procalcitonin and Evaluation and Biochemical Characterization of the Latex Enhanced Immunoturbidimetric Technology for its Clinical Chemistry Use

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Alla mia Patria, “sì bella e perduta”.

E a mio padre, per sempre con me.

PUBLICATIONS

The present doctoral thesis is a cumulative work and is based on the following three publications:

PUBLICATION 1

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PUBLICATION 2

Masetto T., Matzenbach K., Reuschel T., Tölke S.A., Schneider K., Esser L.M., Reinhart M., Bindila L., Peter C., Grimmler M., **Comprehensive Comparison of the Capacity of Functionalized Sepharose, Magnetic Core, and Polystyrene Nanoparticles to Immuno-Precipitate Procalcitonin from Human Material for the Subsequent Quantification by LC-MS/MS,** *International Journal of Molecular Sciences*, 24(13):10963. (2023) doi: 10.3390/ijms241310963

PUBLICATION 3

Tölke S.A., Masetto T., Grimmler M., Bindila L., Schneider K., **Letter to the Editor regarding "Development of an antibody-free ID-LC MS method for the quantification of procalcitonin in human serum at sub-microgram per liter level using a peptide-based calibration"**, *Analytical and Bioanalytical Chemistry*, 413:4917-4919. (2021) doi: 10.1007/s00216-02-03459-5

The author has also contributed to the following publications, which were not directly at the focus of this dissertation:

Dupuy A.M., Bargnoux A.S., Larcher R., Merindol A., Masetto T., Badiou S., Cristol J.P., **Bioanalytical Performance of a New Particle-Enhanced Method for Measuring Procalcitonin**, *Diagnostics*, 10(7):461. (2020) doi: 10.3390/diagnostics10070461

Masetto T., Grimmler M., **Comment to: Extensive analytical evaluation of the performances of the new DiaSys PCT assay and comparison with Elecsys B·R·A·H·M·S PCT test on Roche Cobas and B·R·A·H·M·S PCT-sensitive Kryptor**,

Clinical Chemistry and Laboratory Medicine,62(2):e48-e50. (2023) doi: 10.1515/cclm-2023-0997

Tölke S.A., Masetto T., Reuschel T., Grimmler M., Bindila L., Schneider K., **Immunoaffinity LC-MS/MS Quantification of the Sepsis Biomarker Procalcitonin Using Magnetic- and Polystyrene-Bead Immobilized Polyclonal Antibodies**, *Journal of Proteome Research*, 22(10):3135-3148. (2023) doi: 10.1021/acs.jproteome.3c00082

1 ZUSAMMENFASSUNG

Sepsis ist eine lebensbedrohliche Funktionsstörung eines Organs, die durch eine fehlgesteuerte Immunreaktion des Wirts auf eine Infektion zurückzuführen ist. Sie bedeutet eine große globale Bedrohung mit einer Inzidenz von 49 Millionen Neuerkrankungen im Jahr 2017. Procalcitonin, die Vorstufe des Hormons Calcitonin, gilt als vielversprechender früher Biomarker für die Sepsisdiagnose und als Richtwert für die Antibiotikatherapie. Dennoch ist seine Verwendung durch die Nachteile der fehlenden Standardisierung und der Langsamkeit aktueller PCT-Immunoassays begrenzt.

Die vorliegende Arbeit bietet die Grundlage für die Sepsisdiagnose durch die Analyse von Procalcitonin (PCT), für die Messstandardisierung dieses Proteins und für seine biologische Charakterisierung.

Die umfassende Analyse der Ergebnisse der externen Qualitätsbewertung (EQA) für PCT in Deutschland zeigt eine hohe Heterogenität bei der PCT-Quantifizierung durch verschiedene Immunoassays. Im Extremfall (Abbott vs. Biomerieux) werden dieselben Proben nahezu doppelt so viel gemessen. Eine erstmals durchgeführte Kommutabilitätsstudie für den Parameter PCT zeigt, dass derzeit verwendete Standardmaterialien nicht mit endogenem PCT aus menschlichen Proben vergleichbar sind. Zudem wird die Eignung der partikelverstärkten turbidimetrischen Immunoassay (PETIA)-Technologie für die PCT-Messung im Vergleich zu einem bereits etablierten Lumineszenzimmunoassay demonstriert (Korrelationskoeffizient Spearmans R 0.975). Dieser Teil wird in Zusammenarbeit mit dem Institut für Klinische Chemie/Interdisziplinäres UMG-Labor (Göttingen, Deutschland) durchgeführt.

Darüber hinaus wird die quantitative Immunpräzipitation von PCT aus menschlichen Proben unter Verwendung von der PETIA-Technologie abgeleiteten Polystyrol-basierten Partikeln, Sepharose™ und magnetischen Partikeln beschrieben. Die Immunpräzipitationsmethode unter Verwendung von magnetischen Partikeln scheint für diesen Zweck am besten zu funktionieren, mit einer PCT-selektiven Präzipitation von 100% innerhalb von zwei Stunden und einem massenspektrometrischen (MS)-spezifischen Signal-Rausch-Verhältnis, das fünf bis zehnmal höher ist als das durch die Präzipitation mit Polystyrolpartikeln erreichte. Dieser Prozess ist die Grundlage der MS Methode, die zum einen zur Standardisierung der Messung von PCT-Immunoassays und zur selektiven Immunpräzipitation potenzieller PCT-Bindungspartner verwendet werden soll, um zum anderen die weitere biologische Charakterisierung dieses Proteins zu unterstützen. Dieser Teil wird in Zusammenarbeit mit dem Institut Fresenius (Idstein, Deutschland) und der Johannes-Gutenberg-Universität Mainz (Mainz, Deutschland) durchgeführt.

2 ABSTRACT

Sepsis is the organ dysfunction resulting in a life-threatening risk, caused by the dysregulated immuno-reaction of the host to an infection. Sepsis is a major global threat with an incidence of 49 million new cases in 2017.

Procalcitonin, the precursor of the hormone calcitonin, has been reported as a promising early biomarker for sepsis diagnosis and to guide antibiotic therapy. Nonetheless, its use is limited by the drawbacks of the missing standardisation and the slowness of present immunoassays.

The present work offers the basis for the diagnosis of sepsis by analysis of procalcitonin, for the measurement standardisation of this protein, and for its biological characterisation.

The comprehensive analysis of the results of the external quality assessment (EQA) for PCT in Germany shows a tremendously high heterogeneity of PCT quantification by different immunoassays. In the extreme case (Abbott vs. Biomerieux) the same samples are measured nearly double in quantity. For the first time for the parameter PCT, a commutability study is conducted demonstrating that currently employed standard materials are not comparable with human blood. Furthermore, the suitability of the Particle Enhanced Turbidimetric Immuno-Assay (PETIA) technology for PCT measurement is demonstrated in comparison to an already established luminescence immunoassay (correlation coefficient Spearman´s R 0.975). This part is carried out in cooperation with the Institute for Clinical Chemistry/Interdisciplinary UMG Laboratory (Göttingen, Germany).

Additionally, the quantitative immuno-precipitation of PCT from human samples is reported, employing polystyrene-based particles derived through PETIA technology, Sepharose™, and magnetic-based particles. The immuno-precipitation method employing magnetic particles appears to work best for this purpose, with a PCT selective precipitation of 100% within two hours and mass-spectrometric (MS) specific signal to noise ratio five to ten times higher than those delivered by the polystyrene particle-based precipitation.

This process is the basis of the MS method, which shall be used to standardise the measurement of PCT immunoassays, and to selectively immuno-precipitate potential PCT´s binding partners for the further biological characterisation of this protein. This part is performed in cooperation with the Institute Fresenius (Idstein, Germany) and the Johannes Gutenberg University of Mainz (Mainz, Germany).

3 ABBREVIATIONS

aa: amino acid(s)
ACCP: American College of Chest Physicians
AUC: Area Under Curve
CAPA: CAlcitonin gene-related Peptideamylin-(pro-)calcitonin-Adrenomedullin
CGRP-I/-II: Calcitonin Gene Related Peptide I/II
CI: Confidence Interval
CLIA/E-CLIA: Chemiluminescence Immunoassay and Electro-Chemiluminescence Immunoassay
CLIA: ChemiLuminescent ImmunoAssay
CLSI: Clinical & Laboratory Standards Institute
CRP: C-Reactive Protein
CV%: Coefficient of Variation percentage
ELFA: Enzyme-Linked Fluorescent Assay
EQA: External Quality Assessment
FiO_2: Fraction of inspired Oxygen (L min^{-1})
ICU: Intensive Care Units
IEF: IsoElectric Focusing
IFCC: International Federation of Clinical Chemistry
IL-1β: Interleukin-1 beta
IL-6: Interleukin-6
INF-γ: Interferon-gamma
iNOS: inducible Nitric Oxide Synthase
IP: immuno-precipitation
IVD: In Vitro Diagnostic
IVDR: In Vitro Diagnostic Regulation
LBP: Lipopolysaccharide-Binding Protein
LNE: *Laboratoire national de métrologie et d'essais*
LOD: Limit of Detection
LOQ: Limit of Quantitation
LRTI: low respiratory tract infections
mAb: monoclonal antibody
MagP: Protein G-magnetic particles
MAP: Mean Arterial Pressure
mE: milli Extinction (absorbance)
mRNA: messenger Ribo-Nucleic Acid
MVZLM: *Medizinisches Versorgungszentrum für Labormedizin und Mikrobiologie Ruhr GmbH*
nCD64: neutrophil CD64
pAb: polyclonal antibody
$PaCO_2$: Partial pressure CO_2 (mmHg)
PaO_2: Partial pressure Oxygen (mmHg)
PCT: Procalcitonin
PETIA: Particle Enhanced Turbidimetric Immuno Assay
PGS: Protein G-Sepharose particles
pI: isoelectric point
POCT: Point-of-Care-Testing
PTM: post translational modification
PTX3: Pentraxin-3
qSOFA: quick SOFA
RfB: Referenzinstitut für Bioanalytik
rhPCT: recombinant human PCT
ROC: Receiver Operating Characteristic
RSD: Response Surface Design
SAA: Serum Amyloid A
SCCM: Society of Critical Care Medicine
SDS-PAGE: SDS-PolyAcryamide Gel Electrophoresis
SIRS: Systemic Inflammatory Response Syndrome
SOFA: Sequential Organ Failure Assessment

sTREM-1: soluble Triggering Receptor Expressed on Myeloid cells-1

suPAR: soluble urokinase-type Plasminogen Activator Receptor

TNF-α: Tumour Necrosis Factor-alpha

TRACE: Time-Resolved Amplification of Cryptate Emission

WBC: White Blood Cell

WHO: World Health Organization

4 TABLE OF CONTENTS

5 INTRODUCTION

5.1 Epidemiology of sepsis

Sepsis is one of the major medical concerns worldwide. The WHO reports that the global incidence of sepsis reached 49 million new cases in 2017 (1).

The deep analysis of Rudd et al. about the global, regional, and national sepsis incidence and mortality in the period 1990-2017 (2), reveals that the worldwide sepsis incidence amounts to 48.9 million cases, with related mortality reaching 11.0 million deaths in 2017. The sepsis-related mortality represents 19.7% of all global deaths (2).

In addition, Buchman et al. report an increase of sepsis-related hospitalisations of about 40% in the period 2012-2018 in the USA, which in turn led to a growth of total costs (inpatient admission costs plus any subsequent skilled nursing costs) for the American health system of about $14 billion (3).

Torio and Moore report sepsis to be responsible for 1.3 million hospital stays and to be among the four most costly pathologies in the USA in 2013, encoding aggregate hospital costs of almost $24 billion (4). The 6-month mortality after sepsis has been reported to be about 31% for specific microorganism sepsis, and even to reach 60% following septic shock (3,5). A similar dramatic picture has been described also in Germany. Fleischmann-Struzek et al. state an incidence of 1006/100,000 adult persons (6), with up to 75,000 deaths per year. This data sets sepsis among the three most common causes of death in Germany in 2021 (7).

Furthermore, sepsis remains an extensive problem in developing countries. Recent data by Sankar et. al reports an incidence in India of 3% newborns (8), while Rudd et al. (2) describe that 85% of all global sepsis cases occur in low or middle-income countries. Moreover, the problem of the antimicrobial resistance is becoming more and more compelling. As recently described by the Antimicrobial Resistance Collaborators (9), the six microbes mainly responsible for resistance-associated mortality can reach in some cases more than 80% of the isolated clones and they account potentially for almost one million directly attributable deaths worldwide.

A rapid and thorough diagnosis of sepsis is essential to reduce a negative prognosis for the patient, allowing a prompt antibiotic administration (10). Indeed, Chippendale et al. report that for a patient in septic shock, every hour's delay in antibiotic administration increases the absolute mortality of 7.6% (11,12).

5.2 History of sepsis definition

The first description of sepsis in modern times, contemplating the dysregulated reaction of the immune system, was given in 1913 by William Osler. He noticed that “*the patient seems*

to die because of the body's reaction to infection rather than because of the infection itself" (13).

In 1991 the American College of Chest Physicians (ACCP) and the Society of Critical Care Medicine (SCCM) published a consensus document (14) giving the first sepsis definition (Sepsis-1) and outlining the concept of Systemic Inflammatory Response Syndrome (SIRS). The SIRS implied four criteria:

- tachycardia (heart rate > 90 beats/min)
- tachypnea (respiratory rate > 20 breaths/min) or $PaCO_2$ < 32 mmHg
- fever or hypothermia (temperature > 38 or < 36 °C)
- leukocytosis, leukopenia, or bandemia (white blood cells > 12,000/mm³, < 4,000/mm³ or bandemia ≥ 10%).

A patient meeting at least two of these criteria was classified as SIRS-affected, and sepsis was defined as the infection resulting in SIRS. Furthermore, the new terms "severe sepsis", in case of complication by organ dysfunction, and "septic shock", in case the "sepsis-induced hypotension persists despite adequate fluid resuscitation" were coined.

More recently (2001), a task force of 29 participants from Europe and North America revisited the Sepsis-1 definition, delineating its limitations concerning sensitivity and specificity, and expanding the original list of four diagnostic criteria to hemodynamic and tissue perfusion parameters (Table 1). The work of this task force resulted in the Sepsis-2 definition. Accordingly, a patient presenting at least two SIRS criteria and additionally a suspected or confirmed infection would be diagnosed with sepsis under this new definition (15). The definitions of severe sepsis and septic shock were not changed.

5.3 Current Sepsis definition (Sepsis-3)

In 2016, the Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3) redefined sepsis as the "life-threatening organ dysfunction caused by a dysregulated host response to infection" (16).

The task force introduced many important differences to the past definition. First, the central role of the infection was underlined. Furthermore, the new definition identifies the essential difference between sepsis and infection in the dysregulated host response and the presence of organ dysfunction, previously defined as severe sepsis.

Finally, the lethality rate of sepsis is significantly higher in comparison to infection, which implies the need for urgent recognition and action (16).

The most remarkable change in this new sepsis definition is that any reference to SIRS has been removed, since the definition of SIRS was affected by the following boundaries:

- the extreme focus on inflammation;
- the ambiguous idea that sepsis always leads to severe sepsis and septic shock;

- inadequate diagnostic accuracy (specificity and sensitivity) of the four SIRS criteria;
- simultaneous use of multiple definitions for sepsis, septic shock, and organ dysfunction, and different cut-offs for blood pressure, levels of hyperlactatemia, vasopressor use, and defined fluid resuscitation volume/targets, all resulting in remarkable discrepancies in sepsis incidence and mortality (16,17).

Sepsis-3 further redefines the concept of septic shock as “a subset of sepsis in which underlying circulatory and cellular metabolism abnormalities are profound enough to substantially increase mortality”. In this case, the main difference in the Sepsis-3 definition is the stress on cellular abnormalities, emphasizing a higher sickness severity and consequently a much higher likelihood of adverse outcomes.

The differences between the three modern definitions of sepsis are depicted in a schematic form in Table 1 (adapted from (17)).

Table 1. Schematic comparison of the three sepsis definitions: Sepsis-1, 1991, Sepsis-2, 2001, and Sepsis-3, 2016 (adapted from (17)).

Older definitions		**Newer definition: Sepsis-3**	
Sepsis-1	**Sepsis-2**	**Definition**	**Clinical criteria**
Systemic inflammatory response syndrome (SIRS) = the systemic inflammatory response to a variety of severe clinical insults. -Temperature > 38 °C or < 36 °C -Heart rate > 90 beats/min -Respiratory rate > 20 breaths/min or $PaCO_2$ < 32 mmHg -White blood cell count > 12,000/mm³, < 4,000/mm³, or > 10% immature (band) forms.	**Diagnostic criteria for sepsis** **Infection** Documented or suspected and some of the following: **General parameters** -Fever (> 38.3 °C) -Hypothermia (< 36 °C) -Heart rate (> 90 beats/min) -Tachypnea (> 30 breaths/min) -Altered mental status -Significant edema or positive fluid balance (> 20 mL/kg over 24 h) -Hyperglycemia (glucose > 110 mg/dL)	**Screening for Sepsis** qSOFA (quick Sequential Organ Failure Assessment) scoring system. Accordingly, an increase of 2 or more in the qSOFA score should create a suspicion of sepsis and organ dysfunction	**qSOFA** -Altered mental status (GCS score < 15) -Systolic blood pressure < 100 mmHg -Respiratory rate > 22 breaths/min *qSOFA positive with at least 2 of these criteria positive*
Sepsis = the systemic response to infection, manifested by two or more of the SIRS criteria as a result of infection	**Inflammatory parameters** -Leukocytosis (WBC > 12,000/mm³) -Leukopenia (WBC < 4,000/mm³) -Normal WBC with >10% immature forms-CRP & PCT > 2	Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection	Suspected or documented infection and an acute increase of ≥ 2 SOFA points

Older definitions		Newer definition: Sepsis-3	
Sepsis-1	**Sepsis-2**	**Definition**	**Clinical criteria**
	SD above the normal value		
Severe sepsis = sepsis associated with organ dysfunction, hypoperfusion, or hypotension.	**Hemodynamic parameters** -Arterial hypotension (systolic blood pressure < 90 mmHg, mean arterial pressure < 70, or a systolic blood pressure decrease > 40 mmHg in adults) -Venous oxygen saturation > 70% -Cardiac index > 3.5 L/min m^2	Septic shock is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality	Sepsis and vasopressor therapy are needed to elevate MAP ≥ 65 mmHg and lactate > 18 mg/dL despite adequate fluid resuscitation
Septic shock = sepsis-induced with hypotension despite adequate fluid resuscitation.	**Organ dysfunction parameters** -Arterial hypoxemia (PaO_2/FiO_2 < 300) -Acute oliguria (urine output < 0.5 mL/Kg h for ≥ 2 h) -Creatinine increase ≥ 0.5 mg/dL -Coagulation abnormalities -Ileus (absent bowel sounds) -Thrombocytopenia (platelet count <100,000/μL) -Hyperbilirubinemia (total bilirubin > 4 mg/dL)		
	Tissue perfusion parameters -Hyperlactatemia (> 27 mg/dL) -Decreased capillary refill		

5.4 The diagnosis of sepsis

Numerous works strengthen the importance of the early diagnosis and correct management of sepsis (18–22).

The thorough and immediate diagnosis of sepsis is fundamental to reduce a negative prognosis and to allow the rapid administration of antibiotics (10), as every one hour of delay in starting the therapy can increase the mortality by about 7.6% (11,12).

Even though the new Sepsis-3 definition puts the accent on the use of clinical criteria to identify organ dysfunction and analysis of blood culture, both methods would lead to a late sepsis identification (18).

The blood culture, especially, is considered the gold standard for the identification and the precise characterisation of the sepsis-causing bacterial strains (23). Nonetheless, it presents the drawback of a long turnaround time, which can vary between a few hours and five days. Moreover, between one and two further days can be necessary to test the antibiotic susceptibility (18). Additionally, this technology offers low sensitivity, ranging between 5% and ca. 60% (16,18,24), which is also influenced by the initiation of antibiotic therapy. Supplementary limitations are ascribable to pre-analytical variables (i.e. sample contaminations, affecting up to 56% of the positive samples (24)), the complexity of automatisation, though some attempts have been tried in the last years (25), and the need of large sample volumes (up to 60 mL), particularly cumbersome for pediatric samples. It has been reported that only 18% of the blood culture bottles were filled up with the recommended volume (8-10 mL) and 47% had less than 8 mL (26). In relation to scores systems, like SIRS, SOFA, and qSOFA, proposed by the Sepsis-1 and Sepsis-3 definitions, they are mainly thought to be used for prediction of mortality or in general as prognostic tools (18,27) and not as early diagnostic tools.

5.5 Proposed biomarkers of sepsis

Due to these limitations, in the last decades a plethora of blood biomarkers has been proposed by laboratory medicine for early diagnosis sepsis: lactate, C-reactive protein (CRP), procalcitonin (PCT), soluble CD14-subtypes (sCD14-ST, also known under the commercial name Presepsin), interleukin 6 (IL-6), lipopolysaccharide-binding protein (LBP), neutrophil CD64 (nCD64), pentraxin-3 (PTX3), uncoupling protein-2, serum amyloid A (SAA) soluble triggering receptor expressed on myeloid cells-1 (sTREM-1) and the serum soluble urokinase-type plasminogen activator receptor (suPAR) (18,28–32).

Due to technological limitations (low throughput instruments, high cost-per-test) or unsatisfactory performances, mainly low specificity, many of these biomarkers are weakly represented in the clinical routine. On the other hand, sCD14-ST (28,33,34) and PCT (35,36) are currently considered good biomarkers for early sepsis diagnosis due to their good sensitivity and specificity. Additionally, while sCD14-ST determinations are still carried out mainly on semi-automated/POCT (Point-of-Care-Testing), PCT is available on fully automated high-throughput immunoassay instruments, which facilitates its extensive use (37,38).

5.6 PCT as a biomarker of sepsis and for antibiotic stewardship

The first mention of PCT as a biomarker of sepsis was reported in 1993 in *Lancet* by Assicot et al. (35). They noticed that 19 pediatric patients with severe bacterial infections had PCT serum concentration ranging from 6-53 ng/mL, significantly higher in comparison to 21 patients with no infection showing PCT concentrations < 0.1 ng/mL. Furthermore, PCT concentrations quickly decreased upon antibiotic administration. While calcitonin concentrations remained normal in all these patients, the PCT concentration seemed to correlate with the severity of the infection. Since that time, many works and clinical studies have proven the usefulness of this protein as a biomarker of infection and sepsis and also to guide antibiotic therapy.

The PROcalcitonin to Reduce Antibiotic Treatment in Acutely ill patients (PRORATA) study (39), a multicentre, randomised study, involved 630 patients admitted to intensive care units (ICU) with a suspected infection. For the PCT group, the antibiotic therapy was initiated and stopped according to predefined cut-off values (Figure 1), still valid nowadays, while the control group was administered according to the current practice. The patients in the PCT-guided group showed a significant 2.7-day reduction of the mean number of days with antibiotics, without having a higher 28- and 60-day mortality. This in turn can reduce costs (40) and adverse effects (22,41).

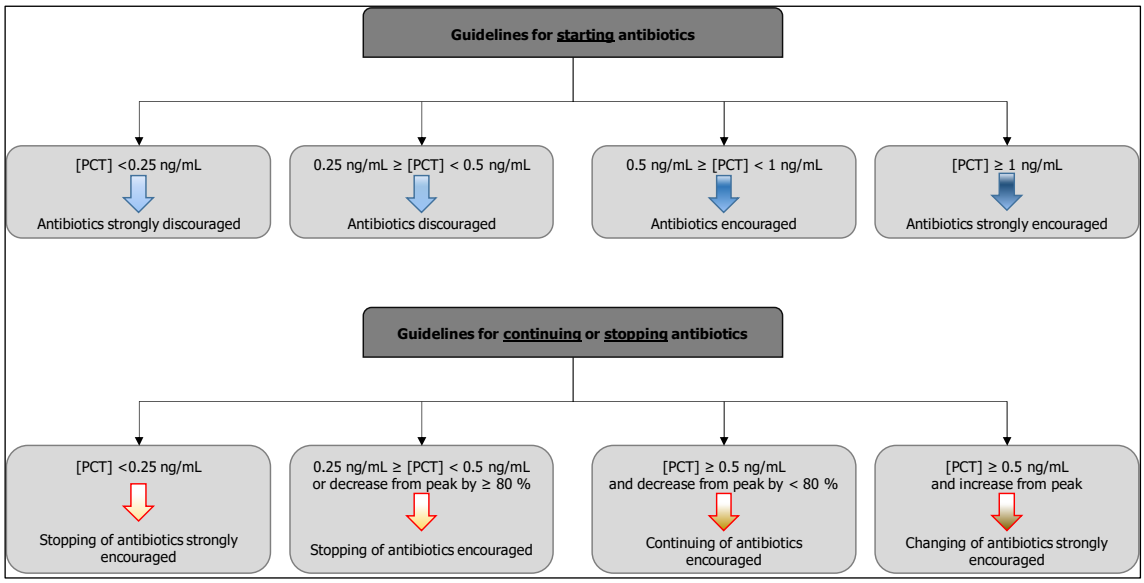

Figure 1. Algorithm for starting, continuing, or stopping of antibiotic therapy according to PCT concentration and/or variation. Adapted from (39).

In the so-called Biomarkers in Acute Heart Failure (BACH) trial, PCT was used to diagnose pneumonia (42). Its AUC was higher than any other diagnostic parameter analysed individually. Furthermore, patients with raised PCT concentration had a worse outcome (lower survival probability) if not treated with antibiotics ($p = 0.046$). On the other hand, a

better outcome was reached by not administering antibiotics to patients with low PCT values ($p = 0.049$).

The Stop Antibiotics on guidance of Procalcitonin Study (SAPS) in 2016 concentrated on the effects of the PCT usage for the discontinuation of antibiotics, however without evaluating its performances in terms of diagnosis (43,44). The authors used the same cut-off values as per PRORATA study. In this case, surprisingly, the PCT-guided group had even a significantly lower 28-day mortality (19.6% vs. 25.0%, $p = 0.0122$) in comparison to the control group. Furthermore, the antibiotic-therapy duration with PCT was 5 days vs. 7 days for the control group ($p < 0.0001$). The authors also report a reduction in antibiotic costs of 34 € per patient.

Similar conclusions were reached by the ProHOSP trial (45) on patients with lower respiratory tract infections. PCT reduced the duration of the antibiotic therapy by 3 days (8.7 vs. 5.7, -34.8%) and the frequency of adverse events of about 8% (28.1% vs. 19.8%).

Not all studies showed a benefit in guiding the antibiotic therapy through PCT. This is the case of PROcalcitonin Antibiotic Consensus Trial (ProACT) (46). This trial didn´t show a significant reduction in treatment duration between the PCT-guided group and the control group. A limitation of this study is that only one specific cut-off value was used (0.1 ng/mL) without developing a discontinuation algorithm as used by the PRORATA and SAPS trials. On the other hand, the study´s population biased the results as well, as it was not likely to benefit from PCT aided therapy (39% had asthma, 91% had PCT < 0.25 ng/mL).

Also in relation to PCT diagnostic accuracy (sensitivity/specificity) different data have been published. A deep examination of the meta-analysis about the most common sepsis biomarker for the sepsis diagnosis has been completed by Lippi et al. (27). In relation to PCT, values of AUC between 0.78 and 1.00 have been reported, as well as sensitivity between 0.71 and 1.00 and specificity 0.61 and 0.88 (Table 2). Lippi et al.´s work was published in 2017, immediately after the introduction of the Sepsis-3 definition. More recent works did confirm their conclusions (36,47–53).

Table 2. Schematic summary of AUC, sensitivity, and specificity for different biomarkers in relation to sepsis diagnosis.

Biomarker	Range AUC	Range sensitivity	Range specificity
PCT	0.78-1.00	0.67-1.00	0.61-0.88
CRP	0.71-0.77	0.75-0.91	0.36-0.67
Presepsin	0.86-0.89	0.77-0.75	0.73-0.88
IL-6	0.70-0.80	0.68-0.72	0.73-0.73
SAA (only neonates)	0.90	0.84	0.89

Similarly, Lippi et al.´s work revealed that PCT can significantly reduce antibiotic therapy duration between 1 and 4.2 days (27) (Table 3). However, one work showed no significant decrease in the length of antibiotic therapy (54).

Table 3. Schematic summary of results (reduction of duration) for the use of PCT for antibiotic stewardship.

Authors	Setting	Result (antibiotic therapy reduction)
Kopterides et al., 2010 (55)	ICU	4.19 days
Heyland et al., 2011 (56)	ICU	2.14 days
Schuetz et al., 2011 (57)	ED, ICU	20-27%
Soni et al., 2013 (58)	ICU	2.05 days
Prkno et al., 2013 (59)	ICU	27%
Andriolo et al., 2017 (60)	Unselected population	1.28 days
Wirz et al., (61)	ICU	1.19 days
Schuetz et al., (62)	ICU	2.4 days
Lam et al., 2018 (63)	ICU	1.26 days
Iankova et al., 2018 (64)	ICU	1.49 days
Pepper et al., 2019 (65)	Critically ill adults	1.31 days
Peng et al., 2019 (66)	Critically ill adults	0.99 days
Meier et al., 2019 (67)	ICU	2.86 days
Gutiérrez-Pizarraya et al., 2022 (68)	ICU	1.98 days

In conclusion, PCT is currently considered the best, even though not perfect, biomarker to diagnose sepsis and to guide antibiotic therapy.

5.7 The CALC gene family and the induction of PCT

Four genes are currently known to be associated with the synthesis of proteins having a sequence similar to calcitonin, undergoing the name of the calcitonin gene family (CALC gene). This family includes the human CALC-I gene, human CALC-II gene, human CALC-III gene, and human CALC-IV (amylin gene) (69,70).

The gene CALC-II does not lead to final calcitonin synthesis (71) but it only produces the protein CGRP-II. CALC-III is a pseudogene and it does not encode for any protein. CALC-IV, on chromosome 12, codifies for the protein amylin, involved in glucose regulation as an antagonist of insulin. Its structure has 46% similarity to calcitonin (70,71). All these genes play negligible or no role at all in relation to PCT/calcitonin synthesis.

On the other hand, the gene CALC-I, on chromosome 11, is one of the first genes for which an alternative splicing has been described, resulting in three different mRNAs. The first

resulting protein is the CGRP-I, which has lost the sequence of calcitonin and is produced in the cells of the peripheral and central nervous system (72). It has no calcium-related functions, but it acts as a vasodilator. The further two mRNAs result in the proteins calcitonin-I and calcitonin-II. Both can originate PCT as the final product (70).

All these proteins have common features, like their initial production as pre-pro form, the disulphide bridge between two cysteine residues, and two main cleavage sites to lead to the final active form. For these reasons, they are usually grouped into the so-called CAlcitonin gene-related Peptideamylin-(pro-)calcitonin-Adrenomedullin (CAPA) protein family.

The gene CALC-I is the source of mature calcitonin. This protein is produced under normal physiological conditions exclusively in the thyroidal C-cells. The same gene encodes for the inflammatory PCT (70). Domenech et al. show that, upon bacterial infection, the mRNA transcription from the CALC-I gene appears to be strongly incremented in almost all body tissues (73). The authors hypothesise the existence of infection-specific elements in the promoter of the CALC-I gene, which they call microbial infection-specific response elements (MISRE) (73). Also the inhibition of PCT expression operated under viral infection by IFN-γ seems to happen through the direct blocking effect on the CALC-I gene expression (74,75). However, these possible specific elements haven´t been further investigated yet and little is known about their biochemical roles and in general about PCT expression intracellular patterns (74).

The proteins resulting from the CALC-I gene appear to be quite highly conserved between different mammalian species (homo sapiens, dog, sheep, mouse, rat, and horse) as reported in UniProt-BLAST comparison (Figure 2). Their similarity, ranging between 61% and 93%, suggests that PCT probably derives from one common ancestor gene, which in turn denotes important shared functions, especially related to calcium regulation and homeostasis (76).

Figure 2. BLAST comparison of primary sequences for the calcitonin protein from different mammals (dog, sheep, mouse, rat, homo sapiens, horse), outlining high similarity. The reported sequence corresponds to the pre-prohormone of calcitonin, including the signal peptide (red). The blue colour indicates the amino acid conservation grade between the species (dark violet: high conservation grade; light violet: low conservation grade).

Finally, there is no specific cellular receptor for PCT, as this protein is normally not released into the bloodstream (see paragraph below). However, Sexton et al. (77) demonstrated that PCT can be active on receptors specific to other proteins derived from the CALC gene. Especially, PCT was reported to be a strong partial agonist of the CGRP-I receptor, with approx. 50% of the CGRP-specific efficacy, and to specifically inhibit further stimulation of this receptor by CGRP itself at concentrations typical of septic settings (approx. 5 nmol/L) (77). PCT appeared to have weak partial agonist activity also on the AMY1 receptor, while it showed no activity at all at the calcitonin receptor (77).

5.8 Pathophysiology of PCT and its structure

PCT is a protein of 116 amino acids (aa) and about 13 kDa molecular weight (78). Its aminoacidic sequence is shown in Figure 3, while no tertiary structure by X-ray crystallography or NMR analysis has been reported so far.

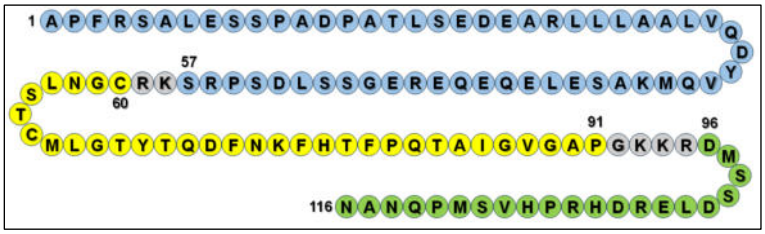

Figure 3. The primary sequence of PCT as per UniProt entry P01258 CALC_HUMAN. Blue: N-terminal moiety, aa 1 to 57; yellow: Calcitonin moiety, aa 60 to 91; green: Katacalcin moiety, aa 96 to 116; grey: protease-specific sequences.

PCT is the precursor of the hormone calcitonin. Under normal physiological conditions, PCT is synthesized exclusively within the thyroidal C-cells as pre-procalcitonin, with a 25-aa long signal sequence at the N-terminus. This hydrophobic signal peptide drives the binding to

the endoplasmic reticulum, where it is cleaved by endopeptidases giving PCT (78,79) (Figure 4). In the thyroidal C-cells, the N-terminal and the C-terminal (Katacalcin) of the PCT molecule are further cleaved by the enzyme prohormone convertase to result in the final 32-aa long calcitonin, involved in calcium regulation (74,79). This is finally processed and stored in secretory granules (70) or secreted into the blood flow. It is important to notice that the calcitonin´s cysteine residues in positions 1 and 7 forms a disulphide bridge and the proline at C-terminus is hydroxylated. These both post-translational modifications, essential for calcitonin receptor recognition, are absent in the PCT molecule (71,78).

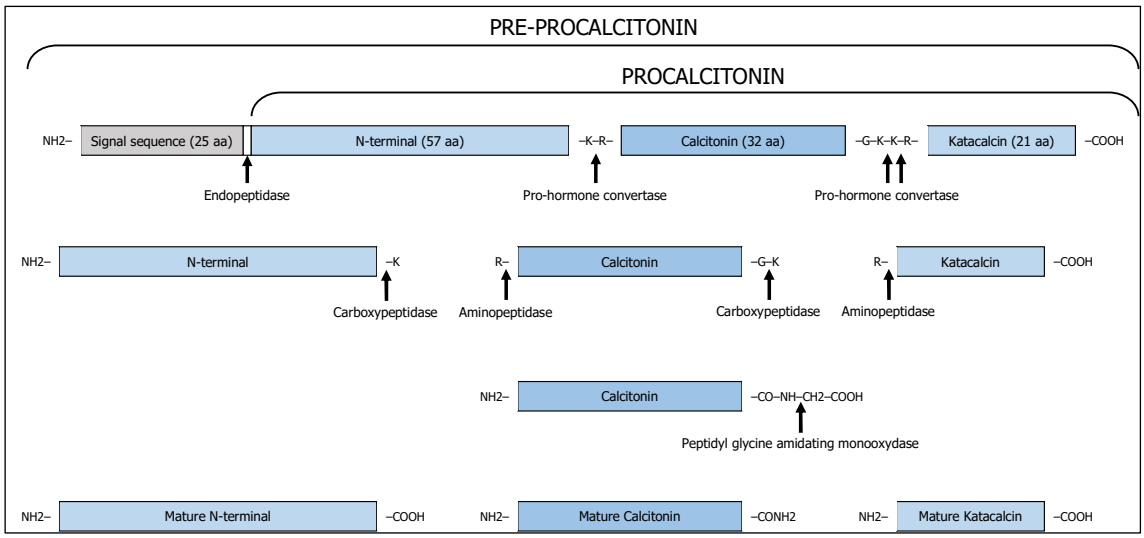

Figure 4. Primary structure and cleavage of (pre)procalcitonin to give mature calcitonin under normal physiological conditions. Adapted from (78).

Cleland et al. report that normal physiological PCT blood concentrations are very low, lower than 0.05 ng/mL (79). However, in concomitance with bacterial infections and sepsis, PCT synthesis can increase up to 1000-fold upon stimulation of endotoxins (lipopolysaccharides, LPS) (74) and/or cytokines, especially IL-6, TNF-α, IL-1β, and IL-2 (79,80). This stimulation, whose cellular mechanisms remain not yet well explained (73), can act on different tissues and is therefore responsible for the extra-thyroidal (constitutional) synthesis of PCT. Infection-related PCT production has been reported especially in liver, pancreas, kidney, lung, spleen, intestine (79), and in adipocytes (74) but interestingly not in leukocytes (70). On the other hand, viral-specific cytokines, e.g. INF-γ, appear to mediate the down-regulation of PCT, suggesting its use for differential diagnosis of bacterial vs. viral sepsis (81), even though this application is still under debate (82). The synthesis of full-length PCT upon bacterial stimuli and its secretion into the blood flow are schematically depicted in Figure 5 (35,70,71,74,79).

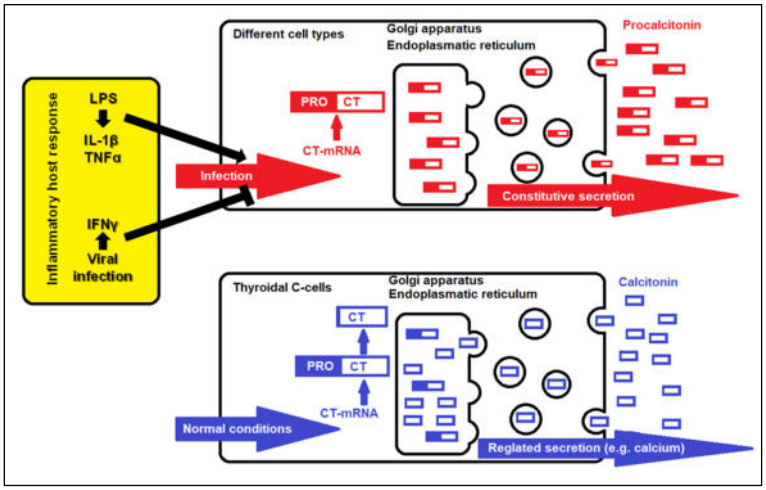

Figure 5. Synthesis and release of calcitonin, under physiological conditions, and PCT, under inflammatory stimuli, from different cells. Bacterial stimuli (LPS) induce the production of PCT, while viral-specific cytokines (e.g. IFNγ) downregulate its expression (yellow box). Adapted from (74).

About the post-translational modifications, both Meisner (70) and Jacobs et al. (83) report PCT to be glycosylated between the two cysteine residues within the calcitonin moiety (position 3, asparagine). Under physiological conditions, the final calcitonin molecule will be de-glycosylated before release (71).

Bartolovic et al. at the contrary postulate that, the inflammatory PCT is not a glycoprotein and this would lead to its direct secretion instead of undergoing the specific cleavage process (84). The same authors furthermore propose the phosphorylation of the signal sequence as the trigger indication for PCT to be released intact into the blood flow.

One further important post-translational modification of the full-length PCT is the dipeptidyl peptidase IV-mediated hydrolysis of the N-terminal dipeptide Ala-Pro, resulting in the PCT form PCT(3-116) (85). This enzyme is located on the surface of the cells of different tissues, particularly renal, epithelial, and endothelial cells, and like PCT itself it is also induced by pro-inflammatory stimuli and endotoxins (70). Consequently, the septic PCT is indeed a truncated protein, comprising the aa 3-116, as proven by mass spectrometry and Edman analysis (70).

Finally, Meisner et al. (70) and Level et al. (86) report that PCT is proteolytically degraded in the blood, leading to the formation of the N-terminal fragment and the calcitonin-katacalcin fragment. Renal elimination seems to play a minor role in the catabolism of PCT (86).

PCT´s blood kinetic (Figure 6) is also an essential aspect of its clinical use as a sepsis biomarker. Indeed, PCT blood concentration has been described to promptly increase two to four hours upon onset of sepsis and six to twelve hours after bacterial infection (70,71,79), which explains why PCT is considered a better parameter than CRP for sepsis (CRP

induction time > 12 hours (71)). PCT´s half-life in blood is also short (20-24 hours), which supports its use in the follow-up of the antibiotic therapy (87,88) especially over CRP (half-life up to several days (71)).

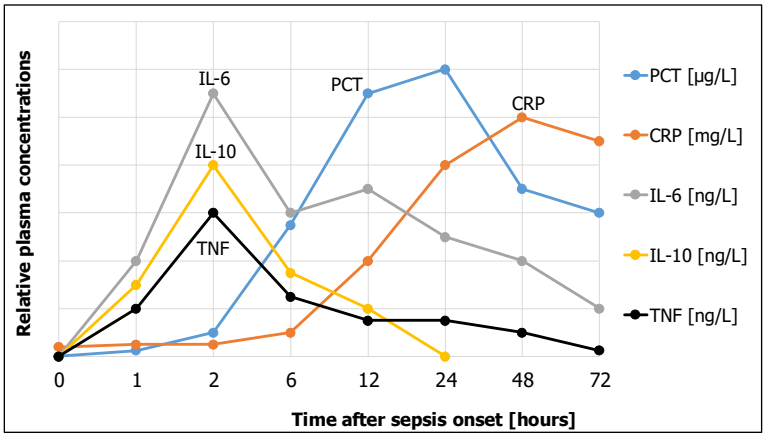

Figure 6. Relative blood concentrations for different proposed biomarkers of sepsis and infection. The corresponding unit is reported in the legend. PCT (light blue curve) is characterised by an early increase upon bacterial infection onset and at the same time rapid blood half-life. Adapted from (71).

Some of the parameters reported in Figure 6 (IL-6, IL-10, and TNF-α), show a faster increase of blood concentration upon infectious stimuli than PCT. However, their application as sepsis biomarkers is limited by their low sensitivity and specificity for bacterial infections. Lippi et al. (27) report for PCT an average sensitivity 0.85 and specificity 0.75, while for IL-6 sensitivity 0.70 and specificity 0.73 (Table 2). IL-10 and TNF-α were not included in Lippi et al.´s study due to their poor performance.

5.9 Proposed biological function for PCT

Different studies have been conducted to try to understand the biological function of PCT, which however remains to date not clear (70,89).

Meisner (70) and Nylén et al. (90) report the effect of PCT on lowering plasma calcium levels. However, this aspect is debated, as the PCT´s calcitonin-like function has not been demonstrated yet, at least in relation to the septic status (91,92).

It has been proposed that PCT has a multifunctional co-factor function, likely modulating different effects during endotoxin shock (70). Furthermore, due to the complexity of CALC-I splicing process, it seems likely that tissue-specific regulations take place (89).

One more proposed effect of PCT is the regulation of the inducible nitric oxide synthase (iNOS) in smooth muscle cells. However, data are conflicting, as low or moderate PCT concentrations seem to inhibit iNOS activity, but on the other hand high concentrations do not (70,93,94).

Finally, endotoxin shock models *in-vivo* (hamster, swine) demonstrate that intravenous injection of PCT increases the mortality of the animals, while antibody-mediated PCT neutralisation leads to a significant increase in the survival rate (95). Nevertheless, also in this case evidence is contrasting. Indeed, as shown by *in-vitro* experiments, PCT could play a role in lowering cytokine expression (e.g. TNF-α (96)), even though this effect does not appear to be relevant *in-vivo*, as high concentrations of this pro-hormone are needed to exert this inhibitory function (70).

5.10 Currently available immunoassays for PCT quantification

In the last decades, the *In-Vitro* Diagnostic (IVD) industry has dealt with blood protein concentrations down to pg/mL as in the case of IL-6 (Figure 7), and with the difficulties connected to their measurement. As one last-generation biomarker, PCT belongs to the proteins whose very low blood concentrations (ng/mL) request high-demanding technologies and analysis methods.

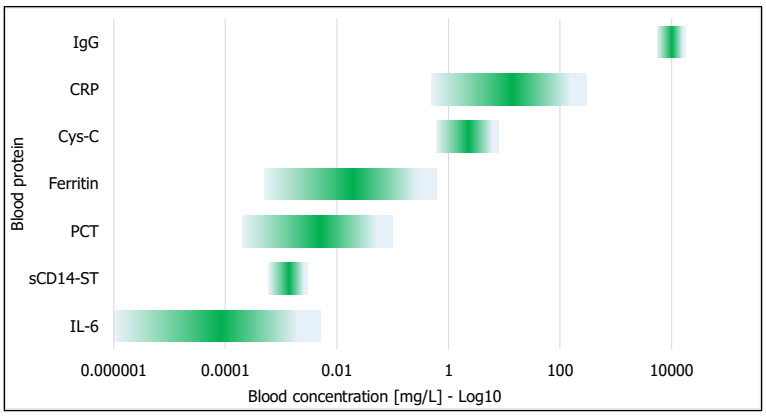

Figure 7. Blood concentrations of some model proteins commonly analysed through immunoassays provided by IVD companies.

Consequently, almost all the immunoassays for the quantification of PCT in human samples so far proposed, are based on heterogeneous technologies like Chemiluminescence Immunoassay and Electro-Chemiluminescence Immunoassay (CLIA/E-CLIA), Time-Resolved Amplification of Cryptate Emission (TRACE), and Enzyme-Linked Fluorescent Assay (ELFA) (97,98). Heterogeneous technologies usually involve the use of a primary antibody (capture antibody) to immobilise the antigen on a solid phase (plate or magnetic beads). After the sample is added, the second antibody (tracer) enters the reaction. This antibody carries the signalling molecule, usually an acridinium ester, ruthenium, or luminol. Heterogeneous techniques consider the separation of the unbound analyte-antibody complex from the rest of the reaction before the final detection of the specific signal (99). This process usually takes place through one or more washing steps, which makes the

method more specific, sensitive, and precise. After the washing step/s the detecting component is added to the reaction giving the final signal (Figure 8).

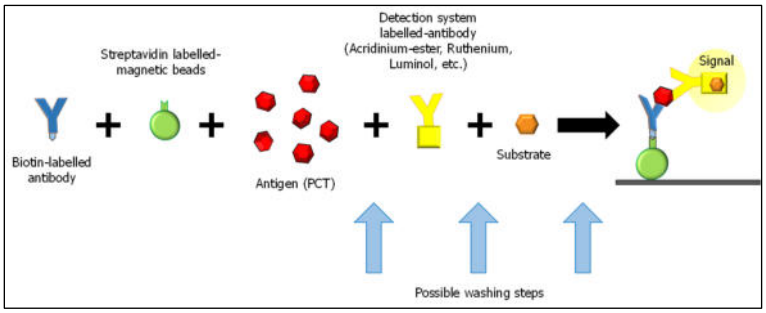

Figure 8. Principle of heterogeneous immunoassays (e.g. CLIA). Magnetic particles (green) are coupled with the so-called capture antibodies (blue), which selectively bind the antigen (red) in the sample mixture. After one or more washing steps (light blue arrows), the tracer antibodies (yellow), carrying the chemiluminescent molecule, are added to the reaction and finally, the signal is measured.

Most of these PCT immunoassays show analytical sensitivity, also defined as the Limit of Detection (LoD) (100), lower than 0.1 ng/mL (79,101–104). However, due to their intrinsic features, they usually offer a lower throughput and longer time-to-result. One more disadvantage is the relatively high sample volume needed, typically 20 to 50 µL (105) but reaching even up to 200 µL per test (106), which represents a limitation, especially in the case of paediatric samples. Moreover, the instruments requested to run heterogeneous assays are very specific, since the fluorescent or luminescent molecules producing the signal and their activation are specific too, and overall quite slow, reaching at most a couple of hundreds tests per hour.

More recently, the company Diazyme Laboratories (Poway, CA, USA) launched an immunoassay for PCT based on the homogeneous technology, Particle Enhanced Turbidimetric Immuno-Assay (PETIA) (107,108). On the contrary of the heterogeneous immunoassays, the homogenous ones do not need any separation of the analyte-antibody compound from the unreacted sample, as they can differentiate between free and antibody-bound antigens within the reaction itself (99). PETIAs are based on the specific agglutination of polystyrene microparticles, adequately coupled with antigen-specific polyclonal (pAb) or monoclonal (mAb) antibodies. The addition of the human sample, containing the antigen to be analysed, triggers the aggregation of the beads, which in turn is quantitatively measured spectrophotometrically through the light absorbance (Figure 9). Usual spectral photometer can be used to run this quantification.

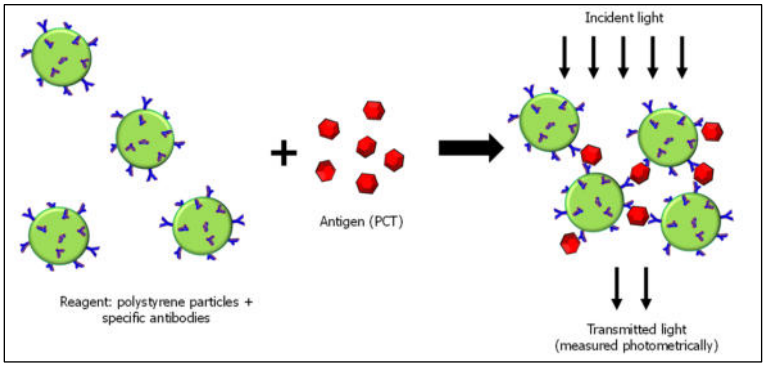

Figure 9. Functional principle of homogeneous immunoassays (e.g. PETIA). The polystyrene particles are showed in green while the coupled antibodies are in blue. The addition of the antigen (red) leads to particle aggregation which in turn is measured photometrically.

Due to the absence of washing steps and the reaction signal, based on the increase of turbidity (109), PETIAs are more prone to interferences and show lower precision and sensitivity. The PETIA technology has been employed so far to analyse proteins with blood concentrations in the order of mg/L (Figure 7). The most common PETIA tests on the market measure CRP (110,111), cystatin C (112,113), anti-streptolysin O antibodies (114), rheumatoid factors (115), and ferritin (116).

Consequently, the adoption of the PETIA technology for PCT, whose functional sensitivity, also referred to as Limit of Quantitation (LOQ) (100), has to reach values down to 0.2 ng/mL or lower, is challenging. The most recent literature reports questionable performances for the Diazyme´s PETIA to PCT, especially in terms of precision and method comparison to the heterogeneous reference technologies (117–120). Table 4 schematically reports the main differences, weaknesses, and strengths of PETIAs and CLIAs in relation to PCT analysis.

Table 4. Features of CLIA and PETIA technologies. The respective strengths are marked in italics.

Parameter	CLIA	PETIA
Precision	++ *(CV% ca. 2% at 0.49 ng/mL)*	+ (CV% ca. 7% at 0.45 ng/mL)
Functional sensitivity (LOQ)	++ *(0.06 µg/L ca.)*	+ (0.2 µg/L ca.)
Analytical range (Δ linearity-LOQ)	++ *(up to 100 µg/L)*	+ (50 µg/L)
Interferences	++	+
Stability	++	++
Cost structure	High	*Low*
Applicability	Low (instrument specific)	*Very high* *(every photometric system)*
Time-to-result	Longer (18-40 min)	*Short (10 min)*

Parameter	CLIA	PETIA
Sample volume	High (average ca. 50 µL)	*Small (ca. 10-15 µL)*
Rubbish production	Disposable materials	*No disposable materials needed*

As resulting from the analysis of the external quality assessment schemes in Germany (121), the currently most used technology to measure PCT is chemiluminescence or its derivatives (approx. 78%), followed by fluorescence (15%), and finally the turbidimetry with only approx. 7% of the total market (Figure 10).

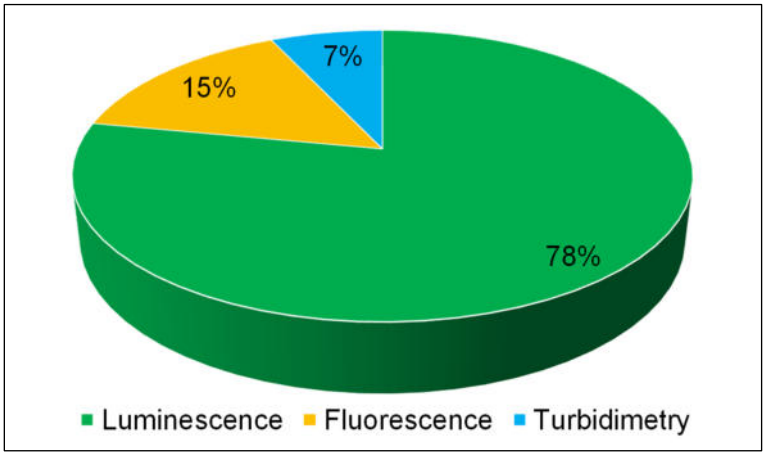

Figure 10. Market shares of the currently used technologies to measure PCT. Green: chemiluminescence (78%). Orange: fluorescence (15%). Light blue: turbidimetry (7%).

5.11 Clinical chemistry analysers: an overview

The immunoassay type here described (PETIA) is commonly run in clinical laboratories on clinical chemistry analysers. These are special instruments based on high-throughput robotics, which can run up to thousands of tests per hour. Even though each company offers its own instrument with different software interfaces and performances, the working principle remains the same.

Most instruments first pipette the sample into one of the cuvettes in the dedicated rotating tray, immediately followed by the addition of the first reagent for the analysis of a specific parameter. After mixing, the reaction is incubated at 37 °C for three to five minutes after which the second reagent is also pipetted into the same cuvette, mixed, and again incubated at 37 °C. The reaction usually proceeds for further five minutes before being discarded and the cuvette washed for the next reaction. Within the total ten minutes of reaction, the cuvette tray keeps rotating leading each cuvette in front of the multiwavelength photometer at preset time intervals (called cycles). Consequently, the absorbance of each cuvette is monitored in real-time to finally return the specific kinetic of each reaction. Utilising the proper cycles, it is possible to calculate the specific reaction absorbance, which in turn

directly correlates with the parameter´s concentration in the sample to be measured. An example of a typical reaction kinetic for a PETIA reagent is shown in Figure 11.

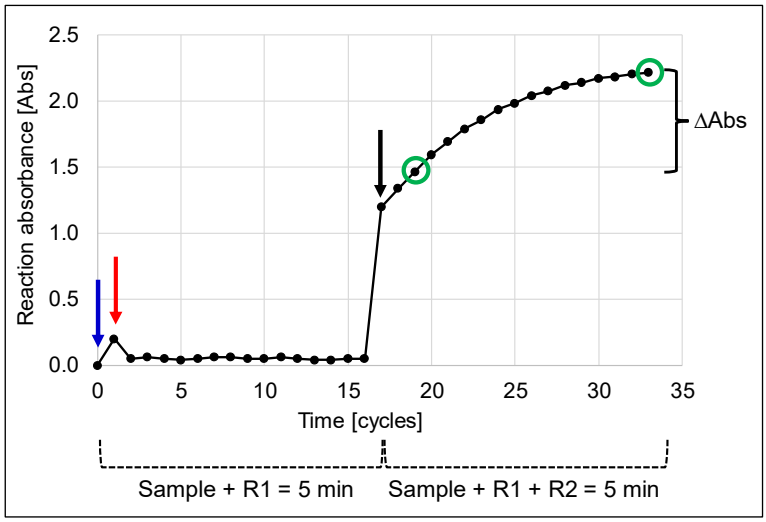

Figure 11. Typical reaction kinetic for a PETIA reagent. At time 0 the predefined sample volume is added (blue arrow), followed by the volume of the first reagent (red line). They are incubated for five minutes before the second reagent (black arrow) is added, too. The two green circles define the cycles whose absorbances are used to calculate the sample specific reaction (ΔAbs).

5.12 The problem with the standardisation of PCT measurements

Besides the technological differences and the specific features of each assay, further issues compromise the widespread application of PCT.

The first and most relevant is the use of considerably different antibodies. Huynh et al. (106) analyse in detail all PCT immunoassays currently available on the market, well depicting this intricated situation. Some assays employ polyclonal antibodies directed either to the whole PCT molecule (DiaSys) or to its calcitonin part (Thermo BRAHMS Sensitive Kryptor). On the other hand, most tests are based on two monoclonal antibodies. These tests however do not show more similar inter-assay quantitation, compared to the pAb-based ones. The first explanation for these dissimilar measurements is that each test is based on different antibodies, sometimes derived even from different animal species (e.g. rat for the Abbott Architect BRAHMS PCT and mouse for the Siemens Healthcare Atellica IM BRAHMS PCT). Different IgG classes come along with different hinge regions, an aspect that leads also to different molecular flexibility and finally immuno-reactivities. Second, the epitopes recognised by these antibodies are only characterised for the Diasorin Liaison BRAHMS PCT and Fujirebio Lumipulse G BRAHMS PCT tests (in both cases mouse anti-katacalcin and mouse anti-calcitonin mAbs). For most other tests, the exact aimed epitopes are unknown. Similarly, the PCT antigen molecules used by the manufacturers to set up their calibrator and control materials are also not always defined. This also plays an important role in increasing uncertainty, as the equivalence between recombinant vs. wild

type and full-length vs. peptide-based antigen has not been demonstrated yet. Moreover, possible isoforms and post-translational modifications of the PCT antigen (e.g. oxidation, glycosylation (77)) could further complicate the measurement variability, affecting the recognition by the antibodies. A similar effect on the antibody recognition has the storage of the internal reference materials (lyophilised, liquid-stable, frozen, etc.). Finally, sample volume (varying in the range 10 - 225 µL), sample matrix (serum, heparin plasma, EDTA plasma, lithium heparin plasma, K2-EDTA plasma, sodium heparin plasma, K3-EDTA plasma, EDTA whole blood), and measuring range (between 0.01 and 500 ng/mL) add further measurement uncertainty sources.

In the scientific literature, high uncertainty at cut-off values (0.5 and 2.0 ng/mL) has been reported (120,122,123). This problem encouraged some authors to highlight the importance of defining the standardisation process. However, as long as no reference material will be available, clinicians should evaluate *in-loco* their PCT assays versus predicate devices, to possibly define test-specific reference values (124).

This heterogenic situation of PCT quantitation with its direct impact on sepsis diagnosis induced the International Federation of Clinical Chemistry (IFCC) to initiate a working group for PCT standardisation (IFCC-WG PCT) (125), aiming to produce a mass spectrometric (MS)-based reference method and certified reference material.

As reported by Huynh et al. (106), the IFCC-WG PCT sets itself the following goals:

1. the development and validation of a reference method for PCT based on absolute quantification by MS;
2. the analysis of measurement variability of current PCT immunoassays;
3. checking the need for PCT measurement standardisation;
4. checking its feasibility;
5. performing PCT immunoassay standardisation, if necessary.

The present work sets in the context of the IFCC WG-PCT project, offering the basis to define the traceability of PCT measurements. In the field of laboratory medicine traceability relates to the ability to link the values of each laboratory measurement to higher-order reference materials and/or standard reference procedures. This guarantees measurement comparability across systems, locations, and times (126).

5.13 Goals of the work

In line with the objectives of the IFCC WG-PCT, this work aimed to characterise the heterogeneous situation of PCT measurements by different immunoassays. This should be performed by the analysis of external quality assessment schemes depicting all the most utilised immunoassays in Germany. Furthermore, the work aimed also to study the commutability of the manufacturer´s standard materials, essential to assure assay

comparability, and to compare the performances of the PETIA for PCT in relation to an already established luminescence immunoassay for the diagnosis of sepsis and to guide the antibiotic therapy (antibiotic stewardship).

Additionally, the present work proposed the PCT antigen selective immuno-precipitation from human samples by polystyrene, Sepharose™, and magnetic particles, also characterising and describing the reduction of unspecific bindings.

Finally, the selective immuno-precipitation method was adopted as the basis for the subsequent mass spectrometric analysis of PCT, as higher-order reference procedure, leading to PCT measurement standardisation. The immuno-precipitation method here described could be employed also for further proteomic analysis of the PCT molecule.

5.14 File number of the ethics vote

All human materials included in the present work were left-over samples, completely anonymized and de-identified. Their use was evaluated and approved by the ethics committee of the Medical Faculty of the University Duisburg-Essen (reference number 17-7838-BO).

6 PUBLICATIONS

6.1 PUBLICATION 1 - National External Quality Assessment and direct method comparison reflect crucial deviations of Procalcitonin measurements in Germany, Masetto, T., Eidizadeh, A., Peter, C., Grimmler, M., *Clinica Chimica Acta*, 529:67-75, (2022)

Title:	National External Quality Assessment and direct method comparison reflect crucial deviations of Procalcitonin measurements in Germany
Authors:	Thomas Masetto, Abass Eidizadeh, Christoph Peter, Matthias Grimmler
Published in:	Clinical Chimica Acta (official journal of IFCC) Volume 529, 2022 April, Pages 67-75
Impact factor:	5.02
Proportional work on this manuscript:	50% Conceptualisation of the EQA data elaboration, selection of the recovery-groups, and the commutability study Statistical analysis of the EQA scheme results and the commutability test Selection and ordered preparation of all data for visualisation, definition of the conversion factors between the immunoassays Graphical representation of all the data through different statistical software Preparation of all figures Contributing of writing the manuscript

6.2 PUBLICATION 2 - Comprehensive Comparison of the Capacity of Functionalized Sepharose, Magnetic Core, and Polystyrene Nanoparticles to Immuno-Precipitate Procalcitonin from Human Material for the Subsequent Quantification by LC-MS/MS, Masetto, T., Matzenbach, K., Reuschel, T., Tölke, S.-A., Schneider, K., Esser, L.M., Reinhart, M., Bindila, L., Peter, C., Grimmler, M., *International Journal of Molecular Sciences*, 24(13):10963, (2023)

Title:	Comprehensive Comparison of the Capacity of Functionalized Sepharose, Magnetic Core, and Polystyrene Nanoparticles to Immuno-Precipitate Procalcitonin from Human Material for the Subsequent Quantification by LC-MS/MS
Authors:	Thomas Masetto, Kai Matzenbach, Thomas Reuschel, Sebastian-Alexander Tölke, Klaus Schneider, Lea Marie Esser, Marco Reinhart, Laura Bindila, Christoph Peter, Matthias Grimmler
Published in:	International Journal of Molecular Sciences Volume 24, Issue 13, 2023 June, Pages 10963
Impact factor:	5.6
Proportional work on this manuscript:	30% Design of the coupling schemes and precipitation experiments (Lx, PGS, MagP) for proteomic and MS application Coupling of beads, optimisation precipitation process, quantitation of PCT and visualisation of unspecific proteins Validation of immunoprecipitation methods Collection and ordered preparation of all data for visualisation Graphical representation of PCT immunoprecipitation visualisation and unspecific binding Preparation of figures for the publication Contributing of writing the manuscript

6.3 PUBLICATION 3 - Letter to the Editor regarding "Development of an antibody-free ID-LC MS method for the quantification of procalcitonin in human serum at sub-microgram per liter level using a peptide-based calibration", Tölke, S.-A., Masetto, T., Grimmler, M., Bindila, L., Schneider, K., *Analytical and Bioanalytical Chemistry*, 413:4917-4919, (2021)

Title:	Letter to the Editor regarding "Development of an antibody-free ID-LC MS method for the quantification of procalcitonin in human serum at sub-microgram per liter level using a peptide-based calibration"
Authors:	Sebastian-Alexander Tölke, Thomas Masetto, Matthias Grimmler, Laura Bindila, Klaus Schneider
Published in:	Analytical and Bioanalytical Chemistry Volume 413, Issue 19, 2021 July, Pages 4917-4919
Impact factor:	4.3
Proportional work on this manuscript:	10% Development of immunoprecipitation methods by Latex particles Validation and robustness proof of immunoprecipitation method Critical revision of the manuscript

7 DISCUSSION

Sepsis is defined as the “life-threatening organ dysfunction caused by a dysregulated host response to infection” (16). Procalcitonin (PCT), the precursor of the hormone calcitonin, has emerged as a promising biomarker (35) for the early diagnosis of bacterial sepsis and for the monitoring of antibiotic treatment (39,43). Nonetheless, its clinical use is jeopardised by the lack of measurement standardisation and the laborious nature of analytical technologies (106).

The first goal of the present work was to dissect and thoroughly depict the high heterogeneity of PCT measurements by the intensive analysis of the External Quality Assessment (EQA) schemes in Germany for the year 2020 (Publication 1).

This work further aimed to propose an easy-to-use and robust immunoassay to measure PCT in human samples for the diagnosis of sepsis and the follow-up of antibiotic treatments. To achieve this goal, the suitability of the Particle Enhanced Turbidimetric Immuno-Assay (PETIA) technology was demonstrated by the direct method comparison of 135 human samples in comparison to an already established chemiluminescence immunoassay. Moreover, in this thesis for the first time a commutability study for the parameter PCT was conducted to analyse the impact of standard material matrices on the immunoassay quantitation (Publication 1).

Finally, the use of the polystyrene particles, derived by the PETIA technology, was proposed for the selective immuno-precipitation of PCT in a sample (Publication 2). The immuno-precipitation served as the upstream step for the subsequent mass spectrometric analysis to be used as the reference method for PCT measurement standardisation (Publication 3).

7.1 Analysis of heterogeneous PCT measurement, comparability of immunoassays, and commutability study

The heterogeneity in immunoassay measurements is a major factor jeopardising the clinical usefulness of PCT (106,120) and has been amply reported in the literature (108,120,123,124,127). These works are performed by measuring human samples containing different PCT concentrations directly with two immunoassays (method comparison), and comparing the respective recoveries by linear regression (128). These comparisons offer the advantage of considering real human materials. However, they have the drawback of correlating from time to time a limited number of tests, leading also to a limited and non-homogeneous picture. Moreover, direct method comparisons do not consider differences in used technology and antibodies. These two factors play an important role in increasing measurement variability. Different technologies are based on diverse signal modes (fluorescence, luminescence, and turbidity), reagents (streptavidin-biotin system, ruthenium, acridinium, isoluminol (129,130)), and detection systems (generated

light for the fluorescence and luminescence assays (97) vs. absorbed photometric light for turbidimetric assays (131)). Moreover, while chemiluminescence- and fluorescence-based methods involve washing steps during the reaction (heterogenous technologies), the turbidimetric methods do not include any washing of the antigen-antibody reaction (homogenous technology), leading to differential background and unspecific binding molecules (heterophilic antibodies, lipids, etc.). The sample volumes can also consistently vary between 10 µL and 200 µL, implying heterogenous antigen-antibody interaction molar ratios and leading to different selectivity, specificity, and matrix effects (106). Regarding the antibodies, the differences include pAb vs. mAb, different immunoglobulin subclasses (IgG1, IgG2, IgG3, and IgG4), and most relevant, different idiotypes. These antibody peculiarities can lead to differential recognition of diverse PCT structural conformations (e.g. as a consequence of PTM), epitopes, isoforms, and fragments, implicating divergent antigen quantitation (127).

In the present work, the immunoassay measurement variability of the parameter PCT was depicted for the first time using EQA scheme data. This part was conducted in cooperation with the Institute for Clinical Chemistry/Interdisciplinary UMG Laboratory (Göttingen, Germany) and published in Clinica Chimica Acta (Publication 1, (98)), the official journal of the IFCC.

This approach offered the important advantage of embracing at the same time all measurement techniques (luminescence, fluorescence, turbidimetry) and all currently available antibodies (mAb vs. pAb, full-length PCT epitope vs. Calcitonin/Katacalcin epitopes). In this analysis, the data of the four EQA schemes of the year 2020 were recovered almost twice as much as each other by different tests based on the same technology. This observation suggested that using the same immunoassay technology did not guarantee homogenous measurements; on the contrary, this parameter represented one source of measurement variability.

Similarly, the EQA-based approach offered the advantage that the data could be easily divided and evaluated with reference to both the immunoassay producer and, subsequently, the employed antibodies. This convenient data organisation showed that the recoveries of the seven PCT tests included in this work hold always the same respective positions relative to each other. Overall deviations implied the use of conversion factors to readily correlate and convert the quantitative recoveries among all manufacturers (Publication 1, Figure 5). This application was possible only due to the high number of data per test provided by the EQA schemes, ensuring the statistical power of the analysis. On the contrary, the direct evaluation of only two tests could lead to misleading conclusions, as reported in the literature. For example, Wang et al. report a good agreement (93%) at cut-off values for the Abbott-BRAHMS and the Biomerieux-BRAHMS PCT immunoassays, with only a mild bias

(20%) in the total analytical range (132). On the other hand, Katz et al. also document good agreement at cut-off values for the Roche-BRAHMS and the Biomerieux-BRAHMS immunoassays but note a 50% discrepancy in the total analytical range (133). Finally, Eidizadeh et al. describe differences up to about 80%, both in the whole-range and at the medical decisional values, for the three BRAHMS-licensed tests by Roche, Diasorin, and Abbott (123).

The PCT measurement situation resulting from this work was extremely heterogeneous and clearly explained the reason why PCT utility as a sepsis biomarker has been reported to be questionable (134). All immunoassays currently on the market are based on the same cut-off values (0.5 ng/mL to diagnose systemic infections, 2 ng/mL to differentiate events of severe sepsis (39,120)). These algorithms however were established on Thermo-BRAHMS Kryptor in 2010 (39) and they are specific for this immunoassay.

As a consequence of this diverging standardisation, opposite medical decisions could be drawn from the use of the same decisional algorithms for all immunoassays. As demonstrated by the present analysis, in the most divergent case between Abbott-BRAHMS and Biomerieux-BRAHMS immunoassays, the relative quantification was almost double (factor 1.74). A direct consequence of this issue was that approximately 74% more samples measured with the Biomerieux-BRAHMS test in comparison to the Abbott-BRAHMS test were recovered in the septic range (PCT > 0.5 ng/mL). In turn, these patients would receive antibiotic treatment if analysed with the Biomerieux-BRAHMS test but not with the Abbott-BRAHMS one. Similar conclusions are reported in the literature by Eidizadeh et al. (135) for the direct comparison between the Abbott-BRAHMS and the DiaSys immunoassays.

This work further showed the effectiveness of the PETIA technology for PCT quantitation through the direct method comparison of 135 human samples compared to the Abbott-BRAHMS chemiluminescence test. The high Spearman´s correlation coefficient both in the total analytical range (0-50 ng/mL, $R = 0.975$) and in the medical decisional range (0-2 ng/mL, $R = 0.925$) proved that this homogenous technology can be successfully employed for PCT measurement. Importantly, the slope of the Passing & Bablok regression line (128) between the two tests resulted very close to the conversion factor obtained from the EQA schemes of 2020 (1.63 vs. 1.56, respectively), confirming the conclusions drawn from that analysis. Interestingly, this work for the first time described the non-linear re-standardisation of immunoassay calibrators, by application of three different conversion factors throughout the whole measuring range. This observation held utmost significance concerning the PCT standardisation process. It indicated that at least three reference materials with different PCT concentrations spread throughout the whole measuring range (0 to approx. 50 ng/mL) are needed to guarantee the complete realignment of immunoassays.

The PETIA technology applied in this study to PCT differed significantly from existing chemiluminescence and fluorescence heterogenous methods. PETIAs are homogenous tests (131), which indicates that the antigen-antibody reaction does not involve any washing steps. This aspect might elevate non-specific interferences from the sample matrix, thereby diminishing the precision and sensitivity of the test. Indeed, whereas the majority of chemiluminescence/fluorescence-based tests currently available on the market exhibit analytical sensitivity as low as 0.02 ng/mL, the PETIA test achieved 0.2 ng/mL (106). The lower sensitivity was also apparent in this study, as reflected in the increased dispersion of the recovered samples in the medical range of the method comparison. However, contrary to Diazyme's PETIA, which has been reported in the literature to have insufficient precision and sensitivity (117–120), the PETIA described here could robustly measure the cut-off 0.5 ng/mL (136), which is pertinent for sepsis diagnosis and antibiotic stewardship. Lower cut-off values (0.1 ng/mL and 0.25 ng/mL) are only requested for diagnosing different pathologies, such as lower respiratory tract infection (LRTI) (45), and are beyond the scope of this work.

On the other hand, the PETIA test for PCT reported in this work offered significant advantages over the heterogeneous assays. The PETIA did not require dedicated equipment; instead, it could be applied to any photometric system, such as clinical chemistry analysers (118,135,137). This allowed for a faster time-to-result and improved throughput. The PETIA technology also provided a more favourable cost structure, which is especially relevant in sepsis diagnosis given that 85% of global cases are identified in low or middle-income countries (2). Lastly, the PETIA did not imply the use of disposable materials (such as plastic cuvettes), thereby reducing the environmental impact.

Finally, for the first time for the parameter PCT, this work examined the commutability of calibrator materials. The concept of commutability, originally described in the 70s in relation to enzymes (138), was then applied to all non-native materials, included calibrators and controls (139). Two enzymatic- or antigen-based calibrator materials are considered commutable if their inter-assay activities are measured similarly to those of the same enzymes or antigens in human native material. Commutability is a critical aspect in clinical chemistry, as non-commutable calibrator materials could adversely affect the immunoassay recovery, leading to differences that are not proportionate to those observed when measuring native human materials (140). Possible sources of non-commutability are synthetic matrices, recombinant proteins, like those employed by the analysed PCT immunoassays, and full-length antigens vs. peptides. According to the CLSI protocols EP14-A3 (141) and EP30-A (142), a calibrator material is considered commutable if it is recovered within the 95% CI of the regression line performed with a sufficient number of original human samples. In this study both DiaSys´ and Abbott-BRAHMS´ calibrators were

detected to be non-commutable, which in turn represented one further factor affecting the measurement variability and increasing the differences in comparison to native materials. This aspect should be carefully considered for the restandardisation of PCT immunoassays. Only commutable materials shall be used to directly compare and realign different tests.

7.2 Employment of the PETIA-derived particles for the selective immuno-precipitation of PCT and the development of a mass spectrometric (MS) reference method for the measurement standardisation of immunoassays

Mass spectrometry (MS) has been proposed in the past years for absolute quantification and standardisation purposes of proteins (143,144), as it offers high selectivity and guarantees low measurement uncertainty. However, the validation of MS as a measurement reference procedure for PCT poses a significant challenge. This is primarily due to the low blood concentrations of this parameter, down to 0.1 ng/mL (79). The immunoassays commonly available on the market can achieve analytical sensitivity down to 0.02 ng/mL (106). Therefore, to match these analytical performances and to serve as a higher-order reference method for PCT measurement standardisation, the MS analysis needs to reach a low limit of quantification (LOQ) of at least 0.1 ng/mL, while maintaining high accuracy.

Huynh et al. (145) propose an MS method for the standardisation of PCT measurement, in which the serum sample is pre-treated and concentrated using the surfactant sodium deoxycholate and two solid-phase extraction steps. The purpose of this treatment is double: first, to simplify the serum matrix by denaturing all proteins through the addition of 0.5% surfactant and subsequent removal via HLB (Hydrophilic-Lipophilic Balance) solid-phase extraction, and second, to concentrate the PCT molecule by precipitating it with 50% acetonitrile and 0.5% surfactant. The authors report their MS method to achieve the LOQ of 0.245 ng/mL in human serum (145).

Differently from the approach described by Huynh et al., in this thesis, the PCT-containing sample for MS analysis was processed by immuno-precipitation as described in Publication 2 (146). The subsequent MS method, reported in Publication 3 (147), was developed and performed by the colleagues of the Institute Fresenius (Idstein, Germany) and the Johannes Gutenberg University of Mainz (Mainz, Germany).

The immuno-precipitation approach was based on specific anti-PCT pAb bound on the surface of different types of microparticles (polystyrene/latex, Sepharose™, and magnetic). Compared to the surfactant-solid phase approach, the antibody-based method provided the advantage of simpler, faster, and more specific antigen recognition in the sample. This method is particularly effective in isolating the antigen from the matrix, offering also other important advantages.

After tryptic digestion, the immuno-precipitation method led to a higher number of PCT-specific peptides detected in the subsequent MS analysis. Huynh et al. report the detection of two PCT tryptic peptides by their MS method (145). These two peptides are the only ones effectively captured by the solid phase extraction steps. Moreover, only one of them (SALESSPADPATLSEDEAR) can be used for quantitative MS analysis, while the second one (FHTFPQTAIGVGAPGK) is reported to be useful only as qualitative control to confirm PCT identification. On the other hand, the immuno-precipitation technique here proposed, due to the employment of pAb, captured seven PCT tryptic peptides detected in the MS analysis at medically relevant concentrations (PCT < 25 ng/mL) and three of them (SALESSPADPATLSEDEAR, LLLAALVQDYVQMK, and DMSSDLER) were used for antigen quantitation (147).

The immuno-precipitation based on pAb further demonstrated a superior recognition of oxidised PCT compared to methods relying on mAb (Publication 2, (146)). Post-translational modifications, in general, are a crucial aspect to consider, as they have the potential to induce alterations in the three-dimensional structure of PCT and the antigenic epitopes recognised by the antibodies of the immunoassays. In the specific case of the oxidation discussed in this study, the molecular structure of PCT revealed two cysteine residues located in the calcitonin moiety. Notably, these cysteine residues do not form a disulphide bond in septic PCT (71). The oxidation of these two amino acids, resulting in the formation of a disulphide bond, induced a change in the conformation of septic PCT, consequently diminishing the epitope recognition by the antibodies. pAbs, by definition, recognise multiple epitopes on the PCT surface, thereby reducing their sensitivity to partial alterations of the antigen structure. This characteristic, in turn, contributed to an efficient immuno-recognition and precipitation process (Publication 2, (146)).

Based on this data, it is intriguing to hypothesise that the immuno-precipitation method based on pAb, as described here, may recognise and precipitate also different potential PCT septic isoforms and PCT complexed with interacting proteins. However, it is important to note that this aspect has not been analysed in the current study, and its confirmation will require additional experiments.

The latex-based immuno-precipitation proved unsuitable for PCT standardisation purposes. This was primarily attributed to challenges in handling and precipitating this type of particles as demonstrated in Publication 2 (146). As a result, the MS analysis following this immuno-precipitation method also exhibited insufficient performance, as only one PCT tryptic peptide (LLLAALVQDYVQMK) showed an LOQ near the septic medical decisional range (0.5 ng/mL), and no peptide matched the sensitivity of the immunoassays (< 0.1 ng/mL) (Publication 3, (147)).

To meet the initial standardisation goals and approach the sensitivity of immunoassays, the present study investigated the alternative immuno-precipitation methods using Sepharose™ (PGS) and magnetic particles (MagP). Similarly to latex, the PGS was deemed unsuitable to achieve the required sensitivity performances for use in the MS standardisation process. This was primarily attributed to low precipitation reproducibility, inadequate antigen-specific recovery, and high binding of non-specific proteins.

On the contrary, MagP met the criteria requested for standardisation purposes, aligning with the findings reported by Whiteaker et al. for the proteins α1-antichymotrypsin and TNF-α (148). The highly specific PCT immuno-precipitation showed by MagP was attributed to the bottom-up oriented coupling of antibodies facilitated by the Protein G on the particle surface. Additionally, optimal handling and robust precipitation were ensured by the magnetic properties intrinsic to this type of particles. On the other hand, MagP also exhibited the lowest non-specific binding by sample matrices, reducing the background in MS and enhancing the signal-to-noise ratio, thus improving the LOQ.

The MS method following the MagP-based immuno-precipitation reached an LOQ of 0.25 ng/mL and a linearity up to 50 ng/mL, as recently published (149). The MS analytical sensitivity was strongly improved by the use of magnetic particles but it couldn´t yet match that of immunoassays (< 0.1 ng/mL). Additional LOQ improvements could be attained by conducting the immuno-precipitation process in larger sample volumes and reducing the chromatography flow rates. However, the coupled MagP immuno-precipitation/MS methodology described here could already be employed in standardisation processes involving PCT concentrations higher than 0.25 ng/mL. These concentrations are typically used for diagnosing sepsis and guiding antibiotic stewardship (39), which were the clinical applications core focus of the present study.

7.3 Conclusions and further perspectives

This work revelad the great PCT measurement heterogeneity for the first time through the EQA scheme analysis. This approach ensured that all potential sources of variability were included in the analysis: immunoassay technology and antibody, commutability of standard materials, and non-linear recovery between different immunoassays. According to this study, the restandardisation of PCT immunoassays appeared highly necessary. The reference method and higher-order reference materials should comprise a minimum of three commutable solutions, with one for each relevant concentration range in PCT analysis (0-2 ng/mL, 2-20 ng/mL, and 20-50 ng/mL). It remains unclear whether these commutable materials can be prepared using recombinant PCT or if real human materials are required. This aspect is currently under evaluation in collaboration with the IFCC PCT standardisation working group.

The present work further demonstrated the suitability of the PETIA technology for clinical chemistry analysis of PCT and the diagnosis of sepsis. However, the current sensitivity limit of the PETIA here described (LOQ approximately 0.2 ng/mL) ensures accurate measurement only for the septic cut-off 0.5 ng/mL. The next steps should explore whether advancements in the PETIA technology (different particles, more sensitive antibodies, etc.) can enable robust measurement of lower cut-offs (≤ 0.25 ng/mL), allowing the diagnosis of clinical conditions other than sepsis (e.g. low respiratory tract infections, pneumonia, etc.).

The polystyrene particle-based method, derived from the PETIA technology, was proposed to immuno-precipitate PCT from a sample for subsequent MS analysis, but it demonstrated insufficient performances, due to handling difficulties and protein unspecific binding on the particles. The alternative method based on magnetic particles resulted suitable for MS standardisation purposes, providing good reproducibility, complete PCT immuno-precipitation in a short time, and low unspecific binding. The next steps in the direction of PCT measurement standardisation shall define the comparability of all currently available immunoassays in comparison with the two MS methods developed by Huynh et al. (PCT precipitation by detergents and solid phase extraction) and Toelke et al. (immuno-precipitation of PCT), as presented here. This round-robin study is being organised by the IFCC PCT standardisation working group, involving approx. 40 samples spread throughout the whole PCT analytical range.

Finally, further analyses of the PCT molecule in different physiological settings should be performed using the proposed immuno-precipitation method based on magnetic particles and pAb. PCT should be identified and separated together with potential interaction partners, isoforms, and post-translational modified variants, to allow subsequent biochemical analyses and the characterisation of potential clinical specific PCT subtypes.

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