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Foreword of the editor

Editor in Chief: Gábor L. Kovács, M.D., Ph.D., DSc

Dr. Tamás Kőszegi presently is a full professor of laboratory medicine at the Department of Laboratory Medicine, University of Pécs, Hungary. He graduated as an MD from the University of Pécs (1979) and obtained his specialty degree in Medical Laboratory Diagnostics (1984). He wrote his PhD thesis on the release kinetics of intracellular ATP using different cellular models (1996). His research interest is wide but in common, he uses mainly those methods that are related to luminescence. One of his pioneering work on procalcitonin (PCT) research began in 1999. He published several papers in collaboration with clinicians on the role of PCT in systemic inflammation (sepsis). He also proved that neutrophil granulocytes might be a potential source of PCT release in septic patients. He is devoted to proteomics and to find protein biomarkers in systemic diseases with a special emphasis on inflammation. He worked out a method to characterize perchloric acid soluble serum proteins in systemic diseases related to inflammation (sepsis, malignancies, autoimmune diseases, Crohn's syndrome, etc.). Recently, his interest has been focusing on serum actin, actin binding proteins (gelsolin and Gc-globulin) and also on urinary orosomucoid, cystatin C and actin detection in systemic inflammatory conditions. His group adapted gelsolin, Gc-globulin, urinary orosomucoid and urinary cystatin C to automated routine laboratory instruments. These biomarkers may give substantial additional help for the clinicians at the intensive care unit to make a quick decision in the treatment of severely ill patients. He also published several papers on the mode of action, molecular and cellular interactions of mycotoxins with a major focus on ochratoxin A. His most recent interest is to capture and characterize circulating tumor cells and cell-free nucleic acids in breast cancer patients. Dr. Kőszegi has published more than 300 papers including abstracts, has a cumulative IF 140, and obtained independent citations close to 500.

The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

eJIFCC

Advances in the diagnosis of sepsis

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EDITORIAL

Inevitably, sepsis has still remained one of the major challenges at the Intensive Care Units (ICUs) (1). About 30 million cases per year are estimated worldwide and this tendency is continuously increasing (2). Although sepsis is known for a long time, its pathomechanism is not completely understood due to the various triggering factors and also to the altered response of the individuals with different underlying diseases.

In sepsis with bacteremia, endotoxins (LPS) of Gram negative microbes and exotoxins from Gram positive microbes play a major role in the development of the symptoms. Currently, it is thought that pathogenesis of sepsis includes microbial interaction with the host defense system before bacteria can enter the bloodstream (3-5). Defense mechanisms in tissues differ from those of the intravascular ones. In the tissues (e.g. first in localized infections) leukocytes are the main antimicrobial factors while in the bloodstream fight against bacteria is mediated by humoral factors on the surface of erythrocytes. Bacteria that can invade the circulatory system possess

antioxidant enzymes (SOD, catalase, etc.) protecting them from oxidative injury exerted by the host. In the bloodstream invading bacteria are attached to the surface of erythrocytes stimulating oxygen release (from oxyhemoglobin) that might kill bacteria by oxidation. If bacteria escape oxidation they enter erythrocytes by permeabilizing the membrane. Once inside the erythrocytes bacteria are most probably to be killed due to the high concentration of oxygen. On the other hand, bacteria might survive inside the RBCs at poor oxygenation or when bacteria are resistant to oxidation. In this way, RBCs may form a bacterial reservoir where they can further proliferate (6,7). Inside the erythrocytes bacteria are protected from most of the antibiotics and the antibacterial factors of the host. Bacterial proliferation damages erythrocytes with a subsequent release of the microbes into the bloodstream (or to other erythrocytes). In case of bacteremia a premature release of oxygen from erythrocytes and an oxidation resistant infection might occur. As a consequence, sepsis and in severe cases, septic shock will develop. Further oxidation of plasma proteins and lack of proper oxygen content in erythrocytes may cause injury of distant organs leading to multi-organ failure (MOF) (8,9).

These events are also strongly related to the development of a misbalance between the inflammatory and anti-inflammatory cascade especially when tissue injury (major surgery, trauma, burns, pancreatitis, etc.) is present. From the laboratory part, only a few parameters are used routinely for early detection of sepsis from the more than 200 sepsis related biomarkers, namely pro-inflammatory and acute-phase proteins (CRP, procalcitonin, interleukines) (10-14), pentraxins (15,16), cytokine/chemokine biomarkers (IL-6, IL-8, IL-10, TNF- α, etc.) (17,18), macrophage migration inhibitory factor (19,20), high-mobility-group box 1 (HMGB1) (21,22), coagulation biomarkers (23,24), triggering receptor expressed on myeloid cells 1 (TREM-1) (25,26) and midregional pro-adrenomedullin (27). Up to now, no single marker or a combination of the above markers proved to be specific and sensitive enough for timely diagnosis of sepsis. Furthermore, the ultimate need to predict the outcome of the disease or to monitor therapeutic efficiency by laboratory testing has not been fulfilled completely.

The uncertainty regarding both clinical and laboratory diagnostic criteria has led to the establishment of new sepsis guidelines in 2016. Among the diverse findings and explanations in sepsis, the only true fact is, that diagnosis with proper decision making should be performed within the shortest possible time. The sooner the antibiotic therapy is begun the higher chance for the patient to survive. In order to fulfil this requirement both clinical and laboratory findings (including microbiological identification) are equally important.

In this issue of the *eJIFCC*, there are four manuscripts which summarize the present knowledge on the major aspects of diagnosis and treatment of sepsis with the introduction of some unconventional new biomarkers. The first manuscript of Trásy and Molnár highlights sepsis management from the point of view of intensive therapy. The paper is focusing on the important aspects of the new sepsis guidelines and on the pathophysiology of the disease. The authors describe the body's immune response to pathogen invasion (pathogen-associated molecular patterns: PAMP and damage-associated molecular patterns: DAMP). The role of procalcitonin (PCT) in the diagnosis and antibiotic treatment is discussed in details. Professor Molnár and his group have been involved in the research of diagnostic and prognostic markers of sepsis for more than 15 years with special emphasis on the clinical usage of PCT (28,29).

In the next paper Rogić and her co-authors, besides the classical CRP and PCT markers highlight the potential use of presepsin as a recent laboratory parameter for early detection of sepsis. Presepsin is a 13 kDa soluble form of CD14 cluster surface glycoprotein derived mainly from membrane bound CD14 on the surface of monocytes (mCD14). Presepsin enables the binding of LPS and the LPS-binding protein (LBP) complex to toll-like receptors (TLRs), augmenting the inflammatory response. Even if the clinical usefulness of presepsin has not been verified in every detail yet, the major advantage of this test lies in the very early rise of presepsin in sepsis (within 1 hour). Another advantage of the test is that measurement of presepsin can be done at the bedside with a POC method. Professor Rogić's basic fields of research and professional activities are evidence-based laboratory medicine, organization and management of medical biochemistry laboratory, point-of-care testing, and organization and management of laboratory parameters of renal diseases. The next review of Kustán et al. deals with unconventional biomarkers with potential clinical usefulness at the ICU. A challenging observation in sepsis and septic shock is the release of large amounts of a physiological intracellular protein, actin into the circulation. Once freed from the cells, excess actin is toxic and enhances the risk for respiratory distress syndrome, forming of micro emboli and development of multiple organ dysfunction syndrome (MODS). Excessive actin release into the bloodstream decreases the level of the actin scavenger proteins gelsolin and Gc globulin. In septic patients, especially with acute kidney injury (AKI) urinary actin level is strongly associated with kidney status. In critically ill patients, urinary alpha-1-acid glycoprotein or orosomucoid (u-ORM) as an inflammatory marker is extremely elevated and may be considered as a non-invasive marker for diagnosis of sepsis. Kustán and his co-authors have worked out an automated immune turbidimetric assay for measuring of u-ORM and that of gelsolin is under development (30). Finally, the manuscript of Miha Košir and Matej Podbregar are discussing the function and clinical usage of a less known gaseous transmitter, hydrogen sulfide. Besides NO and CO, hydrogen sulfide (H_2S) is the third known gasotransmitter molecule influencing many physiological processes such as maintenance of vascular tone, modulating the inflammatory response, scavenging reactive oxygen species, etc. Its plasma concentration has a predictive value for the outcome of sepsis. Interestingly, too high or too low plasma H_2S levels exert unfavorable effects predicting the severity of the disease and indicating a worse outcome. Professor Podbregar and his team are attempting to place successful basic research into clinical context including interest in pathophysiology of shock, hemodynamic stabilization, prediction of severity of shock, cytokine removal techniques/modulation of inflammation and bioactive gases (NO, H_2S). They are also interested in development of point of care prediction tools.

In conclusion, the successful diagnosis and treatment of sepsis is based on the correct interpretation of clinical signs and symptoms and also on the availability of laboratory tests with high specificity and sensitivity. Measurement of one lab parameter is never enough and monitoring of key markers such as procalcitonin is essential. The tendency (rising or falling) of the biomarker is usually more important than the absolute values. Further evaluation of presepsin in comparison with well-established markers (PCT, CRP) and with possible interfering factors (kidney failure) is of utmost importance. The introduction of non-commercially available tests as u-ORM, gelsolin, Gc globulin and H_2S into the routine laboratory palette inevitably would give valuable complementary data for sepsis management.

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In this issue: Advances in the diagnosis of sepsis

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Procalcitonin – assisted antibiotic strategy in sepsis

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ABSTRACT

Sepsis is one of the biggest challenges in critical care nowadays. Defining sepsis is a difficult task on its own and its diagnosis and treatment requires well trained, devoted personnel with interdisciplinary collaboration in order to provide the patients the best chance for survival. Immediate resuscitation, early adequate antimicrobial therapy, source control and highly sophisticated organ support on the intensive care units are all inevitable necessities for successful recovery.

To help fast and accurate diagnosis biomarkers have been measured for decades. Procalcitonin (PCT) is one of the most studied, but the results are conflicting. Sepsis means a very loose cohort of a large heterogeneous patient population, hence defining certain cut off values for PCT to differentiate between different severities of the disease is almost impossible. Clinicians first have to understand the pathophysiological background of sepsis to be able to interpret correctly the PCT results.

Nevertheless, PCT has been shown to have the best sensitivity and specificity to indicate infection, antibiotic appropriateness and stopping therapy.

In this article we will focus on some important aspects of pathophysiology and advice on how to implement that in the everyday clinical practice. We believe that this multimodal evaluation of the clinical picture together with PCT results can be a useful tool to make the most out of the PCT results, and do the best for patients on the ICU.

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INTRODUCTION

One of the most challenging tasks in critical care medicine is the treatment of serious infection related multiple organ dysfunction, termed in general as sepsis, and septic shock. Early detection of infection and the immediate start of resuscitation parallel with adequate antimicrobial therapy undoubtedly give the best possible chance for survival and received strong recommendation by the Surviving Sepsis Campaign guidelines [1]. However, while recognizing organ failure via objective signs is relatively easy, diagnosing infection as the possible underlying cause remains a challenge. Due to the non-specific properties of conventional signs of infection, such as body temperature and white cell count (WCC), biomarkers have been utilized to aid diagnosis for decades. One of the most studied biomarkers is procalcitonin (PCT) [2]. Its role in assisting antibiotic (AB) therapy has been studied extensively, with contradicting results. There are positive studies [3, 4] showing that a PCT-guided patient management reduced antibiotic exposure and length of antibiotic therapy without affecting patient outcomes. There are also negative studies, which could not show this benefit [5-7]. However, to understand the values and limitations of inflammatory biomarkers it is inevitable to understand the immunological background of critical illness determined mainly by the host response. Moreover, putting the results of these studies in context, based on new insights of the pathomechanism of sepsis and systemic inflammation generated mainly by the individuals' host response, may explain the differences between the reported results and help the clinician to interpret PCT data with more confidence at the bedside.

SEPSIS SYNDROME AS A DISEASE

In most surgical and medical specialties we diagnose definitive diseases, which would indicate definitive treatment. However, defining, hence diagnosing sepsis is not that simple.

The term "sepsis syndrome" was invented during the designing of the protocol of one of the first prospective randomized trials in sepsis, performed by a group of scientists led by the late Roger Bone in Las Vegas in 1980 [8]. Several years later a statement paper was published by the same authors titled "Sepsis syndrome: a valid clinical entity" [9], after which the medical society started to deal with sepsis as with a definitive disease, which created false expectations:1) physicians wanted one single test with high sensitivity and specificity to diagnose sepsis, and 2) there was an urge to find an "antisepsis magic bullet". Neither of these wishes have and will never ever come true.

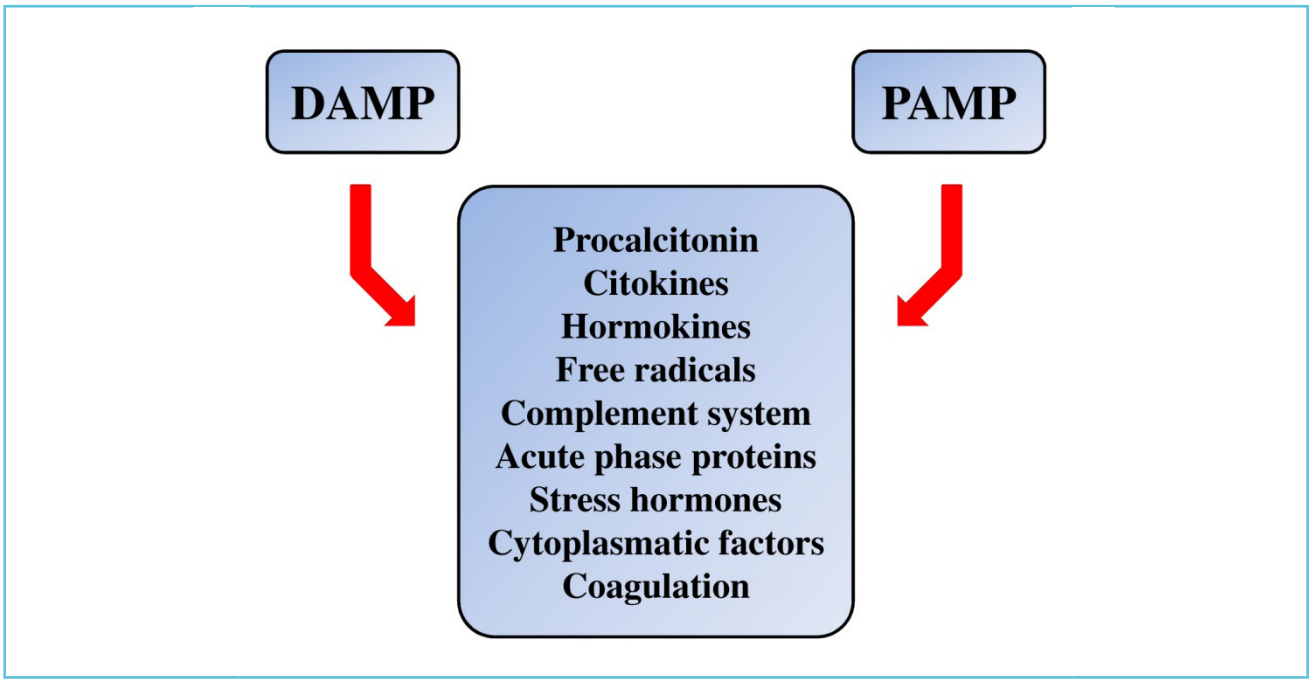
Regarding the definition and diagnosis of sepsis, the classical signs of the "sepsis syndrome" such as fever/hypothermia, leukocytosis/leukopenia, tachycardia and hypotension, meant a very large and non-specific cohort of patients. For this reason, a consensus conference was brought together which defined the so called "consensus criteria" of sepsis, which has been used for decades in research and clinical practice alike [10]. However, the uncertainty about sepsis definitions lingered on that resulted the recently published new definitions as "Sepsis 3" [11]. In this, sepsis is defined as a "life- threatening organ dysfunction caused by a dysregulated host response to infection". As categories only sepsis, septic shock, and organ dysfunction remained.

These efforts clearly show that finding the appropriate definition of sepsis has been a continuous challenge for more than 30 years. The difficulty in defining sepsis originates from its complex pathophysiology, which is affected by numerous individual variations of the host response. Furthermore, in most specialties diagnostic laboratory or radiological tests have very high sensitivity and specificity often reaching almost 95-100% [12]. However, in the case of sepsis, it is different, which makes not just the diagnosis, but the interpretation of the results of clinical trials and also epidemiological data very difficult.

THE IMMUNE RESPONSE FOR AN INSULT

The immune system is a complex network and the immune response to pathogens relies on both innate and adaptive components, dynamically defined as the pro-, and anti-inflammatory forces. The innate immune system (including the complement system, sentinel phagocyte and natural killer cells), is responsible for the eradication of the invaders, while the adaptive immune system's role is to control the process and keep it localized to the site of the insult [13]. Under normal circumstances these mechanisms remain in balance. The innate system acts by broad recognition of antigens, mainly by triggering "pathogen-associated molecular patterns" (PAMP) of lipopolysaccharide elements of the surfaces of invading pathogens. When there is an imbalance due to the dysregulation of the pro-, and anti-inflammatory forces, the local response escalates into a systemic host response also termed as "cytokine storm" [14]. It was a surprising finding, that after trauma, burns, ischemia-reperfusion, pancreatitis, major surgery, etc., same or similar molecules are released mainly from the mitochondria of the injured or stressed cells that are found during PAMPs, and can also cause a cytokine storm. This process accompanying tissue

Figure 1 The molecular responses for damage (DAMP) and pathogen (PAMP) type insults



For further details, see text.

injury is called "damage-associated molecular patterns" (DAMP). In the case of bacterial infection this similarity is due to the fact that the bacteria and the mitochondria (which is more-or-less an encapsulated bacterium) share very similar genetic background. This explains why tissue injury induced DAMP and bacterial infection induced PAMP manifest in similar host responses and clinical manifestations [15]. This similar pathophysiological response is summarized in Figure 1. This indicates that in addition to PAMP, DAMP can also cause the induction of the production of similar cytokines, hormokines and also PCT. This on the one hand provides the potential benefit of PCT in diagnosing infection (PAMP) but also limits its accuracy as levels may increase in scenarios without infection (DAMP). This is the reason, why unexpected PCT values (high or low) are often interpreted as "false negative" or "false positive". However, understanding the nature of PCT production helps a lot in the interpretation of PCT values at the bedside.

THE ROLE OF PCT IN DIAGNOSING INFECTION

The so called "sepsis biomarkers" do not recognize sepsis per se, but inflammation. The reasons have been explained in the previous paragraphs, namely that both damage and pathogen related insults can provoke a very similar inflammatory host response. Therefore, in this context, the right question is: whether the critically ill condition is due to infection or not? Because if it is, we should start anti-microbials or other source control. But if it isn't, then anti-microbial therapy should not be commenced, due to its several undesired effects. Therefore, it is not "sepsis" what we treat, but organ dysfunction and infection.

Diagnosing infection on the ICU is not easy and requires a multimodal approach. Clinical signs are obviously the most important in recognizing critical illness and suspecting infection and even the source of infection, but they cannot prove it on their own. Conventional indicators such as fever/hypothermia, leukocytosis/leukopenia, tachypnea, tachycardia, hypotension, taken from the classical "sepsis-syndrome" criteria are non-specific, and in fact poor indicators of infection. To fill this gap inflammatory biomarker measurements have been developed [2]. Every biomarker has its own merit and limitations, but there is no "ideal" biomarker, and there may never be one. Biomarkers can support decision making but they will never be able to differentiate between inflammatory response for infection from host response for non-infectious insults with a 100% sensitivity and specificity due to the complex, overlapping pathomechanism of PAMP and DAMP. This is in sharp contrast with the diagnostic power of certain biomarkers used in the world of "definitive" diseases, where several laboratory parameters have this ability. Furthermore, learning how to use biomarkers is not easy either.

The two most commonly used markers in infection/sepsis diagnostics and for guiding therapeutic interventions are PCT and CRP [2]. One of the main limitations of CRP is that it moves "slowly", and after a certain insult it reaches its maximum value usually 48 hours later. This is in general unacceptable on the ICU, as every hour delay in starting for example appropriate antibiotic treatment can affect mortality as indicated by the study of Kumar et al. [16]. Furthermore, levels are generally elevated in most ICU patients, making interpretation of CRP very difficult [17].

Procalcitonin is detectable in the serum within a few (4-6) hours after its induction, which is most often bacterial infection. During the "normal" course of an infection it reaches its peak within 24 hours and then starts its decline in the case of adequate treatment with levels reducing by roughly 50% daily according to its half-life [18].

Procalcitonin differentiates bacterial infections from systemic inflammatory response of other etiologies with higher sensitivity and specificity as compared to CRP [19], and also have a good prognostic value regarding survival [20]. However, interpreting PCT values on admission or after the onset of an acute insult, let it be infectious or not, is not simple. But this holds true for any biomarker, as they show a large scatter between patients with a seemingly similar clinical condition, hence single absolute values are difficult to interpret.

There are many studies reporting that PCT values correlate with severity and differ significantly in patients with SIRS, sepsis, severe sepsis and septic shock [21]. Clec'h et al., found that patients with septic shock had more than 10 times higher median PCT levels as compared to those admitted with shock of non-septic origin [22]. However, looking at the data carefully reveals that although there was a remarkable and statistically significant difference, but there is also a huge scatter and overlap of the PCT data between the groups (septic shock: 14 [0.3-767] vs. non-septic shock: 1 [0.15-36] ng/ml, respectively), which makes individual interpretation of a single measurement very difficult - a finding, which is generally true for every biomarker of inflammation. This has been reinforced by the same group in a subsequent study, in which they found that the median PCT value in medical vs. surgical patients differed both in SIRS: 0.3 (0.1-1.0) vs. 5.7 (2.7-8.3) ng/ml, and in septic shock: 8.4 (3.6-76.0) vs. 34.0 (7.1-76.0) ng/ml, respectively [23]. These differences and the large overlap can be explained by the PAMP and DAMP based host response. In certain cases there is a single PAMP or DAMP, but they can also occur in combination as PAMP+DAMP. The latter is bound to have a pronounced inflammatory response reflected in several times higher PCT values. Therefore, it has become clear that the same PCT value, in other words a given "normal" value, cannot be used in every condition. Medical patients with infection in general should have lower PCT values (single insult of PAMP) as compared to surgical patients with infection, where DAMP and PAMP are present at the same time. Moreover, it is also important to acknowledge, that any cellular injury, let it be direct tissue or ischemia-reperfusion injury without infection can result in elevation of PCT induced by a single DAMP type insult.

Although PCT absolute values have the above mentioned limitations, but there is overwhelming evidence that in most cases high PCT values indicate bacterial infection. The shortcomings of PCT absolute values might be compensated when the kinetics of PCT is taken into account to indicate infection.

PCT-ASSISTED ANTIBIOTIC THERAPY

There are three fundamental questions to be answered during our ward rounds when treating patients with suspected or proven infections on the ICU: 1) is there infection, in other words should we start empirical antibiotic therapy; 2) is the commenced antibiotic effective; and finally 3) when should we stop antibiotic treatment?

In this article we are giving some aspects to answer these questions referring to the result of previous studies which were performed at our department in the last few years in the field of procalcitonin and antibiotic therapy [24, 25, 26].

1. Is there infection?

It has been explained earlier that either PAMP or DAMD can induce PCT production. Serum levels of any biomarker show large scatter even in a seemingly homogenous patient population. That is why it is so difficult, almost impossible, to define an exact PCT value that indicates bacterial infection. Indeed, even the most accurate studies can only show 75-85% sensitivity and specificity.

Unfortunately, most clinicians tend to interpret sepsis as a definitive disease, therefore they have false expectations from the role of the biomarkers in the diagnosis of infection, and they become "biomarker sceptic", when they find high levels of PCT after a non-infectious insult, such as surgery, trauma, or after cardiopulmonary resuscitation. But those who know the patophysiology of inflammation (mechanism of DAMP, PAMP) are not surprised by this phenomenon, because they understand that this is due to the etiology and heterogeneity of patients, more precisely due to the individual immune response after a particular insult.

Our recently published results showed that PCT kinetics could give a much more reliable help to the clinicians' decision making than absolute values. As it has a half-life of less than 24 hours, we hypothesized that kinetics may produce different pattern in those who receive adequate treatment as compared to those who don't. In the EProK („Early Procalcitonin Kinetics") study patients were enrolled who were thought to have infection [24]. From the enrolled 209 patients in 114 cases PCT was available from the previous day before the infection was suspected [25]. Throughout this 24 hours we found that PCT elevation was approximately twice higher in patients who turned out to have infection versus those who did not. So a more than 88% PCT elevation in 24 hours refers to infection with 75% (65-84) sensitivity and 79% (60-92) specificity (AUC 77%). It can be an absolute value independent indicator of infection.

It is important to note that if the patient is hemodynamically unstable and infection is likely, by definition he/she has septic shock or at least one cannot exclude it, hence antibiotic therapy shouldn't be delayed but has to be commenced immediately, regardless of the PCT or any biomarker value [16]. However, if the patient is stable hemodynamically, and PCT is "low" or decreasing then we can wait, observe the patient and reassess later. What "low" means as an exact value is difficult to define, as it depends on the etiology and the patient. Therefore, we have to admit, that diagnosing infection with or without PCT remains a challenge.

2. Evaluating antibiotic appropriateness

After commencing empirical antibiotic therapy, it is indispensable to confirm appropriateness to correct treatment if needed as soon as possible because it is upmost vital. In septic shock every hour delay in starting adequate antibiotic therapy could have serious effect on survival [16]. But unnecessary overuse of antibiotics can also cause increased bacterial resistance, invasive fungal infections, side effects and increased costs [27]. Despite international guidelines are available to help in choosing the right medication with the best possible chance, unfortunately it seems that inappropriate empirical antibiotic therapy can be as high as 25-30% on the ICU [28, 29]. The gold standard for proving appropriateness of antibiotic therapy is the microbiological confirmation of the bacteria and its susceptibility. However, these results may come far too late, in reality days after the specimen had been sent, but treatment cannot be delayed. At present there is very little to help the clinicians at the early stage of patient care to confirm appropriate antibiotic treatment.

The above mentioned EProK study showed that there was a significant difference in the early kinetics of PCT between patients receiving appropriate as compared to those getting inappropriate antibiotic therapy (24). Serum PCT levels were measured right after ABs were commenced then 8 hourly in the first day. In those patients who received effective AB therapy PCT reached the highest level at 16 hours and started to decline at the end of the first day while those whose therapy turned out to be inadequate the PCT level continued to increase during the first day (Figure 2).These data suggest that

early response of PCT within the first 24 hours of commencing empirical antibiotics in critically ill patients may help the clinician to evaluate the appropriateness of therapy.

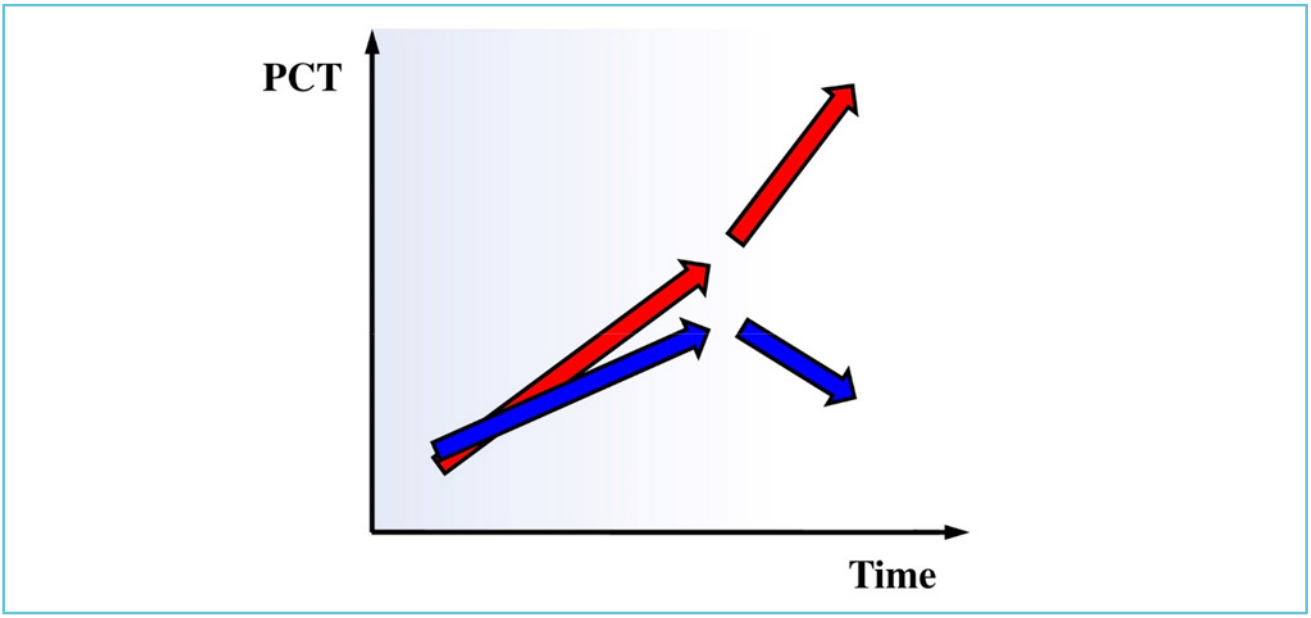
Measuring PCT when antibiotics were commenced, then 12-16 and 24 hours later can reveal a certain kinetics. In the case of continuous increase (red arrows), it is likely that the empirical ABs are inappropriate. However, a "roof-top" type PCT pattern, indicating, the levels peaked somewhere around 12-16 hours can indicate appropriate antibiotic therapy.

Once empirical antibiotics were commenced, daily re-evaluation of the situation is needed. In addition to the clinical picture and PCT levels, microbiology should also be taken into account. Microbiological data usually becomes available 2-3 days after specimen were sent, and then it's time to re-evaluate the situation. The course of the clinical condition, combined with both the microbiological and PCT results, what we term as a multimodal approach, can assist us whether to continue, reconsider or change antibiotics and/or reassess organ support and most importantly, to stop antibiotics even on day 3 if they are considered unnecessary.

Despite no clinical improvement a decrease in PCT still may indicate that the infection is under control, but the patient needs more time to gain benefit from treatment. Therefore, ABs should be continued. On the contrary, if PCT is not decreasing or even increasing, these can be important signs that infection is not under control, hence source of infection and antibiotics (type, dose) should be reassessed. If antibiotics are appropriate, depending on PCT changes ABs should either be continued or other sources of infection should be looked for. In case of inappropriate ABs and no clinical improvement, regardless of the PCT, therapy should be changed.

If there is clinical improvement but no proof of infection (micro: negative), based on PCT changes (↓ or ↑) infection may be excluded and ABs stopped, or continued. Similar algorithms can be applied if ABs are appropriate. If ABs are inappropriate and PCT decreases, then one may consider the microbiology as false positive and stop ABs, because it is highly unlikely that there

Figure 2 PCT kinetics during the first day of effective and ineffective empiric antibiotic therapy [24]



is clinical improvement and decreasing PCT if an infection is not under control due to inappropriate ABs. This scenario happens when there are pathogens (colonization for example), but no infection. Finally, in case of inappropriate ABs and unfavourable PCT changes, consultation with infectologists and microbiologists is recommended.

This multimodal evaluation could help to individualize suspected infection management in the early course of sepsis on the ICU.

3. Stopping antibiotic therapy

Procalcitonin, mainly due to its favourable kinetic profile can potentially be a useful biomarker for also the cessation of antibiotic treatment [30]. In the first trial on 600 ICU patients, the PRORATA study [4], PCT-guided antibiotic management was tested. Antibiotics were encouraged in case of elevated PCT levels, and discouraged when levels were low. The novelty of this trial was that investigators were encouraged to discontinue antibiotics when PCT concentration was less than 80% of the peak value or when absolute concentration of less than 0.5 ng/ml was reached. The same protocol was repeated in a large recent study on 1500 patients by de Jong et al., in a multicenter prospective trial [31]. The results were similar just like the previous one applying this approach shortened the duration of antibiotic treatment and the daily dose antibiotic consumption, in addition the mortality in this group was significantly lower in the PCT-group as compared to conventionally treated patients. In spite of the reinfection rate being higher in the PCT guided group the cumulative cost of antibiotics per patient was significantly lower. Despite the significantly shorter antibiotic therapy, they were unable to show any difference in outcome between the groups, in other words patients did not suffer harm from not receiving antibiotics for the length of time recommended by guidelines.

CONCLUSION

In this deadly battle of fighting the burden of serious infections on the ICU, we often keep missing the point. Although sepsis exists, just like critical illness, but precisely defining it is probably impossible due to its diversity in etiology, pathomechanism and clinical manifestation. Therefore, interpreting the results of sepsis studies is a daunting task. Procalcitonin is definitely one of the most reliable inflammatory markers in the critically ill to date, and there is also convincing evidence that its use to guide antibiotic therapy can rationalize starting, escalating and stopping antibiotic therapy. Furthermore, when the concept, highlighted in this paper is applied, PCT may also become cost effective, by not starting at all, or stopping antibiotic therapy early. However, starting or stopping antibiotic treatment is more complex than just treating one single figure or even the kinetics of PCT values. A multimodal, individualized concept, consisting of a) recognizing organ dysfunction, b) identifying the possible source, c) following the clinical picture and d) taking PCT and PCT-kinetics into account, is necessary to make the most out of your PCT and to do the best for your patients in your everyday practice. Indeed, it requires well-trained, devoted, thinking physicians who dial in all information such as the results of physical examination, laboratory data, and physiologicmeasurements and make the decisions. Therefore, PCT is not the answer, but it can certainly help, considering that we understand what's going on in our patients.

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Advances and pitfalls in using laboratory biomarkers for the diagnosis and management of sepsis

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ABSTRACT

Sepsis is a critical patient condition with high mortality rate caused by a complex and inadequate host response to infection. Since early identification and start of antibiotic therapy in the first few hours after sepsis development dramatically improves outcomes, it is of utter importance to offer fast, reliable and specific early laboratory biomarkers to help clinicians in sepsis recognition. On the other hand, the biomarkers should also be helpful in excluding sepsis and/or confirming therapy effectiveness, and thus prevent overprescribing of antibiotics. In this paper, we discuss the significance and relative merits of three currently available protein biomarkers: C-reactive protein, procalcitonin and presepsin. Although useful, none of these biomarkers has been shown to completely fulfill the roles mentioned above.

INTRODUCTION

The 2016 Guidelines of the Surviving Sepsis Campaign (SSC) classify sepsis and septic shock as medical emergencies and therefore treatment and resuscitation must begin immediately (1). Sticking to the shortest possible time frame for diagnosis and treatment is crucial since it often means life or death for the patient. However, it is –especially in the early stages– not easy to be sure if a patient is septic or not. As a consequence, to be on the safe side, antibiotics are often overprescribed, which generates further problems. As sepsis represents one of the major problems in intensive care units, it is essential for clinicians to have fast, accurate and reliable biomarkers that can help them to make a quick diagnosis and appropriately manage or exclude this life-threatening condition. An ideal biomarker should have all of the following characteristics: fast and specific increase in sepsis, rapid decrease after effective therapy, short half-life and fast and widely available and reliable method of determination. None of the current biomarkers exhibits all of these specifications in full, but the best currently used biomarker available both as a point of care test (POCT) and as a part of several major in vitro diagnostics (IVD) manufacturers' portfolio is procalcitonin (PCT) (2). However, since CRP is still uniformly and inevitably used worldwide, its importance in sepsis diagnosis and management will be briefly discussed. The focus of attention will be placed on the new and rapidly advancing biomarker presepsin whose role is still uncertain, but which might represent a step towards earlier and better sepsis recognition by laboratory means (3). Of course, the golden standard for sepsis confirmation remains within the scope of microbiology laboratory, but the time to result even with the recent advancement of Matrix Assisted Laser Description/Ionization - Time of Flight (MALDI TOF) technology is still inferior to the above mentioned surrogate biomarkers.

C-REACTIVE PROTEIN

C-reactive protein (CRP) belongs to the pentraxin family of calcium-dependent, ligand-binding proteins. Human CRP molecule has a discoid shape and consists of five identical nonglycosylated polypeptide subunits, each containing 206 amino acid residues (4). The *CRP* gene is located on the first chromosome (1q21q23).

CRP was isolated from the sera of patients infected with Streptococcus pneumoniae, and was first described by Tillett and Frances in 1930 (5). The CRP, named for its capacity to precipitate the somatic polysaccharide-F of Streptococcus pneumoniae, was the first acute-phase protein to be described and is an exquisitely sensitive marker of systemic inflammation and tissue damage (4).

CRP is synthesized primarily in the liver, and at lower levels in adipocytes as a response to interleukin-6 (IL-6), interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). CRP is an acute phase protein. During acute-phase response to infection, inflammation or tissue damage, the concentration of CRP increases several thousand times within 48 hours. Authors Pepys and Hirschfield stated that the median concentration of CRP is 0.8 mg/L in healthy young adult volunteer blood donors, the 90th percentile is 3.0 mg/L, and the 99th percentile is 10 mg/L (1) but, following an acute-phase stimulus, the values may increase from less than 50 µg/L to more than 500 mg/L, that is, 10,000-fold (6).

The function of CRP is manifold. The CRP can bind to specific ligands and activate a complement on the classical pathway and thereby participate in non-specific defences against infection and prevent the development of autoimmune diseases. The main ligand for CRP is phosphocholine which is present in the cells of most pathogens, including bacteria and fungi. Furthermore, it is considered that the CRP interacts with damaged endothelial cells or the apoptotic and necrotic cells.

The major CRP drawback in sepsis lies in its lack of specificity. Elevated CRP concentrations in the circulation may indicate inflammation and/or tissue damage of any origin, such as bacterial and viral infections, mycoses, allergic complications of infection, various inflammatory reactions, necrosis, trauma, or malignancies (7-13). The particular problem is a common postoperative CRP elevation with values that might easily overlap with the septic ones, particularly in the initial stages when early recognition is of utter importance.

The CRP values are not affected by diurnal variations, food intake and most medications.

Recent evidence suggests that aging has a significant effect on inflammatory response and the immune system of elderly people, which is to be considered when interpreting CRP values (14).

Acute phase proteins such as CRP, PCT, serum amyloid A, IL-6, and hepcidin have been investigated in multiple studies related to neonatal sepsis. CRP is usually used as an indicator of bacterial sepsis in newborns since PCT values may not be easily interpreted in the first few days of life. However, the determination of CRP has a few drawbacks; it is not useful as a marker for the early phase of infection because it can only be detected about 12 hours after the onset of clinical symptoms, it reaches its maximum after 20 to 72 h (15) and does not show satisfactory specificity (16). However, due to its universal availability and fairly straightforward interpretation of values, CRP is still the most common marker used worldwide in all hospitalized patients, including those with high risk of sepsis development.

PROCALCITONIN

PCT is a calcitonin precursor prohormone which consists of 116 amino acids and is normally expressed by neuroendocrine cells of the thyroid gland, lungs and pancreas. In healthy people PCT values are low, less than 0.046 µg/L (95th percentile) (17,18). PCT values in various non-septic infections are usually lower than 0.5 µg/L (18). This fact, i.e. exclusively bacteria-related increase represents one of the crucial PCT advantages when compared to CRP. In severe septic shock PCT can rise up to 1000-fold. The pathomechanism of blood PCT elevation is a reaction to various exogenous and endogenous stimuli, such as inflammatory interleukins, membrane elements, bacteria lipopolysaccharides or peptidoglycans as well as bacterial endotoxins. In sepsis, PCT is synthesized mostly in liver, but also in other parenchymal organs (19).

Increase in PCT level in patient's blood can be detected approximately 2 to 4 hours after the onset of sepsis (19), which represents another major advantage making it both more suitable and specific than CRP in this clinical context. It is important to know that PCT has a plasma half-life of 20-24 hours (20) Therefore, according to current recommendations, the minimum time before testing needs to be repeated (minimum retesting interval) should be 24 hours (21).

PCT can serve as an aid for clinicians in assessing the risk category for their patient of developing sepsis or septic shock, according to the classification (18) in Table 1.

After initial measurement, PCT can be used to monitor progression of the disease and therapy effectiveness, taking into account minimum retesting interval for PCT of 24 hours.

PCT has also proved to be useful in guiding antibiotic therapy. This approach was mainly evaluated in patients with respiratory tract infections; however, it can also be used in critically ill patients with sepsis or severe sepsis of various origins (21,22). In those patients, daily measurement of PCT is indicated and discontinuation of antibiotic therapy should be considered when PCT levels decrease to less than 80% of the peak value or below 0.5 µg/L (23).

Table 1 Risk categories according to PCT values

PCT value (µg/L)	Risk category
< 0.5	low risk of systemic bacterial or fungal infection
0.5 - 2.0	high risk of systemic bacterial or fungal infection
2.0 – 10	high risk of sepsis and progression to septic shock
> 10	high risk of septic shock

This approach has been shown to significantly reduce the antibiotics use without compromising the patient outcome, which might prove beneficial towards the goal of minimising the antibiotic usage both for the sake of patients and hospital resources. It is important to note that current recommendations state that measurement of PCT levels can be used to support shortening the duration of antimicrobial therapy in sepsis patients; however, as yet this approach is not based on strong evidence (1). Similar strength of recommendations pertains to the fact that PCT levels can be used to support the discontinuation of empiric antibiotics in patients who initially appeared to have sepsis, but have subsequently been proven to have limited clinical evidence of infection (1).

In conclusion, PCT has proven to be a helpful biomarker in early diagnosing of sepsis in emergency departments and intensive care units. It has also been recommended for monitoring effectiveness and modulating duration of antibiotic therapy. It is now widely available both as a laboratory and as a point-of-care test, whereas laboratory methods are preferred due to semiquantitative nature of results on most POCT devices. When ordering PCT, it should be essential that the result provides the clinician with an answer that could not have been resolved by other laboratory tests combined with clinical signs and symptoms, such as complete blood count or CRP measurement. It is important to note that PCT and all other biomarkers can provide only supportive and supplemental data to clinical assessment. Decisions on initiating, altering, or discontinuing antimicrobial therapy should never be made solely on the basis of changes in any biomarker, including PCT (1). The following questions therefore cannot as yet unequivocally be answered exclusively either by PCT or any other available biomarker: Does my patient have sepsis? Is this antibiotic therapy effective for my patient? Can I now safely discontinue antibiotic therapy? PCT seems to be of help in answering those questions in many clinical situations; however, it cannot fulfill this role by itself and should therefore be ordered rationally.

PRESEPSIN

Presepsin is a newly investigated sepsis marker that has been shown to have potential as an early marker of sepsis recognition, for antimicrobial therapy monitoring and as a prognostic marker. Presepsin (sCD14-ST) is a peptide sized 13 kDa that is generated by proteolytic cleavage of soluble forms of CD14 cluster (sCD14). CD14 is a cell surface glycoprotein with molecular mass of 53-55 kDa that is anchored by glycosylphosphatidylinositol (GPI) to cell membrane and represents a membrane form of CD14 (mCD14). The mCD14 as a co-receptor mediates the binding of the bacterial endotoxin,

lipopolysccharides (LPS) and complex LPS-lipopolysaccharide binding protein (LBP) to toll-like receptors (TLRs), causing the activation of inflammatory responses: cell activation, fagocytosis and cytokine production defending host against pathogen (24-26). After the sTLR activation, the mCD14 undergoes the proteolysis, producing two soluble forms (sCD14) of different sizes. The smaller sCD14 is produced by protease cleaving of mCD14 and the bigger one is produced intracellularly and is directly released from the cell in the protease-independent manner (27-29). CD14 is present mostly on monocytes, macrophages, neutrophils, B-lymphocytes and also on chondrocytes, dendritic cells and human epithelial intestinal cells. Hepatocytes can also express CD14, especially during endotoxemia (30). Recent investigation of presepsin kinetics has shown that, when the polymorphonuclear and monocytic cells were exposed to LPS, presepsin could be detected as early as one hour after the exposure, with maximum concentration in the third hour (31). This finding may confirm that presepsin can be a useful marker of host response to bacteria and can be both a specific marker of infection as well as an early indicator, compared to current markers.

Many studies have investigated accuracy of presepsin in diagnosis of system immune response syndrome (SIRS), sepsis and septic shock in different clinical conditions. The systematic reviews and meta-analysis showed that presepsin as a diagnostic marker of sepsis has diagnostic sensitivity and specificity of 0.83 and 0.78, respectively, diagnostic accuracy (expressed as ROC AUC, receiver operating characteristic, area under the curve) of 0.88, and positive and negative likelihood ratios of 3.9 and 0.21, respectively (32).

Multicenter prospective studies have demonstrated a statistically significant difference in presepsin levels between patients with bacterial and non-bacterial infective disease. Presepsin at the cut-off value of 600 ng/L has diagnostic sensitivity and specificity of 87.8% and 81.4%, respectively, which is comparable to PCT at the cut-off of 0.5 μg/L. A study showed that there was no statistically significant difference in presepsin levels between patients with localized and systemic infection, which might preclude its usefulness in sepsis (33).

A multicenter randomized clinical trial which investigated the clinical role of presepsin assay for monitoring disease in relation to the development of complications (34) found that higher presepsin levels on the first day were closely associated with higher incidence of subsequent organ failures (SOFA score). It also studied presepsin role in monitoring the host response to antimicrobial therapy and appropriateness of therapy – the trial confirmed that patients with increasing presepsin concentrations during the first 7 days were less likely to have received early appropriate antibiotic therapy. The study also stated prognostic accuracy of presepsin for early and long-term outcomes; early presepsin was higher in non-survivors than in survivors. Patients with lung infection had lower baseline presepsin levels than patients with abdominal and urinary tract infections (34,35).

Another study has shown that presepsin levels are elevated at an early stage of sepsis and increase with its progression. The plasma presepsin levels reached the highest level in septic shock. ROC analysis of presepsin to differentiate SIRS (systemic inflammatory response syndrome) and sepsis revealed that, at the cut-off of 581 ng/L, sensitivity and specificity were 65% and 100%, respectively, AUC was 0.830. When presepsin is combined with the MEDS (Mortality in Emergency Department Sepsis) scoring system, the AUC was significantly higher - 0.95, with the sensitivity and specificity of 85% and 100%, respectively (36). A similar investigation of the diagnostics of sepsis has shown AUC for

presepsin to be 0.82. For predicting severe sepsis in sepsis patients, the AUC was 0.840 and, with the combination of presepsin and MEDS score the AUC was 0.875 (35).

A very important piece of information about presepsin should be emphasized. As presepsin is a small protein (13 kDa) and is filtered by kidneys, its level is strongly dependent on kidney function. The decreased glomerular filtration causes elevated levels of presepsin in circulation and thus, presepsin levels above the cut-off value in patients with renal failure have to be interpreted with caution (37,38). A study showed no statistical difference in presepsin concentration between septic and non septic oliguric patients (stage failure in RIFLE criteria; RIFLE, concensus classification criteria for acute kidney injury: Risk, Injury, Failure, Loss, End-stage kidney disease), which might preclude its use in patients with renal failure (38). In this regard, however, future studies are necessary to confirm this finding. There is also a recent study (39) which deemed presepsin as a valuable biomarker for diagnosis of infection and sepsis; however, its diagnostic accuracy has not shown any superiority compared to PCT. Therefore, the authors question its validity for introduction into clinical practice. Similarly, its added value has been questioned in patients with pyelonephritis (40).

Despite the somewhat controversial findings about its validity, presepsin might be an effective biomarker for the timely diagnosis of sepsis, particularly due to its early elevation. Besides diagnosis, it might prove useful for monitoring therapy effectiveness and it might serve as an aid in prognosis. It is important to note that its measurement can be performed quickly and easily (41) as a POC test; however, as it is still not widely available on common laboratory platforms, its utility might be hampered. Along with, or –as a better scenario for healthcare resources– instead of other diagnostic inflammatory markers, presepsin might therefore get its place in the septic patient care as a routine laboratory marker used to facilitate current diagnostic strategies, particularly for early recognition of sepsis within the first few hours, which nowadays still represents a diagnostic challenge.

CONCLUSION

So far in clinical practice no single, optimal biochemical marker is available to confirm or exclude the diagnosis of severe infection within the clinically required time frame. Therefore the diagnosis has to include consideration of all important signs of infection. The quest for an ideal sepsis biomarker is still going on. The most reliable one according to current knowledge is still PCT, while the emerging and promising markers such as presepsin still lay in waiting to be unequivocally proven as more useful and effective.

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In this issue: Advances in the diagnosis of sepsis

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Nonconventional markers of sepsis

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ABSTRACT

Sepsis still remains a challenging healthcare problem with high mortality rate. To improve outcome, early diagnosis and monitoring of sepsis is of utmost importance. In this process objective laboratory parameters are the most helpful.

Procalcitonin and C-reactive protein are the most commonly used and recommended markers of sepsis however, more than 200 sepsis biomarkers have already been published. This mini review focuses on nonconventional novel possibilities for the recognition of sepsis severity. Presepsin, actin and actin scavenger proteins (gelsolin and Gc-globulin) and orosomucoid are discussed. Besides serum parameters, the urinary levels of these markers are also elaborated, since urinary biomarkers of sepsis provide new diagnostic implications and are helpful for monitoring both the kidney function and the septic process.

Increasing serum actin levels and decreasing levels of actin binding proteins seem to be associated with sepsis severity and outcome. Actin can be detected in the urine samples of septic patients as well, and strongly elevated levels of it were found in sepsis-related acute kidney injury. Both serum and urinary

orosomucoid might be able to indicate sepsis, however urinary orosomucoid is a more sensitive inflammatory marker.

Novel laboratory tests can provide rapid help for clinical decision making because the key point in successful treatment lies in the early diagnosis of sepsis.

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INTRODUCTION

Although sepsis is one of the oldest syndromes in medicine it is a challenging healthcare problem even nowadays. In spite of the era of modern antibiotics and intensive therapy sepsis is still one of the leading causes of morbidity and mortality (1).

Sepsis is a heterogeneous and complex syndrome with various etiology, severity and prognosis. To our present knowledge the inflammatory response is the key role in the pathophysiology of sepsis however; a kind of uncertainty exists regarding the factors most likely to lead to increased lethality. In spite of the uncertainties one fact is obvious: the earlier the diagnosis of sepsis is raised, the more favorable outcome may be predicted (2, 3).

Based on the novel results and advances of pathobiology, management and epidemiology of sepsis, the definitions of the syndrome have been changed recently. Sepsis-3 consensus defines sepsis as a life-threatening organ dysfunction caused by a dysregulated host response to infection (4).

The diagnosis of sepsis is most often not easy especially in newborns or in patients whose immune response is not adequate. Therefore, it is of utmost importance to introduce diagnostic biomarkers which can predict or verify systemic inflammation as early as possible. These tests should also be applicable for monitoring of the disease progression and efficacy of therapy as well.

Microbiological identification of pathogens is essential for efficient therapy of sepsis, because the clinical signs are nonspecific. Gold standard microbiological culturing methods require quite a long time (days), but new molecular biological techniques, polymerase chain reaction and mass spectrometric methods can shorten pathogen identification in the bloodstream (5). However, these methods can not differentiate between colonization and infection, moreover they need a well trained and equipped laboratory.

The diagnosis and monitoring of sepsis is of utmost importance, in this regard objective laboratory tests may provide rapid information for proper decision making. Up to now, more than 200 sepsis biomarkers have already been studied, most of them belonging to the inflammatory mediators' family (acute phase proteins, cytokines, chemokines, CD markers, adhesion molecules, etc.) (6, 7).

This mini review discusses classical sepsis biomarkers as well but the major focus will be on some of novel interesting nonconventional markers of sepsis.

CONVENTIONAL SEPSIS MARKERS: SERUM PCT AND CRP

The diagnostic and therapeutic guidelines of sepsis management recommend the use of procalcitonin (PCT) and C-reactive protein (CRP) measurements for early recognition of the syndrome (2, 8).

Blood levels of PCT rise 4-6 hours after the onset of systemic infection and PCT's half-life is about one day. Procalcitonin concentrations showed good correlation with the severity of sepsis, higher PCT levels correlated with higher risk of mortality (9). Massive tissue damage could also provoke elevated serum PCT values without infection, but fungal and viral infections do not elevate the PCT concentrations (10).

Monitoring of PCT kinetics is recommended because delta PCT is a better marker of infection than absolute levels and furthermore, early PCT kinetics could indicate the efficacy of antibiotic therapy (11, 12).

CRP is a non-specific inflammatory marker, therefore it increases in many acute and chronic diseases (tissue injury, autoimmune disorders, malignancies), however in sepsis management, CRP could supplement PCT measurements. After infections serum CRP reaches its maximum within 48-72 hours. Strongly elevated CRP levels were found to be severity and mortality predictors in sepsis (13). The measurement of high sensitivity CRP (hsCRP) is recommended.

Since both biomarkers have some limitations, promising other possibilities should be searched for and in fact, are available nowadays.

PRESEPSIN

CD14 molecule is a pattern recognition receptor existing in two forms: as a membrane-bound type (mCD14) and a soluble form (sCD14). Both forms play a role in recognition of LPS and in cell activation. Soluble CD14 subtype (sCD14-ST) also called as presepsin elevates significantly during inflammation and seems to be usable in differentiating between bacterial and nonbacterial infections (14).

Presepsin is normally present in very low concentrations in the serum of healthy individuals. In response to bacterial infections, its concentration increases within 2 hours, according to the severity of the disease (15). Studies have been reported with various diagnostic cut-off levels for sepsis between 400–600 pg/ml (16, 17). Preliminary studies showed that plasma presepsin is a highly sensitive and specific marker of sepsis, and its concentration significantly correlates with the severity of the disorder and in-hospital mortality of patients suffering from severe sepsis and septic shock (18). A novel point of care test is available on the market for rapid presepsin determination, which can help clinicians in rapid decision making.

Due to its 13 kDa molecular weight, presepsin is filtered through the glomeruli, then reabsorbed, and catabolized within proximal tubular cells (19). There is increasing evidence, that presepsin levels are affected by kidney function. Elevated presepsin levels were found in patients with decreased renal function and inverse correlation was described between presepsin and GFR as well (19, 20).Therefore, presepsin levels should be interpreted more attentively in patients with kidney disease.

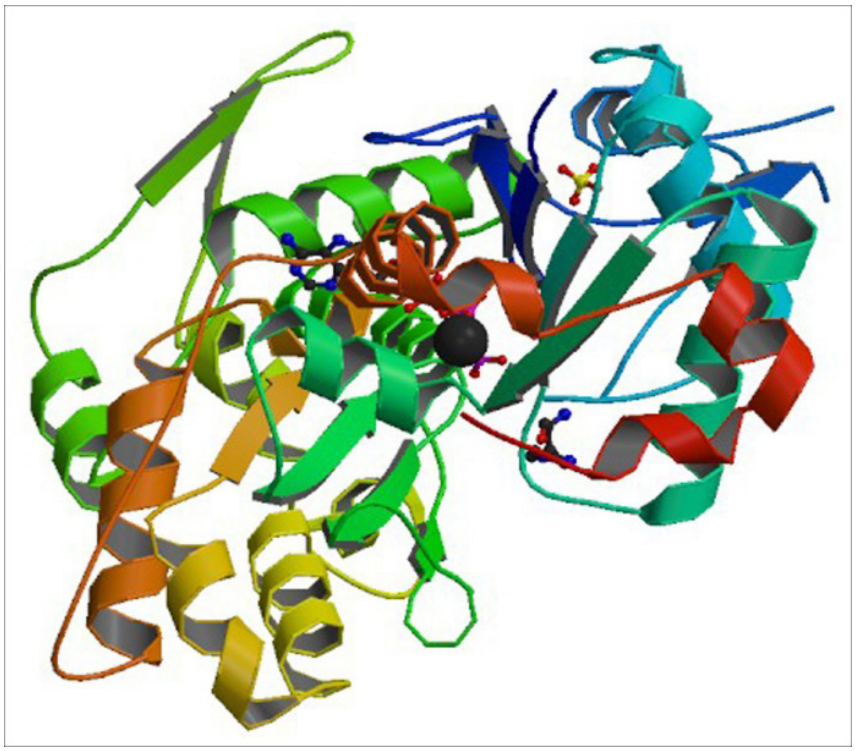
ACTIN

Actin is a multifunctional 43 kDa protein which is present in all eukaryotic cells in monomeric/globular (G-actin) and in polymeric/filamentous (F-actin) form (Figure 1). The two forms dynamically change due to the very rapid polymerization and depolymerization of the molecule. Actin takes a pivotal part in many cellular processes (building up microfilamental cytoskeleton, motility, moving, division, junctions) and in muscle contraction, too (22).

As actin is one the most abundant intracellular protein, during massive cell injury and catabolic conditions high amounts of actin can release into the circulation. Free extracellular actin has toxic effects, since actin filaments are thought to increase blood viscosity, to activate platelets, and cause endothelial cell damage and small blood vessel obstructions. Therefore, high amounts of extracellular actin may contribute to the development of multiple organ failure (23, 24).

The so called actin scavenger system is responsible for the protection of the body from actin toxicity; however the capacity of this defense system can be overwhelmed by massive tissue injury (25).

Figure 1 The structure of native G-actin



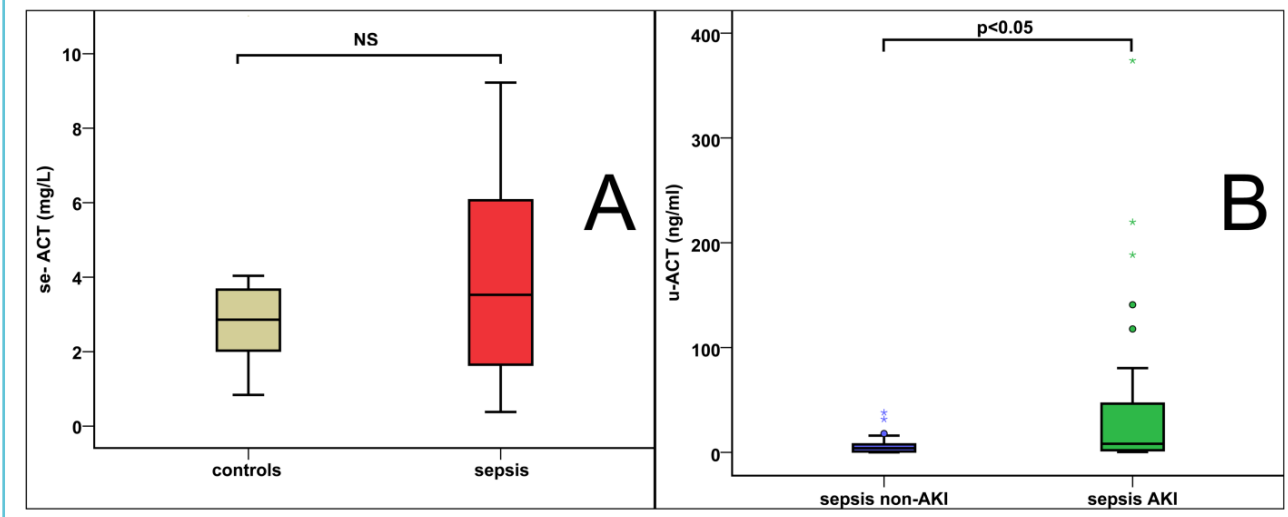
G-actin contains 4 subdomains, and includes one ATP molecule and one Ca^{2+} ion located in the nucleotide cleft, which exists in a closed state (21).

In healthy individuals the major source of extracellular actin is most probably the skeletal muscle with its large mass and high actin content. Circulating actin levels might provide clinically relevant information on disease severity, serum actin (se-ACT) levels were found to be higher in septic patients (3.5 (1.6-6.1) mg/L) than in controls (3.0 (2.1-3.7) mg/L) however did not meet criteria for statistical significance (Figure 2A). The cause of increased se-ACT levels in systemic inflammation and in sepsis might be the extensive tissue injury and detritus of blood cells (26).

There is only scarce data on urinary appearance of actin, however due to its molecular weight free actin could be filtrated through the glomeruli. Recently, our research group has observed the presence of actin in urine samples of septic patients in contrast, actin could not be detected in urine specimens from healthy individuals (27). Urinary actin (u-ACT) levels were determined by quantitative western blot, as in serum. Significantly higher urinary actin was measured in samples of patients with sepsis-related acute kidney injury (AKI, Figure 2B) compared to non-AKI patients (8.17 (2.09-45.53) ng/mL vs. 4.03 (091-10.21) ng/mL). Dialyzed patients showed extremely high u-ACT levels (36.02 (4.7-176.56) ng/ml). U-ACT correlated significantly ($p<0.01$) with kidney function markers (serum creatinine: 0.315, urinary albumin: 0.704) but no correlation was found with se-ACT levels (27).

Previously Kwon et al. found increased u-ACT levels as predictors of kidney failure after ischemic injury in renal allografts (u-ACT/u-Cr were 1095.6 ± 729.6 ng/mg in cadavers with sustained acute renal failure and 355.0 ± 247.0 ng/mg in

Figure 2 Serum and urinary actin levels in septic patients



Based on (26), (27).

cadavers recovering from acute renal failure; p<0.05) (28). However the appearance of actin in urine has not been clarified, u-ACT excretion may reflect overall cellular damage in the kidneys, thus it might provide novel possibility for early diagnosis of AKI, which is the most severe complication of sepsis.

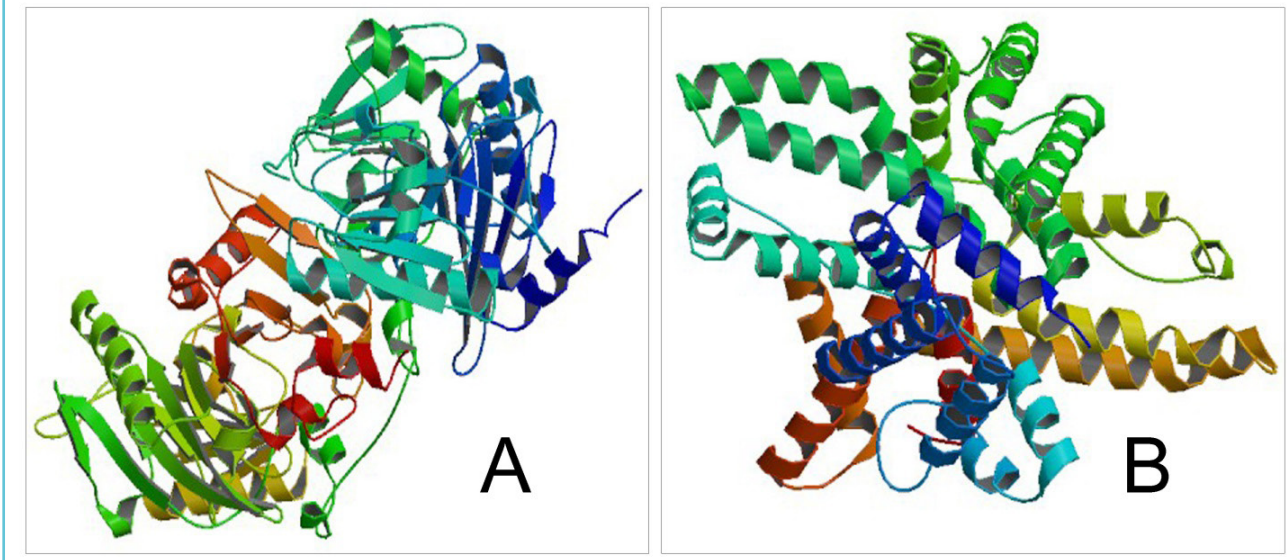
ACTIN-BINDING PROTEINS

In order to protect the body from overwhelming actin toxicity, there are two major extracellular actin-binding proteins called gelsolin (GSN) and Gc-globulin (group specific component, also called vitamin D-binding protein) (Figure 3). Both plasma proteins are essential actin scavengers working in concert. GSN severs and depolymerizes actin filaments originating from disrupted cells, and Gc-globulin frees GSN from actin monomers and sequesters them. The bound actin filaments and monomers are finally cleared from the circulation by the reticulo-endothelial system (31). Furthermore, both GSN and Gc-globulin could modulate inflammatory processes. Under physiological conditions, the concentration of actin in the blood is far less than that of actin binding proteins. Interestingly, in case of severe systemic inflammation, due to excessive tissue injury the excessive amount of extracellular actin and the pro-inflammatory mediators exceed the binding capacity of the scavenger proteins, so the plasma concentration of these drops significantly (25). Both actin-binding proteins are cleared from the circulation by the reticulo-endothelial system, however urinary levels of them are also studied (31).

GELSOLIN

Gelsolin is a ubiquitous, multifunctional protein. Three different isoforms exist in humans, two cytoplasmic forms and one circulatory isoform (32). Circulatory GSN is mainly secreted by muscle tissue (26). Circulatory GSN is a 93 kDa Ca^{2+}-dependent protein and its plasma values range between 190-300mg/L (but these are highly method-dependent) (31,32). Besides actin, plasma GSN may also be able to bind to bioactive molecules (lysophosphatidic acid, sphingosine 1-phosphate, fibronectin and platelet activating

Figure 3 Crystal structures of calcium-free human gelsolin and that of uncomplexed Gc-globulin



A: GSN consists of six domains (G1-G6) indicated by different colors. In the Ca-free, inactive form of GSN, the six similarly folded domains adopt a compact globular structure held together by extensive noncovalent interactions of G2 with both G6 and the C-terminal tail (29).

B: Gc-globulin is built up of 3 three homologous α-helical domains. Domains I and II can be subdivided further into two structurally related subdomains (30).

factor), pro-inflammatory mediators and bacterial wall components (lipoteichoic acid and lipopolysaccharides).

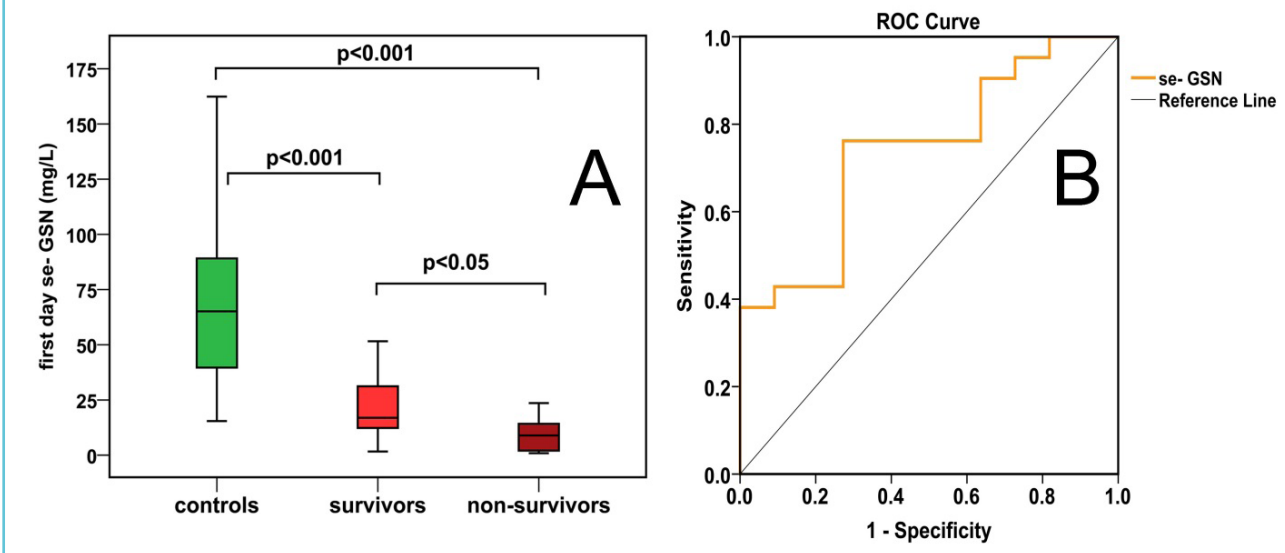
In follow-up studies, first-day GSN levels were proven to have a significant distinguishing ability regarding the septic and the non-septic states furthermore, GSN also predicted the outcome of sepsis (26, 34-36). Non-survivor septic patients showed lower levels of serum GSN (Figure 4). Recently, our research group introduced a new promising marker besides GSN, the serum actin/GSN ratio (derived from the same patients' actin and GSN levels) which had similar prognostic value as APACHE II clinical scores regarding intensive care unit mortality (26). One limiting factor is the lack of a rapid detection method for actin and GSN, which is the current focus of our research.

Higher plasma GSN levels seem to have good prognostic value in sepsis, moreover the protective role of GSN have been proven by administration of exogenous gelsolin to rodents with septicemia and severe injury yielding reduction in mortality (37).

Studies regarding urinary GSN (u-GSN) levels in sepsis have been scarcely performed. Ferreia et al. (38) described u-GSN as a discriminating protein regarding cisplatin- and gentamicin-induced AKI in rats. Another study of Maddens et al. (39) reported increased u-GSN levels in septic mice. Both of these observations based on Western blot analyses indicated that u-GSN originates from the blood by glomerular filtration. In addition, u-GSN seems to be a possible diagnostic marker in patients suffering from type I diabetes mellitus (40). Interestingly, decreased u-GSN levels were found in rheumatoid arthritis patients (41), however they did not offer any predictive value. So far, all studies regarding u-GSN are promising starting points and should be further validated.

Figure 4 A: First-day serum GSN levels in septic survivor and non-survivor patients based on 7-day mortality

B: Receiver operating characteristic curve of serum GSN for predicting 7-day mortality of sepsis



AUC: 0.74, cut-off value: 11.38 mg/L (sensitivity: 76.2%, specificity: 72.7%). Based on (26).

GC-GLOBULIN

Plasma Gc-globulin (52 - 59 kDa) is a member of the albuminoid superfamily. Gc-globulin is mainly produced by the liver (serum level: 300-600 mg/L) owning 3 major isoforms (Gc1f, Gc1s, Gc2) (42). Gc-globulin seems to act as an acute-phase protein after injury. Also, important function of Gc-globulin is binding and transporting 25-OH-D and 1,25-$(OH)_2D_3$ vitamin metabolites. Furthermore it enhances neutrophil chemotaxis and could modulate T cell responses (42).

Admission plasma concentration of Gc-globulin below 134 mg/L (determined by immune nephelometry) was found to be associated with organ dysfunction (hematologic or respiratory failure) and sepsis after traumatic injury (43). Jeng et al. found an association between critical illness and lower 25-OH-D and Gc-globulin levels in critically ill patients when compared to healthy controls (44).

Gc-globulin is filtered freely through the glomeruli because of its low molecular weight. In the kidney, Gc-globulin is involved in the vitamin D biosynthesis process. Under normal circumstances, Gc-globulin is reabsorbed and catabolized by proximal tubular epithelial cells resulting only in a trace urinary excretion (42). Therefore, acute tubular injury is expected to result in exaggerated urinary Gc-globulin excretion. Recently, urinary Gc-globulin (u-Gc-globulin) was reported as a promising novel biomarker of major contrast material induced nephropathy-associated events (u-Gc-globulin/u-Cr in patients developing major adverse renal events (MARE) vs. those without MARE were 125.68 ± 211.62 vs. 14.99 ± 38.10 ng/ml/mmol/l; p<0.001) (45). Shoukry et al. have determined increased u-Gc-globulin levels by ELISA in diabetic patients as an early diagnostic marker of diabetic nephropathy. Urinary Gc-globulin/u-Cr levels were

more than 10 times higher in macroalbuminuric patients compared to controls (1516.3 ± 228.6 ng/mg vs. 123.4 ± 28.2 ng/mg; p<0.001) (46). Investigating the association between sepsis-induced acute kidney injury and Gc-globulin in urine still remains an interesting challenge.

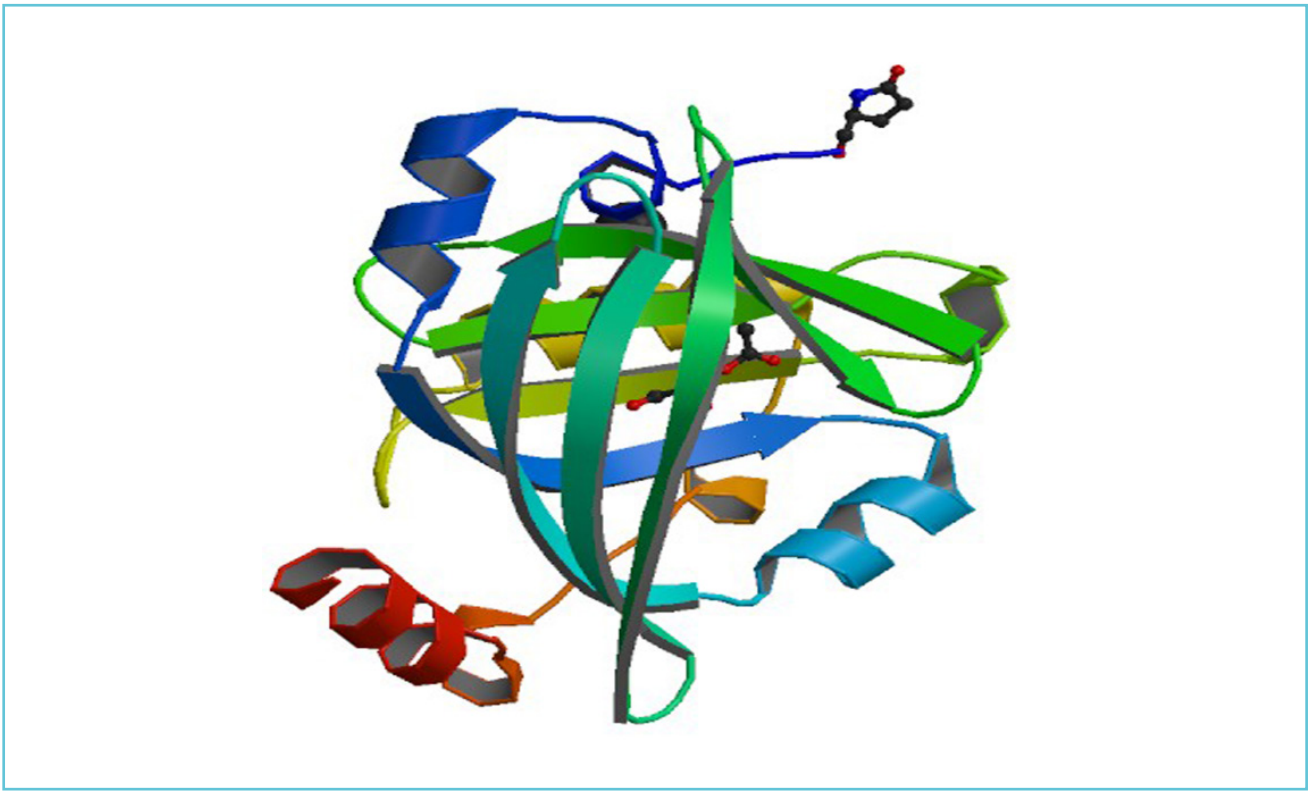
OROSOMUCOID

Orosomucoid (ORM) or α-1-acid glycoprotein is a positive acute phase protein. ORM is a 41-43kDa heavily glycosylated protein (Figure 5) with several transport and immunomodulatory function (47). ORM has been described as part of the non-specific defense system against excessive inflammatory response (48). ORM has anti-neutrophil and anti-complement activity, it can inhibit apoptosis, macrophage activation, lymphocyte proliferation, superoxide generation, and platelet aggregation as well (49). Its protective role was demonstrated also in several rodent models of shock, inflammation and sepsis (50-52).

The normal orosomucoid concentration in human serum ranges between 0.5-1.2 g/L and it can rise during acute and chronic inflammatory diseases (53). In spite of the well-known fact that serum orosomucoid (se-ORM) is a non-specific inflammatory marker, recently it has been described as a potential diagnostic and prognostic biomarker of sepsis. Significantly higher levels were found in sepsis than in SIRS and admission se-ORM levels showed a good prognostic accuracy for sepsis mortality if combined with SOFA score (AUC ROC: 0.878) (54).

ORM is also present in urine, but with much lower concentrations than in serum, normally ORM accounts for about 1-5 % of total proteins in urine (<3 mg/L) (55, 56). Previous studies described slightly elevated u-ORM levels in

Figure 5 Crystal structure of human orosomucoid (alpha1-acid glycoprotein)



ORM contains a typical lipocalin fold with an eight-stranded beta-barrel. This structure is responsible for diverse ligand-binding. Furthermore, ORM structure contains five N-linked glycosylation sites (47).

diseases associated with chronic inflammatory activation, like autoimmune diseases, diabetes mellitus and cancer (57-60). U-ORM excretion can be elevated after acute inflammatory stimuli as well. Recently published data suggest that u-ORM could be a promising non-invasive marker for diagnosis of sepsis (61). About 100-times higher levels were found in sepsis than in controls, and SIRS patients showed 10-fold higher u-ORM levels than controls. U-ORM was referred to urinary creatinine levels and a cut off value at 6.75 mg/mmol with great sensitivity and specificity (94.7% and 90.0%, respectively) has been described for diagnosis of sepsis. The diagnostic accuracy of u-ORM for sepsis (AUC ROC: 0.954) was similar to PCT and higher than se-ORM. Furthermore, u-ORM levels correlated well with conventional inflammatory parameters. In this study, extremely elevated u-ORM levels were found in septic patients with dialysis requirement (61). Another paper demonstrated u-ORM above 40 mg/L as an early predictor for acute kidney injury after cardiac surgery in children (AUC ROC 0.87). U-ORM values were found to be strongly associated with severity of AKI (62).

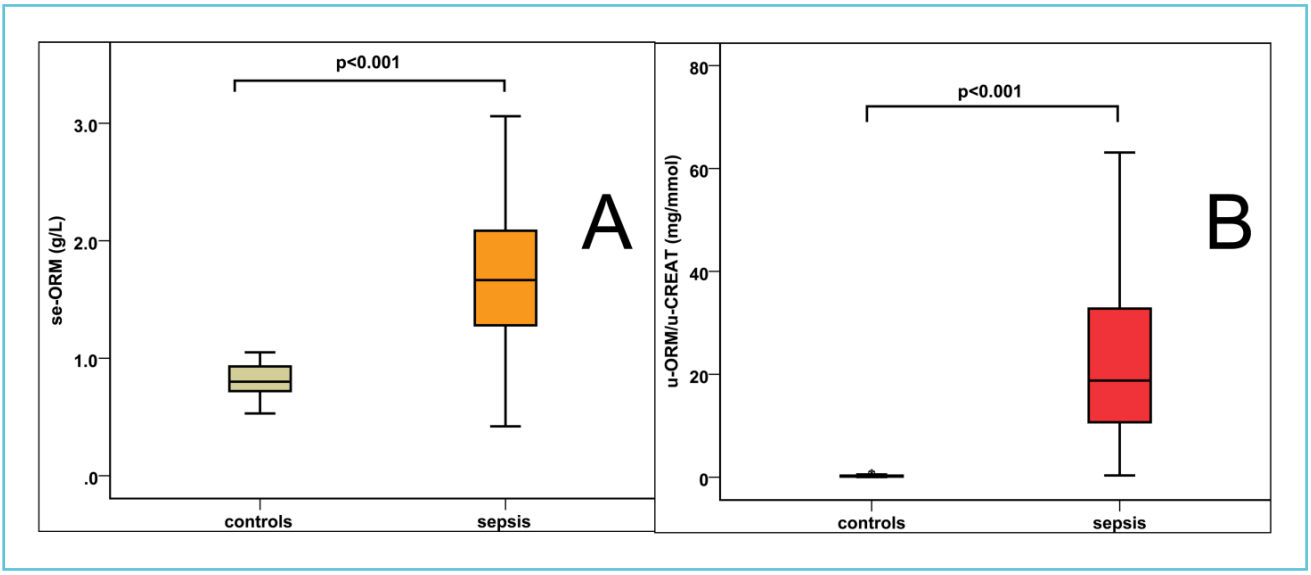
In spite of the promising data, the exact mechanism of u-ORM elevation is not well explored. Local renal processes due to systemic inflammation could play a crucial role, since extrahepatic gene expression of ORM (leukocytes, endothelial cells, kidney, etc.) has been described (63). Furthermore, glomerular and tubular dysfunction also may have a pivotal part.

U-ORM seems to be a more sensitive marker of sepsis than se-ORM (Figure 6), providing clinically relevant information for real-time monitoring of inflammatory activation in a non-invasive manner.

CONCLUSION

The outcome of sepsis largely depends on early diagnosis and the earliest possible beginning of a consecutive adequate antibiotic therapy. For definitive diagnosis, identification of pathogens is still the gold standard however this approach quite often requires several hours or days leading to a delay in decision making.

Figure 6 Serum orosomucoid (A) and urinary orosomucoid (B) levels in sepsis



Urinary orosomucoid levels are referred to urinary creatinine and expressed in mg/mmol. Based on (61).

Therefore, measurement of fast responding protein biomarkers of sepsis has gained a major focus in the last decades. Unfortunately, most of the protein biomarkers do not have proper specificity even if they possess better sensitivity. For the assessment of overall tissue damage, monitoring of the actin-scavenger system is a promising new entity. Urinary markers provide a non-invasive tool for real-time monitoring of septic processes. Orosomucoid determination in urine might be a novel possibility for the early recognition of systemic inflammation. Since sepsis is a heterogeneous clinical syndrome and not a definitive disease a single marker alone should never be satisfactory. Multi-marker approach and complex evaluation of the clinical signs and biomarkers should improve patient management at the bedside.

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In this issue: Advances in the diagnosis of sepsis

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Advances in the diagnosis of sepsis: hydrogen sulfide as a prognostic marker of septic shock severity

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hydrogen sulfide, shock, sepsis

ABSTRACT

Hydrogen sulfide (H_2S) is a third known gasotransmitter. Most of the time it was knows as a poisonous gas. In last 30 years, we are seeing change in its perception. Scientists have discovered its major role in different organ systems. It is endogenously produced in various tissues and its production is influenced by many factors. In normal, physiological conditions only 20% of H_2S is in its free form. The role of H_2S is very wide. It acts as a signaling molecule, has influence on vascular tone, inflammatory response, scavenges reactive oxygen species, can be cytoprotective and can even reduce the extent of myocardial ischemia. Different studies have shown H_2S has considerable influence in pathology of sepsis and its outcome. High free plasma levels of H_2S are predictor of unfavorable outcome. Findings show that moderate free plasma levels of H_2S have protective effect. Paradoxical very low free plasma levels of H_2S, seen in patients with chronic heart failure, are also predictor of severity of disease and poor outcome. We presume that relationship between morbidity/mortality and concentration of H_2S has a wide U-shape curve dependence. New researches with discovery of H_2S agonists and antagonists could open new ways in understanding different pathologies and ability to

treat them. Recent advances in the identification of H_2S agonists and antagonists may help in forwarding our understanding of pathomechanisms and hence their treatment.

❖❖❖❖❖

INTRODUCTION

Hydrogen sulfide (H_2S) is a long-known substance. In normal conditions, it is a gas with a very characteristic odor of rotten eggs (1,2). In the 16th century it was the cause to eye inflammation and bacterial infection in sewer workers. The toxic effect of H_2S is in its ability to bind to cytochrome C oxidase. As such, inhibiting the mitochondrial respiratory chain and consequently inhibiting cell energy production. A change in perspection came in 1989, when H_2S was detected in mammalian brain. At the end of the century it was discovered that hydrogen sulfide has ability to modulate vascular tone, neuronal function and also has cryoprotective abilities during ischemia (3). With these discoveries, the hydrogen sulfide, beside nitric oxide (NO) and carbon monoxide (CO), became a member of so called gasotransmitters (4). In the last decade, numerous studies have investigated the role of H_2S at different diseases, ranging from chronic heart failure to different types of shock. In one of this studies, Goslar and colleagues demonstrated that total plasma sulfide is a marker of shock severity in non-surgical, critically ill adult patients admitted into the medical ICU (5). Other animal and human studies confirm their findings.

ENDOGENOUS PRODUCTION AND METABOLISM OF H_2S

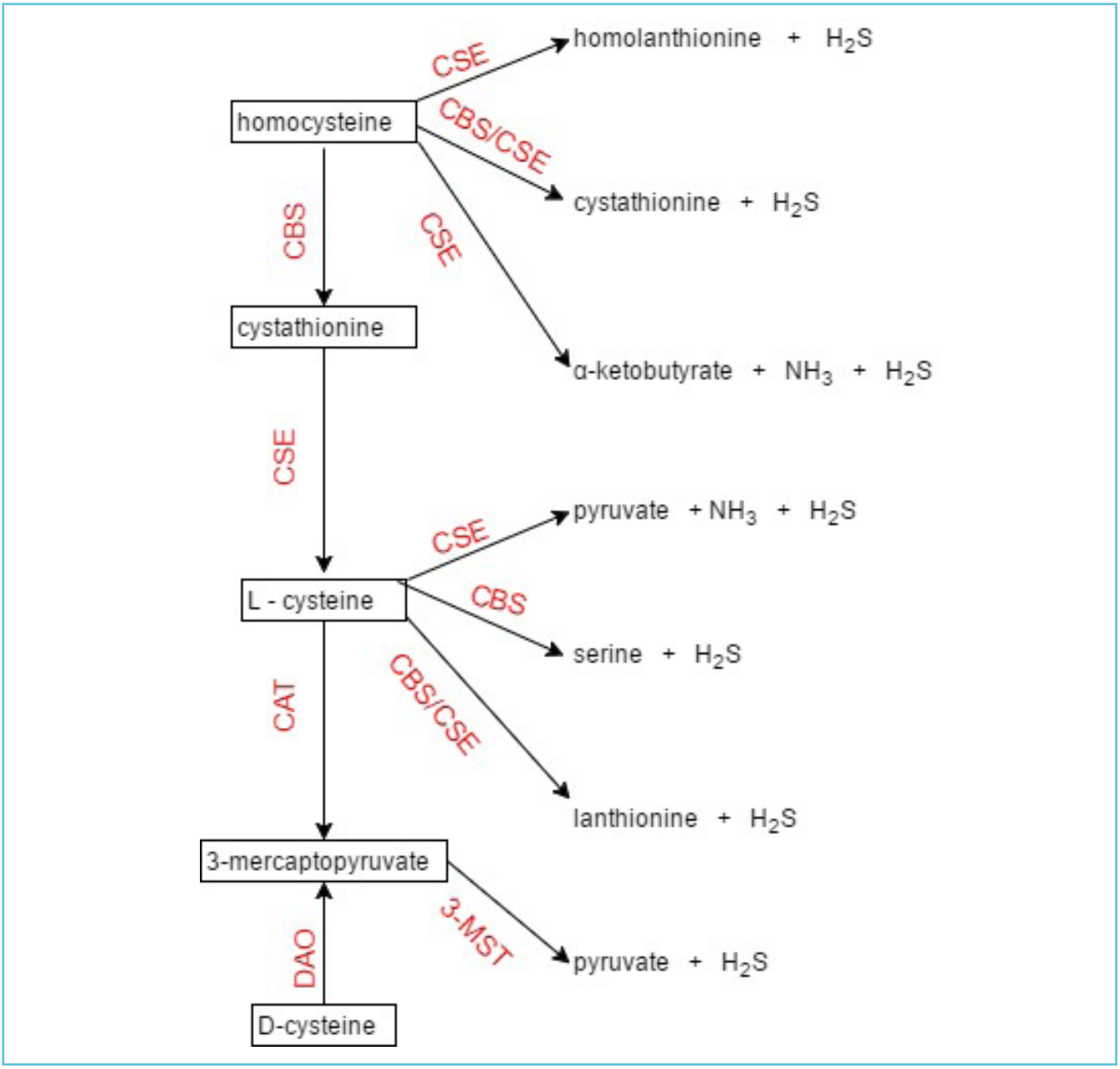
To date we know four different pathways of H_2S production (Figure 1). Two of them are pyridoxal 5'-phosphate (vitamin B_6) dependent enzymatic pathway with cystathionine β-synthase (CBS) and cystathionine γ-lyse (CSE). The substrate in both cases is L-cysteine (6). CBS is the main H_2S synthase in the nervous system, but is also present in kidney, liver, brain, ileum, uterus, placenta and pancreatic islets. The production of H_2S is suppressed by NO and CO and enhanced via CSB by S-adenosyl methionine. CSE is widely present in different peripheral organs (kidney, liver, thoracic part of aorta, ileum, portal vein, uterus, pancreatic islets, placenta), but it was not detected in the brain. The activity of CSE is regulated by concentration of Ca^{2+} (7).

The third pathway consists of two enzymes, cysteine aminotransferase (CAT – aspartate aminotransferase) and 3-mercaptopyruvate sulfurtransferase (3-MST). This way of producing H_2S is present mainly in mitochondria. The base ingredient is L-cysteine. The activity of both enzymes is presumed to be suppressed under oxidative stress conditions, like in mitochondria. Other way to regulate the production of H_2S via CAT/3-MST is with Ca^{2+} ions. In the absence of Ca^{2+} the production of H_2S is maximal and lowered in concentration depended manner (6,7).

The fourth way of H_2S synthesis is synthesis from D-cysteine via D-aminoacid oxidase (DAO) and 3-MST. This is happening in kidney and cerebellum. D-cysteine is not endogenous produced molecule and can be only provided from food – exogenous pathway. Because of this, it is thought, that D-cysteine has a therapeutic potential to increase H_2S production in cerebellum and kidney (6,7).

As already mentioned H_2S can stop (inhibit) mitochondrial respiratory chain by inhibiting cytochrome c oxidase. This happens when H_2S concentration is high. Similar happens with NO, CO and cyanide. On the other side, it is very interesting that it can also be enzymatically metabolized in mitochondria and as that can be a source for generating ATP. This can only happen in very low concentrations (less than 10 µM) of H_2S. In that conditions, it also increases mitochondrial O_2 consumption and ATP production. High concentrations have opposite effect (6).

Figure 1 Pathways of endogenous production of H_2S (simplified scheme)



Legend
CSE: cystathionine γ-lyse; CSB: cystathionine β-synthase; CAT: cysteine aminotransferase; 3-MST: 3-mercaptopyruvate sulfurtransferase; DAO: D-aminoacid oxidase

PHYSIOLOGICAL ROLE OF H_2S

Hydrogen sulfide is known to have a large number of pharmacological effects in various cell types and tissues. Its effect mostly depends on plasma level of H_2S. Higher plasma levels have pros and cons, which will be described in following lines. Also, low levels, below than normal present in healthy individual, can indirectly predict increased morbidity and mortality. This was shown in study by Kovačić and colleagues where they have demonstrated that total plasma sulfide in patients with chronic heart failure (CHF) vary with NYHA stages.

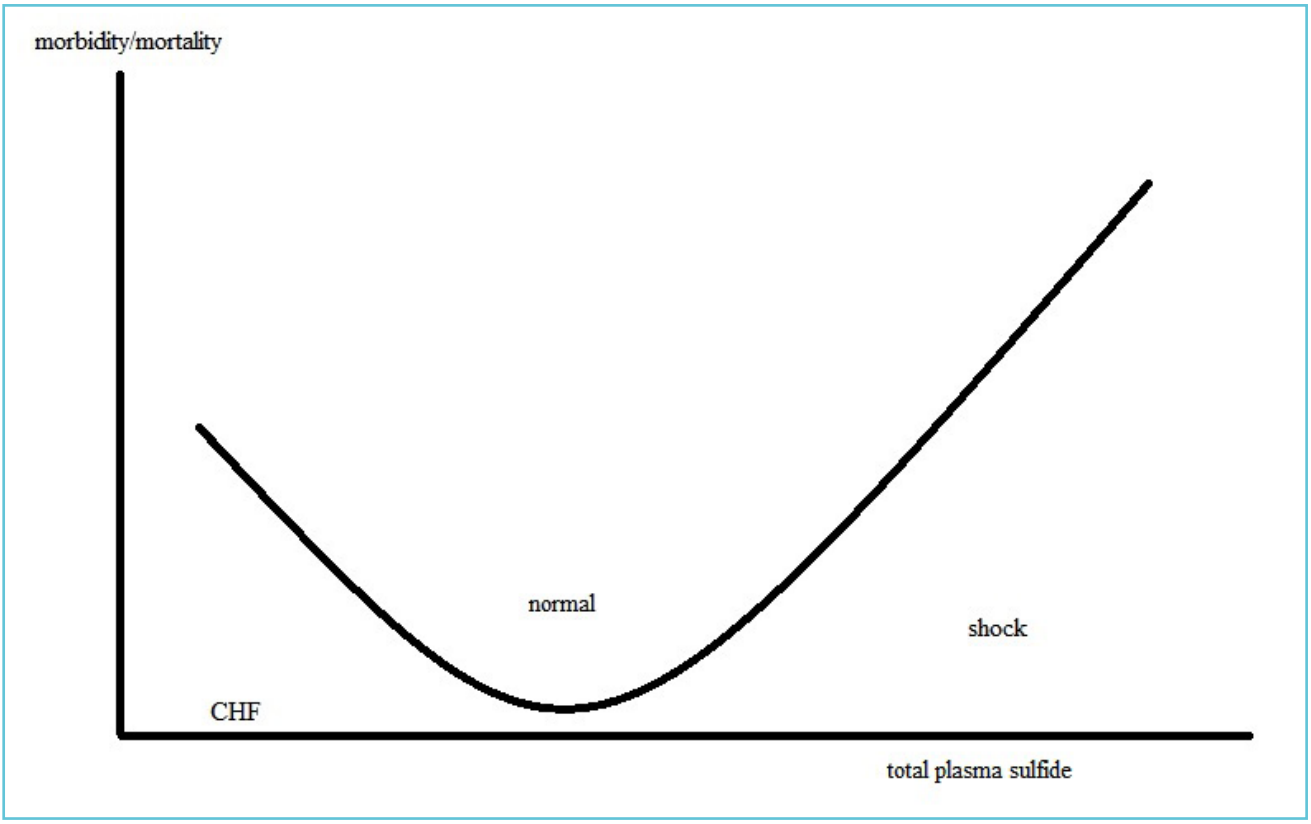
Lowest sulfide levels were present in NYHA class IV (2.67 [2.22-4.31] µM) compared to NYHA class II (5.84 [4.33-8.00] µM), along with negative correlation with pro-BNP and pulmonary artery systolic pressure (8). High levels are often associated with cytotoxic mechanisms, which are associated with generation of free radicals, depletion of glutation, release of intracellular iron and pro-apoptotic actions (8,9). Based on different data we can assume, that morbidity/mortality, among other factors, depends also on plasma concentration of hydrogen sulfide. Correlation can be illustrated with wide U shape curve (Figure 2).

Because of the topic of the article we will focus on the cytoprotective and vascular effects (dysfunction). This is crucial to understanding the H_2S effect during septic shock.

Cytoprotective effect

Evidence shows that H_2S has wide cytoprotective effect in various tissues and organs. Low levels of H_2S are presumed to exert antiapoptotic and antinecrotic mechanisms (9). In nervous system, it increases the levels of glutathione (a major intracellular antioxidant) and, as such, protects cells from oxidative stress. Similar effect is seen in kidney and heart (7). During heart ischemia the energy levels drop. Because there is lack of oxygen the anaerobic respiration starts and lactic acid starts to accumulate. The consequence is reduction of intracellular pH (10). Na/K-ATPase cannot function and there is accumulation of sodium in the cell. This leads to reversal of Na/Ca antiporter and intracellular and mitochondrial calcium accumulation. At the

Figure 2 **Graph presenting allegedly correlation between total plasma sulfide and morbidity/mortality**



CHF: chronic heart failure

reperfusion, oxygen delivery restores, but the respiratory chain complexes are in reduced state and a large quantity of reactive oxygen species is observed. All these events lead to promotion of cell death by necrosis (11). With studies where endogenous production of H_2S was enhanced or exogenous H_2S donors were administered, scientists observed that H_2S was successful in attenuating myocardial infarction following ischemic-reperfusion injury, promoted angiogenic responses and inhibited fibrosis during heart failure. H_2S also acts as a ROS scavenger (7). A study by Elrod JW et al. from 2007 demonstrated that exogenous administration of H_2S or overexpression of CSE, which enhance H_2S production, reduced myocardial infarct size and preserved left ventricular function after ischemic-reperfusion injury. H_2S can reduce mitochondrial respiration to induce a state known as a "suspended-animation" in which cellular respiration and oxygen demand are reduced. Consequently, the oxidative stress is reduced and mitochondrial function is preserved (3).

Vasodilatory effect

Hydrogen sulfide has also vasodilatory effect, which is concentration-dependent. H_2S induces the relaxation of vascular smooth muscle via activation of K_{ATP} channels. The consequence is hyperpolarization of the cell and the effect is relaxation of the cell (4). Although the effect of H_2S is not only dependent on K_{ATP} channels. Its function can also be modified by local oxygen concentration (5). High expression of K_{ATP} channels leads to vascular dysfunction (hypotension) during sepsis.

One other pathway has also been demonstrated to induce vasodilatation with H_2S. H_2S can also increase intracellular concentration of cyclic adenosine monophosphate (cAMP). This causes activation of protein kinase A and relaxation of smooth muscle (12).

Evidence suggest that there is a synergistic action of NO and H_2S in the modulation of vascular tone (13). H_2S can activate nitric oxide synthase located on endothelia (eNOS) and augment NO bioavailability (3). NO donor was shown to increase the expression of CSE and by that increase the conversion of L-cysteine to H_2S (14).

HYDROGEN SULFIDE AND ITS ROLE IN SEPTIC SHOCK

By definition, since 2016, sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. Septic shock should be defined as a subset of sepsis in which particularly profound circulatory, cellular, and metabolic abnormalities are associated with a greater risk of mortality than with sepsis alone (15). During this time, many endogenous factors contribute to development of symptoms we usually see in septic patient. There is substantial production of cytokines, eicosanoids, reactive oxygen species and nitrogen species. All this overproduction leads to severe systemic inflammatory response with hypotension and vital organs hypoperfusion and the end result can be multiorgan failure. Secondary to that there is also loss of vascular responses to endogenous and exogenous catecholamines.

In 2011, Goslar and colleagues reported that during the state of shock plasma sulfide values are very elevated. Based on our results it can be concluded that total plasma sulfide is equivalent to lactate as a predictor of ICU survival. As described above we confirmed the findings that H_2S is involved in regulation of vascular tone and the association between plasma H_2S concentration and extent of tissue damage. Higher total plasma sulfide was inversely correlated with blood pressure and cardiac function. Patients with higher total plasma sulfide during ICU admission had higher mortality compared to patients with lower levels. According to our

findings an increase in total plasma sulfide of 1µM resulted in a 5,8% higher probability of ICU mortality (5). In 2008, Zhang found that endogenous production of H_2S is time-dependent during sepsis. Over production was only present in early stages and peaked 4-8 hours after CLP (*coecal ligation* and *puncture)* (16).

Correlation between plasma H_2S levels and sepsis was also shown in a different study by Zhang and coworkers, where they showed significant increase in plasma H_2S levels in mouse models of LPS (bacterial endotoxin lipopolysaccharide) induced sepsis along with increased CSE gene expression and activity of the enzyme (17). This increase in gene expression, enzymatic activity and consequently H_2S levels was reduced with administration of dexamethasone. Dexamethasone also reduced activity of inducible nitric oxide synthase (iNOS) (18).

The importance of H_2S in pathogenesis of sepsis is also reported in studies where propargylglycine (PAG), a CSE inhibitor, was administered. There was a significant decrease in plasma H_2S levels with better survival rates. Furthermore, high H_2S levels during sepsis may be responsible for hemodynamic collapse in septic shock (4).

Increased endogenous production of H_2S starts in the acute phase of shock. During septic shock, there is also increase of NO levels because of upregulation of iNOS. This higher NO levels can induce CSE expression, which leads to higher H_2S levels – a positive feedback loop (4). Further studies are needed to elucidate the role of H_2S synthesis, where it may mediate inflammation or an anti-inflammatory mechanism.

Contrary to the data above there are some reports which are suggesting that higher (probably moderately high?) H_2S levels act as cytoprotective and may prevent multiorgan failure (4). It is known that H_2S acts as a ROS scavenger in vitro. The reduction of oxidative stress can be result of inactivation of ROS producing enzymes or by direct "rummaging" of ROS (4, 19).

H_2S can also act as an endogenous modulator of inflammation. As already described during sepsis there is an increase in H_2S production. Inhibition of H_2S production decreased systemic inflammatory response and reduced multiorgan failure in sepsis. Suggesting that H_2S has proinflammatory properties (16). Leukocytes and their interaction with endothelium have a main role in pathogenesis of sepsis. Studies utilizing H_2S donors (sodium hydrogen sulfide and sodium sulfide) showed reduced infiltration and adherence of leukocytes to the endothelium through the activation of K_{ATP} channels. H_2S donors also reduced edema formation. Suppression of endogenous H_2S production have the opposite effect. Suppression can be achieved with NSAID, which reduce expression of CSE (14).

MEASUREMENT OF ENDOGENOUS H_2S AND ITS DIFFICULTIES

There are several methods to measure sulfide concentrations in live biological systems – head space gas analysis, derivatization methods (pentafluorobenzyl bromide or N,N-dimethyl-p-phenylenediamine sulfate), spectrophotometry, direct measurement in solution with a silver sulfide or polarographic sensor and most recently developed colorimetric system using a silver-embedded Nafion/polyvinylpyrrolidone (PVP) membrane (20, 21). Different methods give very variable results and there is no consensus which method is the most accurate and best represent the true value of sulfide in living system. Direct and precise measurement in living cells and fluids still remains a challenge.

Sulfide is naturally subjected to oxidation, it has lipophilic property, is slightly soluble in water and acts as a weak acid. Beside free plasma H_2S there are also other forms of sulfide in the

body – mostly sulfane sulfur (elemental sulfur, thiosulfate, persulfide, thiosulfonate, polysulfides, polythionates) and acid-labile sulfur. The latter is mostly present in iron-sulfur clusters in proteins. The level of H_2S is also influenced by pH and temperature – at physiological pH and 37°C, around 20% of sulfide is present as H_2S, and if the temperature drops to 25°C 40% of sulfide is present as H_2S. Main problem today is how to measure the gaseous form of H_2S (5, 20, 21). Measurement of plasma H_2S is still a challenge and new methods have to be developed.

Most widely used method is indirect measurement of H_2S in plasma. In this case plasma is mixed with distilled water, trichloroacetic acid, zinc acetate, N,N-dimethyl-p-phenylenediamine sulfate in 7.2 M HCl and $FeCl_3$ in 1.2 M HCl. This results in a blue color reaction – methylene blue formation. The supernatant is then spectrophotometrically measured. The calibration curve of NaHS (3.125-100 µM) or Na_2S (0.699-139.86 µM) are used to calculate the sulfide concentration (5, 16, 21).

A recently developed method enables selective detection of H_2S in living cells using a silver embedded Nafion/polyvinylpyrrolidone (PVP) membrane and a colorimetric detection method. Silver and H_2S form Ag_2S which is brown in color. Then absorbance at 310 nm is measured (22).

With gas chromatography, we are able to detect sulfide at physiological levels, but it can falsely rise sulfide levels by liberating loosely-bound sulfide because of irreversible sulfide binding or shifts in phase transition equilibria (21).

Hartman and Dcona have described a method for direct measuring of H_2S. It is based on conversion of profluorescent 8-azidopyrene-1,3,6-trisulfonic acid (N3-PTS) to 8-aminopyrene-1,3,6-trisulfonic acid (APTS) by H_2S. Then the fluorescence at 435 nm is measured (23).

CONCLUSION

The knowledge about role of hydrogen sulfide in sepsis is still fragmental. High endogenous (over)production of H_2S during sepsis has multiple implications. It contributes to smooth muscle cell dysfunction, which leads to hypotension. This causes circulatory failure, myocardial dysfunction, organ injury and at the end multi organ failure. Contrary to all negative effect, higher H_2S production also has some beneficial effects. It improves endothelial function, prevents adhesion of leukocytes and thrombocytes, stimulates the host defense system. H_2S acts as reactive oxygen species scavenger and reduces oxidative stress, preserves mitochondrial function consequently leading to less apoptotic cells. H_2S has U-shaped effects curve; in moderate elevation of H_2S production during (early stages of) sepsis can be beneficial in term of host defense and other protective effect; on the other hand, the extensive overproduction has harmful effects.

All physiological stimulations which influence H_2S production and maintain a certain level of substance in the tissue are not known. Next step can be discovering and synthesis of H_2S agonists and antagonists, which will influence the pathogenesis of several diseases where the role of H_2S apparently has been implicated.

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A validation study of after reconstitution stability of diabetes: level 1 and diabetes level 2 controls

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Key words:
HbA1c, six sigma, decision charts, total allowable error, HPLC

Limitation of the study:
Involvement of more participant laboratories could produce better scope of assessment of after reconstitution stability. But laboratories using same commercial lot was not available except one laboratory, the data from which has been supplemented.

Ethical issues:
The laboratories involved in the study used controls exclusively for validation study. For regular use to validate patient results the manufacturer's instruction has been followed.

ABSTRACT

Objective

The after reconstitution stability of L1 & L2 is 7 days when stored at 2°-8°c and tightly capped. The total content of the vial is 500 µL and per test requirement is 5 µL. Hence, in 7 days laboratories would consume only 35 µL wasting 365 µL which is 73% of the expensive control samples and such wastage should be ideally prevented. The study of after reconstitution stability proved the stability of the control samples up to 90 days resulting in proper utilization of L1 & L2.

Materials and methods

The L1 & L2 controls were reconstituted using 500 µL deionized water. The vials were allowed to stand for 5-10 minutes, swirled 8-10 times to maintain the homogeneity. Aliquots of 10 µL were prepared. One aliquot of one control level being used per day, both levels on alternate day. 5 µL control is mixed with 1.5 mL diluent and HbA1c was tested in D10 system by HPLC method. Therefore, 500µL may be utilized up to 45 days approximately provided the extended use of control run is not affecting quality of test results. The minimum number of samples tested by the laboratory from a single vial is 45 and maximum 46.

Results

Three lots were tested of which one lot has been tested in two laboratories. Mean, SD, CV%, TAE, %Bias, z-score and sigma calculations were done. The medical method decision charts were created for all lots based upon normalized operational specifications which showed excellent precision in both control levels. Number of rejections in the study was nil.

Conclusion

The extended use of controls is validated.

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Abbreviations (in alphabetical order)

L1: *Lyphochek Diabetic control level 1*
L2: *Lyphochek Diabetic control level 2*
HbA1c: *Glycosylated Hemoglobin*
HPLC: *High Performance Liquid Chromatography*
TAE: *Total Allowable Error*

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INTRODUCTION

The IUPAC technical committee recommended validation study in a single laboratory provided the study is appropriate to the fitness of purpose and the validated document is continually verified (1). The recommendation suggested to estimate measurement trueness, recovery and linearity. In the present study, the validation of stability of reconstituted analyte was in question. So, the number of rejections, CV% as an indicator of measurement trueness, %bias and z-score are the parameters to be estimated (2). As the values of the analyte have been provided in the manufacturers insert so TAE may be obtained from the same. In 1974, Westgard, Carey and Wold introduced the concept of TAE to provide a quantitative approach for judging the acceptability of method performance (2) and on 2009, Clinical and Laboratory Standards Institute (CLSI) published the TAE of all clinical chemistry analytes (3). It has been recommended to use the CLSI table for TEA or to use 95% confidence interval of the limit of possible analytic error whichever is appropriate/less (4). If the result of reconstituted sample remains within the range provided by manufacturer apparently it may be said that the after reconstitution period may be extended. To validate the extended stability after reconstitution, Sigma should to be calculated and approved finally from medical method decision chart (5, 6). The medical method decision chart is based on sigma calculations and operating point ≥ 5 sigma is the marker of excellence of performance.

MATERIALS AND METHODS

Lyphochek Bilevel Diabetes Controls (L1 &L2) were reconstituted using 0.5 ml deionized water. Calibrated auto pipette was used for reconstitution. The stopper was replaced and the control samples were allowed to stand for 5-10 minutes. The vials were gently swirled 8-10 times before preservation in aliquots to maintain homogeneity. 10 µL reconstituted controls were kept in each aliquot. The aliquots were preserved at 2°C-8°C (7). One aliquot was taken out every day and 5 µL of reconstituted sample was mixed in 1.5 mL diluent. The aliquot was brought to room temperature and swirled before mixing with diluent. The L1 & L2 aliquots were used on alternate day. Hence approximately 45 aliquots of one level control would be consumed in 90 days. Three vials of Lot numbers 33870, 33890 and 33920 were tested for 90 days generating 135 numbers L1 & L2 data i.e., 135 number results generated per level per lot. Lot 33920 have been tested in two different laboratories (incidentally common lot was supplied) hence 135 number data of both levels could have been accumulated from two laboratories. Both level control samples of same lot

were reconstituted and aliquoted on the same day and time. HbA1c was measured in BIORAD D10 system by Ion Exchange HPLC method. As the objective was to establish after reconstitution stability of control samples hence method and mode needed to be user specific and widely accepted.

STATISTICAL CALCULATIONS

Statistical calculations were as per the guideline of Westgard et al (4). Mean, SD, CV%, %Bias of every lot have been calculated. Bias is the difference of laboratory mean and Peer group mean. The manufacturer's mean of Lot 33870 were 5.4% (L1), 9.9 % (L2). The peer mean were 5.38% and 9.79%. The manufacturer's mean for lot 33890 were 5.3% and 9.8%. Peer mean were 5.48 & 9.79. The manufacturer's mean for 33920 were 5.4 & 9.8 and peer mean were 5.42% and 9.84% respectively. Peer means were used for statistical calculations as per CLSI guideline and Westgard's rule. TAE is the percentage of total allowable deviation range. The ratio of manufacturer's allowable deviation and manufacturer's target mean is expressed in % (8). When TAE is more than 20% as per CLSI guideline 20% has been considered as optimum TAE (4). From TAE, %Bias and CV% Sigma is calculated (TAE-%Bias/CV%). Method decision charts were plotted on the basis of calculations from Westgard Website.

RESULTS AND DISCUSSION

The after reconstitution stability was tested in three consecutive lots 33870, 33890 & 33920. The bias was calculated considering peer mean as target mean. As per Multi QC rule ± 3SD would be considered as acceptable allowable error (TAE) keeping the option of one day 2-3SD result in the warning range when the laboratory uses its own laboratory mean [8]. In lot 33870 calculated TAE (from peer mean and SD) of L1 was 20.9 and in 33920 both level values were 28.5 and 20. But as per CLSI guideline optimum TAE should be 20% [Table 1]. So, instead 20.9 & 28.5 the TAE was considered to be 20. No result was found to have exceeded ± 2SD. Hence number of rejection/warning range result in three

Table 1 HbA1c Peer results of lots 33870, 33890, 33920

Control	Lot number	Target (%)		Peer SD		Range (±3SDX2)		TAE (%) = Range x 100/ Peer mean	
Level		Peer	Insert	Peer	Insert	Peer	Insert	Peer	Insert
L1	33870	5.38	5.4	0.184	0.2	1.104	1.2	20.0	22.22
L2	33870	9.79	9.9	0.257	0.4	1.542	2.4	15.6	24.24
L1	33890	5.48	5.3	0.166	0.2	0.996	1.2	18.2	22.64
L2	33890	9.87	9.8	0.245	0.4	1.47	2.4	14.9	24.5
L1	33920	5.42	5.4	0.184	0.2	1.104	1.2	20.0	22.22
L2	33920	9.84	9.8	0.328	0.4	1.968	2.4	20.0	24.5

Table 2 Statistical evaluation of HbA1c values of Lot 33870

N	Mean HbA1c (%)	SD	CV%	Deviation from peer mean	% Bias	Tests per vial	Sigma	z-score	Allow-able bias*	Allow-able impre-cision*	Critical error*
182	5.47	0.127	2.32	0.09	1.67	45+46 +46+45	8.0	0.49	8.3	11.6	-0.641
182	9.52	0.191	2.01	-0.27	2.96	45+46 +46+45	6.3	-1.13	19	13	-1.911

**Allowable Bias- Normal Operational Specifications along Y-axis. Calculated as: %Bias*100/TAE (%)*

**Allowable Imprecision- Normal Operational Specifications along X-axis. Calculated as: CV%*100/TAE (%)*

N- Number of tests performed

Critical error – Calculations obtained from "Quality Control Grid Calculator". Critical error < ±2.0 shows excellent performance

Table 3 Statistical evaluation of Lot 33890

N	Mean HbA1c (%)	SD	CV%	Deviation from peer mean	% Bias	Tests per vial	Sigma	z-score	Allow-able bias*	Allow-able impre-cision*	Critical error*
180	5.56	0.127	2.285	0.08	1.46	45+45 +45+45	7.3	0.48	8	12.5	-0.834
183	9.67	0.262	2.2	-0.20	2.02	46+46 +45+46	5.9	-0.82	13.6	14.7	-1.337

lots was zero. The peer mean & SD were considered for TAE calculation as they are narrower than manufacturer's range (Insert value), based on statistical calculation of worldwide results and system and method specific.

Two lot results showed intra laboratory precision findings. After reconstitution stability of Lot 33870 & 33890 were tested using two L1 & two L2 vials for 90 & 92 days. One aliquot tested on alternated day so 45 aliquots consumed within 90 days and 46 consumed by 92 days. The sigma values were 8.0, 6.3 & 7.3, 5.9 [Tables 2 & 3].

Z-Score calculation is recommended for accuracy check of internal quality control [9] and in both lots both level z-score are within ± 2 [Tables 2 & 3].

The inter performance testing of one lot has been done in two laboratories with lot no. 33920. Both laboratory tested 3 vials of L1 & L2 for 90 days obtaining total 270 results [Tables 4 & 5]. The sigma values were above 6 and z-scores within limit. The sigma and z-score of all three lots satisfies the criteria of standard performance.

Table 4 Statistical evaluation of Lot 33920 (Laboratory 1)

N	Mean HbA1c (%)	SD	CV%	Deviation from peer mean	% Bias	Tests per vial	Sigma	z-score	Allow-able bias*	Allow-able impre-cision*	Critical error*
135	5.49	0.17	2.5	0.07	1.3	45+45 +45	7.5	0.27	6.5	12.5	-0.569
135	9.97	0.203	2.04	0.13	1.32	45+45 +45	9.2	0.39	6.6	10.2	-0.336

Table 5 Statistical evaluation of Lot 33920 (Laboratory 2)

N	Mean HbA1c (%)	SD	CV%	Deviation from peer mean	% Bias	Tests per vial	Sigma	z-score	Allow-able bias*	Allow-able impre-cision*	Critical error*
135	5.55	0.23	2.2	0.13	2.4	45+45 +45	8.0	0.5	12	11	-0.923
135	10.18	0.206	2.02	0.34	3.45	45+45 +45	8.2	1.03	17.2	10.1	-1.37

The use of normalized operation specification chart (NOPSPECs) is being done from the early 1990s. The chart describes the operational limits for imprecision and inaccuracy with respect to a desired level of quality assurance for the particular analyte [10]. The chart is the medical decision chart to select a QC procedure (11, 12). The idea of the medical decision chart is to express the values on the X and Y axis as % of TAE. The X-axis is plotted as imprecision$_{measure}$% TAE i.e., percentage of allowable imprecision and Y axis Bias $_{measure}$% TAE or percentage of allowable bias of the method to be validated (Charts 1-8). The charts may be downloaded from Westgards website. Sigma would be automatically calculated from the input of TAE (%), CV% & % Bias.

The medical decision chart is based on CLSI guideline by Westgard et al where maximum allowable bias is 20% and CV is 10% resulting 2 sigma. Considering allowable Bias $_{measure}$%TAE 20 the colour codes in the charts showed the less the imprecision (CV %) the higher is the sigma value (2-6, colour codes red –green). The method is validated if sigma is ≥4, good if sigma is ≥5, excellent when sigma is ≥ 6. The sigma operating points below 6 are beyond the level of excellence as above 6 sigma no grade has been prescribed yet. Calculations were described below Table 2 and followed for all three lots. The operational specification chart for both controls in 3 groups were evaluated (Tables 1-5, Charts 1-8). Analytical accuracy for all the assay

performance are within optimum excellence decision range (Charts 1-8). Hence, the diabetic controls may be used for 3 months instead of 7 days after reconstitution.

As per the QC calculator rule critical error up to 2 indicates excellent precision. In the present study, the critical errors of all the lots are < ± 2. The stability of the reconstituted control have satisfactorily passed both operating points and critical error criteria. So, the utilization of diabetic controls up to 90 days is acceptable if reconstituted control material is kept in aliquot at 2°-8°C and one aliquot is being used once (13, 14).

CONCLUSION

1. The use of diabetic controls of BIORAD for extended days is validated and verified. The controls may be used for approximately 90 days provided they are preserved properly.
2. The study is having an important practical application value. The laboratory has saved 5-fold expenditure i.e. 1 control in 7 days, hence 6 control vials in 45 days approximately. Such cost saving would be beneficial for the patient care services as the laboratory would be able to perform HbA1c at a patient beneficial rate. HbA1c is a prognostic marker of diabetes so the aim of the laboratories should be to offer the test at a moderate cost.
3. Any waste should be discouraged. The study showed prevention of waste of expensive controls.

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Chart 1 Method decision chart of L1, 33870

Test or Analyte	HbA1c,33870,L1	*Analyst*	Dr.Shyamali Pal
Methodology	Ion Exchange HPLC	*Date*	

Quality Requirement	
Allowable Total Error	20.0
Offset	0.0
Method Performance	
Bias (% diff)	1.7
Imprecision (% CV)	2.3
Sigma Metric	**8.0**

Sigma Limits	*s*
6	3.33
5	4.00
4	5.00
3	6.67
2	10.00

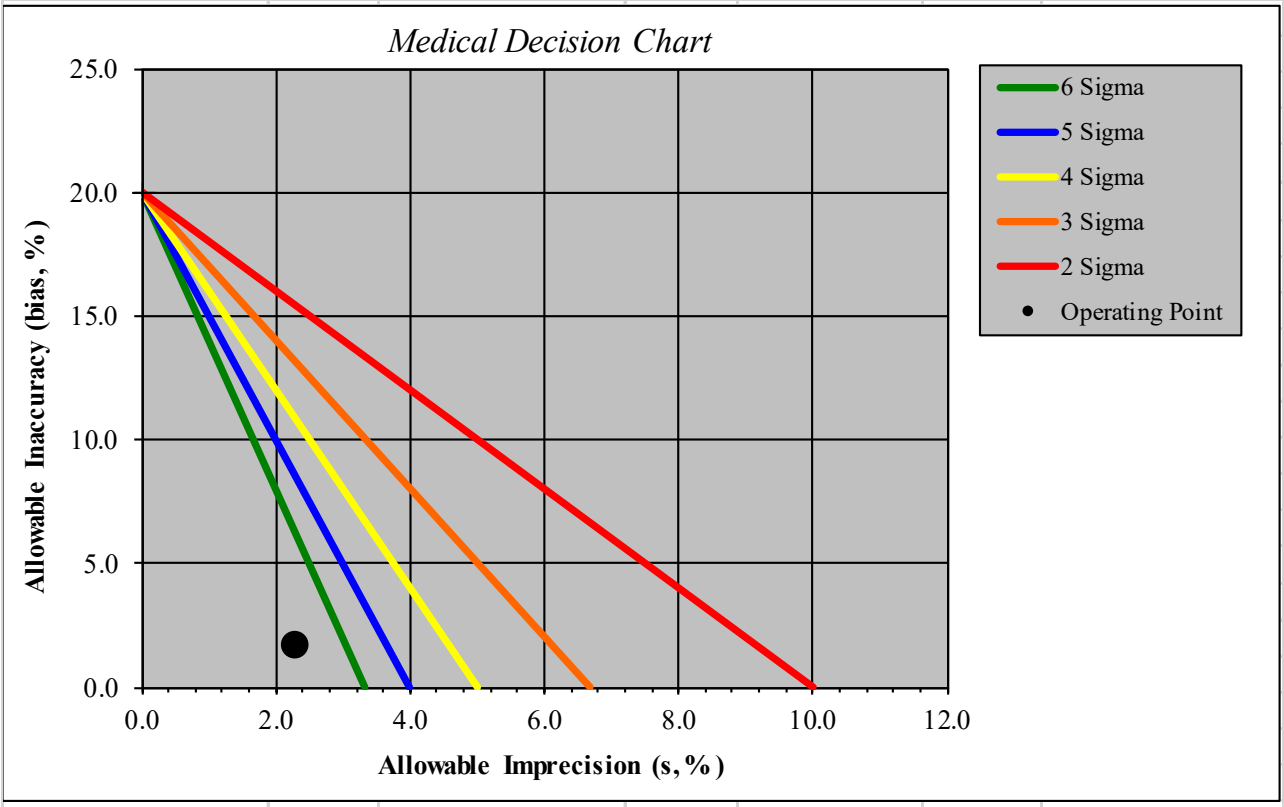


Chart 2 Method decision chart of L2, 33870

Test or Analyte	HbA1c,33870, L2
Methodology	Ion Exchange HPLC

Analyst	Dr.Shyamali Pal
Date	

Quality Requirement	
Allowable Total Error	15.6
Offset	0.0
Method Performance	
Bias (% diff)	3.0
Imprecision (% CV)	2.0
Sigma Metric	**6.3**

Sigma Limits	*S*
6	2.60
5	3.12
4	3.90
3	5.20
2	7.80

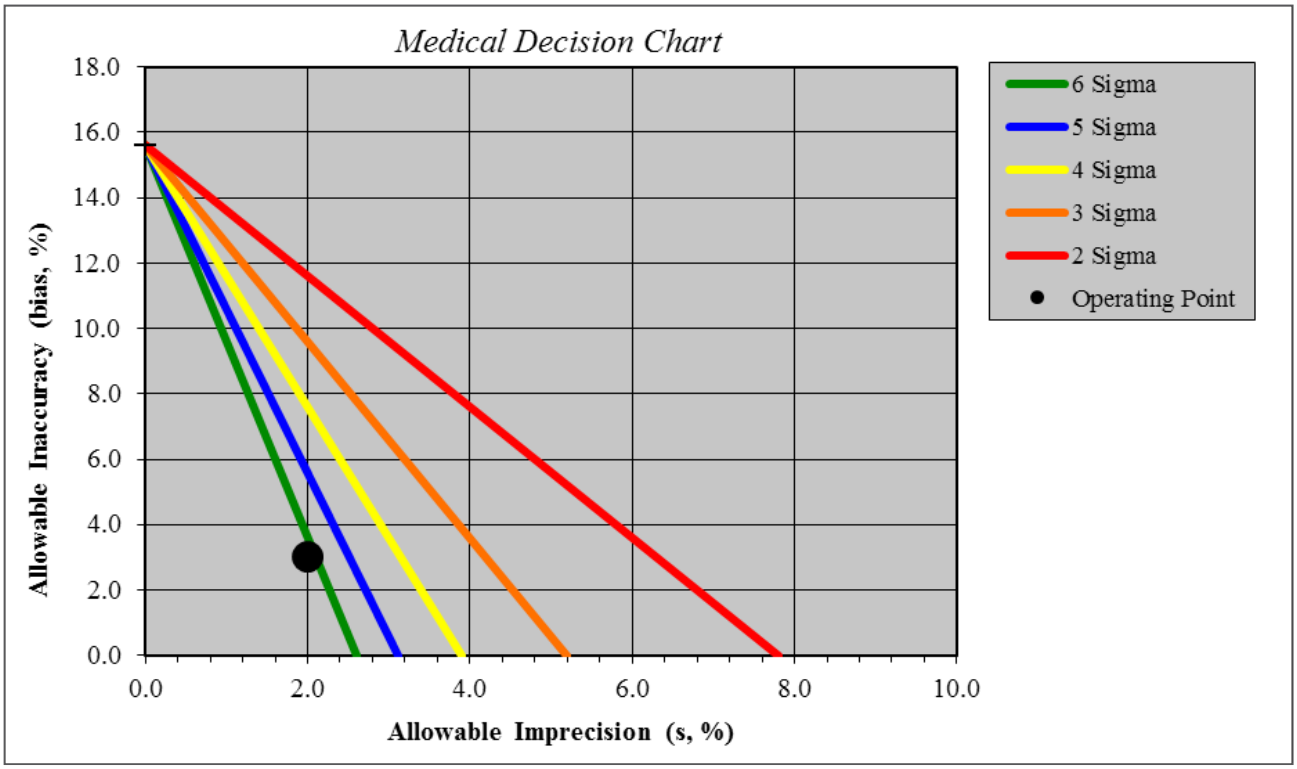


Chart 3 Method decision chart of L1, 33890

Test or Analyte	HbA1c,33890,L1	*Analyst*	Dr.Shyamali Pal
Methodology	Ion Exchange HPLC	*Date*	

Quality Requirement	
Allowable Total Error	18.2
Offset	0.0
Method Performance	
Bias (% diff)	1.5
Imprecision (% CV)	2.3
Sigma Metric	**7.3**

Sigma Limits	*s*
6	3.03
5	3.64
4	4.55
3	6.07
2	9.10

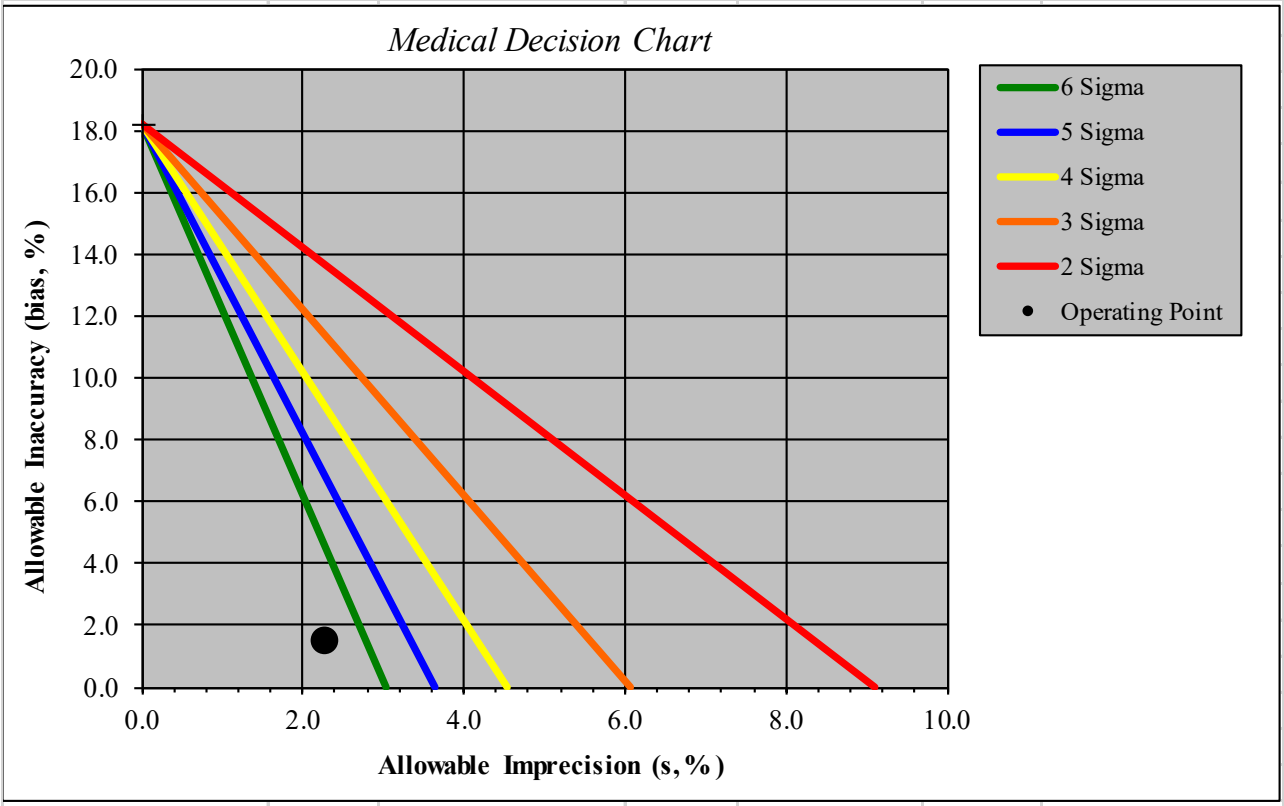


Chart 4 Method decision chart of L2, 33890

Test or Analyte	HbA1c,33890,L2	Analyst	Dr.Shyamali Pal
Methodology	Ion Exchange HPLC	Date	

Quality Requirement	
Allowable Total Error	14.9
Offset	0.0
Method Performance	
Bias (% diff)	2.0
Imprecision (% CV)	2.2
Sigma Metric	**5.9**

Sigma Limits	s
6	2.48
5	2.98
4	3.73
3	4.97
2	7.45

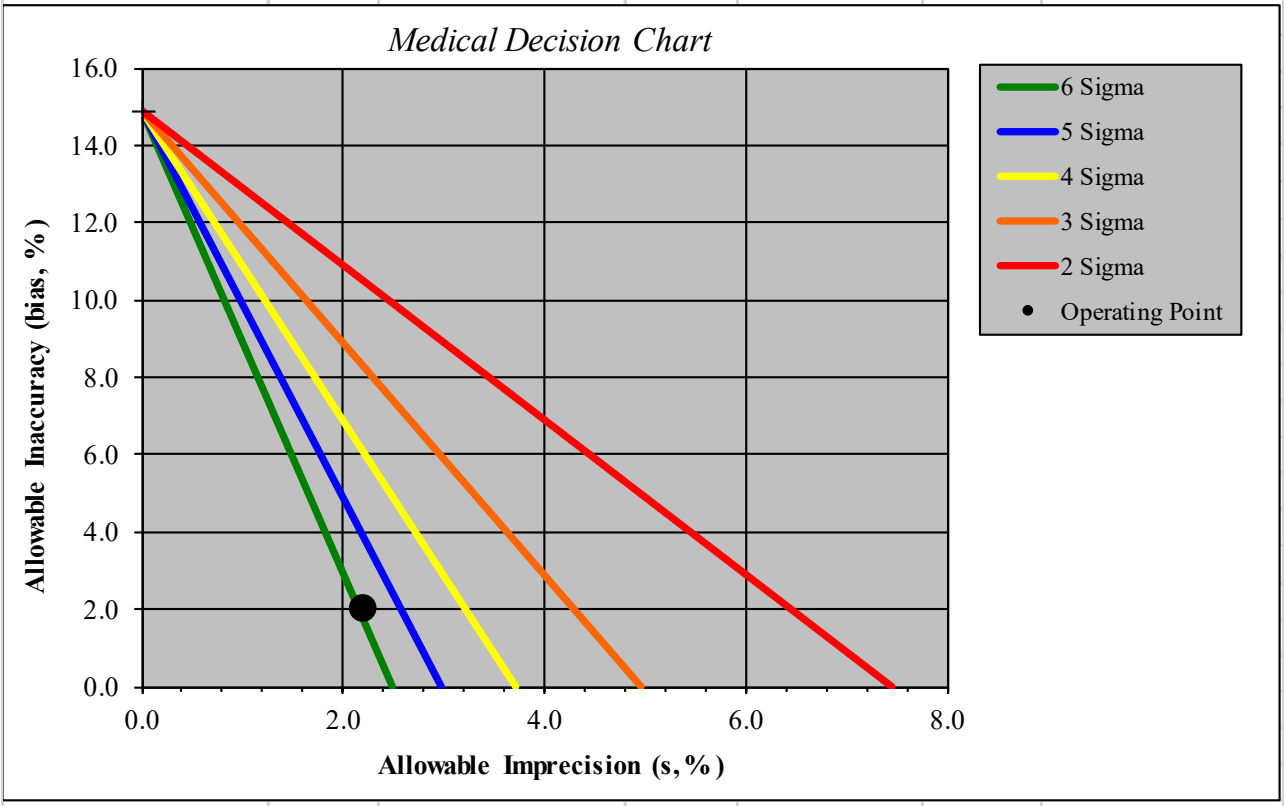


Chart 5 Method decision chart of L1, 33920 (Laboratory 1)

Test or Analyte	HbA1c,33920,L1, Laboratory1	*Analyst*	Dr.Shyamali Pal
Methodology	Ion Exchange HPLC	*Date*	

Quality Requirement	
Allowable Total Error	20.0
Offset	0.0
Method Performance	
Bias (% diff)	1.3
Imprecision (% CV)	2.5
Sigma Metric	**7.5**

Sigma Limits	*s*
6	3.33
5	4.00
4	5.00
3	6.67
2	10.00

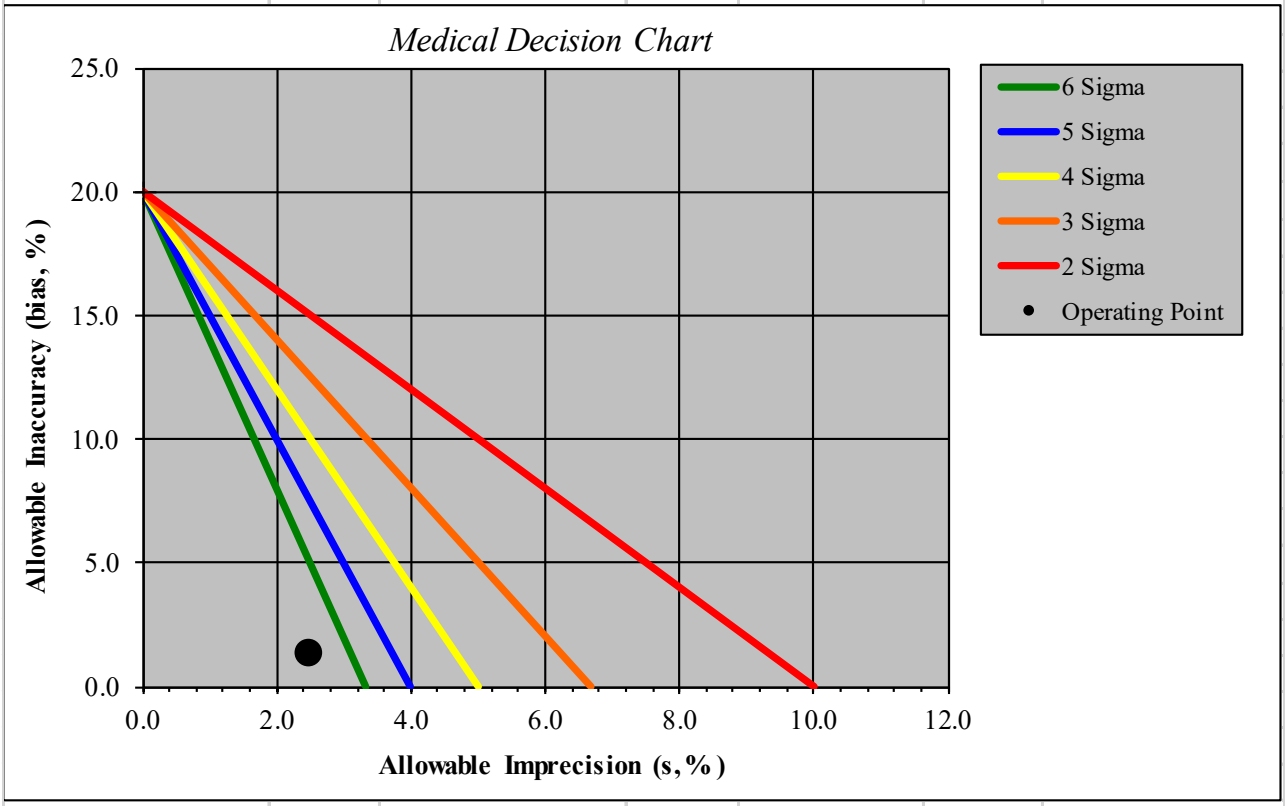


Chart 6 Method decision chart of L2, 33920 (Laboratory 1)

Test or Analyte	HbA1c,33920,L2,Laboratory 1	*Analyst*	Dr.Shyamali Pal
Methodology	Ion Exchange HPLC	*Date*	

Quality Requirement	
Allowable Total Error	20.0
Offset	0.0
Method Performance	
Bias (% diff)	1.3
Imprecision (% CV)	2.0
Sigma Metric	**9.2**

Sigma Limits	*s*
6	3.33
5	4.00
4	5.00
3	6.67
2	10.00

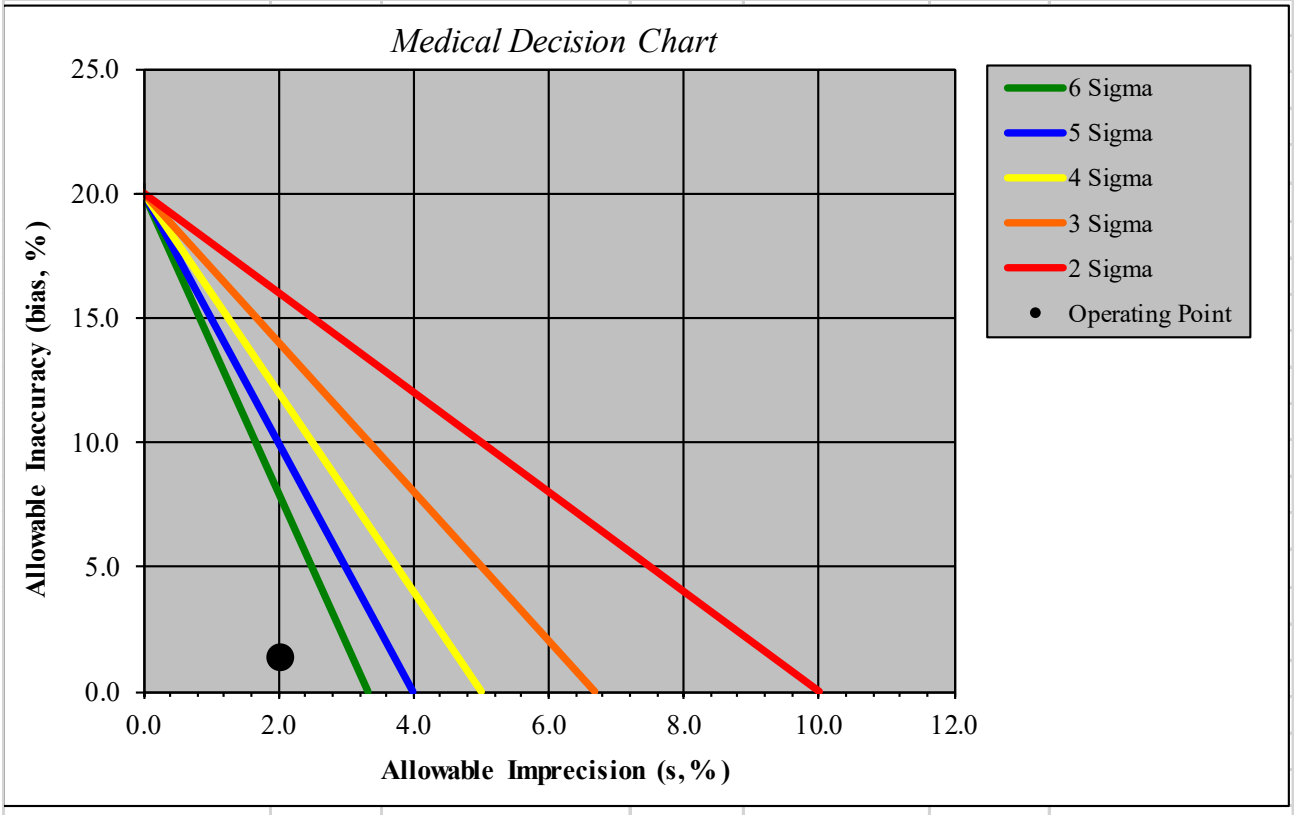


Chart 7 Method decision chart of L1, 33920 (Laboratory 2)

Test or Analyte	HbA1c,33920,L1,Laboratory 2	Analyst	Dr.Shyamali Pal
Methodology	Ion Exchange HPLC	Date	

Quality Requirement	
Allowable Total Error	20.0
Offset	0.0
Method Performance	
Bias (% diff)	2.4
Imprecision (% CV)	2.2
Sigma Metric	**8.0**

Sigma Limits	s
6	3.33
5	4.00
4	5.00
3	6.67
2	10.00

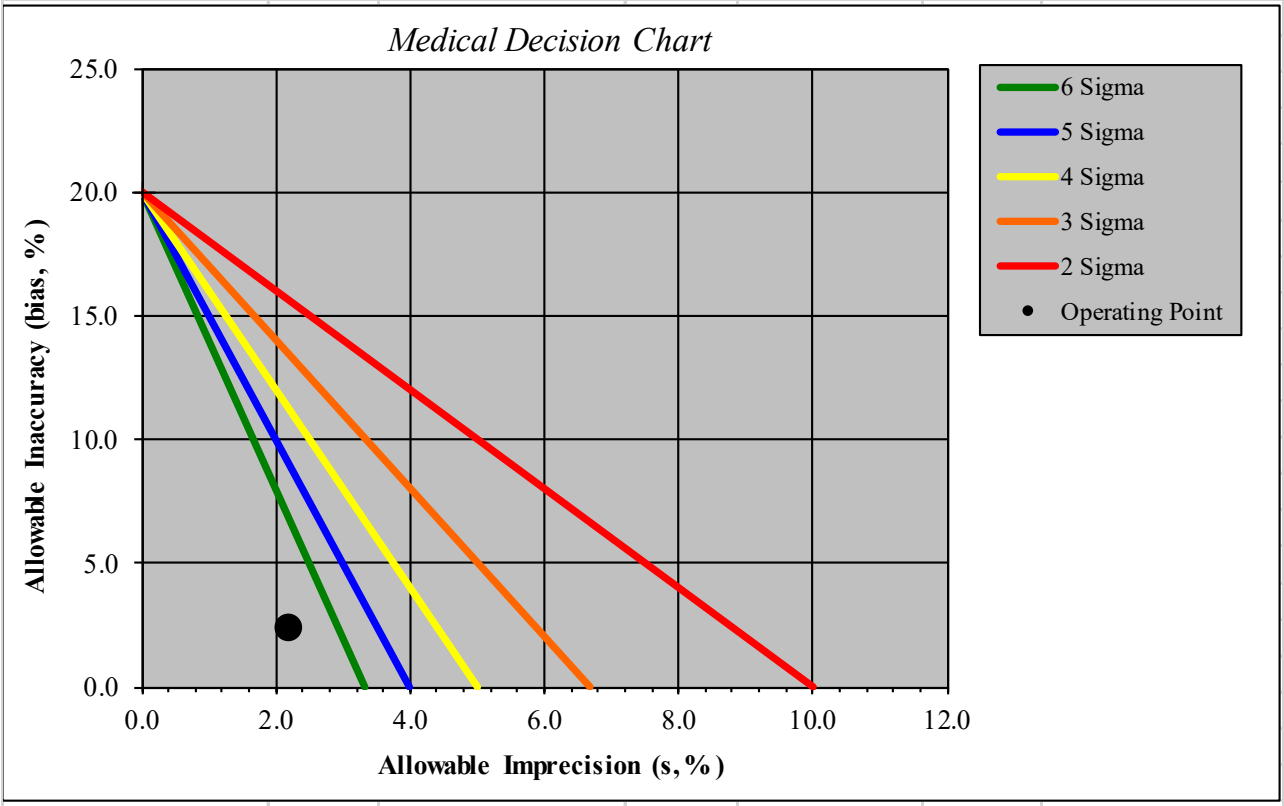
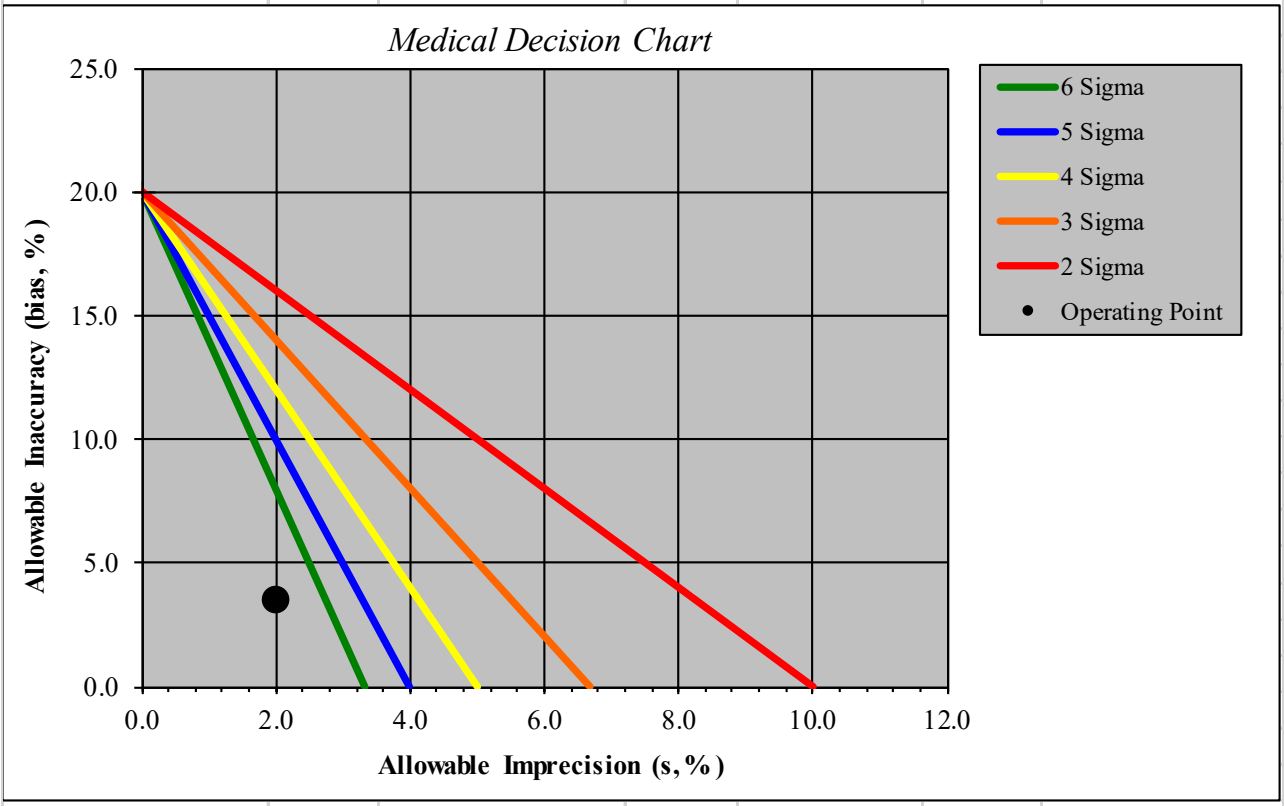


Chart 8 Method decision chart of L2, 33920 (Laboratory 2)

Test or Analyte	HbA1c,33920,L2,Laboratory 2	*Analyst*	Dr.Shyamali Pal
Methodology	Ion Exchange HPLC	*Date*	

Quality Requirement	
Allowable Total Error	20.0
Offset	0.0
Method Performance	
Bias (% diff)	3.5
Imprecision (% CV)	2.0
Sigma Metric	**8.2**

Sigma Limits	*s*
6	3.33
5	4.00
4	5.00
3	6.67
2	10.00



The Journal of the International Federation of Clinical Chemistry and Laboratory Medicine

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Case report: A toddler with anasarca caused by congenital nephrotic syndrome

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TS, OK and TSP conceived the idea and wrote and edited the manuscript. GvB was the paediatrician managing the patient and contributed to the manuscript. KP was the anatomical pathologist who analysed the biopsies and contributed to the manuscript.

ABSTRACT

Congenital nephrotic syndrome is a rare inherited disorder arising from defects in the proteins of the cells in the glomerular basement membrane and develops either in utero or at birth. The clinical presentation is the result of massive protein loss in the urine with associated compensatory mechanisms.

Here we present a clinical case of a female toddler with a history of anasarca (severe generalised edema) from birth and who presents with the classical biochemical laboratory findings of nephrotic syndrome, together with the more pronounced features that arise from protein loss including abnormal thyroid function testing and a marked hypercholesterolaemia. Renal biopsy indicated congenital nephrotic syndrome of the Finnish type. This clinical-diagnostic case report represents an example of the broad spectrum of pathophysiological findings of a severe congenital nephrotic syndrome.

INTRODUCTION

Congenital nephrotic syndrome (CNS)

CNS manifests normally at birth to 3 months of life and is associated with inherited defects in one of the three components of the glomerular basement barrier namely fenestrated endothelium, glomerular basement membrane and podocytes (1-3). Mutations in podocytes lead to congenital nephrotic syndrome of the Finnish type (CNS-F) where podocyte foot processes form filtration slits covered by the slit diaphragm (1-4). The responsible NPHS1 gene was the first described and encodes for nephrin, a type - 1 transmembrane protein of the immunoglobulin superfamily and is a pivotal component of the slit diaphragm. Almost 50 mutations so far have been described in the NPHS1 gene and the autosomal recessive gene maps to 19q13.1 (4, 5). CNS-F presents with massive non-selective proteinuria *in utero* allowing for prenatal screening (1). Other features include premature birth (35 – 38 weeks) with low birth weight and neonatal edema as they were present in this case. Other mutations may occur in podocin (NPHS2) and is associated with autosomal recessive, steroid-resistant focal segmental glomerulosclerosis (FSGS). Autosomal dominant FSGS can result from TRCP6 and CD2AP mutations causing defects in the podocyte. Also, mutations in Wilms´ tumor suppressor gene can occur in the Denys-Drash syndrome (glomerulopathy in combination with Wilms' tumor and male pseudohermaphroditism). Although the common histological finding is mesangial sclerosis, this diagnosis was excluded by the absence of ambiguous genitalia and abdominal mass (2, 4).

CASE REPORT

Clinical features

A 15-month-old first-born female toddler presented with a history of generalised body swelling since birth and neurodevelopmental delay. The first presentation was at 2 weeks after full-term normal vaginal delivery with low birthweight of 2.3 kg. The mother was HIV-positive during pregnancy and received anti-retroviral drugs. She reported unsatisfactory growth of the new-born with motor and speech delay. The child was fed breast milk until 4 months and afterwards with infant formula. On examination, she was below the 3rd percentile for height and weight (weight 6.5 kg; length 64 cm and head circumference 44.5 cm). Despite having Harrison's sulci, the child was un-distressed with no dysmorphic features. There were generalised edema and hypoplastic teeth. Central nervous system examination revealed delayed social and motor milestones with no smile, reaching or sitting and decreased tone and muscle power with normal reflexes. Based on these clinical presentations, congenital nephrotic syndrome was suspected and specific laboratory testing was performed and renal biopsy was done for immuno-histochemical work-up to establish diagnosis.

Laboratory findings

The urine dipstick resulted with 3+ for proteinuria, no signs of haematuria. Blood testing showed a significant depressed C3 level of 0.638 g/L (reference interval 0.9-1.8 g/L) and hypoalbuminaemia of 2.0 g/L (reference interval 27-43 g/L) indicating nephrotic syndrome (NS). Whole blood count revealed anaemia with haemoglobin of 8.0 g/L and there was thrombocytosis with 656 x 10^{9}/L. Plasmatic coagulation tests were found within normal ranges, as well as liver enzymes and renal function testing and electrolytes. Total- and LDL cholesterol levels were markedly increased; TSH and PTH were also significantly elevated together with hypocalcaemia and hyperphosphatemia as outlined in Table 1. Serologic testing for active infections including hepatitis, toxoplasmosis, anti-streptolysin-O titer, cytomegalo virus and syphilis gave negative results, HIV-antibody ELISA was negative and HIV-RNA was not detectable.

Histological and immunochemical findings

A renal biopsy showed mesangial hypercellularity, focal microcystic dilatations of the tubules and a mild interstitial inflammatory infiltrate (Figure 1A). 4+ foot process effacement and increased tubular lipid droplets were visualised on electron microscopy. Immunofluorescence displayed focal 1+ IgM mesangial positivity.

Clinical course

After establishing diagnosis, optimal supportive treatment including Enalapril p.o., intravenous albumin, furosemide, low salt intake, high caloric- and protein diet were given along with calcitriol and thyroxine. Poor compliance to thyroxine contributed to neurodevelopment delay. Treatment was not effective and was hampered by persistent proteinuria.

Unilateral nephrectomy was performed showing massive lymphoplasmocytic inflammatory infiltrates, focal segmental glomerulosclerosis with microcystic tubular dilatation, hypertensive vascular changes and ascending pyelonephritis (Figure 1B). Unfortunately, renal function dramatically deteriorated with volume overload, subsequent bacterial peritonitis and the patient died from septic shock.

Figure 1A Renal biopsy histology showing mesangial hypercellularity and focal microcystic dilatations of tubules

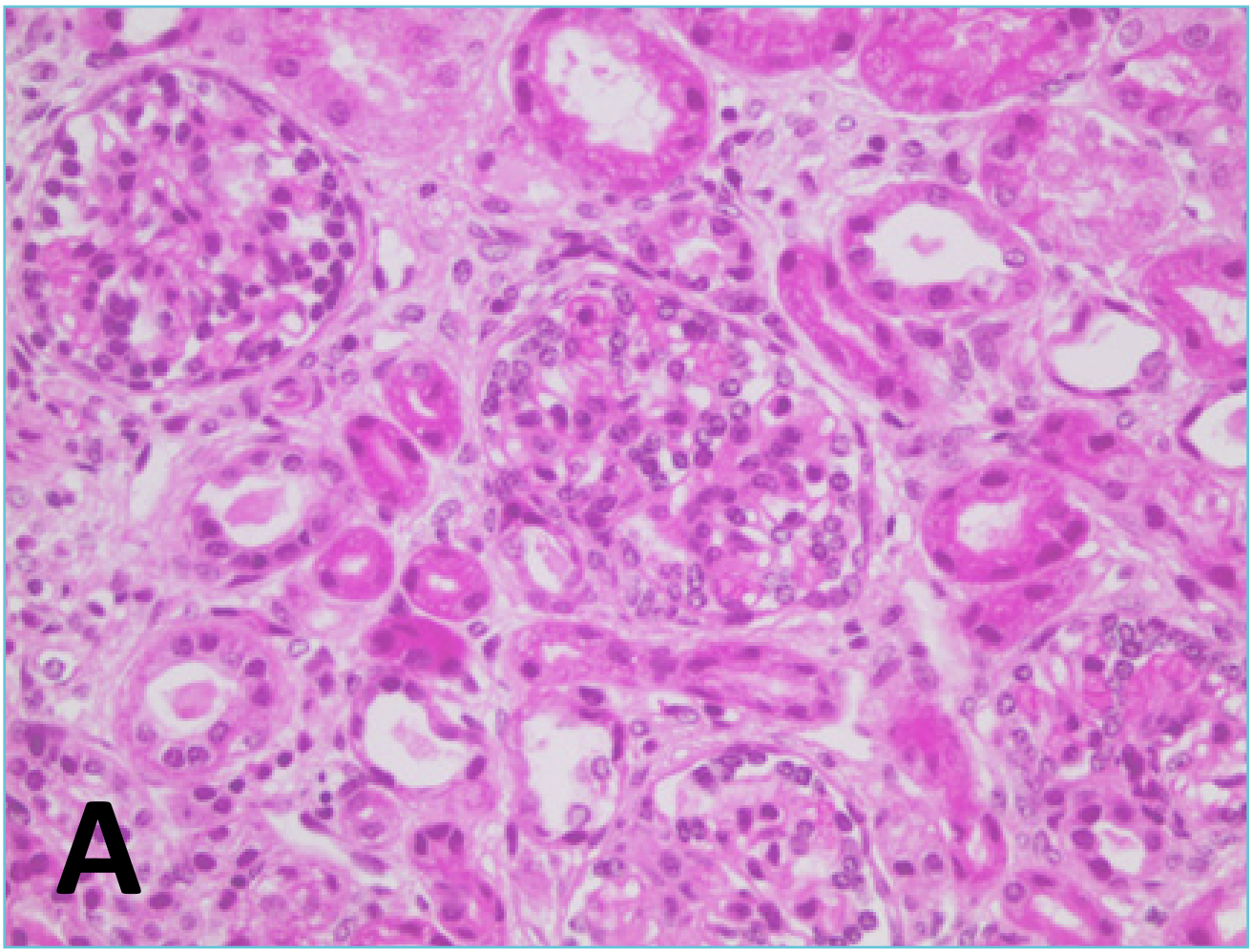
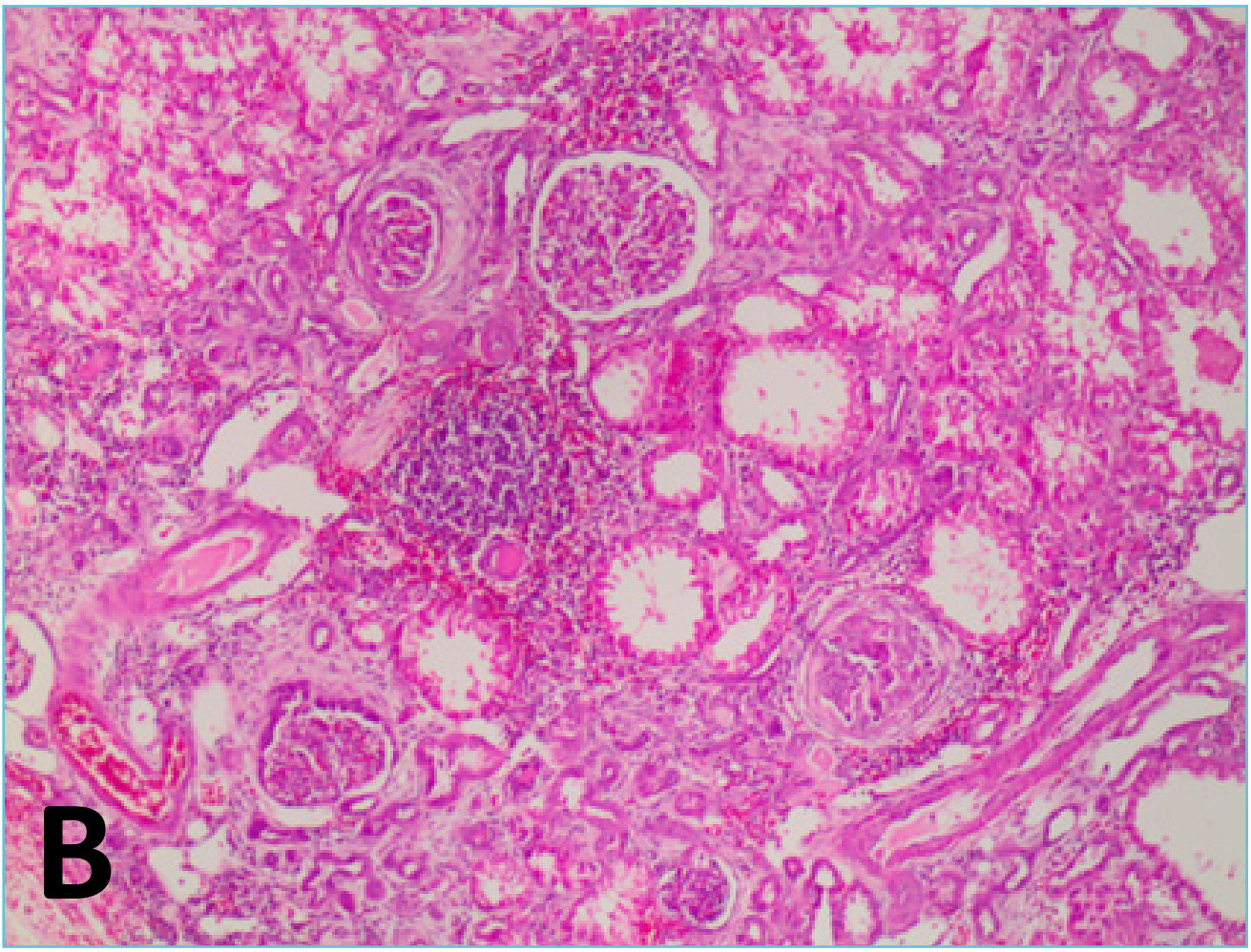


Figure 1B Left kidney histology post nephrectomy demonstrating interstitial lymphoplasmocytic inflammatory infiltrates, microcystic dilatations of tubules, fibrosis and tubular atrophy



DISCUSSION

Generalised edema can result from renal disease, liver disease, allergic reactions, cardiac disease, malnutrition or protein-losing enteropathy. In children, the most common cause is renal disease. First, evaluation can commence with simple tests such as urine dipstick and urine microscopy to identify proteinuria and casts. In our case, edema is a hallmark of NS but the pathogenesis is not fully understood (6, 7). Selective loss of albumin leads to hypoalbuminemia and decreased capillary oncotic pressure resulting in net fluid flux outwards and renal losses not compensated with increased albumin production in the liver. Sequestration of fluid in the interstitium triggers renal sodium reabsorption and fluid retention to maintain intravascular volume and blood pressure balanced (the "underfill" hypothesis) (6, 7).

In NS associated with glomerulonephritis the extent of proteinuria is variable and a reduction in GFR is common. Intravascular fluid volume is typically normal or expanded because of inappropriately stimulated sodium and fluid retention together with decreased GFR results in an "overfill" state. Distinguishing between these two

states is critical to the management of edema in NS. Protein loss in the urine leads to further complications including hypogammaglobulinaemia.

In NS, there is increased risk of thromboembolism owing to increased pro-coagulants and decreased anticoagulants (urinary loss of antithrombin III, plasminogen and protein S) (8). In our patient, we found thrombocytosis which is one of the prothrombotic factors in NS; however the rest of the coagulation profile was unremarkable and there were no clinical signs of a thromboembolic state. As part of nephrotic syndrome workup to exclude secondary causes, relevant infections particularly in the presence of pre-natal HIV exposure were sought and testing was found negative for all pathogens tested.

The markedly elevated total and LDL cholesterol is probably due to over-compensated lipoprotein synthesis for reduced plasma oncotic pressure due to depleted albumin (9). This is associated with an increased risk of cardiovascular events (9). The hypothyroid state in the nephrotic syndrome could also be a contributory factor to the hypercholesterolemia. The elevation of Apo B containing lipoproteins is a prominent feature in nephrotic syndrome and in severe cases is accompanied by hypertriglyceridemia (9). Thyroid hormone alterations in Congenital Nephrotic syndrome of the Finnish type (CNS-F) without renal impairment are well documented (10). Thyroid binding globulin is lost with proteinuria leading to low total T4 and T3. Correspondingly, free thyroid hormones are expected to be normal or even elevated with normal TSH. In severe CNS-F, as in this case, FT4 is lost in urine proportionally with massive proteinuria without commensurate compensation by the thyroid gland. This in turn stimulates the hypothalamic-pituitary axis as demonstrated by high elevated TSH (10). Low FT3 in this case can be explained by the catabolic nature of NS as a non-thyroidal illness characterised by impaired peripheral conversion of T4 to T3.

There was persistent hypocalcaemia with no other overt signs of rickets. She had poor neurodevelopment including motor milestones, Harrison sulci and hypoplastic teeth. Vitamin D-binding protein loss is a likely cause for calcidiol depletion owing to its greater affinity for $25OHD_3$. However, $1.25(OH)2D_3$ and $24,25(OH)2D_3$ can also be low in patients with nephrotic syndrome without renal impairment (11). This can be explained by depletion of the precursor in the form of $25\text{-}OHD_3$ and to a lesser extent to loss of binding protein in severe cases. Tubular damage as a result of exceeded reabsorption of filtered protein can lead to destabilisation of 1α-hydroxylase resulting in low calcitriol level (11). It has been hypothesized that corrected total calcium overestimates hypocalcemia; this was also evident in our case (Table 1). Ionised calcium and vitamin D metabolites were unfortunately not measured. Depleted total calcium was associated with an elevated PTH on more than two occasions (Table 1) indicative of secondary hyperparathyroidism leading to bone demineralization (11).

The histology was typical of CNS-F where immunofluorescence did not detect immune deposits. Microcystic changes of proximal and distal tubules are common findings in CNS of Finnish type, but it may also be present in other histological subtypes of NS. Mostly, CNS-F shows slower progression even with marked proteinuria and renal function tests can be normal early on. If signs of deterioration are present despite optimal supportive treatment, bilateral nephrectomy, dialysis and subsequent transplantation should be the definitive approach to prevent further severe deterioration by reversing the biochemical abnormalities and hormonal disturbances with minimal risk of recurrence after surgery (12). However, in our case a rapid dramatic deterioration occurred with septic shock leading to fatal outcome.

Table 1 Laboratory testing

	Result	Reference interval
Urine		
Protein	4.9 g/L	
Creatinine	0.6 mmol/L	
Protein/creatinine ration	8.17	<0.015 g/mmol
Urea and electrolytes		
Sodium	141	136-145 mmol/L
Potassium	5.2	3.4-4.7 mmol/L
Chloride	112	98-107 mmol/L
Urea	27.0	1.1-5.0 mmol/L
Creatinine	124	15-31 umol/L
Bicarbonate	13	23-29
Anion gap	21	9-16
Liver function tests		
Total protein	26	48-70 g/L
Albumin	<10	27-43 g/L
Total Bilirubin	6	6-21 mmol/L
Direct Bilirubin	1	0-6 mmol/L
Alanine transaminase (ALT)	11	2-25 U/L
Aspartate transaminase (AST)	28	0-49 U/L
GGT	129	15-132 U/L
ALP	191	48-406 U/L
Calcium	1.66	2.12-2.59 mmol/L
Corrected calcium	2.39	2.19-2.64 mmol/L (low albumin)
Magnesium	0.92	0.7-0.99mmol/L
Phosphate	2.13	1.10-1.95 mmol/L

TSH	>100	0.95-6.52mIU/L
FT4	5.8	7.6-16.1pmol/L
FT3	2.9	4.5-10.5pmol/L
PTH	103.4	1.3-9.3pmol/L
Lipids		
Total Cholesterol	13.36 mmol/L	
Triglycerides	6.55 mmol/L	
LDL-Cholesterol	9.19 mmol/L	
HDL-Cholesterol	0.68 mmol/L	
Complete blood count		
WBC	20.56	6-18x10^9L
Hb	8.0	10.7-13g/L
MCV	93.3	70-86fl
Haematocrit	0.255	0.32-0.420
Platelet	656	180-440x10^9L
Iron	4.9	9.0-21.5 umol/L
Transferrin	<0.70	1.49-3.82 g/L
Ferritin	34	36-84 ug/L

TAKE HOME MESSAGES / LEARNING POINTS

- Anasarca in neonates requires a systemic approach to rapidly establish the correct diagnosis. Bedside testing with urine dipstick is an essential screening tool to guide further investigations; a 3+ proteinuria on dipstick is highly suggestive of nephrotic syndrome to be confirmed by appropriate laboratory work-up.
- Nephrotic syndrome is characterised by proteinuria, edema, hyperlipidaemia, hypoproteinaemia and may be associated with coagulopathy. Early diagnosis will necessitate aggressive supportive treatment in preparation for definitive treatment with surgery for restoration of growth and development.
- Always exclude secondary causes of congenital nephrotic syndrome because they tend to occur more frequent than primary causes showing better treatment response.
- Various mutations in CNS have been described; CNS-F resulting from nephrin gene mutations is not uncommon worldwide and affects both sexes equally. Genetic counselling and antenatal screening for congenital nephrotic syndrome - Finnish type with

haplotype analysis is often required and should therefore be considered early on.

- In congenital nephrotic syndrome, thyroid hormones and Vitamin D status must be evaluated.
- Renal biopsy is mandatory in CNS to further differentiate and support diagnosis and to better estimate the severity of clinical progression regarding renal failure.

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