Actin-binding proteins in sepsis

Doctoral (PhD) Thesis

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List of abbreviations

A: actin

ADP: adenosine diphosphate

APACHE II: Acute Physiology and Chronic Health Evaluation II

APTT: activated partial thromboplastin time

ARDS: acute respiratory distress syndrome

ATF3: activating transcription factor 3

AUC: area under the curve

CD: cluster of differentiation

CKD: chronic kidney disease

COPD: chronic obstructive pulmonary disease

CV: coefficient of variation

CVD: cardiovascular disease

C5a: complement component 5a

DAMP: damage-associated molecular pattern

DIC: disseminated intravascular coagulation

DM: diabetes mellitus

EASS: extracellular actin scavenger system

EDTA: ethylenediamine tetraacetic acid

ELISA: enzyme-linked immunosorbent assay

F-actin: filamentous actin

FBS: fetal bovine serum

FiO_2: fraction of inspired oxygen

G-actin: globular actin

GCS: Glasgow Coma Scale

Gc: group-specific component

GSN: gelsolin

γδ T cells: gamma delta T cells

HIS: hospital information system

HMGB1: high-mobility group box-1

hsCRP: high sensitivity C-reactive protein

ICU: intensive care unit

IFN: interferon

IL: interleukin

INR: international normalised ratio

IQR: interquartile range

IT: immune turbidimetry

LIS: laboratory information system

LOB: limit of blank

LOD: limit of detection

LOQ: limit of quantification

LPS: lipopolysaccharide

LTS: lipoteichoic acid

MALDI-TOF MS: matrix-assisted laser desorption/ionization time-of-flight mass spectrometry

MAF: macrophage-activating factor

MAP: mean arterial pressure

MODS: multiple organ dysfunction syndrome

MW: molecular weight

NO: nitrogen monoxide

$PaCO_2$: partial arterial pressure of carbon dioxide

PAF: platelet activating factor

PAMP: pathogen-associated molecular pattern

PCR: polymerase chain reaction

PCT: procalcitonin

PMN: polymorphonuclear leukocyte

qSOFA: quick Sequential Organ Failure Assessment

ROC: receiver operator characteristic

SAPS II: Simplified Acute Physiology Score II

SBP: systolic blood pressure

SD: standard deviation

SIRS: systemic inflammatory response syndrome

SNP: single nucleotide polymorphism

SOFA: Sequential Organ Failure Assessment

ST2: suppression of tumorigenicity 2

TAT: turnaround time

Th: helper T cell

TLR: toll-like receptor

TNF-α: tumor necrosis factor alpha

VDBP: vitamin D binding protein

WB: Western blot

WBC: white blood cell

I. Introduction

I.1. Sepsis

I.1.1. Evolution of definitions and diagnostic criteria

I.1.1.1. Sepsis-1 and Sepsis-2 consensus panels

Diagnosis of sepsis still remains one of the major challenges in medicine. Early recognition and treatment of this life-threatening condition is critical because of its long-term consequences and unacceptably high mortality rate. Since sepsis is a multifaceted syndrome rather than a disease, it is difficult to raise objective diagnostic criteria, for which clinicians and researchers have struggled in the past 30 years.

The first international consensus panel (subsequently named as Sepsis-1) held in Northbrook (Chicago) in 1991 introduced the phrase systemic inflammatory response syndrome (SIRS) which encompasses 4 main criteria, and 2 of them support its diagnosis. Criteria for SIRS are the followings: core temperature: >38°C or <36°C; heart rate: >90 beats/min; respiratory rate: >20 breaths/min or $PaCO_2$ <32 mmHg; white blood cell count >12000/μL, or <4000/μL or > 10% immature (band) forms (1). Sepsis was defined as a systemic inflammatory response to infection. Sepsis and its sequelae (severe sepsis, septic shock) were suggested to represent a continuum of clinical and pathophysiologic severity (Table I, Figure 1).

The prognostic term multiple organ dysfunction syndrome (MODS) was also introduced which was described as a dynamic process and as a continuum of organ dysfunction.

The 2001 International Sepsis Definitions Conference (Sepsis-2) in Washington D.C. reviewed and improved the sepsis terminology which predominantly remained unchanged (2), although the term SIRS was found to be overly sensitive and non-specific. Additional important laboratory (e.g. serum C-reactive protein (CRP), procalcitonin (PCT)) and clinical criteria (e.g. altered mental status, hemodynamic parameters) were offered to support the diagnosis of sepsis in adults (Table II).

Table I. Sepsis-1 diagnostic criteria. Adopted from Bone et al. (1).

	Definitions
Sepsis	Infection + SIRS
Severe sepsis	Sepsis associated with organ dysfunction, hypoperfusion (including lactic acidosis, oliguria, acute alteration in mental status) or hypotension (SBP <90 mmHg or its reduction by ≥40 mmHg from baseline in the absence of other causes for hypotension).
Septic shock	Sepsis-induced hypotension*, persisting despite adequate fluid resuscitation, along with the presence of hypoperfusion abnormalities or organ dysfunction.

Abbreviations: SBP: systolic blood pressure. *: septic shock patients receiving inotropic or vasopressor agents may no longer be hypotensive by the time they manifest hypoperfusion abnormalities or organ dysfunction.

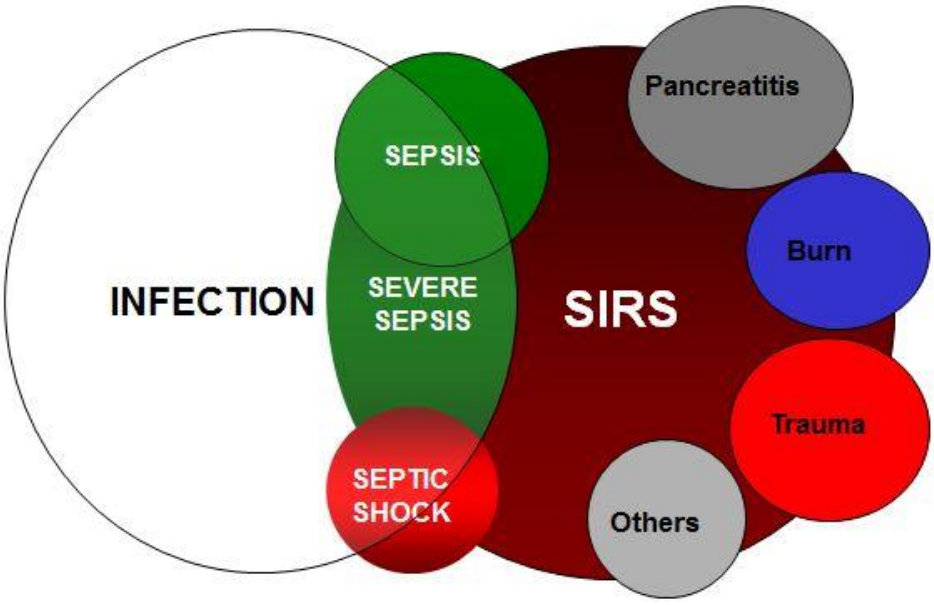

Figure 1. Sepsis definitions established by the Sepsis-1 conference. Based on literature (1).

Table II. Diagnostic criteria of sepsis according to the Sepsis-2 consensus panel (2).

Documented or suspected *infection* and some of the followings:
General parameters • Fever (core temperature >38.3°C) or hypothermia (<36°C) • Heart rate >90 bpm • Tachypnea: >30 bpm • Altered mental status • Significant edema or positive fluid balance (>20 mL/kg over 24 h) • Hyperglycemia (plasma glucose >7.7 mmol/L) in the absence of diabetes
Inflammatory parameters • Leukocytosis (WBC >12,000/μL) or leukopenia (WBC <4,000/μL) or normal WBC with >10% immature forms • Serum CRP >2 SD above the normal value • Serum PCT >2 SD above the normal value
Hemodynamic parameters • Arterial hypotension (SBP <90 mmHg, MAP <70, or an SBP decrease >40 mmHg) • Mixed venous oxygen saturation >70% • Cardiac index >3.5 L/min/m^2
Organ dysfunction parameters • Arterial hypoxemia (PaO_2/FiO_2 <300) • Acute oliguria (urine output <0.5 mL/kg/h for at least 2 h) • Creatinine increase ≥44.2 μmol/L • Coagulation abnormalities (INR >1.5 or APTT >60 s) • Ileus (absent bowel sounds) • Thrombocytopenia (platelet count <100,000/μL) • Hyperbilirubinemia (plasma total bilirubin >70 μmol/L)
Tissue perfusion parameters • Hyperlactatemia (>3 mmol/L) • Decreased capillary refill or mottling

Abbreviations: APTT: activated partial thromboplastin time; FiO_2: fraction of inspired oxygen; INR: international normalised ratio; MAP: mean arterial pressure; PaO_2: arterial partial pressure of oxygen; SD: standard deviation; SBP: systolic blood pressure; WBC: white blood cell count.

I.1.1.2. Sepsis-3 consensus criteria

The definitions of sepsis remained largely unchanged for more than 2 decades. Based on novel pathophysiological findings, advances in therapy and epidemiology, the former definitions have been re-examined in the third (Sepsis-3) consensus panel in 2014/15 (3). The validity of previous definitions was disturbed by an excessive focus on inflammation, the confusing thesis that sepsis follows a continuum through severe sepsis to shock, and the inadequate specificity of the SIRS criteria.

The new panel defined sepsis as life-threatening organ dysfunction caused by a dysregulated host response to infection. Severity of organ dysfunction is recommended to be uniformly assessed by the Sequential Organ Failure Assessment (SOFA) score. The clinical score incorporates respiratory (PaO_2/FiO_2 ratio), coagulation (platelet count), liver function (serum bilirubin concentration), cardiovascular (MAP and vasopressor/inotrope requirement), neurological (Glasgow Coma Scale score) and renal function tests (serum creatinine concentration or daily urine output), as well (4). Organ dysfunction is suggested when having an acute change in total SOFA scores of 2 points or more. This indicates an overall mortality risk of approximately 10% in a general hospital population with presumed infection. Baseline SOFA score is assumed to be zero except the case that the patient has any pre-existing organ dysfunction.

Septic shock has been termed as a subset of sepsis, which diagnostic criteria include persisting hypotension requiring vasopressors to maintain MAP ≥65 mmHg and plasma lactate levels >2 mmol/L despite adequate volume resuscitation.

Another important message of the consensus panel is that a novel bedside scoring system, quick SOFA (qSOFA), has been introduced for screening patients with suspected infection who likely develop poor outcomes. Based on 3 simple clinical parameters, it can be easily checked: GCS (<15), systolic blood pressure (≤100 mmHg) and respiratory rate (≥22/min). Two or 3 “positive” clinical parameters may indicate a poor prognosis and even suggest an undiscovered infection. Quick SOFA can be assessed without any laboratory tests therefore it can also be utilised on the regular floor. The diagnostic tree based on the Sepsis-3 consensus panel is illustrated in Figure 2.

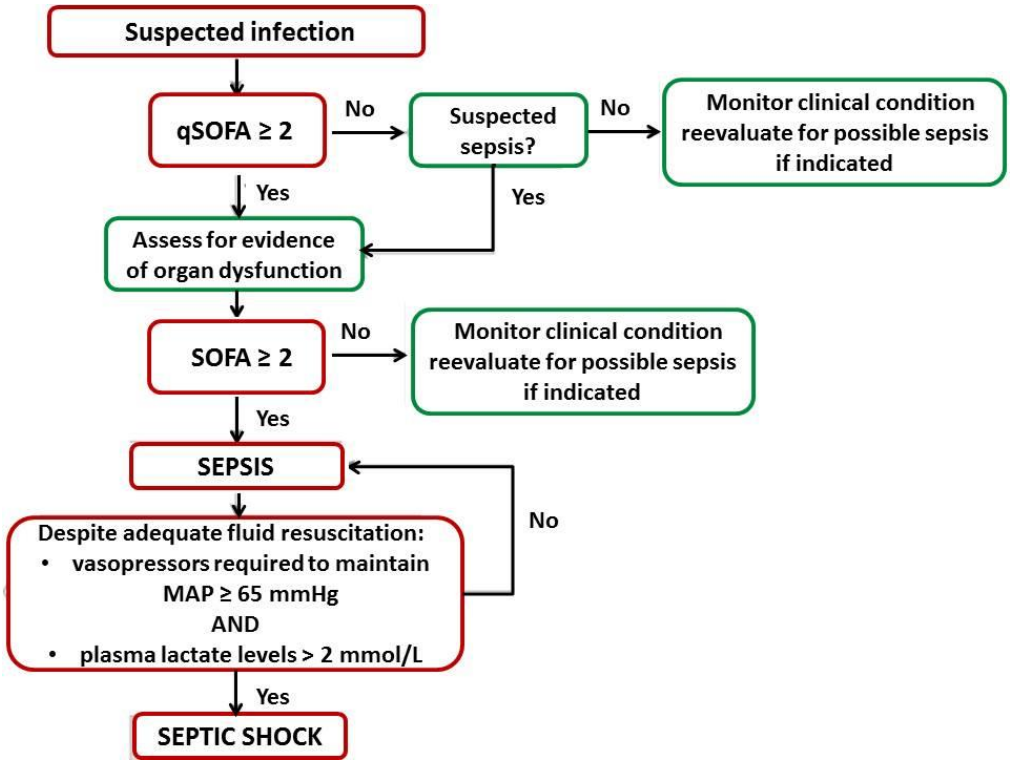

Figure 2. Diagnostic algorithm based on the Sepsis-3 definitions. Partly based on literature (3). MAP: mean arterial pressure; qSOFA: quick Sequential Organ Failure Assessment; SOFA: Sequential Organ Failure Assessment.

I.1.1.3. Sepsis-3 versus Sepsis-2 criteria: pros and cons

There is an ongoing debate regarding the superiority of the new sepsis terms. There are suggestions that organ dysfunction included into the new definitions is not an early sign of the syndrome which could lead to delayed treatment (5, 6). The new sepsis definitions are suggested to be more specific but less sensitive in terms of mortality prediction, when compared to Sepsis-2 criteria (7, 8). Members of the third consensus group (9) emphasized that although qSOFA criteria are clinically valuable but imperfect markers of sepsis, not a part of the sepsis definition and not a replacement for SIRS.

Recent and former septic shock definitions do not detect the same patients. Patients recognized by the former but not by the new septic shock definition might be underrated regarding illness severity and mortality risk (10, 11).

Experts suggest that a prospective, multicentre evaluation of the new sepsis terms and comparison of their validity with the previous definitions is mandated (3, 6, 12). Defining

sepsis must be a continuous process requiring revisions as new findings are present (13), and a middle road is proposed that takes the strengths of each definition (14).

I.1.2. Epidemiology

Global estimates suggest 19 million hospitalized individuals with sepsis per year (15). The syndrome has an increasing incidence (3–10/1000/year in high-income countries) due to aging populations with more comorbidities, growing bacterial drug resistance, more efficient recognition, but acute mortality of sepsis in adults is improving (3, 16, 17). According to a study performed by Csomós et al. (18), there were 2659 patients with septic Diagnosis Related Group (DRG) code in Hungary in 2001 with a mortality rate of 42.7%. Other studies also suggest that mortality of septic shock can still be as high as 40–55% and is accompanied with a 2- to 3-fold longer ICU and hospital stay (3, 19).

Risk factors for developing sepsis are infancy and older age, excessive use of tobacco and alcohol, implanted medical devices. Chronic medical conditions such as cancer, diabetes mellitus, chronic lung and kidney diseases, immunosuppression, peripheral artery and coronary artery diseases, stroke and dyslipidemia also serve as predisposing factors (20-28). Males and non-white races are more prone to develop sepsis (22, 24). Sepsis risk is suggested to increase with the number of chronic medical conditions (21). Functional polymorphisms in genes involved in innate immunity [toll-like receptors (TLR) 1 and 4] also predispose to Gram-negative infections and candidemia (29-33).

Around 70% of sepsis cases are community-acquired (34). According to the Extended Prevalence of Infection in Intensive Care (EPIC II) study, the site of infection is most commonly the lungs (64%), followed by the abdomen (20%), bloodstream (15%), and renal or genitourinary tract (14%). Among patients with positive microbiology, 47% of the microorganisms were Gram-positive (20% *Staphylococcus aureus*), 62% Gram-negative (20% *Pseudomonas spp*., 16% *Escherichia coli*) bacteria, and 19% fungi (35). Although *Streptococcus pneumoniae* is known to be the most frequent cause of bacterial pneumonia worldwide, it is rarely identified because of the difficulty of standard microbiological culturing (36). Patients with *Candida* bloodstream infection had much higher ICU mortality and length of hospital stay than those with bacteremia (37).

Survivor patients often have an increased risk for infection and usually suffer from long-term consequences including cognitive impairments and cardiovascular diseases (38-40).

I.1.3. Pathophysiology

The difficulty in defining sepsis lies in its diversified pathophysiology. It relies on a highly complex, integrated response that includes the activation of a number of cell types, inflammatory mediators, and the hemostatic system (41).

The innate immune system functions by broad recognition of antigens, mainly by detecting pathogen-associated molecular patterns (PAMP) located on the surfaces of common microorganisms (Figure 3) (42, 43). The systemic spread of a local response results in cytokine-chemokine storm (44). Similarly, cellular injury – as a result of burns, pancreatitis, trauma - can release endogenous damage-associated molecular patterns/alarmins (DAMPs; mainly from the mitochondria) that also activate innate immunity (45). At the beginning of SIRS, pro-inflammatory processes overwhelm the anti-inflammatory ones, and the adaptive immune response is initiated by Th1 reaction. Under normal circumstances, the pro-inflammatory process will be reduced after 2-4 days, while the adaptive response switches to a Th2 response. In the case of an unbalanced (pro-inflammatory and anti-inflammatory), dysregulated (unbalance between the relationship of Th1, Th2, Th17, and γδ T cells to each other) response, the localized process becomes systemic which leads to distant organ dysfunction. In the late phase of sepsis, the anti-inflammatory process may overwhelm the pro-inflammatory forces which results in cellular reprogramming: hematopoietic cells become hyporeactive while other cells (e.g. liver, lung, kidney) will be hyperreactive and develop hyperinflammation, especially in the infected organs. Anti-inflammatory responses are responsible for the enhanced susceptibility to secondary viral, and especially pulmonary, infections (42-44, 46, 47).

In sepsis endothelial dysfunction develops, which results in increased leukocyte adhesion, a shift to pro-coagulation, vasodilation, diminished barrier function, and finally in widespread tissue edema (48).

Microcirculatory alterations become manifest such as obstruction of microvessels by microthrombi, plugs of erythrocytes and white blood cells leading to tissue hypoxia (41). Microcirculatory failure is suggested to trigger late adaptive responses at the cellular level,

such as cellular metabolic alterations, mitochondrial dysfunction, and dysregulated apoptosis (49).

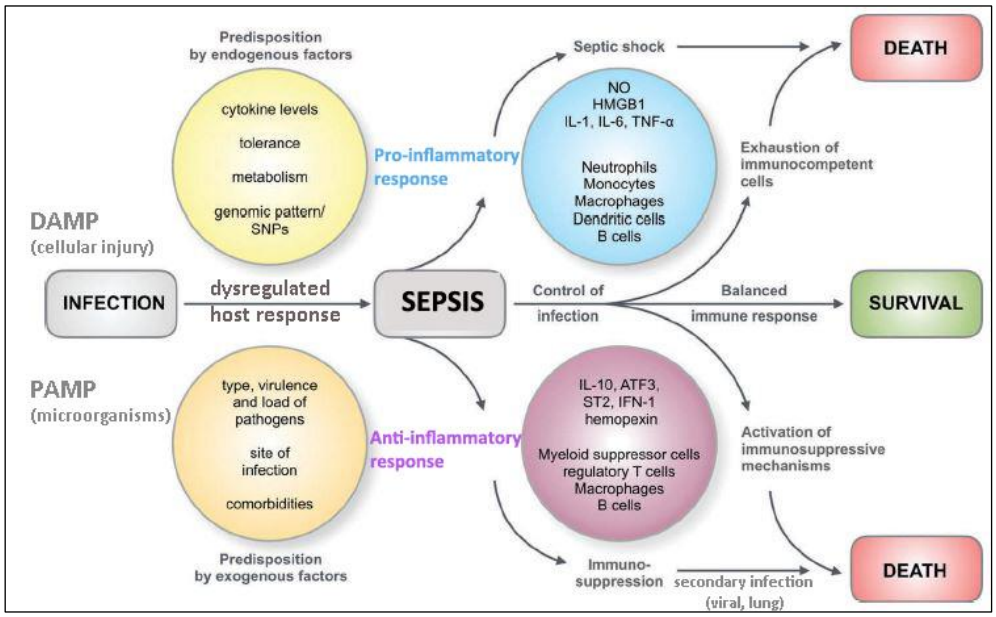

Figure 3. Immunological features in sepsis. Partly adopted from literature (42). ATF3: activating transcription factor 3; DAMP: damage-associated molecular pattern; HMGB1: high-mobility group box 1 protein; IFN-I: type-1-like interferon; IL: interleukin; NO: nitric oxide; PAMP: pathogen-associated molecular pattern; SNPs: single nucleotide polymorphisms; ST2: suppression of tumorigenicity 2; TNF-α: tumor necrosis factor alpha.

The clinical signs of sepsis are heterogenous. Frequently manifested acute organ dysfunction is located in the respiratory system and commonly appears in form of an acute respiratory distress syndrome (ARDS) (43). Cardiovascular system disturbances can be confirmed by the presence of worsening hemodynamic parameters including hypotension, or elevated plasma lactate levels. Later, myocardial dysfunction could develop, too (50). Signs of central nervous system dysfunction are ranging from mild cognitive disorder to coma. Acute kidney injury can be diagnosed by decreased urine output and increased serum creatinine levels. Metabolic and gastrointestinal disturbances as altered glycemic control, elevated aminotransferase levels, paralytic ileus could also develop. Common signs of coagulopathy are disseminated intravascular coagulation (DIC) and thrombocytopenia. Adrenal dysfunction and euthyroid sick syndrome may also be present in septic patients (43, 47).

I.1.4. Role of laboratory diagnosis

I.1.4.1. Microbiological identification

Unfortunately, results of traditional culturing methods become available 24–48 hours at the earliest (46). Every hour of delay in antibiotic treatment over the first 6 hours of presentation to hospital is suggested to increase the mortality of septic shock by 7.6% (51). Therefore, rapid microbiological detection methods with short turnaround time (TAT) are of paramount importance. PCR (polymerase chain reaction) assays performed directly on whole blood samples can rapidly (TAT: 4-8 h) detect several pathogens and some antibiotic resistance markers, however, the reported too many false positive and false negative test results limit their reliability (52, 53). Matrix assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) is well-proved, fast (TAT<1h) and highly accurate method for identifying pathogens, although it better detects Gram-negative bacteria than Gram-positives or yeasts (54). Despite the advantages of the reported methods, blood culture is still considered as the gold standard diagnostic test.

I.1.4.2. Biomarkers

Biomarker measurements are mandatory, partly because of inefficacy of standard culturing methods. Currently, there are almost 200 potential sepsis markers under investigation (55). Serum procalcitonin (PCT) is the most widely used marker in sepsis. It is a prohormone of calcitonin normally produced by the thyroid C cells. Interestingly, in severe systemic inflammation PCT is secreted by many tissues (56). Its clinical advantage lies partly in the fact that it rapidly becomes detectable (2-6 hours) after the onset of severe bacterial infection. Normally, it reaches its peak in 12-48 hours and - in case of adequate treatment – its concentration will be reduced by roughly 50% per day (57, 58). PCT may accurately differentiate SIRS from sepsis (59) moreover, promising studies came to light regarding PCT-guided antibiotic therapy (60). However, PCT levels also rise after burn, cardiogenic shock, surgery, trauma, liver cirrhosis, kidney failure and its concentration does not increase markedly in fungal sepsis.

Frequently investigated acute-phase protein in sepsis is high-sensitivity C-reactive protein (hsCRP). It is a pentraxin secreted by hepatocytes (61). In response to acute inflammatory

events its serum concentration can increase up to 1000-fold, however, it responds slower to the septic insult than PCT and reaches its peak concentration after 2-3 days. Also, the plasma kinetics of hsCRP is slower compared with PCT (46). Even local infections could trigger hsCRP synthesis, which limits its specificity for sepsis. Serial measurements of hsCRP could inform about the adequacy of antibiotic therapy (62).

Increasing interests have been dedicated to studying serum presepsin, the soluble form of CD14, which is released during sepsis. The receptor CD14 is expressed on the membrane of macrophages and monocytes and plays an important role in the activation of toll-like-receptor 4 after lipopolysaccharide stimulation. Serum levels of presepsin rise earlier than those of PCT, and are suggested to inform about the severity of sepsis which makes it a challenging diagnostic and predictive marker (63).

I.2. Actin and actin-binding proteins

I.2.1. Actin

I.2.1.1. Structure, isoforms

Actin (molecular weight [MW]: 42 kDa; 375 amino acid residues) is a ubiquitous, conserved protein expressed in all eukaryotic cells (64, 65). The protein was first isolated in 1942 from muscle extracts at the University of Szeged, Hungary (66). Actin owns 6 species-independent isoforms encoded by different genes located on chromosome 7 in humans: 2 cytoplasmic (β, γ), 2 smooth muscle (α, γ) and 2 striated muscle isoforms (α-skeletal, α-cardiac). The protein exists in two main forms, in globular (G)/monomeric and filamentous (F)/polymeric forms (Figure 4). Globular actin constitutes of one large (residues 145–337; subdomain III and IV) and one small (residues 1–144 and 338–375; subdomain I and II) domain. High-affinity nucleotide and cation binding sites are located in the deep interdomain cleft of G-actin. In addition, globular actin contains multiple cation-binding sites that - when appropriately saturated - initiate polymerization to form actin filaments (67-72).

I.2.1.2. Functions of intracellular actin

In the cell, actin filaments are suggested to be dynamic, responsive elements (71). Intracellular actin is known to be involved in cell motility, structure, turnover, intercellular signaling and

gene expression (73, 74, 75). Furthermore, a well-known fact is that muscle contraction is based on the interaction of filamentous actin with myosin (76). Actin cytoskeleton also contributes in the development of various diseases (e.g. genetic diseases, bacterial invasion, cancer) (77-82).

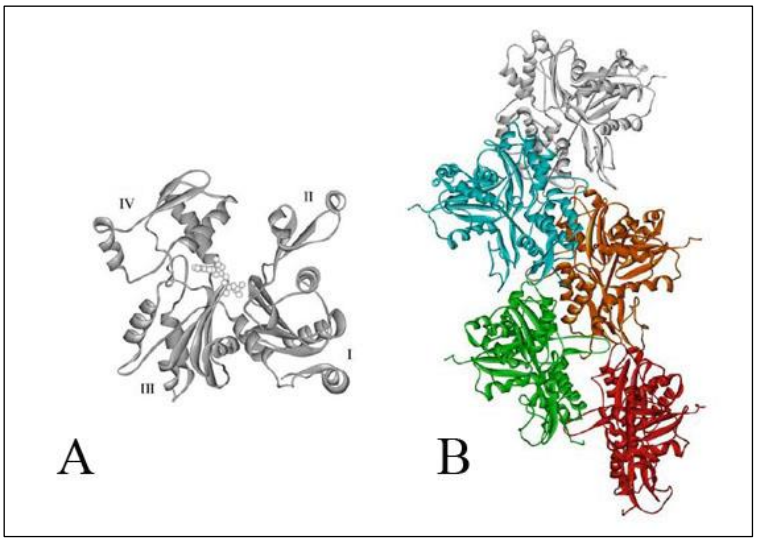

Figure 4. Structure of globular (A) and filamentous actin (B). A: G-actin contains 4 subdomains (I-IV) and includes one ADP (adenosine diphosphate) molecule (indicated by white color). Adopted from (67). B: F-actin is a helical polymer and can develop a length up to several micrometers (70). Adopted from (71).

I.2.1.3. Actin in the extracellular space

Actin is also present in the extracellular environment: at the outer cell surfaces, in the extracellular matrix and in the extracellular fluids, as well (83-91). Actin release from cells could occur as a result of apoptosis/necrosis thereby acting as DAMP (92, 93), but it could also stem from viable, undamaged cells (94). Cell surface and extracellular matrix-associated actin may have several functional roles, as membrane-bound actin could activate plasminogen, and could even inhibit neoplastic progression (95-97).

There is a suggested tendency towards actin microfilament formation in the extracellular space (98, 99). Human studies proved the presence of circulating α-actin in the plasma of patients with angina pectoris and myocardial infarction (85, 100). Furthermore, detectable concentrations of α-actin in the serum of patients with type II diabetes and neuropathy were suggested as potential risk factors of acute myocardial infarction (101). Recently, plasma α-smooth muscle actin was found to be helpful for the diagnosis of intestinal muscle damage in

rats and in humans, too (102). Circulating actin was also detectable in the plasma of septic patients admitted to the ICU, and of chronic hemodialysis patients (103-105).

Excessive amounts of free circulating actin could have toxic effects. Animal study of Haddad et al. reported that injection of G-actin results in intravascular microfilament formation leading to the production of microthrombi and endothelial damage (98). In addition, cell culture experiments revealed that the addition of serum of healthy volunteers saturated by G-actin or serum from ARDS patients to endothelial cells exerted direct toxic effect (106). ADP-bound F-actin was shown to activate platelet aggregation (107). Further in vitro study demonstrated the incorporation of actin microfilaments into fibrin clots which impeded their lysis as a result of plasmin-binding and inhibition (108, 109). Circulating plasma actin may also enhance the severity of *Escherichia coli* infections by promoting alpha-hemolysin production (110).

Clearance mechanism for actin in the systemic circulation occurs by actin-binding proteins: gelsolin (GSN) and group specific component (Gc) globulin. Elimination sites include the reticuloendothelial system and possibly the kidney (91, 98, 99, 103, 111, 112).

I.2.2. Gelsolin

I.2.2.1. Structure, isoforms

There have been reported more than 100 intracellular actin-binding proteins from those the most widely studied are the members of the so-called gelsolin superfamily (71, 113). The eponymous member of the superfamily is GSN, which was first described in 1979 as a calcium-dependent regulatory protein being capable to transform macrophage extracts from viscous gels to fluid sols (114). It is encoded by a gene on chromosome 9 (q32-q34) in humans and composes of six conserved GSN domains (G1 - G6, from the N- to the C-terminus) (113, 115-118) (Figure 5). Monomer actin binding sites are located in domain G1 and G4–6, while segments G2–3 own the highest affinity for F-actin. In the absence of Ca^{2+}, the actin monomer binding sites on G1 and G4 and the filament side-binding site on G2 are hidden within the globular arrangement of the inactive protein. Activation by Ca^{2+} is a multistep process, the details of which have remained largely speculative (111, 115, 116, 119).

There are two main isoforms of GSN in mammals: cytoplasmic and extracellular/secreted/plasma GSN. Cytoplasmic GSN consists of 731 amino acid residues (MW: 80.6 kDa) and is expressed ubiquitously, while plasma GSN is by 24 amino acid

residues longer at its N-terminus (MW: 83 kDa) than cytoplasmic GSN and is mainly synthetized by skeletal muscle cells. Plasma GSN concentration varies between 150-300 mg/L in healthy individuals, but it is highly method dependent (120). Secreted GSN was also detected in urine, lymph- and cerebrospinal fluid (86, 91, 121). In addition, a lesser known isoform, gelsolin-3, had also been identified in oligodendrocytes and in testis, which contains an 11-residue N-terminal extension compared with the cytoplasmic isoform (116, 119, 122, 123).

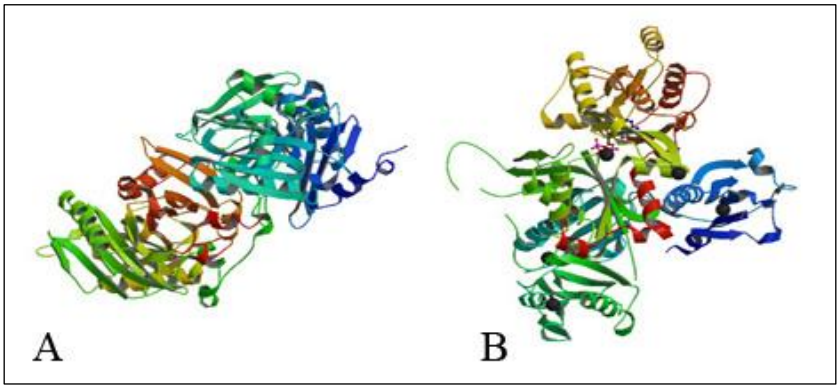

Figure 5. Structures of Ca^{2+}-free human gelsolin (A) and human G1–G3 GSN domains complexed with actin (B). A: GSN consists of six domains (G1-G6) indicated by different colors (PDB ID: 3FFN) (118). B: The structure of human G1–G3 GSN domains bound to actin. Ca^{2+} ions are represented with black spheres (PDB ID: 3FFK) (118).

I.2.2.2. Actin scavenger function

Actin binding and severing activity of GSN is regulated by Ca^{2+}, pH, phosphoinositides and lysophosphatidic acid (114, 124). The conditions of ionic strength, composition, temperature and pH in the extracellular compartment promote actin polymerization (111). As outlined in subsection I.2.1., formation of high amounts of F-actin in the bloodstream could be fatal after actin is released in excess from injured cells in severe systemic inflammation or massive tissue injury.

In order to avert the potential deleterious effects of filament formation, an extracellular actin scavenging system (EASS) has evolved (112, 125-127). In blood plasma, the EASS is built on two proteins: plasma GSN and Gc-globulin or vitamin D binding protein (VDBP). GSN has the ability to sever filamentous actin into short oligomers and Gc-globulin – because of its higher affinity to G-actin – non-covalently sequesters actin monomers at a stoichiometric ratio of 1:1 (Figure 5B, 6B). In addition, one molecule of GSN also can bind 2 molecules of G-actin

thereby directly promoting the depolymerization of actin filaments (125, 126). Under physiological circumstances, the concentration of the two actin-binding proteins is far higher than that of plasma actin. Gc-globulin complexed with monomer actin is suggested to be cleared by Kupffer cells, and removal of filamentous actin (±GSN) occurs by hepatic endothelial cells (128).

In severe systemic inflammation and in tissue injury, the extracellular actin scavenging system gets partly overwhelmed by excessive amounts of intravascular actin (103, 126, 129). GSN and Gc-globulin complexed with actin are suggested to be cleared more rapidly than the free proteins, their expected half-lives being normally 12–24 h (Gc-globulin) and 55.2 hours (GSN), respectively (112). Animal studies demonstrated that the half-life of Gc-globulin complexed with monomer actin is markedly reduced (about 30 min) (112). Since the synthetic rate of plasma GSN in the skeletal muscle cells proved to be unchanged even in severe systemic inflammatory syndromes, its concentration declines rapidly. In contrast to GSN, plasma Gc-globulin behaves similarly to positive acute-phase proteins in inflammatory disorders (130-132).

I.2.2.3. Non-actin scavenger roles in inflammatory processes

Apart from its actin-binding capacity, plasma GSN is suggested to have other protective roles in systemic inflammation, thereby acting as a buffering protein. Plasma GSN binds lipopolysaccharide (LPS) from Gram-negative bacteria with high affinity in vitro, which inhibits its actin-binding and depolymerizing capacity (133). Also, LPS-mediated inhibition of thrombin activity could be reversed by plasma GSN (134). While GSN is able to partially inhibit LPS- or lipoteichoic acid (LTA)-induced release of IL-8 from human neutrophils, it seems to be unable to prevent bacterial growth (135). Animal study also supported the protective role of GSN in endotoxinaemia based on inhibition of cytokines (136).

Plasma GSN binds to sphingosine 1-phosphate, a pleiotropic cellular agonist involved in various immune responses (137). In vitro study by Osborn et al. indicated that recombinant human plasma GSN caused marked inhibition of platelet activating factor (PAF)-mediated platelet and neutrophil inflammatory responses (138).

However, functions of extracellular GSN and the mechanisms leading to its protective nature are not fully understood yet.

I.2.2.4. Serum gelsolin in inflammatory diseases and injury

Reduced serum GSN levels have been found in intensive care-related disorders, autoimmune diseases, chronic kidney diseases, hepatitis, and malaria (129, 139-160).

Patients of intensive care units – including those after trauma or major surgery - with depressed plasma GSN levels were characterized by increased mortality rate, hospital stay, and delayed period of mechanical ventilation (139, 140). Studies regarding burn patients suggested GSN to be a valuable marker for severe complications, including MODS and sepsis (143, 144). Studies which investigated septic and other critically ill patients so far, showed an association between low plasma GSN levels and sepsis occurrence, and mortality (103, 145). Recent proteomic study regarding plasma of septic patients stressed abnormally regulated cytoskeletal proteins, a higher expression of GSN and depletion of actin in mononuclear cells of survivors compared to non-survivors (147). Plasma GSN concentration was significantly lower in patients with ischemic stroke than in healthy controls, and it was proven to predict 1-year mortality (149).

In autoimmune processes, as multiple sclerosis (MS), or IgA nephropathy, GSN levels were found to be significantly lower than in controls (151, 152).

In a study regarding chronic hemodialysis patients, low blood levels of GSN were associated with progressive aortic arch calcification (154).

I.2.2.5. Therapeutic approaches

Based on the potential of actin-scavenging and anti-inflammatory properties of GSN, there are many efforts regarding the replenishment of reduced plasma GSN levels. Recombinant plasma GSN was proven to have beneficial effects in several animal models including those dealing with inflammatory disorders, injury or neurological diseases (161-166). Recombinant human GSN was first administered in humans as an inhalational agent to decrease the viscosity of airway secretions from patients with cystic fibrosis (167, 168). Recent in vitro study revealed that pGSN could even restore lung macrophage defense mechanisms against bacteria by binding free actin (169).

I.2.3. Gc-globulin

I.2.3.1. Structure, isoforms

Serum Gc-globulin (MW = 52–59 kDa; 458 amino acids) is a glycosylated α_2-globulin (170, 171). It is encoded on chromosome 4 (q11-13) and belongs to the albuminoid superfamily, which also includes albumin, α-fetoprotein, and α-albumin/afamin, respectively (172, 173). Gc-globulin is mainly synthetized by hepatocytes and its serum concentration ranges between 200 and 600 mg/L in healthy individuals (similarly to that of GSN it is highly method dependent) (174).

It constitutes three structurally similar α -helical domains (from N to C terminus, domains I-III) (Figure 6A) (175). There are 3 codominant alleles of Gc-globulin, known as Gc1s, Gc1f, and Gc2 (176-178). Additionally, more than 120 unique racial variants and single nucleotide polymorphisms regarding Gc-globulin have been described (178, 179).

The geographical variation of Gc allele frequencies is related to skin pigmentation and relative sunlight exposure: populations with pale skin have low Gc1f and relatively high Gc1s allele frequency. Caucasians own high Gc2 allele frequency, while Gc1f is widespread among Africans (132, 171, 178).

Gc-globulin racial variants are connected with different serum levels of the protein, being relatively high in Gc1-1, intermediate in Gc1-2, and low in Gc2-2 phenotypes (180). Serum Gc-globulin is characterized by a diurnal rhythm with a decrease in the morning, followed by a rapid increase during the day (181). Its concentration seems not to change during the course of aging. Serum Gc-globulin levels also depend on the catabolic ratio of serum vitamin D, and they are positively associated with worsening lipid profile, oral contraceptive use, smoking, and a similar positive association is suggested with body mass index, although contradictory results were also demonstrated (171).

I.2.3.2. Functions in health and diseases

Gc-globulin works in concert with GSN to scavenge free actin from the circulation, where acting as a potent monomer-trapping protein (details in subsection I.2.2.2.), (Figure 6B). Major Gc-globulin phenotypes are reported to have an equal actin binding affinity (171).

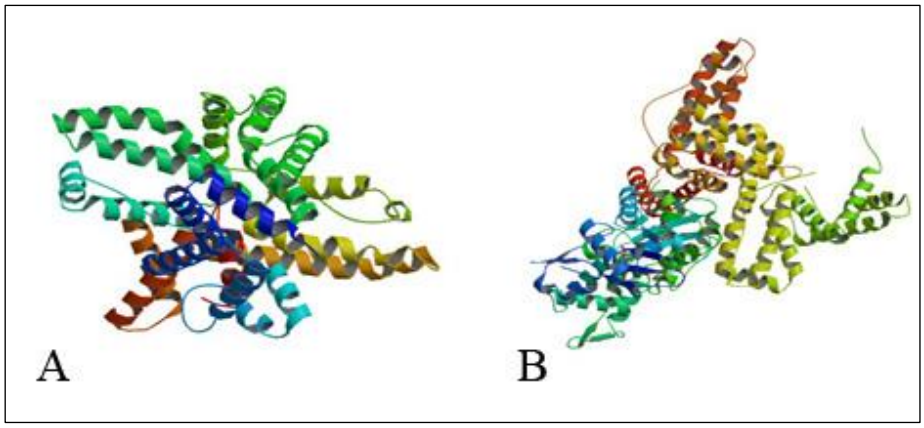

Figure 6. Uncomplexed Gc-globulin (A) and Gc-globulin complexed with skeletal actin (B). A: Gc-globulin is built up of three similar α-helical domains. Domains I and II can be subdivided further into two structurally related subdomains (PDB ID: 1KW2) (175). B: Gc-globulin is suggested to ‘‘clamp’’ onto actin by narrowing the angle between domain I on one side and domains II and III on the other side (PDB ID: 1KXP) (175).

Other key role of Gc-globulin is the transport of vitamin D_3 ligands by domain I (hence its other name, VDBP) (182). It binds 25(OH)-vitamin D_3 and 1,25$(OH)_2$-vitamin D_3 more tightly than albumin, which transports only 10-15% of vitamin D metabolites. Gc-globulin is present in a 20-fold molar excess compared with vitamin D ligands, therefore it is suggested to have a protective role against vitamin D toxicity and may serve as a reservoir for 25(OH)-vitamin D_3 (171). The preservation of serum 25(OH)-vitamin D_3 levels and their conversion to 1,25$(OH)_2$-vitamin D_3 is regulated by megalin-mediated endocytosis of Gc-globulin-bound 25(OH)-vitamin D_3 in the proximal tubules of the kidney (183). In kidney damage, Gc-globulin could be excreted into the urine and may serve as an indicator of kidney failure (184, 185).

Gc-globulin, as the other members of the albuminoid superfamily, can bind and transport fatty acids, although at a less extent than albumin (186).

In case of Gram-negative infections, Gc-globulin can bind and inhibit endotoxins (187). Gc-globulin is also suggested to have co-chemotactic activity for C5a in inflammatory processes (171, 188, 189). Among others, it binds with low affinity to chondroitin sulfate proteoglycans, such as CD44, which, associated with annexin A2 is part of the leukocyte surface binding site complex and mediates the chemotactic cofactor effect (190). On the other hand, it can support neutrophil recruitment by binding oleic acid, a tonic inhibitor of chemotaxis. Actin or 1,25$(OH)_2$-vitamin D_3 binding neutralizes its co-chemotactic function (171). Gc-globulin

could also indirectly influence T cell responses through vitamin D metabolites, which is suggested to be a complex, multifactorial process (191).

Gc-globulin can be converted by enzymes of activated T and B cells through partial deglycosylation to Gc-macrophage activating factor (Gc-MAF) (171). Gc-MAF may have beneficial effect on bone health (171). In addition, it has been proposed that tumor cells efficiently inactivate Gc-MAF by endoglycosidase. Hence, replenishment of Gc-MAF may restore the capability of macrophages to eliminate neoplastic cells (192, 193). Despite of several promising animal and human studies (194), the true positive effect of Gc-MAF administration still remains questionable (171).

There is a growing body of evidence that polymorphisms in the Gc-globulin gene could predict adverse outcomes in several chronic and infectious diseases (195). For example, patients with Gc1f genotype are suggested to have high risk for developing chronic obstructive pulmonary disease (COPD) whereas Gc2 genotype may serve as a protective factor (196).

Apart from serum, Gc-globulin has been detected in a variety of body fluids (urine, breast milk, ascitic fluid, cerebrospinal fluid, saliva, seminal fluid) (171).

I.2.3.3. Serum Gc-globulin in injury and inflammatory diseases

Reduced serum Gc-globulin levels have been observed in trauma patients (131, 141, 197-200), in those suffering from sepsis (198, 200-205), and acute liver failure (206-208).

Studies demonstrated a direct correlation between serum concentration of Gc-globulin and the survival rate of trauma victims, moreover serial measurement of Gc-globulin levels could be used for early identification of patients with increased risk of mortality (131, 199). Further suggested clinical use of Gc-globulin could be to detect trauma patients prone to sepsis.

Clinical studies have demonstrated low Gc-globulin levels in sepsis which were associated with poor survival rates and an increased risk of developing organ dysfunctions (198, 200, 201). Leaf et al. (202) found lower Gc-globulin concentrations in critically ill patients with acute kidney injury than in controls which was not associated with mortality.

Depressed levels of serum Gc-globulin are associated with poor outcome in acute liver failure, where organ failures seem to inversely correlate with its serum levels (206-208).

II. Objectives

II.1. So far, the time dependent changes of both serum actin and gelsolin levels in human sepsis have not been investigated. We aimed to monitor changes and predictive values of serum levels of actin, gelsolin and of a recently defined new marker: actin/gelsolin ratio in SIRS and in severe sepsis.

We carried out a 5-day follow-up study when analysing sera of severe septic (n=32) and SIRS (n=12) patients. Ophthalmologic patients (n=27) served as controls. Serum actin and gelsolin levels were assessed by modified Western blot methods. Besides serum actin, gelsolin and actin/gelsolin ratios, classical laboratory parameters (WBC count, serum PCT, hsCRP) and clinical scores (APACHE II, SAPS II, SOFA) were also assessed. Furthermore, we investigated the predictive values of the studied markers in the diagnosis of sepsis, for 7-day and overall intensive care unit mortalities, too.

II.2. Even up to now, a rapid automated measurement of gelsolin has still remained a challenge. Therefore, our second task was to develop and validate a fast immune turbidimetric assay for serum gelsolin that would be suitable for possible routine clinical use. Our further objective was to adapt a rapid immune turbidimetric assay for serum Gc-globulin.

Validation of serum gelsolin assay was performed on an open developmental channel of the c502 module of Cobas 8000/c502 analyzer (Roche) according to the second edition of Eurachem guidelines. Regarding the clinical evaluation of our gelsolin assay, we studied the diagnostic value of serum gelsolin in sepsis when investigating the first-day sera of septic (n=25), SIRS (n=8) and control patients (n=14). We compared our previously published Western blot data with those of the new turbidimetric assay by Bland-Altman analysis. Performance testing of the adapted serum Gc-globulin assay was also carried out according to the second edition of Eurachem guidelines.

III.3. Simultaneous, rapid determination of the two main serum actin scavenger proteins in sepsis has not been investigated yet. Since rapid immune turbidimetric assays became available for the determination of both gelsolin and Gc-globulin, we aimed to investigate their predictive values together in sepsis.

A 5-day follow-up was performed including 46 septic, 28 non-septic patients while 35 outpatients served as controls. Serum Gc-globulin and gelsolin levels were determined by automated immune turbidimetric assay on a Cobas 8000/c502 analyzer. Intensive care patients were retrospectively categorized according to the Sepsis-3 definitions. Predictive values of the different markers were examined by receiver operating characteristic curves and logistic regression analysis when comparing them with the classic inflammatory parameters (PCT, hsCRP) and SOFA clinical scores.

III. Patients and methods

III.1. Patient categorization

Our study protocol was authorized by the Regional Research Ethical Committee of the University of Pécs (4327.316-2900/KK15/2011) and was performed according to the ethical guidelines of the 2003 Helsinki Declaration. Written informed consent was obtained from all enrolled patients or their appropriate surrogates after detailed information regarding the study design and blood sampling.

In our first investigation, patients with established diagnosis of SIRS or severe sepsis from the Department of Anesthesiology and Intensive Therapy (University of Pécs, Hungary) were enrolled in our follow-up study from January 2013 till December 2014. SIRS and severe sepsis were defined according to the criteria of the American College of Chest Physicians/Society of Critical Care Medicine and the 2012 Surviving Sepsis Guidelines (2). Patients with SIRS fulfilled two or more of the following findings: body temperature >38°C or <36°C, tachycardia (>90/min), tachypnea (respiratory rate >20/min) or $PaCO_2$ <32 mmHg, white blood cell count >12,000/mm^3 or <4000/mm^3 or >10% immature bands. Further SIRS criteria were negative blood culture results and PCT levels <1 ng/mL. Diagnostic criteria for severe sepsis included SIRS plus confirmed or presumed infection, elevated PCT levels (>2ng/mL in bacterial sepsis) and one or more organ dysfunction induced by sepsis. Defined end points were the withdrawal of consent or death during the study period. The control group consisted of ambulatory ophthalmologic patients. Intensive care patients were excluded if they were under 18 years of age or where it was not possible to obtain patient consent or consultee approval. Control patients under the age of 18 years and those suffering from acute inflammation or infectious disease were excluded from the control group. Both 7-day and overall ICU mortalities were investigated.

In our second clinical investigation, patients from the Department of Anesthesiology and Intensive Therapy were enrolled in our follow-up study from January 2013 till August 2016. Septic patients diagnosed according to the former Sepsis-2 guidelines (2) were retrospectively categorized as stated by the Sepsis-3 definitions (3) into sepsis and septic shock groups. Hereafter, we performed our study based on the latter categorization. Organ dysfunction should be determined as an acute change in SOFA score (≥2 points) as a consequence to the

infection. The baseline SOFA scores were assumed to be zero in every patient since no clinical scores were estimated before the ICU admission. Septic shock was defined as a subset of sepsis in which persisting hypotension (requiring vasopressors to maintain MAP ≥65 mmHg) was present and plasma lactate levels were more, than 2 mmol/L despite adequate volume resuscitation. Non-septic ICU patients had negative blood culture results and PCT levels were below 1.0 ng/mL. Voluntary blood donors and preoperative ophthalmologic patients having their blood counts, liver enzyme activities and hsCRP levels in the reference range served as controls. Among septic patients, 14 - day mortality was investigated. Intensive care unit (ICU) patients were excluded if they suffered from any autoimmune disorders, pre-existing hepatic failure or were under 18 years of age. Control patients under the age of 18 years and those with symptoms of acute inflammatory diseases or suffering from autoimmune disorders were also excluded from the study.

Patients were followed in both of our studies during their ICU stay where serum samples were obtained on day 1, 2, 3 and 5 after clinical diagnosis.

III.2. Blood sampling

Venous blood (7.5 mL) was drawn from every patient via central venous catheter or from a peripheral vein into plain tubes with accelerator gel using a closed blood sampling system (BD Vacutainer®). After 30 minutes, clotted blood samples were centrifuged for 10 min at 1500 g and sera were immediately analyzed or stored at −80°C.

III.3. Determination of serum actin and gelsolin levels by Western blot

After the centrifugation (10 min at 1500g) of clotted blood samples, sera were immediately treated with electrophoresis sample buffer and heated at 100°C. Using 10% SDS-PAG electrophoresis by Laemmli (209), serum actin and GSN levels were determined by quantitative chemiluminescence Western blot based on the work of Lee et al. (103). Polyclonal primary antibodies (Rabbit Anti-Human Actin, N-terminal, ref. no: A2103, Sigma-Aldrich Co. LLC; Rabbit Anti-Human Gelsolin, ref.no: A0146, Dako A/S) and horseradish peroxidase-labeled secondary antibodies (Swine Anti-Rabbit Immunoglobulins, ref.no: Z0196,

Dako A/S) were applied. In every gel, the same pretreated control serum sample of a healthy individual was run as internal standard.

Quantification of Western blot was performed by using highly purified G-actin standard obtained from rabbit skeletal muscle (Department of Biophysics, University of Pécs, Hungary (210). For standardization of GSN, purified recombinant human GSN expressed in *Escherichia coli* (His-8) (Department of Biophysics, University of Pécs, Hungary (118)) was used. The Western blots were calibrated by running dilution series of actin and GSN standards of known concentrations on separate gels. Quantification was done after densitometry (Syngene, Cambridge, UK) of the chemiluminescence signal of the standards and establishing a calibration line (light signal vs. protein concentration). The internal standard was applied to every gel in the subsequent experiments therefore patients' data were calculated by interpolation. A/GSN ratios were derived from the same day's actin (mg/L) and GSN (mg/L) levels of each patient.

III.4. Validation of an automated immune turbidimetric assay for serum gelsolin

III.4.1. Reagents, assay conditions

The immune turbidimetric assay for se-GSN measurement was performed on an open developmental channel of the c502 module of a Cobas 8000 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Because of the unavailability of any commercial GSN calibrator for immune turbidimetric assay, we applied recombinant human GSN expressed in *Escherichia coli* (His-8) (Department of Biophysics, University of Pécs, Hungary (118)). Dilution series of the calibrator were prepared using fetal bovine serum (FBS, ref. no. Ph. Euro. 2262, PAN Biotech, Aidenbach, Germany). The FBS did not give any reaction with the anti-human GSN antibody, therefore, we could use it as a serum matrix imitating the physicochemical properties of human serum samples. Pooled human serum from healthy volunteers served as an “in-house” control, due to the lack of commercially available quality control material. We used Polyclonal Rabbit Anti-Human GSN antibody in the assay (ref. no. A0146, Dako A/S, Glostrup, Denmark) pre-diluted (1:4) with Dilution Buffer (ref. no. S2005, Dako A/S); and Reaction Buffer (ref. no. S2007, Dako A/S), based on the previous work of Christensen et al. (211) with modifications.

The sample volume was 7 µL, the volume of the reaction buffer was 100 µL and that of the pre-diluted (1:4) antibody was 50 µL. The wavelength applied for the turbidimetric reaction was 340 nm and delta absorbance was calculated from the data obtained between 41-70 measuring points. The two-point end assay was performed at 37°C with 10-minute reaction time. Full RCM calibration was done by applying a six-point standard curve in the range of 10-260 mg/L gelsolin standards.

III.4.2. Validation of the gelsolin assay

The second edition of Eurachem guidelines (212) was applied for the validation. FBS with GSN buffer (containing no human GSN) was utilized as blank sample (FBS:buffer ratio was similar to that of the highest GSN calibrator). Limit of blank (LOB: mean + 1.645 × SD), limit of detection (LOD: mean + 3 × SD) and limit of quantification (LOQ: mean + 10 × SD) were assessed using the absorbance data of 30 independent blank samples.

Intra-and inter-assay imprecisions were estimated by three different sera (low, middle and high GSN concentrations).

For the assessment of intra-assay variability, 15 parallel measurements were performed on the same day, while for inter-assay variability two parallel measurements were executed on 10 consecutive days. For recovery studies, aliquots of both the low and high GSN concentration serum samples were spiked with two different amounts of human purified GSN standard, thereafter recovery (%) was calculated from the measured (n = 15/level) and calculated values. Linearity was assessed by two parallel measurements for each level of 10 dilutions of a serum sample in the range of 11.18 - 201.2 mg/L GSN.

Stability studies were performed by examining five different serum samples (ranging from 32.94 to 122.14 mg/L) when storing them at +2−8°C. Se-GSN levels were determined on the 1st, 3rd, 5th, 8th and 10th day, respectively. Furthermore, stability of se-GSN was evaluated in the course of 5 freezing-thawing cycles. Aliquots of 5 different sera were frozen at −70°C and subsequently thawed at 20-25° C. After every cycle, se-GSN levels were assessed.

The validated immune turbidimetric GSN assay was compared with the previous Western blot method by Bland-Altman plot procedure.

III.5. Determination of serum Gc-globulin levels by immune turbidimetry

III.5.1. Reagents, assay conditions

The immune turbidimetric assay for serum Gc-globulin measurement was executed on an open developmental channel of the c502 module of a Cobas 8000 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Serum Gc-globulin levels were measured according to the modified protocol of Bangert (213) by using Polyclonal Rabbit Anti-Human Gc-Globulin antibody (ref. no. A0021, Dako A/S, Glostrup, Denmark) pre-diluted (1:5) with Dilution Buffer (ref. no. S2005, Dako A/S); Reaction Buffer (ref. no. S2007, Dako A/S); Human Serum Protein Calibrator (ref. no. X0908, Dako A/S) and Human Serum Protein Low Control (ref. no. X0939, Dako A/S).

The sample volume was 2.5 μL, the volume of the reaction buffer was 100 μL and that of the pre-diluted (1:5) antibody was 50 μL. The wavelength applied for the turbidimetric reaction was 340 nm and delta absorbance was calculated from the data obtained between 9-49 measuring points. The two-point end assay was performed at 37°C with 10-minute reaction time. Full RCM calibration was done by applying a six-point standard curve in the range of 28.89 - 385 mg/L Gc-globulin standards.

III.5.2. Adaptation of the Gc-globulin assay

The second edition of Eurachem guidelines (212) was applied to test the performance of the adapted Gc-globulin assay. Analytical limits were calculated as described above using the absorbance data of 20 independent blank samples.

Intra-and inter-assay imprecisions were estimated by 2 different levels of the control material (ref. no. X0939). For the assessment of intra-assay variability, 10 parallel measurements were performed on the same day, while for inter-assay variability 2 parallel measurements were executed on 10 consecutive days. Linearity was assessed by 2 parallel measurements for each level of 7 dilutions of a serum sample in the range of 8 - 332 mg/L Gc-globulin.

Additional stability studies were performed by examining a serum sample (307.76 mg/L) when storing it at +2−8°C. Serum Gc-globulin levels were determined on 6 consecutive days. Stability of se-Gc-globulin was investigated in the course of 5 freezing-thawing cycles, too. Aliquots of 2 different sera (Gc-globulin concentration: 55 mg/L and 340 mg/L) were frozen

at −70°C and subsequently thawed at 20-25° C. After every cycle, se-Gc-globulin concentrations were determined.

III.6. Determination of routine laboratory parameters and clinical scores

All other laboratory parameters including total blood cell counts, plasma lactate, serum albumin, hsCRP and PCT levels were determined by automated routine laboratory techniques. qSOFA scores were assessed based on ICU admission parameters. APACHE II (Acute Physiology and Chronic Health Evaluation II), SAPS II (Simplified Acute Physiology Score II) and SOFA scores were estimated for the first day of intensive care treatment. Mean arterial pressure (MAP) was assessed by intra-arterial blood pressure monitoring in the ICU.

III.7. Statistical analysis

For statistical analysis IBM SPSS Statistics for Windows, Version 22 and Origin Pro 8 softwares were used. Distribution of data was evaluated by Shapiro-Wilk test. Bland-Altman plot was used for method comparison regarding GSN assay. Power analysis was applied to calculate the required number of patients in each group. Kruskal-Wallis, Mann-Whitney and chi-squared tests were performed for investigating differences between patient groups. Friedman's analysis and post hoc Wilcoxon signed-rank tests were performed for follow-up comparisons. Predictive values were assessed by receiver operating characteristic (ROC) curves, COX regression and logistic regression analyses. Correlations between quantitative parameters were determined by Spearman's rank correlation test. Data are expressed as medians and as interquartile ranges (IQR). Changes in the results were considered to be statistically significant at $p<0.05$.

IV. Results

IV.1. Serum actin, gelsolin and actin/gelsolin ratios in SIRS and in sepsis

IV.1.1. Clinical and laboratory parameters

The clinical and routine laboratory parameters of the enrolled patients are listed in Table III. Control patients were similar in sex and age compared with septic patients. More ICU patients (36.4%) than controls (7.4%) suffered from COPD. The majority of the patients (63.6%) were admitted to the ICU after surgical interventions (e.g. management of acute abdomen, Whipple procedure, etc.) and 36.4% of them after other medical events (e.g. pneumonia). Among the first-day routine laboratory and clinical parameters, we observed significantly higher serum PCT ($p<0.001$), hsCRP levels ($p<0.001$), APACHE II ($p<0.001$), SAPS II ($p<0.001$) and SOFA ($p<0.05$) scores in septic compared with SIRS patients.

Among the routine laboratory and clinical parameters, non-survivor sepsis patients exhibited significantly ($p<0.05$) higher PCT levels and clinical scores than survivors (Table IV). In non-survivors 81.8%, while in survivors 71.4% of the patients developed MODS. Common organ dysfunctions in sepsis were acute renal failure (65.6%) and acute lung injury (50%), in 21.8% of the septic patients we found thrombocytopenia, in 12.5% acute hepatic failure also developed. Hemoculture was positive in 18.8% of the septic patients, in 53.1% of the patients pathogens were detected in other specimen sources (e.g. bronchoalveolar lavage, intra-abdominal abscess, urine) and in 28.1% of the cases the source of infection remained unidentified. Most common pathogens were Gram-positive (coagulase negative *Staphylococci, Enterococci*) and Gram-negative bacteria (*Escherichia coli, Pseudomonas species*), in 28.1% of the patients fungal infections (mostly *Candida species*) were also present.

Table III. Clinical and routine laboratory data of the enrolled patients (first study).

Clinical data	**Control (n=27)**	**SIRS (n=12)**	**Sepsis (n=32)**	**p value**
Age, y	65 (56-74)	67 (55-77)	67 (58-78)	n.s.
Males, n (%)	11 (40.7)	9 (75)	19 (59.4)	n.s.
COPD, n (%)	2 (7.4)	5 (41.6)	11 (34.4)	<0.05
Type II DM, n (%)	4 (14.8)	3 (25)	6 (18.8)	n.s.
CVD, n (%)	19 (70.4)	10 (83.3)	22 (68.8)	n.s.
CKD, n (%)	1 (3.7)	0	1 (3.1)	n.s.
Immunological diseases	0	0	4 (12.5)	
Malignancy, n (%)	0	5 (41.6)	13 (40.6)	<0.05
Cause of admission:				
Internal medicine origin, n (%)		3 (25)	13 (40.6)	
Surgical origin, n (%)		9 (75)	19 (69.4)	
ICU treatment days		2 (2-3.8)	7 (4-12.5)	<0.01
First-day parameters:				
APACHE II scores		9.5 (6.3-14.8)	20.0 (12.3-25.0)	<0.001
SAPS II scores		25.5 (18.0-36.3)	51.0 (34.3-66.0)	<0.001
SOFA scores		6.5 (4.3-9.8)	9.0 (7.0-11.8)	<0.05
se-albumin	46.3 (44-48.1)	30.9 (25.3-33.5)	22.2 (18.5-26.3)	<0.001
se-hsCRP, mg/L	1.4 (0.7-3.9)	85.1 (46.3-201.3)	253.2 (158.2-324.9)	<0.001
se-PCT, ng/mL		0.4 (0.3-0.7)	17.3 (4.4-67.7)	<0.001
se-actin, mg/L	3.0 (2.1-3.7)	2.5 (2-4.1)	3.5 (1.6-6.1)	n.s.
se-GSN, mg/L	64.1 (37.9-88.6)	31.6 (23.6-40.8)	15.2 (5.8-24.1)	<0.01
se-A/GSN	0.04 (0.03-0.08)	0.1 (0.06-0.14)	0.26 (0.1-0.8)	<0.05

Data are expressed as medians, in parentheses percentages and interquartile ranges (25-75%) are given. Level of significance is set at $p<0.05$. Abbreviations: COPD: chronic obstructive pulmonary disease; CKD: chronic kidney disease; CVD: cardiovascular disease; n.s.=non-significant; Type II DM: Type II diabetes mellitus.

Table IV. Clinical, laboratory and microbiological characteristics of septic survivor and non-survivor patients based on 7-day mortality (first study).

Clinical data:	**Survivors (n=21)**	**Non-survivors (n=11)**	**p value**
APACHE II scores	16 (12-20)	28 (24-36.5)	<0.001
SAPS II scores	39 (33-54)	68 (47.5-78)	<0.01
SOFA scores	8 (7-9)	13 (10-18)	<0.001
Organ dysfunctions:			
1	6	2	
2	9	2	
≥3	6	7	
First-day parameters:			
se-albumin, mg/L	26 (22.4-31.2)	19.2 (15.8-22.3)	<0.05
se-hsCRP, mg/L	193.7 (143.9-286.1)	287.6 (216-336.4)	n.s.
se-PCT, ng/mL	10.3 (3.9-21)	59.9 (33.5-101)	<0.05
se-actin, mg/L	3.1 (1.7-5.4)	4.9 (1.6-7.4)	n.s.
se-GSN, mg/L	16.9 (9.5-34.6)	8.9 (1.8-17.9)	<0.05
se-A/GSN	0.15 (0.09-0.46)	0.71 (0.19-3.26)	n.s.
Identified microorganisms:			
Gram-positive	3		
Gram-negative	4	1	
Gram-positive+Gram-negative	4	2	
Gram-negative+fungi	1	1	
Gram-positive+Gram-negative+fungi	5	2	
Unidentified	4	5	

Clinical scores and laboratory data are expressed as medians, in parentheses interquartile ranges (25-75%) are given, organ dysfunctions and microbiological findings are given with numbers. Level of significance is set at $p<0.05$.

IV.1.2. Serum actin, gelsolin levels and actin/gelsolin ratios in critically ill patients and in controls

Analyzing the first-day serum actin levels in sepsis, SIRS and control patient groups, non-significant differences were observed (Table III). First-day serum GSN concentrations were found to be the lowest in the sepsis group, significantly higher GSN levels were observed in SIRS compared to sepsis ($p<0.01$), and the highest values were obtained in controls ($p<0.001$) (Figure 7A). Regarding first-day serum A/GSN ratios, the highest values were observed in sepsis, significantly ($p<0.05$) lower A/GSN ratios were obtained in SIRS and the lowest values were found in controls ($p<0.001$) (Figure 7B).

Well-defined changes were observed regarding the surviving and the non-surviving severe septic patients' parameters (Table IV). Control patients had significantly ($p<0.001$) higher serum GSN levels than survivors and non-survivors, moreover, survivors showed significantly ($p<0.05$) higher GSN levels in their sera than non-survivors (Figure 7C). Non-survivor and survivor patients' A/GSN ratios were significantly ($p<0.001$) higher than those observed in controls (Figure 7D).

Figure 8 illustrates the follow-up of surviving and non-surviving patients. In the first 5 days, 28.1% of the septic patients died while further 21.9% of them required no more intensive therapy and were released from the ICU. Higher median values of serum actin levels were observed in non-survivors than in survivors during the follow-up, although not being statistically significant (Figure 8A). Serum GSN levels were found to be higher in survivors compared with non-survivors on day 1 (Table IV) and on day 3 as well (day 3 median levels in survivors vs. non-survivors: 22.95 mg/L vs. 3.69 mg/L; $p<0.05$; Figure 8B). Patients who failed to survive sepsis had significantly higher 2nd day's A/GSN ratios than survivors (median A/GSN ratios in non-survivors vs. survivors: 2.18 vs. 0.19; $p<0.05$; Figure 8C).

IV.1.3. Spearman's correlation analysis

Serum GSN levels were found to correlate inversely with PCT ($\rho= -0.38$, $p<0.05$), hsCRP levels ($\rho= -0.65$, $p<0.01$), SAPS II ($\rho= -0.37$, $p<0.05$), SOFA clinical scores ($\rho= -0.35$, $p<0.05$) and positively with serum albumin levels ($\rho=0.43$, $p<0.01$). A/GSN ratios positively correlated with hsCRP concentrations ($\rho=0.43$, $p<0.01$) and SOFA clinical scores ($\rho=0.32$, $p<0.05$).

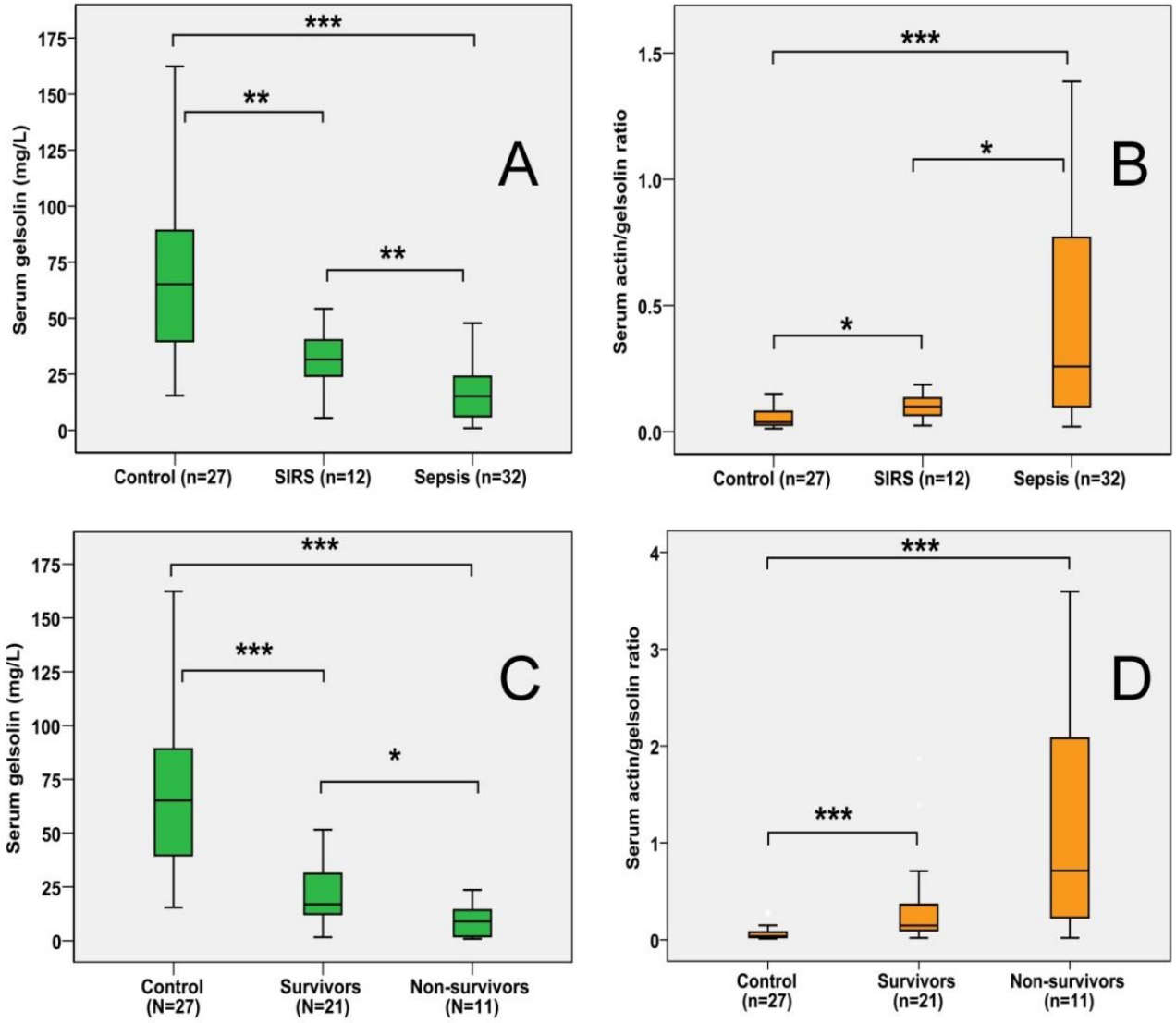

Figure 7. First-day serum GSN levels and A/GSN ratios in septic, SIRS and control patients (A, B), and in septic survivors, non-survivors based on 7-day mortality (C, D). Significance level is set at $p<0.05$ (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$).

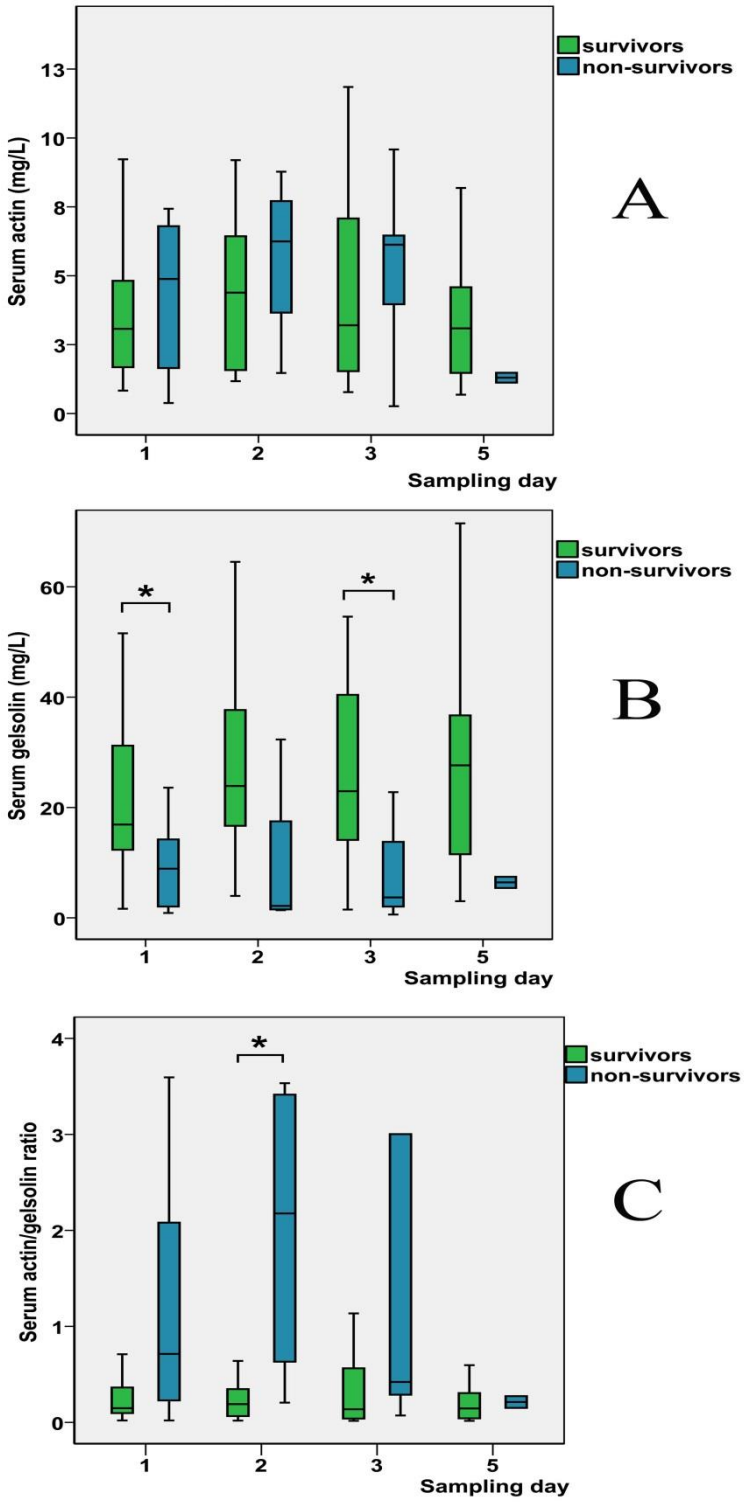

Figure 8. Time course of serum actin (A), GSN levels (B) and A/GSN ratios (C) in sepsis. Septic patients are divided into groups based on 7-day mortality. Significance level is set at $p<0.05$ (*: $p<0.05$).

IV.1.4. Receiver operating characteristics (ROC) and COX regression analyses

Regarding 7-day mortality in sepsis, ROC analysis showed that area under the curve (AUC) value for PCT was found to be 0.75, for GSN it was 0.74 (both significant at $p<0.05$, Figure 9A, B). The derived cut-off value for GSN was found to be 11.38 mg/L (sensitivity: 76.2%, specificity: 72.7%). AUC values for A/GSN (0.70) and for hsCRP (0.66) did not meet criteria for statistical significance. For differentiating patients with sepsis from those with a non-infective systemic inflammatory response, AUC were 0.95 for serum PCT, 0.84 for hsCRP, 0.77 for GSN and 0.70 for A/GSN ratios, respectively (both significant at $p<0.05$, Figure 9C, D). Cut-off values were 21.04 mg/L for GSN (sensitivity: 83.3%, specificity: 68.7%) and 0.10 for A/GSN (sensitivity: 68.8%, specificity: 66.7%).

For determining the predictive value for overall ICU mortality of the studied markers, COX regression analysis was performed. Based on evaluation of the first-day laboratory parameters (hsCRP, PCT, actin, GSN levels, A/GSN ratios) and clinical scores (APACHE II, SAPS II, SOFA) of septic patients we found hazard ratios (HR) and confidence intervals (CI) as follows: HR=1.208; 95% CI =1.083 – 1.347 ($p=0.001$) for A/GSN ratios and HR=1.172; 95% CI=1.079 – 1.273 ($p<0.001$) for APACHE II scores. These two parameters were able to predict the outcome of sepsis.

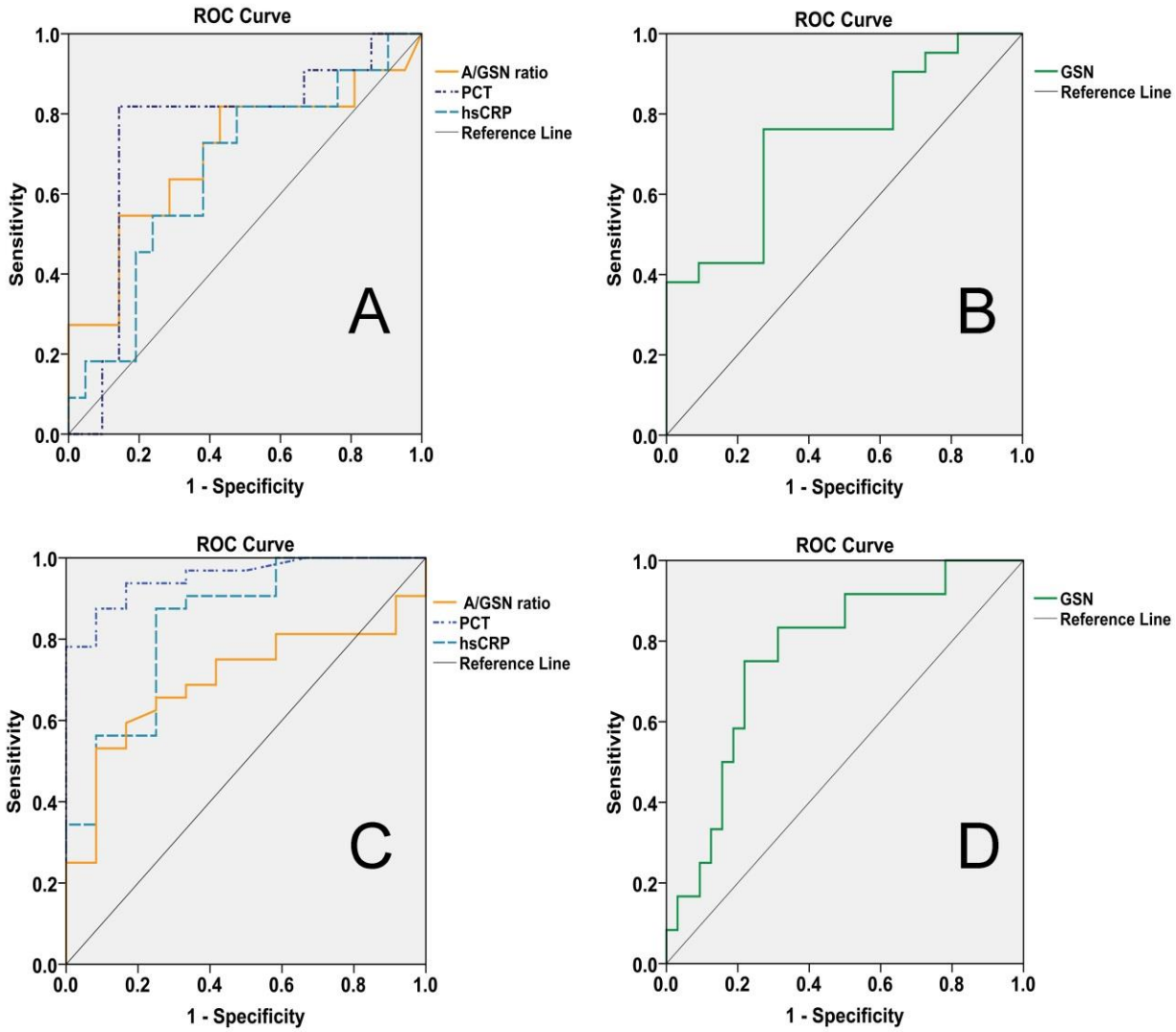

Figure 9. Receiver operating characteristic curves of serum PCT, hsCRP, A/GSN ratios (A) and GSN (B) for predicting 7-day mortality in sepsis and those for distinguishing SIRS from sepsis (C, D).

IV.2. Methodological developments

IV.2.1. Validation results regarding serum gelsolin assay

IV.2.1.1. Validation data and stability studies

Figure 10A represents a cumulative graph of 9 independent calibrations performed during the validation period. LOB, LOD, LOQ were found to be 0.47 mg/L, 0.72 mg/L and 1.99 mg/L, respectively. Coefficient of variation remained below 5% in most of the cases during the intra- and inter-assay variability measurements. Recovery varied between 84.56 - 93.52% when investigating 4 different ranges (Table V).

Table V. Intra-, inter-assay precision and recovery of serum gelsolin assay

	Intra-assay (n=15)				**Inter-assay (n=20)**	
Sample	Mean ±SD (mg/L)	CV%	Recovery% (n=15)		Mean ±SD (mg/L)	CV%
L	20.34±0.79	3.93	87.90	84.56	21.03±1.05	4.99
M	69.91±1.40	2.00	-		69.93±2.06	2.94
H	112.51±3.04	2.71	93.52	92.57	113.30±5.95	5.25

L=low GSN concentration sample, M=middle GSN concentration sample, H=high GSN concentration sample. For recovery study both of the low and the high GSN concentration samples were spiked with purified human GSN.

Linearity study gave an appropriate coefficient by the linear regression analysis (r^2= 0.998) after comparing calculated and measured GSN concentrations regarding 10 different dilutions (Figure 9B).

GSN levels remained almost unchanged (96.70-117.36%) during the 10-day stability period, and no considerable differences were observed even throughout five repeated freezing-thawing cycles (Table VI).

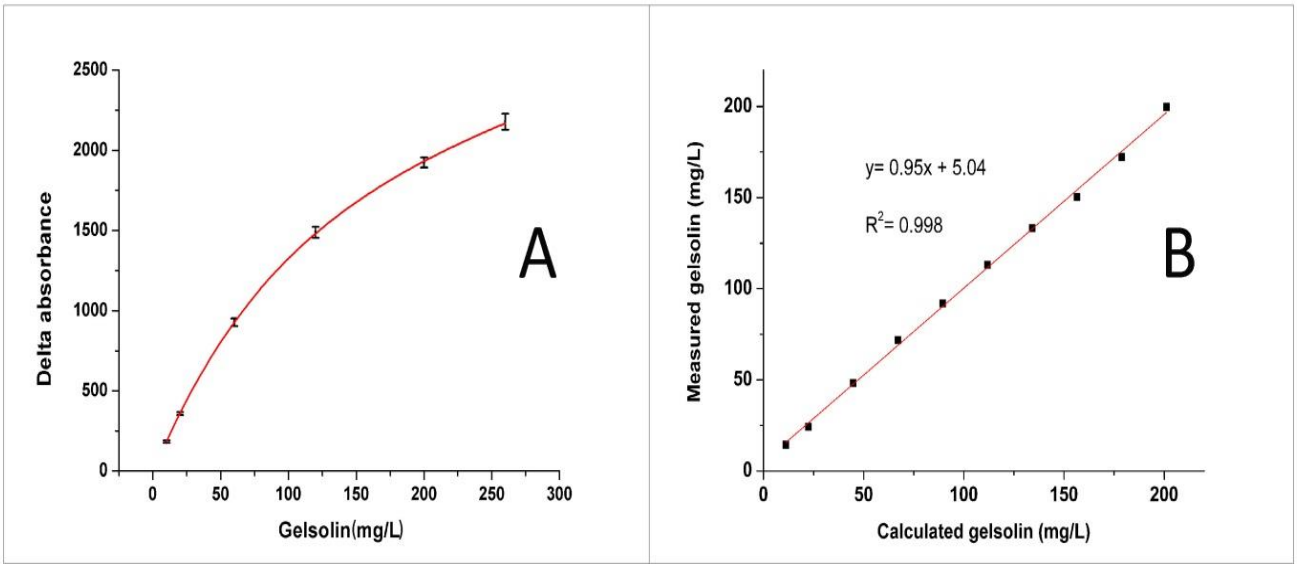

Figure 9. Validation results. **A:** Summarized graph of a 6-point calibration curve of GSN assay in the range of 10–260 mg/L by exponential graph fitting (n=9). **B:** Linearity of serum GSN assay. Dots represent means. R^2: regression coefficient.

Table VI. Stability of se-GSN

Initial GSN concentration (mg/L)	Storage at +4°C				Storage at -70°C				
	Day 3 [%]	Day 5 [%]	Day 8 [%]	Day 10 [%]	Cycle 1 [%]	Cycle 2 [%]	Cycle 3 [%]	Cycle 4 [%]	Cycle 5 [%]
32.94	102.17	100.39	108.58	110.41	100.99	102.97	104.56	106.76	107.37
14.88	96.70	101.84	114.84	109.61	107.01	106.27	106.27	93.78	98.90
77.72	103.16	105.87	105.61	107.97	103.29	103.16	105.35	108.10	105.74
60.89	104.19	112.72	114.05	117.36	106.46	106.32	113.01	114.80	115.10
122.14	101.47	98.99	100.34	104.84	101.47	101.13	101.13	100.68	102.05

IV.2.1.2. Comparison of previous Western blot and recent immune turbidimetric results

Table VII illustrates the main demographic and laboratory parameters of the enrolled patients. Septic and SIRS patients exhibited significantly lower first-day serum GSN levels than controls when measured by immune turbidimetry ($p < 0.001$) (Figure 10A). Furthermore, serum GSN levels were lower in septic than in SIRS patients ($p = 0.015$). Previous Western blot results showed a similar pattern to those of immune turbidimetry except that among the

limited number of subjects there was no significant difference between serum GSN levels of septic and SIRS patients.

Table VII. Data of the studied patients (second study)

	Sepsis (n=25)	**SIRS (n=8)**	**Control (n=14)**	**p value**
Demographic data				
Age, y	73 (30-82)	58 (50-73)	63 (38-78)	n.s.
Male/female	18/7	6/2	7/7	n.s.
1st day clinical scores				
APACHE II	17.50 (12-25)	8 (5.25-13.25)	-	<0.01
SAPS II	51 (37.25-66)	24 (16.50-29.75)	-	<0.001
SOFA	9 (7-12)	5.50 (4.25-9.75)	-	<0.05
1st day laboratory data				
se-hsCRP, mg/L	216.0 (127.75-306.39)	68.50 (46.34-116.19)	1.24 (0.22-1.92)	<0.01
se-PCT, ng/mL	18.64 (4.64-92.10)	0.35 (0.18-0.49)	-	<0.001[a]
se-GSN (IT), mg/L	12.08 (3.89-19.97)	24.67 (13.11-25.94)	58.99 (44.34-63.83)	<0.05[a]; <0.001[b, c]
se-GSN (WB), mg/L	16.74 (8.21-34.57)	26.31 (16.89-38.25)	74.87 (30.67-91.83)	<0.01[b, c]

Data are expressed as medians (25-75% percentiles), except for age (median (min – max)). Level of significance is set at $p < 0.05$. Superscript lowercase letters refer to post-hoc analyses. a: sepsis-SIRS; b: sepsis-control; c: SIRS-control. APACHE II: Acute Physiology and Chronic Health Evaluation II; SAPS II: Simplified Acute Physiology Score II; SOFA: Sequential Organ Failure Assessment); IT: immune turbidimetry; WB: Western blot.

Bland-Altman plot defined a bias of 0.26 and an agreement range from −0.79 to 1.09 units when comparing GSN levels of septic, SIRS and control patients measured by previous Western blot method and by the new turbidimetric assay (Figure 10B).

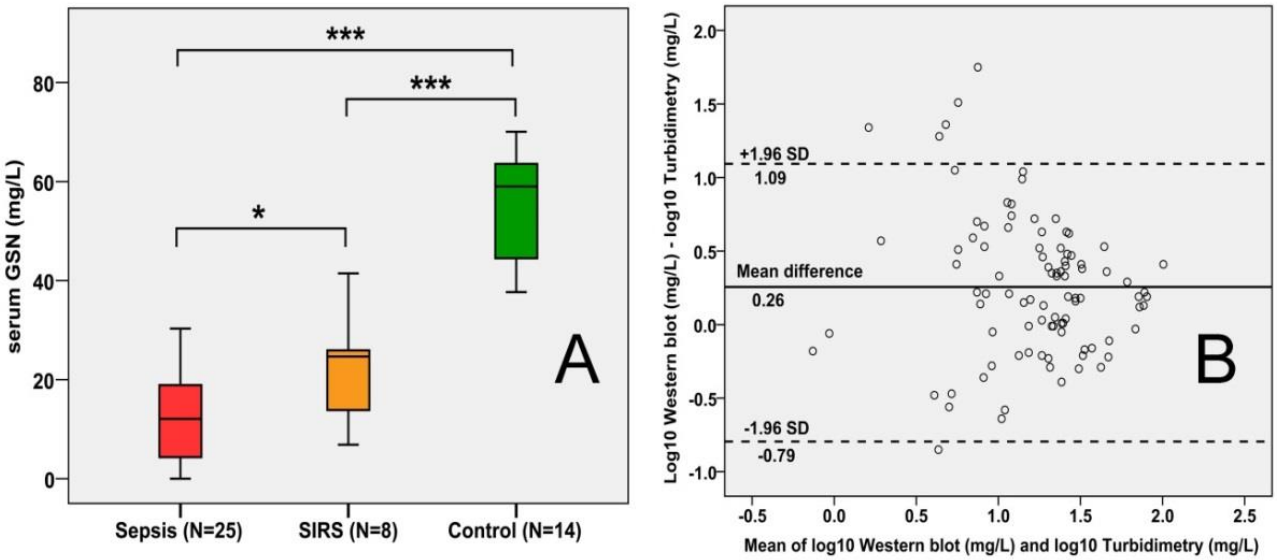

Figure 10. A: Serum GSN levels in septic, SIRS and control patients measured by immune turbidimetry. Significance level is set at $p<0.05$ (*: $p<0.05$, **: $p<0.01$, ***: $p<0.001$). B: Bland-Altman plot: comparing the data of Western blot and immune turbidimetric measurements. Because of the non-normally distributed differences, lg of the data was calculated.

We also investigated whether first-day se-GSN measured by immune turbidimetry could differentiate between the septic and SIRS states. Area under the curve (AUC) value for se-GSN was found to be 0.79, the determined cut-off point was 14.05 mg/L (sensitivity: 75%; specificity: 60%), (Figure 11A). Western blot measurement offered a lower AUC (0.63) for se-GSN when compared to immune turbidimetry. Serum PCT and high-sensitivity C-reactive protein (hsCRP) had an AUC of 0.98 and 0.85, respectively (Figure 11B).

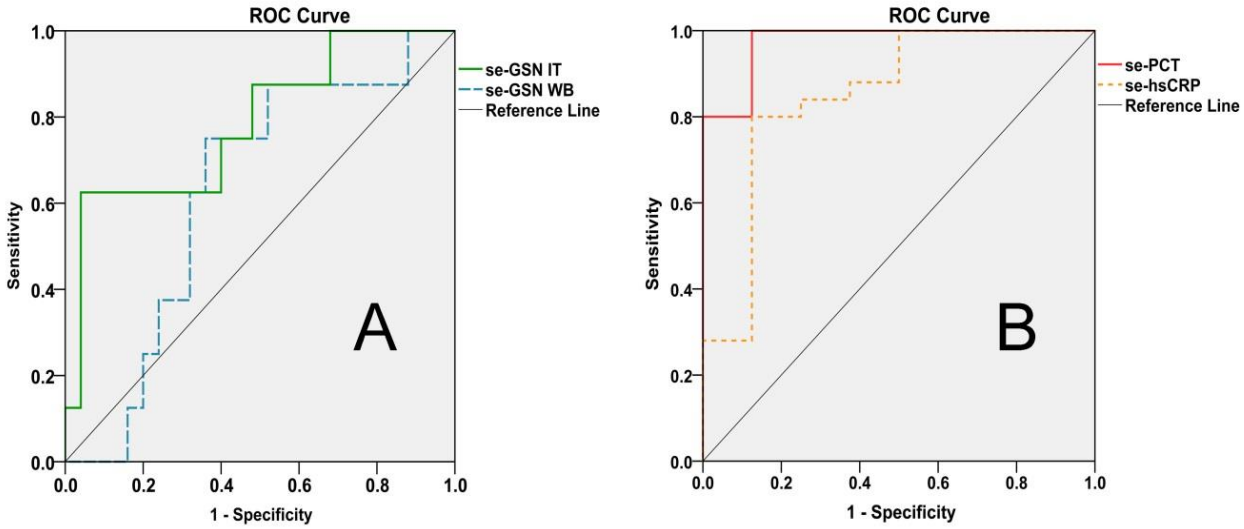

Figure 11. Receiver operating characteristic (ROC) curves of serum laboratory parameters in sepsis. A: ROC of se-GSN. B: ROC of se-PCT and se-hsCRP. IT: immune-turbidimetry; WB: Western blot.

IV.2.2. Performance data of serum Gc-globulin assay and stability studies

LOB, LOD, LOQ were found to be 0.43 mg/L, 0.66 mg/L and 1.85 mg/L, respectively. Intra-assay precision was found to be between 1.38 – 1.64% of CV, while inter-assay imprecision was estimated to be 5.03% of CV (Table VIII).

The method for Gc-globulin determination was found to be linear (r^2=0.995) in the range between 8 mg/L to 332 mg/L after comparing calculated (percentage) and measured Gc-globulin concentrations regarding 7 different dilutions (Figure 12).

No considerable differences of Gc-globulin concentration were noticed during the 6-day stability period and even throughout five repeated freezing-thawing cycles (Table IX).

Table VIII. Intra-, inter-assay precision and recovery of serum Gc-globulin assay

	Intra-assay (n=10)		**Inter-assay (n=20)**	
Sample	Mean ±SD (mg/L)	CV%	Mean ±SD (mg/L)	CV%
L	62.79±1.03	1.64	66.90±3.37	5.03
H	318.26±4.41	1.38	336.38±16.93	5.03

L=low Gc-globulin control (estimated concentration: 60.8 mg/L), H=high Gc-globulin control (concentration: 304 mg/L).

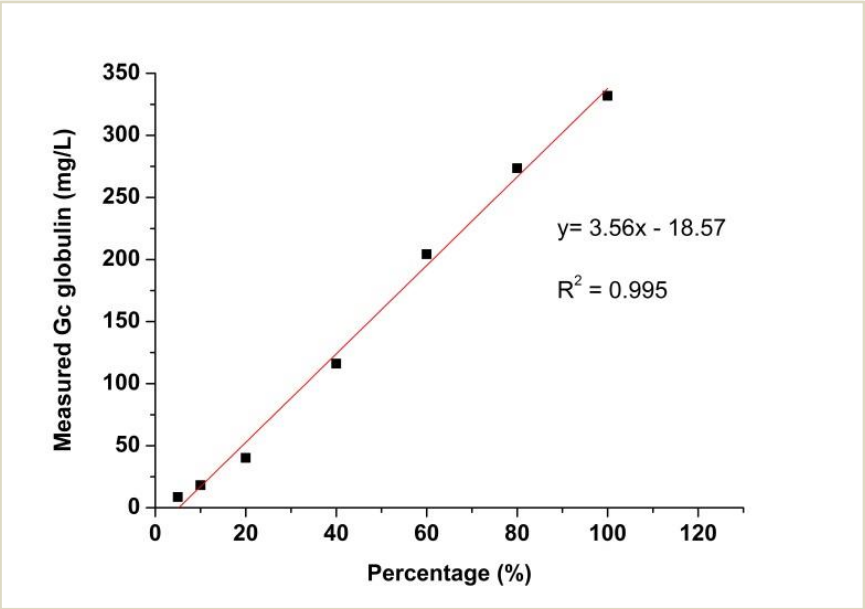

Figure 12. Linearity of serum Gc-globulin assay. Dots represent means. R^2: regression coefficient.

Table IX. Stability of serum Gc-globulin

Initial Gc concentration (mg/L)	Storage at +4°C				Storage at -70°C				
	Day 1 [%]	Day 2 [%]	Day 4 [%]	Day 6 [%]	Cycle 1 [%]	Cycle 2 [%]	Cycle 3 [%]	Cycle 4 [%]	Cycle 5 [%]
340	-	-	-	-	99.97	99.14	101.04	100.44	99.27
55	-	-	-	-	99.46	100.67	98.62	100.56	99.89
307.76	99.43	97.80	97.97	98.02	-	-	-	-	-

IV.3. Assessment of predictive values of both gelsolin and Gc-globulin in sepsis

IV.3.1. Main clinical and laboratory results

We found predominantly males in their 60ies suffering from sepsis and septic shock, and their data were compared with those of non-septic ICU patients (Table X). Septic and septic shock patients were treated about 7.5-times longer at the ICU than non-septic intensive care patients ($p<0.05$). More (74.3%) ICU patients required intensive care treatment after surgical investigations (e.g. management of ileus, pancreatic cancer surgery) than those after other internal medicine complications (e.g. COPD exacerbation, pneumonia-related respiratory insufficiency).

Data of control, non-sepsis and sepsis patients were comparable regarding age, gender and comorbidities.

In the intensive care patients' groups qSOFA scores did not alter significantly. First-day APACHE II scores differed ($p<0.05$) in all three ICU groups, while SAPS II scores were higher ($p<0.01$) in sepsis and in septic shock patients when compared with the non-septic group. SOFA scores were more increased ($p<0.01$) in septic shock compared with sepsis and non-sepsis patients. Serum albumin levels were different ($p<0.01$) in all enrolled patients' groups. Classic inflammatory markers (serum hsCRP, PCT) were significantly ($p<0.001$) higher in septic shock and in sepsis patients compared with non-septic ICU patients.

IV.3.2. Serum gelsolin and Gc-globulin levels in septic shock, septic, non-septic patients and in controls

First-day serum levels of both actin-binding proteins were significantly ($p<0.001$) higher in the control population than in the ICU patients (Table X, Figure 13A, B). Non-septic ICU patients exhibited higher ($p<0.001$) first-day serum GSN concentrations than patients with sepsis and septic shock. First-day Gc-globulin levels were significantly higher in non-septic critically ill patients ($p<0.001$) and in septic patients ($p<0.01$) when compared with those suffering from septic shock.

In the course of the 5-day follow-up period, three septic patients died and another 5 septic patients were discharged earlier from the ICU. Four septic shock patients died during the observation days while 9 of them survived. Patients suffering from sepsis had significantly

($p<0.01$) higher serum Gc-globulin levels during the 5-day follow-up when compared with those seen in septic shock (Figure 13D) (median Gc-globulin concentrations were as follows in sepsis vs. septic shock: 1st day: 237 vs. 82.1 mg/L; 2nd day: 295.6 vs. 197.2 mg/L; 3rd day: 257.8 vs. 188.5 mg/L; 5th day: 267.2 vs. 116.4 mg/L, respectively). Significantly ($p<0.05$) increased 2nd and 5th day's serum Gc-globulin levels were detected in septic patients when compared with the 1st day concentrations.

The two investigated patient groups did not differ significantly regarding serum GSN levels during the 5-day follow-up and no increasing or decreasing tendency was noted (Figure 13C).

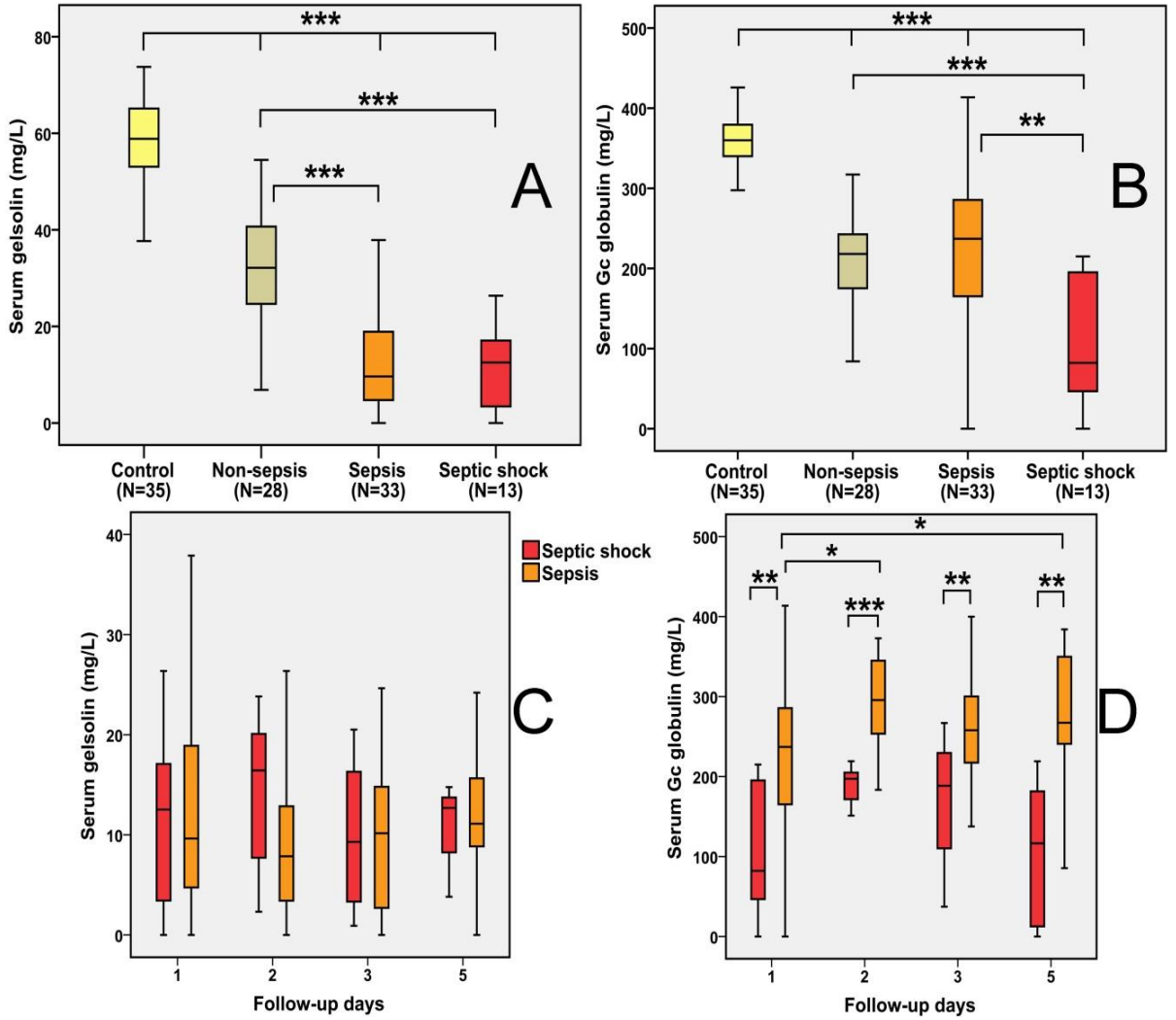

Figure 13. First-day serum gelsolin (A) and Gc-globulin (B) levels in septic shock, septic, non-septic ICU and control patients, and follow-up of serum gelsolin (C) and Gc-globulin (D) concentrations where patients are divided into septic and septic shock groups. *p <0.05, **p <0.01, ***p <0.001.

Table X. Clinical and routine laboratory data of the enrolled patients (third study)

	Control (n=35)	Non-sepsis (n=28)	Sepsis (n=33)	Septic shock (n=13)	p value
Clinical data					
Age, y	60 (56-68)	62 (55-71)	66 (56-76)	65 (47-76)	n.s.
Males (%)	20 (57.1)	20 (71.4)	24 (72.7)	8 (61.5)	n.s.
ICU stay, d	-	1 (1-2)	7 (4.5-12.5)	8 (3.5-15)	$<0.05^{b, d}$
CKD, n (%)	0	3 (10.7)	2 (6.1)	1(7.7)	$<0.05^{f}$
COPD, n (%)	1 (2.9)	7 (25)	10 (30.3)	4 (30.8)	$<0.01^{c, e, f}$
CVD, n (%)	21 (60)	24 (85.7)	24 (72.7)	8 (61.5)	$<0.05^{f}$
DM, n (%)	6 (17.1)	7 (25)	5 (15.2)	0	$<0.05^{b}$
Admission					
Medical	-	1	12	6	
Surgical	-	27	21	7	
qSOFA	-	1.5 (1-2.7)	1 (1-2)	1.5 (1-2)	n.s.
1st day data					
APACHE II	-	8 (5.3-13.3)	14 (10.8-19.3)	20.5 (16-30.3)	$<0.05^{a, b, d}$
SAPS II	-	24 (16.5-29.8)	36.5 (29-53.3)	48 (37.5-74.3)	$<0.01^{b, d}$
SOFA	-	5.5 (4.3-9.8)	7.5 (7-10)	11 (7-15)	$<0.01^{a, b}$
MAP, mmHg	-	62.5 (57.8-74.3)	71 (64.5-85.5)	65 (60.3-75.3)	$<0.01^{a}$
pl-lactate, mmol/L	-	1 (0.8-1.4)	0.9 (0.9-1.7)	3.4 (2.5-5.8)	$<0.001^{a, b}$
se-albumin, mg/L	46.3 (44.4-47.7)	31.6(26.4-33.3)	22.6 (19.5-26.7)	18.7 (13.8-20.2)	$<0.01^{a-f}$
se-hsCRP, mg/L	1.2 (0.5-2)	103.3 (64.3-146.3)	247.4 (124.8-320.3)	226 (143.8-306.4)	$<0.001^{b-f}$
se-PCT, ng/mL	-	0.3 (0.1-0.5)	8.3 (3.7-38.3)	24.8 (10.3-71.3)	$<0.001^{b, d}$
se-Gc, mg/L	359.9 (338.3-381.3)	218.1 (170.2-243.2)	237.0 (160.6-285.6)	82.1 (42.7-195.9)	$<0.01^{a, b, c, e, f}$
se-GSN, mg/L	58.9 (52.6-65.4)	32.1 (24.6-41.1)	9.6 (4.7-19.5)	12.5 (3.3-17.9)	$<0.05^{b, c, d, e, f}$

Data are expressed as medians, in parentheses percentages and interquartile ranges (25–75%) are given. Abbreviations: CKD: chronic kidney disease; COPD: chronic obstructive pulmonary disease; CVD: cardiovascular disease; DM: diabetes mellitus; ICU: intensive care unit; n.s.= non-significant. Level of significance is set at $p < 0.05$. Superscript lowercase letters refer to post-hoc analyses: a: septic shock – sepsis; b: septic shock – non-sepsis; c: septic shock – control; d: sepsis – non-sepsis; e: sepsis – control; f: non-sepsis – control.

IV.3.3. Investigating 14-day mortality in sepsis

IV.3.3.1. First-day parameters and microbiological findings

Septic survivors presented lower ($p<0.05$) qSOFA scores compared with non-survivors (Table XI). All the three investigated clinical scores were different ($p<0.01$) between the two septic groups. MAP and plasma lactate levels were similar in both surviving and in non-surviving patients. Increment of the first-day's hsCRP and PCT levels did not differ significantly in the two septic groups, and albumin levels were also similarly decreased.

Microbiological cultures gave positive results in 76.8% of the septic patients (Table XI). Frequently detected bacteria were Gram-positive *Staphylococcus aureus* and *Enterococcus*, Gram-negative *Escherichia coli* and *Pseudomonas*, whereas for invasive fungal infections *Candida albicans* was most commonly responsible.

IV.3.3.2. Serum gelsolin and Gc-globulin levels in survivors vs. non-survivors

First-day serum GSN levels were higher ($p<0.05$) in survivor than in non-survivor septic patients (Table XI, Figure 14A). No further significant differences or changes were observed in serum GSN concentrations during the 5-day time course of sepsis.

Serum Gc-globulin concentrations did not differ significantly on the 1st day of observation when investigating the two septic patients' groups (Table XI, Figure 14B). However, there was a trend ($p<0.05$) towards increasing Gc-globulin levels, when comparing the 1st with the 2nd day's (median: 212.8 vs. 271.9 mg/L), and the 1st with the 3rd day's (median: 212.8 vs. 235.2 mg/L) Gc-globulin levels in survivors. Similar tendency ($p<0.05$) was seen in non-survivors when comparing the 1st with the 2nd day's serum Gc levels (median: 155 vs. 267.1 mg/L).

From the 1st to the 5th day of ICU follow-up period, 21.4% of the survivors were discharged from the unit because of requiring no more intensive therapy, while 38.9% of the non-survivors died and 11.1% of them were released to other hospital units.

Table XI. First-day data and microbiological findings of septic survivors and non-survivors regarding 14-day mortality (third study)

	Septic survivors (n=28)	**Septic non-survivors (n=18)**	**p value**
First-day data			
qSOFA	1 (1-1.25)	1.5 (1-2)	<0.05
APACHE II	13 (10-18)	23 (16-32)	<0.01
SAPS II	34 (25-51)	53 (43.5-70)	<0.01
SOFA	7 (7-9.5)	11 (9-15.5)	<0.001
MAP, mmHg	70 (65-83.5)	67.5 (59.5-80.5)	n.s.
pl-lactate, mmol/L	1.5 (1-2.2)	1.7 (1.1-5.2)	n.s.
se-albumin, mg/L	22.6 (19.3-26.6)	19.8 (16.2-23.5)	n.s.
se-hsCRP, mg/L	236.7 (151-302.9)	248.3 (121-328)	n.s.
se-PCT, ng/mL	11.2 (4.3-24.8)	26.1 (4.5-77.4)	n.s.
se-Gc, mg/L	212.8 (158.3-284.4)	155 (39.1-243.5)	n.s.
se-GSN, mg/L	12.9 (6.9-21.9)	6.9 (2.8-14.9)	<0.05
Microbiological findings			
Gram-positive	7	2	
Gram-negative	-	6	
Gram-positive& Gram-negative	6	4	
Fungi	1	-	
Gram-positive & fungi	1	-	
Gram-negative & fungi	2	3	
Gram-positive& Gram-negative & fungi	3	-	
Non identified	8	3	

Data are expressed as medians, in parentheses interquartile ranges (25–75%) are given. Microbiological findings are represented by numbers. Abbreviations: MAP: mean arterial pressure. Level of significance is set at $p < 0.05$.

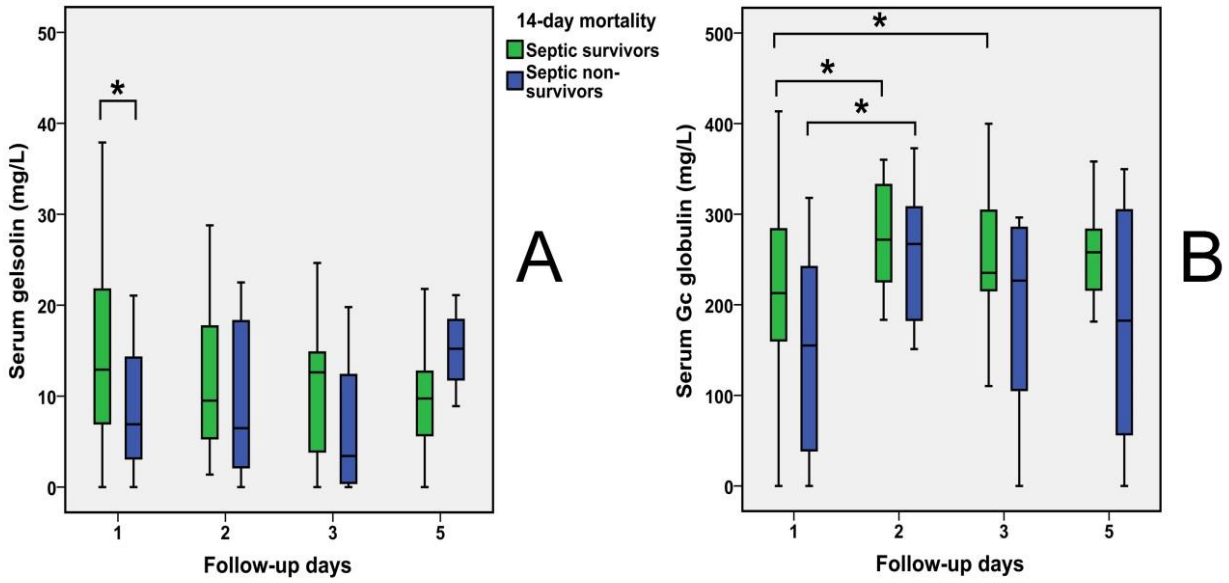

Figure 14. Follow-up of serum gelsolin (A) and Gc-globulin (B) concentrations where patients are divided into septic survivor and non-survivor groups based on 14-day mortality. *p <0.05.

IV.3.4. Spearman's correlation results

Serum GSN and Gc-globulin positively correlated with each other ($\rho = 0.48$, $p < 0.01$), in addition, both of them positively correlated with serum albumin (GSN - albumin: $\rho = 0.54$; Gc - albumin: $\rho = 0.61$, $p < 0.01$) and negatively with hsCRP (GSN - hsCRP: $\rho = -0.68$; Gc - hsCRP: $\rho = -0.43$, $p < 0.01$). Gc-globulin inversely correlated with plasma lactate ($\rho = -0.64$, $p < 0.01$), with PCT ($\rho = -0.34$, $p < 0.01$), and with clinical scores (Gc - SAPS II: $\rho = -0.49$, $p < 0.01$; Gc - APACHE II: $\rho = -0.35$, $p<0.05$; Gc - SOFA: $\rho = -0.52$, $p < 0.01$).

IV.3.5. Results of ROC and logistic regression analyses

In the differentiation of septic patients from other patients suffering from non-infective diseases requiring ICU treatment, besides serum PCT (area under the curve (AUC): 0.98, $p<0.001$) and hsCRP (AUC: 0.80, $p<0.01$), GSN also had significant discriminative value (AUC: 0.88, $p<0.001$) with a cut-off point of 22.29 mg/L (sensitivity: 83.3%, specificity: 86.2%) (Figure 15A, B). Opposite to GSN, Gc-globulin did not have any significant distinguishing role.

The discriminative function of plasma lactate regarding septic shock/sepsis states was proven to be the highest (AUC: 0.99, $p<0.001$; Figure 15D), in addition, Gc-globulin (AUC: 0.76) and MAP (AUC: 0.74) also had significant ($p<0.05$) diagnostic values (Figure 15C). The optimal cut-off point for Gc-globulin was 116.5 mg/L (sensitivity: 78.3%, specificity: 60%). GSN, PCT, hsCRP, qSOFA and SOFA scores did not have any significant informative values in differentiation of septic shock from sepsis.

For predicting 14-day mortality in sepsis, SOFA clinical scores (AUC: 0.88, $p<0.001$) and serum GSN (AUC: 0.71, $p<0.05$) proved to be significant as discriminating factors regarding surviving/non-surviving states (Figure 15E, F). The calculated cut-off value for GSN was 8.7 mg/L (sensitivity: 71.4%, specificity: 58.3%). No significant predictive values were found in cases of MAP, lactate, PCT, hsCRP and qSOFA scores, respectively.

Including the investigated parameters into the logistic regression model, SOFA scores (ß= 0.53; p= 0.03; OR=1.70; 95% CI: 1.03-2.79) and serum GSN (ß=-0.15; p= 0.04; OR=0.87; 95% CI: 0.75-0.99) proved to have predictive capacity regarding 14-day mortality in sepsis. However, in our study, plasma lactate (ß=0.49; p= 0.17; OR=1.64; 95% CI: 0.82-3.28), PCT (ß=0.01; p= 0.39; OR=1.01; 95% CI: 0.99-1.03), hsCRP (ß=-0.00; p= 0.97; OR=1.00; 95% CI: 0.99-1.01) and Gc-globulin (ß=-0.00; p= 0.68; OR=0.99; 95% CI: 0.99-1.01) did not offer any predictive value regarding 14-day mortality.

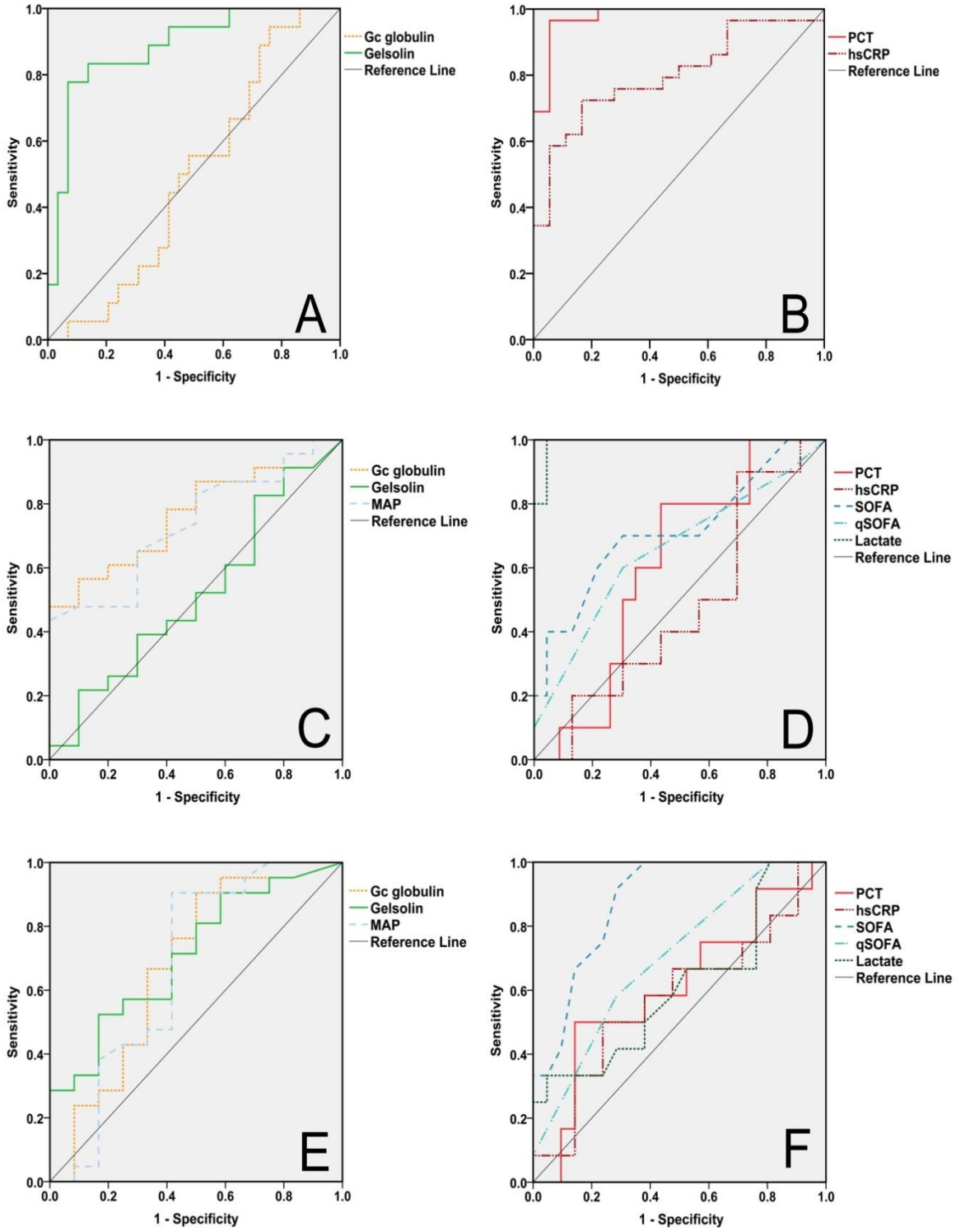

Figure 15. Receiver operating characteristic curves of first-day laboratory and clinical parameters for differentiating non-sepsis from sepsis (A, B), for distinguishing septic shock from sepsis (C, D) and for predicting 14-day mortality in sepsis (E, F).

V. Discussion

V.1. Actinemia in sepsis

The development of a septic process involves early activation of both pro- and anti-inflammatory responses, together with major alterations in non-immunologic pathways (41-44, 46, 47) which partly results in massive cell injury. Besides various other cell components, actin also leaks out in excess from injured cells. In systemic inflammation (e.g. sepsis) and in other pathological processes causing extensive tissue injury actin levels may rise several-fold in contrast to those seen under physiological conditions. High fibrillar actin levels in the plasma that persists non-eliminated may cause various unfavorable processes leading to multi organ failure (98, 104, 106, 109, 120, 126). High levels of extracellular actin are suggested to be toxic, as reported in cell culture experiments by Erukhimov et al. (106) and in an animal model by Li-Chun et al. (165). Possible sources of free extracellular actin in sepsis are microparticles originating from the endothelium and the detritus of blood cells (147), but we also suppose that a sizable portion of free extracellular actin is released from the skeletal muscle tissue. Interestingly, proteomic study performed by Sharma et al. (147) revealed more decreased expression of actin in mononuclear and in polymorphonuclear cells (PMNs) of non-survivors when compared to survivors, moreover, they observed increased expression of GSN in mononuclear cells of survivors and decreased expression in PMNs in all septic patients.

V.2. Serum actin, gelsolin and actin/gelsolin ratios in SIRS and in sepsis

In our first study we elucidated the predictive values of serum GSN and of the newly defined A/GSN ratios quantified by Western blot. We emphasize that in our study septic patients' first-day GSN levels were found to be significantly lower in non-survivors than in survivors based on 7-day mortality, similar to the observation of Lee et al. (103, 140), but in contrast to Wang et al. (145).

Opposite to the studies performed by Lee et al. and Wang et al., we did observe significant difference between septic surviving and non-surviving patients regarding serum albumin levels. Mounzer et al. (139) also reported a positive correlation between serum albumin and GSN levels in trauma patients. Therefore, we suggest that the depletion of GSN is mainly a

result of its scavenging functions in the blood (125, 128, 134, 138), but also the possibility of non-specific protein loss cannot be ruled out.

Investigating both serum and plasma actin concentrations from the same patients, we did not observe any significant differences between the two sample types (data not shown). We observed – in accordance with Mounzer et al. – lower amounts of actin in the sera of septic patients than suggested by Lee et al. (103). Interestingly, Lee et al. identified actin only in 81% of plasma samples from septic patients and in none of normal volunteers, whereas we detected actin in all serum samples of septic and control patients. This could be attributed to our more sensitive actin-detecting method. Interestingly, Belsky et al. (104) found measurable G-actin levels in plasma of controls and intensive care patients as well. Moreover, they detected F-actin in the plasma of intensive care patients but not in those of the controls.

In contrast to Lee et al. (103), who examined only healthy and younger patients as controls compared with septic patients, we investigated age- and gender matched controls vs. septic patients.

Our study is the first which investigates simultaneously the time-dependent changes of both serum actin and GSN levels in human sepsis. Similarly to Belsky et al., we did not observe any significant changes regarding serum actin levels during the follow-up period. We found significantly lower 1^{st} - and 3^{rd} - day GSN levels and 11.4- fold higher median 2^{nd} day A/GSN ratios in non-survivors compared with survivors. Similarly to Lee et al. (140), but contrary to Mounzer et al. (139) and to Wang et al. (145), we observed that GSN levels for all patients in the septic group did not show inter-day significant differences.

V.3. Methodological developments

In our methodological study, we introduced a fast, accurate immune turbidimetric method requiring a very low sample volume for the determination of se-GSN levels, partially based on the previous work of Christensen et al (211). To our best knowledge, no other similarly fast assay for se-GSN is available. For detecting se-GSN, functional nucleation, Western blot, and ELISA are the only available laboratory techniques (120) which require a longer assay time compared with our method. Despite the fact that most ELISA methods have sufficiently low detection limits (214), they are quite user-dependent (e.g. incubation times, pipetting failures) and are not commonly automated. Cobas 8000 analyzer enables an excellent precision and it is

easily connectable to the laboratory informational system (LIS) as well as to the hospital information system (HIS).

Former study of Christensen et al. (211) presented an immune turbidimetric assay for se-GSN on Cobas Mira Plus which offered a slightly lower (4%) total imprecision when compared with our method and a detection limit of 2.7 U/L. However, due to unexpected GSN calibrator and control stability problems, this former assay is no longer available. Study of Dahl et al. (141) presented an immunonephelometric measurement for plasma GSN, but they did not offer any validation data about the assay.

We found se-GSN as a stable protein maintaining its concentration constant at +4°C for 10 days and even throughout 5 repeated freezing-thawing cycles. We did not find any significant differences between serum and plasma levels of GSN (when using sodium citrate tube). However, we do not consider EDTA (ethylenediamine tetraacetic acid)-containing tubes proper for sample collection when measuring GSN levels because plasma GSN drops significantly in a short time (data not shown). One possible explanation to this might be that EDTA binds Ca^{2+} ions more strongly than sodium citrate, in addition, it chelates other metal ions too (215), which results in the destabilization of the GSN domains.

We could confirm the previously published diagnostic capability of first-day se-GSN levels in sepsis by immune turbidimetry, too. Previous Western blot results showed slightly higher GSN levels in all patients when compared with those of our new GSN assay. That difference could be attributed to the fact that Western blot is a more user-dependent method than immune turbidimetry. Similarly to our results, studies of Wang et al. (145) also found significantly lower first-day se-GSN levels in septic patients when compared with non-septic critically ill patients.

In concert with Hamashima et al. (216), we found lower LOD regarding serum Gc-globulin assay when compared with Bangert et al. (213) which possibly arises from different blank samples. Regarding intra- and inter-assay precision we observed no significant differences when comparing the measuring system of Bangert et al. with ours. Hamashima et al. detected similar intra-assay but lower inter-assay imprecision than we obtained in our study. In conjunction with former studies, we also found Gc-globulin as a stable protein in short- and long- term periods, too. Immunoturbidimetric assay of Hamashima et al. required higher sample volume than the assay of Bangert et al. and that of ours.

V.4. Predictive values of gelsolin and Gc-globulin together in critically ill conditions

Since the abovementioned rapid GSN immunoturbidimetric assay became available we sought to investigate the predictive values of GSN and that of the other actin-binding protein, Gc-globulin together in critically ill patients. Our study revealed that serum GSN provides valuable complementary data for the rapid diagnosis of sepsis. Previous studies performed by Lee et al. indicated that first-day GSN levels are significantly higher in survivors than in non-survivors of sepsis (103, 140), which was also confirmed by us. Opposite to Lee et al. (103), Wang et al. (145) and to our previous study, but similarly to Mounzer et al. (139), we did not observe any significant differences between septic survivors and non-survivors regarding serum albumin levels.

For Gc-globulin, we did not find any association between first-day levels and mortality of ICU patients, similarly to Leaf et al. who examined 90-day mortality of critically ill patients (204) and in-hospital mortality in acute kidney injury patients (202). The data obtained from Gressner et al. (205) also indicated no association between Gc-globulin and ICU-/follow-up mortalities.

We did not find any significant differences in first-day's serum Gc-globulin levels when comparing sepsis with non-sepsis patients, contrary to the expectations of Jeng et al. (201). We have demonstrated that first-day's serum Gc levels have valuable predictive capacity when differentiating sepsis from septic shock. In addition, previous study of Dahl et al. (198) confirmed that trauma victims with low admission Gc-globulin concentrations were more prone to develop hematologic and respiratory failures than those with high Gc-globulin levels. Similarly, to their observations, we also noticed a slight increase in Gc-globulin concentrations during the 5 – day follow-up period regarding sepsis patients. That phenomenon can be attributed to the increased synthesis of Gc-globulin as an acute phase reactant after severe injury, reported by Dahl et al. (131). The plasma half-lives of GSN and Gc-globulin when complexed with actin are suggested to be very short (30 minutes in case of Gc-globulin) (112, 162). Opposite to Gc-globulin, no significant increment was noted during the observation period regarding GSN, similarly to the work of Lee et al. (140) and to our previous findings, which could be explained by the lack of newly induced synthesis in the muscle cells after injury (213). However, in survivors, long-term observations performed by Huang et al. (143) and Wang et al. (145) indicated a slow increase of serum GSN levels. The rapid fall in plasma

Gc-globulin and GSN levels at the beginning of severe systemic inflammatory disorders is attributed to the fast consumption of them partly because of the extreme overload by actin (111, 171). However, the cause of their declining levels is suggested to be multifactorial as they own various physiological functions.

Based on literature data of previous studies, we seem to be the first to measure serum GSN and Gc-globulin levels simultaneously by rapid immune turbidimetry during the course of sepsis. We demonstrated a significant positive correlation between the two actin-binding proteins, which supports the hypothesis that they act in concert in the intravascular space. In contrast to Gressner et al. (205), we found significant correlations between Gc-globulin and hsCRP, PCT, furthermore, between Gc-globulin and clinical scores, too. Similarly to our previous data, GSN negatively correlated with hsCRP. Interestingly, Mounzer et al. (139) communicated that in patients after major trauma decreased admission plasma GSN levels were associated with prolonged mechanical ventilation, ICU stay, ARDS, and death. Similarly to our previous study, we investigated age-, gender- and disease-matched control patients, therefore, the comparisons of patients groups regarding serum GSN or Gc-globulin levels were not affected by underlying diseases (e.g. diabetes, chronic kidney disease).

Apart from sepsis, decreased se-GSN levels were found after parenchymal tissue damages including acute lung injury, major trauma, myonecrosis, and acute liver failure, too (111, 120). Depressed serum Gc-globulin levels were found also in patients with hepatic failure and in trauma patients with shock (171). These findings indicate that none of these proteins are specific for sepsis. However, since sepsis is a syndrome rather than a disease, none of the biomarkers would offer 100% specificity (19). The magnitude of the decrease in serum GSN and Gc-globulin levels is of utmost importance, therefore, appropriate cut-off values have to be set.

PCT is the gold standard serum marker of sepsis, with great sensitivity and specificity for the diagnosis of this clinical syndrome. However, we suggest that serum GSN, A/GSN ratios and Gc-globulin give important additional information regarding the outcome and the immune status of the septic patients. GSN is a multifunctional protein which is thought – besides extracellular actin-binding – to be involved in the human body's innate immune response: it can bind bioactive molecules (lysophosphatidic acid, sphingosine 1-phosphate, fibronectin, PAF) and bacterial wall lipids (LTA, LPS) therefore it could localize inflammatory processes.

Animal models supported the hypothesis that repletion of blood GSN during sepsis protects the host from adverse outcomes (163, 166). Gc-globulin also facilitates the host's immune response as it is capable to endotoxin binding and inhibiting, it is involved in the complement-mediated tissue recruitment of neutrophils and it can be converted to Gc-globulin-macrophage activating factor (Gc-MAF) (171).

V.5. Limitations

At present, determination of serum/plasma actin is not possible by rapid laboratory methods and this fact reduces its applicability in clinical use.

Currently, there is no commercially available human GSN calibrator and control for immune turbidimetry. In order to handle this difficulty, we applied highly purified human GSN expressed in *E. coli* (His-8) as a calibrator, and we used pooled serum from healthy individuals as an internal control material. The described rapid and accurate measurement of GSN suggests the ultimate need to develop a commercially available GSN calibrator and control system in the near future.

Other weakness of our studies is the limited number of critically ill patients. Therefore, further studies with a much higher patient number should be performed.

In our third trial, we investigated the Sepsis-3 definitions retrospectively therefore in the future investigations the new sepsis terms should be taken as the primary criteria when defining the patient groups. Also, another limitation is that SOFA scores could not be calculated before the admission of the patients to the ICU. This difficulty most researchers have to face since many of the patients are admitted from out of hospital locations (where no laboratory facilities exist) directly to the ICU.

VI. Summary

We managed to develop a sensitive Western blot method for the detection of serum actin, which serves as a promising starting point for further methodological developments. We simultaneously examined the time dependent changes of both serum actin and gelsolin levels in human sepsis, which is a novelty. The introduced serum actin/gelsolin ratio proved to have a promising predictive capacity regarding overall ICU mortality in sepsis, similarly to that of APACHE II scores. For daily clinical usage, an automated laboratory assay of actin is still needed to be established.

Through the development of a rapid (10 min) and accurate immune turbidimetric method for detecting gelsolin in human blood, we made a step forward towards the routine laboratory application of gelsolin as a predictive marker in the clinical era, especially in the field of intensive care (sepsis) or even internal medicine (chronic inflammatory disorders).

We proved that serum gelsolin and Gc-globulin act in concert and both of them negatively correlate with inflammatory parameters in SIRS and in sepsis, thereby also supporting their immunomodulatory roles. Based on our observations, serum gelsolin may serve as a complementary diagnostic and predictive protein marker in sepsis, while critically low admission Gc-globulin concentration reflects the potential development of septic shock. Immune turbidimetric measurement of gelsolin and Gc-globulin levels gives the possibility to obtain important additional information on sepsis severity within a short turnaround time.

VII. References

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Antagonistic sepsis markers: Serum gelsolin and actin/gelsolin ratio

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ABSTRACT

Objectives: For appropriate sepsis care, prognostic laboratory markers are mandatory. The aim of our study was to evaluate the predictive value of serum actin, gelsolin and the recently defined actin/gelsolin ratio during sepsis by comparison it to classical clinical and inflammatory laboratory parameters.

Design & methods: We analyzed sera of severe septic (n = 32) and SIRS (n = 12) patients for 5 days. Ophthalmologic patients (n = 27) served as controls. Besides serum actin, gelsolin and actin/gelsolin ratios classical laboratory parameters (WBC count, serum procalcitonin, hsCRP) and clinical scores (APACHE II, SAPS II, SOFA), were also assessed.

Results: Septic patients showed significantly decreased first-day gelsolin levels and increased actin/gelsolin ratios compared to SIRS patients ($p < 0.05$), furthermore, non-survivors had significantly lower gelsolin levels compared to survivors ($p < 0.05$). Non-survivors had 11.4-fold higher 2nd day actin/gelsolin ratios than survivors. Besides procalcitonin (PCT) and hsCRP, gelsolin and actin/gelsolin ratios also proved to be useful in discriminating SIRS from sepsis in the ICU ($p < 0.05$). Gelsolin had similar prognostic value to PCT when assessing 7-day mortality and the predictive capacity of the first-day actin/gelsolin ratios was similar to that of APACHE II score regarding ICU mortality in severe sepsis.

Conclusions: Serum gelsolin and actin/gelsolin ratio might serve as efficient complementary prognostic markers in sepsis. However, for daily clinical usage, an automated laboratory assay of actin and gelsolin is still needed to be developed.

1. Introduction

Epidemiological studies suggest an increasing incidence but a decreasing mortality of severe sepsis [1]. However, septic shock remains a leading cause of death at the intensive care units with a mortality rate up to 72% [2,3]. Diagnosis of sepsis is predominantly based on clinical parameters, including general status, inflammatory, hemodynamic, organ dysfunction and tissue perfusion variables [4]. From the laboratory side, early sensitive and specific biomarkers are mandatory for raising the correct diagnosis. Serum procalcitonin (PCT) and highly-sensitive C-reactive protein (hsCRP) are the current gold standard parameters in the sepsis guidelines [4,5]. Monitoring of the septic process is the other essential part of a successful therapy; therefore besides the classic ones, clinicians need novel biomarkers with predictive capacity.

Gelsolin (GSN) is a multifunctional protein, existing in three different isoforms encoded by chromosome 9 in humans [6]. Secreted or plasma GSN (MW = 93 kDa) - mainly originating from the skeletal muscle tissue [7] - is an essential motor of the extracellular actin scavenger system: it severs polymeric or F-actin in the circulation, but also can bind to monomeric or G-actin [8,9]. An increasing body of experimental evidence exists that in severe acute catabolic conditions (e.g. severe sepsis, multiple organ dysfunction syndrome (MODS), extensive trauma) and chronic inflammatory disorders (e.g. chronic kidney disease, multiple sclerosis, rheumatoid arthritis) GSN levels decline in the blood [10–21]. In addition, GSN has other roles in the circulation, as it binds to pro-inflammatory and bioactive molecules and bacterial surface lipopolysaccharides [22,23].

A wide spectrum of knowledge is available on the intracellular functions of actin (MW = 42 kDa) [24], but less attention is paid to actin released into the circulation. In sepsis, high amounts of free actin filaments are thought to increase blood viscosity, to activate platelets

Abbreviations: A, actin; GSN, gelsolin; A/GSN, actin/gelsolin; APACHE II, Acute Physiology and Chronic Health Evaluation II; MODS, multiple organ dysfunction syndrome; SAPS II, Simplified Acute Physiology Score II; SOFA, Sequential Organ Failure Assessment.

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and to slow down fibrinolysis by binding to plasmin and thus obstructing small blood vessels [16,25,26]. Circulating plasma actin may also enhance the severity of *Escherichia coli* infections by promoting alpha hemolysin production and activating purinergic receptors by binding to adenine nucleotides [10]. The continuous presence of large amounts of actin filaments in the blood could promote a condition resembling MODS [18,27]. Gelsolin is a prominent component of the actin scavenger system therefore actin levels depend on its binding capacity.

In our study we hypothesized that the simultaneous measurement of serum actin and GSN might give additional information on the severity of systemic inflammatory response syndrome (SIRS) and the septic process as well. For evaluation of their role we introduced a new marker: serum actin/gelsolin (A/GSN) ratio. This work aimed to monitor changes of serum levels of actin, GSN and our newly defined A/GSN ratio in SIRS and in severe sepsis, and to investigate their diagnostic and predictive values comparing them to the classical parameters widely used in sepsis management.

2. Materials and methods

2.1. Patient enrollment and study design

Our study protocol was authorized by the Regional Research Ethical Committee of the University of Pécs (4327.316-2900/KK15/2011) and was performed according to the ethical guidelines of the 2003 Helsinki Declaration. Written informed consent was obtained from all enrolled patients or their appropriate surrogates after detailed information regarding the study design and blood sampling. Patients with established diagnosis of SIRS or severe sepsis from the Department of Anesthesiology and Intensive Therapy (University of Pécs, Hungary) were enrolled in our follow-up study from January 2013 till December 2014. SIRS and severe sepsis were defined according to the criteria of the American College of Chest Physicians/Society of Critical Care Medicine and the 2012 Surviving Sepsis Guidelines [4,5]. Patients with SIRS fulfilled two or more of the following findings: body temperature >38 °C or <36 °C, tachycardia (>90/min), tachypnea (respiratory rate > 20/min) or $PaCO_2$ < 32 mm Hg, white blood cell count >12,000/mm^3 or <4000/mm^3 or >10% immature bands. Further SIRS criteria were negative blood culture results and PCT levels <1 ng/mL. Diagnostic criteria for severe sepsis included SIRS plus confirmed or presumed infection, elevated PCT levels (>2 ng/mL in bacterial sepsis) and one or more organ dysfunction induced by sepsis. Patients were followed during their ICU stay where serum samples were obtained at day 1, 2, 3, 5 after clinical diagnosis. Defined end points were the withdrawal of consent or death during the study period. Patients were excluded if they were under 18 years of age or where it was not possible to obtain patient consent or consultee approval. Our control group consisted of ambulatory ophthalmologic patients from the Department of Ophthalmology, University of Pécs, Hungary. Ophthalmologic patients under the age of 18 years and those suffering from acute inflammation or infectious disease were excluded from the control group.

2.2. Blood sampling

Venous blood (7.5 mL) was obtained from every patient via central venous catheter into plain tubes with accelerator gel using a closed blood sampling system (BD Vacutainer®). Clotted blood samples were centrifuged for 10 min at 1500*g* and sera were immediately treated with electrophoresis sample buffer and heated at 100 °C (see Section 2.3.). The rest of native sera were stored at −80 °C until further analyses.

2.3. Determination of serum actin and gelsolin levels

Samples were immediately analyzed or were stored at −80 °C. Using 10% SDS-PAG electrophoresis by Laemmli [28], serum actin and GSN levels were determined by quantitative chemiluminescence Western blot based on the work of Lee et al. [13]. Polyclonal primary antibodies (Rabbit Anti-Human Actin, N-terminal, ref. no: A2103, Sigma-Aldrich Co. LLC; Rabbit Anti-Human Gelsolin, ref.no: A0146, Dako A/S) and horseradish peroxidase-labeled secondary antibodies (Swine Anti-Rabbit Immunoglobulins, ref.no: Z0196, Dako A/S) were applied. In every gel, the same pretreated control serum sample of a healthy individual was run as internal standard. Quantification of Western blot was performed by using highly purified G-actin standard obtained from rabbit skeletal muscle (Department of Biophysics, University of Pécs, Hungary [29]). For standardization of GSN, purified recombinant human GSN expressed in *Escherichia coli* [His-8] (Department of Biophysics, University of Pécs, Hungary [30]) was used. The Western blots were calibrated by running dilution series of actin and GSN standards of known concentrations on separate gels. Quantification was done after densitometry (Syngene, Cambridge, UK) of the chemiluminescence signal of the standards and establishing a calibration line (light signal vs. protein concentration). The internal standard was applied to every gel in the subsequent experiments therefore patients' data were calculated by interpolation.

A/GSN ratios were derived from the same day's actin [mg/L] and GSN [mg/L] levels of each patient.

2.4. Assessment of laboratory parameters and clinical scores

All other laboratory parameters including total blood cell counts, serum albumin, hsCRP, and PCT levels were determined by automated routine laboratory techniques in the Department of Laboratory Medicine, University of Pécs, Hungary.

APACHE II (Acute Physiology and Chronic Health Evaluation II), SAPS II (Simplified Acute Physiology Score II) and SOFA (Sequential Organ Failure Assessment) scores were calculated for the first day of intensive care treatment. Both 7-day and overall ICU mortalities were investigated in our study. Septic patients were further subdivided into surviving ($n = 21$) and non-surviving ($n = 11$) groups based on 7-day mortality.

2.5. Statistical analysis

For statistical analysis IBM SPSS Statistics for Windows, Version 22 program was used. Distribution of data was evaluated by Shapiro-Wilk test. Since our data did not show normal distribution, Kruskal-Wallis, Mann-Whitney tests were performed for investigating differences between patient groups. Friedman's two way ANOVA test was used for performing follow-up comparisons. Predictive values of different serum markers were assessed by COX regression analysis and by receiver operating characteristic curves (ROC). Possible correlations between quantitative parameters were determined by Spearman's rank correlation test. Data are expressed as medians and as interquartile ranges (IQR). $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Clinical and routine laboratory data

The clinical and routine laboratory parameters of our patients enrolled in the study are listed in Table 1.

Control patients were similar in sex and age compared to septic patients. More ICU patients (36.4%) than controls (7.4%) suffered from COPD. The majority of the patients (63.6%) were admitted to the ICU after surgical interventions (e.g. management of acute abdomen, Whipple procedure, etc.) and 36.4% of them after other medical events (e.g. pneumonia). Among the first-day routine laboratory and clinical parameters, we observed significantly higher serum PCT ($p < 0.001$), hsCRP levels ($p < 0.001$), APACHE II ($p < 0.001$), SAPS II ($p < 0.001$) and SOFA ($p < 0.05$) scores in septic compared to SIRS patients.

Table 1
Clinical and routine laboratory data of the enrolled patients.

	Sepsis (n = 32)	SIRS (n = 12)	Control (n = 27)	p value
Clinical data				
Age (y)	67 (58–78)	67 (55–77)	65 (56–74)	n.s.
Males, n (%)	19 (59.4)	9 (75)	11 (40.7)	n.s.
COPD, n (%)	11 (34.4)	5 (41.6)	2 (7.4)	<0.05
Type II DM, n (%)	6 (18.8)	3 (25)	4 (14.8)	n.s.
CVD, n (%)	22 (68.8)	10 (83.3)	19 (70.4)	n.s.
CKD, n (%)	1 (3.1)	0	1 (3.7)	n.s.
Immunological diseases	4 (12.5)	0	0	
Malignancy, n (%)	13 (40.6)	5 (41.6)	0	<0.05
Cause of admission				
Internal medicine origin, n (%)	13 (40.6)	3 (25)		
Surgical origin, n (%)	19 (69.4)	9 (75)		
ICU treatment days	7 (4–12.5)	2 (2–3.8)		<0.01
First-day parameters				
APACHE II scores	20.0 (12.3–25.0)	9.5 (6.3–14.8)		<0.001
SAPS II scores	51.0 (34.3–66.0)	25.5 (18.0–36.3)		<0.001
SOFA scores	9.0 (7.0–11.8)	6.5 (4.3–9.8)		<0.05
se-albumin	22.2 (18.5–26.3)	30.9 (25.3–33.5)	46.3 (44–48.1)	<0.001
se-hsCRP (mg/L)	253.2 (158.2–324.9)	85.1 (46.3–201.3)	1.4 (0.7–3.9)	<0.001
se-PCT (ng/mL)	17.3 (4.4–67.7)	0.4 (0.3–0.7)		<0.001
se-actin (mg/L)	3.5 (1.6–6.1)	2.5 (2–4.1)	3.0 (2.1–3.7)	n.s.
se-GSN (mg/L)	15.2 (5.8–24.1)	31.6 (23.6–40.8)	64.1 (37.9–88.6)	<0.01
se-A/GSN	0.26 (0.1–0.8)	0.1 (0.06–0.14)	0.04 (0.03–0.08)	<0.05

Data are expressed as medians, in parentheses percentages and interquartile ranges (25–75%) are given. Level of significance is set at $p < 0.05$. Abbreviations: COPD: chronic obstructive pulmonary disease; CKD: chronic kidney disease; CVD: cardiovascular disease; n.s. = non-significant; Type II DM: Type II diabetes mellitus.

Among the routine laboratory and clinical parameters, non-survivor sepsis patients exhibited significantly ($p < 0.05$) higher PCT levels and clinical scores than survivors (Table 2). In non-survivors 81.8%, while in survivors 71.4% of the patients developed MODS. Common organ dysfunctions in sepsis were acute renal failure (65.6%) and acute lung injury (50%), in 21.8% of the septic patients we found thrombocytopenia, in 12.5% acute hepatic failure also developed. Hemoculture was positive in 18.8% of the septic patients, in 53.1% of the patients pathogens were detected in other specimen sources (e.g. bronchoalveolar lavage, intra-abdominal abscess, urine) and in 28.1% of the cases the source of infection remained unidentified. Most common pathogens were Gram-positive (coagulase negative *Staphylococci*, *Enterococci*) and Gram-negative bacteria (*Escherichia coli*, *Pseudomonas species*), in 28.1% of the patients fungal infections (mostly *Candida* species) were also present.

3.2. Serum actin, GSN levels and A/GSN concentration ratios in SIRS and in sepsis

Analyzing the first-day serum actin levels in sepsis, SIRS and control patient groups, non-significant differences were observed (Table 1). First-day serum GSN concentrations were found to be the lowest in the sepsis group, significantly higher GSN levels were observed in SIRS compared to sepsis ($p < 0.01$), and the highest values were obtained in controls ($p < 0.001$) (Fig. 1A). Regarding first-day serum A/GSN ratios, the highest values were observed in sepsis, significantly ($p < 0.05$) lower A/GSN ratios were obtained in SIRS and the lowest values were found in controls ($p < 0.001$) (Fig. 1B).

Well-defined changes were observed regarding the surviving and the non-surviving severe septic patients' parameters (Table 2). Control patients had significantly ($p < 0.001$) higher serum GSN levels than survivors and non-survivors, moreover, survivors showed significantly ($p < 0.05$) higher GSN levels in their sera than non-survivors (Fig. 1C). Non-survivor and survivor patients' A/GSN ratios were significantly ($p < 0.001$) higher than those observed in controls (Fig. 1D).

Fig. 2 illustrates the follow-up of surviving and non-surviving patients. In the first 5 days, 28.1% of the septic patients died while further 21.9% of them required no more intensive therapy and were released from the ICU. Higher median values of serum actin levels were observed in non-survivors than in survivors during the follow-up, although not being statistically significant (Fig. 2A). Serum GSN levels were found to be higher in survivors compared to non-survivors on day 1 (Table 2) and on day 3 as well (day 3 median levels in survivors

Table 2
Clinical, laboratory and microbiological characteristics of septic survivor and non-survivor patients based on 7-day mortality.

	Non-survivors (n = 11)	Survivors (n = 21)	p value
Clinical data			
APACHE II scores	28 (24–36.5)	16 (12 − 20)	<0.001
SAPS II scores	68 (47.5–78)	39 (33–54)	<0.01
SOFA scores	13 (10–18)	8 (7–9)	<0.001
Organ dysfunctions			
1	2	6	
2	2	9	
≥3	7	6	
First-day parameters			
se-albumin	19.2 (15.8–22.3)	26 (22.4–31.2)	<0.05
se-hsCRP (mg/L)	287.6 (216–336.4)	193.7 (143.9–286.1)	n.s.
se-PCT (ng/mL)	59.9 (33.5–101)	10.3 (3.9–21)	<0.05
se-actin (mg/L)	4.9 (1.6–7.4)	3.1 (1.7–5.4)	n.s.
se-GSN (mg/L)	8.9 (1.8–17.9)	16.9 (9.5–34.6)	<0.05
se- A/GSN	0.71 (0.19–3.26)	0.15 (0.09–0.46)	n.s.
Identified microorganisms			
Gram-positive		3	
Gram-negative	1	4	
Gram-positive + Gram negative	2	4	
Gram-negative + fungi	1	1	
Gram-positive + Gram negative + fungi	2	5	
Unidentified	5	4	

Clinical scores and laboratory data are expressed as medians, in parentheses interquartile ranges (25–75%) are given, organ dysfunctions and microbiological findings are given with numbers. Level of significance is set at $p < 0.05$.

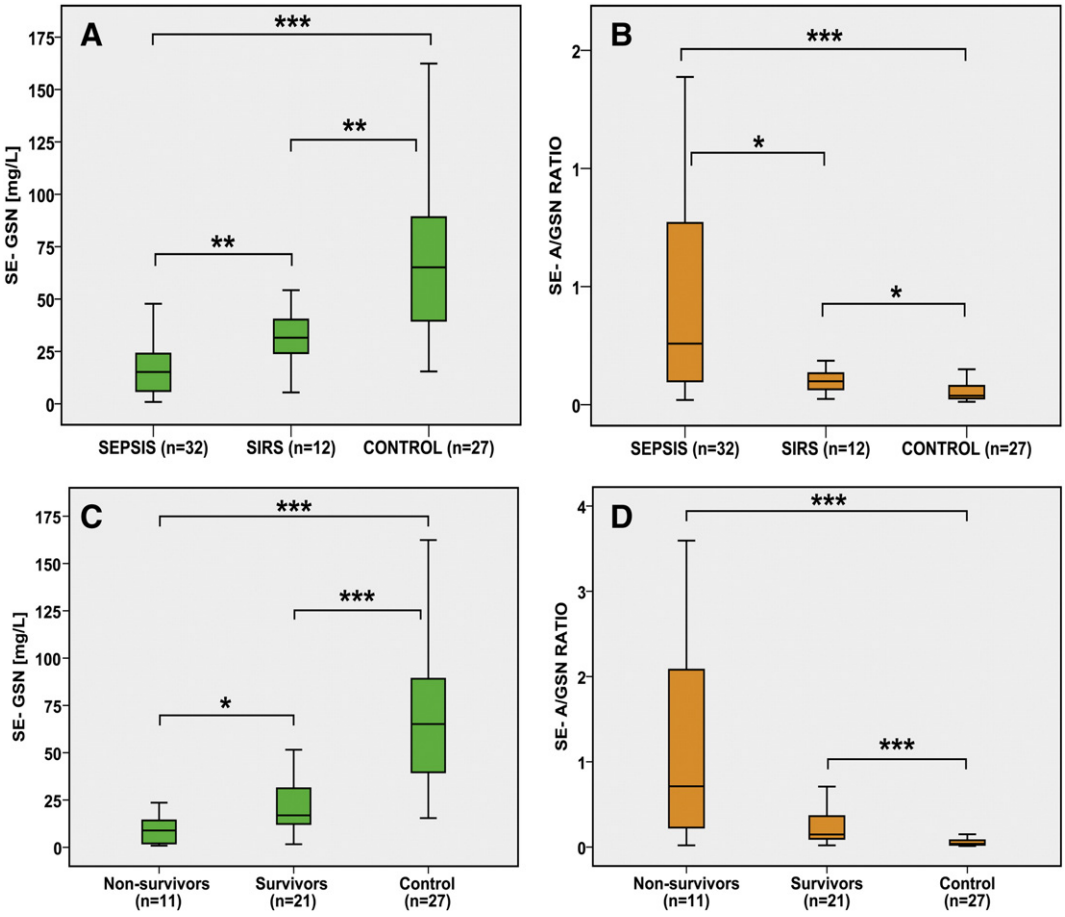

Fig. 1. First-day serum GSN levels and A/GSN ratios in septic, SIRS and control patients (A, B), and in septic survivors, non-survivors based on 7-day mortality (C, D). Significance level is set at $p < 0.05$ (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$).

vs. non-survivors: 22.95 mg/L vs. 3.69 mg/L; $p < 0.05$; Fig. 2B). Patients who failed to survive sepsis had significantly higher 2nd day's A/GSN ratios than survivors (median A/GSN ratios in non-survivors vs. survivors: 2.18 vs. 0.19; $p < 0.05$; Fig. 2C).

3.3. Spearman's correlation analysis and assessment of predictive values

Serum GSN levels were found to correlate inversely with PCT ($\rho = -0.38$, $p < 0.05$), hsCRP levels ($\rho = -0.65$, $p < 0.01$), SAPS II ($\rho = -0.37$, $p < 0.05$), SOFA clinical scores ($\rho = -0.35$, $p < 0.05$) and positively with serum albumin levels ($\rho = 0.43$, $p < 0.01$). A/GSN ratios positively correlated with hsCRP levels ($\rho = 0.43$, $p < 0.01$) and SOFA clinical scores ($\rho = 0.32$, $p < 0.05$).

Regarding 7-day mortality in sepsis, ROC analysis showed that area under the curve (AUC) value for PCT was found to be 0.75, for GSN it was 0.74 (both significant at $p < 0.05$, Fig. 3A, B). The derived cut-off value for GSN was found to be 11.38 mg/L (sensitivity: 76.2%, specificity: 72.7%). AUC values for A/GSN (0.70) and for hsCRP (0.66) did not meet criteria for statistical significance. For differentiating patients with sepsis from those with a non-infective systemic inflammatory response, AUC were 0.95 for serum PCT, 0.84 for hsCRP, 0.77 for GSN and 0.70 for A/GSN ratios, respectively (both significant at $p < 0.05$, Fig. 3C, D). Cut-

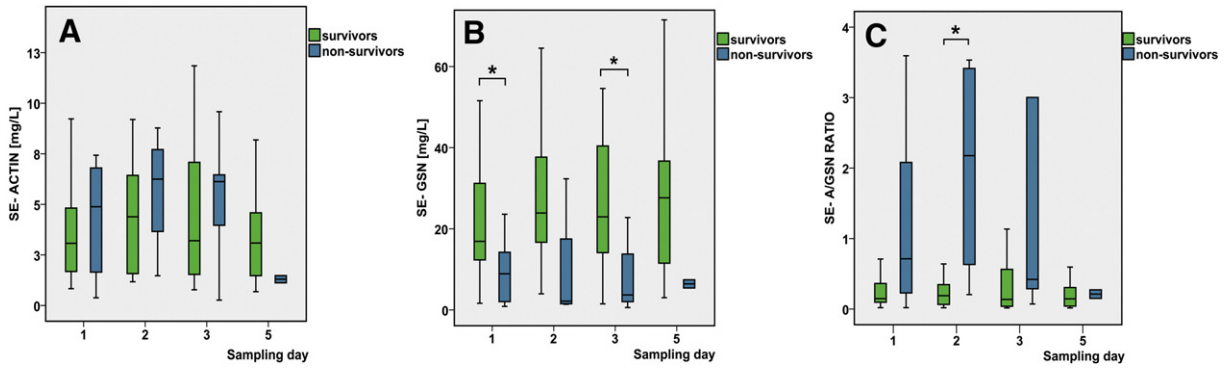

Fig. 2. Time course of serum actin (A), GSN levels (B) and A/GSN ratios (C) in sepsis. Septic patients are divided into groups based on 7-day mortality. Friedman's ANOVA test was performed for comparing the measured parameters of survivors and non-survivors. Significance level is set at $p < 0.05$ (*$p < 0.05$).

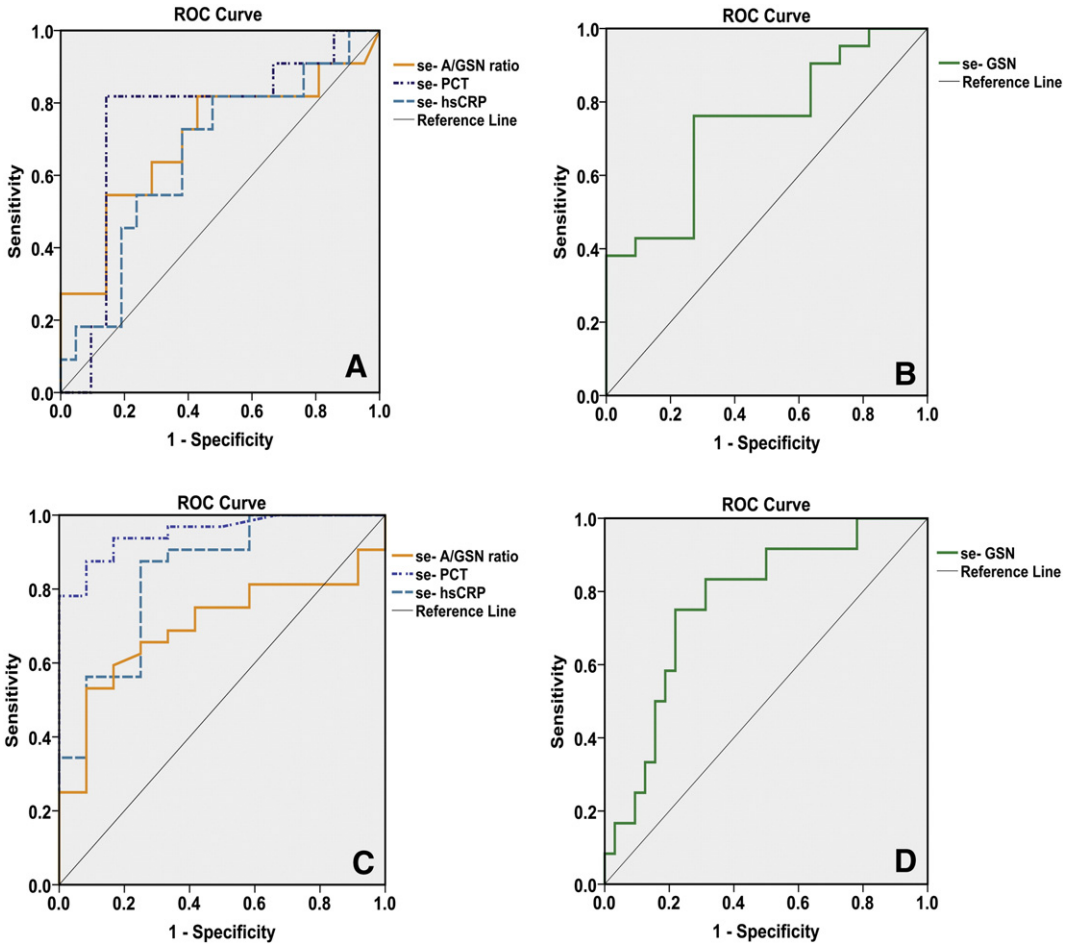

Fig. 3. Receiver operating characteristic curves of serum PCT, hsCRP, A/GSN ratios (A) and GSN (B) for predicting 7-day mortality in sepsis and those for distinguishing SIRS from sepsis (C, D).

off values were 21.04 mg/L for GSN (sensitivity: 83.3%, specificity: 68.7%) and 0.10 for A/GSN (sensitivity: 68.8%, specificity: 66.7%).

For determining the predictive value for overall ICU mortality of the studied markers, COX regression analysis was performed. Based on evaluation of the first-day laboratory parameters (hsCRP, PCT, actin, GSN levels, A/GSN ratios) and clinical scores (APACHE II, SAPS II, SOFA) of septic patients we found hazard ratios (HR) and confidence intervals (CI) as follows: HR = 1.208; 95% CI = 1.083–1.347 ($p = 0.001$) for A/GSN ratios and HR = 1.172; 95% CI = 1.079–1.273 ($p < 0.001$) for APACHE II scores. These two parameters were able to predict the outcome of sepsis.

4. Discussion

The major source of circulating actin in healthy individuals is most probably the skeletal muscle tissue with its large mass and high actin content. However, in systemic inflammation (e.g. sepsis) and in other pathological processes causing extensive tissue injury actin levels may rise several-fold those seen under physiological conditions. High fibrillar actin levels in the plasma that persist non-eliminated, may cause various unfavorable processes leading to multi organ failure [16,18,25–27]. Gelsolin is an actin depolymerizing scavenger protein however, apart from its significance in sepsis, it has a major role in certain types of amyloidosis, autoimmune disorders and cancer [18,31,32]. Interestingly, Mounzer et al. [17] communicated that in patients after major trauma decreased admission plasma GSN levels were associated with prolonged mechanical ventilation, ICU stay, ARDS, and death.

We emphasize that in our study septic patients' first-day GSN levels were found to be significantly lower in non-survivors than in survivors based on 7-day mortality, similar to the observation of Lee et al. [13,15], but in contrast to Wang et al. [33].

Opposite to the studies performed by Lee et al. and Wang et al., we did observe significant differences between septic surviving and non-surviving patients regarding serum albumin levels. Mounzer et al. [17] also reported a positive correlation between serum albumin and GSN levels in trauma patients. Therefore we suggest that the depletion of GSN is mainly a result of its scavenging functions in the blood [8,9,22,23], but also the possibility of non-specific protein loss cannot be ruled out.

Investigating both serum and plasma actin concentrations from the same patients, we did not observe any significant differences between the two sample types (data not shown). We observed – in accordance with Mounzer et al. – lower amount of actin in the sera of septic patients than suggested by Lee et al. [13]. Interestingly, Lee et al. identified actin only in 81% of plasma samples from septic patients and in none of normal volunteers, whereas we detected actin in all serum samples of septic and control patients. This could be attributed to our more sensitive actin-detecting method. In contrast to Lee et al., who examined only healthy and younger patients as controls compared to septic patients, we investigated age- and gender matched controls vs. septic patients.

There can be found an extensive variability in the estimated GSN levels in different studies [13,33] since anti-GSN antibodies applied in recent immunological methods cannot distinguish between cytoplasmic and secretory isoforms of GSN [34].

Our work is the first trial which examines simultaneously the time-dependent changes of both serum actin and GSN levels in human sepsis. We found significantly lower 1st - and 3rd - day GSN levels and 11.4-fold higher median 2nd day A/GSN ratios in non-survivors compared to survivors however, we observed that GSN levels for all patients in the septic group did not show inter-day significant differences. Our findings are similar to those of Lee et al. [15], but are in contrast to Mounzer et al. [17] and to Wang et al. [33].

High levels of extracellular actin are suggested to be toxic, as reported in cell culture experiments by Erukhimov et al. [27] and in an animal model by Kevin Li-Chun et al. [35]. Possible sources of free extracellular actin in sepsis are microparticles originating from the endothelium and the detritus of blood cells [36], but we also suppose that a significant portion of free extracellular actin is released from the skeletal muscle tissue. Procalcitonin is the gold standard serum marker of sepsis, with great sensitivity and specificity for the diagnosis of this clinical syndrome. We suggest that serum GSN and A/GSN ratios cannot replace PCT, but give important additional information regarding the outcome and the immune status of the septic patients. GSN is a multifunctional protein which is thought – besides extracellular actin-binding – to be involved in the human body's innate immune response: it can bind to bioactive molecules (lysophosphatidic acid, sphingosine 1-phosphate, fibronectin, platelet activating factor) and bacterial wall lipids (lipoteichoic acid (LTA), lipopolysaccharide, (LPS)) and therefore it could localize inflammatory processes. Animal models supported the hypothesis that repletion of blood GSN during sepsis protects the host from adverse outcomes [37,38].

Our study has some limitations. In the future, we should study a much larger number of critically ill patients. Since Western blot is a time-consuming and quite expensive method, it is not applicable for the clinical laboratory practice. At present, determination of serum/plasma actin is not possible by rapid laboratory methods and this fact reduces its applicability in clinical use. Currently, we are working on the validation of a fast, automated immune-turbidimetric method for determining serum GSN levels, based on the previous work suggested by DAKO [39].

We are aware that from February 2016, new definitions for sepsis and septic shock are available [40]. These (e.g. quickSOFA) promote earlier recognition and management of patients with sepsis or at risk of developing sepsis. Since our study was performed from January 2013 to December 2014, patients were categorized according to the former sepsis guidelines [4,5].

Our findings strongly support the hypothesis that GSN is the major component of the extracellular actin scavenger system. This study demonstrates that a significant difference is present in surviving vs. non-surviving septic patient groups regarding serum GSN levels. We suppose that the assessment of GSN levels and A/GSN ratios could provide additional information on the actual status of septic patients.

5. Conclusions

To our best knowledge, our study is the first to examine the time dependent changes of serum actin, GSN levels and the recently defined A/GSN ratios. We suggest that the predictive capacity of the first-day A/GSN ratios regarding overall ICU mortality in sepsis is similar to that of APACHE II clinical scores. GSN had similar AUC for assessing 7-day mortality in sepsis as PCT. We verified that serum GSN levels correlated negatively with PCT, hsCRP levels, SAPS II and SOFA clinical scores, whereas A/GSN ratios positively with hsCRP levels and SOFA scores. Therefore, GSN and A/GSN ratio might be considered as complementary prognostic markers in sepsis.

Conflict of interest

The authors declare no conflict of interest.

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RESEARCH ARTICLE

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Validation of an automated immune turbidimetric assay for serum gelsolin and its possible clinical utility in sepsis

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Background: Studies showing the potential predictive value of the actin-binding protein gelsolin, in critically ill patients are scarce. Moreover, even up to now a rapid automated measurement of gelsolin has still remained a challenge. Therefore, we developed and validated an automated serum gelsolin immune turbidimetric assay for possible clinical use.

Methods: Validation of serum gelsolin assay was performed on a Cobas 8000/c502 analyzer (Roche) according to the second edition of Eurachem guidelines. Furthermore, we also studied the diagnostic value of serum gelsolin in sepsis when investigating sera of septic (n = 25), systemic inflammatory response syndrome (SIRS; n = 8) and control patients (n = 14). We compared our previously published Western blot data with those of the new turbidimetric assay.

Results: The sample volume was 7 μL and the assay time was 10 minutes. The detection limit was 0.72 mg/L, intra- and inter-assay imprecision remained in most cases less than 5% expressed as CV. Recovery was found to be 84.56%-93.52% and linearity study gave an appropriate correlation coefficient by linear regression analysis (r^2 = .998). Septic patients exhibited lower (P = .015) first-day serum gelsolin levels than SIRS patients, which confirmed our previous Western blot results. The determined cut-off point for serum gelsolin was 14.05 mg/L (sensitivity: 75%; specificity: 60%) when investigating its diagnostic value in sepsis.

Conclusion: Based on the results, our immune turbidimetric measurement offers a rapid and accurate quantitation of gelsolin in human serum samples. Serum gelsolin seems a promising additional diagnostic marker of sepsis which has to be further investigated.

KEYWORDS
automation, diagnostic marker, immune turbidimetry, sepsis, serum gelsolin

1 | INTRODUCTION

For quick diagnosis and/or decision-making when critically ill patients are considered, the need of fast and accurate laboratory methods for quantification of serum proteins with predictive value is of high importance. Human serum gelsolin (se-GSN) is a Ca^{2+}-dependent 93 kDa protein mainly synthesized by skeletal muscle cells and consisting of 6 gelsolin homolog domains.[1,2] Aside from its actin-binding capacity,[3] se-GSN is suggested to be involved in the modulation of inflammatory processes, too.[4,5] In severe systemic inflammation, as sepsis, se-GSN levels drop significantly. Gelsolin is part of the so called actin scavenger system and therefore a possible reason for its decrease is the

excessive release of filamentous actin into the circulation due to massive cell injury,[3,6,7] and—in case of bacterial infections—the appearance of high amounts of bacterial wall lipids as well.[4,5] Up to now, only a few studies have proven the possible predictive capacity of GSN levels in sepsis,[6,7] severe burns,[8] traumatic brain injury,[9] but also in autoimmune diseases,[10] chronic kidney disease,[11] and HIV-1 disease.[12] However, even at present a rapid automated measurement of this actin-binding protein still remains a challenge. The aim of the present work was to develop and validate a fast immune turbidimetric assay for serum GSN that would be suitable for possible routine clinical use.

2 | MATERIALS AND METHODS

2.1 | Reagents, assay conditions

The immune turbidimetric assay for se-GSN measurement was performed on an open developmental channel of the c502 module of a Cobas 8000 analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Because of the unavailability of any commercial GSN calibrator for immune turbidimetric assay, we applied recombinant human GSN expressed in *Escherichia coli* [His-8] (Department of Biophysics, University of Pécs, Hungary[13]). Dilution series of the calibrator were prepared using fetal bovine serum (FBS, ref. no. Ph. Euro. 2262, PAN Biotech, Aidenbach, Germany). The FBS did not give any reaction with the anti-human GSN antibody, therefore, we could use it as a serum matrix imitating the physicochemical properties of human serum samples. Pooled human serum from healthy volunteers served as an "in-house" control, due to the lack of commercially available quality control material. We used Polyclonal Rabbit Anti-Human GSN antibody in the assay (ref. no. A0146, Dako A/S, Glostrup, Denmark) pre-diluted (1:4) with Dilution Buffer (ref. no. S2005, Dako A/S); and Reaction Buffer (ref. no. S2007, Dako A/S), based on the previous work of Christensen et al[14] with modifications. The sample volume was 7 μL, the volume of the reaction buffer was 100 μL and that of the pre-diluted (1:4) antibody was 50 μL. The wavelength applied for the turbidimetric reaction was 340 nm and delta absorbance was calculated from the data obtained between 41-70 measuring points. The two-point end assay was performed at 37°C with 10-minute reaction time. Full RCM calibration was done by applying a six-point standard curve in the range of 10-260 mg/L gelsolin standards.

2.2 | Validation of the GSN assay

The second edition of Eurachem guidelines[15] was applied for the validation. FBS with GSN buffer (containing no human GSN) was utilized as blank sample (FBS:buffer ratio was similar to that of the highest GSN calibrator). Limit of blank (LOB: mean + 1.645 × SD), limit of detection (LOD: mean + 3 × SD) and limit of quantification (LOQ: mean + 10 × SD) were assessed using the absorbance data of 30 independent blank samples.

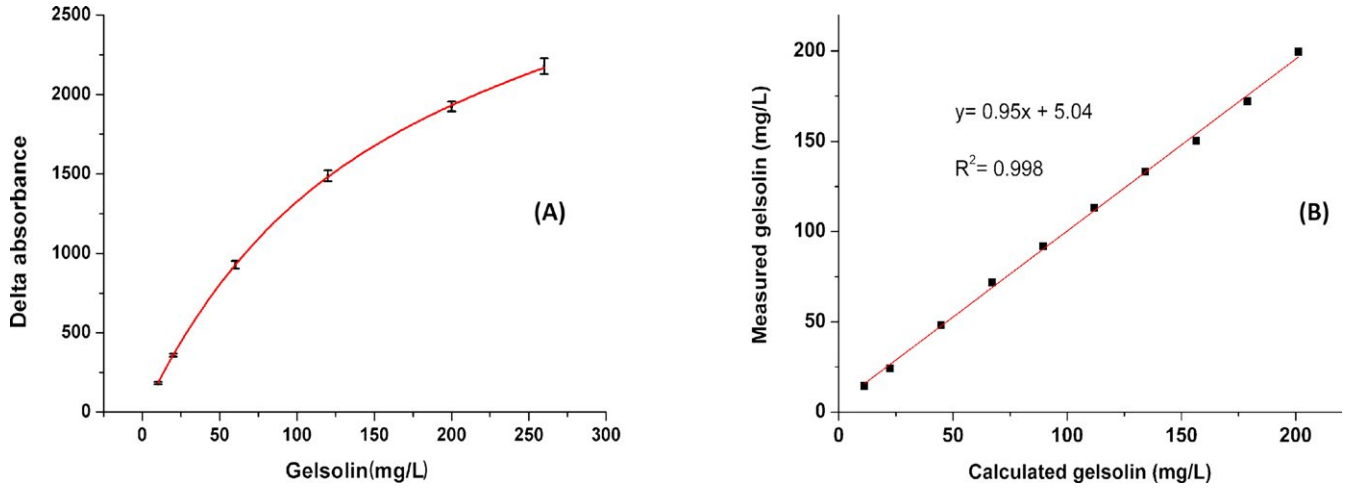

FIGURE 1 Validation results. (A) Summarized graph of a 6-point calibration curve of GSN assay in the range of 10-260 mg/L by exponential graph fitting (n = 9). (B) Linearity of serum GSN assay. Dots represent means. r^2: regression coefficient

TABLE 1 Intra-, inter-assay precision and recovery of serum gelsolin (GSN) assay

Sample	Intra-assay (n = 15)		Recovery% (n = 15)		Inter-assay (n = 20)	
	Mean ± SD (mg/L)	CV%			Mean ± SD (mg/L)	CV%
L	20.34 ± 0.79	3.93	87.90	84.56	21.03 ± 1.05	4.99
M	69.91 ± 1.40	2.00	-	-	69.93 ± 2.06	2.94
H	112.51 ± 3.04	2.71	93.52	92.57	113.30 ± 5.95	5.25

For recovery study both of the low and the high GSN concentration samples were spiked with purified human GSN.

L, low GSN concentration sample; M, middle GSN concentration sample; H, high GSN concentration sample.

Intra- and inter-assay imprecisions were estimated by three different sera (low, middle and high GSN concentrations). For the assessment of intra-assay variability, 15 parallel measurements were performed on the same day, while for inter- assay variability two parallel measurements were executed on 10 consecutive days.

For recovery studies, aliquots of both the low and high GSN concentration serum samples were spiked with two different amounts of human purified GSN standard, thereafter recovery (%) was calculated from the measured (n = 15/level) and calculated values.

Linearity was assessed by two parallel measurements for each level of 10 dilutions of a serum sample in the range of 11.18-201.2 mg/L GSN.

Stability studies were performed by examining five different serum samples (ranging from 32.94 to 122.14 mg/L) when storing them at +2–8°C. Se-GSN levels were determined on the 1st, 3rd, 5th, 8th and 10th day, respectively. Furthermore, stability of se-GSN was evaluated in the course of 5 freezing-thawing cycles. Aliquots of 5 different sera were frozen at −70°C and subsequently thawed at 20-25°C. After every cycle, se-GSN levels were assessed.

2.3 | Clinical evaluation of se-GSN assay

We analyzed a representative number of sera of septic, systemic inflammatory response syndrome (SIRS) and control patients by immune turbidimetry, which had already been investigated by Western blot measurements in our previously published study.[7] The validated immune turbidimetric GSN assay was compared to previous Western blot method by Bland-Altman plot procedure. Western blot and immune turbidimetric results were also studied by receiver operating characteristic curve (ROC) analysis. Regarding the study protocol, the applied sepsis definitions and patients' enrollment we refer to our latest publication.[7] APACHE II (Acute Physiology and Chronic Health Evaluation II), SAPS II (Simplified Acute Physiology Score II) and SOFA (Sequential Organ Failure Assessment) scores were estimated for the first day of intensive care treatment.

2.4 | Statistical analysis

For statistical analysis IBM SPSS Statistics for Windows, Version 22 and Origin Pro 8 softwares were used. Kruskal-Wallis, Mann-Whitney and chi-squared tests were performed for investigating differences between patient groups. Diagnostic values were assessed by receiver operating characteristic (ROC) curves. Bland-Altman plot was used for method comparison. Changes in the results were considered to be statistically significant at $P < .05$.

3 | RESULTS

3.1 | Validation data

Figure 1A represents a cumulative graph of 9 independent calibrations performed during the validation period. LOB, LOD, LOQ were found

TABLE 2 Stability of se-GSN

Initial GSN concentration (mg/L)	Storage at +4°C				Storage at −70°C				
	Day 3 [%]	Day 5 [%]	Day 8 [%]	Day 10 [%]	Cycle 1 [%]	Cycle 2 [%]	Cycle 3 [%]	Cycle 4 [%]	Cycle 5 [%]
32.94	102.17	100.39	108.58	110.41	100.99	102.97	104.56	106.76	107.37
14.88	96.70	101.84	114.84	109.61	107.01	106.27	106.27	93.78	98.90
77.72	103.16	105.87	105.61	107.97	103.29	103.16	105.35	108.10	105.74
60.89	104.19	112.72	114.05	117.36	106.46	106.32	113.01	114.80	115.10
122.14	101.47	98.99	100.34	104.84	101.47	101.13	101.13	100.68	102.05

to be 0.47 mg/L, 0.72 mg/L and 1.99 mg/L, respectively. Coefficient of variation remained below 5% in most of the cases during the intra- and inter-assay variability measurements. Recovery varied between 84.56%-93.52% when investigating four different ranges (Table 1).

Linearity study gave an appropriate coefficient by the linear regression analysis (r^2 = .998) after comparing calculated and measured GSN concentrations regarding 10 different dilutions (Figure 1B).

3.2 | Stability studies

GSN levels remained almost unchanged (96.70%-117.36%) during the 10-day stability period, and no considerable differences were observed even throughout five repeated freezing-thawing cycles (Table 2).

3.3 | First-day serum GSN levels of septic, SIRS and control patients

Table 3 illustrates the main demographic and laboratory parameters of the enrolled patients. First-day clinical scores (APACHE II, SAPS II, SOFA) were proven to be significantly (P < .05) higher among septic than among SIRS patients. Septic patients showed higher levels of serum procalcitonin (PCT) (P < .001) and high-sensitivity C-reactive protein (P < .01) than non-septic critically ill patients. Septic and SIRS patients exhibited significantly lower first-day serum GSN levels than controls when measured by immune turbidimetry (P < .001) (Figure 2A, Table 3). Furthermore, serum GSN levels were lower in septic than in SIRS patients (P = .015). Previous Western blot results showed a similar pattern to those of immune turbidimetry except that among the limited number of subjects there was no significant difference between serum GSN levels of septic and SIRS patients (Table 3).

Bland-Altman plot defined a bias of 0.26 and an agreement range from −0.79 to 1.09 units when comparing GSN levels of septic, SIRS and control patients measured by previous Western blot method[7] and by the new turbidimetric assay (Figure 2B).

3.4 | Diagnostic value of serum GSN levels in sepsis

We also investigated whether first-day se-GSN measured by immune turbidimetry could differentiate between the septic and SIRS states. Area under the curve (AUC) value for se-GSN was found to be 0.79, the determined cut-off point was 14.05 mg/L (sensitivity: 75%; specificity: 60%), (Figure 3A). Western blot measurement offered a lower AUC (0.63) for se-GSN when compared to immune turbidimetry. Serum PCT and high-sensitivity C-reactive protein (hs-CRP) had an AUC of 0.98 and 0.85, respectively (Figure 3B).

4 | DISCUSSION

In the field of life-threatening inflammatory diseases, as sepsis or severe burns, proteins that can be detected rapidly and with a predictive value, are mandatory. In our study, we introduced a fast, accurate immune turbidimetric method requiring a very low sample volume for the determination of se-GSN levels, partially based on the previous work of Christensen et al.[14] To our latest knowledge, no other similarly fast assay for se-GSN is available. For detecting se-GSN, functional nucleation, Western blot, and ELISA are the only available laboratory techniques[16] which require a longer assay time compared to our method. Despite the fact that most ELISA methods have sufficiently low detection limits,[17] they are quite user-dependent (eg, incubation times, pipetting failures) and are

TABLE 3 Data of the studied patients

	Sepsis (n = 25)	SIRS (n = 8)	Control (n = 14)	*P* value
Demographic data				
Age, y	73 (30-82)	58 (50-73)	63 (38-78)	n.s.
Male/female	18/7	6/2	7/7	n.s.
1st day clinical scores				
APACHE II	17.50 (12-25)	8 (5.25-13.25)	-	<.01
SAPS II	51 (37.25-66)	24 (16.50-29.75)	-	<.001
SOFA	9 (7-12)	5.50 (4.25-9.75)	-	<.05
1st day laboratory data				
se-hsCRP, mg/L	216.0 (127.75-306.39)	68.50 (46.34-116.19)	1.24 (0.22-1.92)	<.01
se-PCT, ng/mL	18.64 (4.64-92.10)	0.35 (0.18-0.49)	-	<.001[a]
se-GSN (IT), mg/L	12.08 (3.89-19.97)	24.67 (13.11-25.94)	58.99 (44.34-63.83)	<.05[a]; <.001[b, c]
se-GSN (WB), mg/L	16.74 (8.21-34.57)	26.31 (16.89-38.25)	74.87 (30.67-91.83)	<.01[b, c]

Data are expressed as medians (25%-75% percentiles), except for age (median [min-max]). Level of significance is set at P < .05.
Superscript lowercase letters refer to post-hoc analyses. a: sepsis-SIRS; b: sepsis-control; c: SIRS-control.
APACHE II, Acute Physiology and Chronic Health Evaluation II; SAPS II, Simplified Acute Physiology Score II; SOFA, Sequential Organ Failure Assessment); IT, immune turbidimetry; WB, Western blot.

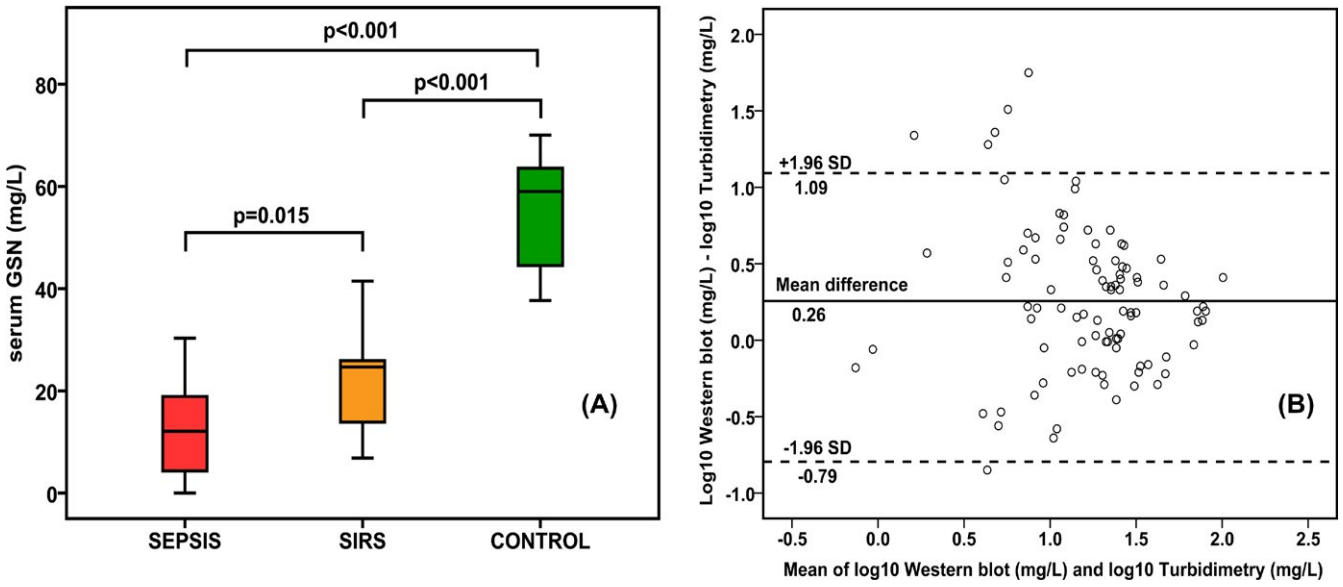

FIGURE 2 Clinical evaluation and method comparison of serum gelsolin determination. (A) Serum GSN levels in septic, SIRS and control patients measured by immune turbidimetry. (B) Bland-Altman plot: comparing the data of Western blot and immune turbidimetric measurements. Because of the non-normally distributed differences, lg of the data was calculated

not commonly automated. Cobas 8000 analyzer enables an excellent precision and it is easily connectable to the laboratory informational system (LIS) as well as to the hospital information system (HIS).

Former study of Christensen et al[14] presented an immune turbidimetric assay for se-GSN on Cobas Mira Plus which offered a slightly lower (4%) total imprecision when compared to our method and a detection limit of 2.7 U/L. However, due to unexpected GSN calibrator and control stability problems, this former assay is no longer available. Study of Dahl et al[18] presented an immunonephelometric measurement for plasma GSN, but they did not offer any validation data about the assay.

We found se-GSN as a stable protein maintaining its concentration constant at +4°C for 10 days and even throughout five repeated freezing-thawing cycles. We did not find any significant differences between serum and plasma levels of GSN (when using sodium citrate tube). However, we do not consider EDTA containing tubes for sample collection when measuring GSN levels because plasma GSN levels drop significantly in a short time (data not shown). One possible reason for that could be that EDTA binds Ca^{2+} ions more strongly than sodium citrate, in addition, it chelates other metal ions too,[19] which results in the destabilization of the GSN domains.

We could confirm the previously published[7] diagnostic capability of first-day se-GSN levels in sepsis by immune turbidimetry, too.

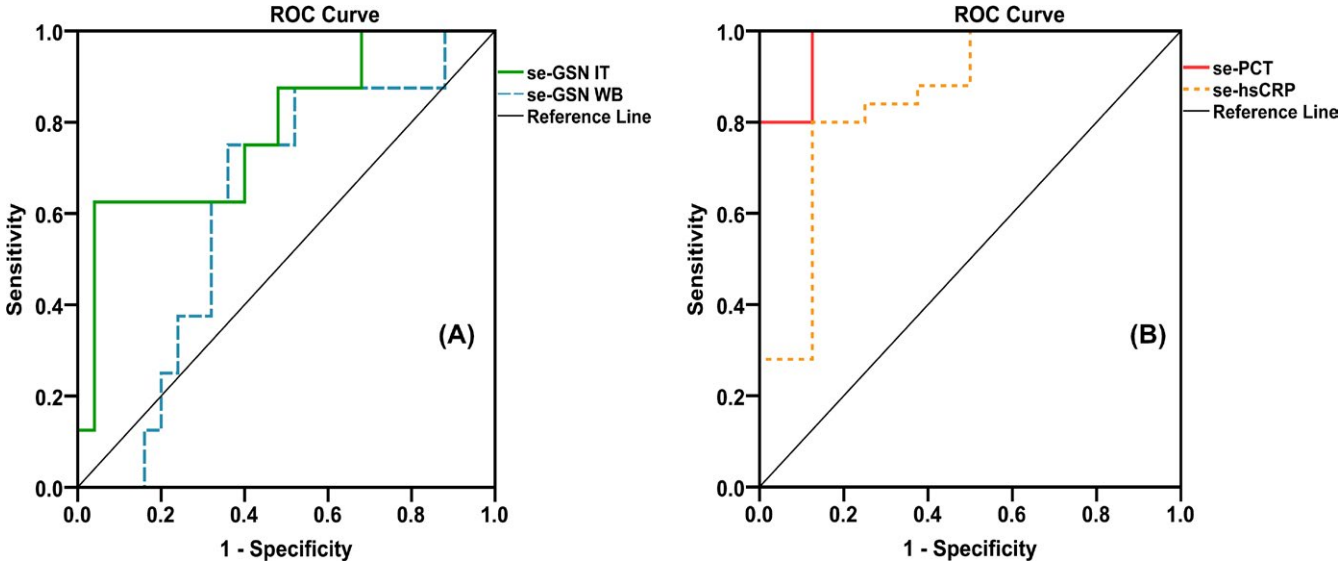

FIGURE 3 Receiver operating characteristic (ROC) curves of serum laboratory parameters in sepsis. (A) ROC of se-GSN. (B) ROC of se-PCT and se-hsCRP. IT, immune turbidimetry; WB, Western blot

Previous Western blot results showed slightly higher GSN levels in all patients when compared to those of our new GSN assay. That difference could be attributed to the fact that Western blot is a more user-dependent method than immune turbidimetry. Similar to our results, studies of Wang et al[6] also found significantly lower first-day se-GSN levels in septic patients when compared to non-septic critically ill patients.

We acknowledge that our study has limitations. Currently, there is no commercially available human GSN calibrator and control for immune turbidimetry. To handle this difficulty we applied highly purified human GSN expressed in E. coli as a calibrator, and we used pooled serum from healthy individuals as an internal control material. The described rapid and accurate measurement of GSN suggests the ultimate need to develop a commercially available GSN calibrator and control system in the near future. Our present work focused mainly on the validation of GSN assay rather than on its clinical assessment, therefore we analyzed a limited number of sera obtained from critically ill patients. A detailed clinical evaluation of the newly developed GSN assay is already under investigation.

Diagnosis of sepsis is still difficult because it comprises partially uncovered pathobiochemical processes and even at present, there are no gold standard diagnostic tests. The diversity of underlying diseases of affected patients and the low specificity of SIRS criteria also hamper correct decision-making. As a consequence, in February 2016, the new Sepsis-3 definitions have been introduced[20] offering a greater consistency and earlier recognition of this life-threatening condition. Therefore, any protein marker investigations focusing on sepsis-including serum GSN studies-should also be performed with respect to the Sepsis-3 criteria.

Immune turbidimetric measurement offers a rapid an accurate way to detect GSN in human serum samples. The diagnostic performance of serum GSN should give additional information for clinicians, and its possible further clinical use has to be further investigated.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Predictive value of serum gelsolin and Gc globulin in sepsis – a pilot study

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Abstract

Background: Simultaneous determination of the two main actin scavenger proteins in sepsis has not been investigated until now. In our pilot study, we elucidated the predictive values of Gc globulin and gelsolin (GSN) in sepsis by comparing them to classic laboratory and clinical parameters.

Methods: A 5-day follow-up was performed, including 46 septic patients, 28 non-septic patients and 35 outpatients as controls. Serum Gc globulin and GSN levels were determined by automated immune turbidimetric assay on a Cobas 8000/c502 analyzer. Patients were retrospectively categorized according to the sepsis-3 definitions, and 14-day mortality was also investigated.

Results: First-day GSN also differentiated sepsis from non-sepsis (AUC: 0.88) similarly to C-reactive protein (AUC: 0.80) but was slightly inferior to procalcitonin (PCT) (AUC: 0.98) with a cutoff value of GSN at 22.29 mg/L (sensitivity: 83.3%; specificity: 86.2%). Only first-day SOFA scores (0.88) and GSN (0.71) distinguished septic survivors from non-survivors, whereas lactate (0.99), Gc globulin (0.76) and mean arterial pressure (MAP) (0.74) discriminated septic shock from sepsis. Logistic regression analyses revealed SOFA scores and GSN being significant factors regarding 14-day mortality. First-day GSN levels were higher ($p < 0.05$) in septic survivors than in non-survivors. Gc globulin levels remained higher ($p < 0.01$) in sepsis when compared with septic shock during the follow-up period.

Conclusions: Both serum GSN and Gc globulin may have predictive values in sepsis. Considering the small sample size of our study, further measurements are needed to evaluate our results. Measurement of Gc globulin and GSN maybe useful in assessment of sepsis severity and in therapeutic decision-making.

Keywords: Gc globulin; gelsolin; immune turbidimetry; predictive value; sepsis-3.

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Introduction

Sepsis is a life-threatening organ dysfunction caused by a dysregulated host response to infection. The syndrome has an increasing incidence (3–10/1000/year in high-income countries) due to aging populations with more comorbidities and more efficient recognition, but acute mortality of sepsis in adults is improving [1–3]. However, survivor patients have an increased risk for infection in the year following their intensive care unit (ICU) discharge and usually suffer from long-term consequences, including cognitive impairments and cardiovascular diseases [4, 5]. The latest sepsis-3 conference in February 2016 highlighted that the diagnosis of sepsis should be based on – apart from a presumed infection – parameters incorporated into quick Sequential Organ Failure Assessment (qSOFA) and Sequential Organ Failure Assessment (SOFA) clinical scores [1].

From the laboratory side, high-sensitivity C-reactive protein (hsCRP) and procalcitonin (PCT) are the preferred markers of sepsis [6]; the latter provides useful information on the efficacy of antibiotic therapy as well [2].

However, there is still an ongoing struggle to find new protein markers of ICU patients with high sensitivity, high specificity and with short assay time, from the methodological side.

Human plasma gelsolin (GSN) (molecular weight [MW] = 93 kDa) is a highly Ca^{2+}-dependent protein encoded by a gene on chromosome 9 consisting of six GSN homologue domains. Plasma GSN is mainly synthesized by skeletal muscle cells [7, 8].

Serum group-specific component (Gc) globulin (or vitamin D binding protein) (MW = 52–59 kDa) is a glycosylated α_2-globulin encoded by a gene on chromosome 4 and mainly expressed by hepatocytes [9, 10]. The protein is built up of three structurally similar domains.

Several similar biological properties have been attributed to GSN and Gc globulin. Both of them are essential actin scavengers working in concert in the extracellular space: GSN severs and depolymerizes actin filaments leaking from disrupted cells, and Gc globulin – because of its high affinity to actin monomers – frees GSN from actin monomers and sequesters them. Then the entrapped actin is subsequently cleared from the circulation by the reticuloendothelial system [8]. In case of severe systemic inflammation, a large-scale cell injury occurs and actin releases in excess to the circulation at an extent that could easily saturate the binding capacities of the capture proteins, depleting the plasma/serum levels of GSN and Gc globulin as well [11–24]. On the other hand, both GSN and Gc globulin could modulate inflammatory processes: GSN binds bacterial wall lipids [25], whereas Gc globulin enhances neutrophil chemotaxis and could modulate T cell responses [10]. In addition, Gc globulin functions also as the main transporter of circulating vitamin D metabolites [10].

Previous studies focused solely on the examination of GSN or that of Gc globulin alone in severe systemic inflammatory disorders [11–24], but – to the best of our knowledge – no previous research investigated quantitatively the two actin-binding proteins together in sepsis. The aim of the present work was to examine the predictive value of serum GSN and Gc globulin in sepsis and to compare them with the classic laboratory markers and clinical scores.

Materials and methods

Blood sampling

Venous blood (7.5 mL) was drawn from every patient via central venous catheter or from a peripheral vein into plain tubes with accelerator gel using a closed blood sampling system (BD Vacutainer®). After 30 min, clotted blood samples were centrifuged for 10 min at 1500*g*, and sera were immediately analyzed or stored at –80 °C.

Determination of serum Gc globulin and GSN levels

Reagents, assay conditions: The immune turbidimetric assay for se-Gc globulin and GSN measurement was executed on open developmental channels of the c502 module of a Cobas 8000 analyzer (Roche Diagnostics GmbH, Mannheim, Germany).

Serum Gc globulin levels were measured according to the modified protocol of Bangert [26] by using Polyclonal Rabbit Anti-Human Gc-Globulin antibody (ref. no. A0021, Dako A/S, Glostrup, Denmark) prediluted (1:5) with Dilution Buffer (ref. no. S2005, Dako A/S); Reaction Buffer (ref. no. S2007, Dako A/S); Human Serum Protein Calibrator (ref. no. X0908, Dako A/S) and Human Serum Protein Low Control (ref. no. X0939, Dako A/S).

Serum GSN assay was carried out according to our previous work [27], thereby using Polyclonal Rabbit Anti-Human GSN antibody (ref. no. A0146, Dako A/S) prediluted (1:4) with Dilution Buffer (ref. no. S2005, Dako A/S) and Reaction Buffer (ref. no. S2007, Dako A/S).

Determination of routine laboratory parameters and clinical scores

Total blood cell counts, plasma lactate, serum albumin, hsCRP and PCT levels were determined by automated routine laboratory techniques. qSOFA scores were calculated based on ICU admission parameters. Acute physiology and chronic health evaluation II (APACHE II), simplified acute physiology score II (SAPS II) and SOFA scores were estimated for the first day of intensive care treatment. Mean arterial pressure (MAP) was assessed by intra-arterial blood pressure monitoring in the ICU.

Categorization of patients and study design

Our study protocol was authorized by the Regional Research Ethical Committee of the University of Pécs (4327.316-2900/KK15/2011) and was performed according to the ethical guidelines of the 2003 Declaration of Helsinki. Written informed consent was obtained from all patients or their families. Patients from the Department of Anaesthesiology and Intensive Therapy were enrolled in our follow-up study from January 2013 to August 2016. Patients were followed during their ICU stay where serum samples were obtained on days 1, 2, 3 and 5 after clinical diagnosis. Septic patients diagnosed according to the former sepsis-2 guidelines [28] were retrospectively categorized as stated by the sepsis-3 definitions [1] into sepsis and septic shock groups. Hereafter, we performed our study based on the latter categorization. Organ dysfunction should be determined as an acute change in SOFA score (≥2 points) as a consequence to the infection. In our study, the baseline SOFA scores were assumed to be zero in every patient since no clinical scores were estimated before the ICU admission. Septic shock was defined as a subset of sepsis in which persisting hypotension (requiring vasopressors to maintain MAP

≥65 mmHg) was present and plasma lactate levels were more than 2 mmol/L despite adequate volume resuscitation.

Voluntary blood donors and preoperative ophthalmologic patients having their blood counts, liver enzyme activities and hsCRP levels in the reference range served as controls.

Non-septic ICU patients had negative blood culture results, and PCT levels were below 1.0 ng/mL.

ICU patients were excluded if they suffered from any autoimmune disorders, preexisting hepatic failure or were under 18 years of age. Control patients under the age of 18 years and those with symptoms of acute inflammatory diseases or suffering from autoimmune disorders were also excluded from the study. Among septic patients, 14-day mortality was investigated.

Statistical analysis

For statistical analysis, IBM SPSS Statistics for Windows, Version 22 was used. Power analysis was applied to calculate the required number of patients in each group. Kruskal-Wallis, Mann-Whitney and χ^2-tests were performed for investigating differences between patient groups. Friedman's analysis and *post hoc* Wilcoxon signed-rank tests were performed for follow-up comparisons. Predictive values were assessed by receiver operating characteristic (ROC) curves and logistic regression analysis. Correlations between quantitative parameters were determined by Spearman's rank correlation test. Data are expressed as medians and as interquartile ranges (IQR). Changes in the results were considered to be statistically significant at $p < 0.05$.

Results

Main clinical and laboratory findings

We observed predominantly males in their 60s suffering from sepsis and septic shock, similar to non-septic ICU patients (Table 1). Septic and septic shock patients were treated about 7.5-times longer at the ICU than non-septic intensive care patients ($p < 0.05$). More (74.3%) ICU patients required intensive care treatment after surgical investigations (e.g. management of ileus, pancreatic cancer surgery) than those after other internal medicine complications (e.g. chronic obstructive pulmonary disease exacerbation, pneumonia-related respiratory insufficiency). Data of control, non-sepsis and sepsis patients were comparable regarding age, gender and comorbidities.

In the intensive care patients' groups, qSOFA scores did not alter significantly. First-day APACHE II scores

Table 1: Clinical and routine laboratory data of the enrolled patients.

	Control (n = 35)	Non-sepsis (n = 28)	Sepsis (n = 33)	Septic shock (n = 13)	p-Value
Clinical data					
Age, years	60 (56–68)	62 (55–71)	66 (56–76)	65 (47–76)	n.s.
Males (%)	20 (57.1)	20 (71.4)	24 (72.7)	8 (61.5)	n.s.
ICU stay, days	–	1 (1–2)	7 (4.5–12.5)	8 (3.5–15)	<0.05[b, d]
CKD, n (%)	0	3 (10.7)	2 (6.1)	1(7.7)	<0.05[f]
COPD, n (%)	1 (2.9)	7 (25)	10 (30.3)	4 (30.8)	<0.01[c, e, f]
CVD, n (%)	21 (60)	24 (85.7)	24 (72.7)	8 (61.5)	<0.05[f]
DM, n (%)	6 (17.1)	7 (25)	5 (15.2)	0	<0.05[b]
Admission					
Medical	–	1	12	6	
Surgical	–	27	21	7	
qSOFA	–	1.5 (1–2.7)	1 (1–2)	1.5 (1–2)	n.s.
First-day data					
APACHE II	–	8 (5.3–13.3)	14 (10.8–19.3)	20.5 (16–30.3)	<0.05[a, b, d]
SAPS II	–	24 (16.5–29.8)	36.5 (29–53.3)	48 (37.5–74.3)	<0.01[b, d]
SOFA	–	5.5 (4.3–9.8)	7.5 (7–10)	11 (7–15)	<0.01[a, b]
MAP, mmHg	–	62.5 (57.8–74.3)	71 (64.5–85.5)	65 (60.3–75.3)	<0.01[a]
pl-Lactate, mmol/L	–	1 (0.8–1.4)	0.9 (0.9–1.7)	3.4 (2.5–5.8)	<0.001[a, b]
se-Albumin, mg/L	46.3 (44.4–47.7)	31.6 (26.4–33.3)	22.6 (19.5–26.7)	18.7 (13.8–20.2)	<0.01[a–f]
se-hsCRP, mg/L	1.2 (0.5–2)	103.3 (64.3–146.3)	247.4 (124.8–320.3)	226 (143.8–306.4)	<0.001[b–f]
se-PCT, ng/mL	–	0.3 (0.1–0.5)	8.3 (3.7–38.3)	24.8 (10.3–71.3)	<0.001[b, d]
se-Gc, mg/L	359.9 (338.3–381.3)	218.1 (170.2–243.2)	237.0 (160.6–285.6)	82.1 (42.7–195.9)	<0.01[a, b, c, e, f]
se-GSN, mg/L	58.9 (52.6–65.4)	32.1 (24.6–41.1)	9.6 (4.7–19.5)	12.5 (3.3–17.9)	<0.05[b, c, d, e, f]

Data are expressed as median (%), and interquartile ranges (25%–75%) are given in parentheses. CKD, chronic kidney disease; COPD, chronic obstructive pulmonary disease; CVD, cardiovascular disease; DM, diabetes mellitus; ICU, intensive care unit; n.s., non-significant. Superscript lowercase letters refer to *post hoc* analyses: [a]septic shock – sepsis; [b]septic shock – non-sepsis; [c]septic shock – control; [d]sepsis – non-sepsis; [e]sepsis – control; [f]non-sepsis – control.

differed ($p < 0.05$) in all three ICU groups, whereas SAPS II scores were higher ($p < 0.01$) in sepsis and in septic shock patients when compared with the non-septic group. SOFA scores were more increased ($p < 0.01$) in septic shock compared with sepsis and non-sepsis patients. Serum albumin levels were different ($p < 0.01$) in all enrolled patients' groups. Classic inflammatory markers (serum hsCRP, PCT) were significantly ($p < 0.001$) higher in septic shock and in sepsis patients compared with non-septic ICU patients.

Serum GSN and Gc globulin levels in septic shock, septic, non-septic critically ill and control patients

First-day serum levels of both actin-binding proteins were significantly ($p < 0.001$) higher in the control population than in the ICU patients (Table 1, Figure 1A, B). Non-septic ICU patients exhibited higher ($p < 0.001$) first-day serum GSN concentrations than patients with sepsis and septic shock. First-day Gc globulin levels were significantly higher in non-septic critically ill patients ($p < 0.001$) and in septic patients ($p < 0.01$) when compared with those suffering from septic shock.

In the course of the 5-day follow-up period, three septic patients died and another five septic patients were discharged earlier from the ICU. Four septic shock patients died during the observation days, and nine of them survived. Patients suffering from sepsis had significantly ($p < 0.01$) higher serum Gc globulin levels during the 5-day follow-up when compared with those with septic shock (Figure 1D) (median Gc globulin concentrations were as follows in sepsis vs. septic shock: first day: 237 vs. 82.1 mg/L; second day: 295.6 vs. 197.2 mg/L; third day: 257.8 vs. 188.5 mg/L; fifth day: 267.2 vs. 116.4 mg/L, respectively). Significantly

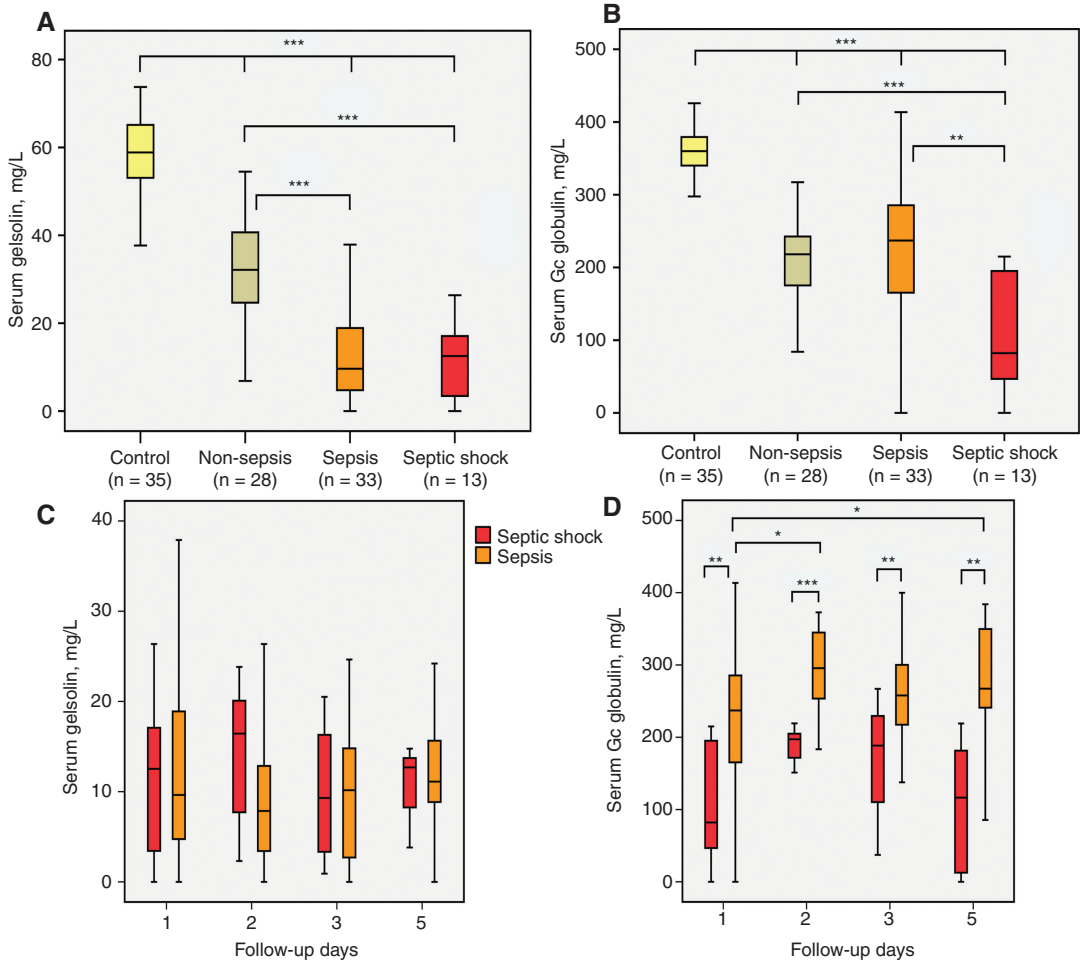

Figure 1: Serum gelsolin and Gc globulin concentrations in controls and in intensive care patients.
First-day serum gelsolin (A) and Gc globulin (B) levels in septic shock, septic, non-septic ICU and control patients, and follow-up of serum gelsolin (C) and Gc globulin (D) concentrations where patients are divided into septic and septic shock groups. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

($p < 0.05$) increased second and fifth day's serum Gc globulin levels were detected in septic patients when compared with the first-day concentrations. The two investigated patient groups did not differ significantly regarding serum GSN levels during the 5-day follow-up, and no increasing or decreasing tendency was noted (Figure 1C).

Data of septic survivors and non-survivors when investigating 14-day mortality

First-day classic parameters and microbiological findings

Septic survivors presented lower ($p < 0.05$) qSOFA scores compared with non-survivors (Table 2). All of the three investigated clinical scores were different ($p < 0.01$) between the two septic groups. MAP and plasma lactate levels were similar in both surviving and in non-surviving patients. Increment of the first-day's hsCRP and PCT levels did not differ significantly in the two septic groups, and albumin levels were also similarly decreased.

Microbiological cultures gave positive results in 76.8% of the septic patients (Table 2). Frequently detected bacteria were Gram-positive *Staphylococcus aureus* and *Enterococcus*, Gram-negative *Escherichia coli* and *Pseudomonas*, whereas for invasive fungal infections, *Candida albicans* was most commonly responsible.

Serum GSN and Gc globulin levels in septic survivor and non-survivor patients

First-day serum GSN levels were higher ($p < 0.05$) in survivor than in non-survivor septic patients (Table 2, Figure 2A). No further significant differences or changes were observed in serum GSN concentrations during the 5-day time course of sepsis.

Serum Gc globulin concentrations did not differ significantly on the first day of observation when investigating the two septic patients' groups (Table 2, Figure 2B). However, there was a trend ($p < 0.05$) towards increasing Gc globulin levels, when comparing the first with the second day's (median: 212.8 vs. 271.9 mg/L) and the first with the third day's (median: 212.8 vs. 235.2 mg/L) Gc globulin levels in survivors. Similar tendency ($p < 0.05$) was seen in non-survivors when comparing the first with the second day's serum Gc levels (median: 155 vs. 267.1 mg/L).

From the first to the fifth day of ICU follow-up period, 21.4% of the survivors were discharged from the unit because of requiring no more intensive therapy, whereas

Table 2: First-day data and microbiological findings of septic survivors and non-survivors regarding 14-day mortality.

	Septic survivors (n = 28)	Septic non-survivors (n = 18)	p-Value
First-day data			
qSOFA	1 (1–1.25)	1.5 (1–2)	<0.05
APACHE II	13 (10–18)	23 (16–32)	<0.01
SAPS II	34 (25–51)	53 (43.5–70)	<0.01
SOFA	7 (7–9.5)	11 (9–15.5)	<0.001
MAP, mmHg	70 (65–83.5)	67.5 (59.5–80.5)	n.s.
pl-lactate, mmol/L	1.5 (1–2.2)	1.7 (1.1–5.2)	n.s.
se-albumin, mg/L	22.6 (19.3–26.6)	19.8 (16.2–23.5)	n.s.
se-hsCRP, mg/L	236.7 (151–302.9)	248.3 (121–328)	n.s.
se-PCT, ng/mL	11.2 (4.3–24.8)	26.1 (4.5–77.4)	n.s.
se-Gc, mg/L	212.8 (158.3–284.4)	155 (39.1–243.5)	n.s.
se-GSN, mg/L	12.9 (6.9–21.9)	6.9 (2.8–14.9)	<0.05
Microbiological findings			
Gram-positive	7	2	
Gram-negative	–	6	
Gram-positive and Gram-negative	6	4	
Fungi	1	–	
Gram-positive and fungi	1	–	
Gram-negative and fungi	2	3	
Gram-positive and Gram-negative and fungi	3	–	
Non-identified	8	3	

Data are expressed as medians, and interquartile ranges (25%–75%) are given in parentheses. Microbiological findings are represented by numbers. MAP, mean arterial pressure.

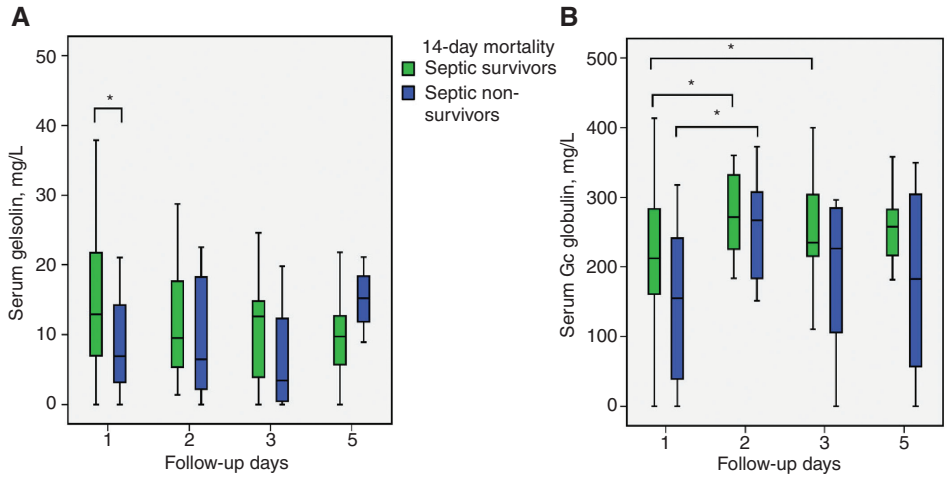

Figure 2: Serum gelsolin and Gc globulin levels in septic survivors and non-survivors.
Follow-up of serum gelsolin (A) and Gc globulin (B) concentrations where patients are divided into septic survivor and non-survivor groups based on 14-day mortality. *$p < 0.05$.

38.9% of the non-survivors died and 11.1% of them were released to other hospital units.

Discriminative values of first-day parameters by investigating ROC analysis

In the differentiation of septic patients from other patients suffering from non-infective diseases requiring ICU treatment, besides serum PCT (area under the curve [AUC]: 0.98, $p < 0.001$) and hsCRP (AUC: 0.80, $p < 0.01$), GSN also had significant (AUC: 0.88, $p < 0.001$) discriminative value with a cutoff point of 22.29 mg/L (sensitivity: 83.3%, specificity: 86.2%) (Figure 3A,B). Opposite to GSN, Gc globulin did not have any significant distinguishing role.

The discriminative function of plasma lactate regarding septic shock/sepsis states was proven to be the highest (AUC: 0.99, $p < 0.001$; Figure 3D); in addition, Gc globulin (AUC: 0.76) and MAP (AUC: 0.74) also had significant ($p < 0.05$) diagnostic values (Figure 3C). The optimal cutoff point for Gc globulin was 116.5 mg/L (sensitivity: 78.3%, specificity: 60%). GSN, PCT, hsCRP, qSOFA and SOFA scores did not have any significant informative values in differentiation of septic shock from sepsis.

For predicting 14-day mortality in sepsis, SOFA clinical scores (AUC: 0.88, $p < 0.001$) and serum GSN (AUC: 0.71, $p < 0.05$) proved to be significant as discriminating factors regarding surviving/non-surviving states (Figure 3E,F). The calculated cutoff value for GSN was 8.7 mg/L (sensitivity: 71.4%, specificity: 58.3%). No significant predictive values were found in cases of MAP, lactate, PCT, hsCRP and qSOFA scores, respectively.

Assessment of predictive values by using logistic regression analysis and Spearman's correlation results

Including the investigated parameters into the logistic regression model, SOFA scores ($\beta = 0.53$; $p = 0.03$; OR = 1.70; 95% CI: 1.03–2.79) and serum GSN ($\beta = -0.15$; $p = 0.04$; OR = 0.87; 95% CI: 0.75–0.99) proved to have predictive capacity regarding 14-day mortality in sepsis. However, in our study, plasma lactate ($\beta = 0.49$; $p = 0.17$; OR = 1.64; 95% CI: 0.82–3.28), PCT ($\beta = 0.01$; $p = 0.39$; OR = 1.01; 95% CI: 0.99–1.03), hsCRP ($\beta = -0.00$; $p = 0.97$; OR = 1.00; 95% CI: 0.99–1.01) and Gc globulin ($\beta = -0.00$; $p = 0.68$; OR = 0.99; 95% CI: 0.99–1.01) did not offer any predictive value regarding 14-day mortality.

Serum GSN and Gc globulin positively correlated with each other ($\rho = 0.48$, $p < 0.01$); in addition, both of them positively correlated with serum albumin (GSN – albumin: $\rho = 0.54$; Gc – albumin: $\rho = 0.61$, $p < 0.01$) and negatively with hsCRP (GSN – hsCRP: $\rho = -0.68$; Gc – hsCRP: $\rho = -0.43$, $p < 0.01$). Gc globulin inversely correlated with plasma lactate ($\rho = -0.64$, $p < 0.01$), with PCT ($\rho = -0.34$, $p < 0.01$) and with clinical scores (Gc – SAPS II: $\rho = -0.49$, $p < 0.01$; Gc – APACHE II: $\rho = -0.35$, $p < 0.05$; Gc – SOFA: $\rho = -0.52$, $p < 0.01$).

Discussion

The development of a septic process involves early activation of both pro- and anti-inflammatory responses, together with major alterations in non-immunologic pathways [1],

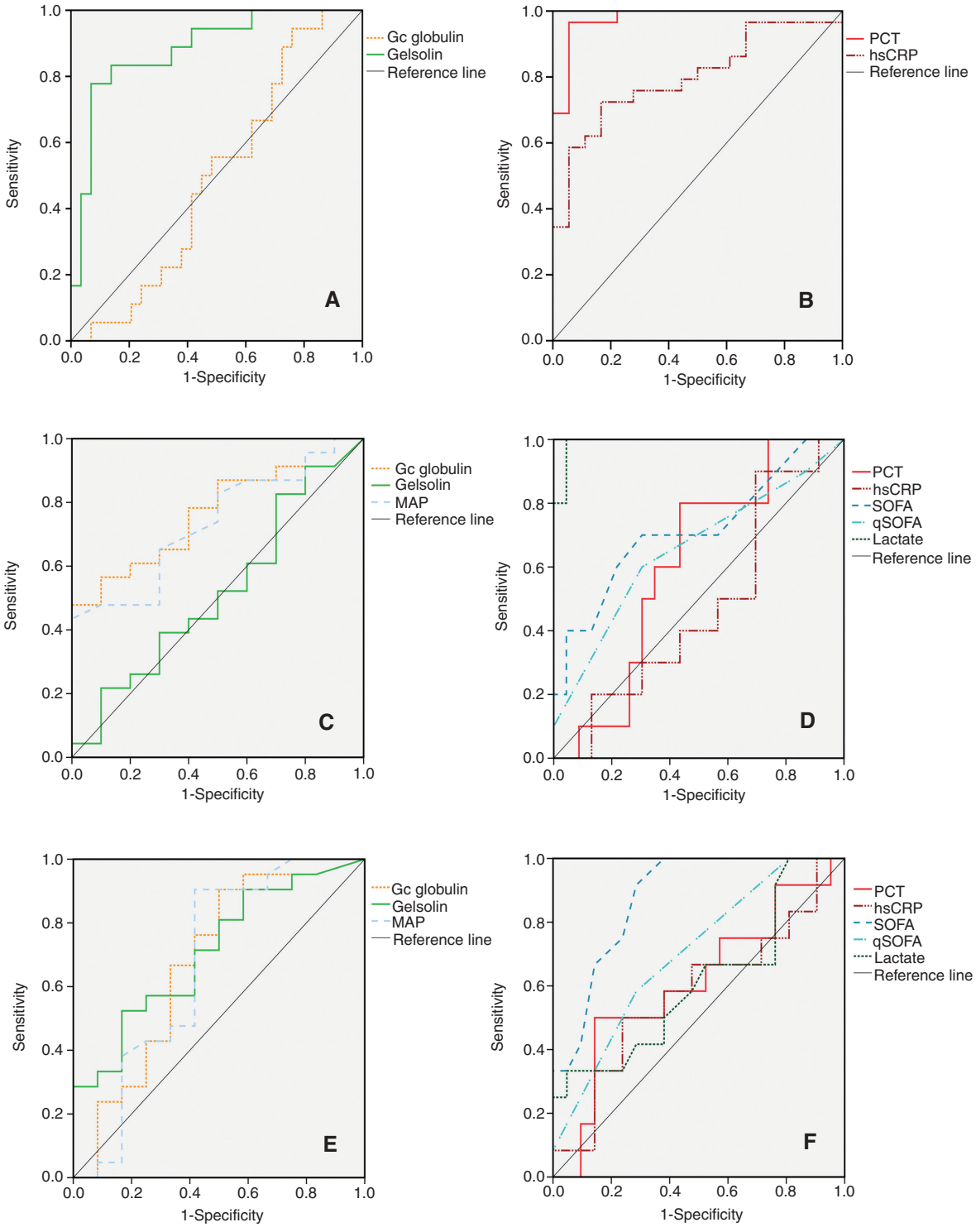

Figure 3: Discriminative values of the investigated parameters.
Receiver operating characteristic curves of first-day laboratory and clinical parameters for differentiating non-sepsis from sepsis (A, B), for distinguishing septic shock from sepsis (C, D) and for predicting 14-day mortality in sepsis (E, F).

which partly results in massive cell injury. Besides various other cell components, actin also leaks out in excess from injured cells in which process is suggested to deplete the levels of the extracellular actin-binding proteins [8, 12, 13, 17, 18, 21, 29]. From that point of view, the simultaneous rapid determination of serum Gc globulin and GSN levels might give important information on the septic process which we have sought to investigate. Our study revealed

that serum GSN provides valuable complementary data for the rapid diagnosis of sepsis, similarly to our latest published results [13]. Previous studies performed by Lee et al. [11, 12] indicated that first-day GSN levels are significantly higher in survivors than in non-survivors of sepsis, which was also confirmed by us [13].

Regarding Gc globulin, we did not find any association between first-day levels and mortality of ICU patients, similarly to Leaf et al. [22] who examined 90-day mortality of critically ill patients and in-hospital mortality in acute kidney injury patients [23]. The data obtained from Gressner et al. [20] also indicated no association between Gc globulin and ICU-/follow-up mortalities.

We did not find any significant differences in first-day's serum Gc globulin levels when comparing sepsis to non-sepsis patients, contrary to the expectations of Jeng et al. [19]. We have demonstrated that first-day's serum Gc levels have valuable predictive capacity when differentiating sepsis from septic shock. In addition, previous study of Dahl et al. [21] confirmed that trauma victims with low admission Gc globulin concentrations were more prone to develop hematologic and respiratory failures than those with high Gc globulin levels. Similarly, to their observations, we also noticed a slight increase in Gc globulin concentrations during the 5-day follow-up period regarding sepsis patients. That phenomenon can be attributed to the increased synthesis of Gc globulin as an acute phase reactant after severe injury, reported by Dahl et al. [18]. Under normal circumstances, the plasma half-life of Gc-globulin unbound to actin varies between 12 and 24 h, whereas that of Gc-globulin-actin complex is suggested to be very short (30 min) [30]. Plasma GSN has a half-life of 2.3 days in healthy individuals [30, 31], which is also suggested to be reduced when complexed with actin. Opposite to Gc globulin, no significant increment was noted during the observation period regarding GSN, similarly to the work of Lee et al. [11] and to our previous findings, which could be explained by the lack of newly induced synthesis in the muscle cells after injury [29]. However, in survivors, long-term observations performed by Huang et al. [14] and Wang et al. [15] indicated a slow increase of serum GSN levels. The rapid fall in plasma Gc globulin and GSN levels at the beginning of severe systemic inflammatory disorders is attributed to the fast consumption of them partly because of the extreme overload by actin [8, 10–18, 20, 21, 29–31].

Based on literature data of previous studies, we seem to be the first to measure serum GSN and Gc globulin levels simultaneously by rapid immune turbidimetry during the course of sepsis. We demonstrated a significant positive correlation between the two actin-binding proteins, which supports the hypothesis that they act in concert in the intravascular space. In contrast to Gressner et al. [20], we found significant correlations between Gc globulin and hsCRP, PCT and, furthermore, between Gc globulin and clinical scores, too. Similarly to our previous data, GSN negatively correlated with hsCRP.

We investigated age-, gender- and disease-matched control patients in our study; therefore, the comparison of patients groups regarding serum GSN or Gc globulin levels was not affected by underlying diseases (e.g. diabetes, chronic kidney disease). Apart from sepsis, decreased pGSN levels were found after parenchymal tissue damages, including acute lung injury, major trauma, myonecrosis, and acute liver failure, too [8, 16, 29]. Depressed serum Gc globulin levels were found also in patients with hepatic failure and in trauma patients with shock [10, 17, 18]. These findings indicate that none of these proteins are specific for sepsis. However, because sepsis is a syndrome rather than a disease, none of the biomarkers would offer 100% specificity [32]. The magnitude of the decrease in serum GSN and Gc globulin levels is of utmost importance; therefore, appropriate cutoff values have to be set.

One of the potential weaknesses of the study is the limited number of critically ill patients. Therefore, further studies with a much higher patient number should be performed. We investigated the sepsis-3 definitions retrospectively; therefore, in the future investigations, the new sepsis terms should be taken as the primary criteria when defining the patient groups. Also, another limitation is that SOFA scores could not be calculated before the admission of the patients to the ICU. This difficulty most researchers have to face because many of the patients are admitted from out of hospital locations (where no laboratory facilities exist) directly to the ICU.

Conclusions

Serum GSN may serve as a complementary diagnostic and predictive protein marker in severe systemic inflammatory syndrome, and critically low admission Gc globulin concentration reflects the development of septic shock. Immune turbidimetric measurement of GSN and Gc globulin levels gives the possibility to obtain important additional information on sepsis severity within a short turnaround time.

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