UNIVERZA V LJUBLJANI

FAKULTETA ZA FARMACIJO

META ZUPANČIČ

MAGISTRSKA NALOGA

MAGISTRSKI ŠTUDIJSKI PROGRAM LABORATORIJSKA BIOMEDICINA

Ljubljana, 2017

UNIVERZA V LJUBLJANI

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METHOD VALIDATION FOR DETERMINATION OF GC GLOBULIN IN SERUM AND ITS SIGNIFICANCE IN PATIENTS WITH SEPSIS

VALIDACIJA METODE ZA DOLOČANJE GLOBULINA GC V SERUMU IN NJEGOV POMEN PRI BOLNIKIH S SEPSO

MAGISTRSKI ŠTUDIJSKI PROGRAM LABORATORIJSKA BIOMEDICINA

Ljubljana, 2017

I performed my master thesis in University of Ljubljana under the supervision of a mentor Prof. Dr. Janja Marc and co-mentor Prof. Dr. Tamas Koszegi, MD, PhD. I completed practical work for my master thesis in Janos Szentagothai Research Centre of University of Pecs in Lab-On-A-Chip research group laboratory and in routine clinical laboratory of Department of Laboratory Medicine, University Medical School of Pecs. Routine measurements were also performed in the Department of Laboratory Medicine.

Acknowledgments:

I would like to gratefully and sincerely thank Prof. Dr. Tamas Koszegi for his guidance, understanding and patience through the master thesis process. I would also like to acknowledge all of the friendly members of the Koszegi research group for all of your acceptance and willingness to help on the professional as well as social area of my Hungarian experience. I am especially indebted to Zoltán Horváth-Szalai for his endless patience and useful advice during my practical work.

I would also like to express my deepest gratitude to my mentor Prof. Dr. Janja Marc for her assistance and guidance, valuable discussions and accessibility.

Finally, I would like express my very gratitude to my beloved boyfriend Jure and my dear mother Barbara for their endless support, continuous encouragement and quiet patience throughout my study years and through the process of researching and writing this thesis.

Statement

Hereby, I testify having performed the experiments to the best of my knowledge and having written this thesis independently under guidance of my supervisors: Prof. Dr. Janja Marc and Prof. Dr. Tamas Koszegi, MD, PhD.

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Abstract

Sepsis is a life-threatening organ dysfunction caused by a systemic deregulation of host response to bacterial, fungal, viral or parasite infections. Sepsis is arising from Systemic Inflammatory Response Syndrome (SIRS) and is the leading cause of death in intensive care units all over the world. The estimated incidence of sepsis reach up to 300 cases per 100,000 inhabitants, with mortality rate around 20%. Diagnostics of sepsis is very difficult and mostly based on physical observations and assessment of scoring systems. Unfortunately, specific laboratory biomarkers for the disease diagnostics, progression and prognosis do not yet play the important role they should in septic patients management, in spite of the fact that more than 170 different new biomarkers have been researched recently. The aim of our work was to test GC globulin as a potential diagnostic marker for sepsis. First, we optimized and validated immune turbidimetric two-point end assay for GC globulin measurement. Full calibration with six calibrators and RCM curve fitting was performed. Validation parameter results were: precision with repeatability (CV% between 1.38 – 1.64 %) and intermediate precision (CV% 5.04 %), limit of detection (0.655 mg/L) and limit of quantification (1.847 mg/L), method working range (1.847 – 385 mg/L), linearity (between range of 8 mg/L to 332 mg/L with r^2=0.9953), short term stability for 6 days at 4°C and long term stability for 3 month at -70°C.

After method validation, clinical study was performed including 51 patients, diagnosed with sepsis or SIRS and 29 healthy individuals. Firstly, significantly lower levels of GC globulin were presented in patients with sepsis and SIRS in comparison to controls. Studying the progression of the disease, the GC globulin values increase significantly from day one to day three after the treatment of sepsis. The comparison of GC globulin with other actin scavenger system, biochemical, hemostasis and hematological markers, positive correlation of GC globulin with gelsolin, serum total protein, creatine kinase and platelet count was found and negative correlation with bilirubin, procalcitonin, creatinine and activated partial thromboplastin time (APTT) was detected. A significant difference in day one GC globulin values between survivors and non-survivors was also found. ROC curve of three-day mortality unveils good predictive value of the test with area under the curve of 0.88 and chosen cut-off value of 38.93 mg/L. ROC curve of seven-day mortality has fair predictive value with area under the curve of 0.783 and cut-off concentration of 50.41

mg/L. Based on our results we can conclude, that GC globulin represents a novel promising diagnostic and prognostic marker for monitoring sepsis.

Key words:

Sepsis, GC globulin, automatized immune turbidimetric assay validation

Razširjen povzetek

Sepsa je življenjsko ogrožujoče stanje, ki nastane kot posledica močno izraženega sistemskega imunskega odziva na vnetje (SIRS) in se odraža s povišano telesno temperaturo, tahikardijo, tahipnejo, povečanim ali znižanim številom levkocitov ali premikom v levo v diferencialni krvni sliki, edemom in zmedenostjo, z razvojem sepse pa tudi kot odpoved različnih organov. Povzročitelji odziva so v večini primerov bakterije, lahko pa tudi virusi, glive in paraziti. Incidenca sepse je ocenjena na 300 primerov na 100,000 prebivalcev s stopnjo smrtnosti okoli 20 % in predstavlja najpogostejši vzrok smrti bolnikov intenzivnih oddelkov po celem svetu. Diagnostika sepse je precej zapletena in danes temelji predvsem na fizičnem pregledu bolnika in na točkovalnih sistemih za opredelitev bolezni. Kljub temu, da je v poteku raziskav več kot 170 različninh specifičnih laboratorijskih označevalcev za diagnostiko, spremljanje, terapijo in prognozo bolezni, se v današnji klinični praksi zelo malo uporabljajo, saj ne dosegajo zadostne specifičnosti ali občutljivosti ter avtomatizacije metode za njihovo določanje. V naši klinični študiji smo preučevali GC globulin kot potencialni označevalec za učinkovitejšo diagnostiko in spremljanje bolezni ter kot prognostični kazalec smrtnosti.

Za merjenje koncentracije GC globulina v vzorcih serumov bolnikov smo najprej optimizirali in validirali novo avtomatizirano imunsko turbidimetrično metodo z dvotočkovno detekcijo na avtomatskem analizatorju, izhajali smo iz predhodno razvite metode (74). Pred postopkom validacije, izvedene po standardih smernic Eurachem, smo izvedli polno šest-točkovno kalibracijo z RCM matematično metodo prileganja krivulje, saj odziv (absorbanca) in koncentracija analita nista v linearni zvezi. Rezultati validacijskih parametrov so bili sledeči: parametri natančnosti, kot so ponovljivost (CV% med 1.38 – 1.64 %) in srednja natančnost (CV% 5.04 %), meja zaznave (0.655 mg/L), meja določanja (1.847 mg/L) in merilno območje (1.847 – 385 mg/L), linearnost metode (v območju med 8 mg/L - 332 mg/L, koeficient korelacije je 0.9953), stabilnost metode, kot sta kratkoročna temperaturna stabilnost (6 dni na 4°C) in dolgoročna temperaturna stabilnost (3 mesece na 70°C).

Po validaciji metode smo izvedli klinično študijo na 51 bolnikih z diagnozo sepse ali SIRSa, nastalih zaradi različnih vzrokov (sladkorna bolezen tipa II, hipertonija, ishemična srčna bolezen, jeterna bolezen, COPB, kronična ledvična bolezen ali rakavo obolenje). Kontrolna skupina je obsegala 29 zdravih posameznikov, brez vnetij (podprto z meritvijo hs-CRP in diferencialne krvne slike) ter kroničnih ali avtoimunih obolenj. Najprej smo dokazali signifikantno razliko v koncentraciji GC globulina med bolniki s sepso ali SIRSom in zdravimi posamezniki, ki je posledica znižanja koncentracije GC globulina zaradi njegove vloge pri odstranjevanju prostega aktina iz sistema pri bolnikih. Najpomembnejši rezulat pri študiju spremembe koncentracije GC globulina pri napredku bolezni je signifikanten porast koncentracije pri bolnikih med prvim in tretim dnem po postavitvi diagnoze, večinoma kot posledica zdravljenja bolezni in pospešene sinteze proteinov. Pri primerjavi med testom za detekcijo GC globulina in testi za označevalce odstranjevalnega sistema aktina, biokemijskimi, hematološkimi testi in testi hemostaze smo odkrili pozitivno korelacijo meritve GC globulina z gelsolinom, celokupnimi serumskimi proteini, keratin kinazo in številom trombocitov in negativno korelacijo z bilirubinom, prokalcitoninom, kreatininom ter APTT (aktiviran delni tromboplastinski čas). Pri preučevanju povezave med koncentracijo GC globulina in smrtnostjo bolnikov smo dokazali signifikantno povezavo med koncentracijo ob razvoju sepse (prvi dan) s smrtnostjo po treh in sedmih dneh od razvoja bolezni. ROC krivulja trodnevne smrtnosti kaže dobro napovedno vrednost s površino pod krivuljo 0.88 in izbrano mejno vrednostjo 38.93 mg/L. ROC krivulja sedemdnevne smrtnosti ima slabšo napovedno vrednost s površino pod krivuljo 0.783 in mejno vrednostjo 50.41 mg/L.

Ključne besede:

Sepsa, GC globulin, validacija avtomatizirane imunoturbidimetrične metode

Abbreviations list

ALT	alanine aminotransferase
APC	antigene presenting cells
APTT	activated partial thromboplastin time
AST	aspartate aminotransferase
C3, C4, C5, C9	complement components
CARS	compensatory anti-inflammatory response syndrome
CK	creatine kinase
CV%	coefficient of varitaion
DIC	disseminated intravascular coagulation
GC globulin	group–specific component globulin
GGT	gamma glutamyl transpeptidase
Hs-CRP	high sensitive C-reactive protein
IFN- γ	interferon gamma
IL	interleukin
INR	international normalized ratio of prothrombin time
LOB	limit of blank
LOD	limit of detection
LOQ	limit of quantification
MARS	mixed antagonist response syndrome
PCR	polymerase chain reaction
PCT	procalcitonin
SIRS	systemic inflammatory response syndrome
SOFA	Sequential Organ Failure Assessment
TLR	Toll-like receptor
TNF- α	tumor necrosis factor alpha

1. Introduction

1.1. Sepsis

Sepsis is defined as a life-threatening organ dysfunction caused by a systemic deregulation of host response to infection. (1) The word 'sepsis' originates from Greek meaning 'decay or decomposition of organic matter'. (2) Sepsis is caused usually by bacterial infection in blood called septicemia, but also by fungi, viruses or parasites. Triggering agents and their products, released into bloodstream, activate mass response of the immune system, which induces sepsis. (3) The disease arise from SIRS – Systemic Inflammatory Response Syndrome, an early response to infection, with symptoms such as high or low body temperature, tachycardia, tachypnea, increased or decreased white blood cell count or left shift, edema and confusion. (4) When systemic infection is confirmed or strongly suspected, SIRS develops into sepsis and after an organ failure is proven, condition turns into severe sepsis. Septic shock arises when severe hypotension after adequate fluid replacement appears and frequently leads to death. (1) New definition of sepsis diagnostics was accepted in 2016 (1) and will be discussed further in subchapter Diagnosis.

1.1.1. Epidemiology

Epidemiology of sepsis is an estimated value because it is hardly detectable due to the high incidence of the disease, different clinical definitions over the world and difficulties to obtain data from undeveloped countries so the fact is that the data available vary a lot. Nevertheless, sepsis is the main cause of death in intensive care units all over the world, estimated to be around twenty million patients annually with mortality rate around 36 %, which means around 20,000 deaths per day. (5) In USA, incidence of sepsis is around 300 cases per 100,000 inhabitants with a mortality rate of 28 %. (6) The latest study from Sweden estimates incidence of sepsis on 678 patients per 100,000 (7) and in Slovenia 118 patients per 100,000 inhabitants. (8) The mortality rate also differs much on the stage of disease: in Europe is estimated to be 20 % in septic patients, 32.2 % in severe septic patients and 54.1 % in patients with septic shock. (9) In reality actual figure can be much higher due to the lack of information from the undeveloped countries, where incidence of

sepsis is the highest due to the low living standards, poor hygienic conditions, malnutrition and consequentially more bacterial, parasitic and HIV infections than in developed countries. (10)

Numerous reports have shown increasing incidence of sepsis in past two decades. This tendency is expected to endure along with growth of the population, increasing incidence of chronic health conditions and increased use of immunosuppressive therapy, transplantation, chemotherapy and invasive procedures. (11)

Evident research progress has been made in sepsis diagnostics and patients' treatment in last 50 years, yet increasing incidence of the disease and high mortality rates display challenge for the researchers worldwide to resolve the etiology of disease, invent better and more specific markers for faster disease recognition and provide more efficient treatment for the patients.

1.1.1.1. Microbial epidemiology of sepsis

Sepsis is defined as the invasion of sterile tissues including bloodstream by one or more microbial pathogens with a resulting systematic inflammatory response. (12) The disease evolves in a broad range of symptoms and different progression in virtue of its distinct sites of infection and wide variety of pathogens causing infection, which also influences the treatment and mortality of the patient. According to the clinical study of the European patients in ICU (intensive care units), lung is the most frequent site of the infection (68 %). Infections of abdomen (22 %), blood (20 %) and urinary tract (14 %) are also quite common, while skin, central nervous system, soft tissue and endocardia represent other infection sites. (11) Bacterial infection is most common in the majority of cases. Gram-positive microorganisms appear in 40 % of septic patients, where most common species represent *Staphylococcus aureus* - Methicillin resistant *Staphylococcus aureus* was isolated from 14 % of patients' samples, *Streptococcus pneumoniae* (4 %) and other *Streptococcus spp.* (12 %). (9) Gram negative bacteremia appears in 38 % of the cases with its most frequent representatives *Pseudomonas spp.* (14 %) and *Escherichia coli* (13 %) and other bacteria: *Klebsiella pneumoniae, Enterobacter spp., Proteus spp.*, *Haemophilus spp.* and *Acinetobacter spp*. (9,11,13) Approximately 17 % of the infections is caused by fungi, mostly with *Candida albicans* (13 % of infections), but some also with viruses or parasites. (9,11,13) Multiple infections also persist in 18 % of patients, especially inside ICU units. (9)

However, it is important to note that in clinical practice, in 40 % of sepsis cases the offending pathogen could not be identified, although infection seemed to be the only plausible initiating agent and the nature of pathogen is predicted through the clinical data to adjust the treatment. (12)

1.1.1.2. Risk factors

The most important risk factors for developing sepsis are age, sex and general heath condition. More than 50 % of severe sepsis arises in adults over 65 years of age and the majority of them also have a chronic health condition: chronic obstructive pulmonary disease, cancer, chronic renal and liver disease or diabetes. (11) Young children are also a risk group in venue of underdeveloped immune and organ systems. (14) Men appear to be at higher risk of evolving sepsis than women. (11)

Race is also an important risk factor for severe sepsis. Higher incidence in black individuals is interpreted by higher infection rate and higher risk of acute organ dysfunction. (14) The underlying mechanism of racial disparities is poorly understood and combines differences in chronic disease burden, particularly subclinical disease, social and environmental factors and genetic predisposition causing differences in the host immune response to infection. (14)

Furthermore, socioeconomic status also associates with increased risk for sepsis due to poor hygiene conditions and availability of health care among poor people. (15) Other risk factors include long term care facilities, malnutrition and use of immunosuppressive therapy and prosthetic devices. (11)

Although understanding of clinical risk factors improved importantly, development and prognosis in some patients does not reflect a predicted course of the disease so it is important to consider the influence of the genetic factors. Sepsis is considered to be under the impact of multiple genes interacting with environmental factors. (11) Promising candidate genes, yet still under research, include tumor necrosis factor (TNF), plasminogen activator inhibitor (PAI-1), and Toll-like receptors (TLR-1 and TLR-4). (16,17,18)

1.1.2. Etiopathogenesis

Interactions between elements of host immune system and macromolecules of the attacking microorganisms are the basic mechanisms, leading to sepsis and septic shock. (19) Normal immune response heads to eradication of pathogens and preserving physiologic integrity of the host. SIRS (Systemic Inflammatory Response Syndrome) occurs for extensive infections, where the whole body is exposed to immune system agents (molecules and cells). (20) In patients with severe sepsis, host response breaks out of control and can lead to the multi-organ failure and death. For the purpose of understanding it can be divided on two stages: Pro-inflammatory response and anti-inflammatory response. (21)

1.1.2.1. Pro-inflammatory response

Innate immune system is the first line of defense, induced by pathogen's surface molecules, which cause the response of complement system through a triggered enzyme cascade, mostly by the alternative way. (19) Firstly, complement system produces large numbers of activated proteins for covalent binding to pathogens. Opsonized pathogen attracts phagocytes bearing the complement receptors (components C3b and C4b). Second, the small component fragments act as a chemoattractants to engage and activate more phagocytes to the infection site (C5a is the most important). Third, the terminal complex from 10-16 C9 components creates pores into the pathogens' membrane and damages them. Finally, components C3a, C4a and C5a induce local inflammatory response with smooth muscle contraction, increased vascular permeability and influence to endothelial cells to reveal adhesion molecules. (22) Chemoattractants intrigue polymorphonuclear leukocytes (PMN), monocytes, macrophages, fibroblasts and endothelial cells to the site of infection. These phagocyte cells as well as lymphocytes T and B release cytokines after the contact with pathogens for regulation of the immunologic and metabolic processes during infection. (19) Cytokine storm is localized in minor infections, but in systemic infections like sepsis, cytokines circulate in the bloodstream, causing endothelial damage resulting in symptoms of the disease. (21) The most important pro-inflammatory cytokines are tumor necrosis factor α (TNF-α), interleukin 1β (IL-1β) and interleukin 6 (IL-6), but IL-8, IL-12 and interferon gamma are also important. TNF-α drives adhesion molecule and chemokine expression to promote leukocyte – endothelial cell adhesion, up-regulate tissue factor and inhibits protein C – coagulation inhibitor. Systemic effects of its excess blood

concentrations are: vasodilatation, myocardial depression, afferent renal artery vasodilatation induced by nitric oxide production. (23,24) IL-1β provokes fever by influencing hypothalamus, reacts with endothelium for hypotension and leukocyte infiltration, activates monocytes, increases adhesion molecule expression, inhibits thrombomodulin secretion and induces acute phase proteins like C-reactive protein (CRP) production in hepatocytes. These effects contribute to pro-coagulant state with disseminated intravascular coagulation (DIC) and possible end-organ ischemia as a consequence. (20,25,26) IL-6 is the most potent activator of acute phase protein synthesis in the liver and induces formation of oxidants in tissues. (27) Pro-inflammatory cytokines also influence the metabolism in venue of high-energy demands of an organism during inflammation. Not only glycogenolysis and gluconeogenesis is accelerated, but also stimulation of muscle catabolism, bone resorption, lipolysis, glutamine synthesis and deposition of glutamine and other amino acids from tissues. (19, 28)

Adoptive immune response is triggered soon after infection by extensive interactions between antigen presenting cells (APC) and lymphocytes. APCs present antigen to T cells in association with other cell-surface proteins and costimulatory molecules. After signal transduction, effector CD4+ T cells excrete cytokines like interferon γ (IFN-γ) to influence phagocyte activation and B cell interaction, which reflects in antimicrobial antibody production. (25)

1.1.2.2. Anti – inflammatory response

While pro – inflammatory response is fundamental for pathogen clearance, anti-inflammatory response must also be encompassed in a regulated manner in order to restore homeostasis and avert cellular and tissue damage. Yet it is a two-edged sword: it may lower the toxic effects of pro - inflammatory response but may also decrease effective host protection from the infection. (28) Septic patients develop SIRS firstly after infection, which is followed temporarily by a compensatory anti-inflammatory response syndrome (CARS) and can often cause increasing risk of nosocomial infections and other adverse effects. (29) CARS characteristic endogenic cytokines include IL-10, interleukin-1 receptor antagonist (IL-1Ra) and TNF soluble receptor. (29) IL-1Ra and soluble TNF receptor are both cytokine antagonists; IL-1Ra blocks IL-1 effects as well as soluble TNF receptors bind and inactivate TNF-α. (20) Circulating IL-10 directly inhibits phagocyte activation and therefore monocyte synthesis of pro – inflammatory cytokines, and raises

expression of anti-inflammatory molecules like IL-1Ra and soluble TNF receptors. (30,31)

Apoptosis also plays an important part in the etiopathogenesis of sepsis. It is induced by the extrinsic, receptor mediated as well as the intrinsic mitochondrial pathway even though the underlying mechanisms for the disease are not fully understood. Although presence of apoptosis has been discovered in many cell types including endothelial, neuron and muscle cell, the most active apoptosis appears in lymphocyte in lymphoid tissues and gastrointestinal endothelial cell populations. (32) Subsequent studies reveal B cells, CD4+ T subsets of lymphocytes and dendritic cells as the most vulnerable immune cells to apoptosis presenting in persisting lymphopenia in septic patients, whereas other T cell subsets and macrophages remain largely unharmed. (25) Degree of apoptosis correlates closely with severity of sepsis symptoms and the outcome of the disease. (33, 34)

It is important to contemplate that sepsis is extremely dynamic process with considerable diversity in human response to infection. Prolonged sepsis presents steady pro-inflammatory and anti-inflammatory equilibrium with the mixed cytokine response pattern, known as MARS – mixed antagonist response syndrome. (29) Graphical display on Figure 1 demonstrates the concept of linear transition of an early hyper-inflammatory SIRS to chronic hypo-inflammatory CARS with possible reoccurrences of both phases via MARS during progression of the disease. (29, 35)

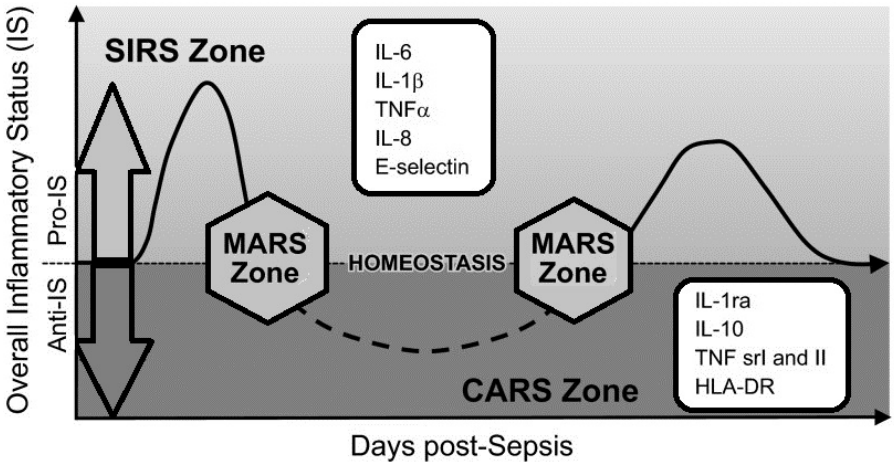

Figure 1: Concept of the bimodal evolution of the systemic immune-inflammatory response in sepsis.

1.1.3. Diagnostics

Diagnosis of sepsis is currently based more on physical observations than on laboratory assessment therefore patients need a careful history and physical examination. Not only environmental flora and the source of infection, but also pre-existing medical conditions, leading to sepsis (for example immunosuppression therapy) might be assumed out of medical history. (12) Physical examination is crucial to set the diagnosis by checking patients' vital signs and possible sources of infection accompanied by laboratory results for markers of inflammation and possible organ dysfunction. First diagnostic recommendations exist from 1991 and are, with some replenishment, widely practiced until today, though new recommendations exist from 2016. 1991 recommendation divide disease on SIRS, sepsis, severe sepsis and septic shock, based on the development of symptoms, and is presented in Table I. (36)

Table I: Clinical definition of SIRS, sepsis, severe sepsis and septic shock. (36)

Term	Criteria
SIRS	2 of the following 4: - Temperature >38°C or <36°C. - Heart rate >90 beats/min. - Respiratory rate >30 breaths/min or arterial CO_2 <32mm Hg. - White blood cell count >12,000 or <4000 cells/μL or >10 % band forms.
Sepsis	Documented or suspected infection plus systemic manifestations of infection: Any of the SIRS criteria count or other possible manifestations include elevations of procalcitonin, C-reactive protein, hyperglycemia in those without diabetes, altered mental status.
Severe sepsis	Sepsis plus evidence of organ dysfunction: - Arterial hypoxemia (PaO_2/FiO_2<300). - Acute oliguria (urine output <0.5 mL/kg per hour for at least 2 h despite adequate fluid resuscitation). - Increase in creatinine >0.5 mg/dL. - Coagulation abnormalities (INR>1.5, aPTT>60 s, platelets <100,000/μL). - Hepatic dysfunction (elevated bilirubin). - Paralytic ileus. - Decreased capillary refill or skin mottling.
Septic shock	Sepsis with hypotension refractory to fluid resuscitation or hyperlactatemia: - Refractory hypotension persists despite resuscitation with bolus intravenous fluid of 30 mL/kg. - Hyperlactatemia >1 mmol/L.

1.1.3.1. Microbiological diagnostics

Microbiological diagnostics is very important for the proper treatment decision and consequently decreased mortality of septic patients. (37) General antimicrobial treatment does not cover all of the pathogens so rare bacteria and fungi may not be covered as well as infections with multi-resistant bacteria. (38) Blood culture is still considered as a golden standard for identification of bloodstream pathogens however it has to admit lack of sensitivity, low pre–test probability and delay in time to result to new commercially available molecular detection methods. (39) Molecular methods for the diagnosis of sepsis are divided on hybridization techniques (FISH – fluorescence in-situ hybridization arrays, probe hybridization), amplification techniques (PCR – polymerase chain reaction, Multiplex PCR, Broad-range PCR), post – amplification detection techniques (PCR + hybridization, PCR + sequencing, PCR + MALDI-TOF mass spectrometry) and non-nucleic acid based techniques (proteomics, spectrometry, phage assays). (38)

1.1.3.2. Novel approach to diagnosis

The SIRS criteria does not necessarily announce a deregulated life threatening response in patients and some of them actually never develop infection, but on the other hand some patients with infection and severe organ failure does not achieve two SIRS criteria to fulfill the old approach to diagnose sepsis. Therefore the obligation for improved criteria arose. (40,41) In 2016, The Third International Consensus Definitions for Sepsis and Septic shock was published, proposing SOFA (Sequential Organ Failure Assessment) scoring system as a predominant option for diagnosing sepsis. (42) A baseline SOFA score can be assumed to be zero in patients without preexisting organ dysfunction nevertheless SOFA score 2 reflects an overall mortality risk of 10 % and defines sepsis. (42) Its calculation is explained in Table II.

Table II: Sequential (sepsis related) Organ Failure Assessment Score (SOFA). (42)

System	Score				
	0	**1**	**2**	**3**	**4**
Respiration					
PaO_2/FiO_2, mm Hg (kPa)	>400 (53.3)	<400 (53.3)	<300 (40)	<200 (28) with respiratory support	<100 (13.3 with respiratory support)
Coagulation					
Platelets x10^3/µL	>150	<150	<100	<50	<20
Liver					
Bilirubin mg/dl (µmol/L)	<1.2 (20)	1.2 – 1.9 (20 – 32)	2.0 – 5.9 (33 – 101)	6.0 – 11.9 (102 – 204)	>12 (>204)
Cardiovascular	MAP >70 mm Hg	MAP <70 mm Hg	Dopamine <5 od dobutamine (any dose)	Dopamine 5.1 – 15 or epinephrine <0.1 or norephinephrine <0.1	Dopamine >15 or epinephrine >0.1 or norepinephrine >0.1
Central nervous system					
Glasgow comma scale score	15	13 - 14	10 – 12	6 - 9	< 6
Renal					
Creatinine mg/dl (µmol/L)	<1.2 (110)	1.2 – 1.9 (111 – 170)	2 – 3.4 (171 – 299)	2.5 – 4.9 (300 – 440)	>5.0 (440)
Urine output mL/day				<500	<200

PaO_2: partial pressure of oxygen, FiO_2: fraction of inspired oxygen, MAP: mean arterial pressure.

The operationalization of SOFA criteria identifying patients with sepsis and septic shock is presented in Figure 2. (42) Quick SOFA (qSOFA) is a screening test for evaluating patients with infection outside ICU units and contains three criteria: alteration in mental status, systolic blood pressure ≤100 mm Hg or respiratory rate ≥22/min. (42) Septic shock is also newly defined: it is a subset of sepsis in which underlying circulatory and cellular/metabolic abnormalities are profound enough to substantially increase mortality. (42)

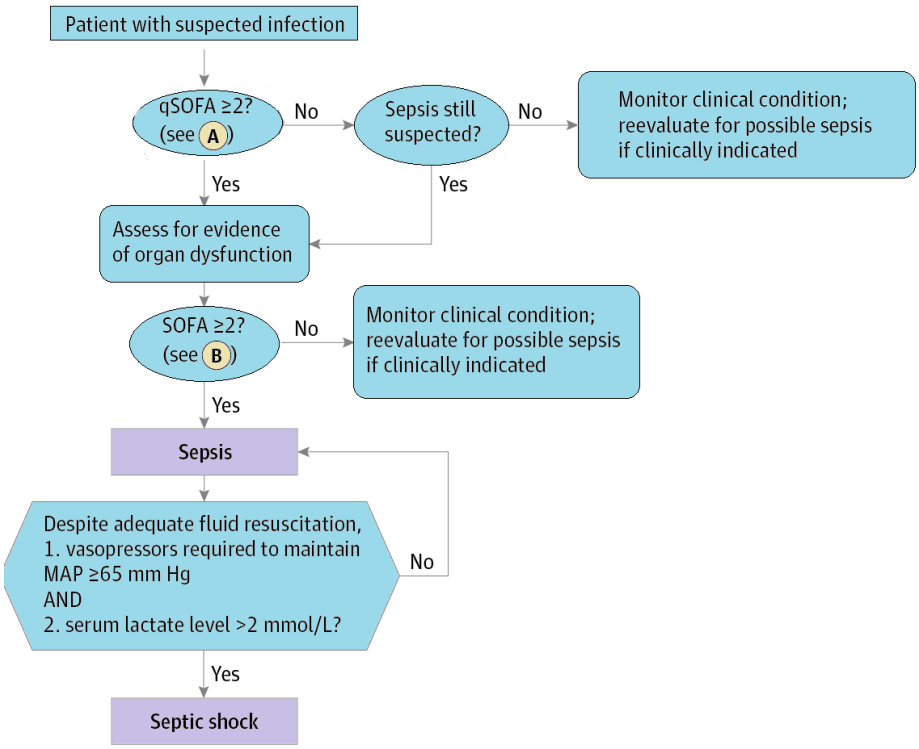

Figure 2: The operationalization of SOFA criteria identifying patients with sepsis and septic shock. (42)

1.1.3.3. Biomarkers

Biomarker is a distinct biochemical, genetic, or molecular characteristic or substance that is an indicator of a particular biological condition or process. Biomarkers are divided to 5 groups, defined by the possible information, obtained from their measurement: screening, diagnostic, stratification, monitoring and surrogate biomarkers. (43)

The exact role of biomarkers in management of septic patients remains undefined due to the highly variable and non-specific nature of the signs and symptoms of the disease. (44) C – reactive protein (CRP) is the only biomarker used in clinical practice as a screening biomarker for inflammation, recently accompanied by procalcitonin (PCT), proposed to be more specific and better prognostic marker, although its value has also been challenged. (45) For estimation of severity of sepsis, organ injury markers: troponin, creatinine, platelet count, liver enzymes, activated partial thromboplastin time, fibrinogen, D-dimer and lactate are measured. (43) More than 175 new biomarkers have been assessed for potential practice in sepsis cases, mostly prognostic, but almost none of them have acceptable specificity or sensitivity to be measured routinely. (46) These markers originate from all main pathophysiological mechanisms: inflammation, coagulation, complement,

contact system activation and apoptosis. Additionally diversity in cell types, tissues and organs affected individually, increase the number of potential candidates. (46)

Combination of several biomarkers may be more effective than single biomarker so future research should be also orientated in development of biomarker panels and if successively combined, biomarkers might still become crucial to an effective and hurried diagnosis, prognosis and treatment. (47)

1.2. GC globulin

GC globulin (group–specific component globulin, also known as vitamin D binding protein) is a multifunctional plasma protein with a molecular mass of 51 000 – 58 000 Da, depending on glycosylation, and belongs to the albumin superfamily of binding proteins as well as albumin, fetoprotein and afamin. (48) Its gene is located on the long arm of chromosome 4 (4q11-13), encoding 458 amino acids, folded into a three-domain protein. Homologous domains I and II contain 186 amino acids though shorter domain III involves only 86 residues. (49) Two binding regions have been recognized within the sequence: vitamin D–binding domain, localized between residues 35 and 49 (domain I) and actin-binding domain, between residues 373 and 403 (domains II and III). (50,51) Protein is presented in vivo as three major isoforms: Gc1F, Gc1S and Gc2 and more than 120 genetic variants are defined by genetic polymorphisms and glycosylation patterns. (52) Synthesis takes place mostly in liver parenchymal cells, but minor contributions fall to non-parenchymal liver cells and extra-hepatic synthesis, confirmed in animal models (kidney, yolk sac and testis). (51,53) GC globulin can be detected in different body fluids: serum, urine, breast milk, ascetic fluids, cerebrospinal fluid, saliva, seminal fluid and on the surface of lymphocytes, neutrophils and monocytes and has a half-life of 48 hours. (49) Serum concentration of GC globulin in healthy individuals totals between 200 to 600 mg/L and is stable from a half year of age for lifetime, with a slight effect of phenotype and diurnal rhythm (lowest at night). (54,55,56) Nevertheless it is decreased in some pathologic conditions and increased in pregnancy. (49) Physiological role is presented in Figure 3 and described below.

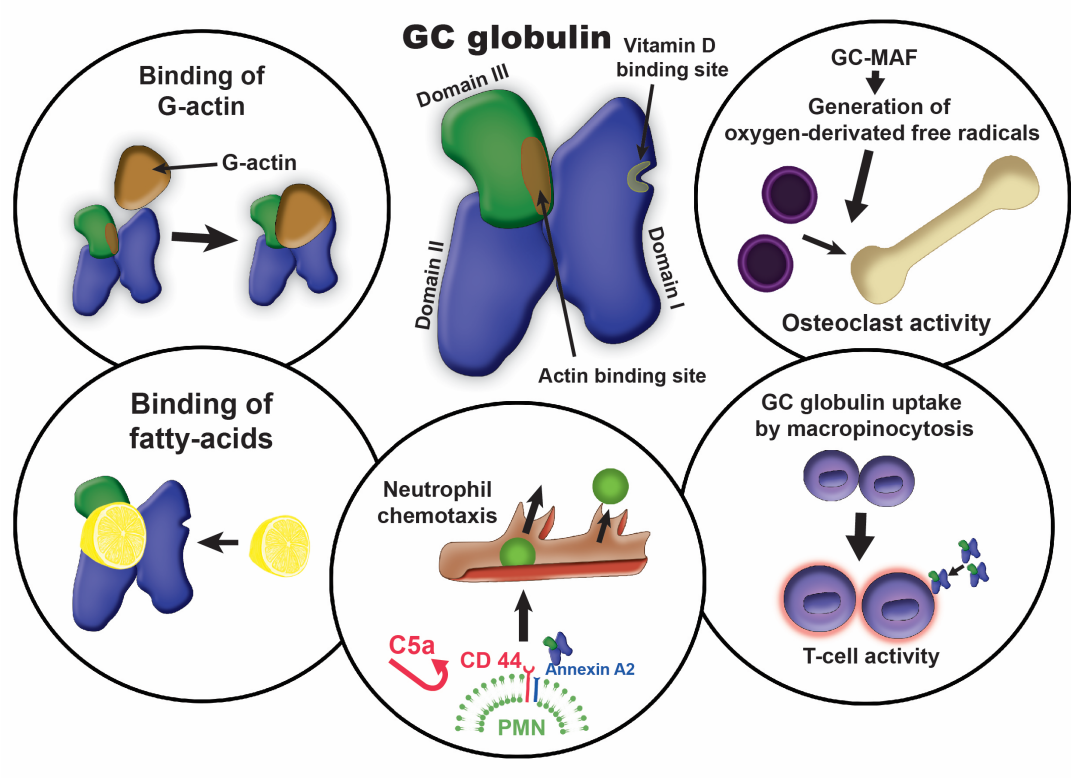

Figure 3: Overview of the different physiological functions of GC globulin.

1.2.1. Extracellular actin scavenger system

GC globulin is one of the two important proteins, participating in scavenging intracellular actin, released into circulation and extracellular space after disruption of cell membrane. (57) Actin is a major cellular protein present in two isoforms: as globular G- and filaments forming F-actin. Spontaneous polymerization of many G-actin molecules under physiological conditions forms filaments and therefore composes cytoskeleton of most cells as well as contractile system in connection with myosin. (58) During excessive tissue damage and cell death, large amounts of actin are released into circulation. Extracellular polymerized actin filaments influence hemostasis and consequently trigger disseminated intravascular coagulation. (51) To prevent this, actin scavenger system molecules GC globulin and gelsolin responds. In the presence of Ca^{2+}, gelsolin separates and caps F-actin filaments at 2:1 molar ratio whereas GC globulin binds highly affinitive to free and gelsolin-capped G-actin with 1:1 molar ratio. (59,60,61) Both G-actin – gelsolin and G-actin – GC globulin complexes are eliminated by the reticuloendothelial system, reducing half-life of proteins to only 30 minutes. (51,62) Low total and actin-free GC-globulin concentrations have been demonstrated to be prognostic markers in situations of severe

organ damage, such as fulminant hepatic failure, acetaminophen overdose, sepsis, multiple trauma and multiple organ failure. (51)

1.2.2. Other functions of GC globulin

1.2.2.1. Vitamin D-binding protein

GC globulin is a main functional protein in the processes of vitamin D solubailization and systemic transport. It binds vitamin D metabolites (primarily 25-OH vitamin D and 1,25-di-OH vitamin D) with different affinities to its binding site, located on N-terminal end of the protein domain I. (63,64) Hence vitamin D metabolites are transported to the target organs. Vitamin D binds on less than 6 % of its possible binding capacity and therefore GC globulin concentration does not correlate with vitamin D metabolites due to the protection against vitamin D toxicity, buffer activity for vitamin D metabolites and GC globulin's other physiological roles. (64,65) Sterol binding does not have an influence on actin binding property or capacity. (66)

1.2.2.2. Precursor for macrophage activation factor

GC globulin plays an important role as a precursor of macrophage activation factor (GC-MAF). It is transformed by combined action of membrane bound galactosidase on activated B cells and salidiase on lymphocytes T which process O-linked carbohydrate side chains on GC globulin transforming it to GC-MAF. (67) GC-MAF stimulates macrophage activity at infection sites but oppositely may induce apoptosis in activated macrophages via caspase induction. (68) GC-MAF is also proposed to stimulate osteoclast activity and bone reabsorption in an extracellular calcium dependent way by the up-regulated oxidative metabolism. (69)

1.2.2.3. Chemotaxin for phagocytic cells

GC globulin enhances the neutrophil chemotactic effect of C5a or C5a des Arg (C5a stable degradation product) for neutrophils and macrophages. Protein seems to bind directly to C5a des Arg, since the GC globulin-C5a des Arg complex increases the number of C5a des Arg molecules on the polymorphonuclear leucocytes. (70) It is possible by the interaction of GC globulin with a binding site complex on the cell surface. (70) Formation of the complex is dynamic, multistep process, requiring cell activation and low affinity bondage creation between GC globulin and surface ligands like CD44 and annexin A. (71)

Besides previously characterized, the most important and described mechanisms in biological role of GC globulin, many others are presumed to exist and research leads to an improvement in revealing them. GC globulin is supposed to participate also in natural killer cell enhancement, arachidonic acid and endotoxin binding, cell differentiation, influencing T cell response, transporting fatty acids and inhibiting actin-induced platelet aggregation. (70)

2. Aim of the work

Differential diagnostics and monitoring of sepsis and SIRS are still challenging in clinical practice, so new diagnostic and prognostic markers are needed to better characterize the full clinical spectrum of the disease and to guide the development and assessment of more effective treatment. GC globulin could be a potential candidate marker for easier recognition, optimizing treatment and reducing the mortality in septic patients. Therefore, the aim of our work was:

1. To compare GC globulin concentrations in patients with sepsis and SIRS and healthy individuals in a clinical trial.
2. To determine the changes of CG globulin concentrations in progress or recovery of the disease, therefore to compare its values on day one, three and five after diagnose is concluded.
3. To correlate GC globulin values with other biochemical, hemostasis and hematological previously used markers.
4. To find the connection between reduced CG globulin concentration and mortality prediction in septic patients.

For the purpose of obtaining GC globulin concentration values from persons of interest, a new method will be introduced to the modular analyzer. Validation of the optimized immune turbidimetric endpoint assay will be performed based on a previously described method (74). During the method validation, evaluation of following parameters is enquired: precision, repeatability, LOB, LOD, LOQ, working range, trueness, linearity and stability of method for GC globulin measurement in our project.

3. Materials and methods

3.1. Optimization and validation of the method for GC globulin serum level determination

3.1.1. Instrument and method description

The turbidimetric method for automated routine GC globulin measurement was developed and validated on Cobas® 8000 modular analyzer (Roche, Mannheim, Germany), module c502 for photometric technology detection. The Cobas® 8000 modular analyzer series is a fully automated system for immunological and clinical chemistry analysis intended for the in vitro quantitative/ qualitative determination of analytes in body fluids. (72) It includes three modules: ISE (ion-selective electrode) technology, c502 module with photometric technology and ECL (electrochemiluminescence) technology module. Module c502, used for our measurements, is capable of performing spectrophotometric and immunological turbidimetric tests with two possible fundamental types of assays: endpoint assays and rate assays. Absorbance is measured in fixed intervals of 7 to 15 seconds. An open developmental channel is available in the module to allow us introduction of new reagents for new method development.

Two-point end assay was introduced for GC globulin measurements, which means readings at two ending points: mp_1 and mp_2. mp_1 is the sample blank measurement and is measured before the final reagent is added. mp_2 measures the absorbance of the final reaction product after the final reagent is added and the reaction is completed as it is graphically presented on Figure 4. (72) To determine the reaction absorbance Ax, the sample blank value is corrected for dilution and then subtracted from the endpoint absorbance:

$$Ax = Amp_2 - d \times Amp_1$$

The calculation of the unknown concentration of the analyte in the sample uses the following formula, where K represents calibration factor, calculated from the calibration curve, Ax is the reaction absorbance, Ab is the absorbance of blank calibrator, Cb is the concentration for blank calibrator, IFA and IFB are instrument constants.

$$Cx = [K(Ax - Ab) + Cb] \times IFA + IFB$$

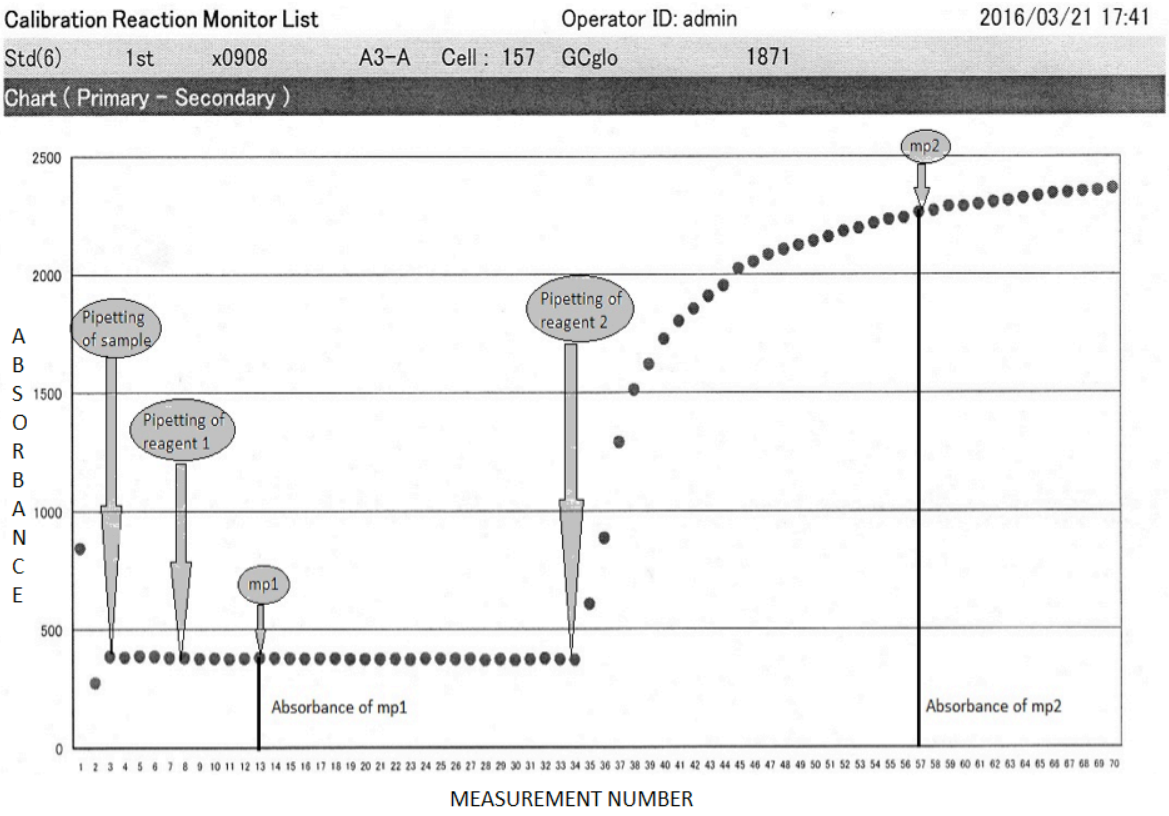

Figure 4: Two-point end assay graph.

3.1.2. Reagents and system settings

Reagent volumes and system settings were obtained from the reagent producer Dako Company recommendations and adjusted during validation procedure. The finalized version of settings is presented below.

Two-point end assay method was applied for 10 minutes reaction time (time from the sample input to results output) with mp_1 set at the 9th and mp_2 at the 49th absorbance point measurement. The wavelength used for the turbidimetric reaction was set at 340 nm. The calibrator/control/sample volume was 2.5 µL. Reagent volumes were 100 µL for the reagent buffer and 50 µL for antibody reagent, diluted in dilution buffer. Antibody dilution was 1:5. Reagent cartridge Cobas C Pack Multi was filled with the proper volumes of reagents according to instructions of the manufacturer. The assay was performed at 37°C. Specification of the reagents is presented in the Table III.

Table III: Raegents.

Reagent	Producer	Reference number
Polyclonal Rabbit Antihuman GC Gobulin	Dako A/S, Glostrup, Denmark	A0021
DAKO Turbidimetry/ Nephelometry Reaction buffer 1	Dako A/S, Glostrup, Denmark	S2007
DAKO Turbidimetry/ Nephelometry Dilution buffer 1	Dako A/S, Glostrup, Denmark	S2005
Human Serum Protein Low Control	Dako A/S, Glostrup, Denmark	X0939
Human Serum Protein Calibrator	Dako A/S, Glostrup, Denmark	X0908

3.1.3. Calibration procedure

The term calibration refers to the determination of a valid relation between the measured signal (absorbance) and the concentration of the analyte of interest. (72) RCM calibration was applied for GC globulin measurement method in accordance to nonlinear increase of absorbance in relation with concentration of analyte. RCM applies working curve approximation based on a mathematical model, where A stands for absorbance, C for concentration of analyte, a for parameter representing absorbance of blank, b for parameter representing the concentration where the absorbance or absorbance rate is 1⁄2 of the span between *Ainf* and *Ab,* c for parameter describing the curvature of the calibration curve and d for parameter representing the predicted absorbance or absorbance rate for infinite concentration – plateau on graph (*Ainf)*. (72)

$$A = \frac{a - d}{1 + (\frac{C}{b})^c} + D$$

Full calibration was performed with 6 calibrators, diluted from Human Serum Protein Calibrator with GC globulin value of 385 mg/L in Dilution buffer, presented in Table IV. Instrument performs two measurements from each calibrator to reduce the impact of intra-

assay variation effect. Full calibration means that all parameters of the calibration curve are updated. Calibration curve with all the parameters is presented on Figure 5.

Table IV: Calibrator dilutions.

	Cal. 1	Cal. 2	Cal. 3	Cal. 4	Cal. 5	Cal. 6
Concentration (mg/L)	385	275	196.43	115.54	57.77	28.88
Dilution (from previous sample)		1:0.4	1:0.4	1:0.7	1:1	1:1

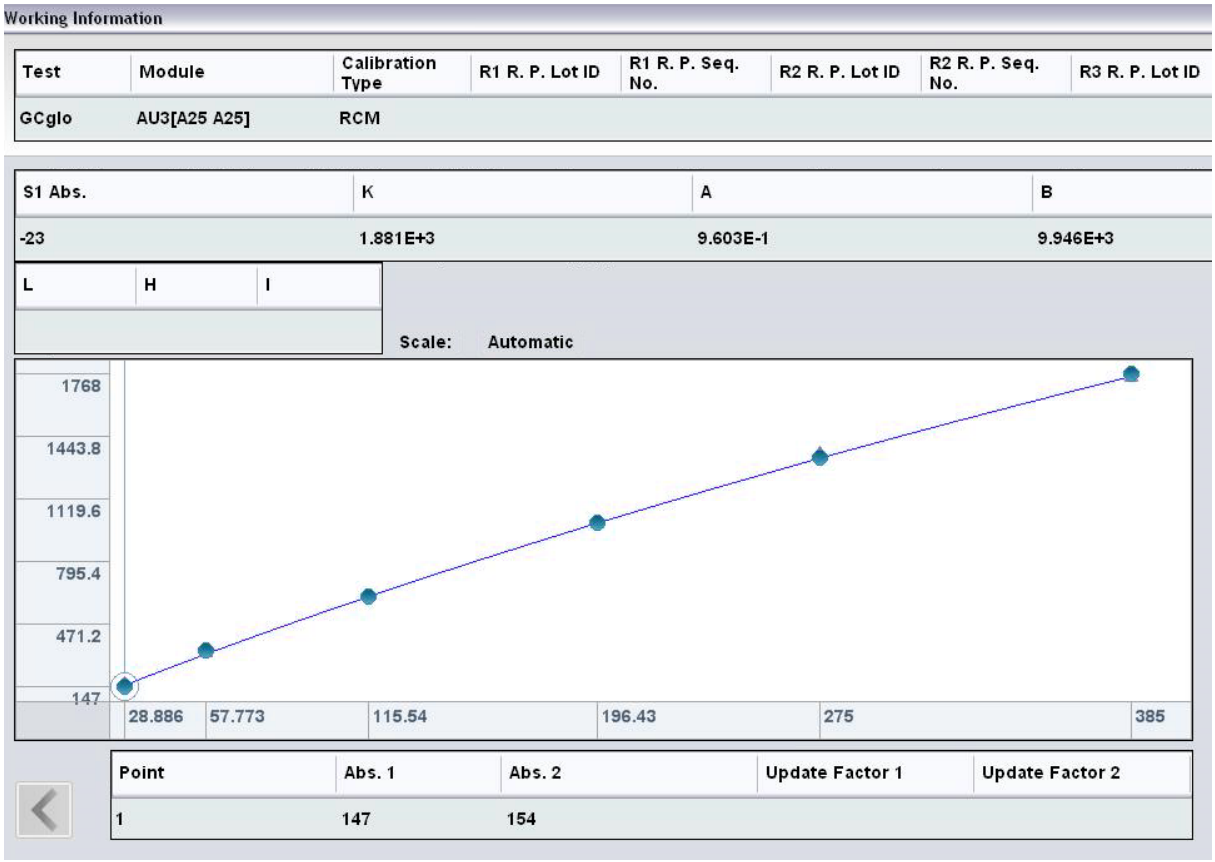

Figure 5: Calibration curve for GC globulin.

3.1.4. Control material

Human Serum Protein Low Control with GC globulin concentration of 304 mg/L was used for daily procedure control and for validation process for parameters, where handling with patients' samples was not appropriate (precision). Dilutions of control material were made with Dilution buffer.

3.1.5. Sample preparation and storage

Full blood samples were collected from the subjects and centrifuged on 1500 g for 10 minutes to obtain serum. Hospitalized patients' samples were collected at the routine morning blood take and the control group samples were collected in morning time to exclude GC globulin circadian rhythm influence. After the measurements of biochemical, hematological and coagulation panel, supernatants were collected into aliquots and stored deeply frozen on -70^{0}C until use. When thawed, serum was directly measured without any diluting.

3.2. Patient selection and sampling

The Ethics Committee of University of Pecs, Medical School, approved the study in accordance to Helsinki declaration. All the patients and controls were circumstantially informed and written consent was obtained from all. 51 patents and 29 healthy people between ages of 25 - 82 were included in our clinical study. Age difference between patient and control group is insignificant even though GC globulin concentrations are not age-related. (55) Patients group includes 30 men and 21 women and control group 13 men and 16 women, so gender differences cannot influence our study results. Including criteria for patient group was confirmed diagnosis of sepsis or SIRS, originating from different primary conditions or combination of them: diabetes mellitus type II, hypertony, ischemic heart disease, liver disease, COPD (chronic obstructive pulmonary disease), chronic kidney disease and malignancy. Control group consisted of healthy individuals without acute inflammation (based on hs-CRP, white blood cell levels and clinical symptoms) or any chronic or autoimmune diseases.

4. Results and discussion:

4.1. Validation of the method for GC globulin serum level determination

Validation of a method is basically the process of defining an analytical requirement and confirming that the method under the consideration has capabilities consistent with what the application requires. (73) Method performance must be validated to define uncertainty of the result and the level of confidence during the interpretation. Method validation is required when it is necessary to demonstrate that its performance characteristics are adequate for use for a particular purpose. According to ISO standard 17025, validation is expected when non-standard method is initiated, new method is developed, standard method is used outside its intended scope and when standard method is modified or amplified. (73)

In our case, the process of method validation was required because of utilization of a non - standard method. Other reasons for single – laboratory approach validation were substitution of laboratory detection system and necessity for lower limit of quantification on account of low GC globulin concentrations in septic patients in comparison with previously developed method. (74) Validation was preformed according to Eurachem guidance: The Fitness for Purpose of Analytical Methods – A Laboratory guide to Method Validation and Related Topics. (73)

4.1.1. Precision

Precision is a measure of how close results are to one another and depends on the concentration of measured analyte so it is necessary to determine it across the whole range of interest. (73) Usually, three types of precision are defined: repeatability, intermediate precision and reproducibility and the main difference between them is measurement series. Repeatability is a measure of the variability in results when a measurement is performed by a single analyst, using the same equipment over a short timescale. (73) Intermediate (measurement) precision gives an estimate of the variation in results when measurements are made in a single laboratory but under conditions that are more variable than repeatability. (73) Reproducibility is a measure of the variability in results between laboratories. (73)

4.1.1.1. Repeatability

Repeatability measurements (known also as precision or interassay precision) were performed on GC globulin control material of two different concentrations within the measuring range, which is 0 to 385 mg/L. Ten parallels of each concentrations was measured as it is prescribed in guidance. Measurement was performed in one day after the routine work (around noon) in one series. Lower control samples were prepared by dilution (1:4) from the manufactory prepared control material – Human Serum Protein Low Control.

Table V: Repeatability data for GC globulin assay.

Target value (mg/L)	304	60.8
Mean (mg/L)	318.26	62.80
Standard Deviaton (mg/L)	4.41	1.03
CV	0.0138	0.0164
CV%	1.38	1.64
Number of meaurements	10	10

Repeatability in turbidimetric GC globulin assay was found to be between 1.38 – 1.64 % as it is seen in Table V. Guidance suggests it to be lower than 5 % in case of protein detection methods. Results near the upper measuring range will have SD around +/- 4.4 mg/L, and the results in the lower measuring quadrant around +/- 1.02 mg/L SD. In conclusion, the results of our assay are precise enough within – run to be credible.

In comparison with the other published validation, where the larger amount of samples were measured, (74) the CV% values of our assay are slightly higher. The difference between them is 0.2 % for higher concentrations and 0.48 % at lower concentrations, which is not a significant difference. Repeatability parameters for both methods lie under the recommended CV percentage so it is possible to conclude that our experimentally defined data are very good and our method is precise in within run term.

4.1.1.2. Intermediate precision

Intermediate precision (known also as day – to day precision or intraassay precision) was assessed at two different concentrations of GC globulin's control material in ten consecutive days. Duplicates were used to lower the intra-assay variation effect and to obtain estimates with sufficient degrees of freedom in case of variance analysis is needed.

Two dilution levels were chosen to include the whole measuring range to our precision determination and ten days measuring time was selected to gain plenty results that precision CV is credible.

Table VI: Intermediate precision data for GC globulin assay.

Target value (mg/L)	**304**	**60.8**
Mean (mg/L)	336.38	66.91
Standard deviaton (mg/L)	16.94	3.37
CV	0.0504	0.0504
CV%	5.04	5.04
Number of meaurements	20	20

Intermediate precision is estimated form the CV, which is a comparison between ten-day values, calculated as the average of two parallels, measured in one day. Table VI shows same variation coefficient, 5.04 % in high and low control data. Guidance recommends intermediate imprecision lower than 8 % for protein measurement assays. Our method is in accordance with that recommendation. Intermediate precision of the GC globulin detection assay is slightly lower than repeatability, which is very plausible because of prolonged period of measuring time or sample storage and different laboratory conditions between the days of detection.

In comparison with Dako's intermediate precision validation, our data are slightly different. CV for upper control material is 2.2 % higher and for the low control material 1.1 % lower. The reason for this deviation is in the different measuring systems, control preparation and different terms and conditions during measuring. Both methods include CV under the critical value for imprecision so we can conclude that our method's precision is credible in between – run term.

The last precision parameter – reproducibility was impossible to measure, because we were the only group, researching GC globulin and this method nearby at these times, so comparison between laboratories was not possible.

4.1.2. Limit of blank, Limit of detection, Limit of quantification

These three parameters are very important part of every validation process. GC globulin concentration detection limit is especially important because its decreased values appear in different diseases including sepsis and SIRS.

First, it is necessary to establish a value of result, which indicates significant difference between analyte, present in sample and zero analyte and is defined as limit of blank (LOB). (1) For GC globulin method, LOB was determined from ten reagent blanks under repeatability conditions and calculated as mean + $1.645 \times SD_{blank}$.

Second, it is important to recognize the lowest concentration of the analyte that can be detected by the method at a specified level of confidence and it is defined as limit of detection (LOD). (73) We determined LOD using reagent blank because of limited possibilities to obtain blank samples or test samples with low concentrations of GC globulin. Ten samples were measured under the repeatability conditions and after that LOD was calculated as mean + $3 \times SD_{blank}$.

Third, it is necessary to establish the lowest level at which the performance is acceptable for a typical application and is defined as limit of quantification (LOQ). (73) LOQ was determined by the same procedure as described above for LOD with a slight difference in calculation. LOQ was assessed as mean + $10 \times SD_{blank}$.

Table VII: Results of LOB, LOD and LOQ calculations.

	Value	SD	Mean	CV%	Number of measurements
LOB	0.426 mg/L	0.170	0.145	117.44	10
LOD	0.655 mg/L	0.170	0.145	117.44	10
LOQ	1.847 mg/L	0.170	0.145	117.44	10

All three parameters for GC globulin measurement assay are very low, which means, that the method is capable to detect pathologically decreased concentrations of GC globulin with a certain level of confidence. That result is very promising for our further clinical

studies with decreased concentrations of GC globulin, where patients' sample amounts possibly declines almost to zero.

Measured LOD is 5,8 mg/L lower in comparison with Dako's validation (74) and it is the only parameter available to compare. The difference is very high and possibly arises from different blank samples. In our validation the reagent blank was utilized and in Dako validation low control dilution was used for LOD specification. Inevitably, the blank samples are not the most appropriate in comparability with the patient samples as they do not contain the true matrix, but in both cases LOD is low enough not to influence the measurements of patient samples.

4.1.3. Working range

Working range is the interval over which the method provides results with an acceptable uncertainty. The lower end of working range is represented by LOQ of the method and the upper end is defined by occurrence of significant anomalies in the analytical performance (e.g. underestimation of high values because of the unfavorable antigen-antibody ratios). (73) It is determined by a reason of establishing a confidence interval for the measurements of patient samples with the less possible deviation from the true value.

The working range of method for the determination of GC globulin is defined as previously specified LOQ, which represents its lower end and as the highest calibration standard presenting the upper end. The working range for the method is therefore between **1.847 mg/L** and **385 mg/L**.

The lower end is very appropriate for the method because of the low concentration of GC globulin in certain patients' samples, but on the other hand the upper end is too low and some sample concentrations exceed the value. The upper end is determined by the highest calibrator in account of impossibility to obtain the standard or calibration material with higher concentration to perform some additional tests above the range. We tried to concentrate samples with centrifugal concentrator, but the results were predictably useless, probably due to the high background. However, in the case of patients with GC globulin concentration higher than the higher standard, the measurement could be repeated after sample dilution. We conclude, that LOB, LOD and LOQ meet our requirements for GC measurement in patients with sepsis and SIRS.

4.1.4. Trueness

Trueness is defined as a validation parameter that expresses, how close the mean of infinite number of results is to a reference or true value according to new guidelines from 2015, (73) in old terminology known as accuracy. Practically it is impossible to measure infinite number of samples so practical assessment of trueness is used and it is called bias. The best way to measure it is by performing the recovery test, but it was impossible to perform it due to the lack of reagents and control material.

4.1.5. Linearity

Linearity of an analytical procedure is its ability to give test results directly proportional to the concentration of analyte in the sample within a given range. (75) The linearity test for GC globulin measurement was performed to detect if the response of the apparatus depends on the concentration of analyte in sample. Patient samples were chosen for the linearity measurements on account of sample background, which reflects the routine measurements more suitable. Seven carefully prepared dilutions from 100 % (original sample) to 5 % with Reagent Dilution buffer were measured in two parallels to decrease the influence of intra – assay deviation and the mean value was used to calculate the linearity. Dilutions cover 85 % of the measuring range - from 8 mg/L to 332 mg/L of GC globulin concentrations and it is comparable to the guidance, which requires 80 – 120 % range covering.

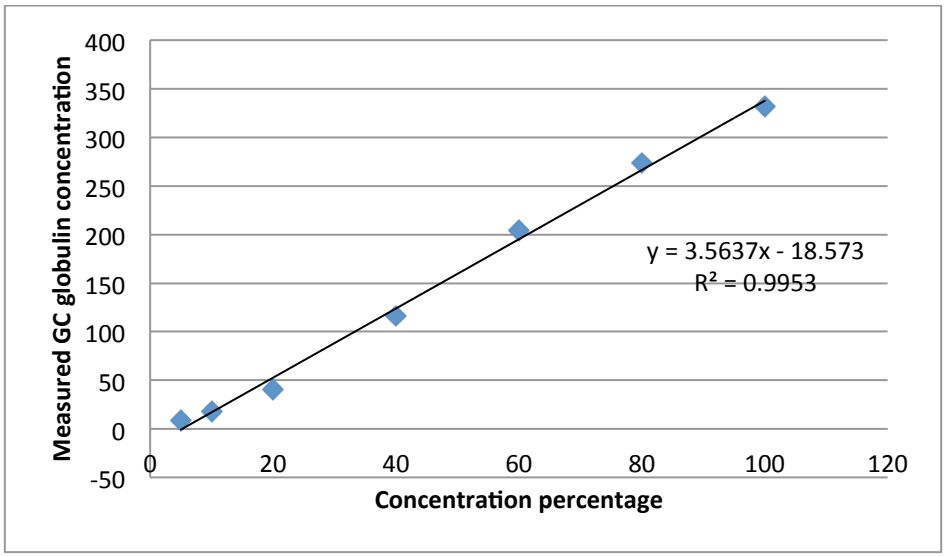

Figure 6: Linearity range of GC globulin determination method.

As it is displayed on Figure 6, the method for GC globulin determination is linear in the range between 8 mg/L to 332 mg/L so it predicts measured GC globulin concentrations for each possible real concentration in sample within this range. Errors of prediction are low across the whole line except of a slight increase by the lowest concentrations, where precision also decreases. Best fitting line minimizes the sum of squad errors of prediction and it is defined as an intercept of -18.573 and the line slope of 3.5637. Coefficient of correlation is imperceptible lower than one but still high enough to indicate the proportion of the variance in the dependent variable that is predictable from the independent one. (75) Coefficient of correlation is a reliable indicator of linearity of GC globulin determination method in a definite range.

Linearity measurements of GC globulin detection method from Dako Company were performed in a wider concentration range between 0 and 476 mg/L on two different patients' serum pools. Results are introduced as percentage of deviation and none of them deviated more than 5 %. Recalculating our results to percentage of deviation, the 5% dilution differs around 10 %, and 20 % dilution differs around 6 % deviation, but the other dilutions are inside 5 % deviation. As mentioned above, our results are slightly imprecise in the lowest part of the measuring range, but in general linearity of our method is acceptable enough to precede our further measurements.

4.1.6. Stability measurements

The general definition presents stability testing as a measure of the assay bias, which is generated during the time interval on account of decomposing of the determined compound. Stability measurement is important for estimating the allowed time span between sample collection and analysis without producing imprecise results. (75) Different types of stability measurements are known, but for GC globulin detection method two types of stability testing were done, short – term for 6 days (at 4°C) and long – term for 3 months (at -70°C) stability, because of necessity for the useful value of the method does not require others.

4.1.6.1. Short - term stability

Six aliquots from one patient sample were measured in six consecutive days in two parallels to decrease the influence of intra – assay deviation. Storage of the aliquots was

the same as the routine sample storage conditions, namely in the refrigerator at 4°C. Selection of number of the days is also associated with the routine procedure, where samples are usually stored for six days and then displaced if not frozen, so it is the time frame of our interest (Figure 7).

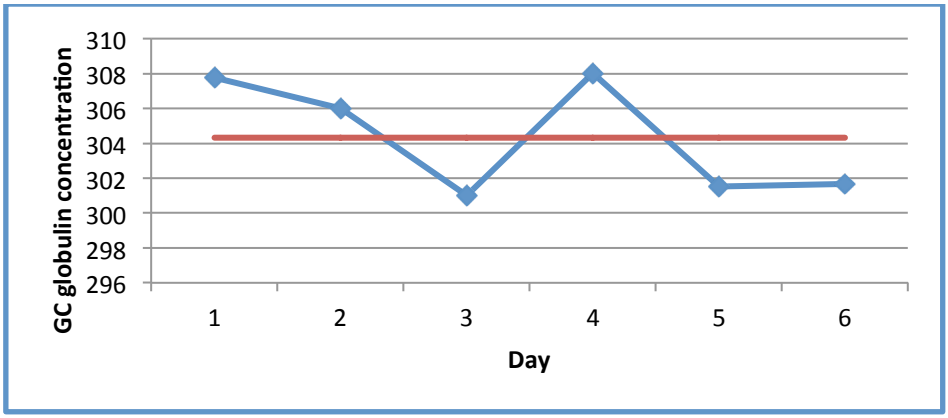

Figure 7: Short – term stability results.

Mean value for measured aliquots is 304 mg/L; SD is 3.29 mg/L and CV% is 1.08 %.

SD and CV are much lower in comparison with intermediate precision. CV% is 3,95 % lower so it is possible to conclude that the error originates from intermediate precision and not from the instability of the GC globulin in sample. GC globulin is stable for six days at 4°C. Samples, analyzed after six days, should be frozen.

The Dako Company's stability measurements specify one week 4 - 8°C stability with a deviation lower than 10 %. Nevertheless the manufacturer does not give an exact CV% for the method, both measurements prove stability of GC globulin in the refrigerator for the time of normal sample storage.

4.1.6.2. Long – term stability

The second type of stability measurement was a combination of freezing – thawing stability and long - term stability measurements. Two different concentrations of patient samples were measured in two parallels. First cycle had been extended for five days with double quantification of thawed sample per day and after that it was frozen to -70°C for next day assessment. For the determination of the long - term stability it was necessary to repeat the same measurements after one and two months of sample storage at -70°C (Table VIII).

Table VIII: Statistical parameters of long – term stability.

	High concentration			Low concentration		
	Freeze and thaw s.	**Long - term s. – 1 month**	**Long - term s. - 2 month**	**Freeze and thaw s.**	**Long -term s. – 1 month**	**Long - term s. - 2 month**
Mean (mg/L)	339.9	361.37	363.58	54.91	44.541	35.19
SD (mg/L)	2.72	4.55	4.85	0.46	0.716	13.25
CV (%)	0.80	1.26	1.34	0.84	1.608	37.66
Number of measurements	11	6	8	12	6	8

Freeze and thaw stability measurements reveal CV% between 0.8 to 0.84 % and it is much lower compared to the intermediate precision CV% and also to short – term stability CV%. This result is expected because freezing preserves sample proteins on the short time scale, but on the other hand it has been demonstrated that freezing and thawing does not decompose GC globulin therefore the freeze and thaw stability of GC globulin is high.

Long - term stability measurements reveal low coefficient of variation for samples with high concentration of GC globulin, 1.26 % after two months and 1.34 % after three months. On the other hand GC globulin declines significantly in samples with low concentration after three months of storage, rising CV% up to 37.66 %. Results point out decomposition of GC globulin in frozen patients' samples after long - term storage, especially when its concentrations are low, so measuring and interpretation of the results after two months storage should be avoided.

Freeze ant thaw stability measurements of Dako Company specify standard deviation less than 10 %, which is comparable with our results. Comparison of the results on long - term stability measurements differentiates firmly because Company claims stability of GC globulin in frozen samples on -20°C for more than eight months with CV% less than 10 %, yet protein in our samples decomposed in three months. There might be a difference in

sample storage on -20°C and - 70°C, on sample preparation and storage conditions, for example direct freezing or freezing after few days, however no data about the procedure is available.

4.2. Determination of clinical importance of GC globulin

GC globulin is anticipated to be an indicator for different types of diseases. It is clinically important in medical conditions like sepsis, nephropathy, liver failure and other liver diseases according to its synthesis and excretion. The main aim of our clinical trial is to define if there is a significant reduction of its concentration in two conditions: sepsis and SIRS. Turbidimetric method for GC globulin determination in patients' serum was validated and then 51 patients' serum samples and 29 healthy people samples were measured. Patients' samples have been assessed on the first day when clinicians diagnosed sepsis or SIRS, on day three and on day five, if the patients survived for that period of time. Three time scale data (day one, three and five) of each patient has been measured to comprise GC globulin values between days and to correlate day-to-day assessments with septic patients' mortality. Other routine biochemical and hematological parameters have been measured at the Department of Laboratory Medicine, University Medical School of Pecs, to detect the relationship between GC globulin levels and their corresponding clinical values. Gelsolin has been measured with laboratory developed Western blot technique because no routine method exists due to the instability of the gelsolin protein calibrators and controls.

Statistics of the obtained data was produced with IBM SPSS Statistics 22 program. Normality tests, Mann - Whitney test, Spearman correlation and ROC curve analysis were used with a significance level of $p =< 0.05$.

4.2.1. Testing for normality

The normality test is performed to determine, if dataset is modeled by the normal distribution of variables. (76). Normality of distribution has great influence on further decision, which statistical test to be performed as they strongly depend on distribution model. Shapiro – Wilk correlation test was selected for testing the normality of GC globulin data because this model gives higher accuracy at lower number of data. Every GC

globulin results from day one, three and five were integrated to define distribution. Two hypotheses are set for normality testing:

H_0: Our distribution originates from normal distribution.

H_1: Our distribution does not originate from normal distribution.

Table IX: SPSS output for normality testing.

Tests of Normality of Distribution

	Status_1: Control=1, SIRS=2, Sepsis=3	Shapiro-Wilk		
		Statistic	df	Sig.
Serum Gc globulin [mg/L]	1	0.660	28	0.000
	2	0.966	17	0.749
	3	0.964	94	0.011

Shapiro – Wilk test results show that only SIRS group is normally distributed while values in sepsis and control group are distributed abnormally ($p<0,05$). In case of sepsis and controls p value is lower so it is possible to reject the null hypothesis and accept the alternative one. In case of SIRS, p value is higher than 0.05 so we cannot reject null hypothesis and our data are normally distributed. For future statistical analysis it is necessary to use nonparametric statistical methods. Although SIRS data are normally distributed, nonparametric tests should be used for comparison with abnormally distributed data (Figure 8).

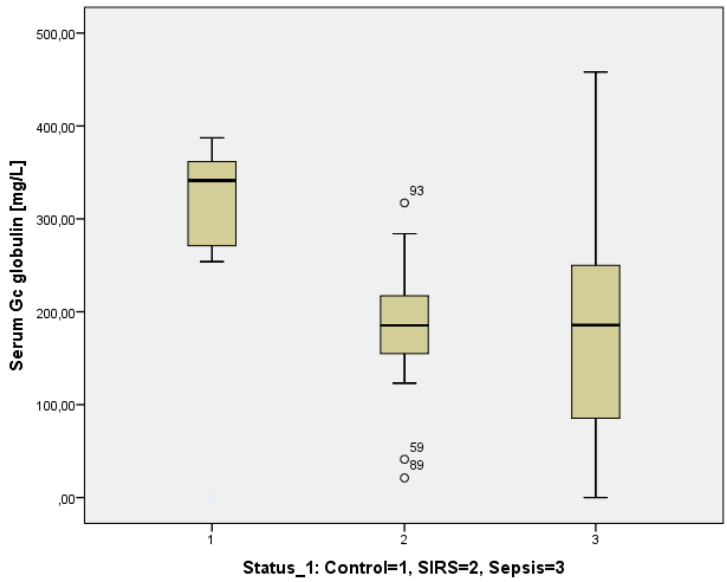

Figure 8: Box and whisker plot of distribution for GC globulin.

Box plots display variation in samples of a certain population without assuming the underlying statistical distribution (76) so that is why they are a good indicator for visual evaluation of the results. From the plots it is possible to estimate the normality of distribution: the second plot has the median in the center of the box and is almost symmetrical however other two plots are not. The median value is much higher in control group than in other two so probably there is a difference between control and patients group, but not an obvious contrast between sepsis and SIRS group. Septic patients' category is very interesting because of wide whisker scope, unveiling large differences between GC globulin values in these cases. The reason for it might be in observance to integrate all day's data to form the plot. The whisker scope is much narrower in SIRS cases but few higher and lower outliers are detected. Recognized outliers should be incorporated in further testing because the reason for their existence cannot be explained by analytical procedure. Box and whisker plot is very useful for a visual evaluation of obtained data nevertheless statistical tests should be performed to achieve a significant result.

Before further statistics was calculated, relationship between gender or age and GC globulin was searched, but no significant difference was found so it is possible to resume that gender or age has no influence on amount of GC globulin in patients' serum. (Data not presented).

In conclusion, only SIRS group has normally distributed values while values in sepsis and control group are distributed abnormally and therefore nonparametric tests should be used for further evaluation.

4.2.2. GC globulin values at occurrence of SIRS or sepsis

Day one GC globulin values are very important to evaluate the clinical importance of GC globulin as a biochemical marker for diagnosing sepsis or SIRS in patient. The significant difference between patient and control group should be proven to consider further comparisons reasonably. Statistics was calculated with 2- sided Mann Whitney test, which is a nonparametric equivalent to parametric t-test and compares two mean values from dissimilar groups of abnormally distributed data to find a significant difference. (76) Significant threshold value is 0.05. Hypotheses for Mann - Whitney test are:

H_0: The distribution of serum GC globulin is the same across categories.

H_A: The distribution of serum GC globulin is not the same across categories.

The results of statistical comparison are gathered in the further Table X.

Table X: SPSS statistics: Comparison of day one measurements of GC globulin.

Categories	Test	Sig.	Decision
Sepsis - SIRS	Independent samples – Mann Whitney U test	0.108	Retain the null hypothesis.
Sepsis - control	Independent samples – Mann Whitney U test	0.000	Reject the null hypothesis.
SIRS - control	Independent samples – Mann Whitney U test	0.000	Reject the null hypothesis.

The difference in GC globulin values between patients with sepsis or SIRS and control samples is determined to be significant. Therefore there is a strong connection between disease and decrease in concentration of GC globulin in patients' blood with a reason of actin scavenging role of GC globulin and its uptake in a bounded form. There is no significant difference between sepsis and SIRS groups therefore on day one, the severity of a patients' state is difficult to recognize. The correct diagnosis depends more on a clinician's opinion or other biochemical markers than on GC globulin. Markers found to distinguish between SIRS and sepsis were hsCRP, serum – PCT, creatinine, serum - CK, albumin, total protein count and gelsolin. Gelsolin is very interesting because as GC globulin it is also a part of actin scavenger system, not used in the clinical practice yet, but also a very promising marker for diagnosing and monitoring sepsis and SIRS.

Significant difference in GC globulin values between septic patients and healthy individuals was also published in different articles. L. Jeng et al found that GC globulin concentrations are significantly lower in critically ill subjects with sepsis compared to critically ill subjects without sepsis and healthy control subjects. (77) Dahl B. et al described significantly lowered GC globulin values in patients, who developed sepsis after injury compared with patents, who did not. (78) Gressner et al proved significant decrease in GC globulin values in critically ill patients compared to those in healthy control subjects yet no association with critically ill patients without sepsis was found. (79)

GC globulin concentration is determined to be significantly lowered in septic patients' samples as well as in serum of SIRS patients, however it is not a marker of differentiation of disease severity in critically ill patients.

4.2.3. GC globulin as a marker of sepsis or SIRS progression

After proving the difference of GC globulin values between patients' samples and control group it is necessary to detect the importance of GC globulin for monitoring the disease progression. For that reason, day one, day three and day five septic and SIRS patients' samples were collected and values compared between the groups. Mann – Whitney test for abnormally distributed data was applied for determination of the value of day – to day comparisons. Hypotheses for day - to day comparison are:

H_0: The distribution for serum GC globulin is the same across categories of sampling day.

H_A: The distribution for serum GC globulin is not the same across categories of sampling day.

Table XI: SPSS: Day to day comparison of GC globulin measurements.

	Day 1 – 3 comparison		Day 3 – 5 comparison		Day 1 – 5 comparison	
	Sig.	Decision	Sig.	Decision	Sig.	Decision
Sepsis	0.031	Reject the null hypothesis.	0.690	Retain the null hypothesis.	0.123	Retain the null hypothesis.
SIRS	0.448	Retain the null hypothesis.	0.800	Retain the null hypothesis.	0.198	Retain the null hypothesis.

The most important finding is that the GC globulin values change significantly from day one to day three during progression of sepsis. GC globulin concentrations increase after the treatment for sepsis or SIRS has started, because of reduction of actin from cell lysis in patients' blood. After day three, increase of GC globulin is slower than in first three days, therefore it is not statistically significant. 1 - 5 day comparison is also not significant due to the slow increase in days after the disease occurrence. GC globulin values of SIRS patients do not change significantly during the 1 – 5 day measurements. Increase occurs, but in most cases the day one measurements are higher than septic patients' values and further days increase is too low to be significantly different.

No similar examination in the literature was found.

We concluded significant increase of GC globulin values in patients from day one to day three after starting the treatment of the disease.

4.2.4. Comparison between GC globulin and routinely used biochemical, hemostasis and hematological parameters

Comparison between GC globulin and some other routinely assessed biochemical, hemostasis and hematological parameters should also be followed to find a possible correlation between currently used markers, and GC globulin as a new marker for sepsis and SIRS. It is important to determine the role and functionality of a new potential biomarker in the clinical practice in different diseases and one of the important points of it is its comparison with other parameters. Correlation between GC globulin and biochemical, hemostasis and hematological markers was calculated with SPSS statistics program and presented as a nonparametric Spearman's rank correlation coefficient. These parameters were measured from patients' serum in the Department of Laboratory Medicine except of gelsolin, which was measured in a research laboratory with a laboratory established Western blot technique. All measured biomarkers for correlation analysis are presented in subgroups in table XII. Biomarkers with significant correlation detected are presented in table XIII, with correlation calculations separated to day one, three and five from sepsis occurrence, while the insignificant are not presented.

Table XII: Biomarkers, measured for correlation analysis.

Actin scavenger system markers:	GC globulin, gelsolin, actin, actin/gelsolin ratio.
Biochemical markers:	Total protein count, albumin, uric acid, AST (aspartate aminotransferase), ALT (alanine aminotransferase), GGT (gamma-glutamyl transferase), total bilirubin, LDH (lactate dehydrogenase), creatine kinase, creatinine, estimated glomerular filtration rate, PCT, hs-CRP.
Markers of hemostasis:	APTT, INR.
Hematological markers:	Red blood cell count, hemoglobin, hematocrit, neutrophil granulocyte count, leukocyte count, lymphocite count, monocyte count, trobmocyte count (Tct).

Table XIII: Significant correlations of GC globulin with other biomarkers.

			Serum GC globulin [mg/L] Day 1	Serum GC globulin [mg/L] Day 3	Serum GC globulin [mg/L] Day 5
Spearman's correlation coefficient	**Serum GC globulin [mg/L]**	Correlation Coefficient	1.000	1.000	1.000
		Sig. (2-tailed)			
		N	52	36	23
	Serum gelsolin [mg/L]	Correlation Coefficient	0.526**	0.315	0.103
		Sig. (2-tailed)	0.001	0.117	0.725
		N	36	26	14
	Serum actin/gelsolin ratio	Correlation Coefficient	-0.385*	-0.134	-0.226
		Sig. (2-tailed)	0.020	0.513	0.436
		N	36	26	14
	Serum total protein [g/L]	Correlation Coefficient	0.367**	0.369*	-0.399
		Sig. (2-tailed)	0.008	0.027	0.060
		N	52	36	23
	Serum total bilirubin [µmol/L]	Correlation Coefficient	-0.289*	-0.621**	-0.582**
		Sig. (2-tailed)	0.038	0.000	0.004
		N	52	36	22
	Serum PCT [µg/L]	Correlation Coefficient	-0.459**	0.079	-0.086
		Sig. (2-tailed)	0.001	0.691	0.761
		N	52	28	15
	Serum CK [U/L]	Correlation Coefficient	0.543**	0.200	0.444
		Sig. (2-tailed)	0.001	0.398	0.112
		N	33	20	14
	Serum creatinine [µmol/L]	Correlation Coefficient	-0.361**	-0.120	-0.280
		Sig. (2-tailed)	0.009	0.484	0.196
		N	52	36	23
	APTT [sec]	Correlation Coefficient	-0.445**	-0.091	-0.333
		Sig. (2-tailed)	0.003	0.710	0.420
		N	43	19	8
	Tct count [G/L]	Correlation Coefficient	0.259	0.680**	0.364
		Sig. (2-tailed)	0.064	0.000	0.114
		N	52	33	20

** Correlation is significant at the 0.01 level (2- tailed).

* Correlation is significant at the 0.05 level (2 – tailed).

Actin scavenger system parameters: Observing the correlation between actin scavenger system parameters, only day one is important. The positive correlation exists between GC globulin and gelsolin, because actin scavenging is distributed proportionately among them so concentrations of both proteins decrease similarly. Few days after sepsis or SIRS is recognized and treatment introduced, actin concentration in blood decreases but liver synthesis of GC globulin and skeletal muscle synthesis of gelsolin is not simultaneous so correlation disappears. There is no correlation between GC globulin and serum actin values because actin excretion from necrotic cells is not regulated process yet GC globulin synthesis is. There is a negative correlation between GC globulin and actin/gelsolin ratio on account of gelsolin correlation with GC globulin.

Biochemical markers: Positive correlation on day one between serum total protein and GC globulin has been demonstrated with a logical biochemical background of inclusion of GC globulin into protein level. The connection is strong on day one, when GC globulin values are lowest and affects protein level the most. Association between GC globulin and albumin was not detected because albumin values are not directly connected with sepsis or SIRS. The negative correlation between GC globulin and serum total bilirubin exists for all five days of observation. Sepsis and SIRS usually causes intravascular hemolysis with high hemoglobin release to be transformed to bilirubin so bilirubin concentrations are elevated. LDH could be also connected with hemolysis however it does not have association with GC globulin. Focus on the classic biochemical markers of inflammation, notably negative association with procalcitonin (PCT) prevails on day one nevertheless there is no connection between GC globulin and hsCRP. There is also a negative correlation on day one between creatinine and GC globulin and positive correlation with serum creatine kinase. Lowered CK values are not clinically important, but the parameters are connected by the biochemical reaction in high energy demanding cells. Panel of liver function tests does not correlate with GC globulin values at any day. The tests compared were AST, ALT and GGT. The correlation does not exist because the patients, developing sepsis, have different background diseases, not only acute liver failure nevertheless in those patients, association is possible. (80)

Markers of hemostasis: Strong negative correlation between GC globulin values and activated partial thromboplastin time (APTT) exists, but only on day one. In sepsis and SIRS, free actin is released from necrotic cells and in the state of GC globulin and gelsolin

being exhausted; free actin forms clots (81) and consumes factors of intrinsic and common coagulation pathways. As a result, APTT is prolonged and notable association with decreased GC globulin values is found. It is interesting that there is no correlation between GC globulin and INR – extrinsic pathway marker at any day, therefore it is possible to assume that free actin reacts more with factors of intrinsic and common pathways. There is a significant positive association between GC globulin and platelet count on day three due to the forming of clots in the first days when actin is released so thrombocytes have been consumed. (82)

Hematological markers: Besides of platelet count, none of the measured hematological parameters correlate with GC globulin values.

Gressner et al found significant correlation between C5a and 1,25-vitamin D3 that we did not evaluate, but comparable to our results no correlation with AST, ALT, and GGT was detected. (79) Charalambos et al discovered correlation with markers of hemostasis: APTT, INR, thrombocyte count and fibrinogen. (82) In our study correlation with INR does not appear, but in both studies different methods for detection of compared parameters were utilized and it can affect the results.

To sum up, correlations between GC globulin and actin scavenging system markers exist in first day measurements in gelsolin and actin/gelsolin ratio. Biochemical markers' correlations prevail on day one between GC globulin and total protein count, PCT, CK, creatinine and all five days with bilirubin. Among markers of hemostasis, negative correlation is determined with APTT on day 1 and positive correlation with thrombocyte count.

4.2.5. GC globulin as a marker of the mortality in sepsis and SIRS patients – ROC curves

When testing comparison of GC globulin between patients' groups for the first day after sepsis occurs, a significant difference between survivors and non – survivors was found. From the beginning of our clinical study proposition exists that GC globulin is a potential marker of disease outcome therefore the patients' mortality was observed. The patients were divided into groups of survivors and non-survivors, so it is possible to compare GC

globulin values in different measuring days within three days-, seven days-, fourteen days- and twenty days mortality. Association with mortality was calculated on the basis of evaluating quality of GC globulin test with ROC curves. Area under the curve and significance of comparison were calculated with SPSS statistics program and an optimal concentration of GC globulin for clinical usage was chosen.

4.2.5.1. GC globulin values on day 1 and patients' mortality

Area Under the Curve

Test Result Variable(s): Serum Gc globulin [mg/L]

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.880	0.047	0.003	0.789	0.972

a. Under the nonparametric assumption

b. Null hypothesis: true area = 0.5

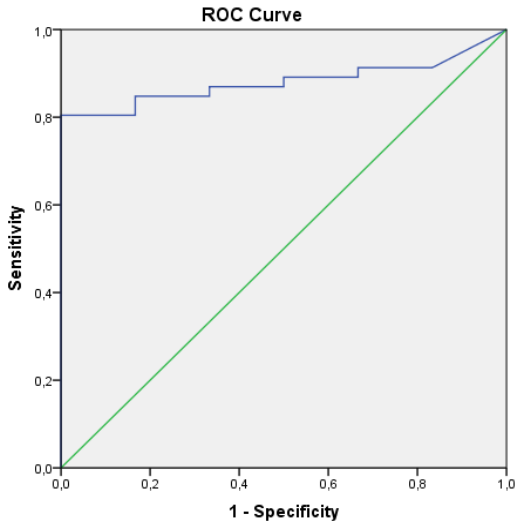

Figure 9: ROC curve of three-day mortality (GC globulin data from day one).

Area Under the Curve

Test Result Variable(s)

Area	Std. Error[a]	Asymptotic Sig.[b]	Asymptotic 95% Confidence Interval	
			Lower Bound	Upper Bound
0.783	0.079	0.003	0.628	0.939

Under the nonparametric assumption$_a$

Null hypothesis: true area = 0.5$_b$

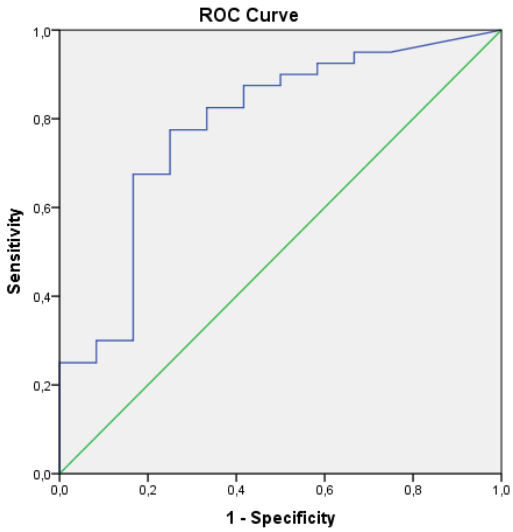

Figure 10: ROC curve of seven-day mortality (GC globulin data from day one).

In both cases p value is lower than significant value of 0.05 so it is possible to assume, that notable association between three- and seven-day mortality and lowered values of GC globulin exists in patients' samples.

ROC curve of three-day mortality unveils high sensitivity of the test along all GC globulin values of non-surviving patients. Area under the curve is 0.880 therefore predictable value of surviving among the septic patients is good. It is necessary to have high sensitivity when defining mortality prediction tests on account of specificity. The reason is that unveiling true positive patients and cure them more efficiently has priority from false positive patients, whom more aggressive treatment do not induce more harm in case of sepsis. In account of this fact the cut-off value is determined. The selected cut-off value for three-day mortality is **38.925 mg/L** of GC globulin concentration. Sensitivity for this value is 0.891 and specificity is 0.5, which means that it is possible to discover 89 % of patients with higher probability of death, but also 50% of false positive cases. Our results have one disadvantage: only six patients were included into a clinical study.

ROC curve for seven-day mortality does not have such high predictable value, as a curve for three-day mortality however there is a significant association between mortality and GC globulin values. Area under the curve is 0.783, for that reason the GC globulin test for determination of seven days mortality is classified as fair. Cut off value is assessed on the basis of high sensitivity, but there are two possibilities to choose. The first one is GC globulin concentration of **38.92 mg/L** with a sensitivity of 92.5 % and specificity of 41.7 % and it is good to be comparable with cut-off value for three days mortality. However it has very high rate of false positive cases. Probably it is better to choose GC globulin value of **50.41 mg/L,** where we detect 90 % of patients, susceptible to death and 50 % of false positive cases.

The significance of association decreases in predictability of mortality in prolonged time after the first day measurement. Consequently, fourteen- and twenty-day mortality areas under the ROC curves do not exceed a value of 0.65 and the p value of comparison retains the null hypothesis of weak connection between the variables.

4.5.2.2. GC globulin values on day 3 and 5 correlated with patients' mortality

Comparison of GC globulin values and seven-, fourteen- and twenty-day mortality provides no predictable value and area under the curve does not exceed over 0.58. Consequentially GC globulin values from patients' samples, obtained three or more days after sepsis had been recognized, are not a valuable prognostic marker for possible negative outcome of treatment.

The only published mortality study is from Gressner et al however they did not find a significant difference between survivors and death cases not in case of GC globulin comparison but also comparison with C3a depend impact on mortality. (79)

First day measurements of GC globulin after sepsis has emerged are good marker for predictability of disease outcome for next seven days however not so accurate for a longer period of time.

5. Conclusions

New immune turbidimetric two-end point assay for GC globulin concentration measurement was successfully introduced to automatic analyzer. Full validation was performed, proving that the method is completely acceptable for GC globulin measurements in routine laboratories in everyday clinical practice with estimated:

- Assay precision,

  Repeatability and intermediate precision are both lower than general guideline demands for protein measurement assays (CV % up to 5 % for repeatability and 8 % for intermediate precision), therefore method is considered precise.

- Assay LOB, LOD and LOQ,

  Low values of method parameters LOB (0.426 mg/L), LOD (0.655 mg/L) and LOQ (1.847 mg/L) provide precise measurements of lowered GC globulin values in septic patients.

- Assay working range,

  Working range of GC globulin concentration (1.847 - 385 mg/L) covers mostly all of the measurements in samples of healthy individuals as well as septic patients. In case of higher GC globulin concentrations, sample dilution is possible to fit in the working range.

- Assay linearity range,

  Linearity range from 8 mg/L to 332 mg/L covers the majority of normal and pathologic values of GC globulin in clinical samples.

- Sample short term and long term stability

  Samples are short-term stable for 6 days at 4^0C and long-term stabile for 2 months at -70^0C.

Furthermore, clinical trial on 51 patients and 29 healthy subject control group indicates GC globulin as a potential marker for sepsis and SIRS. GC globulin is statistically decreased in patients with both sepsis and SIRS, especially on the disease outbreak, when actin is released from necrotic cells, binding with actin scavenger system proteins, including GC globulin, which is removed from the system in bound form. Therefore GC globulin is

presumed to be a diagnostic marker for sepsis. In progression of disease, its concentrations significantly increase in connection with patients' treatment and enhanced protein synthesis; increase is most noticeable till day three from the disease outbreak.

Comparison between newly established test for GC globulin concentration measurement and currently used biochemical, hemostasis and hematological parameters reveals us strong negative correlation with bilirubin concentration through all five days of disease monitoring. Strong positive correlation was found also with serum total protein count, where GC globulin is also measured and with gelsolin, which is also part of the actin scavenger system. Other positive (creatine kinase, platelet count) and negative (procalcitonin, creatinine, APTT) correlations found, appear only on the outbreak of the disease and disappear after in the course of the disease.

Finally, the most important finding from our research is connection of GC globulin concentration at the onset of sepsis with the three- and seven-days mortality. Cut-off concentration of GC globulin for three-days mortality is set on **38.93 mg/L** with sensitivity for this value of 0.891 and specificity 0.5, which means that it is possible to discover 89 % of patients with higher probability of death, but also with 50% of false positive cases. Cut off- concentration for seven-days mortality is set on **50.41 mg/L,** where we detect 90 % of patients with higher probability of death and 50 % of false positive cases – its predictable value is slightly poorer than for three days mortality.

Based on our results we can conclude, that GC globulin is a promising marker for predicting sepsis outcome.

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