A PERSONALIZED RANDOMIZED TRIAL OF VALIDATION AND RESTORATION OF IMMUNE DYSFUNCTION IN SEVERE INFECTIONS AND SEPSIS (THE PROVIDE TRIAL)

STUDY PROTOCOL

Evangelos J. Giamarellos-Bourboulis, MD, PhD and Mihai G. Netea, MD, PhD
Authors

Sponsor and CRO:
Hellenic Institute for the Study of Sepsis
88 Michalakopoulou str., 11528 Athens, Greece
insepsis@otenet.gr
tel 00302107480662

EudraCT number: 2017-002171-26
Protocol version: 4.0
Protocol date: 12 July 2018

DISCLOSURE OF PRINCIPAL INVESTIGATOR

Protocol Study Title: A PERSONALIZED RANDOMIZED TRIAL OF VALIDATION AND RESTORATION OF IMMUNE DYSFUNCTION IN SEVERE INFECTIONS AND SEPSIS (THE PROVIDE TRIAL)

The herein protocol became known to myself by the Study Sponsor. I understand that the protocol remains as yet unpublished; I certify that all disclosed information to myself for this protocol will remain strictly confidential.

The Principal Investigator,

Print Name

Signature Date

Table of contents **Page**

LIST OF ABBREVIATIONS

AC: acute cholangitis

AE: adverse event

aPTT: activated partial thromboplastin time

BSI: primary bacteremia

CAP: community-acquired pneumonia

DIC: disseminated intravascular coagulation

EDTA: ethylene-diamene-tetracetic acid

HAP: hospital-acquired pneumonia

HBD: hepatobilliary dysfunction

HIV: human immunodeficiency virus

HLA-DR: histocompatibility complex DR

G-CSF: granulocyte-colony stimulating factor

IL: interleukin

INR: international normalized ratio

IV: intravenous

LPS: lipopolysaccharide

MAP: mean arterial pressure

MALS: macrophage activation-like syndrome

OR: odds ratio

PBMCs: peripheral blood mononuclear cells

RCT: randomized clinical trial

rhIFNγ: recombinant human activated interferon-gamma

SAE: serious adverse event

sc: subcutaneous

SIRS: systemic inflammatory response syndrome

SOFA: sequential organ failure assessment

TB: tuberculosis

TNFα: tumour necrosis factor-alpha

VAP: ventilator-associated pneumonia

SYNOPSIS

Aim	Our aim is to conduct one RCT of personalized immunotherapy in sepsis targeting patients who lie either on the predominantly hyper-inflammatory arm or on the predominantly hypo-inflammatory arm of the spectrum of the host response. These patients will be selected by the use of a panel of biomarkers and laboratory findings and they will be randomly allocated to placebo or immunotherapy treatment according to their needs.
Design	Prospective, multicenter, randomized, controlled trial
Inclusion criteria	• Age equal to or above 18 years • Male or female gender • In case of women, unwillingness to remain pregnant during the study period. • Written informed consent provided by the patient or by one first-degree relative/spouse in case of patients unable to consent • Community-acquired pneumonia or hospital-acquired pneumonia or ventilator-associated pneumonia or primary bacteremia or acute cholangitis. • Sepsis defined by the Sepsis-3 definitions. • Patients with laboratory diagnosis of MALS or hypo-inflammation (immune-paralysis) based on two consecutive blood sampling with 24 hours apart. MALS is defined as the presence of ferritin >4,420 ng/ml and hypo-inflammation as HLA-DR expression on CD14-monocytes (co-expression) less than 30%
Study groups	• **Standard therapy** Patients will receive the standard type of treatment decided by the attending physicians. They will also receive 1ml intravenous (IV) 0.9% saline (N/S) three times daily (every eight hours) for seven days and 1 ml subcutaneous (sc) 1ml 0.9% N/S every other day for a total of 15 days. • **Immunotherapy** Patients will receive the standard type of treatment decided by the attending physicians. They will also receive IV anakinra 200 mg three times daily (every eight hours) or sc rhIFNγ 100 μg once every other day. More precisely, patients randomized for MALS will receive anakinra three times daily (every eight hours) for seven days and sc 1ml N/S 0.9% every other day for 15 days. Patients having hypo-inflammation will receive IV 1ml N/S 0.9% three times daily (every eight hours) for seven days and sc rhIFNγ every other day for 15 days. Especially for patients randomized to anakinra and suffering from renal disease, the following dosage modification will take place: because of lack

	of data from the study in which anakinra was given to patients with septic shock [3] that the drug contributes to kidney dysfunction, anakinra will be given in half dose (ie 100 mg three times daily) to those patients with a creatinine clearance lower than 30 ml/min, as calculated by the Cockcroft Gault equation [(140-age in years)/ (72 χ serum creatinine in mg/dl) for men and multiplied χ 0,85 for women].
Primary study endpoint	The comparative efficacy of the applied immunotherapy versus standard therapy on mortality after 28 days
Secondary study endpoints	• Mortality after 90 days • Time to decrease of SOFA score by more than 50% • Time to infection resolution • Duration of hospitalization • Development of secondary infections • Change of cytokine stimulation between days 0 and 4 and between days 0 and 7 • Change of gene expression between days 0 and 7 • Change of gut microbiome between days 0 and 7 • Epigenetic changes on day 7 • Classification of the immune function of screened patients not characterized with MALS neither with hypo-inflammation The above secondary endpoints will also be analyzed separately to study the specific effect of anakinra and or rhIFNγ.
Power of the study	We anticipate mortality of placebo-treated patients exposed to MALS to be decreased from 60% to 35% and we hypothesize that mortality of cases exposed to hypo-inflammation will be decreased from 50% to 35%. If the true within-stratum odds ratio for disease in exposed subjects relative to unexposed subjects is 0.497, we will need to study 139 cases and 139 controls to be able to reject the null hypothesis that this odds ratio equals 1 with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05. An interim analysis will take place when the first 130 (65 cases and 65 controls) patients will be enrolled. The criterion for this analysis will be the achievement of OR below 0.618 for more than 25% decrease of SOFA score by day 7 with treatment.

BACKGROUND

Sepsis is a life-threatening organ dysfunction that results from the dysregulated host response to an infection[1]. Accumulating knowledge suggests that this dysregulated host response has a broad spectrum where some patients lie to the two extremes of this spectrum whereas the majority of patients lie in between. The first extreme encompasses patients who are dominated from a hyper-inflammatory response to an infectious insult. On the other extreme lie patients who do not have any hyper-inflammatory response; instead these patients are dominated by an exhausted immune response to an infectious stimulus. The remaining patients have features of both hyper- and hypo-inflammatory responses[2].

Randomized clinical trials (RCTs) that have investigated the effects of immunotherapy in sepsis have all failed to establish beneficial effects for the patients. The reasons for that are multiple but one of the most important is the current notion that sepsis is a complex disorder with heterogeneity regarding patient characteristics. Thus, it is necessary to try and find ways to personalise the immunomodulatory treatment of sepsis. In the clinical proposed here, two personalised approached will be investigated.

Some 25 years ago, there were high expectations of the blockade of interleukin (IL)-1 in sepsis using the human recombinant IL-1 receptor antagonist, anakinra. The expectations were based on animal experiments as well as positive results in a single-centre clinical trial. However, in a large international trial, anakinra did not show benefit over placebo. Still, it became clear from this study, enrolling 906 patients that intravenous anakinra was a very safe drug: there was neither excess mortality in these critically ill patients, nor increased susceptibility to secondary infections[3]. In a post-hoc analysis of this trial published in 2016, it was demonstrated that a subgroup of 34 patients showed a clinical picture compatible with macrophage activation syndrome. Since bone marrow was not performed in these patients, we prefer to call this macrophage activation like syndrome (MALS). MALS is a dreaded complication with a mortality rate in the order of 70%[4]. The post-hoc analysis showed that patients receiving anakinra had 30% significant survival benefit compared to those receiving placebo. From these data we can conclude that it is important to

recognize patients with this complication of sepsis and that anakinra might be a beneficial drug.

A survey of the database of sepsis patients in the Hellenic Sepsis Study Group revealed that 5% of the patients with septic shock suffered from MALS. It was found in this study that MAS can be easily and reliably diagnosed by measuring ferritin in the blood. A cut off of 4,420ng/ml had specificity more than 97%.[5]

Another important clinical phenomenon in sepsis is that patients may run into a phase of immunoparalysis. In this situation, the immune cells do not produce any more proinflammatory cytokines and switch to production of anti-inflammatory cytokines such as IL-10; they also loose important functional markers such HLA-DR. Patients with immunoparalysis have a 50% risk of dying in the subsequent 28 days. There is evidence from preclinical studies and from the endotoxin challenge model in human volunteers that immunoparalysis is reversible at least to some extent. The best candidate drug for this would be interferon gamma (IFNγ). Immunosuppression established in healthy volunteers after experimental endotoxemia was reversed after administration of recombinant human interferon-gamma (rhIFNγ)[6]. rhIFNγ was also investigated for this purpose in nine patients at septic shock in a small open-label and non-randomized clinical trial; reversal of immunoparalysis was achieved[7]. The extensive experience with IFNγ teaches that it is a safe drug, the main side effect being fever and flu-like syndrome, which can be mitigated by premedication with a prostaglandin inhibitor like paracetamol. In patients with autoimmune diseases like systemic lupus erythematosus (SLE) and multiple sclerosis flares of the disease induced by IFNγ have been described. So these diseases are contraindications for the drug.

For the present study, we propose to investigate in a randomised placebo-controlled clinical trial with a double-dummy design in patients with septic shock, whether personalised immunotherapy directed against either MALS or immunoparalysis is able to change the perspective for these critically ill patients. We consider MALS as a more direct life-threatening manifestation of sepsis than immunoparalysis. For that reason we will randomise all patients with evidence of MALS for anakinra of placebo, irrespective the state of immunity as measured by HLA-DR positivity.

AIM OF THE STUDY

Our aim is to conduct one RCT of personalized immunotherapy in sepsis targeting patients who lie either on the predominantly hyper-inflammatory arm or on the predominantly hypo-inflammatory arm of the spectrum of the host response. These patients will be selected by the use of a panel of biomarkers and laboratory findings and they will be allocated to placebo or immunotherapy treatment according to their needs.

STUDY DESIGN

This will be a prospective RCT that will take place for 18 months in 13departments of Internal Medicine or Intensive Care Units in Greece. These study sites are recorded at Appendix VI.

The study protocol will be approved by the Ethics Committees of the participating hospitals, by the National Ethics Committee of Greece and by the National Organization for Medicines of Greece. The study will be registered at Clinicaltrials.gov before enrolment of the first patient.

Study population

Patients who meet ALL the following inclusion criteria and who do not meet any of the following exclusion criteria are allowed to be enrolled:

Inclusion criteria

- Age equal to or above 18 years
- Male or female gender
- In case of women, unwillingness to remain pregnant during the study period.
- Written informed consent provided by the patient or by one first-degree relative/spouse in case of patients unable to consent

- Community-acquired pneumonia (CAP) or hospital-acquired pneumonia (HAP) or ventilator-associated pneumonia (VAP) or primary bacteremia (BSI) or acute cholangitis (AC). These are defined[8-11] in Table 1 below.
- Septic shock defined by the Sepsis-3 definitions[1]. The definition is provided in Table 2 below.
- Patients with either signs of MALS or hypo-inflammation as evidenced in Figure 1. MALS and hypo-inflammation are defined in Table 3.

Exclusion criteria

- Age below 18 years
- Denial for written informed consent
- Acute pyelonephritis or intraabdominal infection other than AC, meningitis or skin infection. It is explicitly stated that in the case of a patient with both AC and any other type of intraabdominal infection, the patient cannot be enrolled.
- Any stage IV malignancy
- Any do not resuscitate decision
- In the case of BSI, patients with blood cultures growing coagulase-negative staphylococci or skin commensals or catheter-related infections cannot be enrolled.
- Active tuberculosis (TB) as defined by the co-administration of drugs for the treatment of TB
- Infection by the human immunodeficiency virus (HIV)

- Any primary immunodeficiency
- Oral or IV intake of corticosteroids at a daily dose equal or greater than 0.4 mg prednisone or greater the last 15 days.
- Any anti-cytokine biological treatment the last one month
- Medical history of systemic lupus erythematosus
- Medical history of multiple sclerosis or any other demyelinating disorder.
- Pregnancy or lactation. Women of child-bearing potential will be screened by a urine pregnancy test before inclusion in the study

Screening for eligibility

Once a patient is presenting with at least two of the signs of the systemic inflammatory response syndrome (SIRS) or at least one sign of the quick SOFA, then he (or legal representative in case the patient cannot consent) are been asked for written informed consent (Appendix I). Patients are screened on the basis of the signs of either SIRS or quick SOFA because a retrospective analysis coming from 4517 patients showed them to be complementary[12]. Once the written informed consent is provided, then the patient is screened for eligibility using the following screening steps. No study related procedure will be performed prior obtaining written inform consent form.

- Step 1: The patient is screened for the exclusion criteria. If he meets any of them, he cannot be enrolled. If he does not meet any of them, he remains eligible and screening proceeds to step 2
- Step 2: The patient is screened for the underlying infection. This mandates thorough clinical and laboratory investigation comprising history, vital signs, physical examination, white blood cell counting, biochemistry, blood gasses, microbiology, chest X-ray and abdominal ultrasound and computed tomography if considered necessary. If the patient does not meet the criteria for any of the infections listed in Table 1, he cannot be enrolled. If he meets the criteria for only one of the infections defined in Table 1, he remains eligible and screening proceeds to step 3.
- Step 3: The patient is screened for the definitions of septic shock once all laboratory findings are available. If he does not meet this criterion as defined in Table 2, he cannot be enrolled. If the patient is classified at septic shock, he remains eligible and screening proceeds to step 4.
    - Step 4: 13.5 ml of whole blood is drawn after venipuncture of one forearm vein under aseptic conditions. The blood is poured into three vials; 2.5 ml into one PAXgene tube; 3ml into one pyrogen-and anticoagulant free tube and centrifuged for serum preparation and another 8 ml into one EDTA-coated tube. The second tube is centrifuged for serum preparation. Another venipuncture of 13.5ml is taking place after 24 hours and it is processed as above. Then all the PAXgene tube, serum samples and tubes with EDTA-blood are transported via courier on the same day to the central lab that is the

Research Laboratory of Immunology of Infections of the 4th Department of Internal Medicine at ATTIKON University Hospital. Upon arrival at the lab, ferritin is measured in the supernatant by an enzyme immunosorbent assay within six hours. Ferritin more than 4,420 is diagnostic for MALS. The tube containing EDTA-blood is used for flow cytometry, plasma separation and buffy coat separation. Cells are then analyzed through an FC500 flow cytometer against cells stained with anti-CD45 PC5 and anti-idiotypic IgG1. If the expression of HLA-DR molecules on CD14/CD45 positive cells is less than 30%, the test for hypo-inflammation is positive. Enrolment and allocation to blind treatment is then decided based on the algorithm presented in Table 3. Remaining serum, prepared plasma and buffy coat are kept stored at -800C to be analyzed as described in page 20. From the content of the PAXgene tube, a volume of 0.5 ml of whole blood and 37.5μl of the isolated RNA will be shipped to bioMérieux S.A. facilities located in Grenoble (Centre Christophe Mérieux) for the measurements of CD74 mRNA R-gene, CX3CR1 mRNA R-gene, HPRT1 mRNA R-gene and FilmArrayTMIt is anticipated that only one out of four patients reaching step 4 of screening will eventually be enrolled in the study (Appendix IV).

- Patients screened until Step 4 but not randomized in the study, provided that they remain under need for intravenous administration of vasopressors seven days after the second sampling described above, will be re-screened with two similar blood draws exactly as described above at Step 4 and following the algorithm described in Table 3.

Allocation to blind treatment

Once a patient is considered eligible for enrolment, he will be blindly allocated at 1:1 ratio to one of the following groups of treatment. This allocation takes into consideration the type of infection. For this strata, patients are divided into two groups: those with VAP and those with all other types of infection.

- **Standard therapy** Patients will receive the standard type of treatment decided by the attending physicians. They will also receive 1ml intravenous (IV) 0.9% saline

(N/S) three times daily (every eight hours) for seven days and 1 ml subcutaneous (sc) 1ml 0.9% N/S every other day for a total of 15 days.

- **Immunotherapy** Patients will receive the standard type of treatment decided by the attending physicians. They will also receive IV anakinra 200 mg three times daily (every eight hours) or sc rhIFNγ 100 μg once every other day. More precisely, patients randomized for MALS will receive anakinra three times daily (every eight hours) for seven days and sc 1ml N/S 0.9% every other day for 15 days. Patients having hypo-inflammation will receive IV 1ml N/S 0.9% three times daily (every eight hours) for seven days and sc rhIFNγ every other day for 15 days. Especially for patients randomized to anakinra and suffering from renal disease, the following dosage modification will take place: because of lack of data from the study in which anakinra was given to patients with septic shock [3] that the drug contributes to kidney dysfunction, anakinra will be given in half dose (ie 100 mg three times daily) to those patients with a creatinine clearance lower than 30 ml/min, as calculated by the Cockcroft Gault equation [(140-age in years)/ (72 χ serum creatinine in mg/dl) for men and multiplied χ 0,85 for women].

Table 1 Definitions of eligible infections

Infection	All the following	At least 2 of the following	At least 1 of the following
AC[8]	• Pain at the right upper quadrant • Fever (tympanic or oral temperature ≥38^{0}C, rectal ≥38.3^{0}C)	None	Consistent ultrasound or CT findings
BSI[8]	• At least 1 positive blood culture • Failure to identify a primary infection site despite thorough clinical and radiology investigation	None	None
CAP[9]	New or evolving infiltrate on chest X-ray	• New onset or worsening of cough • Dyspnea • Auscultatory findings consistent with pulmonary consolidation	• PCT ≥0.25 ng/ml • Hypoxemia pO2≤60mmHg or oxygen saturation ≤90% in room air • Respiratory rate ≥20 breaths/min
HAP[10]	• Onset >48 hours from hospital admission • New or evolving infiltrate on chest X-ray	• New onset or worsening of cough or dyspnea • Purulent tracheobronhchial secretions • Auscultatory findings consistent with pulmonary consolidation	• PCT ≥0.25 ng/ml • Hypoxemia pO2≤60mmHg or oxygen saturation ≤90% in room air • Respiratory rate ≥20 breaths/min
VAP[10,11]	• Onset >48 hours from start of mechanical ventilation • New or evolving infiltrate on chest X-ray	• Purulent tracheobronhchial secretions • Auscultatory findings consistent with pulmonary consolidation	• PCT ≥0.25 ng/ml • Clinical pulmonary infection score (CPIS)* ≥6 (see Appendix II)

Table 2 Definition of sepsis according to the Sepsis-3[1]

- Total SOFA score of 2 or more points for patients who are admitted with infection at the emergency department OR
- Increase of admission SOFA by 2 or more points for patients already hospitalized

AND ALL THE FOLLOWING:

- Mean arterial pressure less than 65 mmHg
- Treatment with nor-epinephrine or other vasopressor
- Plasma lactate more than 2 mmol/l

Calculation of SOFA score is provided in the Appendix II.

Table 3 Combinations of immunotests guiding randomization

First sample								
Ferritin (ng/ml)	>4,420	≤4,420	>4,420	>4,420	≤4,420	≤4,420	≤4,420	≤4,420
HLA-DR (%) on CD45/CD14	≥30	≥30	≥30	<30	<30	≥30	<30	≥30
Second sample after 24 hours								
Ferritin (ng/ml)	>4,420	>4,420	>4,420	>4,420	>4,420	≤4,420	≤4,420	≤4,420
HLA-DR (%) on CD45/CD14	≥30	≥30	≤30	<30	<30	≥30	<30	<30
ACTION	MALS RANDOMIZE				NOT RANDOMIZE		HYPOINFLAMMATION RANDOMIZE	

Abbreviations MALS: macrophage activation-like syndrome; >: more than; <: less than; ≤: less than or equal to

Study drug

The active study drug i.e. anakinra and rhIFNγ and placebo will be provided to each site in the form of pre-filled ready-to-use syringes by the Sponsor. All study drugs need to be stored at 2-8^{0}C at the study site at a refrigerator with recording of temperature. In case recording indicates deviation of temperature below 0^{0}C or above 10^{0}C for more than a day, stored drugs need to be replaced by the Sponsor. The unblinded site designee will prepare the test article according to instructions provided at IB. Syringes with active drug or placebo will be covered to conceal the identity of the test article. Covering materials will be provided by Sponsor. At the exterior of each syringe after coverage will be placed a label with a letter and a 6-digit number. The letter refers to the study site, the first two numbers of the digit refer to the serial number of enrolled patient at the respective study site, the middle two numbers of the digit refer to the day of treatment and the last two numbers of the digit refer to the time of treatment. For example, the digit A010202 refers to study site A, patient number 01 at that study site, treatment day 2 and second injection for that day. The unblinded designee will provide the covered syringes to the blinded nurse or blinded investigator who will administer the infusion.

The randomization sequence will be generated for each study site. It will comprise 1:1 allocation into standard therapy and immunotherapy but it will also comprise one 1:4 allocation within each group into (anakinra and respective placebo) or (rhIFNγ and respective placebo) respectively.

Patients' visits and interventions (Appendix V)

Day 1

This visit will take place on the morning of the day of the start of treatment with the study drug. The following procedures will be done on that day:

- 19.5 ml of blood will be collected either from venipuncture of an antecubital vein or directly from a central vein catheter under sterile conditions and distributed as follows: a) 2.5ml into one PAXgene tube to be used for transcriptomic analysis; b) 7ml into one sterile and pyrogen-free tube for serum isolation and cell pellet for DNA isolation; and c) 10 ml into one EDTA-coated tube for isolation of peripheral blood mononuclear cells (PBMCs) and cytokine stimulation, plasma separation, buffy coat separation and flow

cytometry for anti-CD14 FITC, anti-HLA-DR-PE and anti-CD45 PC5 and Quantibrite HLA-DR/anti-monocyte PerCP-Cy5.5. This will be done before the start of blind treatment.

- 1-2ml of stool by using a rectal swab will be collected into one sterile and pyrogen-free tube to be used for microbiome analysis. This will be done before the start of blind treatment.
- Administration of study drug
- Recording of co-morbidities, co-administered drugs, past-history, SOFA score, vital signs, absolute blood cell count (if available) and biochemistry (if available)

Day 2

This visit will take place on the morning of the second day from the start of treatment with the study drug. The following procedures will be done on that day:

- Administration of study drug
- Recording of co-administered drugs, SOFA score, vital signs, absolute blood cell count (if available), biochemistry (if available), microbiology and antibiogram (if available)

Day 3

This visit will take place on the morning of the third day from the start of treatment with the study drug. The following procedures will be done on that day:

- Administration of study drug
- Recording of co-administered drugs, SOFA score, vital signs, absolute blood cell count (if available), biochemistry (if available), microbiology and antibiogram (if available)

Day 4

This visit will take place on the morning of the fourth day from the start of treatment with the study drug. The following procedures will be done on that day:

- Administration of study drug
- Recording of co-administered drugs, SOFA score, vital signs, absolute blood cell count (if available), biochemistry (if available), microbiology and antibiogram (if available)

- 15 ml of blood will be collected either from venipuncture of an antecubital vein or directly from a central vein catheter under sterile conditions and distributed as follows: a) 5ml into one sterile and pyrogen-free tube for serum isolation; and b) 10 ml into one EDTA-coated tube for isolation of peripheral blood mononuclear cells (PBMCs) and cytokine stimulation, plasma separation, buffy coat separation and flow cytometry for anti-CD14 FITC, anti-HLA-DR-PE and anti-CD45 PC5 and Quantibrite HLA-DR/anti-monocyte PerCP-Cy5.5. In the case the patient is treated with any of meropenem, tigecycline or colistin, drug concentrations will be measured in plasma.

Day 5

This visit will take place on the morning of the fifth day from the start of treatment with the study drug. The following procedures will be done on that day:

- Administration of study drug
- Recording of co-administered drugs, SOFA score, vital signs, absolute blood cell count (if available), biochemistry (if available), microbiology and antibiogram (if available)

Day 6

This visit will take place on the morning of the sixth day from the start of treatment with the study drug. The following procedures will be done on that day:

- Administration of study drug
- Recording of co-administered drugs, SOFA score, vital signs, absolute blood cell count (if available), biochemistry (if available), microbiology and antibiogram (if available)

Day 7

This visit will take place on the morning of the seventh day from the start of treatment with the study drug. This is also the last day of administration of the study drug. The following procedures will be done on that day:

- Administration of study drug

- Recording of co-administered drugs, SOFA score, vital signs, absolute blood cell count (if available), biochemistry (if available), microbiology and antibiogram (if available)
- Clinical state of the infection
- 17.5 ml of blood will be collected either from venipuncture of an antecubital vein or directly from a central vein catheter under sterile conditions and distributed as follows: a) 2.5ml into on PAXgene tube to be used for transcriptomic analysis; b) 4ml into one sterile and pyrogen-free tube for serum isolation; c) 10 ml into one EDTA-coated tube for isolation of peripheral blood mononuclear cells (PBMCs) and cytokine stimulation, plasma separation, buffy coat separation and flow cytometry for anti-CD14 FITC, anti-HLA-DR-PE and anti-CD45 PC5 and Quantibrite HLA-DR/anti-monocyte PerCP-Cy5.5. In the case the patient is treated with any of meropenem, tigecycline or colistin, drug concentrations will be measured in plasma.1-2ml of stool by using a rectal swab will be collected into one sterile and pyrogen-free tube to be used for microbiome analysis

Days 8-28

These visits will take place on the morning of days 8-28 until day 28. The following procedures will be done on each day:

- Recording of co-administered drugs, SOFA score, vital signs, microbiology and antibiogram (if available)
- Clinical state of the infection
- Survival state
- Administration of study drug on days 9, 11, 13 and 15

The clinical state of the infection on any of the evaluable days will be considered as[13]:

- Resolved, if all signs of infection leading to study enrollment have been resolved on that day
- Failure, if infection persists or relapses
- Superinfection, if a new infection due to a new pathogen emerges
- Intermediate, if the outcome cannot be classified in one of the above categories.

LABORATORY PROCEDURES

Isolation of PBMCs

PBMCs will be isolated after gradient centrifugation of whole blood over Ficoll. After serial washing, counting and exclusion of dead cells, they will be stimulated for 24 hours and for five days with LPS, phytohemagglutin and heat-killed *Candida albicans* for the production of TNFα, IL-1β, IL-6, IL-10, IL-17, IL-22 and IFNγ.

A total of 2 x 10^6 PBMCs will be used for the isolation of CD14(+) cells using magnetic beads. After cell centrifugation, the cells will be stored in specific medium for either RNA and for ATAC-sequencing for chromatin accessibility for epigenetic assessment.

Microbiome, epigenome, genome, transcriptome and metabolome analysis

Stored PAXgenes, serum and plasma samples and buffy coats coming from screened patients who will not be enrolled in the study will be analyzed for transcriptome, epigenome and cytokines in both the Research Laboratory of Immunology of Infections of the 4th Department of Internal Medicine at ATTIKON University Hospital and in Department of Internal Medicine at Radboud University Medical Center, Nijmegen, The Netherlands. Stored PAXgenes, DNA, serum and plasma samples, buffy coats, supernatants of PBMCs and stool coming from enrolled patients will be analyzed for transcriptome, microbiome, metabolome, epigenome and cytokines in both the Research Laboratory of Immunology of Infections of the 4th Department of Internal Medicine at ATTIKON University Hospital and in Department of Internal Medicine at Radboud University Medical Center, Nijmegen, The Netherlands. Measurements of drug levels of meropenem, tigecycline and colistin will be done at the Research Laboratory of Immunology of Infections of the 4th Department of Internal Medicine at ATTIKON University Hospital. From the content of the PAXgene tubes collected from both screened and enrolled patients, a volume of 0.5 ml of whole blood and 37.5μl of the isolated RNA will be shipped to bioMérieux S.A. facilities located in Grenoble (Centre Christophe Mérieux) for the measurements of CD74 mRNA R-gene, CX3CR1 mRNA R-gene, HPRT1 mRNA R-gene and FilmArrayTM

STUDY ENDPOINTS

The primary study endpoint will be the comparative efficacy of the applied immunotherapy versus standard therapy on mortality after 28 days

The secondary study endpoints will be the efficacy of the applied immunotherapy versus standard therapy on each of the following:

- Mortality after 90 days
- Time to decrease of SOFA score by more than 50%
- Time to infection resolution
- Duration of hospitalization
- Development of secondary infections
- Change of cytokine stimulation between days 0 and 4 and between days 0 and 7
- Change of gene expression between days 0 and 7
- Change of gut microbiome between days 0 and 7
- Epigenetic changes on day 7
- Classification of the immune function of screened patients not characterized with MALS neither with hypo-inflammation

The above secondary endpoints will also be analyzed separately to study the effect of anakinra and or rhIFNγ. Subgroup analysis will also be done for patients who have circulating through drug levels of meropenem, tigecycline and colistin within the international acceptable therapeutic range.

POWER OF THE STUDY

The study is powered for the primary endpoint. According to this, we are planning a study of cases and controls who will be divided into 2 strata with 20% of cases selected from stratum 1 (MAS), and 80% of cases selected from stratum 2 (hypo-inflammation). We will select 1 control(s) per case in stratum 1, and 1 control(s) per case in stratum 2. We anticipate that 60% of controls exposed to stratum 1 will die, and 50% of controls exposed to stratum 2 will die. We anticipate mortality of cases exposed to stratum 1 to be decreased to 35% as described by others[4] and we hypothesize that mortality of cases exposed to stratum 2 will be decreased to 35%. If the true within-stratum odds ratio for mortality in treatment-

exposed subjects relative to unexposed subjects is 0.497, we will need to study 110 cases and 110 controls to be able to reject the null hypothesis that this odds ratio equals 1 with probability (power) 0.8. The Type I error probability associated with this test of this null hypothesis is 0.05. We will use a continuity-corrected Mantel-Haenszel chi-squared statistic to evaluate this null hypothesis. Taking into consideration the risk of drop-outs and missing data, 139 patients will be allocated into each arm of blind treatment i.e. 278 patients in total.

STATISTICAL ANALYSIS AND INTERIM ANALYSIS

The primary endpoint will be compared between the two groups by the Fisher exact test and by the log-rank test. Secondary endpoints will be compared between the two groups by the Fisher exact test for quantitative variables and by non-parametric statistics for qualitative variables. The time to an event will be compared between the two groups by the log rank test. Any p-value below 0.05 will be considered significant.

An interim analysis is planned when the first 130 patients are enrolled. This analysis will be done by an independent board of three investigators. At that interim analysis, patients will be analyzed only for the change of SOFA score from the baseline. That that analysis, the odds ratio for decrease of SOFA on day 7 more than 30% among patients allocated to the immunotherapy arm compared to patients allocated to the placebo treatment will be calculated. If this is below 0.618, then the study will continue as planned.

ADVERSE EVENTS

Adverse events (AEs) and Serious Adverse Events (SAEs) will be collected from baseline until the last patient’s evaluation. Investigators should monitor subjects for adverse events and are responsible for recording ALL adverse events and adverse reactions occurring to a patient during the trial. Mortality will not be reported as an SAE since this is the study primary endpoint.

An adverse event is any undesirable and unintended medical occurrence in a subject administered a pharmaceutical product and which does not necessarily have a causal relationship with this treatment. The adverse event may be a sign, a symptom or an abnormal laboratory finding.

An adverse reaction is any undesirable and unintended reaction due to investigational medicine product administration, related with any dose administrated. The time relationship is defined from the moment the AE occurs during therapeutic treatment until 30 days or 5 half-lives after treatment discontinuation.

If an adverse event/adverse reaction meets any of the following criteria, it is considered SAE:

- **Life-threatening situation** The subject was at risk of death at the time of the adverse event/experience. It does not refer to the hypothetical risk of death if the adverse event/adverse reaction were more severe or were to progress.
- **Inpatient hospitalization** or prolongation of existing hospitalization.
- **Persistent or significant disability/incapacity** Any AE having an outcome that is associated with a substantial disruption of the ability to carry out normal life functions, including the ability to work. This is not intended to include transient interruption of daily activities.
- **Congenital anomaly/birth defects** Any structural abnormality in subject's offspring that occurs after intrauterine exposure to treatment.
- **Important medical events/experiences** that may not result in death, be life-threatening, or require hospitalization may be considered a serious adverse event/experience when, based upon appropriate medical judgment, **they may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above,** i.e., death, a life-threatening adverse event/experience, inpatient hospitalization or prolongation of existing hospitalization, a persistent or significant disability/incapacity, or a congenital anomaly/birth defect. Examples of such medical events/experiences include allergic bronchospasm requiring intensive treatment in an emergency room or at home, blood dyscrasias or convulsions that do not result in inpatient hospitalization, or the development of drug dependency or drug abuse.
- **Spontaneous and elective abortions** experienced by study subject.

Grading of severity

The severity of the adverse events shall be graded as:

- **Mild** the adverse event/reaction is transient and well tolerated by the patient

- **Moderate** the adverse events/reactions causes discomfort and affects the usual activities of the patient.
- **Severe** the adverse events/reactions affects the usual activities of the patient to an important degree and may cause disability or be life-threatening.

Relationship to the drug

The investigator will use the following definitions to assess the relationship of the adverse event to study drug:

- **Probably Related**: The adverse event has a strong time relationship to the drug or relapses if re-induced, and another etiology is improbable or clearly less probable.
- **Possibly Related**: The adverse event has a strong time relationship to the drug and an alternative aetiology is as probable or less probable.
- **Probably not Related**: The adverse event has a slight or no time relationship to the drug and/or there is a more probable alternative aetiology.
- **Unrelated**: The adverse event is due to an underlying or concomitant disease or to another pharmaceutical product and is not related to the drug (no time relationship and a much more probable alternative aetiology).

If an investigator's opinion of possibly related, probably not related or not related to study drug is given, an alternate etiology must be provided by the investigator. Please note that a severe adverse event/experience is not necessarily serious, as the term severe is a measure of intensity while a serious adverse event is determined based on the aforementioned regulatory criteria. Individual un-blinding thought to be necessary for the management of an adverse event will be documented in the subject Case Report Form.

All Investigator must report every adverse event and evaluate the severity and possible causality with the study drug according to aforementioned criteria. All adverse events/reactions are reported to Sponsor. The sponsor is responsible for evaluation of all AEs. All Serious Adverse Events/ Serious Adverse Reactions must be reported within 24 hours by completion of the SAE and faxing to Hellenic Institute for the Study of Sepsis.

The Sponsor must evaluate whether an adverse event is expected or not. A SAE may qualify for expedited reporting to regulatory authorities if it is determined to

be a suspected, unexpected serious adverse reaction (SUSAR). The Sponsor is responsible for submitting expedited safety reports to the appropriate regulatory agency for all confirmed SUSARs. In the case of a fatal or life-threatening SUSAR, the Sponsor will notify the appropriate regulatory agency as soon as possible but in no case later than 7 calendar days after The Sponsor's initial receipt of the information. For a non-life-threatening SUSAR, the report will be submitted no later than 15 days after the Sponsor is made aware of the event.

The Sponsor has the obligation to submit annually a drug safety updated report (DSUR) according to global experience to appropriate regulatory authorities. The electronic submission to Eudravigilance will be performed through the Organisation ID: HISS.

The above pharmacovigilance procedures will be performed for the Sponsor (Hellenic Institute for the Study of Sepsis) by the Consultant Company «SUSTCHEM Engineering P.Braimiotis-P. Scarlatos LTD», 144 3rd Septemvriou str, 11251, Athens, and the Qualified Person for Pharmacovigilance (QPPV) will be Mrs Areti Voulomenou, Chemical Engineer (contact details in Appendix VI).

QUALITY CONTROL AND ASSURANCE

Quality control and assurance checks are performed by sponsor in order to allow periodic review of adequacy of the study activities and practices and allow for revising for revising such practices as needed so the data and process are maintained, the study meets the protocol and procedural requirements, and is reproducible.

Before enrolling any subject in this study, sponsor personnel and the investigator review the protocol, the IB, The CRFs and instructions for their completion, the procedure for obtaining inform consent, and the procedure for reporting AEs and SAEs.

A qualified representative of the sponsor monitors the conduct of the study by visiting the site and by conduct of the study by visiting the site and by contacting the site by telephone and e-mail. During these site visits, all source documents are reviewed and information recorded in the CRFs is verified against them.

Beside routine monitoring quality assurance will be documented through independent auditing of the quality control activities and where applicable, by regulatory authorities through inspections.

ETHICAL CONSIDERATIONS

Prior to the initiation of this study, the study design will receive ethical, scientific, and where applicable, regulatory review. Investigators will conduct this study in accordance with the principles of the Declaration of Helsinki, GCP, and applicable regulatory requirements.

Regarding Inform Consent Form obtaining procedures, any procedures before any procedures specified in the protocol are performed, a subject must:
Be informed of all pertinent aspects of the study and all elements of inform consent
Be given time to ask questions and tome to consider the decision to participate
Voluntarily agree to participate in the study
Sign and date the updated and approved by IRB/IEC/REB ICF version.

PROTOCOL ADHERENCE AND AMENDMENTS

Investigators ascertain that they will apply due dilligence to avoid protocol deviations. All significant protocol deviations will be recorded and reported in the clinical study report (CSR). Any change or addition to the protocol can only be made in a written protocol amendment that must be approved and signed by the sponsor, health authorities where required, and the IRB/IEC/REB.

REFERENCES

1. Singer M, Deutschman CS, Seymour CW, Shankar-Hari M, Annane D, Bauer M, et al. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis-3). *JAMA* 2016; 315: 801-810
2. Hotchkiss RS, Monneret G, Payen D. Sepsis-induced immunosuppression: from cellular dysfunctions to immunotherapy. *Nat Rev Immunol* 2013; 13: 862-874
3. Opal SM, Fisher CJ Jr, Dhainaut JF, Vincent JL, Brase R, Lowry SF, et al. Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: a phase III, randomized, double-blind, placebo-controlled, multicenter trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group. Crit Care Med 1997; 25: 1115-1122
4. Shakoory B, Carcillo JA, Chatham WW, Amdur RL, Zhao H, Dinarello CA, et al. Interleukin-1 receptor blockade is associated with reduced mortality in sepsis patients with features of macrophage activation syndrome: reanalysis of a prior phase III trial. *Crit Care Med* 2016; 44: 275-281
5. Kyriazopoulou E, Leventogiannis K, Norrby-Teglund A, Dimopoulos G, Pantazi A, Orfanos S, et al. Macrophage activation-like syndrome: an immunological entity associated with rapid progression to death in sepsis. *BMC Med* [in press]
6. Leentjens J, Kox M, Koch RM, Preijers F, Joosten LA, van der Hoeven JG, et al. Reversal of immunoparalysis in humans in vivo: a double-blind, placebo-controlled, randomized pilot study. *Am J Respir Crit Care Med* 2012; 186: 838-845
7. Döcke WD, Randow F, Syrbe U, Krausch D, Asadullah K, Reinke P, et al. Monocyte deactivation in septic patients: restoration by IFN-γ treatment. Nat Med 1997; 3: 678-81
8. Calandra T, Cohen J. The international sepsis forum consensus conference on definitions of infection in the intensive care unit. *Crit Care Med* 2005; 33: 1538-1548
9. Christ-Crain M, Jaccard-Stolz D, Bingisser R, Müller C, Miedinger D, Huber PR, et al. Effect of procalcitonin-guided treatment on antibiotic use and outcome in lower respiratory tract infections: cluster-randomized, single-blinded intervention trial. Lancet 2004; 363: 600-607
10. Kalil A, Metersky M, Klompas M. Management of Adults With Hospital-acquired and Ventilator-associated Pneumonia: 2016 Clinical Practice Guidelines by the

Infectious Diseases Society of America and the American Thoracic Society. *Clin Infect Dis* 2016; 63: 1-51

11. Zilberberg M, Shorr A. Ventilator-Associated Pneumonia: The Clinical Pulmonary Infection Score as a surrogate for diagnostics and outcome. *Clin Infect Dis* 2010; 51: 131-135
12. Giamarellos-Bourboulis EJ, Tsaganos T, Tsangaris I, Lada M, Routsi C, Sinapidis D, et al. Validation of the new sepsis-3 definitions: proposal for improvement in early risk identification. *Clin Microbiol Infect* 2016; E-pub Nov 14
13. Pontikis K, Karaiskos I, Bastani S, Dimopoulos G, Kalogirou M, Katsiari M, et al. Outcomes of critically ill intensive care unit patients treated with fosfomycin for infections due to pandrug-resistant and extensively drug-resistant carbapenemase-producing Gram-negative bacteria. *Int J Antimicrob Agents* 2014; 43: 52-59

APPENDIX I The criteria of SIRS and of quick SOFA to be used for the screening of the patients

Written informed consent is asked for screening for eligibility among patients who have either SIRS criteria or quick SOFA criteria or both

Criteria of SIRS

A patient has to meet at least two of the following:

- Core temperature >38^0C or <36^0C
- Heart rate >90 beats/minute
- Breath rate >20 breaths/minute or p_{co2}<32 mmHg
- Total white blood cell count >12,000/mm^3 or <4,000/mm^3 or >15% bands

Criteria of quick SOFA score

A patient has to meet at least two of the following:

- Breath rate >22 breaths/minute
- Altered mental state defined as Glasgow Coma Scale less than 14
- Systolic blood pressure <100mmHg

APPENDIX II The SOFA score[1]

Variable	**0 points**	**1 point**	**2 points**	**3 points**	**4 points**
PaO_2/FiO_2(mmHg)	≥400	<400	<300	<200	<100
Platelets (per mm^3)	≥150	<150	<100	<50	<20
Hypotension	MAP≥ 70 mmHg	MAP<70 mmHg	Dobutamine whatever dose	Adrenaline ≤0.1* or Noradrenaline≤ 0.1*	Adrenaline>0.1* or Noradrenaline >0.1*
Glasgow Coma Scale	15	13-14	10-12	6-9	<6
Bilirubin (mg/dl)	<1.2	1.2-1.9	2.0-5.9	6.0-11.9	≥12
Creatinine (mg/dl) or Urine output	<1.2	1.2-1.9	2.0-3.4	35-4.9 or <500ml/day	≥5.0 or <200ml/day

*μg/kg/min

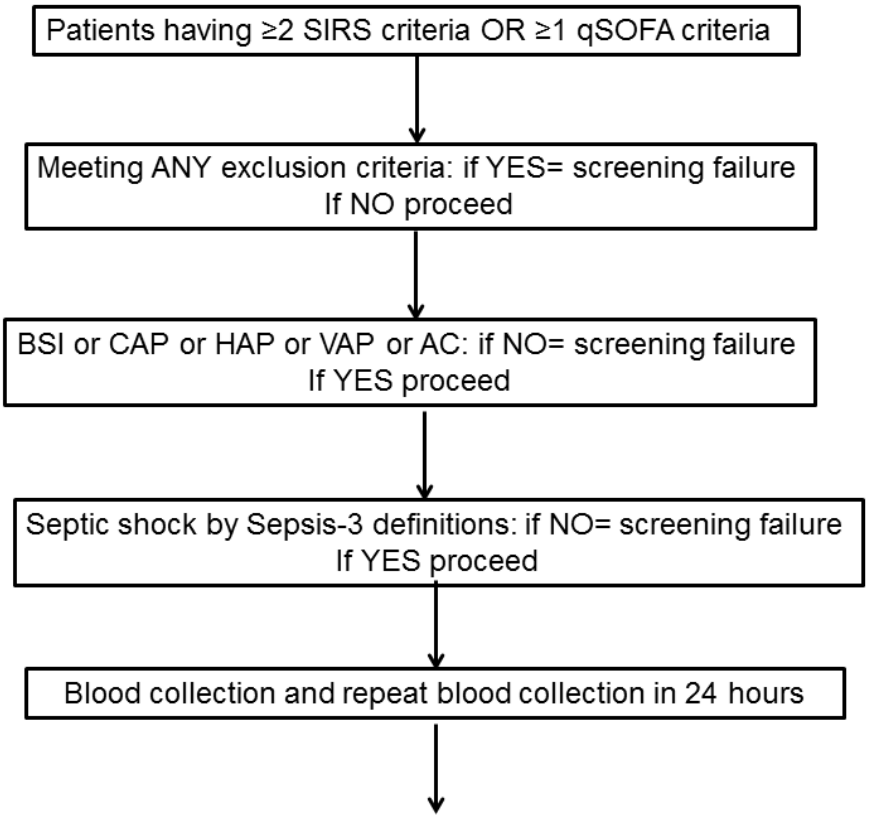
Each variable is scored between 0 and 4. The SOFA score is the sum of the score of each variable

APPENDIX III The clinical pulmonary infection score (CPIS)[10]

Variable	**0 points**	**1 point**	**2 points**
Tracheal secretions	Rare	Abundant	Purulent
Leukocyte count (/mm³)	>4.000 and <11.000	<4.000 and >11.000	<4.000 or >11.000+band forms
Temperature (ºC)	>36.5 and <38.4	>38.5 and <38.9	>39 or <36
pO_2/FiO_2 ratio (mmHg)	>240 or ARDS	-	≤240 and no ARDS
Chest radiograph	No infiltrate	Diffuse infiltrate	Localised infiltrate
Culture of tracheal aspirate	Negative	-	Positive

Each variable is scored between 0 and 2. The CPIS is the sum of the score of each variable

APPENDIX IV Screening procedure of patients until study randomization



First blood sample								
Ferritin	↑	=	↑	↑	=	=	=	=
HLA-DR	=	=	=	↓	↓	=	↓	=
Second blood sample								
Ferritin	↑	↑	↑	↑	↑	=	=	=
HLA-DR	=	=	↓	↓	↓	=	↓	↓
	MALS RANDOMIZE				NOT RANDOMIZE		HYPOINFLAMMATION RANDOMIZE	

Abbreviations MALS: macrophage activation-like syndrome

For ferritin ↑: >4,420 ng/ml; =: ≤ 4,420 ng/ml

For HLA-DR on CD45/CD14 cells =: ≥30%; ↓: <30%

APPENDIX V Study visits

	Study visits																											
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**	**17**	**18**	**19**	**20**	**21**	**22**	**23**	**24**	**25**	**26**	**27**	**28**
Obtain ICF	X																												
Study drug		x	x	X	x	x	x	x		x		x		x		x													
SOFA scoring		x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Clinical information		x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Clinical state of infection								x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Survival		x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Vital signs		x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Microbiology		x	x	X	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x	x
Blood collection		x		X				x																					
Stool collection		x		X				x																					

APPENDIX VI Participating Sites

- 4th Department of Internal Medicine, ATTIKON University Hospital, Athens
- 2nd Department of Critical Care Medicine, ATTIKON University Hospital, Athens
- 1st Department of Pulmonary Medicine and Intensive Care Unit, Sotiria General Hospital, Athens
- Department of Internal Medicine, Patras University Hospital, Rion
- Department of Internal Medicine, Larissa University Hospital
- Intensive Care Unit, “KAT” General Hospital of Attika, Athens
- Intensive Care Unit, Ioannina University Hospital
- Intensive Care Unit, Alexandroupolis University Hospital
- Intensive Care Unit, “G.Gennimatas” Thessaloniki General Hospital
- Intensive Care Unit, “Aghios Dimitrios” Thessaloniki General Hospital
- Intensive Care Unit, “Tzanio” Piraeus General Hospital
- Intensive Care Unit, Koutlimbaneio & Triantafylleio Larissa General Hospital
- Latsio Intensive Care Unit, “Thriasio” Elefsis General Hospital
- 5th Department of Internal Medicine, “Evangelismos” General Hospital of Athens

Monitor of the study as assigned by the Sponsor is Mrs Kotsaki Antigoni, MD, PhD
e-mail: antigonebut@yahoo.com, tel. number: 00306946637164, 21058312562

QPPV of the study as assigned by the Sponsor is Mrs Areti Voulomenou, MSc,
e-mail: voulomenou@suschem.gr; tel. number: 00302108252510