Study Protocol.

Title of the study: Granzyme A in patients with *E. coli* Bacteremic Urinary Tract Infections (GABEC)

Updated on June, the 5th, 2019.

1. Background

Sepsis is a high incidence, high morbidity and mortality, life-threatening organ dysfunction caused by a dysregulated response to infection that constitutes a public health priority. Improving sepsis diagnosis and finding pathogenically-relevant targets for pathophysiological therapy are opportunities to reduce sepsis burden.

Despite not being critical to contain microorganisms, granzyme A (GZM A), a serine protease enzyme that is present in cytotoxic T lymphocyte granulesmight be a pathogenically relevant sepsis mediator. We found that GzmA knocked-out mice had significantly prolonged survival as compared with wild-type mice in an induced polymicrobial peritonitis (cecal ligation and puncture) murine model (Arias et al., 2018. Unpublished data). To the best of our knowledge, the pathogenic role of GZM A in humans with sepsis has been barely explored.

In order to ascertain the pathogenic role of GZM A in sepsis we designed a nested case-control study in patients with bacteremic urinary tract infections caused by *E. coli* This human model of infection-sepsis homogenizes other factors (patient, infection and microorganism related factors) that could be pathogenically associated with sepsis in order to minimize confounding.

2. Research hypothesis

The research team has explored the role of GZM A

- **Conceptual hypothesis:**
  - Granzyme A is a pathogenic sepsis mediator.
  - Granzyme A gene polymorphisms determine the serum concentration of GZM A in patients with systemic infections.
  - Granzyme A gene polymorphisms determine the risk of sepsis among patients with systemic infections.

- **Operational hypothesis**:
  - Among patients with bacteremic (*E. coli*) urinary tract infections (UTIs), GZM A levels are significantly higher in those patients who develop sepsis as compared with those who do not develop sepsis.
  - There are significant differences in the GZM A gene polymorphism profile of patients with bacteremic (*E. coli*) UTIs who develop sepsis as compared with those who do not develop sepsis.

3. Aims and objectives

3.1. Aims

1. To assess the pathogenic role of GZM A in sepsis in patients with bacteremic (*E. coli*) UTIs.
2. To explore the capability of GZM A polymorphisms to anticipate the risk of

developing sepsis in patients with bacteremic (*E. coli*) UTIs.

3. To evaluate the potential usefulness of GZM A as a diagnostic biomarker of sepsis in patients with bacteremic (E. coli) UTIs.
4. To characterize *E. coli* "virulome" among circulating uropathogenic strains.

3.2. Objectives

1. To evaluate the correlation between serum levels of GZM A and systemic inflammatory response in patients with bacteremic (*E. coli*) UTIs.
2. To characterize GZM A gene polymorphisms among patients with bacteremic (E. coli) UTIs
3. To assess GZM A serum kinetics among patients with bacteremic (*E. coli*) UTIs
4. To phenotypically and molecularly characterize *E. coli* strains causing bacteremic UTIs, including their virulence factors ("virulome").

Expected outcomes. Relevance and transformative approach of the proposal.

1. **Characterization of the pathogenic role of GZM A in sepsis in patients with systemic infections**

1.1. Evaluation of the potential association between GZM A serum levels and sepsis.

- The association between GZM A serum levels and the extent of systemic inflammatory response is a non-sufficient, albeit necessary condition to establish a pathogenic (causal) relationship between GZM A and sepsis.

1.2. Identification of gzmA gen polymorphisms associated with an increased risk of developing sepsis among patients with infections:

- Significant differences in serum GZM A concentrations between patients with and without sepsis would suggest a potential pathogenic role of GZM A.
- If certain gzmA gene polymorphisms are associated with lower sepsis risk through lower GZM A serum levels, gzmA based sepsis risk could be estimated so that certain decisions could be personalized.
- GZM A could be a new potential target for pathophysiological therapy of sepsis.

2. **Characterization of GZM A as a sepsis biomarker in a human model of infection-sepsis.**

2.1. Evaluation of GZM A kinetics in the aforementioned human model of infection-sepsis:

- If the GZM A serum level is found to be sensitive and specific enough, determination of GZM A could assist diagnostic and therapeutic decisions in patients with suspected sepsis, which indeed is a difficult-to-diagnose entity due to its common non-specific presentation.
- The correlation between serum concentration of GZM A and the clinical course

of the infection could assist therapeutic decisions, such as antibiotic streamlining, the switch from sequential intravenous to oral antibiotics and establishing the optimal duration of antibiotic therapy.

3. **Phenotypical and molecular characterization of uropathogenic *E. coli* causing bloodstream infections:**
   - Phenotypic and molecular characterization of *E. coli* isolates involved in bacteremic UTIs has a great epidemiological interest
   - Identification of patients infected with hypervirulent strains could potentially influence the monitoring and therapeutic approach.

- Discrimination of *E. coli* isolates according to their virulence (virulome)/potential pathogenicity is important to exclude strain-specific factors, when trying to establish the causal/pathogenic relationship between GZM A and sepsis.

4. Methods

4.1. Design and project scope

- Prospective, exploratory nested case-control study to be conducted at one academic hospital (Hospital Clínico Universitario Lozano Blesa) affiliated with the Instituto de Investigación Sanitaria Aragón (IIS Aragón).

4.2. Study period: June 2019 – December 2020.

4.3. Patients and sample size

Inclusion criteria (To meet all):

- Age≥ 18 years old.
- *E. coli* bloodstream infection
- Urinary source. Urinary source should be considered if (any)
  - Urinary source is clinically suspected and both *E. coli* isolates (in blood and in urine culture) share the same phenotype (antibiogram).
  - Urinary source/origin is clinically suspected, and the urine culture is negative, but the patient had received at least one dose of any systemic antibiotic with antimicrobial activity against the *E. coli* strain causing the BSI before blood cultures were obtained.
  - Both isolates (in blood and in urine culture) share the same phenotype (antibiogram) and there is no alternative source.

Exclusion criteria:

- Use of systemic antibiotics for >48h in the two months preeeding the episode.

- Immunocompromised hosts
  - Patients receiving systemic steroid use (>10 mg prednisone/day during 10 or more days in the previous 2 months).
  - Patients receiving biological therapy (previous 2 months).
  - Active solid or hematological cancer.
  - HIV +.
  - Neutropenia < 500 PMN/microl.

- Basal urinary tract abnormalities or locally modified vesical microbiome (any):
  - Ureteroileostomy, ureterosigmoidostomy, ureterostomy (Bricker) or nephrostomy.
  - Indwelling urinary catheter (in the last two months)
  - Urological surgery (in the last two months).
  - Intravesical chemotherapy (in the last two months).
  - Intravesical BCG instillation (in the last two months).

Potential candidates will be detected daily by the microbiologists on the research team. Inclusion criteria will be verified in the multidisciplinary meeting that antimicrobial stewardship teams (AST) conduct on a daily basis at the participating hospital.

Estimated size of the study population. Matching:

- 50 patients with a sepsis/ non sepsis 1:1 ratio will be included.
- Septic and non-septic patients will be matched on gender, age (+/- 10 years), comorbidity (Charlson score +/-1), time symptom onset to blood culture (+/- 24h)

4.4. Definitions

Cases (sepsis / septic shock):

- Sepsis or shock septic are defined as life-threatening organ dysfunction caused by a dysregulated host response to infection according to the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definition Conference.

Controls:

- Absence of sepsis or septic shock according to the 2001 SCCM/ESICM/ACCP/ATS/SIS International Sepsis Definition Conference.

4.5. Variables

- Patient-related variables:

- Demographical: gender, age.
- Comorbidity: Modified Charlson Index
- Baseline serum creatinine.

- Infection-related variables.
- Time from the onset of symptoms to the start of antimicrobial treatment.
- Time from the onset of symptoms to the start of appropriate antimicrobial

treatment.
- Time form the onset of symptoms to the surgical therapy (if needed).
- Clinical severity at time 0 (blood culture), and day 2- 3 and day 30: sepsis score.

- Inflammation, sepsis mediators and biomarkers.

The following biomarkers will be determined during patient enrollment:
- White blood cell count and differential.
- Platelet count.
- Fibrinogen.
- Prothrombin activity.
- C reactive protein.
- Procalcitonin.
- GZM A.

GZM A serum levels will be obtained in all patients during the enrollment visit, the 2-3 day and the 30 day visits (GZM A kinetics). GZM A levels will be determined by an ELISA commercial assay (Human Granzyme A ELISA development kit{HRP]; Mabtech).

GzmA gene polymorphisms, as well as other potentially associated mutations, will be screened by Whole Exome Sequencing (WES). To this aim, we will use DNA isolated from peripheral blood cells and the AmpliSeq technology kit on the Ion Torrent platform following the manufacturer's instructions. This platform is available at the Genomics Central Research Unit (CRU) at CIBA from University of Zaragoza/IIS Aragon. All kits for isolating and analyzing DNA samples are commercially available and optimized by Thermo Scientific. Bioninformatic analysis will be performed by an agreement established between Genomics CRU and Micromics SL led by Pedro Gonzalez at CRG in Barcelona.

- Microbiological variables

  - Minimum inhibitory concentrations (MIC) of the *E. coli* isolates retrieved from urine and blood cultures will be determined through automated microdilution panels as routinely performed by the Microbiology Laboratory of participating hospitals.

  - Whole genome sequencing (WGS) of the E. coli strains isolated in blood and urine cultures. The presence of E. coli known virulence factors will be analyzed and a virulence score for each strain will be calculated (Mora-Rillo et al., 2015).

4.6. Study plan detailing the procedures.

	Day 0	Day 2 - 3	Day 30
Screening (inclusion and exclusion criteria)	X		
Informed Consent	X		
Anamnesis	X		
Charlson Modified	X		
Sepsis score	X	X	
Serum	X	X	X
Plasma	X	X	X
Microbiological samples	X		
Mortality			X
New hospitalization			X

5. Ethical Aspects

Surveillance of patients with bacteremia is a routine activity of the Antimicrobial Stewardship Team in our hospital, that depends on the Sistema Aragonés de Salud. Demographical, clinical and microbiological data registry is part of this activity.

The study protocol has been reviewed and approved by the Comité Ético de Investigación Clínica de Aragón (CEICA): approval Number: PI19/070

The enrollment of patients to determinate GZM A, gzmA gen polymorphisms will require signed informed consent.

6. Planned Schedule

January to Jun 2019:

- Local Ethics Committee (CEICA) approval request: Compilation of administrative documentation and forms, including information sheet and informed consent
- Statistical consultation.
- Design of the case report form (CRF) and database
- Biobank arrangements.
- Recruitment of the study coordinator.
- Training of research staff.
- Clinical Trials Register.

July 2019 to August 2020.

- Screening and inclusion of patients with bacteremic (E. coli) UTIs.
- Data capture (demographical, clinical and basic laboratory parameters).

- Storage of patient samples and E. coli strains (blood and urine).
- GZM A determination.
- Whole exome sequencing (gzmA polymorphisms).
- Whole genome sequencing (E. coli strains).

September 2020 to December 2020.

- Data analysis
- Manuscripts preparation.
  Manuscript submission.

7. References

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