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Research Paper

A Transcriptomic Biomarker to Quantify Systemic Inflammation in Sepsis — A Prospective Multicenter Phase II Diagnostic Study

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ABSTRACT

Development of a dysregulated immune response discriminates sepsis from uncomplicated infection. Currently used biomarkers fail to describe simultaneously occurring pro- and anti-inflammatory responses potentially amenable to therapy.

Marker candidates were screened by microarray and, after transfer to a platform allowing point-of-care testing, validated in a confirmation set of 246 medical and surgical patients. We identified up-regulated pathways reflecting innate effector mechanisms, while down-regulated pathways related to adaptive lymphocyte functions. A panel of markers composed of three up- (Toll-like receptor 5; Protectin; Clusterin) and 4 down-regulated transcripts (Fibrinogen-like 2; Interleukin-7 receptor; Major histocompatibility complex class II, DP alpha1; Carboxypeptidase, vitellogenic-like) described the magnitude of immune alterations. The created gene expression score was significantly greater in patients with definite as well as with possible/probable infection than with no infection (median (Q25/Q75): 80 (60/101)) and 81 (58/97 vs. 49 (27/66), AUC-ROC = 0.812 (95%-CI 0.755–0.869), $p < 0.0001$). Down-regulated lymphocyte markers were associated with prognosis with good sensitivity but limited specificity.

Quantifying systemic inflammation by assessment of both pro- and anti-inflammatory innate and adaptive immune responses provides a novel option to identify patients-at-risk and may facilitate immune interventions in sepsis.

1. Introduction

Severe sepsis and shock are among the leading causes of death globally, accounting for more than 210,000 deaths annually in the United States and more than 15 million cases worldwide (Angus et al., 2001; Kumar et al., 2011; Adhikari et al., 2010). Sepsis results from a dysregulated response to invasive infection reflected in damage to the host's tissues and organs (Singer et al., 2016). Monitoring of that response may, therefore, provide diagnostic and prognostic information. Multiple circulating proteins have been studied as biomarkers (Pierrakos and Vincent, 2010), based on the assumption that changes in their expression, may reflect eradication or propagation of pathogens. However, none of these is widely accepted or used.

An increasing body of evidence suggests that sepsis with organ failure is associated with an impaired adaptive immune response in which circulating monocytes secrete reduced amounts of pro-inflammatory cytokines (Adib-Conquy and Cavaillon, 2009), antigen-presentation fails, and apoptosis of lymphocytes predominates (Hotchkiss et al., 2013; Giamarellos-Bourboulis and Raftogiannis, 2012). These complex changes require high dimensional approaches, such as functional

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genomics to describe the differing aspects of the host response (Feezor et al., 2005; Desai et al., 2011).

We used a three-stage transcriptomic approach to develop a quantitative real-time polymerase chain reaction (PCR) assay of individual genes to characterize immune alterations associated with sepsis. The objective of this strategy was to assess i) infectious origin, ii) severity of systemic inflammation and iii) its association with outcome. First, patients with extreme disease phenotypes were subjected to transcriptomic analysis to identify transcripts that differentiate non-infectious systemic inflammation from bacterial infection with organ failure or shock. Results were evaluated in a second cohort of subjects representing a continuum from health to high-grade inflammation, identifying clusters of up- and down-regulated pathways that increased with disease severity. Having established a final biomarker panel and a corresponding composite score, we validated the tool regarding identification of infection and prediction of outcome in a pragmatic study in two independent patient cohorts from Germany and Greece covering a broad spectrum of medical and surgical patients with diverse comorbidities in differing health care systems.

2. Patients and Methods

Patients and healthy controls were enrolled at eight investigational sites in four countries (Appendix, Text S1). All study protocols were approved by the respective institutional review boards and written informed consent was provided by patients or their legal representatives.

Gender, age, underlying infections, reason for ICU admission, isolated pathogen, white blood cell count, Acute Physiology and Chronic Health Evaluation (APACHE) II score, Sequential (Sepsis-related) Organ Failure Assessment (SOFA) score, and mortality, respectively, were recorded as pertinent clinical information. For the initial training set, the verification set and the German cohort of the confirmation set C-Reactive Protein (CRP) and procalcitonin (PCT) were recorded as well.

For transcriptomic analyses, blood was sampled and collected into PaxGene tubes (PreAnalytiX, Becton Dickinson, Cockeysville, Md), and stored at −80 °C until assayed. Cases and samples were grouped into cohorts by medical experts and data analysts by pre-specified criteria and definitions (Appendix, Text S2). We excluded patients with immunodeficiency disorders (Appendix, Text S2).

2.1. Study Design

The study was designed in three stages consisting of a training set, a verification set and a confirmation set after transfer of the marker set to a RT-qPCR platform possibly to facilitate its use at the point-of-care (Fig. 1).

The training set identified differences in the transcriptomic profile between patients with extreme phenotypes; i.e. systemic inflammation and organ failure/shock in the absence or presence of infection. Systemic inflammation was diagnosed based on the presence of at least two of four SIRS criteria. In a cohort of 364 patients hospitalized in the ICU of the Jena University Hospital (JUH) between 2002 and 2007 blood sampling was done within the first 24 h after presenting signs of systemic inflammation. Then patients were screened for eligibility. An adjudication committee of two ICU experts selected patients according to pre-specified criteria (Appendix, Text S2). Ninety-six patients met these criteria and their samples were used in order to select qualitative molecular marker candidates and to develop an appropriate classification function, which discriminated cases with and without infection. Demographic characteristics are summarized in Table S1 (Appendix).

Results of the training set were reevaluated in a verification set to validate the marker candidates on a broad spectrum of phenotypes representing a continuum from health to high-grade systemic inflammation and to characterize its suitability to quantify inflammation.

In this sub-study, patients representing six clinical phenotypes were enrolled, i) subjects and preoperative patients for scheduled operations with no signs of infection and no signs of inflammation (controls); ii) patients with local sterile inflammation, iii) patients with local infection but absent signs of systemic inflammation, iv) patients presenting signs of systemic inflammation but without evidence of infection, v) patients with local infection simultaneously fulfilling criteria for systemic inflammation, and vi) patients with bloodstream infection (BSI)-associated severe sepsis/septic shock. Samples were collected before initiation of anti-infective therapy for patients of groups v) and vi) and for patients of group iv) within the first 24 h of presentation of signs of inflammation. The demographic characteristics of the cohorts are summarized in Supplementary Table S2 (Appendix).

The confirmation set comprised two prospectively enrolled cohorts in which a subset of 7 transcripts suitable to assessing the host response to infection was tested after transfer to a RT-qPCR platform. The German cohort was enrolled between May 2009 and October 2010 from the ICU of JUH. Inclusion criteria were systemic inflammation and/or severe sepsis/septic shock with infection ruled out for patients with uncomplicated systemic inflammation and confirmed for patients with severe sepsis/septic shock according to standard definitions at time of enrolment (Levy et al., 2003; Calandra and Cohen, 2005). The Greek cohort was enrolled between October 2012 and January 2013 in three departments of the Hellenic Sepsis Study Group. Inclusion criteria were: a) diagnosis of severe sepsis or septic shock based on standard definitions; (Levy et al., 2003) b) diagnosis of acute pyelonephritis, community-acquired or ventilator-associated pneumonia (CAP or VAP), intra-abdominal infection (IAI) or BSI. For patients enrolled in the confirmation cohort sequential blood samples were obtained; the first on the day of diagnosis and the second 24 h later; in the German cohort sampling was continued on a daily basis until ICU-discharge or death for a maximum of ten days.

For the confirmation of the genomic score, patients were independently classified according to the current clinical gold standard into three groups: 'no infection', 'possible/probable infection' and 'definite infection' (Calandra and Cohen, 2005). In both cohorts, patients were followed up to assess 100-day mortality. Demographic data are presented in Tables S3 and S4 (Appendix).

In all patient sets, classification according to degree of inflammation or presence of infection status was made independently of the genomic score or the use of serum biomarkers.

For a detailed description of the used laboratory techniques see Appendix (Text S3).

2.2. Statistical Analysis

The study consisted of two microarray experiments with a marker screening and of a RT-qPCR evaluation for the marker confirmation. For the design and evaluation of the microarray trials specific methods were employed, including data preprocessing and transformation.

For the training set, 96 RNA samples from 96 ICU patients were hybridized against the in-house research microarray addressing 5308 transcripts. For the classification of cases with and without infection the linear discriminant analysis (LDA) was applied with up to 100 transcripts as classification markers, selected by p-values and estimates of Wilcoxon test. Marker candidates were chosen corresponding to the best concordance between molecular and clinical classification.

For the verification set, 72 RNA samples from 72 cases were hybridized against a genome-wide microarray addressing ca. 50,000 transcripts. One-way analysis of variance with 6 groups was applied gene by gene and evaluated by the estimation of the false discovery rate. The gene expression pattern of 4761 selected transcripts was visualized by a heatmap and quantified by a genomic score (GES), developed for this approach.

In the confirmation set, 7 transcripts, representing an overlap of signatures obtained in the training and verification sets were used to assess the host response to infection in 246 patients, after transfer to a RT-qPCR platform. The GES and its components of up- and down-regulation