Classification of sepsis patients as blood genomic endotypes: a prospective cohort study

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Word count manuscript: 4280

ABSTRACT

Background: The host response during sepsis is highly heterogeneous, which hampers the identification of patients at high-risk of mortality and selection for targeted therapies. We here aimed to identify biologically relevant molecular endotypes in patients with sepsis.

Methods: Prospective observational study comprising consecutive admissions for sepsis in two intensive care units (ICUs) in the Netherlands from January 2011 to July 2012, from which a discovery (n=306) and first validation cohort (n=216) were derived. A second validation cohort consisted of patients with sepsis caused by community-acquired pneumonia from 29 ICUs in the United Kingdom (n=265). Genome-wide blood gene expression profiles were generated from admission samples and analyzed by unsupervised consensus clustering and machine learning. Sepsis patient endotypes were evaluated against clinical traits and outcome by Kaplan Meier and cox regression analysis.

Findings: Four sepsis molecular endotypes, designated Mars1-Mars4, were identified in the discovery cohort, which associated with 28-day mortality (logrank p = 0.022). The worst outcome was found for patients classified as Mars1, with 39% 28-day mortality; for Mars2-4 28-day mortality was 22%, 23% and 33% respectively. The hazard ratio (HR) for death within 28-days of Mars1 patients equated to 1.86 (95% CI: 1.21-2.86, p=0.0045) ; HRs for the other endotypes were 0.64 (95% CI: 0.4-1.04, p=0.061) for Mars2, 0.71 (95% CI: 0.41-1.22, p=0.19) for Mars3, and 1.13 (95% CI: 0.63-2.04, p=0.69) for Mars4. Evaluation of the net reclassification improvement using a combined clinical and endotype model significantly improved risk prediction (0.33, 95% CI: 0.09-0.58, p=0.008). A 140 gene expression signature reliably stratified sepsis patients to four endotypes in both the first and second validation cohort. The HR for death within 28 days of patients classified as Mars1, Mars2, Mars3 and Mars4 in the first

validation cohort equated to 1.97 (95% CI: 1.11-3.54, p=0.024), 1.12 (95% CI: 0.62-2.03, p=0.69), 0.55 (95% CI: 0.25-1.17, p=0.097) and 0.21 (95% CI: 0.03-1.55, p=0.092), respectively; in the second validation cohort these HRs were 2.02 (95% CI: 1.07-3.82, p=0.031) for Mars1, 1.12 (95% CI: 0.66-1.9, p=0.66) for Mars2, 0.47 (95% CI: 0.25-0.88, p=0.018) for Mars3 and 1.65 (95% CI: 0.66-4.13, p=0.29) for Mars4. Hence, only Mars1 consistently associated with 28-day mortality and may be of clinical relevance. To facilitate possible clinical use a biomarker was derived for each endotype, with *BPGM* and *TAP2* reliably identifying Mars1 patients.

Interpretation: This study provides data for the molecular classification of sepsis patients as endotypes at ICU admission. Detection of sepsis endotypes may assist in providing precise patient management and selection for trials.

Funding: Center for Translational Molecular Medicine (CTMM) (http://www.ctmm.nl), project MARS (grant 04I-201).

Evidence before this study

Pronounced heterogeneity in the host response to sepsis complicates the identification of critically ill patients at high-risk of mortality as well as those who would benefit from precise adjuvant therapy. We searched for blood genomic studies of critically ill patients with sepsis prior to January 31st, 2017, using the following search terms: ((“sepsis” OR “severe sepsis” OR “septic shock”) AND (“genomics” OR “gene expression profiling” OR “microarray”) AND (subtype OR endotype OR subclassification OR cluster OR subgroup OR prospective cohort)). We subsequently added “patient” to a second search field. We identified 38 studies of peripheral blood leukocytes in patients. Out of these 38 studies, 3 studies applied genome-wide blood transcriptional profiling coupled with clustering techniques showing the existence of population substructures, also known as endotypes, in pediatric sepsis (n=81), adult sepsis patients (n=126) and sepsis patients with community-acquired pneumonia (n=265). No study has comprehensively investigated the occurrence of patient endotypes in a consecutively enrolled all-cause sepsis adult population and validated in multiple independent datasets.

Added value of this study

In this prospective observational study, which included 522 consecutively enrolled (all-cause) sepsis patients from the Netherlands and 265 patients with sepsis caused by community-acquired pneumonia from the United Kingdom, we identified four blood gene expression endotypes, of which one (Mars1) consistently that significantly associated with acute (28-day) and late (1 year) mortality. Furthermore, combining APACHE IV scores and endotype membership significantly improved patient risk stratification. The four patient endotypes could not be predicted by demographic nor clinical covariates. We also showed that the blood transcriptomes

of these four endotypes had distinct host response signatures, for example endotypes attuned to immunesuppression, hyperinflammation or adaptive immune functions, revealing great potential for more precise patient management and clinical trial design.

Implications of all the above evidence

The substantial heterogeneity in the host response to sepsis has hindered patient management and therapeutic discoveries. Our study and others have demonstrated that leveraging the concepts of unsupervised blood genomic analysis patients can be classified as molecular endotypes with important prognostic and pathophysiological value. The distinct host response signatures between the four endotypes have important implications that include development of precision therapeutics and practices of clinical trial design.

INTRODUCTION

Sepsis remains a remarkable adversary to medicine, characterized by poor prognosis and high mortality rates.[1,2] Despite the burden on patients, their families and the health care system, treatment remains mainly supportive.[1] Unrecognized population substructures and the heterogeneity in the host response complicate the identification of high-risk patients who would benefit from specific adjuvant therapy.[3]

Blood transcriptional profiling has provided substantial advances in the context of sepsis.[4] Although promising new diagnostic biomarkers have emerged from the application of blood genomics to sepsis,[5-7] patient selection for interventional trials and prognostication in sepsis continue to be driven by clinical criteria. While supervised analysis of sepsis patients discordant for survival have identified candidate protein and gene expression prognostic markers,[8,9] substantial heterogeneity remained unexplained. Unsupervised learning coupled with adequate validation metrics have been successfully applied in the field of oncogenomics to dissect the heterogeneity in cancer,[10,11] which revealed important patient endotypes that would have otherwise stayed unnoticed. A comprehensive assessment of the heterogeneity in the adult host response due to all-cause sepsis in consecutive intensive care unit (ICU) admissions has not been performed.

The primary objective of this study was to identify subgroups (endotypes) of sepsis patients based on whole blood RNA expression profiles. For this we leveraged the concepts of unsupervised consensus clustering and machine learning in a discovery cohort of patients admitted to the intensive care unit (ICU) with sepsis, and subsequently tested robustness across two independent validation cohorts from different hospitals. Moreover, we derived candidate blood genomic biomarkers for sepsis endotype classification and evaluated these in all cohorts.

METHODS

Patients

The study was performed within the context of the Molecular Diagnosis and Risk Stratification of Sepsis (MARS) project, a prospective observational study in the mixed ICUs of two tertiary teaching hospitals (Academic Medical Center in Amsterdam and University Medical Center in Utrecht) in the Netherlands (ClinicalTrials.gov identifier NCT01905033).[5,12,13] All patients above 18 years of age admitted to the two ICUs between January 2011 and July 2012 with an expected length of stay longer than 24 hours were included via an opt-out method approved by the medical ethical committees of the participating hospitals.[5,12,13] For every admitted patient the plausibility of an infection was assessed in retrospect using a four point scale (ascending from *none*, *possible*, *probable* to *definite*) using Center for Disease Control and Prevention[14] and International Sepsis Forum consensus definitions[15], as described in detail.[12] The current study comprised consecutive patients admitted to the ICU with sepsis defined as the presence of infection with a *probable* or *definite* likelihood (for site-specific criteria see ref. 12), accompanied by at least one additional general, inflammatory, hemodynamic, organ dysfunction or tissue perfusion parameter as described in the 2001 International Sepsis Definitions Conference (**Table S1**).[16] Patients admitted in Amsterdam were used as discovery cohort; those admitted in Utrecht as first validation cohort. 42 healthy subjects (age 35 (30-63) years, median with interquartile ranges; 57% male) were also enrolled after providing written informed consent. The second validation cohort was from the United Kingdom (UK) Genomic Advances in Sepsis (GAinS) study of adult patients with sepsis due to community-acquired pneumonia

(CAP)[17]. Pediatric sepsis patients derived from a prospective observational study of children ≤ 10 years old admitted to multiple pediatric ICUs in the United States were used as a comparative cohort.[18] Details of the inclusion criteria of the GAinS and pediatric cohorts can be found in appendix 1.

Blood RNA and microarrays

Of patients enrolled in MARS blood was collected in PAXgene blood RNA tubes (Becton-Dickinson, Breda, The Netherlands) within 24 hours of ICU admission. Blood from 42 healthy subjects was also collected in PAXgene blood RNA tubes. Gene expression profiles of patients and healthy subjects were generated using Human Genome U219 96-array plates and the GeneTitanR instrument (Affymetrix) as described.[5,13] MARS gene expression data are available in the Gene Expression Omnibus under accession number GSE65682. Gene expression data for UK GAinS (ArrayExpress accession number E-MTAB-4421) were generated using Illumina Human-HT-12 version 4 Expression BeadChips[17]. Gene expression data of pediatric sepsis patients (GSE13904) were generated using the Affymetrix Human Genome U133 Plus 2.0 Array[18].

Unsupervised clustering and classifier derivation

For endotype discovery (**Figure S1**), probes were ranked by median absolute deviation across 306 patient samples (discovery cohort). The top 5000 ranked probes were selected and analyzed by means of the consensus clustering method.[19,20] We selected the agglomerative hierarchical clustering algorithm on 1-Pearson correlation distances, 99% item (sample) resampling, 1000 iterations and cluster range $k = 2$ to $k = 12$. To estimate k (number of

endotypes) we combined cumulative distribution functions,[19,20] silhouette width analysis[21] available in the cluster package[22] and cophenetic distance correlation analysis to evaluate clustering stability.[23] To construct the *k* endotype classifier we selected patient samples with positive silhouette widths, representing core patients per endotype.[19,21] The 5000 probes were subsequently ranked by non-parametric (kruskal-wallis rank sum test) significance. 2994 unique gene probes were filtered by selecting for highest significance. Using a random forest classifier[24] (supervised classification with high dimensional data methods),[25] we evaluated sepsis endotype classification with 10-fold cross-validation of step-wise increments in gene numbers. We settled on the number of genes that yielded a cross-validation misclassification error rate < 10%. The sepsis endotype classifier gene set was then used to perform random forest prediction of endotypes in the validation cohorts. The 42 healthy subjects were utilized solely for comparing gene expression profiles to those of patients classified as Mars endotypes. See appendix 1 for further description of clustering methodology and differential gene expression analysis.

Derivation of the molecular endotype biomarkers

Endotype biomarkers were assessed using previously described methods.[5,6] For details see appendix 1.

Differential gene expression and Ingenuity pathway analysis

See appendix 1.

Statistics

Statistical analysis was performed using the R statistical computing environment (version 3.1.2; R Foundation for Statistical Computing, Vienna, Austria; http://www.R-project.org/). The Cramer's V measure of effect size was used for a chi-square goodness of fit test. Correlation analysis of continuous data was performed using Spearman's method. Survival analysis was performed by Kaplan-Meier estimation (log-rank test) and Cox proportional hazards regression implemented in the survival method (version 2.37). Hazard ratios and 95% confidence intervals were calculated for each endotype with reference to all other endotypes. Net reclassification improvement was assessed by means of a continuous model using the predictABEL method (version 1.2-2).[26] One model encompassed only Acute Physiology and Chronic Health Evaluation (APACHE) IV scores[27] (clinical), while a second model encompassed both APACHE IV scores and sepsis endotype stratification (clinical + molecular). Significance was demarcated at $p < 0.05$.

Role of the funding source

The funders of the study had no role in the study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data and the final responsibility to submit for publication.

RESULTS

Classification of sepsis patients as four molecular endotypes

To explore the molecular fingerprints that underlie the heterogeneity in sepsis we assessed genome-wide blood gene expression in blood of adult patients admitted to the ICUs of two hospitals in the Netherlands (discovery cohort, n=306; first validation cohort, n=216) and of patients with sepsis caused by CAP admitted to 29 ICUs in the UK (n=265; second validation cohort)[17] (**Figure 1** and **Table 1**). Gene expression data from the discovery cohort were analyzed using a previously developed unsupervised consensus clustering method (**Figure S1**).[19,20,28] Considering cluster (endotype) quality and stability,[21,23] we reached a consensus in partitioning at four molecular endotypes (**Figure 2A, B** and **Figure S2)**, designated Mars1-4. The vast majority of patients were Caucasian (**Table 1**) and we found no association between ethnicity and endotype membership (**Table S2**). Patients who had positive silhouette widths[21] (81 Mars1, 94 Mars2, 63 Mars3 and 29 Mars4), indicative of their high intra-endotype similarity,[19,21,28] were subsequently used. In so doing, we identified 140 genes (appendix 2) that clearly stratified Mars1 to Mars4 endotypes (**Figure 2C** and **Table S2**).

Sepsis endotypes did not show an association with comorbidities and clinical scores including APACHE IV (**Table S2**); however septic shock prevalence and Sequential Organ Failure Assessment (SOFA) scores[29] showed significant dependencies to sepsis molecular endotype classification (**Figure 2D, E**). The estimated effect size (Cramer's V) of septic shock was 0.23, indicating moderate dependency. Kaplan-Meier analysis revealed an association with mortality (**Figure 2F**). The worst outcome at 28 days was found for those patients classified to Mars1 with 39% mortality (n=35 out of 90), 22% (n=21 out of 105) for Mars2, 23% (n=16 out of 71) for Mars3 and 33% (n=13 out of 40) for Mars4. Mars1 classified patients had the worst

outcome until 1 year of patient follow-up (logrank p = 0.023; **Figure S3A**). The hazard ratio (HR) for death within 28 days of patients classified as Mars1, Mars2, Mars3 and Mars4 equated to 1.86 (95% CI: 1.21-2.86, p=0.0045), 0.64 (95% CI: 0.4-1.04, p=0.061), 0.71 (95% CI: 0.41-1.22, p=0.19), 1.13 (95% CI: 0.63-2.04, p=0.69), respectively. The multivariate HR (incorporating Charlson comorbidity indices) for death within 28-days of Mars1 classification equated to 1.79 (95% CI: 1.16-2.75, p=0.0084). To test whether the combination of a molecular and clinical scoring system may be of benefit to patient risk stratification, we evaluated the net reclassification improvement and integrated discrimination improvement [26,30] using a combined APACHE IV score (clinical) and sepsis endotype classification (molecular) model. This clinico-molecular model significantly improved 28-day mortality risk prediction (net reclassification improvement (continuous) [95% CI]: 0.33 [0.09 - 0.58], p=0.008; integrated discrimination improvement [95% CI]: 0.015 [0.0002 - 0.03], p=0.047) when compared to 28-day mortality risk prediction by APACHE IV scores alone. The Hosmer–Lemeshow test showed proper model calibration (**Figure S3B**). Altogether, these results provide for a molecular classification of sepsis patients that possesses prognostic value either in isolation or in combination with established clinical scores.

Independent cohort validation of sepsis molecular endotype classification

In order to ascertain robustness of our findings we evaluated two validation cohorts (**Figure 1** and **Table 1**). Applying the 140 gene classifier clearly identified four sepsis endotypes in the first validation cohort consisting of all cause sepsis (**Figure 3A** and **Figure S4A**). Consistent with the discovery cohort, SOFA scores and septic shock were significantly

associated to sepsis endotype membership (**Figure 3B, C**), with moderate dependency to septic shock (Cramer's V = 0.29). In concordance with the discovery cohort APACHE IV scores did not associate with endotype classification (**Table S3**), while Kaplan Meier analysis showed a significant association to mortality (**Figure 3D** and **Figure S4B**). Again the highest mortality rate was found for patients classified as Mars1, with 32% mortality (n=19 out of 60) at 28 days, while risk stratification by Mars2-4 showed more variance when compared with the discovery cohort, especially Mars4, that is 28-day mortality of patients classified as Mars2, Mars3 and Mars4 was 23% (n=18 out of 79), 14% (n=8 out of 58) and 5% (n=1 out of 19), respectively. The association with mortality was also evident until 1 year of patient follow-up, with Mars1 classified patients having the worst outcome (logrank p = 0.0031; **Figure S4B**). The HR for death within 28 days of patients classified as Mars1, Mars2, Mars3 and Mars4 in the first validation cohort equated to 1.97 (95% CI: 1.11-3.54, p=0.024), 1.12 (95% CI: 0.62-2.03, p=0.69), 0.55 (95% CI: 0.25-1.17, p=0.097) and 0.21 (95% CI: 0.03-1.55, p=0.092), respectively. The multivariate HR (incorporating Charlson comorbidity indices) for death within 28-days of Mars1 classification equated to 1.91 (95% CI: 1.05-3.47, p=0.034). Combining clinical (APACHE IV) and molecular (sepsis endotype classification) data significantly improved 28-day mortality risk prediction (net reclassification improvement (continuous) [95% CI]: 0.38 [0.01 - 0.66], p-value = 0.008; integrated discrimination improvement [95% CI]: 0.028 [0.0018 - 0.055], p=0.036) as compared to APACHE IV scores alone. The Hosmer–Lemeshow test showed proper model calibration (**Figure S4C**).

We also detected four endotypes (**Figure 3E**) with favorable stability (**Figure S5A**) in patients admitted to ICUs in the UK with sepsis caused by CAP.[17] Evaluation of the association to SOFA scores, septic shock and mortality revealed statistically significant associations; Mars1

classified patients had relatively worst prognosis with 34% mortality (n=12 out of 35) at 28 days (**Figure 3F-H**). Risk stratification by Mars2-4 was not fully consistent with the discovery cohort, that is, 28-day mortality of patients classified as Mars2, Mars3 and Mars4 was 22% (n=26 out of 117), 13% (n=13 out of 97) and 31% (n=5 out of 16), respectively (**Figure 3F-H**). The HR for death within 28 days of ICU admission for those patients classified as Mars1, Mars2, Mars3 and Mars4 in the second validation cohort equated to 2.02 (95% CI: 1.07-3.82, p=0.031), 1.12 (95% CI: 0.66-1.9, p=0.66) , 0.47 (95% CI: 0.25-0.88, p=0.018) and 1.65 (95% CI: 0.66-4.13, p=0.29), respectively. No association was uncovered for demographics and APACHE II scores (**Table S4**). Notably, APACHE IV scores were collected in the discovery and first validation cohort, whereas APACHE II scores in the second validation cohort. Considering that the second validation cohort only consisted of CAP patients, as well as the consistent association of Mars endotypes to abdominal and pneumonia diagnosis in the discovery and first validation cohort (**Table S2, S3**), we combined the two all-cause sepsis cohorts (discovery plus first validation cohort) to show that the four endotypes were present irrespective of the primary site of infection; for this we separately analyzed the two main causes of sepsis, i.e., pneumonia (n=215) and peritonitis (n=123). We detected four sepsis endotypes in both subgroups with favorable stability (**Figure S5B-E**). Of note, we detected different proportions of patients classified to each endotype, with more patients classified as Mars3 or Mars4 in the pneumonia cohort in contrast to the abdominal sepsis subgroup (**Figure S5B, C**). This suggests that although the four endotypes were clearly identified, Mars3 and Mars4 endotypes may be more attuned to pneumonia patients. In a cohort of pediatric sepsis patients (GSE13904, n=81)[18] (**Table S5**) we found three endotypes (Mars1, Mars2 and Mars4) with favorable stability (**Figure S6A, B**); in this cohort Mars3 was not reliably detected (**Figure S6B**) and evaluation of 28-day mortality as well as pediatric risk of

mortality scores (PRISM)[31] revealed no significant dependencies on sepsis endotype classification (**Figure S6C**, **D**).

Collectively, these data provide robustness to the classification of adult sepsis patients as high-risk Mars1 endotype. Risk stratification of patients as Mars2-4 may not be as clinically relevant for prognosis, especially the Mars4 endotype with differing mortality counts in the first validation cohort, as compared to the discovery and second validation cohort. Furthermore, these findings suggest Mars1-4 endotype classification is only partially applicable to children with sepsis.

Biological interpretation of the four sepsis endotypes

To understand the biological underpinnings of the molecular subtypes we firstly evaluated the association of sepsis endotypes to leukocyte counts and differentials. A consistent association was uncovered for monocyte counts in both discovery and first validation cohort (**Table S2, S3**), with an overall effect size (eta-squared) equating to 4.3%. Total leukocyte counts and lymphocyte counts were associated to sepsis endotypes only in the discovery and first validation cohort, respectively. No association was found with neutrophil counts. We subsequently turned our attention to differential gene expression analysis and biological pathway inference within the four endotypes. Each endotype in the discovery cohort revealed substantial alterations relative to health (**Figure 4A**), with 77% of the gene expression responses common to all endotypes and Spearman's rho > 0.7 (**Figure 4B**). Gene expression signatures specific to each endotype were also evident, most particularly in the high-risk Mars1 endotype (**Figure 4C**). The Mars1 endotype was characterized by a pronounced decrease in expression of genes

corresponding to key innate and adaptive immune cell functions, for example Toll-like receptor, NF-kB signaling, antigen presentation and T cell receptor signaling, concomitant with increased expression of specific metabolic pathway genes that included heme biosynthesis pathways (**Figure 4D** and **Figure S7A**). The Mars2 endotype was characterized by heightened expression of genes involved pattern recognition, cytokine, cell growth and mobility pathways, including NF-kB, IL-6, iNOS and fMLP signaling. The Mars4 endotype was also associated with elevated expression of genes involved in pattern recognition and cytokine pathways, specifically interferon signaling, RIG1-like receptor and TREM1 signaling (**Figure 4D** and **Figure S7A**). Finally, the Mars3 endotype was significantly associated with elevated expression of predominantly adaptive immune pathway genes, which included T helper cell, natural killer cell, IL-4 signaling and B cell development pathways cell (**Figure 4D** and **Figure S7A**). Of interest, the Mars3 endotype, which was associated with a relatively low risk when compared to Mars1, exhibited a significant association to the previously described low-risk "Sepsis Response Signature" (SRS)2 group in the UK sepsis cohort (**Figure S7B**)[17].

Derivation of sepsis molecular endotype scores

In order to facilitate potential translation to the clinic we sought to derive sepsis endotype scores using a previously described and validated combinatorial approach[5,6]. To this end, we assessed 77,840 combinations of genes in the 140 gene classifier for classification of the four molecular endotypes and identified 8 genes that in specific combinations reliably stratified patients from the discovery cohort as sepsis molecular endotypes (**Figure 5A**). Gene expression combinations of *BPGM* (bisphosphoglycerate mutase):*TAP2* (transporter 2, ATP binding cassette

subfamily B member), *GADD45A* (growth arrest and DNA damage inducible alpha):*PCGF5* (polycomb group ring finger 5), *AHNAK* (AHNAK nucleoprotein):*PDCD10* (programmed cell death 10) and *IFIT5* (interferon induced protein with tetratricopeptide repeats 5):*GLTSCR2* (glioma tumor suppressor candidate region gene 2) were used to classify patients as Mars1, Mars2, Mars3 and Mars4 endotypes, respectively (**Figure 5B** and **Table S5**). These candidate biomarkers of Mars endotype membership also accurately classified patients in the two validation cohorts (**Figure 5C-F** and **Figure S8A, B**). Collectively, these findings provide candidate molecular biomarkers for the identification of sepsis molecular endotypes at ICU admission.

DISCUSSION

We identified four endotypes in three heterogeneous sepsis cohorts based on blood leukocyte genome-wide expression profiles on ICU admission. These sepsis endotypes (Mars1-4) had pathophysiologic implications, and were not easily discernable by clinical characteristics; the Mars1 endotype was consistently associated with the highest mortality rate. Both common and distinct biological signatures characterized the four sepsis endotypes. The poor prognosis Mars1 sepsis endotype was associated with a dramatic decrease in expression of genes involved in innate and adaptive immune functions; whereas the relatively low risk Mars3 endotype had elevated expression of adaptive immune/T cell functions. Finally, 8 genes were derived as candidate biomarkers for the identification of sepsis endotypes at ICU admission, with *BPGM* and *TAP2* transcripts delineating the poor prognosis Mars1 sepsis endotype. *BPGM* encodes for a small molecule, 2,3-diphosphoglycerate, which binds to hemoglobin in red blood cells thereby decreasing the oxygen affinity of hemoglobin[32]. *TAP2* is a member of the superfamily of ATP-binding cassette transporters involved in antigen presentation[33].

Constant improvements in the precision and breadth of "omics" data that can be observed in sepsis patients have set the stage for sophisticated methods to better understand these sources of high-dimensional data especially in relation to clinical traits. Through the use of consensus-based clustering techniques important substructures in disease populations have been identified.[10,11,19,28] An overarching observation of patient endotypes (or subtypes in the oncogenomics field) was their association to varying degrees of disease severity and mortality. Our findings showed a consistent association of the Mars1 sepsis endotype with high mortality across the cohorts. These results extend previous studies in critically ill patients with and without

sepsis[34] as well as CAP.[17] While the former study[34] investigated only neutrophil transcriptional profiles from both sepsis and non-septic patients, the latter study (UK GAinS) enrolled sepsis patients due to CAP identifying two sepsis endotypes with prognostic value.[17] Our analysis of the GAinS CAP cohort, applying an ensemble of methods for rigorously measuring quality and stability of sample partitioning, as well as classification by machine learning, showed that a four sepsis endotype model was favorable in this adult CAP cohort. Of note, the low-risk SRS2 endotype[17] was highly correlated to the low-risk Mars3 sepsis endotype, both characterized by heightened expression of genes predominantly involved in adaptive immune (mainly T cell) functions. The underdeveloped nature of adaptive immunity in children,[35,36] possibly together with the high proportion of shock cases enrolled in the pediatric sepsis cohort we evaluated,[18] may explain, at least in part, the unstable classification of pediatric sepsis patients to Mars3 sepsis endotype.

While all four endotypes were present in patients with either pneumonia or abdominal sepsis, pneumonia was relatively overrepresented in Mars3 and Mars4. A conclusive explanation for this finding is lacking. Possibly, surgical interventions directly before or after ICU admission, which are common in patients with abdominal sepsis but not in those with pneumonia, could partially influence endotype classification.

Our study has limitations. We enrolled only patients with an expected length of ICU stay > 24 hours, mainly to exclude elective (cardiopulmonary) surgical patients who routinely stay on the ICU for a few hours up to one night. Nonetheless, this selection may impair generalizability of our finding. For the discovery and first validation cohort only patients with an infection likelihood of definite and probable were included; although in the second validation cohort infection likelihood was not scored, inclusion of patients with an infection likelihood of possible

in the discovery cohort may have yielded different results. The discovery cohort was admitted to a single ICU in the Netherlands; yet, validation was done in two separate cohorts, one of which composed of patients admitted to 29 ICUs in the United Kingdom. The clinical value of the candidate endotype biomarkers was only assessed by ROC analysis using microarray gene expression data.

Classification of heterogeneous sepsis populations into molecular endotypes may in the future provide clues for targeted therapies for specific subgroups. The poor prognosis sepsis endotype, Mars1, was characterized by decreased expression in genes that function in both innate and adaptive immune mechanisms concomitant to high expression of specific cellular metabolic pathways including heme biosynthesis. Glycine accumulation, biosynthesized by serine derived from the glycolysis pathway intermediate 3-phosphoglycerate, fuels heme biosynthesis and in turn modulates ATP synthesis via oxidative phosphorylation in mitochondria.[37] Defects in immunometabolic circuits, including glycolysis and oxidative phosphorylation, have been shown to underlie immunoparalysis in sepsis.[38] Therefore, these findings suggest that Mars1 classified patients may represent an "immunoparalyzed" endotype with poor prognosis. The Mars2 and Mars4 endotypes were characterized by high expression of genes involved in pro-inflammatory and innate immune reactions, for example NF-kB signaling and interferon signaling, respectively. Thus, Mars2 and Mars4 classified patients may represent distinct hyper-inflammatory endotypes. Genes with elevated expression in the relatively lowest risk Mars3 endotype were over-represented for adaptive immune/T cell pathways, which lends weight to the concept that intact T cell functions improve sepsis outcome[39]. Clinical trials for sepsis seeking to modify the host response have thus far yielded no beneficial effect on outcome.[40] A growing body argues for the re-assessment of clinical trial designs[41] to include biomarkers reflecting the

status of the host response.[40,42] We envisage that endotype classification may provide more homogeneity to the notoriously heterogeneous sepsis population: the molecular endotypes described here show that "sepsis" indeed presents a heterogeneous syndrome with distinct pathophysiological profiles in patients that clinically are not distinguishable. By deriving two-gene biomarkers for each endotype we provided evidence that molecular subtyping of sepsis patients (using rapid bedside polymerase-chain reaction based tests) is feasible in the clinic. The technology to produce such tests with automated generation of results within several hours already exists. Future research is required to identify targetable pathways within these endotypes that could be modulated as part of personalized therapies in subgroups of sepsis patients. This research should involve prospective validation and longitudinal analyses of biomarkers during the course of the disease in order to establish whether patients can switch endotypes. The way to go forward with implementation of personalized medicine in clinical sepsis management is to combine the measurement of a biomarker set that provides insight into the activity of a immunological pathway, with a specific intervention targeting that pathway. This approach, which has been named "theranostics", would allow the use of molecular biomarkers both for selection of patients for a specific therapy and for monitoring thereof.

Acknowledgments

The authors acknowledge all members of the MARS consortium for the participation in data collection, Marek Franitza, PhD, and Mohammed R. Toliat, PhD (Cologne Center for Genomics, University of Cologne, Cologne, Germany) for microarray data collection, none of whom received financial compensation for this study. The authors also acknowledge all patients and healthy volunteers who participated in the study.

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Declaration of interests

BPS and TvdP report a patent pending. The other authors declared no conflicts of interest.

Author contributions

BPS and TvdP conceived the study and design. BPS, LAvV and MAW did the laboratory work. BPS, LAvV and AHZ contributed to the statistical analysis. EED, KLB, PN, MJS, JH, OLC, MJB, CJH, HRW and JCK provided administrative, technical, or material support. BPS and TvdP drafted the paper. TvdP and MJB were responsible for obtaining funding. BPS and TvdP led the study.

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582 **Table 1**. Baseline characteristics and mortality of patients in the discovery and validation cohorts

	Discovery cohort the Netherlands	**First validation cohort the Netherlands**	**Second validation cohort United Kingdom**
Patients, n	306	216	265
Demographics			
Gender, males (%)	166 (54)	131 (61)	145 (55)
Age, median [Q1-Q3]	63 [52-72]	63 [55-71]	64 [52-75]
Ethnicity, Caucasian (%)	241 (78.8%)	208 (96.3%)	-
Chronic comorbidity, n (%)			
None	124 (40.5%)	37 (17.1%)	-
Cardiovascular compromise	53 (17.3%)	55 (25.5%)	118 (44.5%)
COPD	39 (12.7%)	30 (13.9%)	62 (23.4%)
Diabetes	54 (17.6%)	45 (20.8%)	51 (19.2%)
Hypertension	66 (21.6%)	76 (35.2%)	-
Malignancy	36 (11.8%)	74 (34.3%)	17 (6.4%)
Renal insufficiency	41 (13.4%)	36 (16.7%)	28 (10.6%)
Respiratory insufficiency	47 (15.4%)	33 (15.3%)	128 (48.3%)
Charlson comorbidity index	4 [2-5]	5 [3-6]	-
Site of infection, n (%)			
Lung	130 (42.5%)	96 (44.4%)	265 (100)
Abdominal	79 (25.8%)	51 (23.6%)	-
Urinary	25 (8.2%)	24 (11.1%)	-
Skin	24 (7.8%)	6 (2.8%)	-
Cardiovascular	11 (3.6%)	7 (3.2%)	-
Central nervous system	5 (1.6%)	4 (1.9%)	-
Other*	32 (10.5%)	28 (13/0%)	-
Severity on ICU admission			
APACHE Score, median [Q1-Q3]	77 [60-97]†	85 [69-103]†	18 [14-22]‡
SOFA score, median [Q1-Q3]	8 [6-10]	6 [4-9]	6 [4-9]

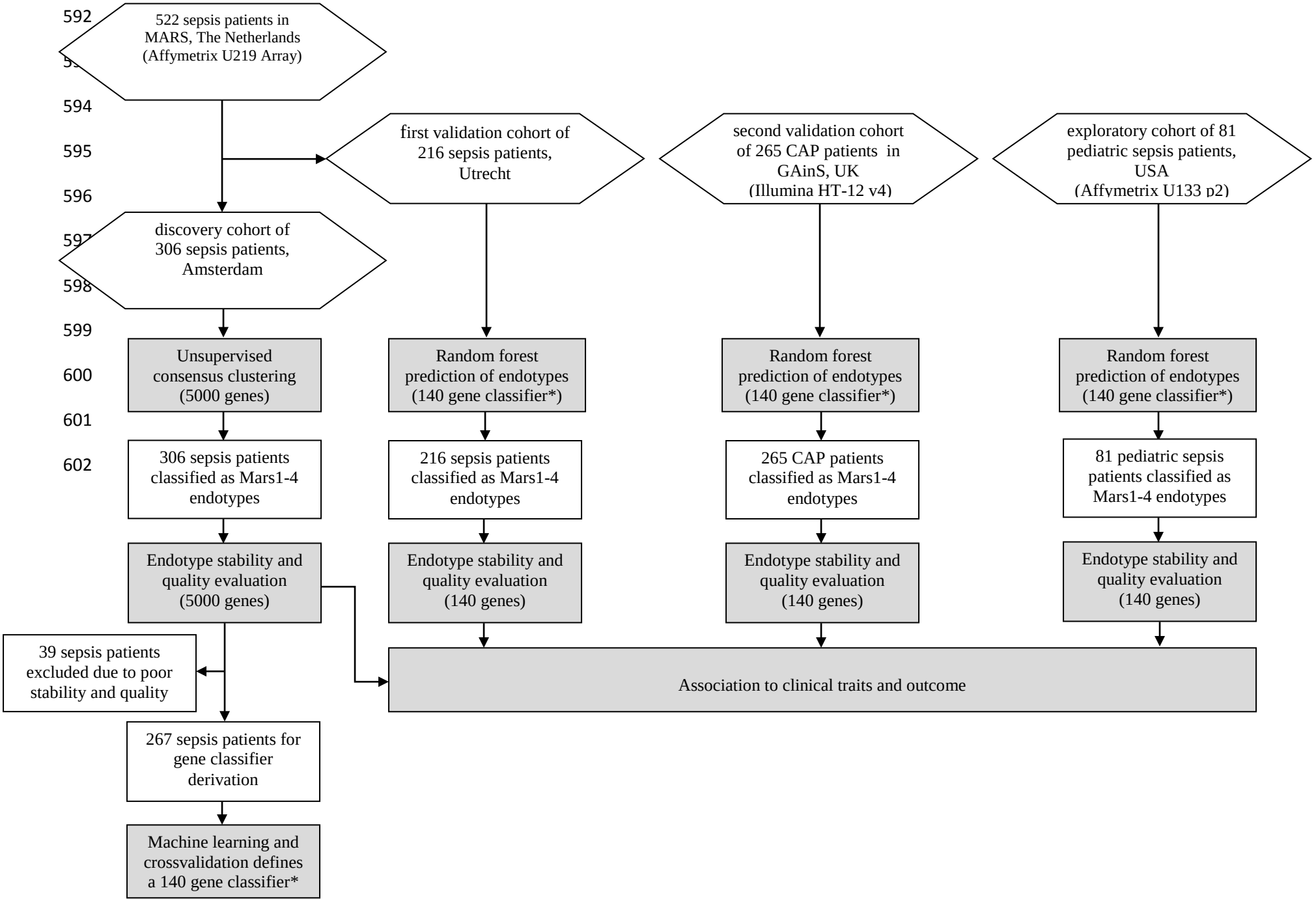
	Discovery cohort the Netherlands	First validation cohort the Netherlands	Second validation cohort United Kingdom
Patients, n	306	216	265
Shock, n (%)	108 (35.3)	73 (33.8)	79 (29.8)
Acute kidney injury, n (%)	132 (43.1)	55 (25.5)	52 (19.6)
Acute lung injury, n (%)	101 (33.0)	52 (24.1)	-
Outcome			
Length of stay, median [Q1-Q3]	4 [2-9]	6 [2-12]	7 (4-15)
ICU mortality, n (%)	58 (19.0)	37 (17.1)	49 (18.5)
Hospital mortality, n (%)	100 (32.7)	57 (26.4)	68 (25.7)
14 day mortality, n (%)	73 (23.9)	36 (16.7)	40 (15.1)
28 day mortality, n (%)	87 (28.4)	46 (21.3)	56 (21.1)
90 day mortality, n (%)	113 (36.9)	70 (32.4)	-
1 year mortality, n (%)	139 (45.4)	97 (44.9)	-

583

584 Clinical characteristics of patients included in the discovery and validation cohorts.
585 Abbreviations: Q1-Q3, 1st quartile-3rd quartile. APACHE, Acute Physiology and Chronic
586 Health Evaluation. SOFA, sequential organ failure assessment. COPD, chronic obstructive
587 pulmonary disease. * Other includes: bone joint infection, endocarditis, mediastinitis,
588 myocarditis, ear infection, oral infection, pharyngitis, post-operative wound infection and lung
589 abscess.† APACHE IV score. ‡ APACHE II score.

590

Figure 1


522 sepsis patients in MARS, The Netherlands (Affymetrix U219 Array)
first validation cohort of 216 sepsis patients, Utrecht
second validation cohort of 265 CAP patients in GAinS, UK (Illumina HT-12 v4)
exploratory cohort of 81 pediatric sepsis patients, USA (Affymetrix U133 p2)
discovery cohort of 306 sepsis patients, Amsterdam
Unsupervised consensus clustering (5000 genes)
Random forest prediction of endotypes (140 gene classifier*)
Random forest prediction of endotypes (140 gene classifier*)
Random forest prediction of endotypes (140 gene classifier*)
306 sepsis patients classified as Mars1-4 endotypes
216 sepsis patients classified as Mars1-4 endotypes
265 CAP patients classified as Mars1-4 endotypes
81 pediatric sepsis patients classified as Mars1-4 endotypes
Endotype stability and quality evaluation (5000 genes)
Endotype stability and quality evaluation (140 genes)
Endotype stability and quality evaluation (140 genes)
Endotype stability and quality evaluation (140 genes)
39 sepsis patients excluded due to poor stability and quality
Association to clinical traits and outcome
267 sepsis patients for gene classifier derivation
Machine learning and crossvalidation defines a 140 gene classifier*

603 **Figure legends**

604

605 **Figure 1. Patient cohorts, samples and analysis**. MARS, Molecular diagnosis and Risk
606 stratification of Sepsis cohort. GAinS, Genomic Advances in Sepsis cohort.

607

608 **Figure 2**. **Unsupervised classification of sepsis patients and the association to clinical**
609 **characteristics and outcome (discovery cohort).** (**A**) Consensus clustering of the discovery
610 cohort (n=306) shows optimal partitioning to four clusters, labeled as Mars1, Mars2, Mars3 and
611 Mars4. (**B**) Silhouette width analysis illustrating stable partitioning to four molecular endotypes.
612 Percent of patients assigned to each subtype is denoted. (**C**) Gene expression heatmap illustrating
613 the 140 gene classifier derived to categorize the patients to endotypes. Red, over-expression;
614 turquoise, under-expression (heatmap rows). Endotypes are color coded and labeled (top bar).
615 (**D-F**) Endotypes were evaluated for their association to clinical severity indices, (**D**) septic
616 shock, (**E**) Sequential Organ Failure Assessment (SOFA) scores, (**F**) 28-day mortality by
617 Kaplan-Meier survival analysis. X^2, chi-squared.

618

619 **Figure 3**. **Assessment of sepsis molecular endotypes in the validation cohorts.** (**A**) Random
620 forest prediction of Mars1, Mars2, Mars3 and Mars4 endotypes in the first validation cohort
621 (n=261). Heatmap illustrating the 140 gene expression classifier. Red, over-expression;
622 turquoise, under-expression (rows). (**B-D**) Stratification of first validation cohort as Mars sepsis
623 endotypes was evaluated for the association against (**B**) total Sequential Organ Failure
624 Assessment (SOFA) scores, (**C**) septic shock, and (**D**) 28-day mortality by Kaplan Meier

survival analysis. (**E**) Random forest prediction of sepsis endotypes and heatmap representation of the second validation cohort (United Kingdom, E-MTAB-4421, n=265)[17]. (**F**-**H**) Stratification of the second validation cohort as Mars sepsis endotypes was evaluated for the association against (**F**) total Sequential Organ Failure Assessment (SOFA) scores, (**G**) septic shock and (**H**) 28-day mortality by Kaplan Meier survival analysis.

Figure 4. Biological interpretation of sepsis molecular endotypes. (**A**) Volcano plot representation of differential gene expression in the discovery cohort categorized to Mars1, Mars2, Mars3 and Mars4 endotypes each compared to healthy subjects (n=42). Plots integrate gene expression (log_2 fold expression of subtype versus healthy subjects, x-axis) and multiple-comparison adjusted P values (y-axis). Within plots, pie charts illustrate the extent of gene expression changes with red slices denoting the number of significantly over-expressed genes (adjusted P value < 0.05 and fold expression > 1.5), blue slices denoting significant under-expression (adjusted P value < 0.05 and fold expression < -1.5) and grey slices illustrating significantly differential gene expression (adjusted P value < 0.05) but outside of the fold expression < -1.5 and > 1.5 cutoff. (**B**) Correlograms illustrating the relationship of the gene expression changes in sepsis patients classified as Mars1-4 endotypes relative to healthy subjects. Rho, Spearman's correlation estimate. All correlations were significant ($p < 1x10^{-10}$). (**C**) Venn-Euler diagram illustrating the relationship in gene expression changes across the four sepsis molecular endotypes relative to healthy subjects. Red arrows denote significant gene over-expression, blue arrows denote significant gene under-expression. The high-risk Mars1 endotype is labeled to illustrate a portion of the gene expression response that is unique. (**D**) Ingenuity

pathway analysis of unique canonical signaling gene sets per endotype (columns in heatmaps). Canonical signaling pathways were grouped into super pathways. Heatmaps represent over-representation Fisher's test probabilities (considering multiple comparison adjusted $P < 0.01$). Red spectrum, significantly over-expressed canonical pathways; turquoise spectrum, significantly under-expressed canonical pathways (rows).

Figure 5. Derivation, validation of candidate sepsis molecular endotype biomarkers.

(**A**) Receiver-operator characteristics (ROC) area-under-the-curve (AUC) analyses of gene expression sepsis endotype classifier scores for Mars1 (*BPGM*:*TAP2*), Mars2 (*GADD45A*:*PCGF5*), Mars3 (*AHNAK*:*PDCD10*) and Mars4 (*IFIT5:GLTSCR2*) in the discovery cohort. 95% CI, bootstrap resampled 95% confidence interval. (**B**) Stripcharts illustrating the significant differences in sepsis endotype scores. Black horizontal line denotes median. Red horizontal line denotes the threshold defined by the best coordinate on the ROC curve. (**C**) Stripchart illustrating the Mars1 sepsis endotype score in the first validation cohort from the Netherlands. (**D**) ROC AUC analysis of the Mars1 sepsis endotype score in the first validation cohort. 95% CI, bootstrap resampled 95% confidence interval (**E**) Stripchart illustrating the Mars1 sepsis endotype score in the second validation cohort from the United Kingdom. (**F**) ROC AUC analysis of the Mars1 sepsis endotype score in the second validation cohort. 95% CI, bootstrap resampled 95% confidence interval