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BLOOD BIOMARKER AND RAMAN ANALYSIS OF LEUKOCYTES IN THE MULTICENTER INTELLIGENCE TRIALS: AT THE INTENSIVE CARE UNIT (INTELLIGENCE-1) AND THE EMERGENCY DEPARTMENT (INTELLIGENCE-2)

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ABSTRACT—**Background:** Sepsis, defined as life-threatening organ dysfunction caused by a dysregulated host response to infection, goes along with a complex and not yet fully understood host response. Despite many different biomarkers, optimal screening tools to identify patients with sepsis are still needed. In a previous, single-center clinical trial with well-defined and well-separated patient cohorts, a new biophotonic marker based on the Raman spectroscopic characterization of leukocytes showed added value to stratify patients and identify infection and sepsis. **Results:** In the INTELLIGENCE studies, 279 patients from six centers in two countries were analyzed, and the Raman spectra of ~1500 leukocytes per patient were measured within only 1 hour. This marks a huge step from bench to bedside regarding usability and technology readiness level of Raman spectroscopy in multicentre clinical trials. With a discriminatory power comparable to individual conventional biomarkers (C-reactive protein, procalcitonin, interleukin-6, and soluble urokinase-type plasminogen activator receptor), the Raman score of leukocytes could not provide added value to the clinical discrimination of sepsis in the INTELLIGENCE-1 study cohorts of intensive care patients with infections. When translating the classification model from INTELLIGENCE-1 to the heterogeneous patient cohort recruited at the emergency department of the double-blinded INTELLIGENCE-2 trial, the Raman score failed to provide added value. **Conclusions:** In theory, Raman assessment of leukocytes might still be a promising tool for sepsis diagnosis and patient stratification, but there is more basic, translational, and clinical research needed to refine its usability and clinical role, probably also taking questions of the pathophysiology of the dysregulated host response for a phenotype stratification into account.

KEYWORDS—Raman spectroscopy; biomarkers; sepsis; diagnostic algorithm; leukocytes; intensive care unit; emergency department

Abbreviations: AUC: area under the curve; CRP: C-reactive protein; EDTA: Ethylenediaminetetraacetic acid, HT: high-throughput; IL-6: interleukin-6 (IL-6), PCT: procalcitonin; qSOFA: quick sepsis-related organ failure assessment; suPAR: soluble urokinase-type plasminogen activator receptor; ED: Emergency Department; ICU: Intensive Care Unit; rcf: relative centrifugal force, ROC curve: receiver operating characteristic curve; RT: room temperature.

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Both Intelligence-1 and Intelligence-2 parts of the study were approved by the local ethics committees.

The two individual parts of the study have been registered: Clinical trials.gov identifier NCT03306186 (Intelligence-1) and NCT03350113 (Intelligence-2).

Data availability: The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

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BACKGROUND

Sepsis is defined as life-threatening organ dysfunction caused by a dysregulated host response to infection (1). The host response is complex and not fully understood (2–4). As patients present very heterogeneously, the classification of sepsis subtypes and endotypes has been suggested (4–6). Many different molecular biomarkers have been proposed to map this complexity; however, the reliable differentiation of sepsis from the immune response to severe, noninfectious stimuli or from localized infection with a regulated host response is still limited (7–9). "What markers identify a dysregulated host response to infection" has therefore been named an important research question by the Surviving Sepsis Campaign in 2021 (2). The question "What is the best strategy for screening and identification of patients with sepsis?" was identified by the Surviving Sepsis Campaign Research 2023 as the top clinical research priority (10).

A potential marker of interest is the Raman spectrum of leukocytes (11). By analyzing the inelastically scattered light from the cells after laser illumination, it can characterize their biochemical composition and give a molecular fingerprint of the whole cell (12–14). The method is label-free, fast, and has advanced to high-throughput devices (15). *In vitro* studies could reveal characteristic Raman spectral differences of activated and nonactivated leukocytes, allowing also a differentiation based on the type of stimuli (12,16,17). Our first translational pilot trial with a small cohort of well-defined patients showed that leukocyte Raman spectra could be used to differentiate between sterile inflammation, localized infection, and sepsis (11). Combining complementary information from the Raman score in addition to conventional biomarkers yielded improved diagnostic accuracy (11).

In this contribution, we performed the next translational step employing a larger patient cohort in two multicentre clinical trials. In those two clinical trials, named INTELLIGENCE-1 and INTELLIGENCE-2, we aimed to establish a workflow, how to included Raman leukocyte analysis in the workflow of clinical assessment and evaluate the diagnostic performance of Raman spectrometry in combination with established molecular biomarkers, vital signs, and demographic information for the diagnosis of sepsis and infection in the intensive care unit (ICU) (INTELLIGENCE-1) and unselected emergency room patients (INTELLIGENCE-2). To benchmark the Raman score, conventional biomarkers (7–9), in particular, C-reactive protein (CRP), interleukin-6 (IL-6), procalcitonin (PCT), and soluble urokinase-type plasminogen activator receptor (suPAR) were used in this study.

METHODS

Clinical trial design and trial centers

INTELLIGENCE-1 and -2 are diagnostic, prospective, and observational, multicentre clinical trials to validate and improve the performance of the Raman based diagnostic approach for the rapid assessment of critically ill patients in two settings: INTELLIGENCE-1 aimed to identify sepsis patients among critical ill patients at the ICUs while INTELLIGENCE-2 aimed to stratify patients at the Emergency Department (ED) with the risk of a poor outcomes based on at least one point in the quick sepsis-related organ failure assessment (qSOFA) score. Both studies were approved by the local ethics committees and registered at clinical trials.gov (INTELLIGENCE-1: NCT03306186 and INTELLIGENCE-2: NCT03350113). Written informed consent was obtained from the patient or first-degree relatives (Athens) or the patient or legal guardian (Jena).

INTELLIGENCE-1 recruited between October 2017 and January 2018 at five ICUs in Greece (1. First Department of Propedeutic Surgery, Ippokration General Hospital, Athens; 2. Intensive Care Unit, Ippokration General Hospital, Athens; 3. Fourth Department of Internal Medicine, ATTIKON University Hospital, Athens; 4. Intensive Care Unit, Aghia Olga Konstantopouleion General Hospital, Athens; 5. Intensive Care Unit, Tzanio Hospital of Piraeus, Piraeus). Critically ill patients admitted to the ICUs with acute pancreatitis or post operative or with clinical signs of infection a minimum qSOFA of 1 were included within 24 hours after the first symptom appeared.

INTELLIGENCE-2 recruited between October 2017 and January 2018 at the University of Athens, Athens, Greece, and after transfer of the Raman device between February 2018 andApril 2018 at the Jena University Hospital, Jena, Germany. Patients were included within 24 hours of admission to the ED if they had a minimum qSOFA of 1. Patients with acute myocardial infarction or severe trauma as an admission diagnosis were excluded. In Jena recruitment was done as an accompanying project to a running study with the same inclusion and exclusion criteria (18).

Sample collection

Blood samples were collected following standard protocols. Blood was collected into Ethylenediaminetetraacetic acid containing tubes (4.9 ml Sarstedt, Nuembrecht, Germany) within 1 hour of inclusion into the study.

Biomarker analysis

Full blood count and analysis of IL-6, procalcitonin (PCT), CRP was performed during routine clinical analysis. While

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Author contributions: M.B., E.J.G.-B., J.P., and U.N. designed the work, secured funding, and interpreted the data. E.J.G.-B., K.T., A.P., S.K., C.M., D.T.R., J.W., M.B., and F.B. supervised clinical trial and recruited patients. J.R., F.K., and I.W.S. developed Raman system, collected, and approved clinical data. A.R., D.T.R., A.P., N.A., J.R., J.E.O., M.K., A.K., S.C., N.A., G.D., and K.L. performed tasks in the clinical trial and recorded data. A.R., D.T.R., O.R., K.S.F.A., T.B., and E.J.G.-B. analyzed data. All authors discussed and interpreted the results. U.N., E.J.G.-B., A.R., and D.T.R. wrote the manuscript. All authors corrected and approved the manuscript.

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suPAR is included in routine diagnostics in the Greek hospitals, it was performed at the end of the study for all samples of INTELLIGENCE-2 included in Germany in a batch analysis. All analysis of suPAR was carried out using the suPARnostic ELISA, ViroGates, Denmark. Measurements below or above the limit of detection had the respective limits assigned as values for further analysis.

Raman-based analysis of blood-derived leukocytes

Leukocytes were isolated from full blood and investigated within 1 hour after collection using protocols established and published previously (11). Briefly, 500 µl of Ethylenediaminetetraacetic acid-blood was transferred into a falcon tube and centrifuged for 10 minutes with 2000 rcf at room temperature. From the cell pellet, leukocytes were isolated by lysing erythrocytes with BD lysis buffer (Beckman Coulter GmbH). Immediately after isolation, the leukocytes were chemically fixed for 20 minutes with a formaldehyde-based fixative solution. Afterwards, the cells were washed successively with PBS and resuspended in PBS with a cell concentration of 4×10^6/ml. Approximately 20 µl of cell suspension was placed on a calcium fluoride slide (Crystal GmbH, Berlin, Germany) in a petri dish filled with distilled water. The cells were allowed to settle down for a few minutes (~5 min).

Raman measurements spectra of the leukocytes were recorded on a high-throughput Raman device (19) with an upright microscope equipped with a 785 nm single-mode excitation laser giving a power of 100 mW at the plane of the objective (60 × /1.0 NA water immersion Nikon). The spectrometer is equipped with a grating of 400 grooves/mm, blazed at 750 nm, and allows a spectral resolution of 9 cm^{-1}. A custom-made algorithm (19) allowed for capturing of bright-field images, automatic selection of the cells followed by the acquisition of the Raman spectra by moving the motorized x-y translation. Approximately, 1500 leukocytes/patient were recorded in an automated manner with a laser exposure of 1 seconds per cells, resulting in a total measurement time of less than 1 hour/patient.

Raman analysis to obtain a Raman spectroscopy score

The Raman spectra of the leukocytes were preprocessed to make the Raman data collected from different cells and patients comparable. Briefly, preprocessing involved spike removal (20), wavenumber calibration based on 4-acetamidophenol standard (21), sensitive nonlinear iterative peak baseline correction (22), and vector normalization. Outliers were removed by excluding the spectra that had a Pearson correlation coefficient below 0.95 when correlated with the average of the dataset. Afterwards, spectra were averaged per each patient, as each patient initially had over 1000 spectra.

The preprocessed Raman spectra of the leukocytes from INTELLIGENCE-1 were subjected to a two-level cross-validation (CV) approach (23) using Support Vector Machine to build a binary model to differentiate sepsis *versus* no sepsis. In the first level (in the inner CV), we optimized parameters that affect how the model works (i.e., "cost" parameter and "kernel"). After fine-tuning, the model was tested using a second, outer level (outer CV) of CV. This step involved using new data that the model had not seen before, ensuring that the results are accurate and can be trusted when applied to real-world scenarios. The predictive Raman model was evaluated using leave-one patient-out CV yielding prediction probabilities for the positive group (e.g., sepsis) with values between 0 and 1 that were used as Raman spectral marker scores in further analysis. To obtain class labels from the marker directly, a threshold of 0.5 was used on the predicted Raman score. The trained model was then applied to the blinded spectral data from INTELLIGENCE-2 clinical trial to obtain Raman spectral scores and predict sepsis patients.

Data analysis

Patients were grouped into sepsis, infection without sepsis, and no infection by experienced physicians with experience in chart review for sepsis diagnosis. Patients' demographic and clinical characteristics are presented by descriptive statistics depending on the structure and distribution of data. To assess the classification performance of molecular biomarkers, qSOFA, and Raman score to predict sepsis, receiver-operating characteristic curves with area under the curve (AUC) were calculated. To assess the benefit of Raman we calculated a binary logistic regression model with sepsis as the dependent variable and molecular biomarkers and qSOFA as covariates and an additional model with the Raman score as an additional covariate. Both model's classification performances were assessed by receiver-operating characteristic with AUCs.

A P value of less or equal to 0.05 was considered statistically significant for all tests. Estimated values are presented with 95% confidence intervals. Missing values were handled by pairwise deletion. Analyses were performed using IBM SPSS Statistics 28 (IBM, Armonk, NY).

RESULTS

The prospective, observational clinical studies INTELLIGENCE-1 and INTELLIGENCE-2 included 278 patients, 168 critically ill patients admitted to ICU were recruited within the INTELLIGENCE-1 and 111 patients from the ED with a qSOFA of ≥ 1 were included in the INTELLIGENCE-2 clinical study (Fig. 1). The patient assessment along with their baseline characteristics and comorbidities are presented in Table 1 (and separately for INTELLIGENCE-2 Athens and Jena cohorts in Table S1). Sepsis diagnosis information was not available to the Raman data analysis team in INTELLIGENCE-2 as it was a blinded study.

INTELLIGENCE-1

Discriminatory power of qSOFA, conventional biomarkers (CRP, IL-6, PCT, and suPAR) and Raman score for the discrimination of sepsis and infection in the ICU-treated cohort was relatively low with AUCs ranging from 0.62 to 0.73 (Fig. 2A) with the lowest performance for the Raman score. A model containing all four biomarkers performed slightly better (AUC 0.79) and was not significantly improved by the addition of the Raman score (AUC 0.81) (Fig. 2B).

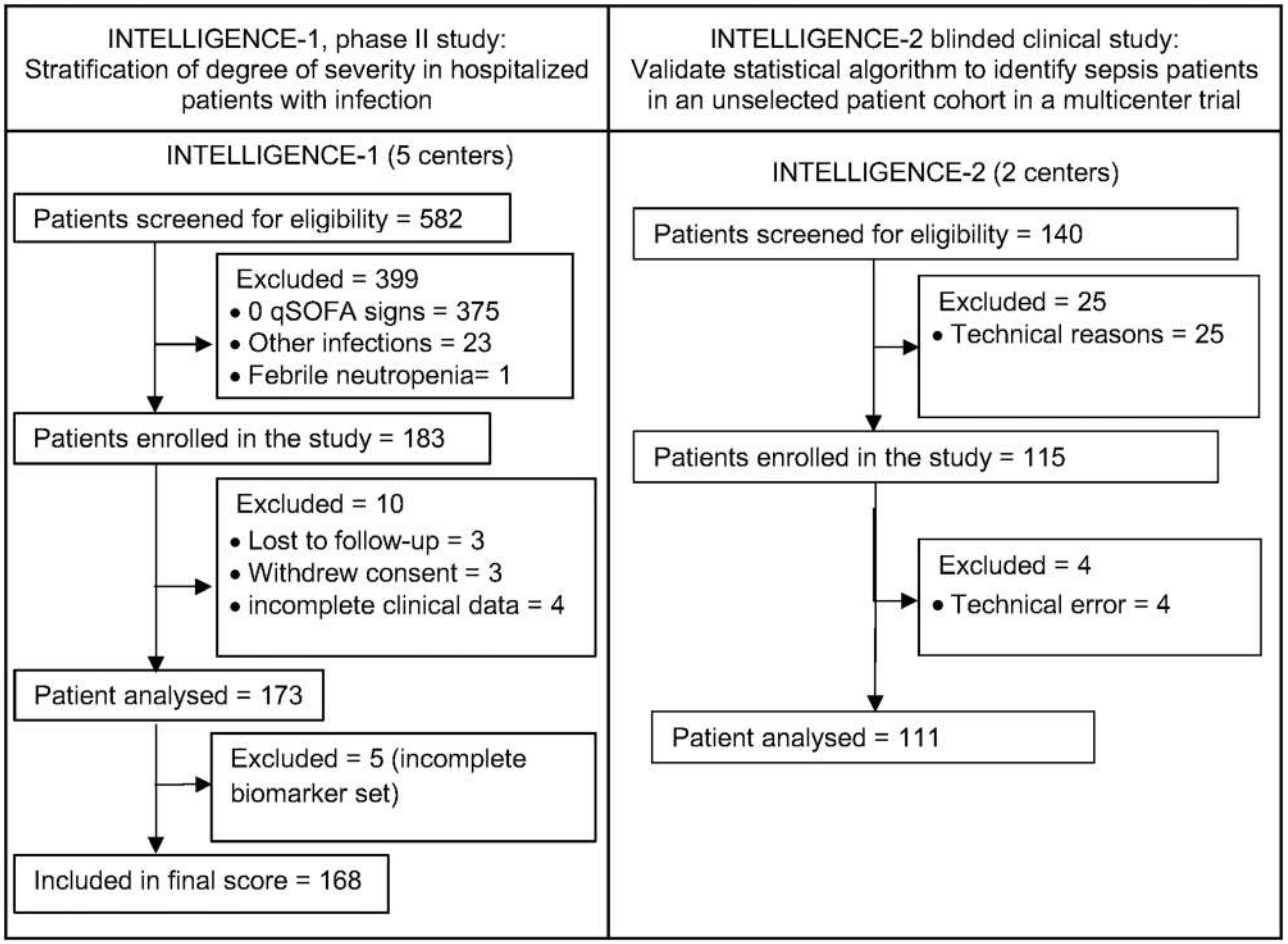

Fig. 1. **Flow chart of patient recruitment for INTELLIGENCE-1 at the intensive care units of five centers in Greece and INTELLIGENCE-2 at the emergency departments at two centers (one in Greece and one in Germany).**

INTELLIGENCE-2

In the INTELLIGENCE-2 cohort, comprising patients from the ED with sepsis, infection, or sterile conditions associated with a qSOFA ≥ 1, the discriminatory power of qSOFA, conventional biomarkers (CRP, IL-6, PCT, and suPAR) and Raman score for the identification of sepsis diagnosed within 96 hours after emergency room admission was similarly low (AUC 0.56–0.78) with the lowest performance for the Raman score derived from the INTELLIGENCE-1 cohort (Fig. 3A). A model containing all four biomarkers performed not better than PCT alone (AUC 0.77) and was not significantly improved by the addition of the Raman score (AUC 0.79) (Fig. 3B).

DISCUSSION

Our study failed to show an additional diagnostic value of our Raman assay for sepsis detection in the assessed cohorts, but established a usability of the technology beyond the research laboratory.

Our initial pilot study (11), which showed promising results, looked at three distinct groups: sterile SIRS, mostly after cardiac surgery, ICU-treated sepsis, and infection treated on a normal ward. While all these patients have systemic inflammation, they are still clinically clearly distinct. Even though we planned to include post surgical patients and pancreatitis as noninfectious patients in INTELLIGENCE-1, nearly all recruited patients had infections treated in the ICU. In those patients, the differentiation of infection with or without sepsis is somewhat arbitrary as those are distributed more along a clinical and pathophysiological continuum and not two dichotomous groups (24). Therefore, the low performance of Raman and classical biomarkers is not to surprising and most likely a result of unlucky recruitment.

As the Raman score was developed in a cohort of infected patients, its low performance in INTELLIGENCE-2, where half of the patients were not infected, is not surprising in hindsight. We plan to reanalyze this cohort to see whether Raman is able to distinguish between qSOFA positive emergency room patients with and without infection. This question is highly relevant as it has therapeutic consequences. As widely discussed, delayed antibiotic therapy in infection and antibiotic overtreatment of patients without infection are both problematic (25).

Beyond the relatively simple questions of infection *versus* no infection and sepsis *versus* infection without dysregulated host response the pathophysiology and phenotype of the dysregulated host response has gained increased interest in recent years (26,27). Distinguishing them would allow precision medicine aimed at the individual pathophysiology (26,28–30).

TABLE 1. **Clinical characteristics of the patient cohorts**

	INTELLIGENCE-1 (multicenter, n = 5)		INTELLIGENCE-2 (multicenter, n = 2)	
Condition	Infection	Sepsis	No sepsis	Sepsis*
Number of patients, n (%)	62 (36.9)	106 (63.1)	83 (74.7)	28 (25.3)
Reason for inclusion				
Signs of infection, n (%)	56 (90.3)	97 (91.5)		
Post operative, n (%)	4 (6.5)	7 (6.6)		
Acute pancreatitis, n (%)	2 (3.2)	2 (1.9)		
Infection diagnosis, n (% of the subgroup)*			26 (31.3)	28 (100)
Sex, Female, n (%)	37 (59.7)	48 (45.3)	45 (54.2)	10 (35.7)
Age	71 (47–83)	79 (66–85)	74 (64–83)	77 (68.5–82)
APACHE II	7 (5–10) (1)	14,5 (11–20.5) (2)	–	–
qSOFA	1 = 57	1 = 63	1 = 81	1 = 20
	2 = 3	2 = 32	2 = 2	2 = 6
	3 = 2	3 = 11	3 = 0	3 = 2
Charlson's comorbidity index	4 (1–6)	6 (4–7)	2 (1–5)	2.5 (0–5)
Leukocyte Gpt/l	9.9 (7.1–11.9)	12.7 (9.2–17.5)	8.5 (6.8–10.8) (3	10.9 (7.8–14.9)
Relative neutrophil count %	77 (70–87)	84 (76–89)(2)	-	-
Relative lymphocyte count %	14 (7–20)	9 (5–17)(2)	-	-
Relative monocyte count %	7 (5–10)	6 (4–8)(2)	-	-
CRP mg/l	26.3 (6.4–92.1)	93.9 (25.3–161)	17.0 (5.7–46.6)	79.2 (12.2–174.6)
IL-6 pg/ml	3.12 (3.12–22.5)	29 (3.12–114.6)	4.9 (0–28.1)	15.6 (0–108.0)
PCT ng/ml	0.1 (0.1–0.3)	0.4 (0.1–3.7)	0.1 (0.1–0.2)	0.4 (0.1–3.1)
suPAR ng/ml	4.4 (2.9–6.2)	7.1 (4.9–13.3)	4.4 (3.2–5.8)	5.5 (3.8–8.4)
28 day mortality	6 (9.7)	40 (37.7)	5 (6.0)**	3 (10.7)**

Data are presented as median (IQR) or n (%).
*Diagnosed within 96 hours from emergency room visit;
**Information only available for patients admitted from the emergency room.
Number in parenthesis indicates the number of missing values (if any) in each group.
APACHE, acute physiology and chronic health evaluation; CRP, C-reactive protein; IL-6, interleukin-6; PCT, procalcitonin; qSOFA, quick sepsis-related organ failure assessment; suPAR, soluble urokinase-type plasminogen activator receptor.

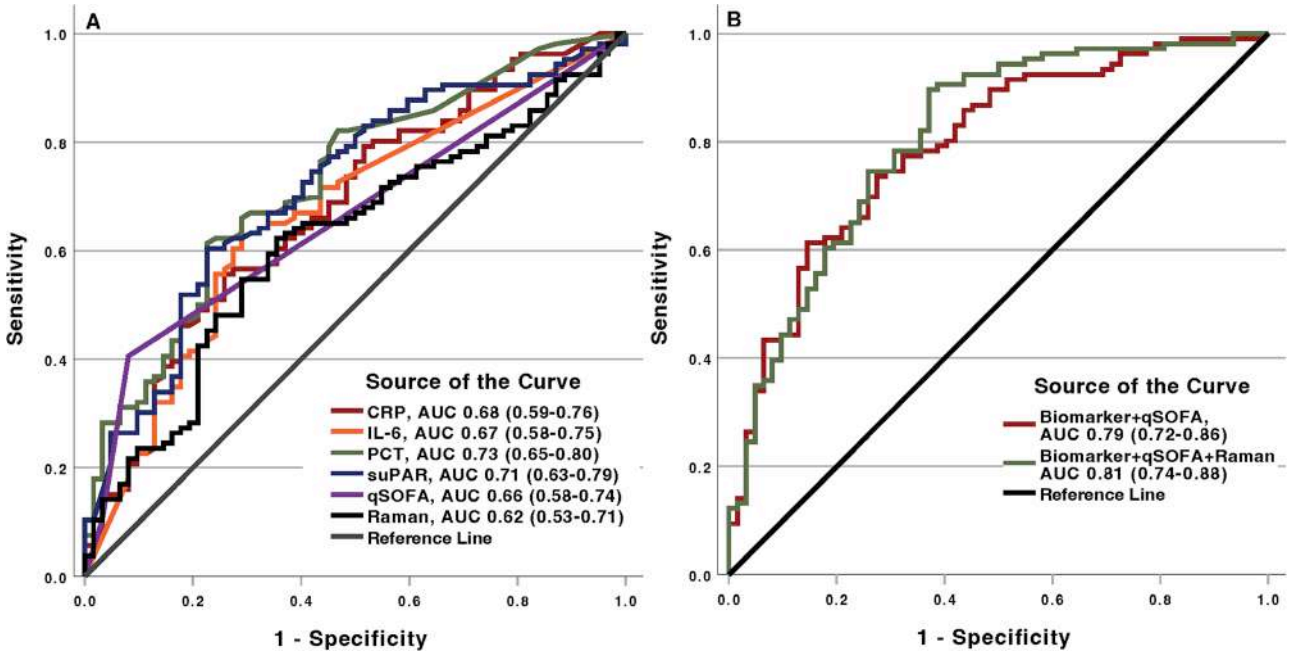

FIG. 2. **Receiver operating characteristic (ROC) curve to discriminate sepsis and infection in INTELLIGENCE-1 (ICU-treated) cohort.** A, displayed for all individual molecular biomarkers, qSOFA score, and Raman score. B, displayed for a model containing all four molecular biomarkers (CRP, IL-6, PCT, and suPAR) and qSOFA combined with and without the addition of the Raman score. qSOFA, quick sepsis-related organ failure assessment; suPAR, soluble urokinase-type plasminogen activator receptor. PCT, procalcitonin.

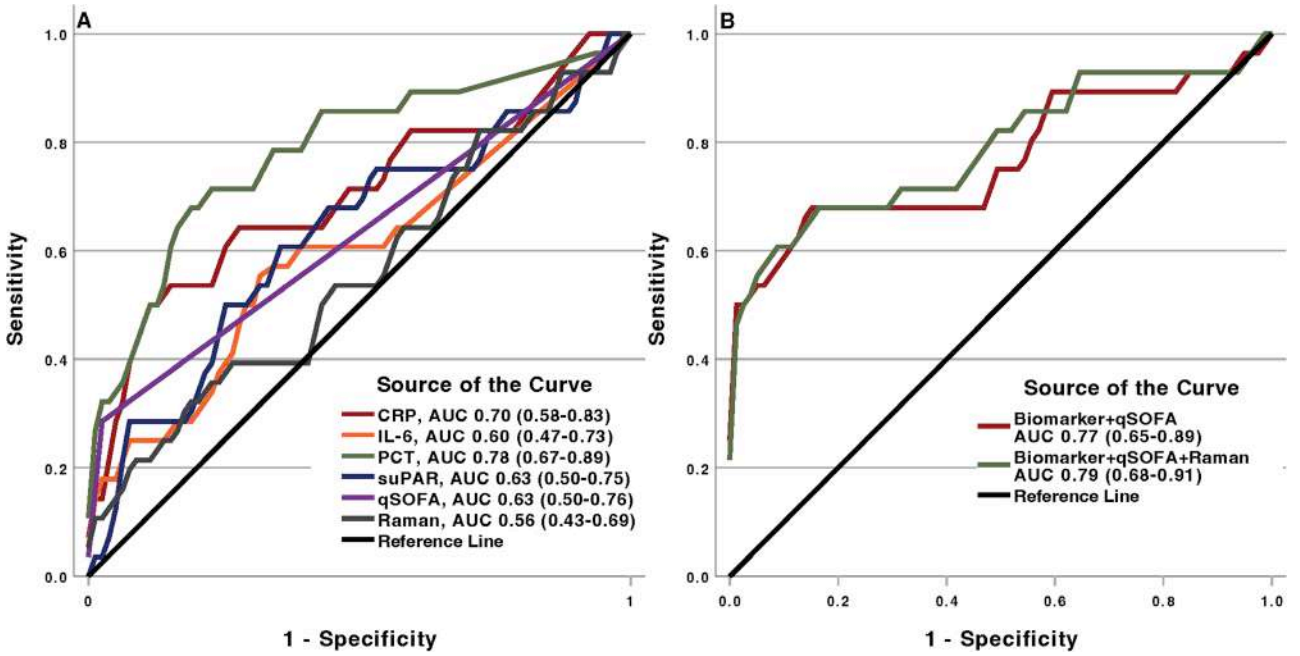

Fig. 3. **Receiver operating characteristic (ROC) curve to diagnose sepsis in INTELLIGENCE-2 (Emergency department) cohort.** A, displayed for all individual molecular biomarkers, qSOFA score, and Raman score. B, displayed for a model containing all four molecular biomarkers (CRP, IL-6, PCT, suPAR) and sSOFA combined with and without the addition of the Raman score. qSOFA, quick sepsis-related organ failure assessment; PCT, procalcitonin; suPAR, soluble urokinase-type plasminogen activator receptor.

Raman measurements of leukocytes, by providing a molecular fingerprint of the activated cell, could be a promising additional tool in rapidly phenotyping patients and future research should be directed at this goal.

Our intended translational step to bring Raman from bench to bedside failed when you looked at diagnostic accuracy only. However, our trials showed a huge step from bench to bedside regarding usability and technology readiness level (31). Our previous pilot study (11) was performed in a single-center only with the device set up in a research laboratory, operated by Raman researchers and we could analyze only 30 cells per patient. In the INTELLIGENCE studies presented here, we recruited patients from six centers in two countries, the Raman device was set up in the clinical chemistry laboratories of the participating hospitals and was operated by newly trained lab personnal (partly, without previous Raman experience), and we measured about 1500 cells per patient within only 1 hour. This opens the door for future multicenter studies with large and diverse patient cohorts.

CONCLUSIONS

In theory, Raman assessment of leukocytes might still be a promising tool for sepsis diagnosis and patient stratification, but there is more basic, translational, and clinical research needed to refine its usability and clinical role.

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