Development and validation of IVDMIA as a novel biomarker of early sepsis using metabolomics approach

SUN YOUNG AHN

Department of Medicine

The Graduate School, Yonsei University

Development and validation of IVDMIA as a novel biomarker of early sepsis using metabolomics approach

Directed by Professor Sang-Guk Lee

The Doctoral Dissertation submitted to the Department of Medicine,
the Graduate School of Yonsei University
in partial fulfillment of the requirements for the degree of Doctor of Philosophy

SUN YOUNG AHN

June 2020

This certifies that the Doctoral
Dissertation of SUN YOUNG AHN
is approved.

Thesis Supervisor: Sang-Guk Lee

Thesis Committee Member#1: Moo Suk Park

Thesis Committee Member#2: Nam Su Ku

Thesis Committee Member#3: Young-Min Hyun

Thesis Committee Member#4: Sail Chun

The Graduate School
Yonsei University

June 2020

< ACKNOWLEDGEMENTS >

I believe that we derive gratification from the action, and not the result. Although there were certain moments when I felt my work was challenging and wanted to give up, I am assured that my journey toward earning a doctoral degree has helped me grow and develop my potential.

First, I would like to offer my gratitude to my supervisor, Professor Sang-Guk Lee, who has encouraged and led me to this position with his sincere guidance and continuous assistance. I am also grateful to members of the thesis committee, Professor Moo Suk Park, Professor Nam Su Ku, Professor Young-Min Hyun, and Professor Sail Chun for their valuable advice.

In particular, I would like to thank to Professor Yong-Sik Kim, who has been a constant companion since I became a medical practitioner. He offered assistance and advice whenever I faced any adversity.

Finally, I would like to express my gratitude to my family members—my father, Chung Mo Ahn, my mother, Dong Suk Seo, and my younger brother, Hyojin Ahn. They have stood by me each time I went through highs and lows in my journey.

I also extend my gratitude to all individuals who supported me during my course for completing my thesis, including Jihye Ha.

I am about to complete the long journey toward earning a doctoral degree. The moments seemed to have passed in the blink of an eye, like a midsummer night's dream. I am convinced that from this moment, a new dream awaits me. I know that I am at the starting point yet again, and I will keep doing my best to become a qualified and dedicated professional.

Thanking you,

Sunyoung Ahn
June 2020

< TABLE OF CONTENTS >

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Development and validation of IVDMIA as a novel biomarker of early sepsis using metabolomics approach

Sunyoung Ahn

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Sang-Guk Lee)

Background: Sepsis is a syndrome that is influenced by pathogens and host factors and is characterized by an aberrant or dysregulated host response and organ dysfunction. Sepsis is the primary cause of death from infection, especially if not recognized and treated promptly. Its recognition mandates urgent attention.[1] However the underlying mechanisms of sepsis are not completely understood. Therefore, a multimarker strategy may be helpful for improving the understanding of the complex pathogenesis of sepsis and its evolution, and especially for facilitating early risk stratification and implementing personalized therapies. The use of emerging metabolomics tools is particularly promising for the diagnosis of complex and heterogeneous conditions such as sepsis and septic shock.

Methods: Several different biomarkers are known to aid the diagnosis of sepsis and are

used as severity or outcome indexes for patients with sepsis. For the application of metabolomics to sepsis research, we recruited individuals and categorized them in three groups – normal healthy group, systemic inflammatory response syndrome (SIRS) group, and sepsis group. We assayed the serum levels of amino acids and the respective metabolites using liquid chromatography-tandem mass spectrometry (LC-MS/MS) and analyzed the result to screen promising biomarkers of sepsis. Lastly, an In Vitro Diagnostic Multivariate Index Assay (IVDMIA) was developed by formulating the indexes using the amino acids selected previously.

Results: Principle component analysis (PCA) was performed using the results of metabolomics research. Based on the score plot and loading plot, we confirmed that the three aforementioned groups were distinguishable and formed clear clusters. We could also infer that amino acid derangements exist in patients with sepsis, and that these metabolomic derangements could be employed as biomarkers using LC-MS. Based on the PCA results, we selected the candidate amino acids that could be used in IVDMIA. We selected four index amino acids (kynurenine (KYN), tryptophan (TRP), phenylalanine (PHE), and arginine (ARG)) and one ratio (KT ratio). We developed various formulas using the five variables mentioned. After assessing the sensitivity, specificity, accuracy, and area under the ROC curve (AUC) from each formula, the formula with the highest performance was selected for developing the IVDMIA for sepsis. The generated IVDMIA was subjected to validation processes, which included performance comparison with preexisting sepsis markers such as White Blood Cell (WBC), C-Reactive Protein (CRP), and procalcitonin (PCT). The selected IVDMIA formula is as follows: ARG × (-0.0513) + PHE × 0.0642 + KT ratio × 27.6591 - 5.4765. We observed that the developed IVDMIA had similar or better potential than procalcitonin as a sepsis marker.

Conclusions: This study demonstrated that the amino acid composition of the body in

patients with sepsis differs significantly from those in normal individuals or in patients with SIRS. We developed an IVDMIA specific for sepsis and validated it. The IVDMIA developed in this study can be used to diagnose sepsis in patients. Small-scale or point-of-care testing MALDI-TOF can be used instead of LC-MS/MS for amino acid analysis, which improves the applicability of the test. It is expected to be ready for clinical practice in the near future. In addition, the variations in the concentrations of amino acids we assessed improved our understanding of metabolic alterations in sepsis.

Key words: sepsis, septic shock, biomarker, IVDMIA (In vitro Diagnostics Multivariate Index Assay), amino acid derangement, principle component analysis (PCA), liquid chromatography-tandem mass spectrometry (LC-MS/MS), matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF), procalcitonin (PCT), kynurenine (KYN), tryptophan (TRP), kynurenine/tryptophan ratio (KT ratio), arginine (ARG), phenylalanine (PHE), white blood cell (WBC), C-reactive protein (CRP), procalcitonin (PCT)

Development and validation of IVDMIA as a novel biomarker of early sepsis using metabolomics approach

Sunyoung Ahn

Department of Medicine

The Graduate School, Yonsei University

(Directed by Professor Sang-Guk Lee)

I. INTRODUCTION

Sepsis is a syndrome that is influenced by pathogenic and host factors and is characterized by aberrant or dysregulated host response and organ dysfunction. Patients with acute organ dysfunction are considered to have severe sepsis.[2] Care of patients with sepsis costs as much as $50,000 per patient, which consequently leads to an annual economic burden of nearly $17 billion in the United States alone.[3] Sepsis is often lethal, and 20 to 50 percent severely affected patients are killed. It is the second leading cause of death among patients in non-coronary intensive care units (ICUs) and the tenth leading cause of death overall in the United States.[4] Furthermore, sepsis substantially reduces the quality of life in survivors. Both immune and endothelial dysfunction are thought to contribute to the high mortality rate in sepsis[5]; however, the exact underlying mechanisms are yet to be understood completely.

Septic shock is the most severe complication resulting from sepsis and is one of the major causes of deaths in ICUs.[6] Although treatment methods have improved over the past decades, the outcomes are poor and difficult to predict. Indeed, the majority of patients who suffer from septic shock develop multiple organ dysfunction syndrome (MODS), a condition in which organs that are not directly affected by the original infection become dysfunctional, and this ultimately forms the primary cause of death.[7] The interrelationship among a series of septic courses that comprise inadequate oxygen delivery to peripheral tissues, ischemia and reperfusion injury in organs, hemodynamic instability, inflammation, and development of MODS has been extensively investigated; however, the underlying molecular mechanisms which ultimately trigger tissue functional injury remain largely undetermined.[8]

Therefore, a multimarker strategy may improve our understanding of the complex pathogenesis in sepsis and its evolution, and may especially help us facilitate early risk stratification and implement personalized therapies.[9] The use of emerging omics tools that can be used to evaluate physiological responses at the system level are particularly promising for the diagnosis of complex and heterogeneous conditions such as sepsis.

Recently, several studies have been published that have focused on the investigation of plasma metabolomic profiles as predictive signatures of ICU mortality in adult patients.[10-12] Different patterns of metabolite composition have been identified using nuclear magnetic resonance or mass spectrometry. Although these methods have different intrinsic metabolomic coverage potential, they clearly highlight the widespread metabolic abnormalities in patients with sepsis and septic shock and the interplay of several different biochemical pathways.

To date, several different bioactive molecules have been proposed as severity or outcome biomarkers in patients with sepsis.[13] Among these, bacterial products such as

endotoxin and bacterial DNA, acute phase proteins (protein C, procalcitonin (PCT), LPS-binding protein (LBP)), coagulation factors (fibrin degrading products, antithrombin III, D-dimer), membrane cell markers (HLA-DR, CD-64, Eselectin), cellular processes (apoptosis), hormones (cortisol, ACTH), soluble receptors (sCD-14, sTNFRI, sTNF-RII), and cytokines (TNF, IL-6, IL-8, IL-10) are the ones used most commonly. Nevertheless, only a limited number of biomarkers have been used in clinical practice; among the above-mentioned ones, C-reactive protein (CRP) and PCT are currently used as biomarkers in clinical practice.

Although CRP and PCT are considered useful biomarkers in sepsis, their precise role (if any) in the pathophysiology of sepsis and organ dysfunction remains unclear.[13] Besides infection, other noninfectious diseases, such as autoimmune diseases, trauma, burns, major surgeries, and malignant diseases, are important inducers of high CRP plasma levels. Interestingly, viral infections usually do not cause a significant increase in plasma CRP or PCT levels.

Recently, several studies have suggested that tryptophan, kynurenine, and the kynurenine/tryptophan ratio (KT ratio) could serve as novel biomarkers for measuring disease severity and predicting prognosis, particularly in sepsis.[14-16] However, a different study also showed a contradictory result.[17] Therefore, it is necessary to determine whether excessive tryptophan catabolism is specific to sepsis, and whether the biomarkers related to tryptophan catabolism could serve as reliable indicators in clinical practice. Also it is necessary to determine if there are amino acids apart from kynurenine and tryptophan that are metabolically related to sepsis.

In this study, the preemptive goal was to verify the feasibility of the preliminary metabolomics approach to identify candidate amino acids as biomarkers in sepsis. The biomarkers selected in the preliminary study were intended to be used in an ongoing

large-scale study, which aims to develop novel markers for sepsis based on metabolomics and compare the performance of newly developed biomarkers with that of biomarkers currently used in clinical practice, such as white blood cells (WBCs), CRP, and PCT. In addition, the use of these biomarkers could reveal the metabolic pathways involved in sepsis, which is a necessary step in the development of targeted therapy.

The ultimate aim of our study is the generation and validation of the applicability of the sepsis marker developed using the metabolomics approach. First, we sorted and selected the candidate amino acid biomarkers through our metabolomics study. Thereafter, the candidate amino acids were combined into single index using the In Vitro Diagnostic Multivariate Index Assay (IVDMIA) method.[18,19]

Currently, the Sequential Organ Failure Assessment (SOFA) score is most commonly used in the evaluation of sepsis patients. However, the SOFA score is not a user-friendly tool, because to calculate the score, a number of vital signs and key clinical features of a patient, such as the Glassgow coma scale, need to be recorded and entered in the equation.[1] The IVDMIA can be used as an alternative to this method. The IVDMIA can be used with candidate amino acids, and a highly sensitive and patient-specific marker that is useful for the screening, diagnosis, and prognosis of sepsis can be developed.[18-20] The sepsis IVDMIA index can be easily used by clinicians, as the concentrations of only a few amino acids are to be determined, which can easily be performed using liquid chromatography-tandem mass spectrometry (LC-MS/MS) or Matrix Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry (MALDI-TOF), which are commonly used techniques in front-line laboratories and are also available at a smaller-scale or for point-of-care testing. Most importantly, the IVDMIA only requires 50 μL of patient serum for the test.

In this study, we attempted to develop an IVDMIA specialized for sepsis by

analyzing the serum samples of sepsis patients and to validate the newly developed IVDMIA index. The entire process of this study is outlined in Figure 1.

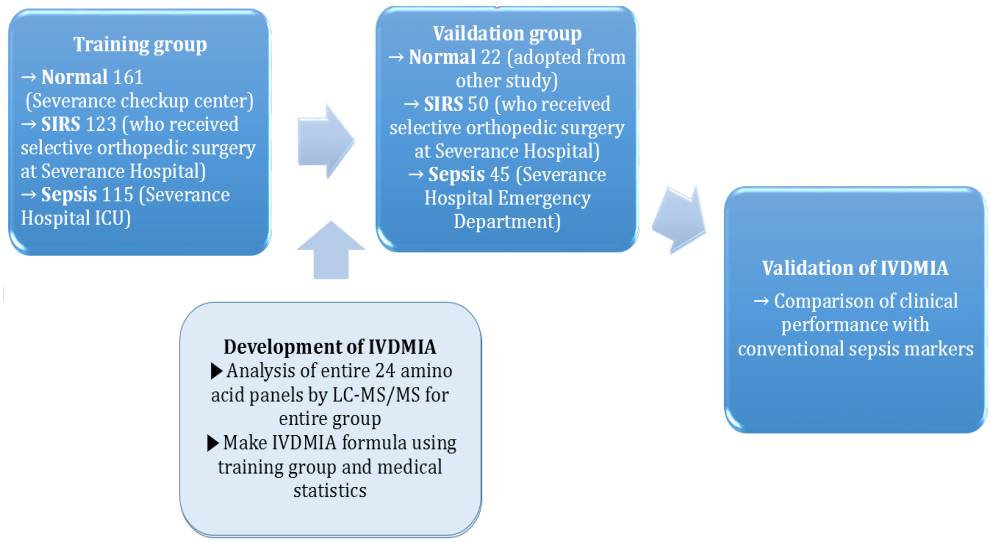

Fig 1. A schematic of the study. Summary of the development process of sepsis IVDMIA through metabolomics approach and validation of the newly developed IVDMIA based on various perspectives.

II. MATERIALS AND METHODS

1. Preliminary study

In our exploratory study, we adopted a targeted mass spectrometry-based quantitative metabolomics approach. Seventy-one participants were recruited for the study; ten participants were normal healthy controls, twelve were patients with pneumonia, and forty-nine were patients with sepsis. The serum samples of patients with sepsis were collected from the two cohorts: one from ICUs and the other from the emergency department (emergency room, ER) at Severance Hospital (Seodaemoon-gu, Seoul, Republic of Korea). Sample collection was conducted after the review and approval were conducted by the Institutional Review Board of Severance Hospital (IRB approval No. 4-2016-0605, 4-2017-0654). Sepsis was defined based on the standards of "Sepsis-3", which was published as a journal in 2016 and is commonly used worldwide.[1]

Liquid chromatography tandem mass spectrometry assay (AbsoluteIDQ 180 kit, Biocrates, Innsbruck, Austria), which is a targeted quantitative method involving the use of a combined direct flow injection, was used for the metabolomics analysis of serum samples collected from normal healthy controls and from patients with pneumonia or sepsis, which had been stored at -80℃.

This strategy allows the simultaneous quantification of 186 metabolites (40 amino acids and biogenic amines, 40 acylcarnitines, 90 glycerophospholipids, 15 sphingomyelins, and 1 monosaccharide), which is adequate for screening candidate metabolites. We focused on several series of metabolites, such as glycerophospholipids, amino acids, biogenic amines, and acylcarnitines, some of which have been identified previously as participants in key biochemical pathways in sepsis.[14,21]

2. Main study for metabolomics and IVDMIA development

A. Recruitment of the research subjects

In this study two groups were formed initially. One was the training group and the other was the validating group. In the training group, three subgroups were formed: healthy controls (N = 161), SIRS patients (N = 123), and sepsis patients (N = 115). The validating group was completely independent from the training group, and comprised three subgroups: healthy controls (N = 22), SIRS patients (N = 50), and sepsis patients (N = 45). This group was to be used for validating the IVDMIA developed in this study. The detailed features of each group are outlined in Table 1 and Table 2.

Table 1. Baseline characteristics of training group participants divided into three subgroups according to septic status. Data (except those for gender) are expressed in terms of median [IQR].

	Normal (N = 161)	SIRS (N = 123)	Sepsis (N = 115)	p value
Gender = M (%)	85 (52.8)	53 (43.1)	49 (42.6)	0.149
Age (yr)	46.00 [37.00, 54.00]	58.67 [44.54, 70.98]	69.40 [59.67, 77.64]	<0.001
Height (cm)	163.10 [158.30, 170.00]	166.00 [159.70, 173.00]	163.50 [157.25, 170.00]	0.062
Weight (kg)	59.80 [54.10, 69.60]	69.10 [59.50, 76.60]	58.50 [49.85, 68.50]	<0.001
BMI	23.05 [20.85, 25.09]	24.77 [21.88, 27.29]	21.77 [19.65, 25.61]	<0.001
Glucose (mg/dL)	91.00 [86.00, 99.00]	106.00 [94.50, 134.50]	136.00 [114.00, 194.00]	<0.001
Creatinine (mg/dL)	0.75 [0.64, 0.88]	0.82 [0.66, 1.04]	1.23 [0.71, 2.75]	<0.001
Total protein (g/dL)	7.30 [7.10, 7.60]	6.40 [5.95, 6.70]	5.10 [4.70, 5.95]	<0.001
Albumin (g/dL)	4.50 [4.40, 4.68]	3.70 [3.30, 4.00]	2.50 [2.20, 2.90]	<0.001
AST (IU/L)	22.00 [18.00, 26.00]	19.00 [15.00, 24.00]	34.00 [22.00, 62.00]	<0.001
ALT (IU/L)	17.00 [13.00, 26.00]	16.00 [11.00, 24.00]	19.00 [11.50, 34.00]	0.079
Bilirubin (mg/dL)	1.00 [0.80, 1.30]	0.70 [0.45, 0.90]	0.50 [0.30, 1.10]	<0.001
Hb (g/dL)	13.60 [12.80, 14.50]	12.20 [10.60, 13.50]	9.80 [8.35, 11.55]	<0.001
Hct (%)	41.50 [39.40, 44.10]	37.10 [33.10, 40.50]	30.60 [25.30, 35.50]	<0.001
Platelet (10^3/μL)	244.50 [204.50, 279.00]	237.00 [196.00, 300.00]	179.00 [79.50, 288.00]	<0.001
WBC (10^3/μL)	5.16 [4.36, 5.81]	7.65 [6.05, 9.35]	10.91 [7.22, 15.25]	<0.001
Procalcitonin (ng/mL)	0.02 [0.02, 0.03]	0.04 [0.02, 0.09]	1.99 [0.52, 11.24]	<0.001
CRP (mg/L)	0.56 [0.33, 1.23]	7.09 [1.47, 42.88]	128.90 [47.90, 227.80]	<0.001

Table 2. Baseline characteristics of validating group participants divided into three subgroups according to septic status. Data (except those for gender) are expressed in terms of median [IQR].

	Normal (N = 22)	SIRS (N = 50)	Sepsis (N = 45)	p value
Gender = M (%)	3 (4.5)	31 (62.0)	11 (42.3)	<0.001
Age (yr)	58.00 [55.75, 60.00]	64.18 [50.33, 72.95]	72.48 [59.33, 78.13]	0.001
Height (cm)	155.00 [150.00, 156.50]	169.00 [163.00, 172.07]	164.00 [158.25, 175.00]	<0.001
Weight (kg)	60.00 [55.00, 64.50]	67.95 [61.00, 71.70]	60.00 [48.00, 63.30]	<0.001
BMI	24.39 [23.52, 26.45]	24.06 [22.26, 24.77]	20.17 [18.45, 22.84]	<0.001
Glucose (mg/dL)	24.39 [23.52, 26.45]	109.50 [94.00, 132.75]	116.00 [104.00, 212.00]	<0.001
Creatinine (mg/dL)	16.20 [14.40, 19.90]	0.90 [0.71, 1.29]	1.30 [0.90, 1.54]	<0.001
Total protein (g/dL)	4.30 [4.20, 4.50]	6.20 [5.80, 6.60]	5.35 [5.00, 6.00]	<0.001
Albumin (g/dL)	4.32 [3.40, 4.58]	3.50 [3.10, 3.90]	3.00 [2.70, 3.20]	<0.001
AST (IU/L)	17.00 [14.00, 21.25]	19.00 [14.00, 22.75]	40.00 [22.00, 75.50]	<0.001
ALT (IU/L)	0.65 [0.57, 0.83]	17.50 [10.25, 25.00]	25.50 [14.25, 38.50]	<0.001
Bilirubin (mg/dL)	13.50 [12.83, 14.22]	0.50 [0.40, 0.78]	0.85 [0.40, 1.30]	<0.001
Hb (g/dL)	12.60 [12.45, 13.05]	11.70 [9.72, 13.17]	10.25 [8.75, 12.00]	0.021
Hct (%)	36.90 [36.30, 37.85]	36.10 [29.12, 39.72]	30.55 [27.07, 35.10]	0.016
Platelet (10^3/μL)	247.00 [224.00, 248.50]	238.50 [181.50, 306.00]	196.00 [157.00, 218.00]	0.005
WBC (10^3/μL)	6.28 [5.43, 7.69]	7.90 [5.73, 10.09]	10.28 [5.25, 15.81]	0.011
Procalcitonin (ng/mL)	0.02 [0.02, 0.06]	0.04 [0.02, 0.33]	11.24 [1.17, 28.35]	<0.001
CRP (mg/L)	1.00 [0.85, 7.21]	9.25 [1.43, 43.22]	106.95 [35.50, 184.45]	<0.001

The healthy controls were enrolled after we obtained informed consent from examinees at the Severance Checkup Center (tongil-ro 10, jung-gu, Seoul, Republic of Korea) under the authorization of the Institutional Review Board (IRB) of Severance Hospital. The specimens were collected after the scheduled examination was completed. The residual sera samples from health screening were collected, aliquoted, and frozen at -80℃ until the commencement of the primary experiment. The participants were recruited between 2018-07-23 and 2019-01-28, and 161 samples were collected during this period that were assigned to the training group. For the validating group, 22 healthy control samples were collected from the other cohort.

The SIRS patient group was recruited from among patients who underwent selective orthopedic surgery at Severance Hospital and did not have any other underlying disease or conditions, including infection. Sera from 173 patients was collected between 2018-07-20 and 2019-05-16. Among these, 50 samples were assigned to the validating group, and the remaining 123 samples were assigned to the training group.

Lastly, the specimens for the sepsis group were collected from two pre-existing cohorts at Severance Hospital; (1) Cohort study of Patient with Severe Sepsis and Septic shock who admitted via emergency department (ER) (IRB approval No. 4-2016-0605) (2) Cohort study of Patients with Sepsis in Intensive Care Unit (ICU) (IRB approval No. 4-2017-0654). We collected 115 specimens from the Intensive Care Unit Cohort and 45 specimens from the ER Cohort; totally 160 specimens were collected, which was comparable to the number of specimens in the healthy control group. The specimens from sepsis patients admitted to the ICU were assigned to the training group, and those from sepsis patients treated in the ER were assigned to the validating group. The participants of the sepsis group were recruited between 2018-06-04 and 2019-05-05.

B. Selection of candidate amino acids using metabolomics approach

After specimen collection was completed, the amino acid were analyzed using LC-MS/MS. For the whole metabolomics analysis conducted in this study, an AB Sciex QTRAP 5500 (Beckman Coulter, Atlanta, Georgia, United States) LC-MS/MS system was used. The ZiVak amino acids kit (Zivak Teknoloji San Tic, Turkey), which is a quantitative LC-MS/MS analysis kit specific for twenty-four amino acids in biological fluids such as human serum, plasma, urine, and cerebrospinal fluid, was used for LC-MS/MS analysis in the main study.

The analysis of amino acids in serum involves the following steps: in brief, frozen serum samples were thawed at room temperature. The serum samples and the standard for calibration, in which various dosages of target acids are dissolved with MeOH, were vortexed for 1 min and centrifuged (14,000 rpm, 10 min, 2-8℃). Next, 10 µL of the supernatants were vortexed with 90 µL of 0.1% formic acid in water. After preparation, the samples were injected into a column (100 mm × 3 mm, 2.6 µm C18 100Å, Kinetex PFP) for the LC-ESI/MS/MS analysis. The analyses were performed by isocratic elution of the injected samples. Sheathless electrospray tandem mass spectrometry in the MRM was used mode for detection.

C. Development of IVDMIA and validation of its potential

After LC-MS/MS analysis was completed, we performed statistical analysis using the results to identify the candidate amino acid markers. Using data on these candidate markers, the IVDMIA was developed through a series of statistical analysis and validation processes which confirmed the potential of the marker for diagnosis under various conditions, for sepsis-severity assessment, and for determining the prognostic index in sepsis.

3. Clinical and laboratory information

Additional clinical and laboratory information of the study participants was obtained from the Electronic Medical Record (EMR) of Severance Hospital with formal authorization. Data were collected under the following categories: age, sex, height, weight, basic physiologic indexes (including body temperature, respiratory rate, blood pressure, and pulse rate), diagnosis, complete blood count test results, general chemistry test results, microbial culture results, SOFA score, date of admission and discharge, drugs administered during hospitalization, and date of death (if applicable). In addition, CRP and PCT were quantitated in almost all specimens to compare the diagnostic performance with that of amino acids. Immunoturbidimetric assay was conducted to measure the serum levels of CRP using Cobas c 702 (Roche, Basel, Switzerland). Absorbance was measured both at 570 nm and at 800 nm. Using the absorbance data, the CRP concentration was calculated. To measure the serum levels of PCT, ECLIA (Electrochemiluminescence Immunoassay) and sandwich method were performed using Cobas e 601 (Roche, Basel, Switzerland).

4. Statistical analysis

A. Analysis of comparison groups

As we were not certain about the normality of the groups we analyzed, the Mann-Whitney U test and the Kruskal-Wallis test were used for comparing the difference in medians of several independent groups. A statistically significant test result indicated that at least one group was significantly different from the other groups.

B. Principal component analysis (PCA)

PCA is a statistical procedure that uses orthogonal transformation to convert a set

of observations of possibly correlated variables into a set of values of linearly uncorrelated variables that are known as principal components. The number of distinct principal components is equal to the lesser of the number of original variables or the number of observations minus one. This transformation is defined in a manner such that the first principal component has the largest possible variance and each succeeding component has the highest variance possible under the constraint that it is orthogonal to the preceding components. The resulting vectors form an uncorrelated orthogonal basis set. PCA is sensitive to the relative scaling of the original variables. The candidate amino acids were selected based on the results of the PCA performed using the metabolomics data of the main study with LC-MS/MS analysis, and the candidates were analyzed subsequently using Kruskal-Wallis analysis and Mann-Whitney analysis.

C. Partial least squares-discriminant analysis (PLS-DA)

PLS-DA is a versatile algorithm that can be used for predictive and descriptive modeling as well as for discriminative variable selection. PLS-DA is type of supervised analytical variation of PCA, which we performed to confirm that the groups of the main study were well separated. In addition, it was used to identify the variable that exerted a significant influence.

D. Confusion matrix analysis

In the current study, confusion matrix analysis was used to confirm that the performance of the PLS-DA model generated by twenty-four amino acid variables and that generated by four candidate amino acid variables did not differ statistically. The result of the analysis was not statistically significant. Therefore, we could use the four candidate amino acids for developing IVDMIA instead of using all the twenty-four amino acids because the two models appeared to perform similarly.

E. Development of In Vitro Diagnostics Multivariate Index Assay (IVDMIA)

According to FDA guidelines, IVDMIA is defined as a test that "combines the values of multiple variables using an interpretation function to yield a single, patient-specific result that is intended for use in the diagnosis of disease or other conditions, or in the cure, mitigation, treatment or prevention of disease, and provides a result whose derivation is nontransparent and cannot be independently derived or verified by the end user".[22]

To develop such IVDMIA for sepsis, we selected several candidate amino acids such as kynurenine, tryptophan, arginine, and phenylalanine, as well as the kynurenine/tryptophan ratio (KT Ratio). We established several IVDMIA formulae using the amino acid concentrations with logistic regression. To screen the most efficient IVDMIA developed using the multiple formulae generated that could diagnose sepsis specifically and effectively, each formula was assessed based on its performance in distinguishing sepsis patients from SIRS patients or from normal healthy individuals.

F. ROC-AUC comparison between the newly-developed IVDMIA and existing sepsis biomarkers for assessing diagnostic performance in sepsis

Receiver operating characteristic (ROC) curves were generated to distinguish between patients from the two subgroups (sepsis and non-sepsis (the combination of healthy control group and SIRS group)) and to compare the diagnostic performances of existing sepsis markers (WBC, CRP, PCT) currently used in clinical practice to that of IVDMIA developed in this study, which IVDMIA was acquired from this research. Additionally, the potential of IVDMIA to distinguish between SIRS and sepsis was evaluated.

G. Pearson's correlation coefficient analysis for the assessment of the newly-developed IVDMIA as a sepsis severity biomarker

Pearson's correlation coefficient analysis is a measure of the linear correlation between two variables. The coefficient has a value between +1 and −1, where 1 indicates complete positive linear correlation, 0 indicates non-linearity, and −1 indicates complete negative linear correlation.

The SOFA score was selected as a sepsis severity index in this study, and its correlation with IVDMIA and preexisting markers such as WBC, CRP, and PCT was analyzed.

H. Statistics programs used for data analysis

Excel 2010 (Microsoft, NY, USA), Analyse-it (version 5.40.2, Analyse-it Software, Ltd., United Kingdom), R studio (version 1.1.453, Boston, USA), and SPSS (version 18, IBM, USA) were used for statistical analyses performed in this study. Differences were considered statistically significant for p**-value** < 0.05.

III. RESULTS

1. Candidate amino acids selected through preliminary metabolomics analysis

A preliminary experiment was conducted with samples from healthy individuals and patients with pneumonia or sepsis using LC-MS/MS, and the concentrations of 186 metabolites were measured. Next, the initial PCA analysis and the Kruskal-Wallis test were conducted to identify the candidate metabolites specific to sepsis. Three metabolites (phosphatidylcholine diacyl C34:1, kynurenine, and tryptophan) and one ratio (kynurenine/tryptophan ratio) were tentatively selected as potential markers. The test results are outlined in Table 3, Fig.2, and Fig.3

Table 3. Key results of Kruskal-Wallis test using metabolite concentrations in the preliminary study.

Metabolite (μM)	Normal	Pneumonia	Sepsis	*p-value* of Kruskal-Wallis Test
Phosphatidylcholine diacyl C34:1	146179.000	158228.500	227234.000	4.09118.E-03
Kynurenine / Tryptophan ratio	0.020	0.035	0.108	4.47689.E-09
Symmetric_ dimethylarginine	537.000	802.000	1332.000	1.42155.E-06
Tryptophan	94629.500	68973.000	39369.000	1.55847.E-06
Kynurenine	1918.000	2173.000	4525.000	8.55281.E-06
Total_ dimethylarginine	917.000	1155.000	1660.000	1.89222.E-05
Glutamine	712225.500	434411.000	409472.000	4.49512.E-05
Histidine	115866.000	76877.500	91704.000	2.66849.E-04
Taurine	136549.500	80627.000	101514.000	7.33068.E-04
Phenylalanine	105633.000	168839.500	155852.000	3.28058.E-03

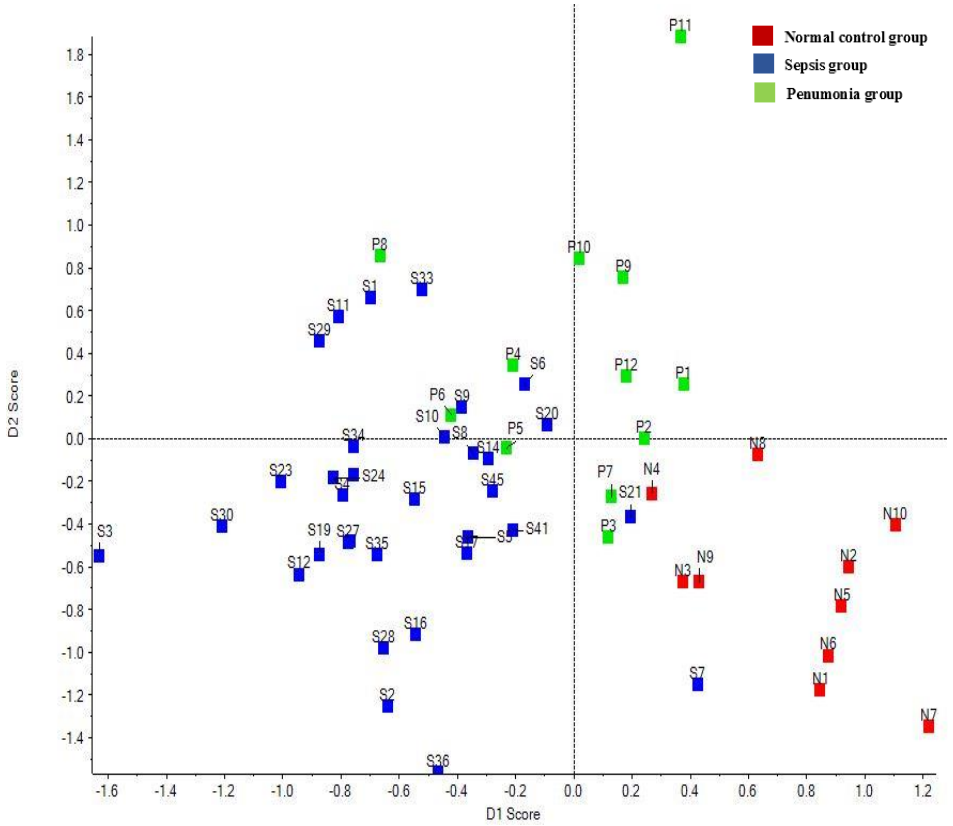

Fig. 2. Principal component analysis (PCA) score plot of amino acids that were analyzed in preliminary experiments. Red square, healthy control group; green square, pneumonia group; blue square, sepsis group

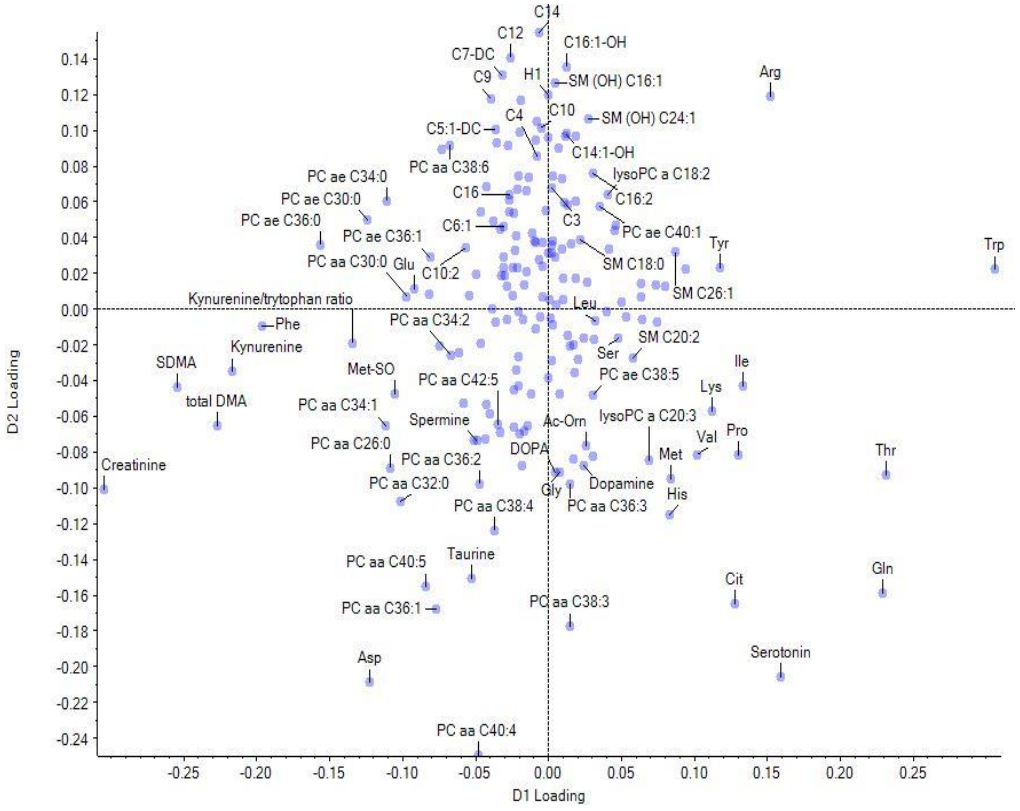

Fig. 3. Principal component analysis (PCA) loading plot of amino acids that were analyzed in preliminary experiments. The blue spots represent different metabolites. The locations of the dots were determined based on the degree of association with sepsis. The names of the amino acids are indicated by the respective three letter codes.

Based on the results of the PCA analysis, we decided that the metabolomics approach would be appropriate for sepsis research. Based on the score plot, we confirmed that the three groups (normal healthy group, pneumonia group, and sepsis group) were clearly distinguishable and formed distinct clusters. Additionally, based on the loading plot, we selected the candidate amino acids that would be analyzed in the main study. A greater distance from the origin in the loading plot corresponded to greater association with sepsis. Metabolites such as creatinine, SDMA, total DMA, phenylalanine, kynurenine, and tryptophan were positioned relatively far from the starting point. In the Kruskal-Wallis test (results presented in Fig.4), the concentrations of amino acids such as phenylalanine, serine, glutamine, and threonine among others, in addition to those of tryptophan and kynurenine, were significantly different among groups. Based on this result, we surmised that the concentrations of amino acids apart from kynurenine and tryptophan could also indicate significant differences between the groups. Therefore, in the main study, we reduced the number of study items and primarily analyzed the amino acids. Additionally, we increased the number of study subjects.

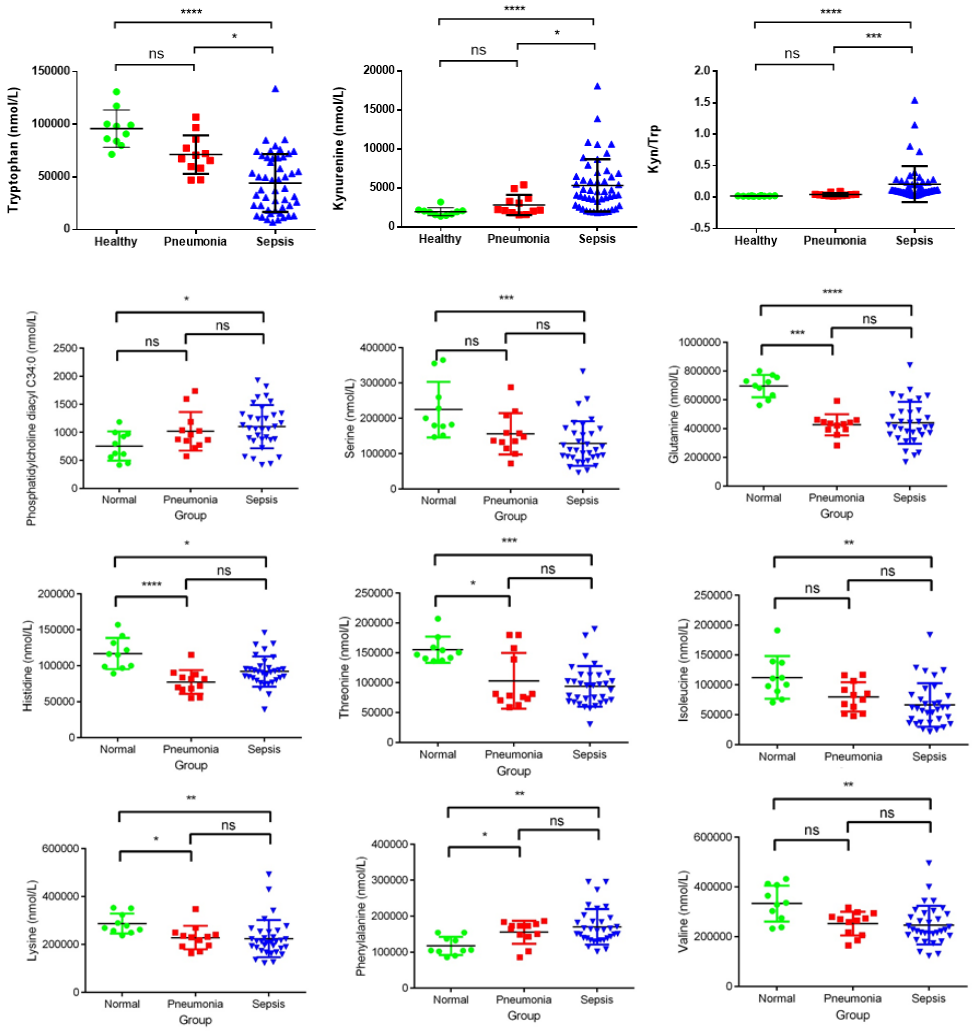

Fig. 4. The results of Kruskal-Wallis tests of the concentrations of amino acids that were analyzed in preliminary experiments. The three groups (normal, pneumonia, sepsis) were compared to each other. Statistical significance was indicated by the number of stars. (ns: not significant)

2. Main metabolomics study to identify and confirm sepsis candidate markers in reference to results from the preliminary study

After analyzing the results from the preliminary study, we performed the main study with samples from the training and validating groups using the ZiVak kit; even though this kit can detect fewer amino acids (24 amino acids) than the Absolute IDQ kit, we used it because it could detect all the candidate amino acids selected in the preliminary study. Additionally, the ZiVak kit has a shorter turnaround time and a lower cost. The concentration of the amino acids are outlined in Table 4 and Table 5. As observable, the concentrations of kynurenine and tryptophan, which were significantly different from those in the preliminary study, had a significantly low ***p-value*** (< 0.001) in this experiment. Moreover, the concentration of arginine differed significantly among the three groups, and it tended to decrease from the normal group to the sepsis group. Additionally, the concentration of phenylalanine followed the opposite trend, as it tended to increase from the normal group to the sepsis group, and the values in the three groups differed significantly.

Table 4. Concentration of amino acids in the training group participants divided into three subgroups according to septic status. Data are expressed in terms of median [IQR].

	Normal (N = 161)	SIRS (N = 123)	Sepsis (N = 115)	*p* value
Alpha-aminobutyric acid, μM	26.58 [21.10, 33.97]	25.39 [18.83, 32.76]	21.63 [13.92, 32.38]	0.003
Alanine (ALA), μM	450.66 [388.32, 520.14]	449.67 [361.30, 535.51]	369.60 [294.02, 498.55	0.0002
Arginine (ARG), μM	126.59 [109.23, 138.14]	105.52 [87.37, 124.37]	68.03 [37.86, 95.71]	<0.001
Asparagine (ASN), μM	54.00 [50.46, 59.21]	52.52 [47.34, 61.40]	50.98 [41.45, 64.19]	0.244
Aspartic acid (ASP), μM	25.03 [22.29, 28.66]	22.33 [15.59, 31.31]	38.05 [26.82, 53.66]	<0.001
Citrulline (CIT), μM/L	24.72 [22.03, 27.24]	26.75 [22.15, 33.73]	19.93 [14.29, 29.64]	<0.001
Glutamic acid (GLU), μM	99.28 [88.03, 113.29]	107.60 [87.28, 122.02]	127.58 [102.09, 167.09]	<0.001
Glutamine (GLN), μM	542.06 [472.00, 613.40]	542.79 [457.83, 645.82]	451.68 [345.50, 544.77]	<0.001
Glycine (GLY), μM	288.03 [262.54, 326.23]	247.91 [225.57, 298.81]	276.12 [221.16, 357.12]	<0.001
Histidine (HIS), μM	86.82 [77.64, 95.38]	74.56 [60.32, 87.85]	62.74 [48.04, 82.58]	<0.001
Hydroxyproline (HYP), μM	8.10 [6.21, 10.82]	12.10 [10.22, 14.79]	12.28 [9.63, 16.13]	<0.001
Isoleucine (ILEU), μM	59.33 [51.46, 69.26]	65.67 [57.43, 75.74]	52.09 [36.36, 68.81]	<0.001
Leucine (LEU), μM	129.07 [115.90, 146.42]	134.67 [116.99, 160.69]	114.66 [87.92, 143.95]	<0.001
Lysine (LYS), μM	210.98 [189.39, 233.11]	193.11 [160.00, 227.07]	165.37 [129.36, 232.80]	<0.001
Methionine (MET), μM	27.26 [24.77, 30.05]	26.88 [22.39, 31.23]	28.97 [19.45, 39.74]	0.505
Ornithine (ORN), μM	61.30 [51.78, 69.45]	78.14 [61.03, 101.59]	96.45 [68.16, 140.87]	<0.001
Phenylalanine (PHE), μM	83.59 [75.50, 91.23]	97.38 [82.20, 114.99]	145.86 [118.19, 189.51]	<0.001
Proline (PRO), μM	134.54 [111.18, 163.76]	142.60 [107.41, 172.51]	148.80 [107.84, 225.73]	0.073
Serine (SER), μM	162.31 [146.00, 183.05]	154.26 [127.91, 172.90]	124.62 [92.18, 155.87]	<0.001
Threonine (THR), μM	122.85 [108.72, 140.65]	120.86 [100.97, 140.48]	96.31 [70.66, 125.98]	<0.001
Tyrosine (TYR), μM	58.58 [50.97, 65.59]	58.10 [50.53, 67.64]	64.37 [49.39, 87.09]	0.044
Valine (VAL), μM	231.40 [207.84, 251.04]	233.55 [202.74, 273.67]	200.27 [160.05, 243.07]	<0.001
Kynurenine (KYN), μM	1.63 [1.44, 1.92]	1.68 [1.34, 2.31]	4.47 [2.71, 6.89]	<0.001
Tryptophan (TRP), μM	58.17 [50.73, 64.54]	50.29 [39.81, 60.30]	29.13 [19.83, 37.55]	<0.001
Phosphatidylcholine diacyl C34:1 (PC aa C34:1), μM	150.91 [131.62, 174.36]	148.04 [123.53, 179.52]	168.22 [140.04, 229.07]	<0.001

Table 5. Concentration of amino acid in the validating group participants divided into three subgroups according to septic status. Data are expressed in terms of median [IQR].

	Normal(N=22)	SIRS(N=50)	Sepsis(N=45)	*p* value
Alpha-aminobutyric acid, μM	20.92 [17.47, 24.60]	22.92 [16.15, 27.79]	20.42 [10.34, 25.83]	0.19
Alanine (ALA), μM	513.70 [467.31, 571.19]	455.42 [332.78, 534.77]	443.56 [332.78, 601.09]	0.1021
Arginine (ARG), μM	107.87 [89.88, 114.79]	122.04 [92.67, 141.02]	65.44 [54.34, 92.73]	<0.001
Asparagine (ASN), μM	65.31 [60.12, 69.58]	55.86 [50.44, 63.24]	45.87 [32.22, 57.71]	<0.001
Aspartic acid (ASP), μM	18.24 [15.86, 24.79]	31.77 [26.68, 41.70]	21.33 [13.09, 32.76]	<0.001
Citrulline (CIT), μM	30.98 [28.20, 37.67]	26.77 [22.96, 33.05]	22.53 [18.04, 29.06]	0.001
Glutamic acid (GLU), μM	105.00 [85.03, 138.17]	115.35 [101.39, 140.64]	81.99 [67.86, 138.34]	0.019
Glutamine (GLN), μM	643.33 [588.74, 680.82]	466.43 [411.22, 572.16]	297.02 [234.16, 384.19]	<0.001
Glycine (GLY), μM	272.54 [245.67, 299.48]	297.68 [247.01, 324.36]	172.18 [127.69, 239.71]	<0.001
Histidine (HIS), μM	85.75 [79.74, 89.04]	73.02 [49.78, 91.56]	53.17 [46.62, 63.01]	<0.001
Hydroxyproline (HYP), μM	13.98 [11.91, 17.09]	11.88 [10.71, 14.82]	13.06 [11.63, 17.41]	0.04
Isoleucine (ILEU), μM	90.00 [67.67, 111.42]	64.06 [54.48, 76.57]	42.18 [34.21, 54.58]	<0.001
Lysine (LYS), μM	214.38 [190.16, 237.46]	188.78 [166.25, 232.78]	115.13 [100.87, 139.50]	<0.001
Methionine (MET), μM	25.25 [23.01, 27.93]	24.33 [21.62, 27.59]	19.36 [14.36, 25.35]	0.007
Ornithine (ORN), μM	85.07 [65.63, 111.52]	76.05 [62.81, 102.30]	62.55 [44.54, 110.20]	0.301
Phenylalanine (PHE), μM	72.63 [65.12, 81.95]	106.93 [92.64, 135.57]	135.49 [99.40, 162.76]	<0.001
Proline (PRO), μM	177.69 [139.30, 229.53]	156.81 [139.51, 184.80]	92.06 [72.00, 228.40]	0.035
Serine (SER), μM	149.58 [130.47, 160.63]	158.12 [131.66, 181.08]	101.26 [62.36, 115.83]	<0.001
Threonine (THR), μM	144.42 [127.70, 160.43]	118.53 [99.20, 143.18]	69.28 [53.34, 97.88]	<0.001
Tyrosine (TYR), μM	63.71 [55.28, 70.70]	57.67 [46.23, 64.54]	65.27 [49.50, 86.43]	0.154
Valine (VAL), μM	272.67 [216.94, 285.85]	233.77 [185.89, 269.71]	150.02 [132.51, 282.43]	0.002
Kynurenine (KYN), μM	1.30 [1.15, 1.63]	2.02 [1.78, 2.68]	3.99 [1.97, 5.86]	<0.001
Tryptophan (TRP), μM	57.53 [52.91, 61.07]	46.02 [33.19, 58.39]	29.38 [17.97, 44.12]	0.001

After the main experiment was conducted using the metabolomics approach using LC-MS/MS, PCA was performed using the amino acid concentration data obtained from the training group. The result of the statistical analysis is presented in Fig.5 and Fig.6.

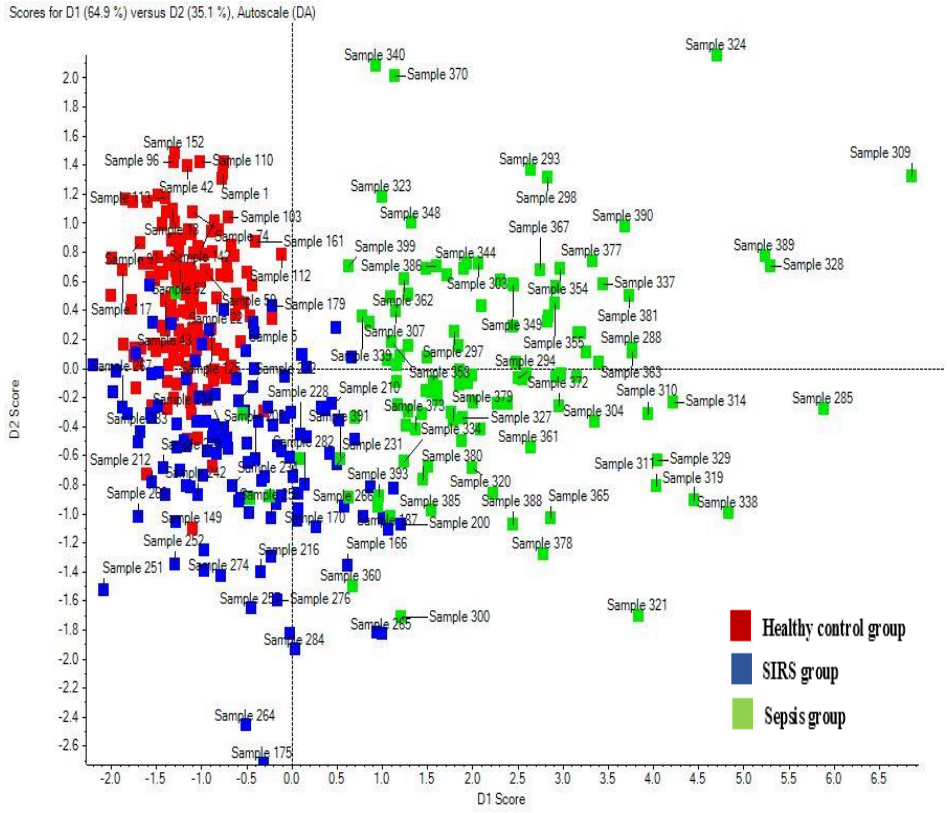

Fig. **5**. Score plot of amino acid concentrations from the training group acquired using Principal component analysis (PCA) that were analyzed in the main study. Red square, healthy control group; blue square, SIRS (systemic inflammatory response syndrome) group; green square, sepsis group

Based on the score plot presented in Fig. 5, we re-confirmed that the three subgroups (normal, SIRS, and sepsis) were clearly distinguishable using metabolomics approach and formed distinct clusters. This result suggests that the metabolomics method using LC-MS/MS could be applied successfully in the analysis of serum samples from sepsis as well as non-sepsis (SIRS and normal healthy) patients. The analysis of amino acid concentration could be useful for distinguishing sepsis patients from non-sepsis patients who exhibit similar clinical characteristics typical in sepsis, such as SIRS, after its successful implementation in clinical settings.

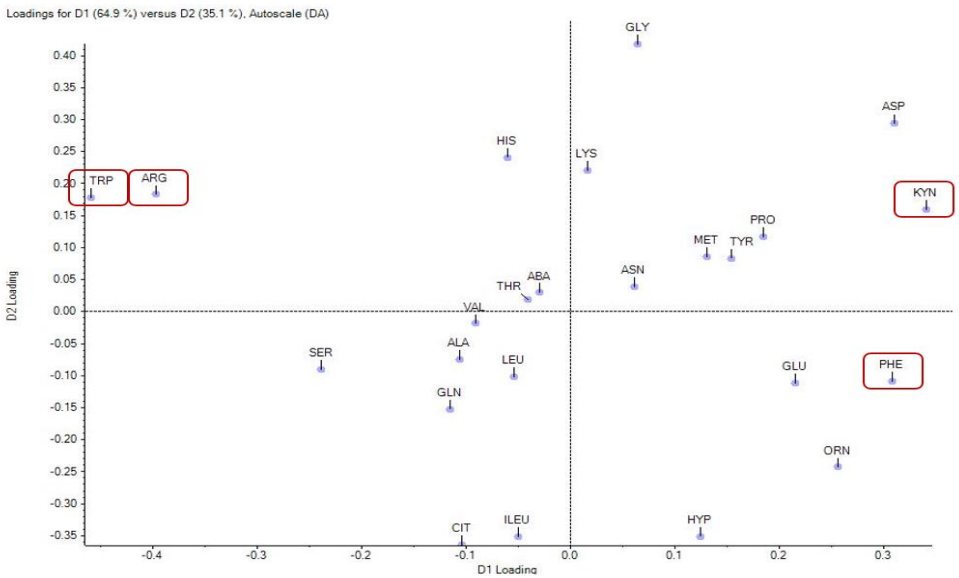

Fig. 6. Loading plot of amino acid concentrations in the training group acquired using Principle component analysis (PCA) that were analyzed in the main study. The blue spots represent individual amino acids. The locations of the dots were determined based the degree of association with sepsis. A greater distance from the center (origin) corresponded to a greater degree of association with sepsis. The amino acids are indicated by the respective three letter codes. Candidate amino acids are marked with a red box around the three-letter code.

Based on the loading plot (Fig. 6.) from the main study, we categorized the amino acids according to the distance from the plot origin. The candidate amino acids finally selected were tryptophan (TRP), kynurenine (KYN), arginine (ARG),[23-25] and phenylalanine (PHE).[26,27] Based on to recent studies, these amino acids are closely associated with metabolic processes in sepsis patients; for example, the catabolic conversion of tryptophan to kynurenine that occurs in sepsis.[15-17,28] However, the kynurenine/tryptophan ratio (KT ratio), which is not an amino acid, was also observed to be highly relevant as a sepsis marker and could be used effectively to calculate the concentration of the two amino acids; KYN and TRP.

The KYN, TRP, and KT Ratio are widely used as indicators of sepsis, and are closely associated with indoleamine-2,3-dioxygenase (IDO) metabolism in patients with sepsis patients.[14,21] IDO is an intracellular, non-secretory enzyme, which catabolizes the production of kynurenine derivatives from tryptophan.[29] Recent findings have clearly revealed that elevated IDO expression is a hallmark of viral and bacterial infections such as sepsis, and is also associated with cancer.[14,30]

However, the two other amino acids, ARG and PHE, which were identified as biomarkers in this study, have not been used commonly as biomarkers.[23,25,26] Although the two amino acids were not considered as common biomarkers of sepsis, they exhibited excellent results in the loading plot in this study, similar to those of KYN, TRP, and KT Ratio. Therefore, we decided to use all the five abovementioned indicators for developing the IVDMIA for sepsis.

3. PLS-DA for comparing the PLS model 4 with the PLS model 24 using McNemars test

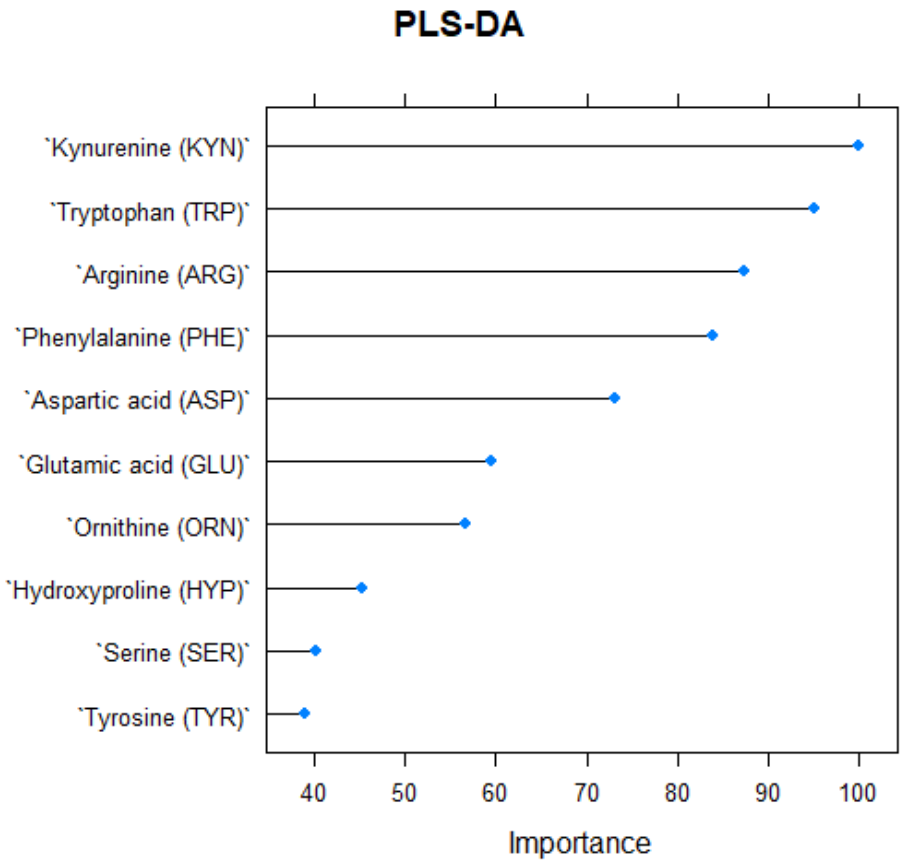

Fig. 7. The importance plot from PLS-DA analysis in the training group. Four candidate amino acids are located at the top of the plot.

As shown in Fig.7, the PLS-DA analysis revealed that the selected candidate amino acids (KYN, TRP, ARG, and PHE) are important for distinguishing between sepsis and non-sepsis patients. As described in the Materials and Methods section, before developing IVDMIA, we performed PLS-DA and confusion matrix analysis

to confirm that there were no statistically significant differences when IVDMIA was executed with 24 variables or 4 variables. Through PLS-DA, we obtained the results for parameters such as confidential interval (CI) of sensitivity, specificity, and accuracy of statistical models. Next, using McNemars test, we compared the results of the two models. In all cases, the ***p-values*** were greater 0.05. Specifically, the ***p-values*** for sensitivity, specificity, and accuracy were 0.4142, 0.1797, and 0.7893, respectively. These results confirmed that, statistically, there were no issues in the application of IVDMIA using four candidate amino acids instead of all the 24 amino acids. Therefore, for developing the IVDMIA for sepsis, we decided to use the four candidate amino acids and one ratio, the KT Ratio.

4. Comparing the candidate amino acid concentrations between two subgroups or among three subgroups of the training and validating groups

To confirm that the concentrations of the amino acids selected in this study differed significantly between the normal, SIRS, and sepsis groups, or between the non-sepsis and sepsis groups, we compared the concentrations of the four amino acids using Kruskal-Wallis test or Mann-Whitney test. We found that the concentrations of the candidate amino acids between the two groups or among the three groups were significantly different (***p-value*** <0.0001). These results suggest that the four amino acids selected by us could meaningfully indicate sepsis. The results of Kruskal-Wallis and Mann-Whitney tests are presented in Fig 8, 9, 10, and 11.

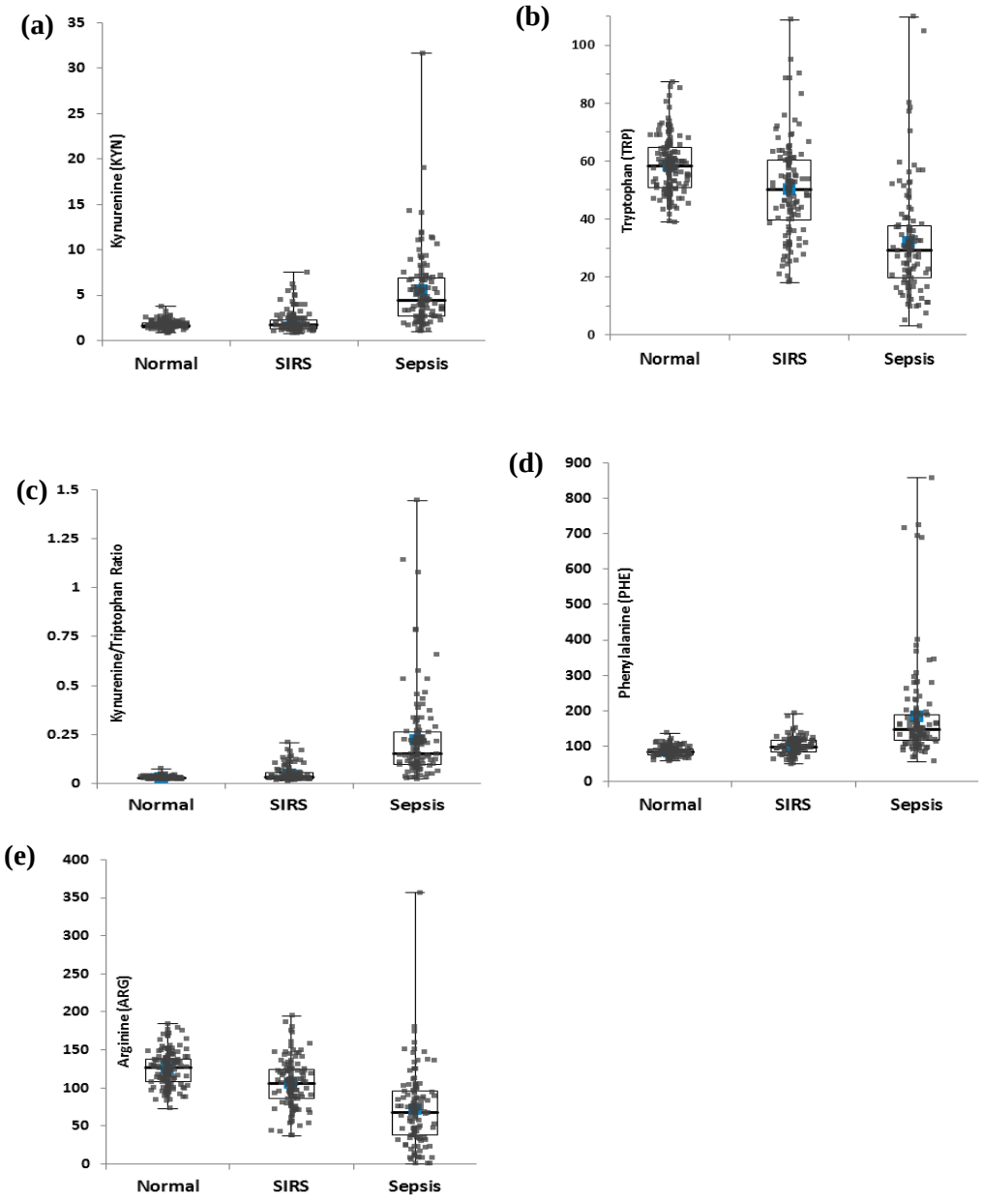

Fig. 8. Results of Kruskal-Wallis analysis of amino acid concentrations in the training set. The concentrations of amino acids in the three groups (normal, SIRS, sepsis) were significantly different (***p-value*** < 0.0001). (a) kynurenine (b) tryptophan (c) KT ratio (d) phenylalanine (e) arginine. The concentrations are expressed in μM.

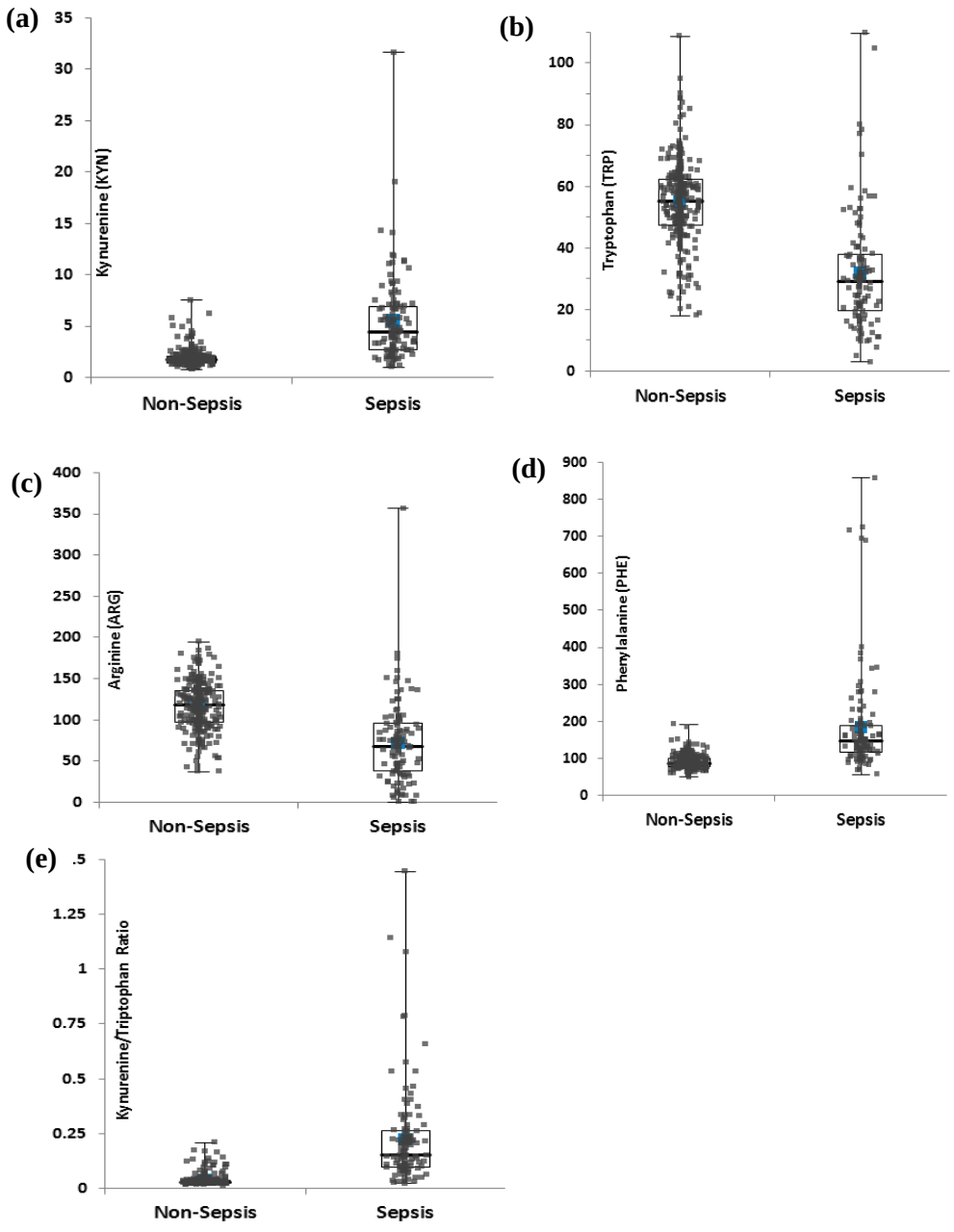

Fig. 9. Results of Mann-Whitney analysis. The concentrations of amino acids in the training set between the two groups (Non-sepsis, sepsis) were significantly different (***p-value*** < 0.0001). (a) kynurenine (b) tryptophan (c) KT ratio (d) phenylalanine (e) arginine. The concentrations are expressed in μM.

We also assessed the concentrations of the candidate amino acids in the participants of the validating set, which had fewer participants than the training set.

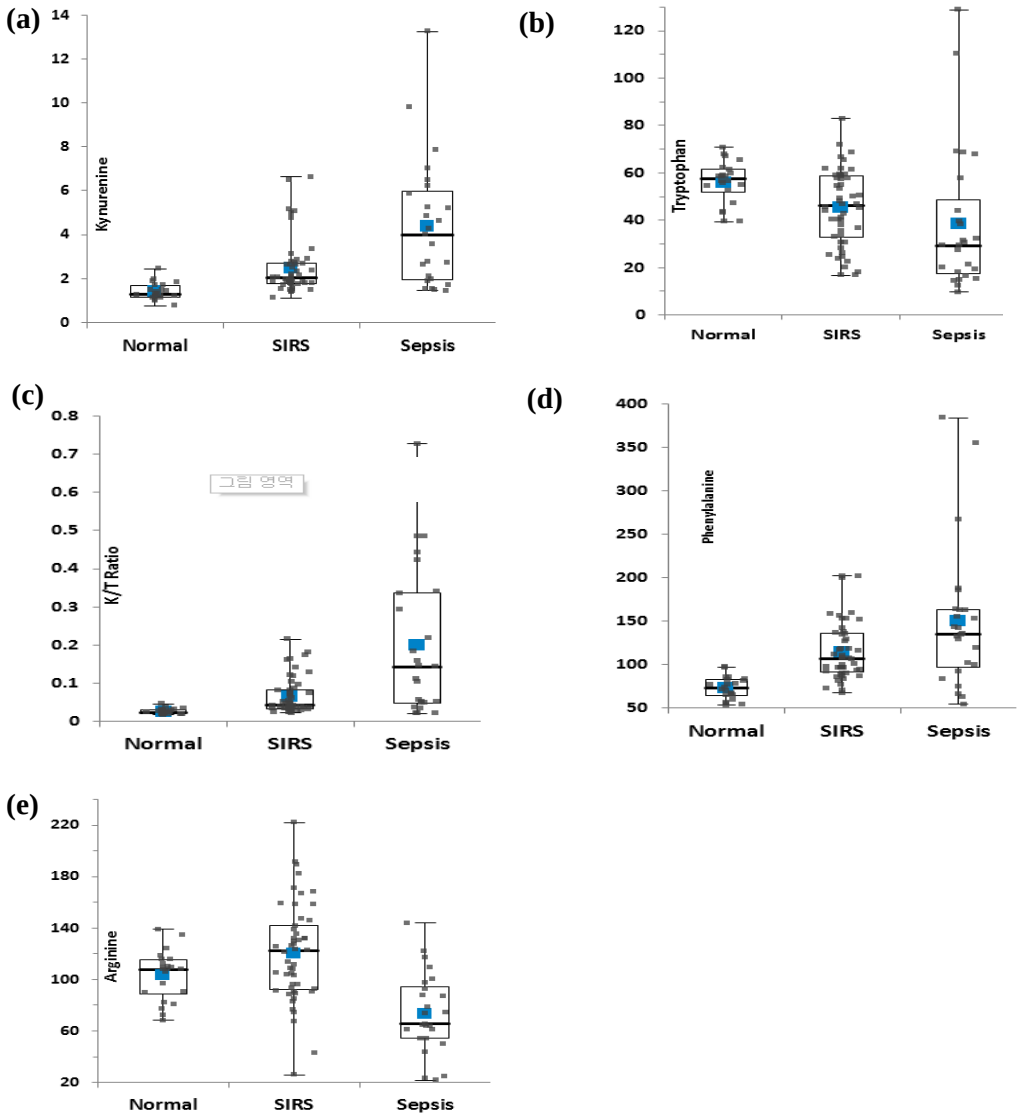

Fig. 10. Results of Kruskal-Wallis analysis of amino acid concentrations in the validating set. The concentrations of amino acids in the three groups (normal, SIRS, sepsis) were significantly different (***p-value*** < 0.0001) (a) kynurenine (b) tryptophan (c) KT ratio (d) phenylalanine (e) arginine. The concentrations are expressed in μM.

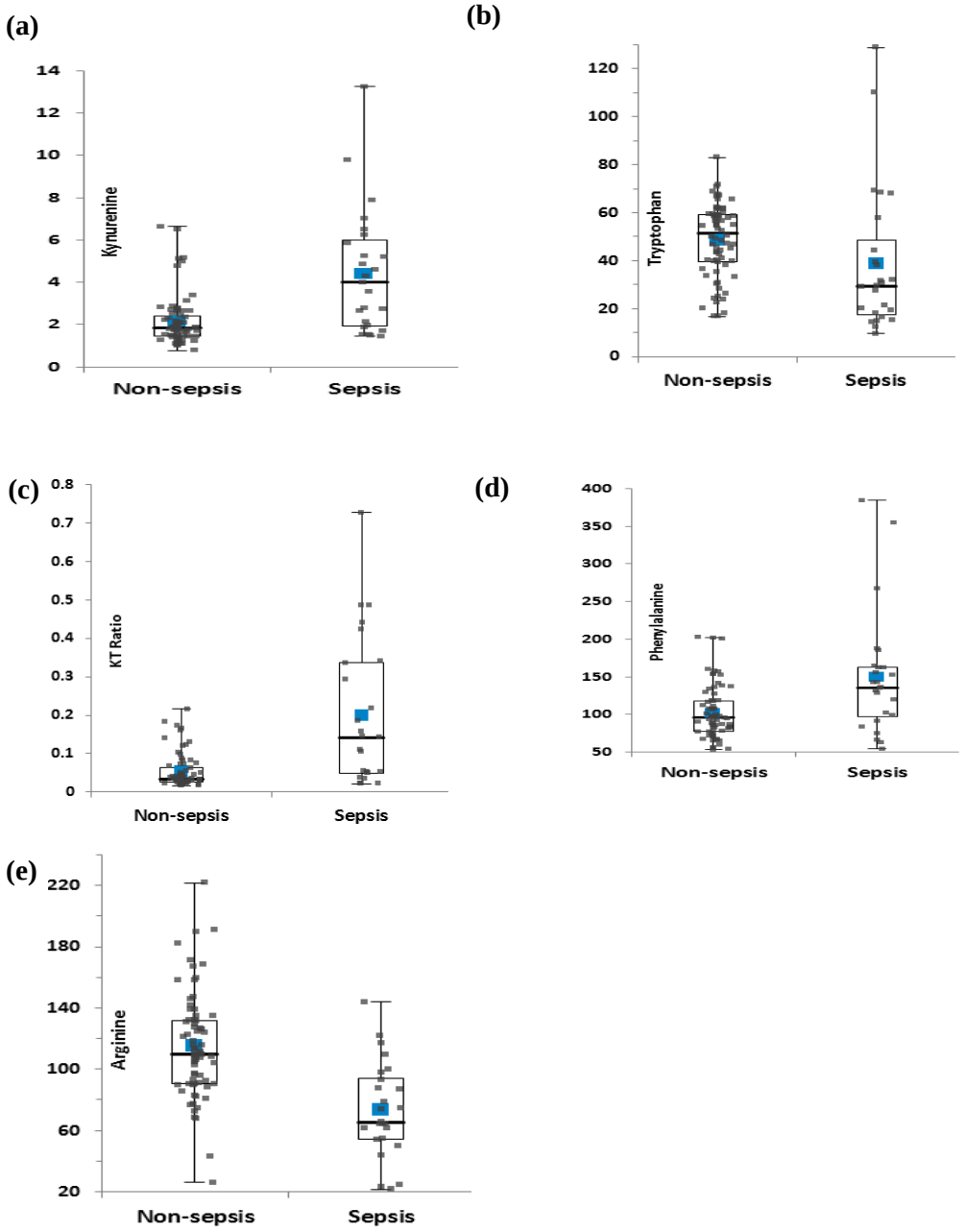

Fig. 11. Results of Mann-Whitney analysis of amino acid concentrations in the validating set (categorized into non-sepsis and sepsis groups) were significantly different (***p-value*** < 0.0001) between two groups. (a) kynurenine (b) tryptophan (c) KT ratio (d) phenylalanine (e) arginine. The concentrations are expressed in μM..

5. Development of IVDMIA for sepsis using the serum concentration data of four candidate amino acids by logistic regression

In the IVDMIA, only biomarkers that collectively outperform a single marker can be included. For the development of the IVDMIA in this study, the training group was used. The number of subjects from the training group who were included in the development of IVDMIA was relatively high (normal: 161, SIRS: 123, and sepsis: 115).

After performing PLS-DA and confusion matrix analysis, we performed logistic regression to generate the formulae that would be used for developing IVDMIA for sepsis. For this process, we used the "lrm" command from "caret" package in the statistics program R.

First, we selected certain amino acids from among the candidate amino acids, which would be included in the generation of the formula. Next, we performed logistic regression using the selected amino acids. We acquired the coefficients and the intercept of the selected amino acids from the generated formula, which is a type of IVDMIA with a single index.

Among the several formulae involving candidate amino acids, we selected an equation involving arginine, phenylalanine and the KT ratio as an IVDMIA for sepsis based on the corresponding AUC, sensitivity, specificity, and accuracy. Additional details about the AUC, sensitivity, specificity, and accuracy are outlined in the following section in a Table 6. We fixed the cut-off value from the ROC curves and configured the AUC, sensitivity, specificity, and accuracy. After the process was completed, we generated six IVDMIA formulae for the selection of sepsis patients from among normal and SIRS patient using various combinations of the four candidate amino acids and the KT ratio.

Eventually, the fourth IVDMIA was selected based on its performance in the determination of sepsis considering the AUC, sensitivity, specificity and accuracy. The generated equations are presented in Table 6. The ROC curves corresponding to each formula are presented in Fig. 12. After selecting the fourth formula as the IVDMIA index for sepsis, we performed the validation of established IVDMIA formula using data from the validating group to evaluate its diagnostic potential under various circumstances and also as a marker of sepsis severity and as a prognosis factor.

Table 6. Results of IVDMIAs determined by logistic regression after application to the validating group. All the formulae and the corresponding AUC, cut-off, sensitivity, specificity, and accuracy are outlined. The formula in bold (formula No.4) was selected as an IVDMIA index for sepsis in this study.

Formula No.	Factor and Formula	AUC	cut-off	Sensitivity	Specificity	Accuracy
1	**Kyn, Trp, Arg, Phe** Kyn x 0.3925 - Trp x 0.0937 - Arg x 0.0516 + Phe x 0.0688 - 1.0310	0.9110	0.4360	0.9300	0.7600	0.8660
2	**Kyn, Trp, Arg** Kyn x 0.8093 - Trp x 0.0764 - Arg x 0.0207 + 2.0834	0.8010	-0.4270	0.8610	0.7600	0.8351
3	**Kyn, Trp, Phe** Kyn x 0.5258 - Trp x 0.0914 + Phe x 0.0412 -3.2856	0.7930	-3.1070	0.5280	0.9600	0.6392
4	**Arg, Phe, KT ratio** **Arg x (-0.0513) +** **Phe x 0.0642 +** **KT ratio x 27.6591 - 5.4765**	**0.9270**	**-0.5510**	**0.8750**	**0.8800**	**0.8763**
5	**KT ratio** KT ratio x 38.5811 - 3.8783	0.7800	-2.0580	0.6940	0.8000	0.7216
6	**KT ratio, Phe** KT ratio x 29.6186 + Phe x 0.0421 - 8.0721	0.8330	-1.4900	0.7080	0.9200	0.7629

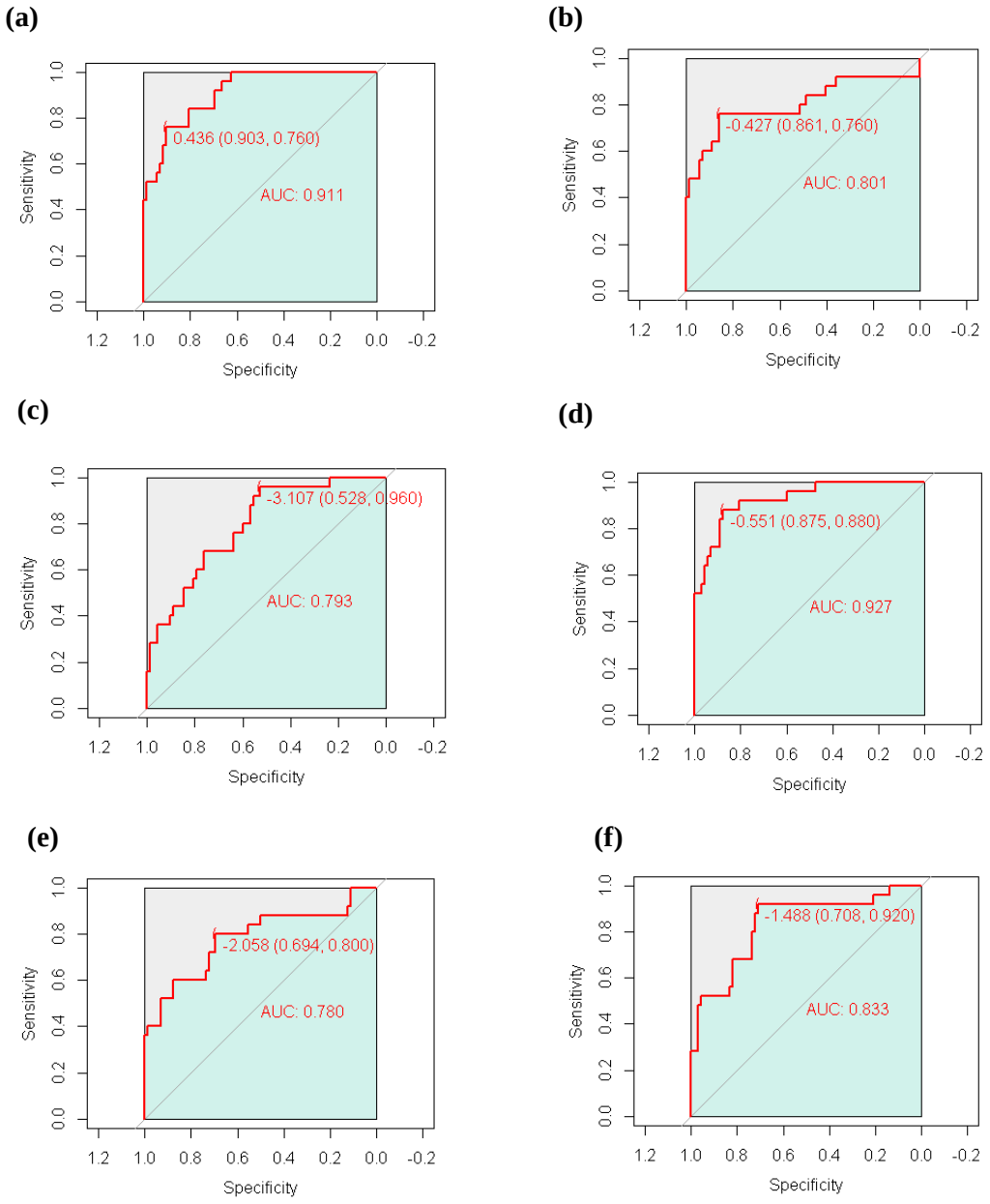

Fig. 12. . The ROC curves obtained when the IVDMIA formulae were applied to the validating group. ROC curve corresponding to the application of (a) formula 1, (b) formula 2, (c) formula 3, (d) formula 4, (e) formula 5, and (f) formula 6 (the detailed description of the formula number is outlined in Table 6)

6. Validation of the newly developed IVDMIA as a sepsis diagnostic marker compared to existing markers

For the validation of the developed IVDMIA, it was applied in the validating group based on ROC curve, sensitivity, specificity, and comparison of diagnostic power between IVDMIA and existing markers.

A. Validating the diagnostic potential of the newly-developed IVDMIA compared to existing sepsis markers

From the six aforementioned formulas, we selected the fourth one, which was composed of ARG, PHE, and the KT ratio. This formula yielded the highest AUC and had considerable sensitivity, specificity, and accuracy. To evaluate the performance of the selected IVDMIA and existing sepsis markers, the Kruskal-Wallis test was performed using data from the three subgroups (normal, SIRS, and sepsis) of the validating group. The test results are outlined in Fig. 13. ***p-value*** < 0.0001 was observed in all the tests, which suggests that there is a significant difference between the values of markers, including IVDMIA, between the three groups.

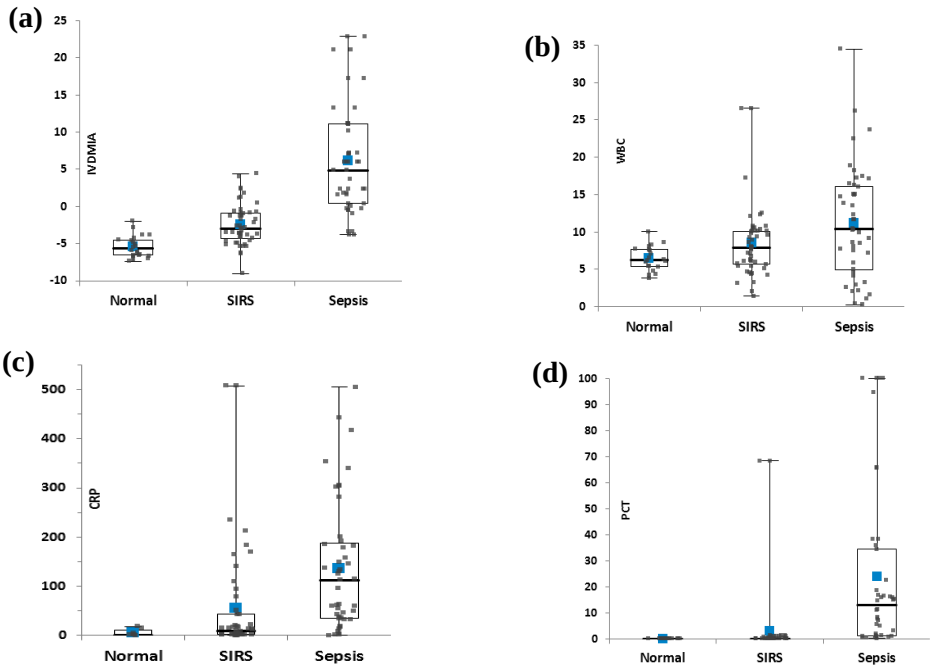

Fig. 13. The results of Kruskal-Wallis test in the three subgroups (normal, SIRS, sepsis in the order mentioned) from the validating group. In all cases, the ***p-value*** was < 0.0001. (a) comparison of IVDMIA, (b) comparison of WBC (10^3/μL), (c) comparison of CRP (mg/L), (d) comparison of PCT (ng/mL)

We also performed the Mann-Whitney test to evaluate the performance of IVDMIA, WBC, CRP and PCT in the two subgroups (non-sepsis and sepsis) of the validating group. The non-sepsis group is a combination of the normal and SIRS subgroups. As observed in the previous comparison of the three groups (Kruskal-Wallis test), a ***p-value*** < 0.001 was obtained in all analytical results. This indicates that there are significant statistical differences between the two subgroups and these markers can be used for diagnosing sepsis. The graphs are shown in Fig.14.

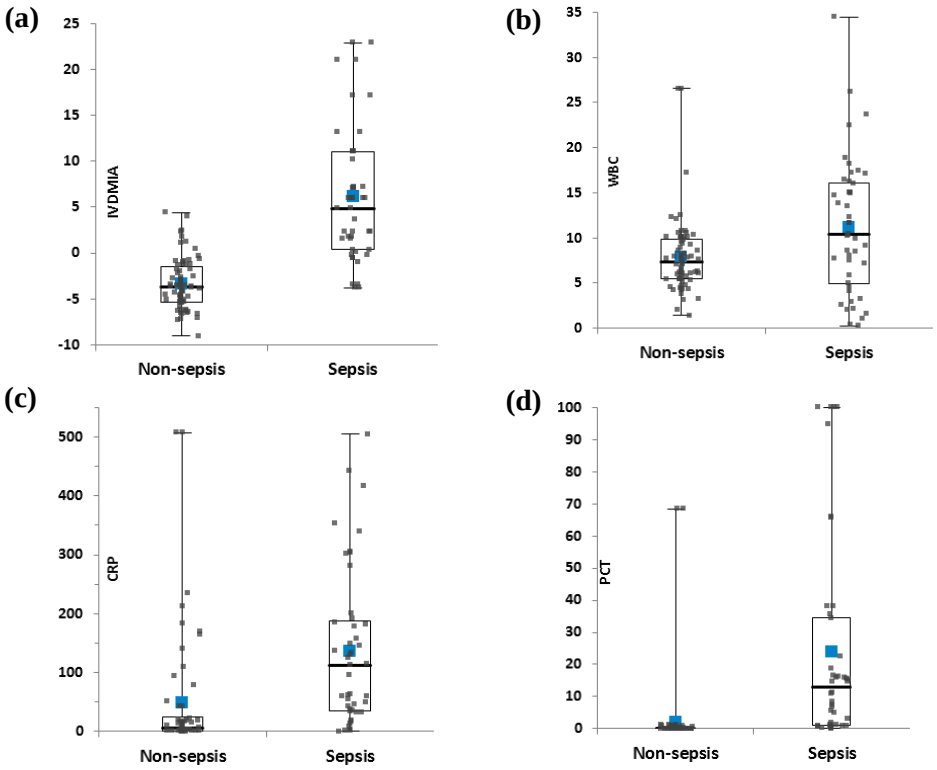

Fig. 14. Results of Mann-Whitney analysis in the validating group. The means of IVDMIA, WBC, CRP, and PCT were significantly different between the two groups (non-sepsis and sepsis, ***p-value*** < 0.0001). (a) comparison of IVDMIA, (b) comparison of WBC (10^3/μL), (c) comparison of CRP (mg/L), (d) comparison of PCT (ng/mL)

For further evaluation, we plotted the ROC curves for evaluating the performances of various existing biomarkers of sepsis and of IVDMIA. First, we plotted the ROC curves of the biomarkers to analyze and compare the AUCs and the 95% CI in the validating group. The results of this analysis are presented in Fig.15 and Table 7.

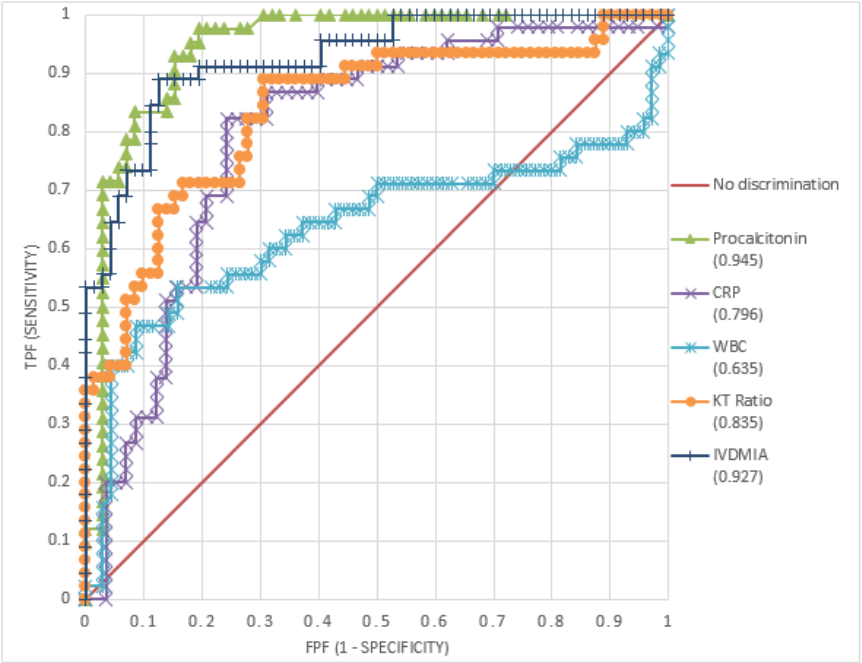

Fig. 15. The comparison of ROC curves for WBC, CRP, PCT (Procalcitonin), KT ratio, and IVDMIA in the validating group. PCT and IVDMIA have a relatively high AUCs, which indicates that the two markers perform remarkably in the diagnosis of sepsis

Table 7. The AUCs, 95% CIs of AUCs, and SEs in the validating group. The CIs for PCT (Procalcitonin) and IVDMIA overlap, which indicates there may not be significant difference between the AUCs of the two markers.

	AUC	95% CI	SE
Procalcitonin	0.945	0.904 to 0.986	0.0209
CRP	0.796	0.708 to 0.887	0.0457
WBC	0.635	0.504 to 0.745	0.0614
KT Ratio	0.835	0.759 to 0.917	0.0403
IVDMIA	0.927	0.876 to 0.970	0.024

Although all the ***p-values*** were less than 0.0001 in the previous Kruskal-Wallis and Mann-Whitney tests, the ROC comparison yielded a contradictory result; only the IVDMIA and PCT curves had AUCs > 0.9, which is a significantly high score. This result indicates that IVDMIA or PCT are better suited as diagnostic biomarkers in sepsis than WBC or CRP. Additionally, we performed DeLong's test because the two 95% CI of the AUCs of IVDMIA and PCT were overlapping. The ***p-value*** of the test in the validating group was 0.495, which indicates there is no statistically significant difference between the two AUCs.

However, the ***p-value*** of the DeLong's test in the training group was 0.01574, which indicates that there is a statistically significant difference between the AUCs of PCT and IVDMIA, and therefore, IVDMIA could possibly perform better than PCT in the diagnosis of sepsis.

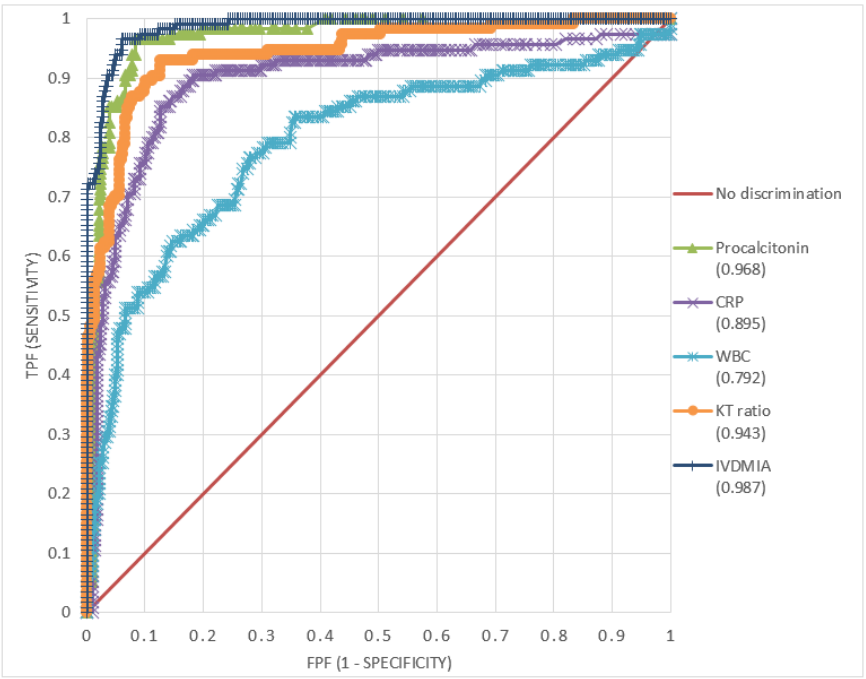

Fig. 16. The comparison of ROC curves for WBC, CRP, Procalcitonin (PCT), KT ratio, and IVDMIA in the training group. IVDMIA has an AUC that is marginally higher than that of PCT, which indicates that it performs remarkably in the diagnosis of sepsis.

Table 8. The AUCs, 95% CIs of AUCs, and SEs in the training group. IVDMIA performs slightly better than Procalcitonin (PCT).

	AUC	95% CI	SE
Procalcitonin	0.968	0.952 to 0.985	0.0085
CRP	0.895	0.854 to 0.936	0.0210
WBC	0.792	0.738 to 0.847	0.0277
KT ratio	0.943	0.916 to 0.969	0.0135
IVDMIA	0.987	0.979 to 0.995	0.0040

B. Distinguishing between sepsis and SIRS

We also assessed the performance of the biomarkers in terms of their ability to distinguish between SIRS and sepsis. In clinical practice, the diagnosis of sepsis demands prompt medical action. Therefore, it is extremely important to distinguish between the two diseases.[8,9,25,31] However, the symptoms of SIRS and sepsis are considerably similar in the initial stages, which makes it difficult to ensure rapid diagnosis. For the evaluation, we used the SIRS and sepsis subgroups while excluding the normal subgroup of the validating group. The demographic features in each group are outlined in Table 9. The ROC curve and AUC table are presented in Fig. 17 and Table 10, respectively.

Table 9. Baseline characteristics of the participants of the modified subgroup (consisting of SIRS and sepsis subgroups) of the validating group. The normal healthy group was not included. Data (except those related to gender) are expressed in terms of median [IQR].

	Sepsis (N = 45)	**SIRS (N = 50)**	***p-value***
Gender = M (%)	11 (24.4)	30 (60.0)	<0.001
Age (yr)	71.00 [61.00, 78.00]	64.00 [50.00, 73.00]	0.016
Height (cm)	164.00 [159.00, 176.00]	169.00 [163.00, 172.07]	0.546
Weight (kg)	60.00 [48.00, 63.00]	67.95 [61.00, 71.70]	<0.001
BMI	20.34 [18.34, 23.57]	24.07 [22.26, 24.77]	<0.001
Glucose (mg/dL)	99.00 [32.40, 166.00]	109.50 [94.00, 132.75]	0.047
Creatinine (mg/dL)	2.67 [1.23, 42.00]	0.90 [0.71, 1.29]	<0.001
total protein (g/dL)	4.80 [3.00, 5.60]	6.20 [5.80, 6.60]	<0.001
Albumin (g/dL)	3.60 [2.90, 49.00]	3.50 [3.10, 3.90]	0.156
AST (IU/L)	35.00 [18.00, 60.00]	19.00 [14.00, 22.75]	<0.001
ALT (IU/L)	10.00 [0.90, 28.00]	17.50 [10.25, 25.00]	0.032
Bilirubin (mg/dL)	1.05 [0.60, 7.83]	0.50 [0.40, 0.78]	<0.001
WBC (10^3/μL)	10.37 [5.05, 16.05]	7.90 [5.73, 10.09]	0.068
Hemoglobin (g/dL)	10.30 [8.50, 12.00]	11.70 [9.72, 13.17]	0.027
Hematocrit (%)	30.60 [26.90, 35.10]	36.10 [29.12, 39.72]	0.01
Platelet (10^3/μL)	196.00 [157.00, 218.00]	238.50 [181.50, 306.00]	0.002
CRP (mg/L)	112.70 [35.10, 185.20]	9.25 [1.43, 43.22]	<0.001
Procalcitonin (ng/mL)	12.96 [1.15, 31.34]	0.04 [0.02, 0.34]	<0.001
IVDMIA	4.84 [0.38, 11.06]	-2.98 [-4.27, -0.89]	<0.001
KT.Ratio	0.14 [0.06, 0.34]	0.04 [0.03, 0.08]	<0.001

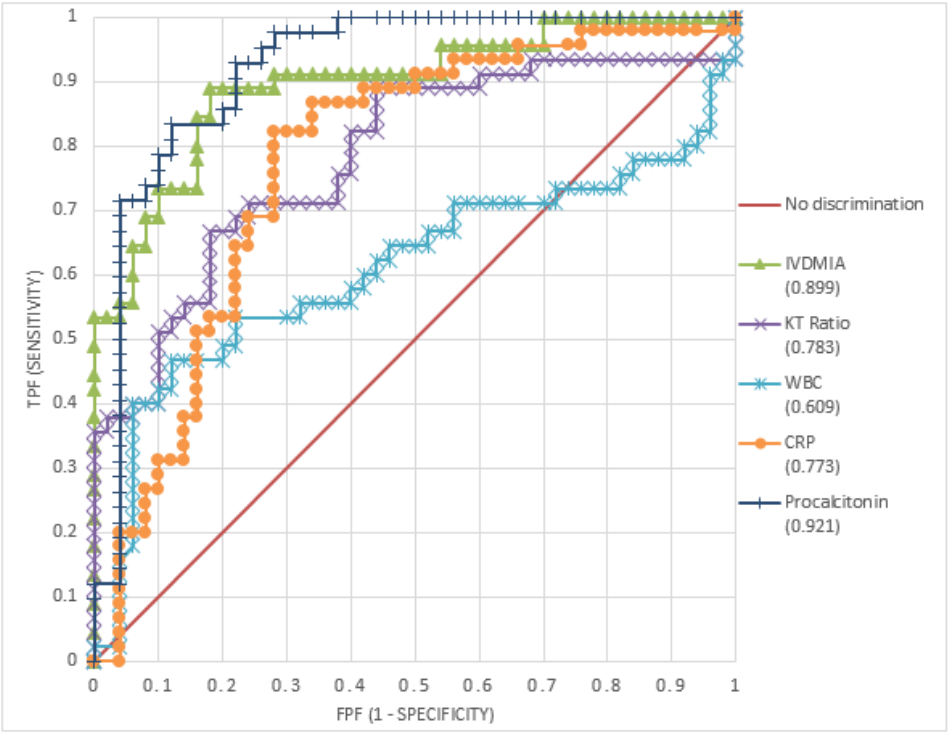

Fig. 17. The comparison of ROC curves for WBC, CRP, Procalcitonin (PCT), KT ratio, and IVDMIA in the modified validating group (including the SIRS and sepsis subgroups). PCT had the highest AUC and IVDMIA had the second-highest AUC. However, the difference is negligible.

Table 10. The AUCs, 95% CI of AUCs, and SEs for the modified validating group (including the SIRS and sepsis subgroups). 95% CIs of Procalcitonin (PCT) and IVDMIA AUCs are within a similar range, and almost overlapping with each other.

	AUC	95% CI	SE
IVDMIA	0.899	0.836 to 0.962	0.0321
KT Ratio	0.783	0.687 to 0.879	0.0489
WBC	0.609	0.486 to 0.731	0.0625
CRP	0.773	0.676 to 0.871	0.0498
Procalcitonin	0.921	0.864 to 0.979	0.0294

The AUC of PCT was higher than that of IVDMIA. However, the respective 95% CIs overlapped; therefore, it was unknown whether the two figures are statistically different. Accordingly, we repeated DeLong's test and obtained a *p-value* of 0.5331, which indicates that it cannot be ascertained whether there is a significant difference between the two AUCs.

C. Diagnostic differences between "simple" sepsis and septic shock

We assessed the sepsis biomarkers, including IVDMIA, with respect to the differences in diagnosis based on the septic status of patients. In this regard, there are certain studies that mention that PCT is more sensitive as a biomarker of septic shock than of "simple" sepsis.[32] In addition, it is also thought that PCT can be used as a severity marker of sepsis.[32] Based on these, we planned to assess the diagnostic potential of sepsis markers in cases of septic shock and "simple" sepsis in the sepsis subgroup of the validating group.

We selected the sepsis subgroup from the validating group and categorized the subjects as patients with "simple" sepsis and septic shock. The definition of septic shock was based on that used globally[1]: vasopressors required to maintain mean arterial pressure (MAP) $\geq$ 65 mmHg and lactate level more than 2 mmol/L.

Table 11. Baseline characteristics of validating group participants divided into "simple" sepsis and septic shock groups based on septic severity. Data (except those related to gender) are expressed in terms of median [IQR].

	"simple" sepsis (N = 7)	septic shock (N = 39)	*p-value*
Age (yr)	73.00 [58.50, 79.50]	67.00 [61.00, 78.00]	0.854
Gender = M (%)	2 (28.6)	9 (23.1)	<0.001
Height (cm)	164.00 [161.50, 171.00]	164.00 [158.00, 176.00]	0.866
Weight (kg)	53.00 [48.00, 60.00]	60.00 [48.00, 65.00]	0.284
BMI	19.47 [18.28, 20.18]	20.89 [18.52, 24.23]	0.174
Glucose (mg/dL)	117.00 [72.70, 199.00]	83.00 [30.55, 116.50]	0.284
Creatinine (mg/dL)	2.55 [1.54, 21.12]	2.82 [1.12, 46.00]	0.691
Total protein (g/dL)	5.40 [4.15, 5.65]	4.50 [2.95, 5.35]	0.298
Albumin (g/dL)	3.20 [2.85, 26.05]	4.00 [2.95, 79.00]	0.327
AST (IU/L)	41.00 [33.50, 58.50]	28.00 [16.00, 56.50]	0.291
ALT (IU/L)	24.00 [6.70, 37.00]	8.70 [0.95, 23.00]	0.491
Bilirubin (mg/dL)	0.65 [0.45, 1.38]	1.30 [0.70, 11.00]	0.161
WBC (103/μL)	9.17 [7.97, 10.91]	10.47 [4.83, 16.13]	0.657
Hemoglobin (g/dL)	10.00 [9.25, 12.05]	10.30 [9.00, 12.00]	0.963
Hematocrit (%)	30.60 [28.60, 36.10]	30.50 [27.25, 35.00]	0.691
Platelet (103/μL)	215.00 [192.00, 309.50]	188.00 [135.50, 217.00]	0.087
Procalcitonin (ng/mL)	0.87 [0.43, 0.92]	14.74 [2.24, 31.34]	0.058
CRP (mg/L)	48.70 [37.85, 113.60]	125.60 [35.90, 196.55]	0.251
IVDMIA	4.50 [2.31, 6.87]	7.84 [5.45, 10.54]	0.251
SOFA score	6.00 [6.00, 7.00]	11.00 [10.00, 12.50]	<0.001

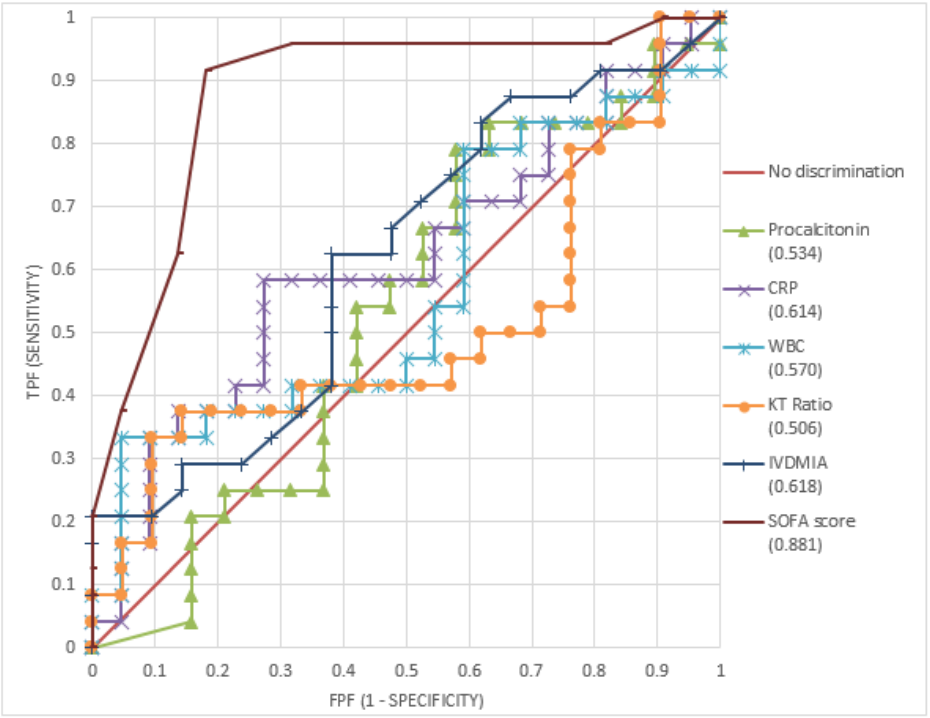

Fig. 18. The comparison of ROC curves for WBC, CRP, PCT (Procalcitonin), KT ratio, and IVDMIA in the modified validating group (including the "simple" sepsis and septic shock cases).

Table 12. The AUCs, 95% CIs of AUCs, and SEs in the modified validating group (including the "simple" sepsis and septic shock cases).

	AUC	95% CI	SE
PCT	0.534	0.35 to 0.718	0.0938
CRP	0.614	0.447 to 0.780	0.0849
WBC	0.570	0.398 to 0.742	0.0876
KT Ratio	0.506	0.328 to 0.684	0.0908
IVDMIA	0.618	0.45 to 0.786	0.0857
SOFA score	0.881	0.775 to 0.987	0.0542

The test results are outlined in Fig. 18 and Table 12. In the comparison of the ROC curves, the SOFA score had the highest AUC, possibly owing to to the fact that SOFA score is used for assessing the severity of organ failures in patients with sepsis. From Table 12, we could compare the AUC of PCT to the AUC of IVDMIA, which were 0.534 and 0.618, respectively. The AUC of IVDMIA was the second highest, which implies that it could be used as a sepsis severity marker as well as a diagnostic marker. Overall, neither PCT nor IVDMIA had significant potential for differential diagnosis between “simple” sepsis and septic shock. This could be attributed to the fact that the two diseases have similar physiology and phenotypes. Nevertheless, distinguishing between septic shock and "simple” sepsis is of significant importance, as patients with septic shock require immediate and critical care that involves elaborate procedures.

7. Evaluation of newly developed IVDMIA and existing sepsis biomarkers as a sepsis severity marker

We primarily focused on the diagnostic potential of sepsis biomarkers, including IVDMIA in the initial stages of the study. However, we next evaluated the potential of IVDMIA as a sepsis severity marker similar to the SOFA score, which is currently used in clinical practice as a severity index of sepsis.

To assess the potential of IVDMIA as a sepsis severity marker, we used the specimens from sepsis patients from the training and validating groups. To evaluate the severity of sepsis, we categorized the sepsis patients according to the respective SOFA scores[33] ≤ 8, 9 - 12, and ≥ 13. Next, we performed Kruskal-Wallis analysis of IVDMIA and PCT values in each group. The graph of the analysis is presented in Fig. 19 and the detailed information of the SOFA score groups is outlined in Table 13.

Table 13. Baseline characteristics of validating group participants divided into three groups based on SOFA scores. Data (except those related to gender) are expressed in terms of median [IQR].

	SOFA score ≤8 (N = 15)	SOFA score 9-12 (N = 20)	SOFA score ≥ 13 (N = 10)	*p-value*
Age (yr)	71.00 [61.00, 79.00]	73.50 [58.75, 78.50]	79.00 [65.25, 82.00]	0.808
Gender = M (%)	7 (43.7)	5 (19.2)	1 (33.3)	0.452
Height (cm)	163.00 [157.00, 173.50]	162.50 [158.00, 168.50]	160.50 [154.75, 163.50]	0.381
Weight (kg)	58.00 [50.50, 63.00]	58.00 [50.00, 61.50]	61.00 [53.50, 64.50]	0.766
BMI	21.00 [19.00, 23.00]	20.00 [18.00, 24.00]	24.00 [20.25, 25.00]	0.4
Glucose (mg/dL)	111.00 [102.50, 145.00]	136.00 [104.25, 219.50]	114.00 [90.25, 169.50]	0.627
Creatinine (mg/dL)	1.15 [0.73, 1.35]	1.34 [0.95, 2.21]	2.25 [1.47, 2.87]	0.02
Total protein (g/dL)	5.00 [5.00, 6.00]	6.00 [5.00, 6.00]	5.00 [5.00, 5.00]	0.008
Albumin (g/dL)	3.00 [2.50, 3.00]	3.00 [3.00, 3.25]	3.00 [2.25, 3.00]	0.146
AST (IU/L)	33.00 [18.00, 46.50]	43.00 [34.75, 74.50]	39.00 [18.00, 139.50]	0.293
ALT (IU/L)	17.00 [13.50, 28.50]	28.00 [16.25, 37.25]	15.50 [12.25, 57.75]	0.414
Bilirubin (mg/dL)	0.60 [0.35, 1.15]	0.85 [0.57, 1.02]	0.75 [0.40, 3.15]	0.598
WBC (103/μL)	10.00 [7.50, 16.50]	10.00 [5.00, 15.00]	9.00 [2.25, 16.50]	0.746
Hemoglobin (g/dL)	10.00 [9.50, 11.50]	11.00 [9.75, 12.00]	8.50 [8.00, 11.00]	0.66
Hematocrit (%)	31.00 [28.00, 34.00]	32.50 [28.75, 35.50]	27.00 [23.25, 33.00]	0.39
Platelet (103/μL)	209.00 [163.00, 258.50]	164.00 [121.00, 200.00]	115.50 [57.75, 186.25]	0.017
CRP (mg/L)	115.00 [40.00, 170.00]	60.00 [17.50, 146.50]	165.00 [79.00, 199.00]	0.284
SOFA score	7.00 [6.00, 7.00]	11.00 [11.00, 12.00]	14.00 [13.00, 16.50]	<0.001
IVDMIA	5.95 [0.96, 9.08]	3.04 [1.10, 11.07]	6.60 [1.26, 9.45]	0.928
Procalcitonin (ng/mL)	3.33 [0.76, 19.55]	15.00 [2.82, 21.78]	15.71 [6.33, 80.04]	0.211

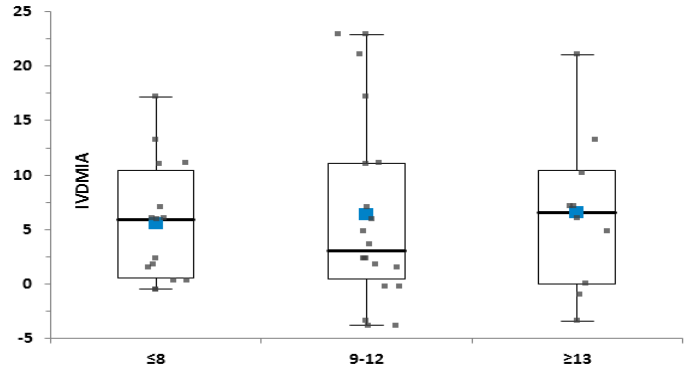

Fig. 19. The results of Kruskal-Wallis analysis of sepsis severity (represented by the SOFA score) and IVDMIA in the validating group. As shown in the figure, the SOFA score and the IVDMIA score were completely unrelated. Consistent with this, the difference was found to be statistically insignificant (***p-value*** = 0.9275).

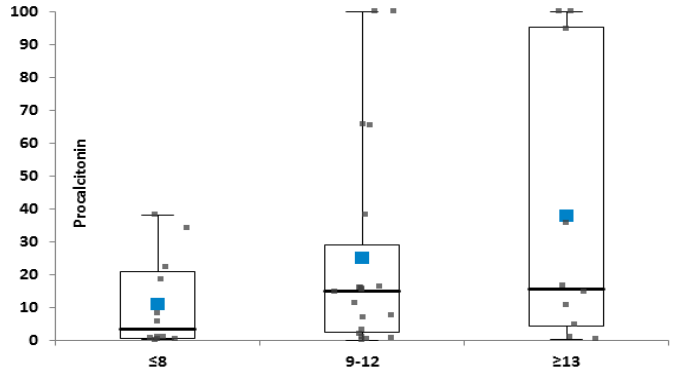

Fig. 20. The results of Kruskal-Wallis analysis of sepsis severity and PCT (Procalcitonin) in the validating group. The ***p-value*** was 0.2109, which implies that PCT concentration is not suitable as a sepsis severity marker. The data are expressed in ng/mL.

Table 14. Baseline characteristics of training group participants divided into three groups based on SOFA scores. Data (except those related to gender) are expressed in terms of median [IQR].

	SOFA score ≤ 8 (N = 71)	SOFA score 9-12 (N = 23)	SOFA score ≥ 13 (N = 21)	*p-value*
Age (yr)	69.00 [57.50, 76.50]	76.00 [69.00, 79.00]	65.00 [60.00, 79.00]	0.134
Gender = M (%)	65.42 (15.58)	72.26 (12.25)	66.67 (16.73)	0.176
Height (cm)	163.00 [156.00, 170.00]	165.00 [156.50, 170.00]	165.00 [159.75, 170.00]	0.762
Weight (kg)	58.50 [49.00, 70.75]	56.00 [50.00, 64.00]	60.00 [50.00, 66.60]	0.683
BMI	21.80 [19.05, 25.75]	21.50 [19.70, 25.70]	23.20 [19.88, 24.98]	0.97
Glucose (mg/dL)	135.00 [114.00, 198.00]	172.00 [124.50, 197.00]	136.00 [106.00, 169.00]	0.418
Creatinine (mg/dL)	1.00 [0.60, 1.85]	2.60 [1.20, 3.55]	1.60 [1.20, 3.10]	<0.001
Total protein (g/dL)	5.20 [4.95, 6.00]	4.90 [4.50, 5.70]	5.10 [4.50, 5.70]	0.126
Albumin (g/dL)	2.70 [2.30, 3.15]	2.30 [2.15, 2.60]	2.10 [1.90, 2.80]	<0.001
AST (IU/L)	33.00 [22.00, 50.00]	26.00 [19.50, 88.50]	43.00 [26.00, 86.00]	0.312
ALT (IU/L)	22.00 [11.50, 33.00]	20.00 [11.50, 36.50]	19.00 [13.00, 34.00]	0.767
Bilirubin (mg/dL)	0.50 [0.30, 0.80]	0.60 [0.30, 1.20]	1.20 [0.40, 3.40]	0.013
WBC (10^3/μL)	10.60 [7.10, 14.45]	13.60 [7.55, 18.45]	9.00 [7.40, 15.00]	0.533
Hemoglobin (g/dL)	10.50 [8.85, 11.70]	8.60 [7.80, 10.15]	8.80 [8.30, 11.30]	0.052
Hematocrit (%)	31.90 [27.10, 35.50]	26.90 [23.85, 32.10]	26.70 [24.60, 36.00]	0.068
Platelet (10^3/μL)	227.00 [163.00, 314.00]	132.00 [77.50, 205.00]	53.00 [40.00, 80.00]	<0.001
CRP (mg/L)	131.30 [48.20, 218.65]	125.20 [59.45, 275.40]	123.20 [44.40, 215.10]	0.933
SOFA score	7.00 [5.00, 8.00]	11.00 [10.00, 11.50]	14.00 [13.00, 15.00]	<0.001
Procalcitonin (ng/mL)	1.20 [0.30, 6.40]	2.80 [0.85, 16.40]	5.60 [1.80, 16.10]	0.006
IVDMIA	5.52 [3.59,7.85]	11.18 [8.25,13.52]	15.05 [13.05,17.03]	<0.001

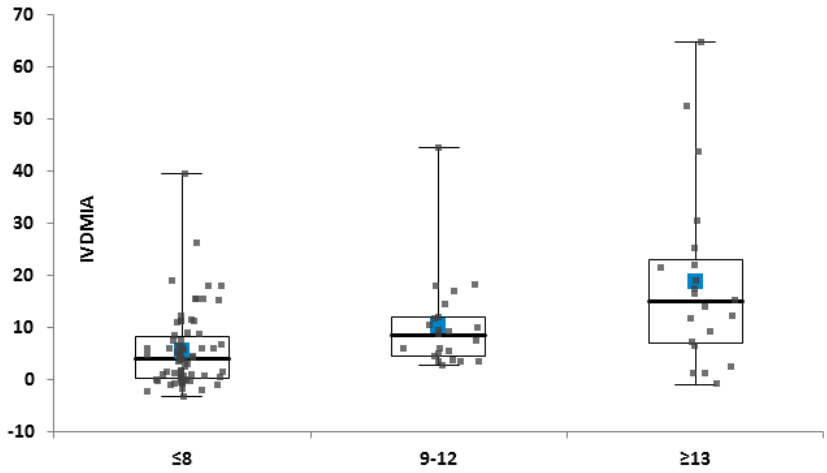

Fig. 21. The results of Kruskal-Wallis analysis of sepsis severity and IVDMIA in the training group. As shown in the figure, IVDMIA tends to share a positive correlation with the SOFA score. Statistical significance was observed as well (***p-value*** < 0.0001).

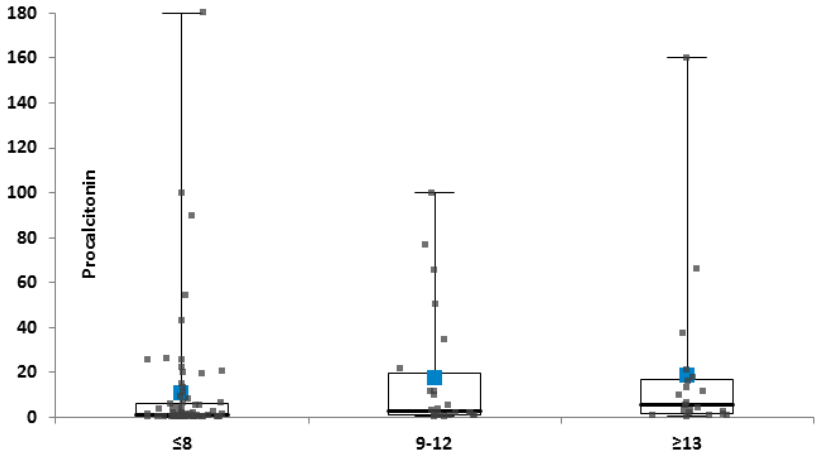

Fig. 22. The results of Kruskal-Wallis analysis of sepsis severity and PCT (Procalcitonin) concentration in the training group. As shown in the figure, PCT tends to share a positive correlation with the SOFA score. Statistical significance was observed as well (***p-value*** = 0.006). The data are expressed in ng/mL.

On the contrary, in the training group, the analysis results indicated that both IVDMIA and PCT concentration could be used as severity markers in sepsis. The reason for these contradictory results in the two groups could be attributed to the fact that the groups do not have a similar composition. Also, there were fewer participants in the validating group than in the training group. Additionally, the sepsis patients in the validating group mostly had septic shock and were not evenly distributed with "simple" sepsis patients. Therefore, it was difficult to derive meaningful results from this analysis. This topic warrants further research.

To evaluate the potential of the indexes as sepsis severity marker using alternate methods, we calculated Pearson's r coefficient between the SOFA score and the sepsis biomarkers, including PCT and IVDMIA. The SOFA score indicates the severity of sepsis. The results of the analysis were presented collectively in Table 15.

Table 15. Pearson's correlation coefficient analysis between existing and newly developed sepsis biomarkers and the SOFA score, which is an index of sepsis severity.

Biomarker	**Pearson's r**	***p-value***
IVDMIA	0.070	0.6488
PCT	0.130	0.4049
CRP	0.052	0.7337
KT ratio	0.105	0.4937
kynurenine	0.171	0.2623
tryptophan	-0.044	0.7746
arginine	0.110	0.4729
phenylalanine	0.159	0.2967

In general, we did not observe significant correlation between the sepsis biomarkers and the SOFA score. In other words, it could not be confirmed if the existing markers and the newly-developed IVDMIA markers can serve as markers for sepsis severity in clinical practice. The values of Pearson's r were mostly distant from one or -1, and were close to zero, which indicates the absence of significant correlation with the SOFA score. Additionally, ***p-value*** > 0.05 were observed in all biomarkers. In conclusion, based on the present study, all the sepsis biomarkers, dealed in this study including newly-developed IVDMIA, cannot be considered efficient biomarkers of sepsis severity.

8. Effect of infecting microorganisms on sepsis biomarkers, including IVDMIA

Several factors affect the performance of sepsis biomarkers. Among them, we focused on the infecting microorganism, which included gram-positive bacteria, gram-negative bacteria, and fungi. Certain studies state that patients typically infected by gram-negative bacteria have higher PCT concentration than patients infected by gram-positive bacteria. In addition, there is a related opinion which states that PCT concentrations are higher in cases of bacteremia than in cases of non-bacteremia infections.[32]

Based on the above, the history of infection of patients with sepsis from both training and validating groups was investigated and the patients were divided into groups according to the type of infecting microbe: gram-positive bacteria, gram-negative bacteria, and fungi.

The Kruskal-Wallis test was performed to determine if the values of the sepsis biomarkers were affected by the type of infecting microorganism. The results are outlined in Table 16 and Table 17.

Table 16. The values of sepsis markers in the training group divided into three groups based on the infecting organism. None of the markers showed statistically significant differences based on the infecting microorganisms.

	Fungus (N=24)	gram-negative bacterium (N=50)	gram-positive bacterium (N=35)	Non-cultured (N=6)	*p-value*
WBC (10^3/μL)	13.92 (8.91)	11.88 (6.58)	13.26 (11.33)	9.67 (2.80)	0.620
CRP (mg/L)	180.79 (108.16)	148.42 (126.26)	138.57 (102.41)	54.67 (89.44)	0.108
Procalcitonin (ng/mL)	16.12 (39.63)	14.30 (30.20)	11.69 (21.01)	5.17 (7.86)	0.840
KT Ratio	0.23 (0.19)	0.21 (0.19)	0.21 (0.21)	0.31 (0.56)	0.779
IVDMIA	8.12 (7.81)	9.16 (13.08)	8.95 (9.52)	7.88 (15.56)	0.980

Table 17. The values of sepsis markers in the validating group based on the type of infecting organisms. None of the markers showed statistically significant differences based on the infecting microorganisms

	Fungus (N=8)	gram-negative bacteria (N=12)	gram-positive bacteria (N=19)	Non-cultured (N=6)	*p-value*
WBC (103/μL)	7.88 (8.17)	12.42 (9.07)	11.16 (6.70)	12.33 (5.01)	0.575
CRP (mg/L)	115.50 (137.08)	113.25 (85.59)	165.47 (148.23)	122.67 (155.73)	0.679
Procalcitonin (ng/mL)	31.88 (34.28)	10.64 (14.47)	25.44 (34.84)	35.40 (45.18)	0.395
KT Ratio	0.11 (0.10)	0.22 (0.19)	0.26 (0.19)	0.16 (0.19)	0.216
IVDMIA	4.86 (10.28)	4.08 (4.79)	7.94 (7.90)	6.16 (3.95)	0.511

In Table 16 and Table 17, all the analysis results indicate that ***p-value*** > 0.05 was obtained in all cases, which indicates that the type of infecting microorganism did not affect the performance of the sepsis diagnostic markers. However, these results, which is opposite to those published in literature, could be attributed to the relatively small number of cases evaluated or to simultaneous infection by multiple microorganisms in certain patients.

Overall, the IVDMIA could not be ranked above other sepsis biomarkers in terms of its potential for diagnosing sepsis depending on the infecting microorganism. In addition, the difference in performance between the two groups is not significant. However, provided that additional evidence can be used to validate this, IVDMIA could be considered more effective than PCT as a sepsis marker.

9. IVDMIA as a prognostic factor in sepsis

Lastly, we compared the IVDMIA scores between the sepsis survivor and expired subgroups. We used the data of sepsis patients from the training group, wherein there were 54 cases of survival and 61 cases of expiration. Additionally, we also analyzed the data of surviving and expired patients from the validating group. The detailed characteristics of each group are outlined in Table 18 and Table 19.

Table 18. Baseline characteristics of training group participants subgrouped as "surviving patients" and "expired patients" in sepsis. Data (except those related to gender) are expressed in terms of median [IQR].

	Expired patients (N = 61)	**Surviving patients (N = 54)**	***p-value***
Gender = M (%)	29 (47.5)	20 (37.0)	0.343
Age (yr)	68.70 [58.55, 76.75]	70.53 [60.73, 79.88]	0.516
Height (cm)	164.50 [157.75, 170.25]	162.50 [157.25, 168.75]	0.532
Weight (kg)	57.00 [49.50, 65.70]	64.00 [51.05, 70.83]	0.122
BMI	34.86 [30.42, 39.88]	38.42 [32.43, 43.77]	0.082
Glucose (mg/dL)	149.00 [115.00, 196.00]	132.50 [112.50, 178.25]	0.207
Creatinine (mg/dL)	1.44 [0.86, 3.06]	0.98 [0.59, 2.29]	0.014
Total protein (g/dL)	5.10 [4.60, 5.70]	5.20 [4.93, 6.10]	0.057
Albumin (g/dL)	2.50 [2.20, 2.80]	2.60 [2.23, 3.00]	0.238
AST (IU/L)	33.00 [22.00, 65.00]	34.50 [22.50, 57.75]	0.801
ALT (IU/L)	23.00 [11.00, 36.00]	17.00 [12.00, 32.50]	0.446
Bilirubin (mg/dL)	0.50 [0.30, 1.10]	0.55 [0.30, 1.05]	0.917
WBC (103/μL)	10.22 [7.32, 15.06]	11.52 [7.07, 15.48]	0.984
Hemoglobin (g/dL)	9.50 [8.40, 11.10]	10.50 [8.17, 11.60]	0.428
Hematocrit (%)	27.90 [25.50, 34.70]	31.40 [24.95, 35.70]	0.521
Platelet (103/μL)	171.00 [61.00, 261.00]	207.00 [118.50, 302.25]	0.047
SOFA score	10.00 [7.00, 13.00]	7.00 [5.00, 9.00]	<0.001
CRP (mg/L)	127.30 [55.40, 217.90]	130.85 [35.65, 237.55]	0.844
KT Ratio	0.18 [0.11, 0.26]	0.13 [0.08, 0.25]	0.147
Procalcitonin (ng/mL)	3.07 [0.82, 16.12]	1.09 [0.26, 6.34]	0.005
IVDMIA	5.98 [3.49, 15.02]	4.72 [0.61, 11.00]	0.039

Table 19. Baseline characteristics of validating group participants subgrouped as "surviving patients" and "expired patients" in sepsis. Data (except those related to gender) are expressed in terms of median [IQR].

	Expired patients (N = 16)	**Surviving patients (N = 29)**	***p-value***
Gender = M (%)	3 (18.8)	8 (27.6)	<0.001
Age (yr)	75.87 [61.27, 79.87]	66.90 [60.93, 75.81]	0.351
Height (cm)	165.00 [159.00, 176.00]	163.00 [158.00, 172.00]	0.802
Weight (kg)	51.00 [47.00, 60.00]	60.00 [49.00, 63.40]	0.274
BMI	20.08 [17.26, 22.31]	21.33 [18.82, 24.03]	0.255
Glucose (mg/dL)	72.00 [35.80, 117.00]	104.00 [26.90, 166.00]	0.856
Creatinine (mg/dL)	2.82 [1.35, 42.02]	2.67 [1.14, 46.50]	0.82
Total protein (g/dL)	4.00 [2.50, 5.40]	4.80 [3.00, 5.60]	0.274
Albumin (g/dL)	4.00 [2.70, 62.00]	3.30 [3.00, 49.00]	0.891
AST (IU/L)	22.00 [14.00, 40.00]	36.00 [24.00, 74.00]	0.072
ALT (IU/L)	8.00 [0.90, 19.00]	13.00 [1.30, 33.00]	0.4
Bilirubin (mg/dL)	0.90 [0.40, 1.00]	0.70 [0.50, 1.50]	0.855
WBC (10^3/μL)	10.37 [8.04, 15.09]	10.19 [4.09, 16.05]	0.641
Hemoglobin (g/dL)	8.35 [4.30, 10.05]	8.10 [4.00, 12.00]	0.594
Hematocrit (%)	30.00 [27.60, 31.00]	31.10 [26.30, 37.10]	0.054
Platelet (10^3/μL)	218.00 [182.00, 292.00]	188.00 [157.00, 215.00]	0.155
SOFA score	10.00 [8.00, 11.00]	11.00 [7.00, 13.00]	0.442
CRP (mg/L)	101.20 [32.60, 200.50]	112.70 [45.10, 178.40]	0.882
KT Ratio	0.11 [0.05, 0.16]	0.16 [0.08, 0.42]	0.168
Procalcitonin (ng/mL)	15.76 [3.11, 22.38]	9.64 [0.95, 29.76]	0.427
IVDMIA	4.77 [0.22, 12.59]	4.84 [1.54, 7.10]	0.776

The results indicate that there is significant difference between the IVDMIA scores of expired and surviving patients in the training group, as indicated by ***p-value*** = 0.039. This result implies that IVDMIA can be used as a prognostic factor for sepsis. However, in the validating group, none of the markers showed statistically significant results, which implies that both the existing markers and IVDMIA are unsuitable as prognostic markers. This difference in the results could be attributed to the small size of the validating group.

We also performed Mann-Whitney test for IVDMIA scores in the training and validating groups. The graph is presented in Fig. 23 and Fig. 24.

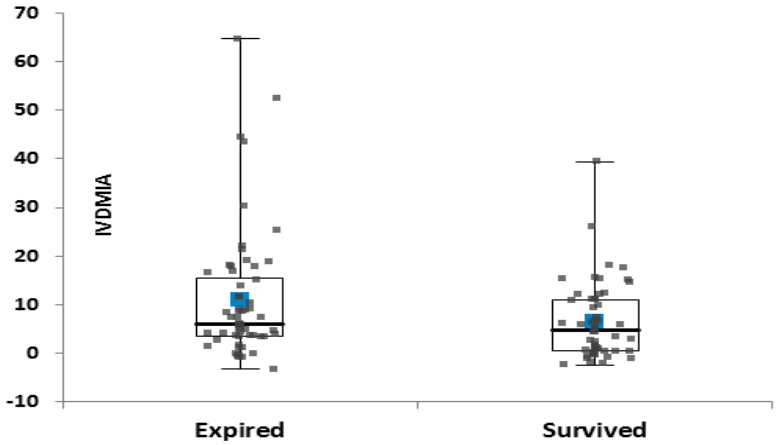

Fig. 23. Results of the comparison of IVDMIA scores between surviving patients and expired patients in the training group. The ***p-value*** was 0.0392, which implies that there was statistically significant difference between the two groups.

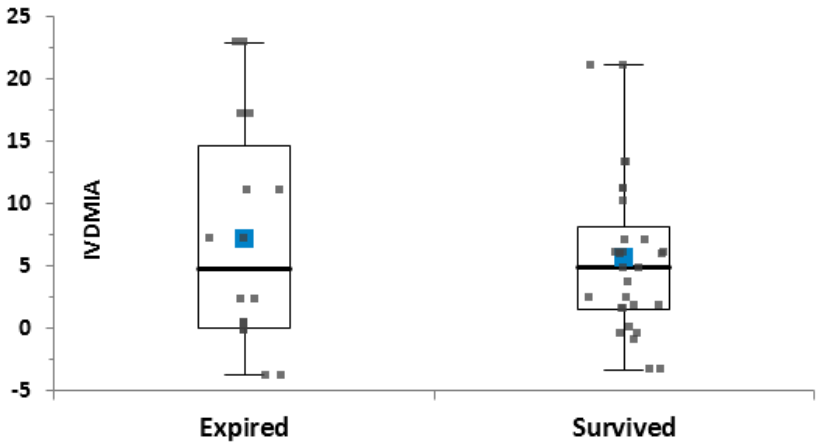

Fig. 24. Results of the comparison of IVDMIA scores between surviving patients and expired patients in the validating group. The ***p-value*** was 0.7759, which indicates that there was no statistically significant difference between the two groups.

In contrast to that in the training group, there was no statistically significant result in the validating group. This could be attributed to the small sample size of the validating group, among other causes. Therefore, re-analysis of the data in a larger sample size in the validating group is recommended in further studies.

IV. DISCUSSION

The advances in metabolomics and the demand for personalized medicine have driven the development and application of biomarkers for risk assessment, early detection, diagnosis, treatment selection, prognosis, and monitoring. In clinical practice, patients with sepsis typically exhibit significant heterogeneity with respect to variables such as age, the presence of underlying or secondary disease, the state of the immune system, and the severity of the infection. Additionally, the term “sepsis” covers a wide variety of pathophysiological processes that occur in an infected individual.[34] Such features of heterogeneity in sepsis increases the challenges of developing personalized treatment methods. Owing to its heterogeneous nature, a metabolomics approach is preferred in the treatment of sepsis. In particular, there are certain studies on the treatment of the sepsis using metabolomics techniques.[10-12,35]

Sepsis is a major catabolic insult that leads to increased muscle breakdown and nitrogen loss.[36-38] Energy deficit occurs in sepsis owing to insulin resistance in the peripheral tissues, primarily in the muscle,[39] and causes muscle breakdown and oxidation of amino acids, mainly branched chain amino acids (BCAAs), to fulfill the energy requirements of the body. Therefore, the utilization of BCAAs for energy generation is increased. The selective use of BCAAs to fulfill energy requirements involves extensive muscle breakdown and proteolysis, which, when coupled with relative hepatic insufficiency, significantly increases the plasma levels of most amino acids present in muscles, particularly the aromatic amino acids (phenylalanine and tyrosine) and the sulfur-containing amino acids (taurine, cysteine, and methionine) which are muscle-rich amino acids.[40] Conversely, the levels of BCAAs (valine, leucine, and isoleucine) are usually within normal limits. Patients with sepsis exhibit a marked increase in plasma phenylalanine and tyrosine levels, whereas arginine levels are observed to be exceptionally low.[40] Considering the severe changes in the

concentrations of amino acids in sepsis, metabolomics research could serve as an efficient solution.

Currently, we are in urgent need of effective biomarkers and reliable measurements that can be used for the diagnosis and risk stratification of patients with sepsis and would help in effective identification of clinically significant patients who are at the highest risk of a poor outcome. Such markers would be of fundamental importance for decisions regarding early intervention therapy or for the designing of clinical trials of sepsis. Furthermore, the availability of more specific therapies may clarify the pathophysiologic process underlying the development of sepsis and minimize their sequela. Appropriate biomarkers will be useful at this point in the treatment process, for screening of patients with sepsis and identifying those eligible for specific therapies. However, the efficacy of biomarkers developed to date has been insufficient.

In the current situation, IVDMIA could serve as an important alternative. The advantages of IVDMIA compared to those of a single biomarker assay are based on the premise that the single-valued index, which is aggregated information from several complementary biomarkers, will outperform each of the component biomarkers used individually.[13] The IVDMIA index developed in this study can serve as a promising biomarker for sepsis. Additionally, it also offers highghly sufficient sensitivity and specificity.

To develop IVDMIA as a biomarker for sepsis, we selected four candidate amino acids: arginine, phenylalanine, tryptophan, and kynurenine. The concentrations of these amino acid candidates used effectively to differentiate between sepsis and non-sepsis patients in principle component analysis, both in the preliminary study as well as in the main study. Using these four amino acids, we performed logistic regression and obtained several equations, from which we selected one based on the AUC, sensitivity, and specificity values.

Tryptophan, one of these four amino acids, is an essential amino acid and plays

a key role in mammals as a precursor of physiological substances including serotonin and melatonin. Approximately 95% of tryptophan is metabolized via the kynurenine pathway. IDO is a rate-limiting enzyme in this reaction that is localized in hepatic tissues. IDO is induced by interferon-gamma, toll-like receptor, and bacterial DNA, which are molecules related to infection and inflammation. IDO-mediated acceleration of tryptophan metabolism to kynurenine in sepsis is relatively well-known.[14,28] The increase in the enzymatic activity of IDO, which occurs as a part of the immune defense mechanism during infection, leads to the breakdown of tryptophan to kynurenine. The levels of IDO and kynurenine exhibited a strong positive correlation with those of PCT, whereas they were negatively correlated with the levels of tryptophan.[41]

Arginine is a nonessential amino acid under normal physiological conditions that is derived from food intake,[42,43] and is also synthesized ***de novo*** from citrulline in the kidney.[44] However, arginine becomes a semi-essential amino acid as an important initiator of immune response. Arginine is catabolized through various pathways.[44] First, arginine is incorporated in proteins used in the body. Second, arginine is a substrate for the synthesis of urea and ornithine mediated by arginase.[45] Third, arginine is the only substrate in the synthesis of nitric oxide (NO) by NO synthase. However, arginine deficiencies may occur in multiple events associated with sepsis, including the reduction of arginine synthesis in kidney and the increase in the synthesis of proteins such as acute phase proteins, which contain arginine. Although the balance between arginine anabolism and catabolism is maintained in moderate inflammation, arginine catabolism possibly overrides anabolism in severe inflammation such as that occurring in sepsis, and consequently, the plasma levels of arginine reduce. The findings from studies, wherein plasma arginine concentration was observed to reduce in sepsis, are consistent with the findings of our research.[23,24,46,47]

Phenylalanine, which is an essential amino acid and a necessary precursor in the

synthesis of catecholamines and thyroid hormone, is converted to tyrosine in the liver by phenylalanine hydroxylase (PAH), which is a sensitive hepatic enzyme.[48] The concentration of phenylalanine has been observed to increase following infectious disease.[49] The serum concentrations of phenylalanine could increase as a result of the increase in the release of phenylalanine from skeletal muscle proteins, the reduction in the conversion of phenylalanine to tyrosine in the liver, or alterations in renal clearance. In our study, we observed that the concentration of phenylalanine increased with the progression of sepsis. Although the elevation in the levels of phenylalanine in the blood of patients with trauma and sepsis has been noted commonly, the reason remains unexplained. However, recently, a study revealed that increased phenylalanine levels are indicative of insufficient conversion to tyrosine by PAH owing to damage caused to the cofactor 5,6,7,8-tetrahydrobiopterin by oxidative stress resulting from immune activation.[27] Specifically, in macrophages that are activated in infections, the formation of neotriptin, which is an immune activation marker,[27] is paralleled by the release of toxic reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2) or hydrochlorous acid (HOCl).[50]

In this study, a relatively significant number of participants were included compared to that in similar studies published previously. This strengthens the statistical significance of our study, especially with respect to the validity of IVDMIA. In addition, we could acquire abundant data about the participants using checkup questionnaires and the EMR system at Severance Hospital. This enabled us to acquire valuable information about underlying diseases, vital signs, abnormal results of laboratory tests, and the drugs administered among others. Based on these characteristics of our study, we can reasonably conclude about the novelty and superiority of this study compared to those published to date on the same topic.

However, there are certain limitations to this study. First, experiments based on serial sampling from one sepsis patient could be useful for determining the pathophysiology in sepsis and suggesting solutions. Second, since the number of

validation set was relatively small, especially with respect to the number of patients with sepsis, the power of validation in this study may be relatively weak. Therefore, the results should be validated and refined through rigorous analysis in additional validation test sets.

Additionally, IVDMIA, which is the single-valued index derived in this study, would not always guarantee better clinical performance results compared to a single biomarker test.[51,52] For a example, A large-scale study revealed that the combined use of biomarkers for ovarian cancer did not guarantee better performance than the CA125 test,[51] and it remains debatable whether the combined use of HE4 and CA125 offers better clinical performance than the CA125 test alone.[52]

The IVDMIA we developed in this study showed significantly better results and had greater potential than preexisting sepsis markers apart from PCT. The performance of PCT in sepsis diagnosis was similar to that of IVDMIA. Therefore, it is necessary to conduct further studies to compare the performance of PCT and IVDMIA, especially in patients with neutropenia. PCT is produced by WBCs to some extent,[53] owing to which its diagnostic value in patients with conditions such as febrile neutropenia or fungal infection is questionable, since these conditions are not well-diagnosed using PCT.[54] In particular, it is known that the plasma levels of PCT are significantly higher in patients with bacterial infections than in patients with viral infections.[55,56] Therefore, a comparative study with neutropenia or with viral infection patients would help demonstrate the potential of IVDMIA.

Although the developed IVDMIA did not perform encouragingly in experiments on sepsis classification, it demonstrated remarkable diagnostic potential and possible applicability as a prognostic predictor. We consider that a few additional steps are required before the IVDMIA can be applied in clinical practice. Lastly, we hope that our research will pave the way for further studies on the potential of IVDMIA as a diagnostic marker in other heterogeneous diseases, such as cancer.

V. CONCLUSION

Precision medicine is an emerging field for disease prevention and treatment as it considers the individual variability in the molecular signatures of each patient. However, treatment success and survival rates in sepsis are relatively poor compared to those in other diseases because individualized treatment is difficult owing to the significant heterogeneity in the conditions. The majority of patients with sepsis exhibit broad and varied clinical characteristics, and it is difficult to screen patients and facilitate early diagnosis in sepsis. Therefore, the implementation of emergent individualized therapies that are optimized for each patients is important in sepsis. In practice, the efficient treatment and prevention of complications in sepsis rely on early and precise diagnosis.

In such scenarios, metabolomics technologies could serve as an excellent alternative. Recently, the development of metabolomics technologies has led to the recognition of their significance in clinical work in the field of critical care medicine.[57-60] Nevertheless, identification of sepsis biomarkers that are reliable and easily applicable in clinical practice is an ongoing process.

To date, several omics-based approaches have been used to develop effective biomarker candidates. The results of such metabolomics studies in sepsis patients suggests that there is an overall derangement of energy circuits and both amino acid and lipid metabolism in sepsis pathophysiology.[11,61] In addition, the results also suggest that the magnitude of these changes might possibly serve as an indicator of sepsis diagnosis and severity, and could also provide useful information for the treatment of sepsis.

Based on these factors, we adopted a metabolomics approach and identified several reliable candidate amino acid biomarkers of sepsis, such as kynurenine, tryptophan, KT ratio, phenylalanine, and arginine using LC-MS/MS. After statistical

analysis using tests such as Mann-Whitney test, Kruskal-Wallis test, PCA, PLS-DA, and logistic regression, the single-value index IVDMIA was developed, using the four selected amino acid biomarkers, which could be applied in the clinical settings.

In conclusion, this study demonstrated that a metabolomics approach in the diagnosis of sepsis could yield promising results because it is based on the individual characteristic metabolomic states, which would enable clinicians to use tailored medicine, ensure early diagnosis, provide adequate treatment, and predict prognosis precisely. Using the results of preceding metabolomics experiments, IVDMIA was used to generate simple and high-quality indexes that were highly specific for sepsis.

To the best of our knowledge, this is the first metabolomics study that addresses the topic of sepsis and includes patients with SIRS, which is a condition that is barely distinguishable from sepsis. We have confirmed that the metabolic processes, particularly amino acid metabolism, in sepsis patients are different from those in healthy individuals, and the concentrations of selected amino acids differed significantly from those in healthy individuals. These candidate amino acids were used to formulate a sepsis index using IVDMIA, and the newly developed IVDMIA for sepsis performed remarkably well as a diagnostic tool. Therefore, we opine that the IVDMIA index will be able to assist in the diagnosis and treatment of sepsis. Furthermore, this study might aid clinicians and researchers by improving their understanding of the applicability of metabolomics in medicine and the development of IVDMIA.

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;315:801-10.

2. Bone RC, Balk RA, Cerra FB, Dellinger RP, Fein AM, Knaus WA, et al. Definitions for sepsis and organ failure and guidelines for the use of innovative therapies in sepsis. The ACCP/SCCM Consensus Conference Committee. American College of Chest Physicians/Society of Critical Care Medicine. Chest 1992;101:1644-55.

3. Martin GS, Mannino DM, Eaton S, Moss M. The epidemiology of sepsis in the United States from 1979 through 2000. N Engl J Med 2003;348:1546-54.

4. Pittet D, Rangel-Frausto S, Li N, Tarara D, Costigan M, Rempe L, et al. Systemic inflammatory response syndrome, sepsis, severe sepsis and septic shock: Incidence, morbidities and outcomes in surgical ICU patients. Intensive Care Medicine 1995;21:302-9.

5. Hotchkiss RS, Karl IE. The pathophysiology and treatment of sepsis. N Engl J Med 2003;348:138-50.

6. Russell JA. Management of sepsis. N Engl J Med 2006;355:1699-713.

7. Cohen J, Vincent JL, Adhikari NK, Machado FR, Angus DC, Calandra T, et al. Sepsis: a roadmap for future research. Lancet Infect Dis 2015;15:581-614.

8. Angus DC, van der Poll T. Severe sepsis and septic shock. N Engl J Med 2013;369:840-51.

9. Ferrario M, Cambiaghi A, Brunelli L, Giordano S, Caironi P, Guatteri L, et al. Mortality prediction in patients with severe septic shock: a pilot study using a target metabolomics approach. Sci Rep 2016;6:20391.

10. Schmerler D, Neugebauer S, Ludewig K, Bremer-Streck S, Brunkhorst FM,

Kiehntopf M. Targeted metabolomics for discrimination of systemic inflammatory disorders in critically ill patients. J Lipid Res 2012;53:1369-75.

11. Rogers AJ, McGeachie M, Baron RM, Gazourian L, Haspel JA, Nakahira K, et al. Metabolomic derangements are associated with mortality in critically ill adult patients. PLoS One 2014;9:e87538.

12. Mickiewicz B, Duggan GE, Winston BW, Doig C, Kubes P, Vogel HJ. Metabolic profiling of serum samples by 1H nuclear magnetic resonance spectroscopy as a potential diagnostic approach for septic shock. Crit Care Med 2014;42:1140-9.

13. Bozza FA, Bozza PT, Castro Faria Neto HC. Beyond sepsis pathophysiology with cytokines: what is their value as biomarkers for disease severity? Memórias do Instituto Oswaldo Cruz 2005;100:217-21.

14. Schmidt SV, Schultze JL. New Insights into IDO Biology in Bacterial and Viral Infections. Frontiers in Immunology 2014;5:384.

15. Darcy CJ, Davis JS, Woodberry T, McNeil YR, Stephens DP, Yeo TW, et al. An observational cohort study of the kynurenine to tryptophan ratio in sepsis: association with impaired immune and microvascular function. PLoS One 2011;6:e21185.

16. Changsirivathanathamrong D, Wang Y, Rajbhandari D, Maghzal GJ, Mak WM, Woolfe C, et al. Tryptophan metabolism to kynurenine is a potential novel contributor to hypotension in human sepsis. Crit Care Med 2011;39:2678-83.

17. Zeden JP, Fusch G, Holtfreter B, Schefold JC, Reinke P, Domanska G, et al. Excessive tryptophan catabolism along the kynurenine pathway precedes ongoing sepsis in critically ill patients. Anaesth Intensive Care 2010;38:307-16.

18. Zhang Z. An In Vitro Diagnostic Multivariate Index Assay (IVDMIA) for Ovarian Cancer: Harvesting the Power of Multiple Biomarkers. Rev Obstet Gynecol 2012;5:35-41.

19. Joon-Seok C. Study on Development of IVDMIA Medical Device Evaluation

Technology for Personalized Medicine. Regulatory Research on Food, Drug and Cosmetic 2015;10:189-95.

20.Jeong TD, Cho EJ, Ko DH, Lee W, Chun S, Kwon HJ, et al. A new strategy for calculating the risk of ovarian malignancy algorithm (ROMA). Clin Chem Lab Med 2017;55:1209-14.

21.Munn DH, Mellor AL. Indoleamine 2,3 dioxygenase and metabolic control of immune responses. Trends Immunol 2013;34:137-43.

22.Services UDoHaH. Draft Guidance for Industry, Clinical Laboratories, and Staff: In Vitro Diagnostic Multivariate Index Assays.; April 2, 2012.

23.Wijnands KA, Castermans TM, Hommen MP, Meesters DM, Poeze M. Arginine and citrulline and the immune response in sepsis. Nutrients 2015;7:1426-63.

24.Luiking YC, Poeze M, Ramsay G, Deutz NE. The role of arginine in infection and sepsis. JPEN J Parenter Enteral Nutr 2005;29:S70-4.

25.O'Dwyer MJ, Dempsey F, Crowley V, Kelleher DP, McManus R, Ryan T. Septic shock is correlated with asymmetrical dimethyl arginine levels, which may be influenced by a polymorphism in the dimethylarginine dimethylaminohydrolase II gene: a prospective observational study. Critical Care 2006;10:R139.

26.Huang SS, Lin JY, Chen WS, Liu MH, Cheng CW, Cheng ML, et al. Phenylalanine- and leucine-defined metabolic types identify high mortality risk in patients with severe infection. Int J Infect Dis 2019;85:143-9.

27.Ploder M, Neurauter G, Spittler A, Schroecksnadel K, Roth E, Fuchs D. Serum phenylalanine in patients post trauma and with sepsis correlate to neopterin concentrations. Amino Acids 2008;35:303-7.

28.Logters TT, Laryea MD, Altrichter J, Sokolowski J, Cinatl J, Reipen J, et al. Increased plasma kynurenine values and kynurenine-tryptophan ratios after major trauma are early indicators for the development of sepsis. Shock 2009;32:29-34.

29.Suzuki Y, Suda T, Yokomura K, Suzuki M, Fujie M, Furuhashi K, et al. Serum activity of indoleamine 2,3-dioxygenase predicts prognosis of community-

acquired pneumonia. J Infect 2011;63:215-22.

30. Suzuki Y, Suda T, Furuhashi K, Suzuki M, Fujie M, Hahimoto D, et al. Increased serum kynurenine/tryptophan ratio correlates with disease progression in lung cancer. Lung Cancer 2010;67:361-5.

31. Park DW, Chun BC, Kim JM, Sohn JW, Peck KR, Kim YS, et al. Epidemiological and Clinical Characteristics of Community-Acquired Severe Sepsis and Septic Shock: A Prospective Observational Study in 12 University Hospitals in Korea. J Korean Med Sci 2012;27:1308-14.

32. Yunus I, Fasih A, Wang Y. The use of procalcitonin in the determination of severity of sepsis, patient outcomes and infection characteristics. PLoS One 2018;13:e0206527.

33. Desai S, Lakhani JD. Utility of SOFA and APACHE II score in sepsis in rural set up MICU. J Assoc Physicians India 2013;61:608-11.

34. Jensen JU, Bouadma L. Why biomarkers failed in sepsis. Intensive Care Med 2016;42:2049-51.

35. Mickiewicz B, Tam P, Jenne CN, Leger C, Wong J, Winston BW, et al. Integration of metabolic and inflammatory mediator profiles as a potential prognostic approach for septic shock in the intensive care unit. Crit Care 2015;19:11.

36. Cuthbertson D, Tilstone WJ. Metabolism during the postinjury period. Adv Clin Chem 1969;12:1-55.

37. Biolo G, Toigo G, Ciocchi B, Situlin R, Iscra F, Gullo A, et al. Metabolic response to injury and sepsis: changes in protein metabolism. Nutrition 1997;13:52s-7s.

38. O'Donnel TF, Clowes GH, Jr., Blackburn GL, Ryan NT, Benotti PN, Miller JD. Proteolysis associated with a deficit of peripheral energy fuel substrates in septic man. Surgery 1976;80:192-200.

39. Ryan NT, George BC, Egdahl DH, Egdahl RH. Chronic tissue insulin resistance following hemorrhagic shock. Annals of surgery 1974;180:402-7.

40. Freund HR, Ryan JA, Fischer JE. Amino Acid Derangements in Patients With Sepsis: Treatment With Branched Chain Amino Acid Rich Infusions. Annals of Surgery 1978;188:423-9.

41. Meier MA, Ottiger M, Vogeli A, Steuer C, Bernasconi L, Thomann R, et al. Activation of the tryptophan/serotonin pathway is associated with severity and predicts outcomes in pneumonia: results of a long-term cohort study. Clin Chem Lab Med 2017;55:1060-9.

42. Heys SD, Gardner E. Nutrients and the surgical patient: current and potential therapeutic applications to clinical practice. J R Coll Surg Edinb 1999;44:283-93.

43. Visek WJ. Arginine needs, physiological state and usual diets. A reevaluation. J Nutr 1986;116:36-46.

44. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. Biochem J 1998;336 (Pt 1):1-17.

45. Cynober L. Can arginine and ornithine support gut functions? Gut 1994;35:S42-5.

46. Garcia-Martinez C, Llovera M, Lopez-Soriano FJ, Argiles JM. The effects of endotoxin administration on blood amino acid concentrations: similarities with sepsis. Cell Mol Biol (Noisy-le-grand) 1993;39:537-42.

47. Bruins MJ, Lamers WH, Meijer AJ, Soeters PB, Deutz NE. In vivo measurement of nitric oxide production in porcine gut, liver and muscle during hyperdynamic endotoxaemia. Br J Pharmacol 2002;137:1225-36.

48. Herndon DN, Wilmore DW, Mason AD, Jr., Pruitt BA, Jr. Abnormalities of Phenylalanine and Tyrosine Kinetics: Significance in Septic and Nonseptic Burned Patients. Archives of Surgery 1978;113:133-5.

49. Wannemacher RW, Jr., Klainer AS, Dinterman RE, Beisel WR. The significance and mechanism of an increased serum phenylalanine-tyrosine ratio during infection. Am J Clin Nutr 1976;29:997-1006.

50. Nathan CF. Peroxide and pteridine: a hypothesis on the regulation of

macrophage antimicrobial activity by interferon gamma. Interferon 1986;7:125-43.

51. Timmerman D, Van Calster B, Vergote I, Van Hoorde K, Van Gorp T, Valentin L, et al. Performance of the American College of Obstetricians and Gynecologists' ovarian tumor referral guidelines with a multivariate index assay. Obstet Gynecol 2011;118:1179-81; author reply 81.

52. Van Gorp T, Cadron I, Despierre E, Daemen A, Leunen K, Amant F, et al. HE4 and CA125 as a diagnostic test in ovarian cancer: prospective validation of the Risk of Ovarian Malignancy Algorithm. Br J Cancer 2011;104:863-70.

53. Oberhoffer M, Stonans I, Russwurm S, Stonane E, Vogelsang H, Junker U, et al. Procalcitonin expression in human peripheral blood mononuclear cells and its modulation by lipopolysaccharides and sepsis-related cytokines in vitro. J Lab Clin Med 1999;134:49-55.

54. Dornbusch HJ, Strenger V, Kerbl R, Lackner H, Schwinger W, Sovinz P, et al. Procalcitonin--a marker of invasive fungal infection? Support Care Cancer 2005;13:343-6.

55. Assicot M, Gendrel D, Carsin H, Raymond J, Guilbaud J, Bohuon C. High serum procalcitonin concentrations in patients with sepsis and infection. Lancet 1993;341:515-8.

56. Al-Nawas B, Krammer I, Shah PM. Procalcitonin in diagnosis of severe infections. Eur J Med Res 1996;1:331-3.

57. Liu XR, Zheng XF, Ji SZ, Lv YH, Zheng DY, Xia ZF, et al. Metabolomic analysis of thermally injured and/or septic rats. Burns 2010;36:992-8.

58. Izquierdo-Garcia JL, Nin N, Ruiz-Cabello J, Rojas Y, de Paula M, Lopez-Cuenca S, et al. A metabolomic approach for diagnosis of experimental sepsis. Intensive Care Med 2011;37:2023-32.

59. Xu PB, Lin ZY, Meng HB, Yan SK, Yang Y, Liu XR, et al. A metabonomic approach to early prognostic evaluation of experimental sepsis. J Infect 2008;56:474-81.

60. Lin ZY, Xu PB, Yan SK, Meng HB, Yang GJ, Dai WX, et al. A metabonomic approach to early prognostic evaluation of experimental sepsis by (1)H NMR and pattern recognition. NMR Biomed 2009;22:601-8.

61. Remick DG. Pathophysiology of Sepsis. The American Journal of Pathology 2007;170:1435-44.

ABSTRACT (IN KOREAN)

Metabolomics 기법을 적용한 체외진단다지표분석 (IVDMIA)을 이용한 패혈증에서의 새로운 조기 바이오마커의 개발 및 검증

<지도교수 이상국> 연세대학교 대학원 의학과

안 선 영

배경: 패혈증은 미생물에 감염되어 전신에 걸친 염증 반응이 나타나는 상태이며, 이러한 상태가 지속되거나 악화하여 다발성 장기 부전을 거쳐 사망에 이를 수 있는 주요한 질환이다. 그러나 패혈증의 병태생리학이나 대사 작용에 대해서는 알려진 바가 많지 않다. 패혈증은 다양하고 복잡한 특징을 지니기 때문에 각 경우에 맞는 적절한 진단과 치료가 필요하지만, 맞춤 의학이 발전하는 최근에도 이를 패혈증에 적용하기는 쉽지 않은 것이 현실이다. 따라서 여러 가지의 바이오마커를 병합하여 이용하는 방안인 체외진단다지표분석 (In Vitro Diagnostic Multivariate Index Assay, IVDMIA)이 제기되었고, 본 연구에서는 Metabolomics 기법을 비용한 패혈증 환자에서의 새로운 조기 바이오마커의 개발 및 검증이 시행되었다.

방법: 연구에 동의한 건강검진 대상자, 전신염증반응증후군 환자, 패혈증 환자의 세 그룹 참여자들로부터 검사 후 잔여 혈청을 획득하였다. 이 검체들을 대상으로 액체 크로마토그래피 질량 분광법을 이용해 Metabolomics 를 시행하였고, 그 결과를 주성분 분석 통계법으로

분석하였다. 분석 결과를 통해 패혈증에서의 체외진단다지표분석에 이용할 패혈증 조기 마커들을 선정하였고, 이들을 적용해 패혈증 지표를 생성하였다.

결과: 세 그룹에 대해 시행한 Metabolomics 결과 분석을 거쳐 kynurenine, tryptophan, KT ratio, phenylalanine, 그리고 arginine 등 5 개의 아미노산 및 그 계산치를 패혈증 바이오마커로 선정하였고, 선정된 바이오마커들을 이용해 패혈증에서의 체외진단 다지표분석을 거쳐 임상에서 사용이 용이한 패혈증 지표-IVDMIA-를 만들어내었다. 생성된 패혈증 지표는 실제 환자 그룹에 적용되어 진단, 중증도, 예후 평가 및 예측 등에서 그 임상적 성능이 검증되었다.

결론: 본 연구는 Metabolomics 기법을 통해 패혈증 환자에서 아미노산 구성을 포함한 신진대사가 유의하게 교란됨을 입증하였으며, 혈청의 아미노산 분석을 통해 패혈증에 유의한 아미노산들을 발굴하였다. 이를 기반으로 패혈증 지표가 생성되었고 그 성능이 검증되어 임상에서 유용하게 쓰일 것으로 기대된다.

핵심되는 말: Metabolomics, 패혈증, 바이오마커, 액체 크로마토그래피 질량 분광법, 체외진단다지표분석 (IVDMIA), kynurenine, tryptophan, phenylalanine, arginine, KT Ratio