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Article

Point-of-care neutrophil and monocyte surface markers differentiate bacterial from viral infections at the emergency department within 30 min

Bernard N. Jukema,[1,2,†] Titus A.P. de Hond,[3,†] Martijn Kroon,[2] Anna E. Maranus,[2] Leo Koenderman,[1,2,‡] and Karin A.H. Kaasjager[3,*‡]

[1]Department of Respiratory Medicine, University Medical Centre Utrecht, Utrecht University, Heidelberglaan 100, Utrecht, 3584 CX, The Netherlands
[2]Centre for Translational Immunology, University Medical Centre Utrecht, Utrecht University, Heidelberglaan 100, Utrecht, 3584 CX, The Netherlands
[3]Department of Internal Medicine and Acute Medicine, University Medical Centre Utrecht, Utrecht University, Heidelberglaan 100, Utrecht, 3584 CX, The Netherlands
*Corresponding author: Department of Internal Medicine and Acute Medicine, University Medical Centre Utrecht, Utrecht University, Heidelberglaan 100, Utrecht, 3584 CX, The Netherlands. Email: h.a.h.kaasjager@umcutrecht.nl

Abstract

Rapid discrimination between viral and bacterial infections in a point-of-care setting will improve clinical outcome. Expression of CD64 on neutrophils (neuCD64) increases during bacterial infections, whereas expression of CD169 on classical monocytes (cmCD169) increases during viral infections. The diagnostic value of automated point-of-care neuCD64 and cmCD169 analysis was assessed for detecting bacterial and viral infections at the emergency department. Additionally, their value as input for machine learning models was studied. A prospective observational cohort study in patients suspected of infection was performed at an emergency department. A fully automated point-of-care flow cytometer measured neuCD64, cmCD169, and additional leukocyte surface markers. Flow cytometry data were gated using the FlowSOM algorithm. Bacterial and viral infections were assessed in standardized clinical care. The sole and combined diagnostic value of the markers was investigated. Clustering based on unsupervised machine learning identified unique patient clusters. Eighty-six patients were included. Thirty-five had a bacterial infection, 30 had a viral infection, and 21 had no infection. neuCD64 was increased in bacterial infections ($P < 0.001$), with an area under the receiver operating characteristic curve (AUROC) of 0.73. cmCD169 was higher in virally infected patients ($P < 0.001$; AUROC 0.79). Multivariate analyses incorporating additional markers increased the AUROC for bacterial and viral infections to 0.86 and 0.93, respectively. The additional clustering identified 4 distinctive patient clusters based on infection type and outcome. Automated neuCD64 and cmCD169 determination can discriminate between bacterial and viral infections. These markers can be determined within 30 min, allowing fast infection diagnostics in the acute clinical setting.

Keywords: acute disease, bedside, CD169, CD64, diagnostic, differentiation

1. Introduction

Slow and/or incorrect diagnosis of infections including early sepsis is one of the biggest causes of preventable deaths of hospitalized patients at the emergency department (ED).[1] Incorrect diagnoses leads to inappropriate use of antibiotics and consequently complications such as anaphylactic reactions, *Clostridioides* infections, and increased antibiotic resistance. Additionally, these complications can lead to increased length of stay and increased healthcare costs. Current techniques for identifying infections such as polymerase chain reaction, bacterial culturing, or rapid urinary antigen tests can be inconclusive and definite results (e.g. for blood cultures) can take up to 5 days. Therefore, new approaches for rapidly and adequately diagnosing infections in patients presenting at the ED are of major importance.

Research has pointed out several cellular markers that are promising for diagnosing and discriminating bacterial and viral infections: specifically, expression of CD64 (FcgRI) on neutrophils (neuCD64) and CD169 (Siglec-1) on classical monocytes (cmCD169).[2–6] Other surface markers are known to be correlated with outcome: a low HLA-DR expression on monocytes is an important marker for severe outcome, reflecting the immunoparalysis in the subacute phase of sepsis.[7] This reduced HLA-DR expression correlates with impaired release of proinflammatory cytokines and a reduction in antigen-presenting capabilities, resulting in increased rates of nosocomial infections and death.[8,9]

The assessment of these surface markers is complicated, as they are measured by using flow cytometry. This technique requires specialized lab personnel, is time-consuming (it often takes 4–6 h before results can be interpreted), and is usually only performed during office hours. The time-consuming character also leads to a relatively long bench time, which is correlated with upregulation of activation markers, decreasing assay reliability. A bench time of 1 h already leads to significant upregulation and increasing variance in neutrophil and monocyte markers.[10] These limitations have restricted the clinical implementation of flow cytometric analyses based on innate immune biomarkers.

The previously mentioned difficulties can be resolved by using an automated flow cytometer that automatically prepares and analyzes blood samples in a point-of-care setting. Results are

† These authors have contributed equally to this work and share first authorship.
‡ These authors have contributed equally to this work and share last authorship.

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available within 30 min after placing the blood collecting tube in the machine, which is a breakthrough for the clinical application of these markers. Especially in an ED setting, in which time is scarce, an automated point-of-care system could have significant clinical value for diagnosing infections, potentially accelerating and improving antibiotic treatment strategies. The application of automated flow cytometry analysis of neuCD64 as stand-alone marker for bacterial infections has already been demonstrated.[6] A limitation was that this stand-alone marker could not be used to rule out bacterial infections. Adding other leukocyte markers, such as cmCD169, to the automated flow cytometry analyses resolves this limitation. Multiple intensive care unit studies showed that patient clusters identified by machine learning algorithms could be used to identify sepsis patient groups with specific treatment requirements.[11–13] Combining automated point of care–measured cellular markers in patients at the ED could also identify patients with different treatment strategies.

In this explorative study, the use of several neutrophil and monocyte surface markers was investigated in two ways. First, the diagnostic value of automated analysis of neuCD64 and cmCD169 and other point of care–determined leucocyte markers for bacterial and viral infection in a point-of-care setting was examined. Second, the value of these markers as input for machine learning models was assessed.

2. Methods

2.1 Study design

We performed a monocenter prospective, observational cohort study, approved by the medical research ethics committee of UMC Utrecht (research proposal number 21/539). All clinical data were collected via the structure of the SPACE (SePsis in the ACutely ill patient in the Emergency room) cohort (METC number 16-594/C).[14] This is an ongoing cohort of adult patients suspected of an infection at the ED.

2.2 Study population and data collection

Patients were included from October 2021 to January 2022. All patients (age >18 yr) presenting at the ED of UMC Utrecht for internal medicine and pulmonology with suspicion of infection were eligible for inclusion. Suspicion of infection included the following criteria: need for antimicrobial therapy, microbiological cultures (including viral diagnostics), or suspicion based on radiographic findings. The final decision whether a patient was suspected of infection was left to the treating physician. The likelihood of infection (none, possible, probable, definite) was listed according to the SPACE cohort structure.[15] All clinical results (including cultures, radiological features, and blood tests) in combination with likelihood of infection were evaluated independently by 2 physicians. All cases with disagreement were discussed until agreement, resulting in a unanimous, confirmed/reliable final type of infection (no infection, viral infection, or bacterial infection) for each patient.

2.3 Diagnostic performance

First, the optimal performance of neuCD64 and cmCD169 in patients with confirmed/reliable final infectious diagnosis was investigated. Confirmed final diagnosis was effectuated by excluding viral-bacterial co-infection, fungal or parasitic infection, and uncertain final diagnosis. Second, to also observe the diagnostic performance in an unselected population with the presence of unconfirmed/unreliable final diagnosis, a sensitivity analysis with all potential patients was performed.

2.4 Automated flow cytometry

From each patient, an extra 4 mL sodium heparin (Vacuette; Greiner Bio-One) blood collection tube was collected during the standard laboratory blood testing procedure and was immediately placed into the automated AQUIOS CL "Load & Go" flow cytometer (Beckman Coulter) by emergency room personnel. This device performs fully automated preparation and flow cytometry analysis of the full blood samples. Detailed operating procedures of this machine have been reported before.[10] Each sample was measured with 2 separate antibody panels: one neutrophil antibody panel (CD16-FITC, CD11b-PE, CD62L-ECD, CD10-PE-Cy5, and CD64-PE-Cy7) and one monocyte antibody panel (CD11b-FITC, CD169-PE, CD16-ECD, CD14-PE-Cy5, and HLA-DR-PE-Cy7). All mentioned antibodies were obtained from Beckman Coulter. Antibody concentrations were titrated for both panels. The compensation was performed by measuring cells with single stains applying the AQUIOS designer software (Beckman Coulter).

2.5 Automated clustering of flow cytometry data

Flow cytometry files (.lmd) were exported from the flow cytometer and analyzed in Cytobank (Beckman Coulter; www.cytobank.org), a Web-based flow cytometry analysis platform. The granulocyte population was identified based on forward and sideward scatter. The granulocytes were automatically gated into 64 clusters and 6 metaclusters by the FlowSOM algorithm.[16] The mature neutrophil metacluster (green) was identified based on containing $CD11b^{high}$/$CD16^{high}$ cells.[17] The total monocyte population was gated based on forward and sideward scatter. This gate partially overlapped with the lymphocyte and granulocyte population to ensure that no monocytes were excluded. Next, the monocytes were automatically gated into 100 clusters and 8 metaclusters by the FlowSOM algorithm. Three clusters with a separate monocyte subset were identified based on CD14 and CD16 expression, while lymphocytes and granulocytes were successfully separated into other metaclusters.[18] The gating strategies are depicted in Fig. 1. The median fluorescence intensity was determined for the aforementioned markers on mature neutrophils and monocyte (subsets).

2.6 Statistical analyses

Flow cytometry data and data from SPACE were combined and analyzed with IBM SPSS version 27 (IBM) and GraphPad Prism 9 (GraphPad Software). Surface marker expression between groups was compared by using the Kruskal-Wallis test with post hoc Dunn's multiple comparison test. Sensitivity, specificity, and receiver-operating characteristic (ROC) curves were calculated to estimate diagnostic value. Sensitivity and specificity were based on the optimal cutoff value of the markers based on the Youden index. Multivariate binary logistic regression was performed via backward stepwise selection.

A machine learning automated clustering method called partitioning around medoids (PAM), was used to investigate whether such algorithm would be able to find clinically relevant clusters based on the measured leukocyte surface markers. To find the optimal number of clusters, the average silhouette width was calculated. The highest silhouette score for >2 clusters was used for analysis, as we hypothesized that the algorithm could distinguish at least 3 clusters (bacterial, viral, and noninfectious). All clustering analyses were done in RStudio (version 1.3.1093; http://www.rstudio.com/) using R Statistical Software, version 4.0.3 (R Foundation for Statistical Computing). The following packages were used: haven, factoextra, cluster, ggplot2, dplyr, tidyverse, Rtsne, readxl, skimr, knitr, janitor, Rcpp, compareGroups, and ggpubr.

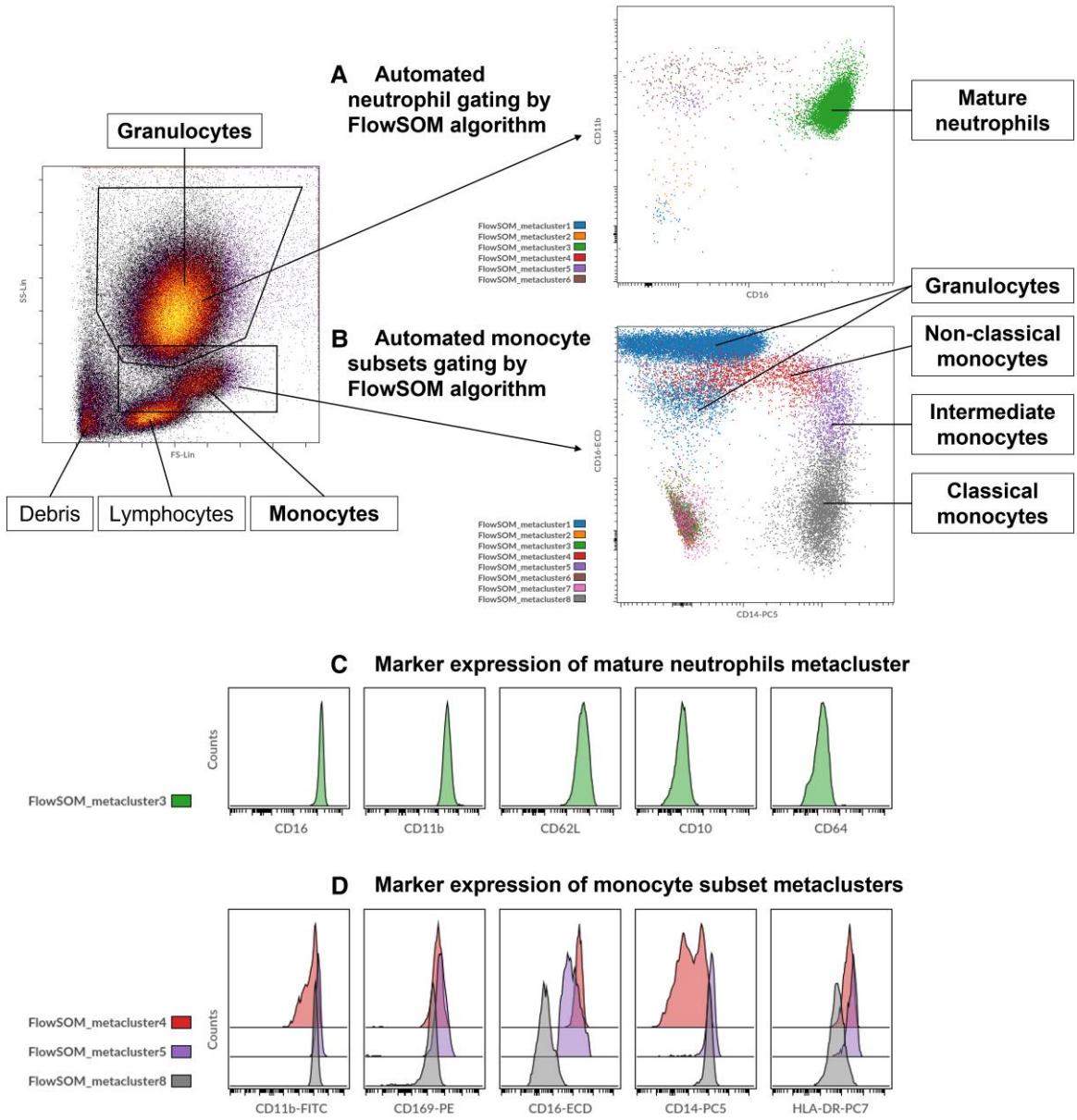

Fig. 1. Automated gating strategy for mature neutrophils (metacluster 3) by using the FlowSOM algorithm with 64 clusters and 6 metaclusters on the neutrophil antibody panel (A). Automated gating strategy for classical monocytes (metacluster 8), intermediate monocytes (metacluster 5), and nonclassical monocytes (metacluster 4) by using the FlowSOM algorithm with 100 clusters and 8 metaclusters on the monocyte antibody panel (B). The expression of the measured markers for mature neutrophils (metacluster 3; neutrophil panel) and the monocyte subsets (metaclusters 4, 5, and 8; monocyte panel) (C,D).

2.7 Ethics statements

The Medical Ethics Review Committee Utrecht concluded that the study did not meet the scope of the Dutch Law for Medical Scientific Research with humans (Wet Medisch Wetenschappelijk Onderzoek met mensen), as the participants were not subjected to any additional, study-specific procedures.

3. Results

3.1 Study population and sample collection

Blood was collected from 107 eligible patients. Eighty-six patients remained for final analyses after applying exclusion criteria (Fig. 2). Patients were classified as having bacterial infection ($n = 35$), viral infection ($n = 30$), or no infection ($n = 21$). The baseline characteristics are listed in Table 1. Patients with bacterial infection were slightly older, were more frequently admitted to the hospital, and had higher mortality rates when compared with patients with no infection or viral infection. Patients with no infection had lower serum C-reactive protein (CRP) levels and lower body temperature than patients in the other 2 groups.

3.2 Diagnostic value of neutrophil CD64

neuCD64 was higher in patients with bacterial infection when compared with patients with viral or no infection ($P < 0.001$). Post hoc analysis showed significant differences for both bacterial vs no infection ($P < 0.001$) and bacterial vs viral infection ($P = 0.025$) (Fig. 3A). Based on the Youden index, the optimal sensitivity was 0.69 and optimal specificity was 0.73. Univariate ROC

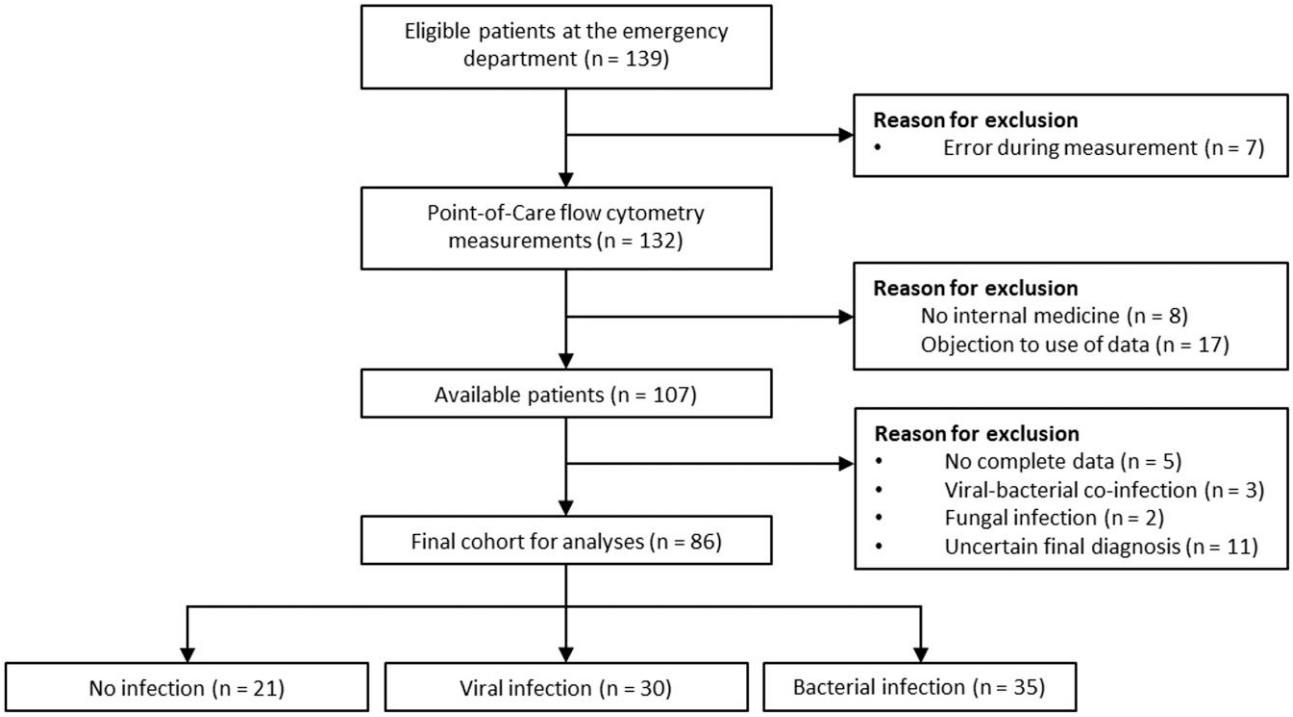

Fig. 2. Flowchart of study inclusions.

Table 1. Baseline characteristics of the study population.

	All (*N* = 86)	No infection (*n* = 21)	Viral infection (*n* = 30)	Bacterial infection (*n* = 35)
Demographics				
Age, y	63 (41–73)	61 (34–67)	59 (41–69)	69 (49–74)
Male	49 (57)	10 (48)	20 (67)	19 (54)
Charlson Comorbidity Index	4 (2–6)	3 (2–5)	4 (2–6)	5 (2–6)
Vital parameters				
Temperature, °C	37.9 (37.1–39.1)	37.1 (36.8–37.9)	38.2 (37.6–38.9)	38.2 (37.1–39.7)
Heart rate, beats/min	96 (82–112)	88.0 (80–103)	103 (85–113)	97 (84–115)
Respiratory rate, breaths/min	18 (16–23)	18 (15–18)	20 (18–25)	18 (16–24)
Systolic blood pressure, mmHg	129 (113–146)	133 (125–144)	131 (114–143)	125 (107–149)
Glasgow coma scale	15 (15–15)	15 (15–15)	15 (15–15)	15 (15–15)
Clinical scores				
SIRS ≥2	52 (60)	9 (43)	22 (73)	21 (60)
qSOFA ≥2	7 (8)	2 (10)	0 (0)	5 (14)
Inflammation parameters				
CRP, mg/L	44 (17–123)	8 (2–32)	50 (18–117)	72 (34–188)
Leukocytes ($\times 10^9$/L)	9.8 (6.5–12.8)	8.7 (6.8–11.9)	8.1 (4.1–11.4)	11.3 (8.1–16.0)
Admission data				
Admitted in hospital	56 (65)	11 (52)	17 (57)	28 (80)
Length of stay, days	4 (3–7)	3 (2–4)	4 (2–7)	4 (3–8)
Died within hospital	4 (5)	0 (0)	0 (0)	4 (11)
Immune compromised	34 (40)	5 (24)	12 (40)	17 (49)

Values are median (interquartile range) or n (%).
Abbreviations: CRP, C-reactive protein; qSOFA, quick sepsis related organ failure assessment; SIRS, systemic inflammatory response syndrome.

analysis for neuCD64 to diagnose bacterial infection is shown in Fig. 3B with an area under the ROC curve (AUROC) of 0.73. For comparison, CRP showed an AUROC of 0.69. After combining several leukocyte surface markers (neuCD64, cmCD11b, cmCD14, intermediate monocyte CD11b [imCD11b], imCD14, imCD16, non-classical monocyte CD169 [ncmCD169]) in a multivariate analysis, the AUROC increased to 0.86 to diagnose bacterial infection (Fig. 3C). The expression of all measured markers for the different subgroups is displayed in Supplementary Fig. 1A.

3.3 Diagnostic value of classical monocyte CD169

cmCD169 was higher for patients with a viral infection when compared with the other 2 groups ($P < 0.001$). This difference persisted in post hoc analysis for viral infection vs no infection ($P < 0.001$) and viral vs bacterial infection ($P < 0.001$) (Fig. 3D). The optimal sensitivity and specificity were 0.67 and 0.91, respectively. The ROC analysis for cmCD169 to diagnose viral infection is shown in Fig. 3E with a corresponding AUROC of 0.79, while this increased to 0.93 in a multivariate model with other leukocyte surface

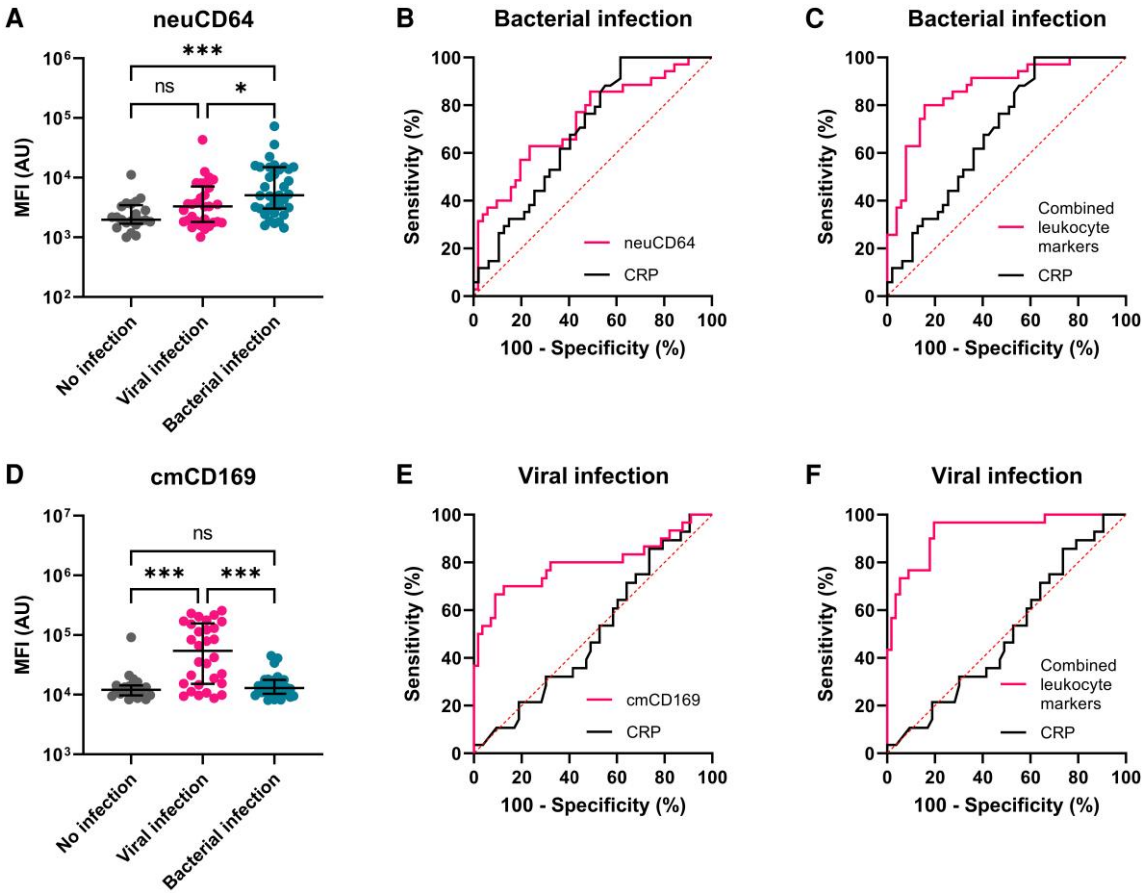

Fig. 3. The diagnostic value of neuCD64 for bacterial infection and cmCD169 for viral infection compared with no infection. neuCD64 expression was significantly higher for patients with bacterial infection ($P < 0.001$, Kruskal-Wallis test) (A). In a univariate ROC analysis, neuCD64 had an AUROC of 0.73. The AUROC of CRP was 0.69 (B). In a multivariate analysis with other leukocyte surface markers, this diagnostic value increased to an AUROC of 0.86 (C). cmCD169 expression was significantly increased for patients with viral infection ($P < 0.001$, Kruskal-Wallis test) (D). In univariate analysis, cmCD169 had an AUROC of 0.79 (E). In multivariate analysis with other leukocyte surface markers, this AUROC increased to 0.93 (F). The bars in panels A and D indicate the median value with interquartile range. AU, arbitrary units; MFI, median fluorescence intensity; ns, not significant.

markers (neuCD64, neuCD10, imCD14, imHLA-DR, ncmCD14, ncmCD16, ncmCD169, ncmHLA-DR) (Fig. 3F). The expression of all measured markers for all monocyte subsets is displayed per subgroup in Supplementary Fig. 1B–D.

3.4 Sensitivity analysis with total cohort

Potentially, the AUROC for both bacterial and viral infection was high due to excluding patients with viral-bacterial co-infection, fungal infection, or uncertain final diagnosis. Therefore, the same diagnostic analyses were performed for an extended cohort, including all patients with complete flow cytometry measurements ($n = 102$). neuCD64 was increased in patients with bacterial infection ($P = 0.003$) (Supplementary Fig. 2A) and cmCD169 was increased in patients with viral infection ($P < 0.001$) (Supplementary Fig. 2D). In univariate analysis, neuCD64 scored an AUROC of 0.70 to diagnose bacterial infections (Supplementary Fig. 2B), while cmCD169 scored an AUROC of 0.78 to diagnose viral infections (Supplementary Fig. 2E). Next, the multivariate models were tested for this extended cohort, resulting in an AUROC of 0.81 for bacterial infection (Supplementary Fig. 2C) and 0.92 to diagnose viral infection (Supplementary Fig. 2F).

3.5 Clustering data

To explore the potential of leukocyte cell membrane markers in identifying patient clusters with specific diagnostic and outcome characteristics, a PAM algorithm was used. Markers with significant differences among at least 2 of the 3 infectious groups were used: cmCD11b, cmCD16, cmCD169, imHLA-DR, and neuCD64. Utilizing a silhouette method (Supplementary Fig. 3A), 4 clusters were used and are represented in a 2-dimensional principal component analysis (Supplementary Fig. 3B). For each cluster, patient characteristics are summarized in Table 2, while marker values are shown in Supplementary Fig. 4. Cluster 1 predominantly comprised bacterial infections (85.7%), while cluster 4 primarily consisted of viral infections (91.7%). Although the numbers were limited, cluster 1 exhibited higher mortality rates and quick Sepsis Related Organ Failure Assessment (qSOFA) scores compared with other clusters. Although less distinct in infection type, clusters 2 and 3 displayed differences in clinical outcomes. Cluster 2 comprised patients with worse clinical outcome regarding mortality, admission rates, and length of stay compared with cluster 3, highlighting the clinical relevance of these clusters (see Supplementary Fig. 5 for a simplified representation).

4. Discussion

To our knowledge, this is the first study that applied automated point-of-care assessment of neuCD64 and cmCD169 to discriminate between different types of infection. It demonstrated high diagnostic value in distinguishing bacterial from viral infections

Table 2. Characteristics of the 4 clusters as constructed via the PAM clustering method.

	All (N = 86)	Cluster 1 (n = 14)	Cluster 2 (n = 47)	Cluster 3 (n = 13)	Cluster 4 (n = 12)
Infection source					
No infection	21 (24)	0 (0)	18 (38)	2 (15)	1 (8)
Viral infection	30 (35)	2 (14)	10 (21)	7 (54)	11 (92)
Bacterial infection	35 (41)	12 (86)	19 (40)	4 (31)	0 (0)
Outcome					
ICU <3 d after admission	3 (4)	1 (7)	1 (2)	0 (0)	1 (8)
Mortality during admission	4 (5)	2 (14)	2 (4)	0 (0)	0 (0)
Hospital characteristics					
Admitted from ED	56 (65)	10 (71)	32 (68)	6 (46)	8 (67)
Length of stay, days	4 (3–7)	4 (3–18)	4 (3–5)	1 (1–7)	7 (3–19)
Immune compromised	34 (40)	7 (50)	16 (34)	6 (46)	5 (41)
Clinical scores					
SIRS ≥2	52 (60)	8 (57)	26 (55)	9 (69)	9 (75)
qSOFA ≥2	7 (8)	3 (21)	2 (4)	1 (7)	1 (8)
Laboratory results					
CRP, mg/L	44 (17–123)	114 (43–237)	46 (11–170)	40 (8–54)	26 (8–81)
Leukocytes ($\times 10^9$/L)	9.8 (6.5–12.9)	11.3 (7.5–15.0)	11.2 (8.0–16.1)	7.0 (3.9–11.3)	6.3 (3.9–9.3)

Values are n (%) or median (interquartile range).
Abbreviations: CRP, C-reactive protein; ICU, intensive care unit; ED, emergency department; qSOFA, quick sepsis related organ failure assessment; SIRS, systemic inflammatory response syndrome.

from no infections. Additionally, we uniquely employed leukocyte surface markers for identification of patient clusters. These innovative immunological profiles effectively identified 2 clusters associated with predominantly viral or bacterial infections and revealed distinct outcome characteristics for both clusters. While the remaining 2 clusters have lower diagnostic value, they still discriminated between patients with better and worse clinical outcome.

The cell membrane markers were chosen based on published studies. neuCD64 is known as a sensitive and specific marker for bacterial infections and sepsis.[19–21] Also, neuCD64 is able to differentiate between infection and sterile inflammation.[22] In the current study, neuCD64 was significantly higher for patients with bacterial infection. While the univariate diagnostic performance was already fairly good and performed better than CRP, which currently is the only broadly accepted biomarker in this context, the multivariate analysis showed a much better diagnostic performance. Likewise, cmCD169 (Siglec-1) was recently discovered as a good viral infection marker.[5] In our study, cmCD169 showed excellent specificity with a good AUROC for diagnosing viral infection. Additionally, the diagnostic performance was excellent in the multivariate model.

The unsupervised machine learning algorithm was able to identify a cluster with high neuCD64 values, which predominantly consisted of patients with bacterial infection. Likewise, a viral cluster showed high levels of cmCD169 expression, which is a type I interferon–inducible receptor.[5,23] Also, monocyte HLA-DR was included in our clustering method, serving as a marker for monocyte antigen-presenting capabilities. Low HLA-DR expression is a reliable indicator of functional monocytic deactivation.[24,25] This deactivation results in an impaired innate immune system, associated with a higher risk of lethal nosocomial infections due to reduced proinflammatory cytokine production.[25,26] Accordingly, our study had one cluster that showed significantly lower imHLA-DR expression and a relatively high disease severity (qSOFA) and mortality rate when compared with the other clusters. Despite our study's relatively small sample size, it is unlikely that these differences resulted from chance, as HLA-DR is a known marker for disease severity and outcome in immunoparalysis associated with (severe) infections and sepsis.[27–30]

Other activation/maturation markers were included in our cluster analysis. cmCD11b expression was relatively high in the bacterial cluster with severe clinical outcome, indicating that this might be a marker for patients at risk for severe bacterial infection. Accordingly, other studies reported monocyte CD11b expression to be correlated with early mortality and (systemic) inflammation.[31,32] Concurrently, cmCD16 was higher in the viral cluster. This is remarkable because classical monocytes are characterized by $CD16^{dim}$ expression.[33] This implies that CD16 can be found very lowly expressed on classical monocytes, which is in line with previous research investigating optimal gating strategies for monocyte subsets.[34]

It is noteworthy that the PAM algorithm was not based on clinical data: the clusters were formed based on the leukocyte markers instead of outcome. Because certain bacterial (e.g. neuCD64) and viral (e.g. mCD169) markers were included in our analyses, one anticipates that some clusters would be predominantly viral or bacterial. Nonetheless, it is encouraging to see how well PAM is able to identify clusters based on infectious agent. More importantly, it simultaneously identified patients at risk for a bad outcome. For example, patients in cluster 1 had a higher qSOFA score at the ED and had a higher mortality rate compared with the other clusters. As this cluster is dominated by bacterial infections, these patients would benefit from early antibiotic treatment. Although cluster 1 did not capture all patients with in-hospital mortality, the 2 deceased patients in cluster 2 both had strikingly low imHLA-DR expression levels (among the 3 lowest of 47 patients in this cluster). Thus, individual markers in undifferentiated clusters have potential clinical value for identifying patients at risk of a severe outcome. Also, PAM clustering identified a cluster with patients unlikely to benefit from antibiotic treatment. Cluster 4 consisted almost exclusively of viral infections with no bacterial infections. At the same time, still 25% of patients in this cluster were, in hindsight, unnecessarily treated with antibiotics at the ED.

The current study has developed several improvements in early diagnostics for putative infections in the ED. First, all measurements were performed on an automated flow cytometer in a point-of-care setting. This is a major advantage for future clinical implementation: (1) these markers can be automatically measured within 30 min of blood collection without needing

specialized/trained personnel and (2) it enables physicians to delay antibiotic administration pending these initial results, especially because no outcome benefit has been shown for direct (<1 h after ED presentation) vs early antibiotics (1 to 3 h after ED presentation).[35] The point-of-care approach also ensured that ex vivo activation of the measured cells was kept to a minimum.[10] Furthermore, the flow cytometry data were (for a greater extent) analyzed by using a self-organizing FlowSOM clustering algorithm. This analysis technique surpasses outdated 2-dimensional gating strategies by using all measured markers (5 for each panel), while simultaneously minimizing analysis bias and human error. Using the SPACE cohort structure is another strength of this study. The SPACE cohort allowed us to use a structured dataset in which likelihood of infection was determined based on predefined criteria. The SPACE structure also resulted in very low missing data rates (<2%). Only respiratory rate was missing in a significant amount of patients (16%), which is a known restriction for this parameter at the ED, as it is the most neglected vital sign in daily practice.[36,37] Last, compared with other partitioning techniques, PAM clustering is a proven method that results in a good representation of true clustering patterns, even in the presence of noise and outliers.[38] Moreover, calculation of the average silhouette width identifies the optimal number of clusters, optimizing the distinctiveness between the clusters with regard to clinical diagnosis and prognosis.

This study also has limitations. First, some patients were excluded for the initial analyses due to several circumstances including uncertain final diagnosis, viral-bacterial co-infections, and patients with fungal infections. These patient categories were excluded to ensure good quality endpoints to test diagnostic performance. Patients with uncertain final diagnosis were excluded because they lack the primary endpoint. Fungal infections are rare but more common in a large tertiary hospital such as the UMC Utrecht. Including these patients would introduce another category of infection, lacking clinical/statistical significance due to small numbers. Likewise, we excluded 3 patients with viral-bacterial co-infection. To investigate the effect of these exclusions in the initial analyses, a sensitivity analysis that also included these patients was performed. The results were similar, with an only slightly impaired performance of the models that did not differ substantially from the original analyses. Another potential limitation is the relatively small cohort size, potentially introducing a lack of statistical power for some comparisons. Likewise, sensitivity analysis on localized infections vs sepsis could not be performed. Nonetheless, neuCD64 and cmCD169 showed clear diagnostic performance and the clustering method showed relevant clinical clusters for identifying patients with different type of diagnosis and prognosis. Therefore, it is not to be expected that a larger sample size will dramatically influence our results. Regarding the relatively small sample size, the number of variables in our multivariate models might have led to overfitting of the multivariate models. Validation on an external dataset would be required for further model evaluation. However, this was considered beyond the scope of this explorative study. Finally, the identification of the type of infection (e.g. type of pathogen) might have imperfections. Because this parameter was based on infection likelihood combined with all available clinical data, it is possible that patients were misclassified. Nonetheless, this approach was used to optimize quality of the data, which has resulted in robust data and consequently reliable cluster forming. This point is strengthened by the fact that our results can be substantiated with data from other studies without notable differences.

5. Conclusions

This study shows that point-of-care determination of neuCD64 and cmCD169 possesses great diagnostic value for differentiating between bacterial and viral infections. Especially in multivariate models based on multiple leukocyte surface markers, the diagnostic performance is very good. Moreover, leukocyte surface marker–based PAM clustering reduces heterogeneity in infectious patients at the ED regarding clinical outcome. To our knowledge, this is the first study that has investigated these markers in this point-of-care, automated context. This is a very important first step toward individualized care for patients suspected of infectious diseases at the ED, based on rapid, bedside immune phenotyping.

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Author Contributions

B.N.J., T.A.P.d.H., L.K., and K.A.H.K. prepared and reviewed the manuscript. B.N.J. and T.A.P.d.H. performed the methodology for the study. B.N.J., T.A.P.d.H., M.K., and A.E.M. performed the analysis of the data. B.N.J. and T.A.P.d.H. visualized the data. L.K. and K.A.H.K. supervised the project. All authors have contributed, read, and agreed to the published version of the manuscript.

Supplementary material

Supplementary materials are available at *Journal of Leukocyte Biology* online.

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References

1. Lu TC. Preventable deaths in patients admitted from emergency department. *Emerg Med J*. 2006:23(6):452–455. https://doi.org/10.1136/emj.2004.022319
2. Nuutila J, Hohenthal U, Laitinen I, Kotilainen P, Rajamäki A, Nikoskelainen J, Lilius E-M. Simultaneous quantitative analysis of FcγRI (CD64) expression on neutrophils and monocytes: a new, improved way to detect infections. *J Immunol Methods*. 2007:328(1–2):189–200. https://doi.org/10.1016/j.jim.2007.09.002
3. Herra CM, Keane CT, Whelan A. Increased expression of Fc receptors on neutrophils and monocytes may reflect ongoing bacterial infection. *J Med Microbiol*. 1996:44(2):135–140. https://doi.org/10.1099/00222615-44-2-135
4. Bourgoin P, Soliveres T, Ahriz D, Arnoux I, Meisel C, Unterwalder N, Morange PE, Michelet P, Malergue F, Markarian T. Clinical

research assessment by flow cytometry of biomarkers for infectious stratification in an emergency department. *Biomark Med*. 2019:13(16):1373–1386. https://doi.org/10.2217/bmm-2019-0214
5. Bedin AS, Makinson A, Picot MC, Mennechet F, Malergue F, Pisoni A, Nyiramigisha E, Montagnier L, Bollore K, Debiesse S, *et al.* Monocyte CD169 expression as a biomarker in the early diagnosis of coronavirus disease 2019. *J Infect Dis*. 2021:223(4):562–567. https://doi.org/10.1093/infdis/jiaa724
6. van de Ven NLM, Bongers SH, Spijkerman R, Koenderman L, Leenen LPH, Hietbrink F; COVPACH study group. Point-of-care neutrophil CD64 as a rule in diagnostic test for bacterial infections in the emergency department. *BMC Emerg Med*. 2023:23(1):28. https://doi.org/10.1186/s12873-023-00800-2
7. van der Poll T, van de Veerdonk FL, Scicluna BP, Netea MG. The immunopathology of sepsis and potential therapeutic targets. *Nat Rev Immunol*. 2017:17(7):407–420. https://doi.org/10.1038/nri.2017.36
8. Asadullah K, Woiciechowsky C, Döcke WD, Egerer K, Kox WJ, Vogel S, Sterry W, Volk HD. Very low monocytic HLA-DR expression indicates high risk of infection—immunomonitoring for patients after neurosurgery and patients during high dose steroid therapy. *Eur J Emerg Med*. 1995:2(4):184–190. https://doi.org/10.1097/00063110-199512000-00003
9. Döcke WD, Randow F, Syrbe U, Krausch D, Asadullah K, Reinke P, Volk HD, Kox W. Monocyte deactivation in septic patients: restoration by IFN-γ treatment. *Nat Med*. 1997:3(6):678–681. https://doi.org/10.1038/nm0697-678
10. Spijkerman R, Hesselink L, Hellebrekers P, Vrisekoop N, Hietbrink F, Leenen LPH, Koenderman L. Automated flow cytometry enables high performance point-of-care analysis of leukocyte phenotypes. *J Immunol Methods*. 2019:474:112646. https://doi.org/10.1016/j.jim.2019.112646
11. Seymour CW, Kennedy JN, Wang S, Chang CH, Elliott CF, Xu Z, Berry S, Clermont G, Cooper G, Gomez H, *et al.* Derivation, validation, and potential treatment implications of novel clinical phenotypes for sepsis. *JAMA*. 2019:321(20):2003. https://doi.org/10.1001/jama.2019.5791
12. Bai Y, Xia J, Huang X, Chen S, Zhan Q. Using machine learning for the early prediction of sepsis-associated ARDS in the ICU and identification of clinical phenotypes with differential responses to treatment. *Front Physiol*. 2022:13:1050849. https://doi.org/10.3389/fphys.2022.1050849
13. Scicluna BP, van Vught LA, Zwinderman AH, Wiewel MA, Davenport EE, Burnham KL, Nürnberg P, Schultz MJ, Horn J, Cremer OL, *et al.* Classification of patients with sepsis according to blood genomic endotype: a prospective cohort study. *Lancet Respir Med*. 2017:5(10):816–826. https://doi.org/10.1016/S2213-2600(17)30294-1
14. Uffen JW, Oomen P, de Regt M, Oosterheert JJ, Kaasjager K. The prognostic value of red blood cell distribution width in patients with suspected infection in the emergency department. *BMC Emerg Med*. 2019:19(1):76. https://doi.org/10.1186/s12873-019-0293-7
15. de Hond TAP, Oosterheert JJ, van Hemert-Glaubitz SJM, Musson REA, Kaasjager KAH. Pancreatic stone protein as a biomarker for sepsis at the emergency department of a large tertiary hospital. *Pathogens*. 2022:11(5):559. https://doi.org/10.3390/pathogens11050559
16. Jukema BN, Smit K, Hopman MTE, Bongers CCWG, Pelgrim TC, Rijk MH, Platteel TN, Venekamp RP, Zwart DLM, Rutten FH, *et al.* Neutrophil and eosinophil responses remain abnormal for several months in primary care patients with COVID-19 disease. *Front Allergy*. 2022:3:942699. https://doi.org/10.3389/falgy.2022.942699
17. Grieshaber-Bouyer R, Nigrovic PA. Neutrophil heterogeneity as therapeutic opportunity in immune-mediated disease. *Front Immunol*. 2019:10:346. https://doi.org/10.3389/fimmu.2019.00346
18. Wong KL, Yeap WH, Tai JJY, Ong SM, Dang TM, Wong SC. The three human monocyte subsets: implications for health and disease. *Immunol Res*. 2012:53(1–3):41–57. https://doi.org/10.1007/s12026-012-8297-3
19. Davis BH, Bigelow NC. Comparison of neutrophil CD64 expression, manual myeloid immaturity counts, and automated hematology analyzer flags as indicators of infection or sepsis. *Lab Hematol*. 2005:11(2):137–147. https://doi.org/10.1532/LH96.04077
20. Bourgoin P, Soliveres T, Barbaresi A, *et al.* CD169 and CD64 could help differentiate bacterial from CoVID-19 or other viral infections in the emergency department. *Cytometry A*. 2021:99(5):435–445. https://doi.org/10.1002/cyto.a.24314
21. Fjaertoft G, Håkansson LD, Pauksens K, Sisask G, Venge P. Neutrophil CD64 (FcgRI) expression is a specific marker of bacterial infection: a study on the kinetics and the impact of major surgery. *Scand J Infect Dis*. 2007:39(6–7):525–535. https://doi.org/10.1080/00365540601113693
22. Allen E, Bakke AC, Purtzer MZ, Deodhar A. Neutrophil CD64 expression: distinguishing acute inflammatory autoimmune disease from systemic infections. *Ann Rheum Dis*. 2002:61(6):522–525. https://doi.org/10.1136/ard.61.6.522
23. York MR, Nagai T, Mangini AJ, Lemaire R, Van Seventer JM, Lafyatis R. A macrophage marker, siglec-1, is increased on circulating monocytes in patients with systemic sclerosis and induced by type I interferons and toll-like receptor agonists. *Arthritis Rheum*. 2007:56(3):1010–1020. https://doi.org/10.1002/art.22382
24. Manjuck J, Saha DC, Astiz M, Eales LJ, Rackow EC. Decreased response to recall antigens is associated with depressed costimulatory receptor expression in septic critically ill patients. *J Lab Clin Med*. 2000:135(2):153–160. https://doi.org/10.1067/mlc.2000.104306
25. Haveman JW, Muller Kobold AC, Cohen Tervaert JW, van den Berg AP, Tulleken JE, Kallenberg CG, The TH. The central role of monocytes in the pathogenesis of sepsis: consequences for immunomonitoring and treatment. *Neth J Med*. 1999:55(3):132–141. https://doi.org/10.1016/S0300-2977(98)00156-9
26. Lukaszewicz AC, Grienay M, Resche-Rigon M, Pirracchio R, Faivre V, Boval B, Payen D. Monocytic HLA-DR expression in intensive care patients: interest for prognosis and secondary infection prediction. *Crit Care Med*. 2009:37(10):2746–2752. https://doi.org/10.1097/CCM.0b013e3181ab858a
27. Lekkou A, Karakantza M, Mouzaki A, Kalfarentzos F, Gogos CA. Cytokine production and monocyte HLA-DR expression as predictors of outcome for patients with community-acquired severe infections. *Clin Diagn Lab Immunol*. 2004:11(1):161–167. https://doi.org/10.1128/CDLI.11.1.161-167.2004
28. Hershman MJ, Cheadle WG, Wellhausen SR, Davidson PF, Polk HC Jr. Monocyte HLA-DR antigen expression characterizes clinical outcome in the trauma patient. *Br J Surg*. 1990:77(2):204–207. https://doi.org/10.1002/bjs.1800770225
29. Pandey K, Malviya D, Awasthi NP, Nath SS, Harjai M. Comparison of neutrophil CD64 and monocytic HLA-DR with existing biomarkers for the diagnosis and prognosis of sepsis. *Anaesthesiol Intensive Ther*. 2021:53(4):304–311. https://doi.org/10.5114/ait.2021.108579
30. Tschaikowsky K, Hedwig-Geissing M, Schiele A, Bremer F, Schywalsky M, Schüttler J. Coincidence of pro- and anti-inflammatory responses in the early phase of severe sepsis: longitudinal study of mononuclear histocompatibility leukocyte antigen-DR expression, procalcitonin, C-reactive protein, and changes in T-cell subsets in septic and postoperative patients.

Crit Care Med. 2002:30(5):1015–1023. https://doi.org/10.1097/00003246-200205000-00010

31. Pfluecke C, Wydra S, Berndt K, Tarnowski D, Cybularz M, Barthel P, Linke A, Ibrahim K, Poitz DM. CD11b expression on monocytes and data of inflammatory parameters after transcatheter aortic valve implantation in dependence of early mortality. *Data Brief*. 2020:31:105798. https://doi.org/10.1016/j.dib.2020.105798
32. Duan M, Steinfort DP, Smallwood D, Hew M, Chen W, Ernst M, Irving LB, Anderson GP, Hibbs ML. CD11b immunophenotyping identifies inflammatory profiles in the mouse and human lungs. *Mucosal Immunol*. 2016:9(2):550–563. https://doi.org/10.1038/mi.2015.84
33. Kapellos TS, Bonaguro L, Gemünd I, Reusch N, Saglam A, Hinkley ER, Schultze JL. Human monocyte subsets and phenotypes in major chronic inflammatory diseases. *Front Immunol*. 2019:10:2035. https://doi.org/10.3389/fimmu.2019.02035
34. Thomas GD, Hamers AAJ, Nakao C, Marcovecchio P, Taylor AM, McSkimming C, Nguyen AT, McNamara CA, Hedrick CC. Human blood monocyte subsets. *Arterioscler Thromb Vasc Biol*. 2017:37(8):1548–1558. https://doi.org/10.1161/ATVBAHA.117.309145
35. de Groot B, Ansems A, Gerling DH, Rijpsma D, van Amstel P, Linzel D, Kostense PJ, Jonker M, de Jonge E. The association between time to antibiotics and relevant clinical outcomes in emergency department patients with various stages of sepsis: a prospective multi-center study. *Crit Care*. 2015:19(1):194. https://doi.org/10.1186/s13054-015-0936-3
36. Elliott M. Why is respiratory rate the neglected vital sign? A narrative review. *Int Arch Nurs Health Care*. 2016:2(3):050. https://doi.org/10.23937/2469-5823/1510050
37. Cretikos MA, Bellomo R, Hillman K, Chen J, Finfer S, Flabouris A. Respiratory rate: the neglected vital sign. *Med J Aust*. 2008:188(11):657–659. https://doi.org/10.5694/j.1326-5377.2008.tb01825.x
38. Van der Laan MJ, Pollard KS, Bryan J. A new partitioning around medoids algorithm. *J Stat Comput Simul*. 2003:73(8):575–584. https://doi.org/10.1080/0094965031000136012