

CAT Critically Appraised Topic

Titel: Potential hemocytometric parameters for diagnosis and prognostic stratification of COVID-19 patients

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CLINICAL BOTTOM LINE

In a literature study we identified hemocytometric changes that are characteristic of COVID-19. We selected the best performing diagnostic markers and benchmarked them in a retrospective cohort study. Receiver Operating Characteristic (ROC) curve analysis demonstrated area-under-the-curve (AUC) values of 0.66 for lymphopenia, 0.68 for eosinopenia, 0.71 for monocytopenia, 0.67 for the MECOR score, 0.74 for the PARIS score, and 0.78 for a modified algorithm that quantifies patterns of lymphocyte distribution on the Sysmex XN WDF scattergram (the so-called 'sandglass' pattern). We find that diagnostic performance of these parameters is, generally, less than reported in the literature. We show that hematologic changes that are deemed characteristic of COVID-19 are more pronounced in severe illness. Therefore, the sensitivity of these markers is significantly less in COVID-19 cohorts that include patients with milder illness. In addition, we find decreasing specificity of these markers when comparing COVID-19 with other common viral respiratory infections (such as influenza, RSV and miscellaneous viral infections). This undermines the diagnostic utility of these parameters when used in settings with a high prevalence of respiratory viral illness, such as during flu season. We demonstrate that various analytical variables contribute to the discrepancies in diagnostic performance reported in the literature. Among these are the use of different analytical cutoff values for defining cytopenias, technical variables in flow cytometry and microscopy methods, and technical issues with newly introduced flow cytometry parameters.

CLINICAL/DIAGNOSTIC SCENARIO

The ongoing COVID-19 pandemic presents significant challenges in triage and admission of large patient inflows in emergency departments. Molecular diagnostic testing is hampered by imperfect sensitivity, significant turn-around times, and by limited availability of these assays in smaller hospitals and low-resource countries. A plethora of diagnostic and prognostic prediction tools have been published to aid diagnosis. These tools include demographic, anamnestic, clinical, imaging and/or laboratory features.¹ In addition, several hemocytometry-based laboratory parameters or composite scores have been proposed for presumptively differentiating COVID-19 patients from non-infectious illness or other respiratory viral infections.^{2,3} These are particularly attractive, as a complete blood count and leukocyte differential are readily available in almost all medical centers, are rapidly obtained and are inexpensive. We appraised the literature on the performance of hematological parameters and composite scores for diagnosing COVID-19. We identified the most promising diagnostic markers and benchmarked them in a retrospective cohort study in Jessa Ziekenhuis, Hasselt. The study population consisted of laboratory data from 257 COVID-19 patients and of a control cohort of 1261 patients with diverse respiratory illness (including influenza, RSV, CMV/EBV), patients with bacterial infections and with non-infectious illness.

QUESTION(S)

1) What is the diagnostic value of hemocytometric alterations in COVID-19?

- a. Literature study on hematological changes in COVID-19 (see Appendix A)
- b. Markers validated in our retrospective cohort study:
 - i. Lymphopenia
 - ii. AS-LYMP, RE-LYMP, HFLC
 - iii. Eosinopenia
 - iv. Monocytopenia and lymphocyte-monocyte ratio
 - v. MECOR score
 - vi. PARIS score
 - vii. 'Sandglass' pattern on the Sysmex XN WDF scattergram

2) What analytical factors affect hemocytometric parameters in COVID-19?

- a. Varying analytical cutoff values
- b. Variables between flow cytometry and microscopy
- c. Technical issues in newly introduced flow cytometrical parameters

RELEVANT EVIDENCE/REFERENCES

1. Wynants, L. *et al.* Systematic review and critical appraisal of prediction models for diagnosis and prognosis of COVID-19 infection. *medRxiv* 2020.03.24.20041020 (2020) doi:10.1101/2020.03.24.20041020.
2. Tordjman, M. *et al.* Pre-test probability for SARS-Cov-2-related Infection Score: the PARIS score. <http://medrxiv.org/lookup/doi/10.1101/2020.04.28.20081687> (2020) doi:10.1101/2020.04.28.20081687.
3. Osman, J. *et al.* Rapid screening of COVID-19 patients using white blood cell scattergrams, a study on 381 patients. *Br. J. Haematol.*
4. Huang, I. & Pranata, R. Lymphopenia in severe coronavirus disease-2019 (COVID-19): systematic review and meta-analysis. *J. Intensive Care* **8**, 36 (2020).
5. Martens, R. J. H. *et al.* Hemocytometric characteristics of COVID-19 patients with and without cytokine storm syndrome on the sysmex XN-10 hematology analyzer. *Clin. Chem. Lab. Med. CCLM* **59**, 783–793 (2021).
6. Rutkowska, E. *et al.* Usefulness of the New Hematological Parameter: Reactive Lymphocytes RE-LYMP with Flow Cytometry Markers of Inflammation in COVID-19. *Cells* **10**, 82 (2021).
7. Sun, S. *et al.* Abnormalities of peripheral blood system in patients with COVID-19 in Wenzhou, China. *Clin. Chim. Acta* **507**, 174–180 (2020).
8. Outh, R. *et al.* Eosinopenia <100/ μ L as a marker of active COVID-19: An observational prospective study. *J. Microbiol. Immunol. Infect.* **54**, 61–68 (2021).
9. Soni, M. Evaluation of eosinopenia as a diagnostic and prognostic indicator in COVID-19 infection. *Int. J. Lab. Hematol.*
10. Davido, B. *et al.* Eosinopenia in COVID-19: What we missed so far? *J. Microbiol. Immunol. Infect.* (2021) doi:10.1016/j.jmii.2021.01.013.
11. Khartabil, T. A., Russcher, H., van der Ven, A. & de Rijke, Y. B. A summary of the diagnostic and prognostic value of hemocytometry markers in COVID-19 patients. *Crit. Rev. Clin. Lab. Sci.* **57**, 415–431 (2020).
12. Wang, L. *et al.* Differentiating Between 2019 Novel Coronavirus Pneumonia and Influenza Using a Nonspecific Laboratory Marker-Based Dynamic Nomogram. *Open Forum Infect. Dis.* **7**, ofaa169 (2020).
13. Song, L. *et al.* Differential diagnosis and prospective grading of COVID-19 at the early stage with simple hematological and biochemical variables. *Diagn. Microbiol. Infect. Dis.* **99**, 115169 (2021).
14. Tordjman, M. *et al.* Pre-test probability for SARS-Cov-2-related infection score: The PARIS score. *PLOS ONE* **15**, e0243342 (2020).
15. Cazzaniga, M. *et al.* Eosinopenia is a reliable marker of severe disease and unfavourable outcome in patients with COVID-19 pneumonia. *Int. J. Clin. Pract.* **n/a**, e14047.
16. Xie, G. *et al.* The role of peripheral blood eosinophil counts in COVID-19 patients. *Allergy* **76**, (2020).
17. Liu, F. *et al.* Patients of COVID-19 may benefit from sustained Lopinavir-combined regimen and the increase of Eosinophil may predict the outcome of COVID-19 progression. *Int. J. Infect. Dis.* **95**, 183–191 (2020).
18. Lin, A. *et al.* Early risk factors for the duration of SARS-CoV-2 viral positivity in COVID-19 patients. 14.
19. Li, Q. *et al.* Eosinopenia and elevated C-reactive protein facilitate triage of COVID-19 patients in fever clinic: A retrospective case-control study. *EClinicalMedicine* **23**, 100375 (2020).
20. Huang, J. *et al.* Absolute Eosinophil Count Predicts Intensive Care Unit Transfer Among Elderly COVID-19 Patients From General Isolation Wards. *Front. Med.* **7**, (2020).
21. Ndieugnou Djangang, N. *et al.* Eosinopenia in COVID-19 Patients: A Retrospective Analysis. *Microorganisms* **8**, 1929 (2020).
22. Tanni, F. *et al.* Eosinopenia and COVID-19. *J. Am. Osteopath. Assoc.* **120**, 504–508 (2020).
23. Chen, N. *et al.* Epidemiological and clinical characteristics of 99 cases of 2019 novel coronavirus pneumonia in Wuhan, China: a descriptive study. *The Lancet* **395**, 507–513 (2020).
24. Huang, I. & Pranata, R. Lymphopenia in severe coronavirus disease-2019 (COVID-19): systematic review and meta-analysis. *J. Intensive Care* **8**, 36 (2020).
25. Lippi, G. & Plebani, M. Laboratory abnormalities in patients with COVID-2019 infection. *Clin. Chem. Lab. Med. CCLM* **58**, 1131–1134 (2020).
26. Gérard, D., Henry, S. & Thomas, B. SARS-CoV-2: a new aetiology for atypical lymphocytes. *Br. J. Haematol.* **189**, 845–845 (2020).
27. Chong, V. C. L. *et al.* Reactive lymphocytes in patients with COVID-19. *Br. J. Haematol.* **189**, 844–844 (2020).
28. Weinberg, S. E., Behdad, A. & Ji, P. Atypical lymphocytes in peripheral blood of patients with COVID-19. *Br. J. Haematol.* **190**, 36–39 (2020).
29. Zini, G., Bellesi, S., Ramundo, F. & d’Onofrio, G. Morphological anomalies of circulating blood cells in COVID-19. *Am. J. Hematol.* **95**, 870–872 (2020).
30. van Mirre, E. *et al.* Sensitivity and specificity of the high fluorescent lymphocyte count-gate on the Sysmex XE-5000 hematology analyzer for detection of peripheral plasma cells. *Clin. Chem. Lab. Med.* **49**, 685–688 (2011).
31. Oehadian, A. *et al.* New parameters available on Sysmex XE-5000 hematology analyzers contribute to differentiating dengue from leptospirosis and enteric fever. *Int. J. Lab. Hematol.* **37**, 861–868 (2015).
32. Prodjosoewojo, S. *et al.* A novel diagnostic algorithm equipped on an automated hematology analyzer to differentiate between common causes of febrile illness in Southeast Asia. *PLoS Negl. Trop. Dis.* **13**, e0007183 (2019).
33. Yun, H. *et al.* Laboratory data analysis of novel coronavirus (COVID-19) screening in 2510 patients. *Clin. Chim. Acta* **507**, 94–97 (2020).

34. Merino, A. *et al.* Atypical lymphoid cells circulating in blood in COVID-19 infection: morphology, immunophenotype and prognosis value. *J. Clin. Pathol.* jclinpath-2020-207087 (2020) doi:10.1136/jclinpath-2020-207087.
35. Boulanger, M. *et al.* Peripheral Plasma Cells Associated with Mortality Benefit in Severe COVID-19: A Marker of Disease Resolution. *Am. J. Med.* (2021) doi:10.1016/j.amjmed.2021.01.040.
36. Linssen, J. *et al.* A novel haemocytometric COVID-19 prognostic score developed and validated in an observational multicentre European hospital-based study. *eLife* **9**, e63195 (2020).
37. Wang, Z. *et al.* High-fluorescent lymphocytes are increased in patients with COVID-19. *Br. J. Haematol.* bjh.16867 (2020) doi:10.1111/bjh.16867.
38. Yip, C. Y. C. *et al.* Temporal changes in immune blood cell parameters in COVID-19 infection and recovery from severe infection. *Br. J. Haematol.* (2020) doi:10.1111/bjh.16847.
39. Rolla, R. *et al.* Reduced activity of B lymphocytes, recognised by Sysmex XN-2000™ haematology analyser, predicts mortality in patients with coronavirus disease 2019. *Int. J. Lab. Hematol.* **43**, e5–e8 (2021).
40. Zhang, J.-J. *et al.* Clinical characteristics of 140 patients infected with SARS-CoV-2 in Wuhan, China. *Allergy* **75**, 1730–1741 (2020).
41. Abidi, K. *et al.* Eosinopenia is a reliable marker of sepsis on admission to medical intensive care units. *Crit. Care* **12**, R59 (2008).
42. Lippi, G. & Henry, B. M. Eosinophil count in severe coronavirus disease 2019. *QJM Int. J. Med.* **113**, 511–512 (2020).
43. Lippi, G., Sanchis-Gomar, F. & Henry, B. M. Response to: Eosinophil count in coronavirus disease 2019: more doubts than answers. *QJM Int. J. Med.* **114**, 70–71 (2021).
44. Le Borgne, P. *et al.* Do Blood Eosinophils Predict in-Hospital Mortality or Severity of Disease in SARS-CoV-2 Infection? A Retrospective Multicenter Study. *Microorganisms* **9**, (2021).
45. Yan, B., Yang, J., Xie, Y. & Tang, X. Relationship between blood eosinophil levels and COVID-19 mortality. *World Allergy Organ. J.* **14**, 100521 (2021).
46. Glickman, J. W., Pavel, A. B., Guttman-Yassky, E. & Miller, R. L. The role of circulating eosinophils on COVID-19 mortality varies by race/ethnicity. *Allergy* **76**, 925–927 (2021).
47. Stegeman, I. *et al.* Routine laboratory testing to determine if a patient has COVID-19. *Cochrane Database Syst. Rev.* (2020) doi:10.1002/14651858.CD013787.
48. Qin, C. *et al.* Dysregulation of Immune Response in Patients With Coronavirus 2019 (COVID-19) in Wuhan, China. *Clin. Infect. Dis.* **71**, 762–768 (2020).
49. Mertoglu, C., Huyut, M. T., Arslan, Y., Ceylan, Y. & Coban, T. A. How do routine laboratory tests change in coronavirus disease 2019? *Scand. J. Clin. Lab. Invest.* **81**, 24–33 (2021).
50. Merekoulis, G., Alexopoulos, E. C., Belezos, T., Panagiotopoulou, E. & Jelastopulu, Dr. med. E. Lymphocyte to monocyte ratio as a screening tool for influenza. *PLoS Curr.* **2**, RRN1154 (2010).
51. Luo, Y. *et al.* Using a diagnostic model based on routine laboratory tests to distinguish patients infected with SARS-CoV-2 from those infected with influenza virus. *Int. J. Infect. Dis.* **95**, 436–440 (2020).
52. Lv, D. *et al.* Differential diagnosis of coronavirus disease 2019 pneumonia or influenza A pneumonia by clinical characteristics and laboratory findings. *J. Clin. Lab. Anal.* **35**, (2021).
53. Yu, G., Zhang, Q., Wang, R. & Jiang, S. Predictive value of neutrophil-to-lymphocyte ratio and other inflammatory indicators in estimating clinical severity of coronavirus disease. *World J. Emerg. Med.* **12**, 79 (2021).
54. Kalabin, A., Mani, V. R. K., Valdivieso, S. C. & Donaldson, B. Role of neutrophil-to-lymphocyte, lymphocyte-to-monocyte and platelet-to-lymphocyte ratios as predictors of disease severity in COVID-19 patients. 8.
55. Ramos-Peñafiel, C. O. *et al.* Utilidad de los índices neutrófilo/linfocito, monocito/linfocito y linfocito/plaqueta para el pronóstico de complicaciones asociadas a COVID-19. *Gac. M^éxico* **156**, 4687 (2020).
56. Asan, A. *et al.* Do initial hematologic indices predict the severity of COVID-19 patients? *Turk J Med Sci* **7**.
57. Biamonte, F. *et al.* Combined lymphocyte/monocyte count, D-dimer and iron status predict COVID-19 course and outcome in a long-term care facility. *J. Transl. Med.* **19**, 79 (2021).
58. Lippi, G., Sanchis-Gomar, F. & Henry, B. M. Pooled analysis of monocyte distribution width in subjects with SARS-CoV-2 infection. *Int. J. Lab. Hematol.* **n/a**.
59. Crouser, E. D. *et al.* Monocyte Distribution Width: A Novel Indicator of Sepsis-2 and Sepsis-3 in High-Risk Emergency Department Patients*. *Crit. Care Med.* **47**, 1018 (2019).
60. Lin, H.-A. *et al.* Clinical impact of monocyte distribution width and neutrophil-to-lymphocyte ratio for distinguishing COVID-19 and influenza from other upper respiratory tract infections: A pilot study. *PLoS One* **15**, e0241262 (2020).
61. Zeng, X. *et al.* Monocyte volumetric parameters and lymph index are increased in SARS-CoV-2 infection. *Int. J. Lab. Hematol.* (2020) doi:10.1111/ijlh.13323.
62. Higgins, P., Runnegar, N., Bird, R. J. & Markey, K. A. Rates of neutropenia in adults with influenza A or B: a retrospective analysis of hospitalised patients in South East Queensland during 2015. *Intern. Med. J.* **46**, 1328–1332 (2016).
63. Ye, Z., Ai, X., Liao, Z., You, C. & Cheng, Y. The prognostic values of neutrophil to lymphocyte ratio for outcomes in chronic obstructive pulmonary disease. *Medicine (Baltimore)* **98**, e16371 (2019).
64. Gao, X. *et al.* Association of Neutrophil to Lymphocyte Ratio With Pulmonary Function in a 30-Year Longitudinal Study of US Veterans. *JAMA Netw. Open* **3**, e2010350 (2020).
65. Li, Y., Hou, H., Diao, J., Wang, Y. & Yang, H. Neutrophil-to-lymphocyte ratio is independently associated with COVID-19 severity: An updated meta-analysis based on adjusted effect estimates. *Int. J. Lab. Hematol.* ijlh.13475 (2021) doi:10.1111/ijlh.13475.
66. Urrechaga, E., Aguirre, U., España, P. P. & Guadiana, L. G. de. Complete blood counts and cell population data from Sysmex XN analyser in the detection of SARS-CoV-2 infection. *Clin. Chem. Lab. Med. CCLM* **59**, e57–e60 (2021).

67. Birben, B. *et al.* Efficacy of the delta neutrophil index in predicting 30-day mortality in COVID-19 patients requiring intensive care. *Int. J. Clin. Pract.* (2021) doi:10.1111/ijcp.13970.
68. Kazancioglu, S., Bastug, A., Ozbay, B. O., Kemirtlek, N. & Bodur, H. The role of haematological parameters in patients with COVID-19 and influenza virus infection. *Epidemiol. Infect.* **148**, (2020).
69. Page, E. M. & Ariëns, R. A. S. Mechanisms of thrombosis and cardiovascular complications in COVID-19. *Thromb. Res.* **200**, 1–8 (2021).
70. Raadsen, M. *et al.* Thrombocytopenia in Virus Infections. *J. Clin. Med.* **10**, 877 (2021).
71. Pranata, R. *et al.* Thrombocytopenia as a prognostic marker in COVID-19 patients: diagnostic test accuracy meta-analysis. *Epidemiol. Infect.* **149**, (2021).
72. Zong, X., Gu, Y., Yu, H., Li, Z. & Wang, Y. Thrombocytopenia Is Associated with COVID-19 Severity and Outcome: An Updated Meta-Analysis of 5637 Patients with Multiple Outcomes. *Lab. Med.* **52**, 10–15 (2021).
73. Jiang, S.-Q., Huang, Q.-F., Xie, W.-M., Lv, C. & Quan, X.-Q. The association between severe COVID-19 and low platelet count: evidence from 31 observational studies involving 7613 participants. *Br. J. Haematol.* **190**, e29–e33 (2020).
74. Amgalan, A. & Othman, M. Hemostatic laboratory derangements in COVID-19 with a focus on platelet count. *Platelets* (2020).
75. Liu, Y. *et al.* Association between platelet parameters and mortality in coronavirus disease 2019: Retrospective cohort study. *Platelets* **31**, 490–496 (2020).
76. Eslamijouybari, M. *et al.* Neutrophil-to-Lymphocyte and Platelet-to-Lymphocyte Ratios in COVID-19 Patients and Control Group and Relationship with Disease Prognosis. *Casp. J. Intern. Med.* **11**, (2020).
77. Nair, P. R. *et al.* Neutrophil-to-lymphocyte Ratio and Platelet-to-lymphocyte Ratio as Predictors of the Early Requirement of Mechanical Ventilation in COVID-19 Patients. *Indian J. Crit. Care Med. Peer-Rev. Off. Publ. Indian Soc. Crit. Care Med.* **24**, 1143–1144 (2020).
78. Asghar, M. S. *et al.* Hematological parameters predicting severity and mortality in COVID-19 patients of Pakistan: a retrospective comparative analysis. *J. Community Hosp. Intern. Med. Perspect.* **10**, 514–520 (2020).
79. Renshaw, A. A., Drago, B., Toraya, N. & Gould, E. W. Respiratory syncytial virus infection is strongly correlated with decreased mean platelet volume. *Int. J. Infect. Dis.* **17**, e678–e680 (2013).
80. Ozcelik, N., Ozyurt, S., Kara, B. Y., Gumus, A. & Sahin, U. The value of the platelet count and platelet indices in differentiation of COVID-19 and influenza pneumonia. *J. Med. Virol.* **93**, 2221–2226 (2021).
81. Gumus, H., Demir, A. & Yükkaldıran, A. Is mean platelet volume a predictive marker for the diagnosis of COVID-19 in children? *Int. J. Clin. Pract.* **75**, (2021).
82. Koç, E. M. E. *et al.* Comparison of hematological parameters and perinatal outcomes between COVID-19 pregnancies and healthy pregnancy cohort. *J. Perinat. Med.* **49**, 141–147 (2021).
83. Güçlü, E. *et al.* Effect of COVID-19 on platelet count and its indices. *Rev. Assoc. Médica Bras.* **66**, 1122–1127 (2020).
84. Bommenahalli Gowda, S. *et al.* Prognosis of COVID-19: Red Cell Distribution Width, Platelet Distribution Width, and C-Reactive Protein. *Cureus* (2021) doi:10.7759/cureus.13078.
85. Guner Ozenen, G. *et al.* Demographic, clinical, and laboratory features of COVID-19 in children: The role of mean platelet volume in predicting hospitalization and severity. *J. Med. Virol.* (2021) doi:10.1002/jmv.26902.
86. Zhang, S. *et al.* SARS-CoV-2 binds platelet ACE2 to enhance thrombosis in COVID-19. *J. Hematol. Oncol. J Hematol Oncol* **13**, 120 (2020).
87. Faber, J., Hvas, A.-M., Kristensen, S. D., Grove, E. L. & Adelborg, K. Immature Platelets and Risk of Cardiovascular Events among Patients with Ischemic Heart Disease: A Systematic Review. *Thromb. Haemost.* (2020) doi:10.1055/s-0040-1721386.
88. Cohen, A. *et al.* Immature platelets in patients hospitalized with Covid-19. *J. Thromb. Thrombolysis* (2020) doi:10.1007/s11239-020-02290-6.
89. Sambataro, G. *et al.* The Model for Early COvid-19 Recognition (MECOR) Score: A Proof-of-Concept for a Simple and Low-Cost Tool to Recognize a Possible Viral Etiology in Community-Acquired Pneumonia Patients during COVID-19 Outbreak. *Diagnostics* **10**, 619 (2020).
90. Schneider, C. A., Rasband, W. S. & Eliceiri, K. W. NIH Image to ImageJ: 25 years of image analysis. *Nat. Methods* **9**, 671–675 (2012).

1) What is the diagnostic value of hemocytometric alterations in COVID-19?

Based on a literature study, we identified hemocytometric parameters with potential diagnostic value for COVID-19 (see Appendix A). These are the following: lymphopenia, the antibody-secreting lymphocyte count (AS-LYMP), the reactive lymphocyte count (RE-LYMP), the highly-fluorescent lymphocyte count (HFLC), eosinopenia, monocytopenia and the lymphocyte-monocyte ratio, the MECOR score, and the PARIS score. For a description of study design, see Appendix C.

Lymphopenia

Lymphopenia has been named as a hemocytometric feature of severe COVID-19.⁴ We investigate the diagnostic performance of this parameter in mild and severe COVID-19.

Sensitivity

The sensitivity of lymphopenia (as defined at our laboratory at ≤ 1.1 lymphocytes/ μ L) was 61.4%. There are at least two sources of bias that likely have caused an overestimation of sensitivity in our cohort. First, since we did not acquire chest CT imaging data, only patients that tested positive with RT-PCR were counted as true positive COVID-19 cases. It is known that RT-PCR has imperfect sensitivity in patients with milder illness and low viral load. Second, our cohort consisted of all consecutive patients that were tested at our laboratory in the period of March until May 2020. National case definitions and testing guidelines are now known to have been too narrow early in the pandemic, so that patients that were (more severely) symptomatic were more likely to get tested. Many patients with mild or absent symptomatology likely went undetected. Therefore, it is probable that our cohort was selected for more severe illness, and that the true sensitivity of lymphopenia would be lower in a broader SARS-CoV-2-infected population with milder or absent clinical illness. This undermines the use of lymphopenia as a test for guiding quarantine measures, as patients with mild illness and normal lymphocyte counts might yet be contagious.

Considering lymphopenia as a predictor of ICU-admission, we find limited differences in lymphopenia rates between an ICU-admitted and a non-ICU admitted cohort (70.8% and 59.3%, respectively). However, paradoxically, mortality and median age were higher in the non-ICU admitted COVID-19 cohort than in ICU-patients. This is not unexpected. In a pandemic setting with constrained critical care resources, intensive medical care may be withheld from patients with poor prognosis. However, even among those that do receive intensive medical care, about one third of patients do not exhibit lymphopenia, so that, as a test for severe disease, lymphopenia has but mediocre sensitivity.

Specificity

We determined median lymphocyte counts and the proportion of lymphopenic patients in a COVID-19 population and in control cohorts of diverse infectious and non-infectious etiologies. COVID-19 caused relatively high rates of lymphopenia in ICU-admitted and non-ICU admitted patients (as noted above, respectively 70.8% and 59.3%). However, the proportion of lymphopenic patients with both influenza infection and bacterial sepsis surpassed that of COVID-19 (81.3% for ICU-admitted influenza patients, 62.3% in non-ICU admitted influenza patients, and 73.3% in bacteremic patients) (see Figure 1). The median lymphocyte counts and the distribution of lymphocyte counts are similar between critically ill patients with COVID-19, influenza, and bacterial sepsis. This implies that lymphopenia is not useful as a parameter for distinguishing COVID-19 from influenza infection or from severe bacterial infections. As a test in a broader hospital-admitted population, lymphopenia similarly has limited specificity: half of geriatric patients that are admitted for reasons other than COVID-19 or influenza exhibit lymphopenia, and one-third of patients admitted to a surgical ward exhibit lymphopenia. These findings indicate that the use of lymphopenia as a single hematologic parameter to predict COVID-19 has poor specificity.

We find limited sensitivity of lymphopenia for detection of both mild and severe COVID-19. In addition, we find that lymphopenia is a non-specific finding in severe illness of various (infectious and non-infectious) etiology.
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% Lymphopenia and median lymphocyte counts in COVID-19 and control cohorts

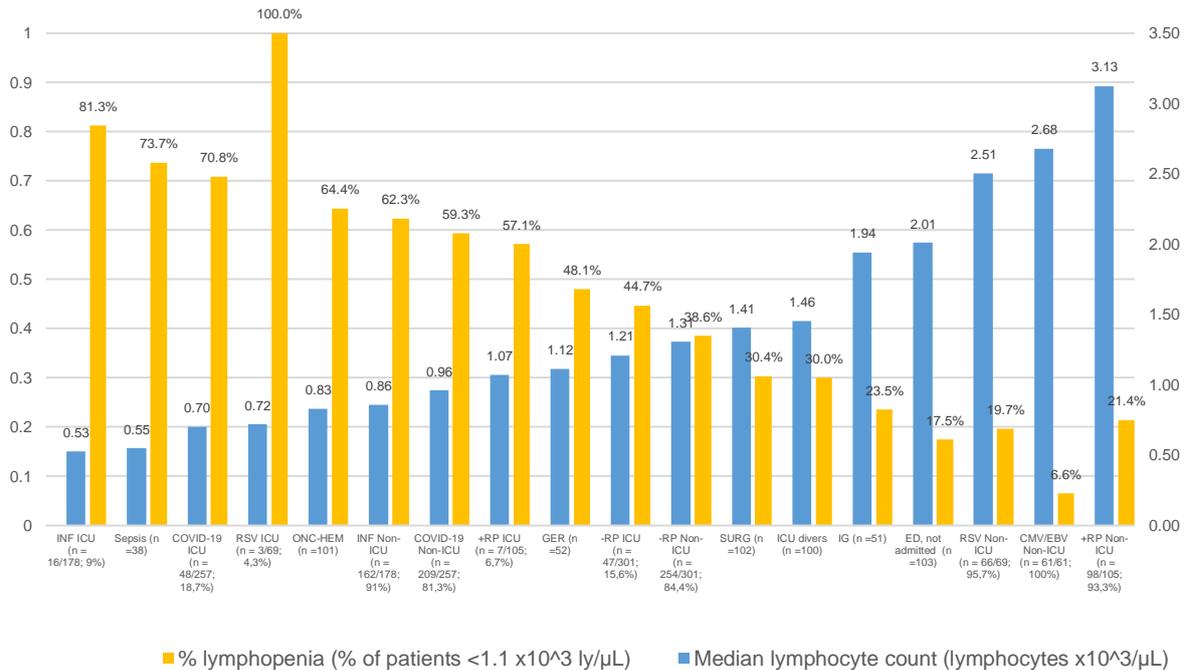


Figure 1. The proportion of patients per cohort with lymphocyte counts $1.1 \times 10^3/\mu\text{L}$ (yellow) and median lymphocyte counts per cohort (blue).

Antibody-secreting lymphocytes (AS-LYMP), highly-fluorescent lymphocytes (HFLC), and reactive lymphocytes (RE-LYMP)

Various alterations in lymphocyte subsets have been described in SARS-CoV-2 infection, reflecting the development of an adaptive immune response. Some groups of lymphocytes may be presumptively differentiated using fluorescence flow cytometry. Some parameters that quantify these subsets have gained interest as possible diagnostic tools for COVID-19.

The reactive lymphocyte count on Sysmex XN (Sysmex Corporation, Kobe, Japan) analyzers quantifies all lymphocytes with an above-normal fluorescence; this concerns lymphocytes with increased nucleic acid contents and these often represent activated or reactive lymphocytes. Previous studies found increased RE-LYMP counts in COVID-19 compared to negative controls, but lower counts than in some other viral infections.^{5,6} Our data suggest a similar pattern: we find lower median RE-LYMP counts (RE-LYMP%, expressed as a percentage of total WBC count) in COVID-19 than in, for example, RSV and acute CMV/EBV infection, but higher median counts than in a cohort with influenza infection or with a negative respiratory multiplex PCR panel, or in patients presenting at the Emergency department with diverse illnesses. This precludes use of RE-LYMP% as a specific marker for COVID-19 infection. As mentioned below, we find that the proportion of invalid results ('vote out' results, reported as '----') reaches as high one in four in our population with RSV infection and in our cohort with non-influenza, non-RSV, non-COVID-19 respiratory illness. This also impedes clinical use of this parameter.

The Highly-Fluorescent Lymphocyte Count (HFLC) and the Antibody-Secreting Lymphocyte count (AS-LYMP) quantify a subset of lymphocytes with the highest fluorescence signal. As reported by Martens et al. and Yun et al., we also find higher median HFLC% values in COVID-19 than in a negative control population (see Fig. 2) However, similarly to the reactive lymphocyte count, we find the highest median values in our RSV-infected cohort. We find an identical median HFLC% in COVID-19 (n = 257) as in the cohort with infection with other respiratory pathogens (influenza and RSV excluded; n = 105). Median HFLC% of a cohort with bacterial sepsis is remarkably low, compared to viral etiologies of illness. This implies that HFLC% may be of some use in differentiating viral from bacterial illness (see Appendix D), but is not a specific parameter for diagnosing COVID-19.

Similar to the RE-LYMP% parameter, the AS-LYMP% parameter is often not calculable or is 0%, resulting in median values of 0% for all cohorts. Mean values are highest in RSV and COVID-19 cohorts (respectively 0.34% and 0.32%). The use of this parameter in light of the analytical issues that go along with it, is discussed in section 2 (*What analytical factors affect hemocytometric parameters in COVID-19?*).

AS-LYMP, RE-LYMP and HFLC quantify subsets of activated lymphocytes. Elevated values for HFLC are suggestive of viral rather than bacterial infection. Elevated values are not specific to COVID-19; these are equally seen in RSV, CMV/EBV and miscellaneous respiratory viral infections.

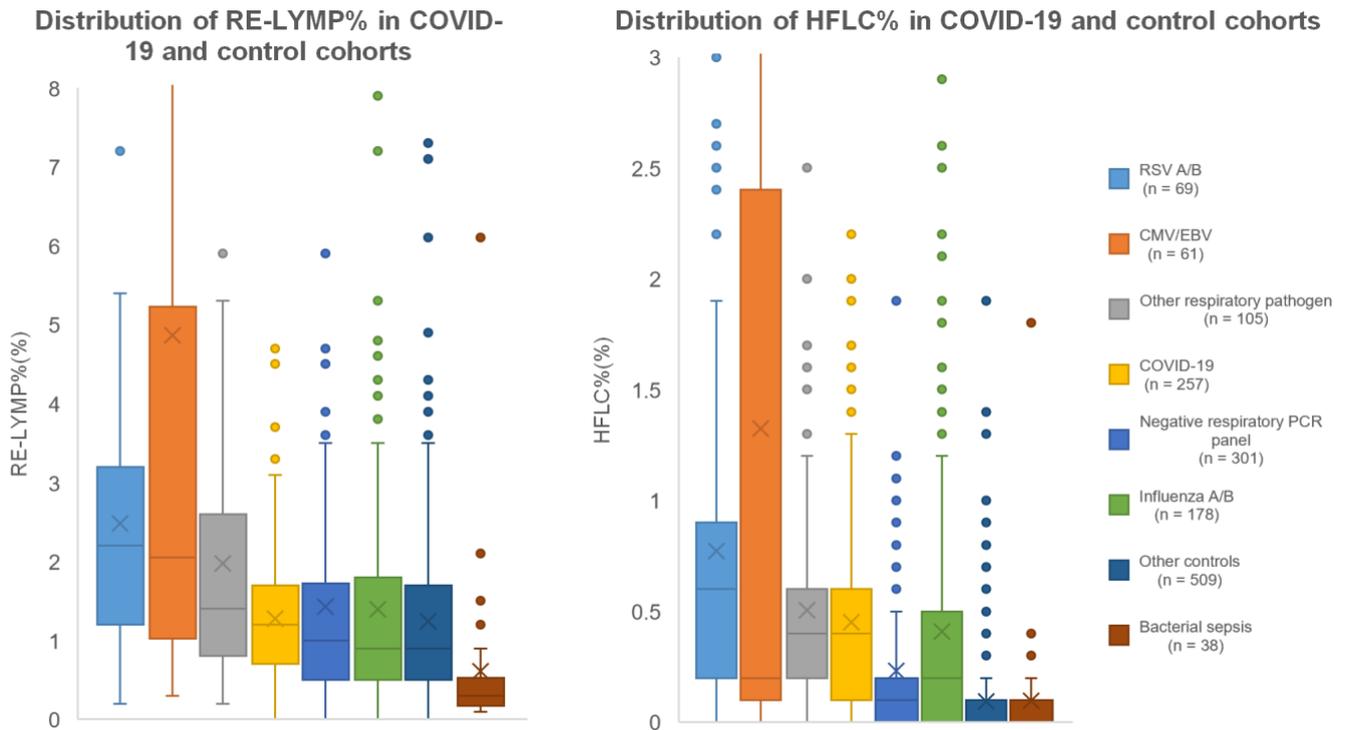


Figure 2. *Left panel:* distribution of RE-LYMP% in COVID-19 and in control cohorts. *Right panel:* distribution of HFCLC% in COVID-19 and in control cohorts. Horizontal bars indicate median values; cross-marks indicate mean values.

Eosinopenia

Eosinopenia has drawn attention as a possible diagnostic marker for COVID-19, with some groups reporting a high discriminatory ability (AUCs of 0.84, 0.85 and 0.97).⁷⁻⁹ Others have questioned the specificity of this marker.¹⁰

We find an AUC of 0.68 of the eosinophil count as a marker for COVID-19, when using a control cohort composed of all infectious and non-infectious control patients included in this study (n = 1261) (see Fig. 3). For the same reasons as mentioned above, our COVID-19 cohort was likely selected for more severe illness, and therefore the diagnostic performance of the eosinophil count may be overestimated. Supporting the notion that eosinopenia is predictive of more severe illness, we find (slightly) improved AUCs when considering eosinopenia as a diagnostic marker in the ICU-admitted population (AUC 0.73) (see Fig. 3).

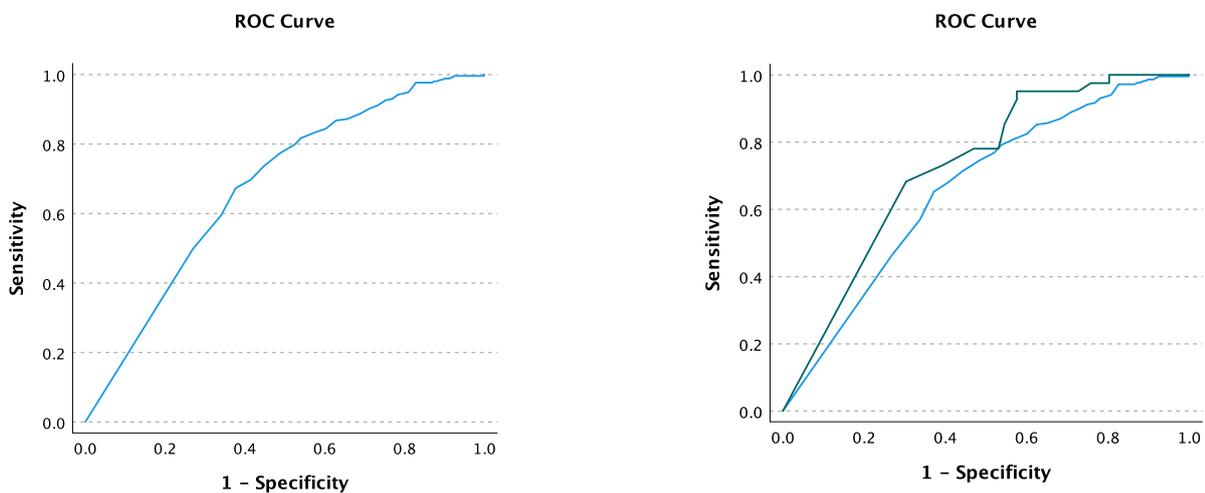


Figure 3. *Left panel:* ROC Curve of the absolute eosinophil count for prediction of COVID-19 positive PCR (n = 257) compared to 1261 controls (AUC 0.68). *Right panel:* ROC Curve of the absolute eosinophil count for prediction of COVID-19 positive PCR in 48 ICU admitted patients (turquoise line, AUC 0.73) and 209 non-ICU admitted patients (AUC 0.66, blue line) compared to 1261 COVID-19-negative controls.

Investigating the source of diagnostic errors for this marker, we investigated the distribution of eosinophil counts and in our different control cohorts (see Fig. 4). We find the lowest median eosinophil counts in ICU-admitted cohorts with COVID-19 (n = 47), RSV infection (n = 3) and in the cohort with bacterial sepsis (n = 38) (median eosinophil counts of 0 eosinophils/ μL) (see Table 1 in Appendix B). Of cohorts including non-ICU admitted patients, we find the lowest median eosinophil counts in the COVID-19 cohort, the influenza cohort (each median counts of $0.01 \times 10^3/\mu\text{L}$), and in RSV-infected patients ($0.02 \times 10^3/\mu\text{L}$). This suggests that that eosinopenia is not specific to COVID-19, but a common finding in other viral respiratory illnesses and in severe bacterial infection.

Eosinopenia is a non-specific finding in various severe viral and bacterial infections.

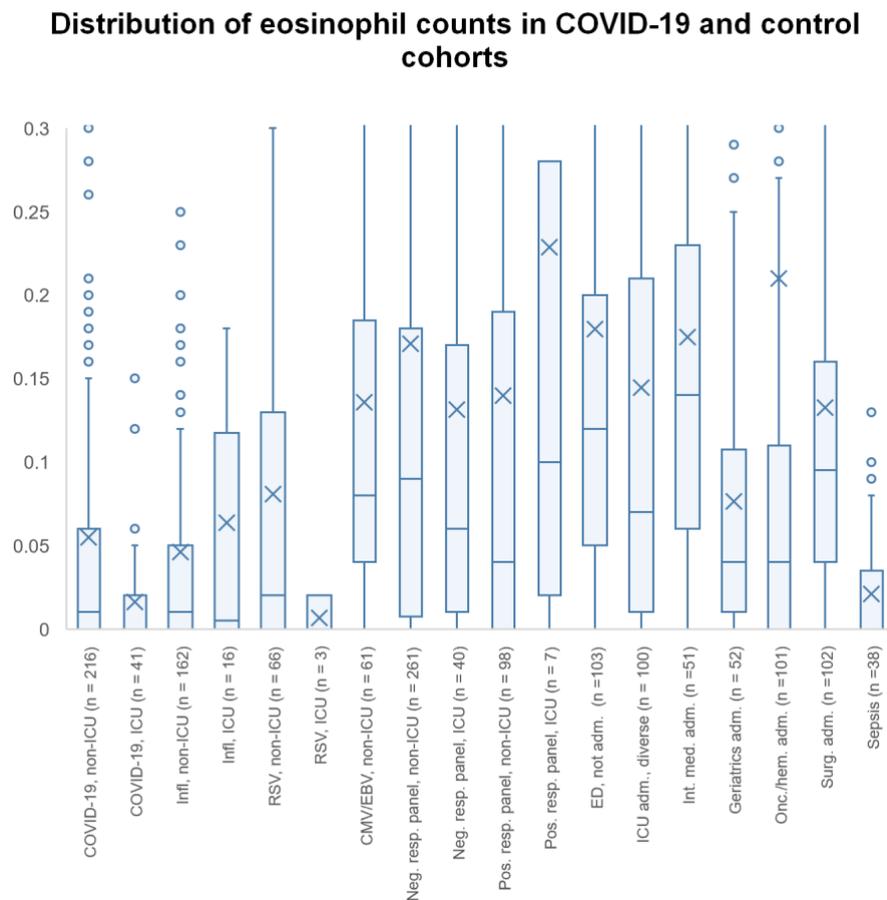


Figure 4. The distribution of eosinophil counts (eosinophils $\times 10^3/\mu\text{L}$) in COVID-19 and in control cohorts. For median values, see data table in Appendix B). Horizontal bars indicate median values; cross-marks indicate mean values.

Monocytopenia and lymphocyte-monocyte ratio (LMR)

Literature on the monocyte counts in COVID-19 is conflicting.¹¹ Monocyte counts have inconsistently been described to be both elevated, decreased or in the normal range in COVID-19 patients. Our data confirm that monocyte counts in COVID-19 patients span the range of low, normal and increased counts (reference range at our laboratory: $0-0.51 \times 10^3$ monocytes/ μL). However, median monocyte counts in COVID-19 patients seem to be lower than in other respiratory infections and in non-infectious illness (see Fig. 5). Therefore, we investigated the diagnostic performance of monocytopenia as a diagnostic marker for COVID-19.

We find an AUC of 0.71 of monocytopenia for differentiating COVID-19 from controls (see Fig. 6, left panel), which suggests that monocytopenia has better diagnostic performance in our study population than both lymphopenia (AUC 0.66) and eosinopenia (AUC 0.68) for a diagnosis of COVID-19.

Differences have been noted in monocyte counts between COVID-19 and influenza infection. The latter is associated with higher monocyte counts, which has sparked interest in using monocyte counts in differential diagnosis of these two common respiratory infections.¹² In our ICU-admitted COVID-19 population, the mean monocyte count was half that of the ICU-admitted influenza-infected cohort, potentially implying specificity of monocytopenia for COVID-19. However, the AUC for differentiating COVID-19 from influenza is 0.63, which implies only modest diagnostic value (Figure 6, right panel).

Some groups have used the lymphocyte-to-monocyte ratio (LMR) as a marker for distinguishing COVID-19 from influenza infection, based on the finding that lymphopenia is more pronounced in influenza infection, and monocytopenia more prominent in COVID-19. One group reports an AUC of 0.895 of an elevated LMR for predicting influenza infection rather than COVID-19.¹³ Although we confirm a pattern of higher lymphocyte-monocyte ratios in influenza compared to COVID-19, we find that the AUC of this ratio for differentiating these illnesses is not much better than that of the monocyte count alone (an AUC of 0.64 compared to 0.63, respectively — ROC curve of LMR not shown).

In our study population, monocytopenia is the best performing single diagnostic marker for COVID-19, with an AUC of 0.71. The monocyte count and lymphocyte-monocyte ratio has no significant value in differentiating COVID-19 from influenza infection.

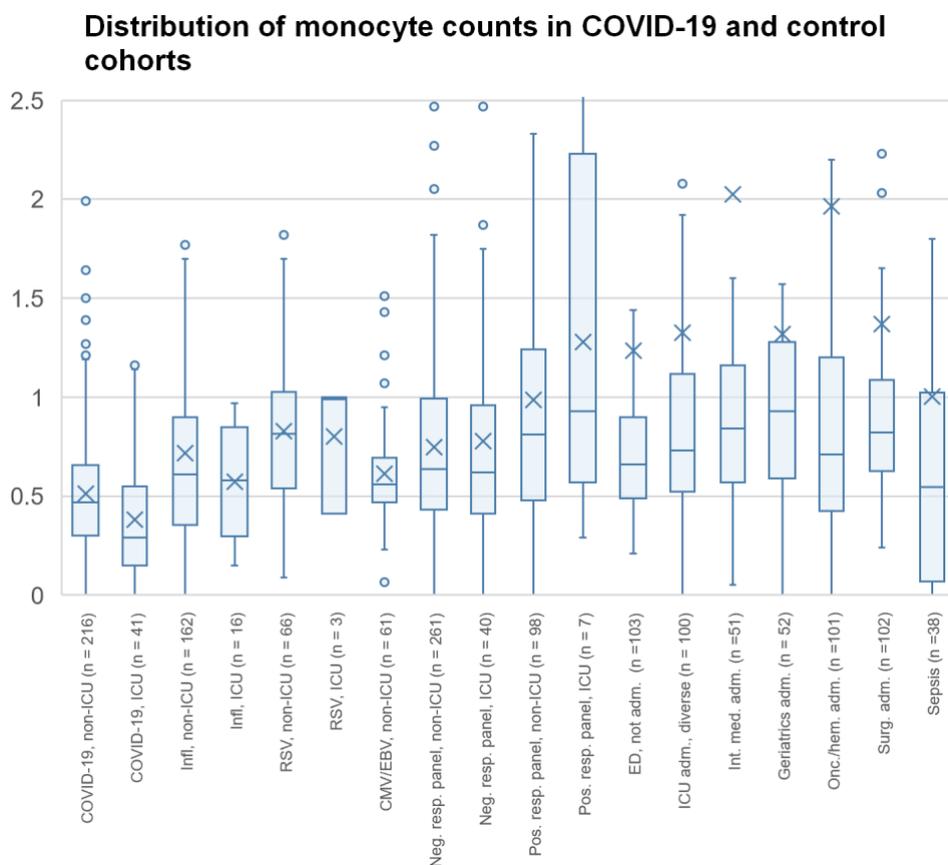


Figure 5. The distribution of monocyte counts (monocytes $\times 10^3/\mu\text{L}$) in COVID-19 and in control cohorts. For median values, see Table 1). Horizontal bars indicate median values; cross-marks indicate mean values.

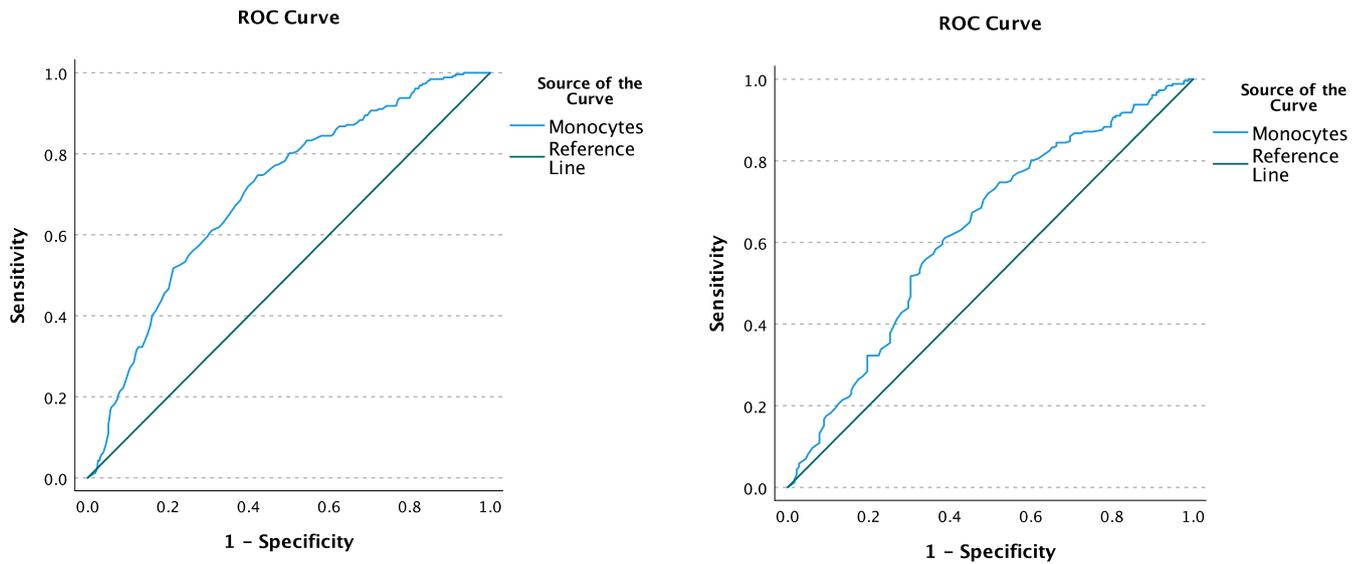


Figure 6. Left panel: ROC Curve of the absolute monocyte count for prediction of COVID-19 positive PCR (n = 257) compared to 1261 controls (AUC 0.71). Right panel: ROC Curve of the monocyte count for differentiating COVID-19 from influenza infection (AUC 0.63).

The MECOR score

Sambataro et al. describe the ‘Model for Early COVID-19 Recognition’ (MECOR) score, specifically for differentiating SARS-CoV-2 infection from other causes of community-acquired pneumonia (CAP). The score is based on total white blood cells, and lymphocyte, monocyte, neutrophil and platelet counts, which are predicted to be lower in COVID-19 than in other causes of CAP (AUC is 0.84). We determined the predictive accuracy of this model both in a population with suspected or confirmed respiratory illness and in a broader population that also included non-infectious pathology. The control cohort of the first study population consisted of influenza- and RSV-infected patients, and all patients that underwent testing with respiratory multiplex PCR (whether any pathogen was detected or not) (n = 653). The control cohort of the second population consisted of the aforementioned cohorts and all other (including the non-infectious) cohorts (n = 1257; four patients were excluded due to incomplete data or an invalid formula result). **We found identical AUCs of 0.67 in both study populations, suggesting that the MECOR score has limited diagnostic value both for differentiating COVID-19 from other causes of pneumonia, and from non-infectious causes** (see Fig. 7). The MECOR score performs less well than eosinopenia (AUC 0.68) and monocytopenia (AUC 0.71) as single predictors for COVID-19 in our study population. These findings are compatible with ‘overfitting’ of the MECOR model in the population used to establish it, resulting in lesser performance of the model in differently composed study populations, and impeding the model’s generalizability to other populations.

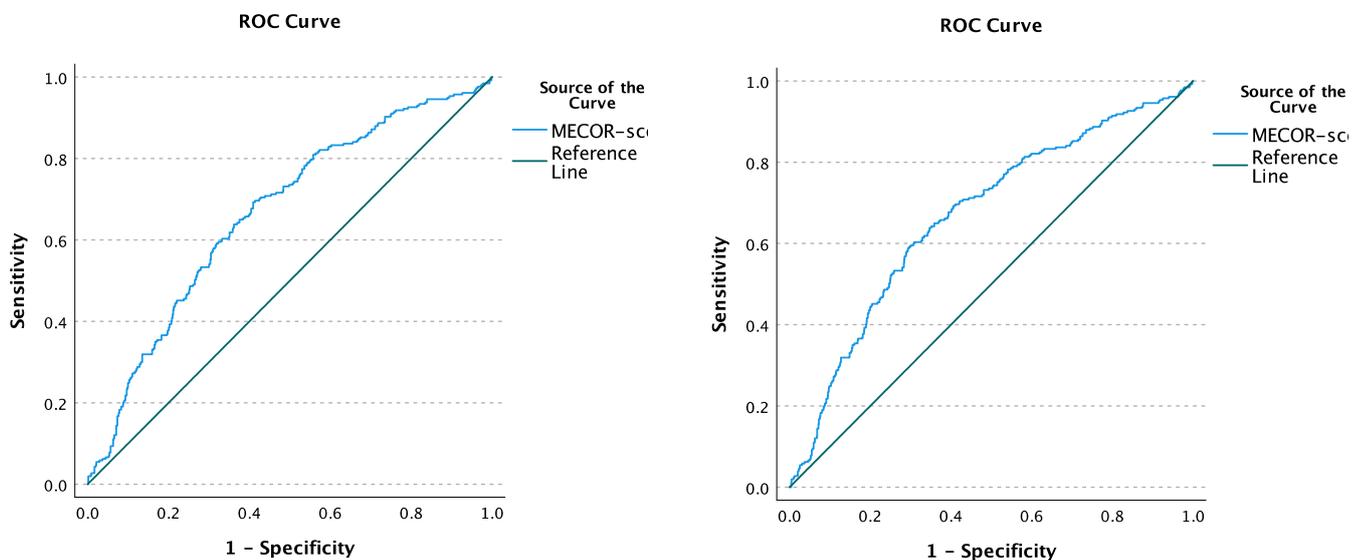


Figure 7. *Left panel:* ROC Curve of the MECOR score for prediction of COVID-19 positive PCR (n = 257) compared to 653 controls with respiratory illness (AUC 0.67). *Right panel:* ROC Curve of the MECOR score for differentiating COVID-19 from other infectious and non-infectious control cohorts (n = 1257) (AUC 0.67).

The PARIS score

Tordjman et al. propose the so-called ‘Pre-test probability for SARS-CoV-2 Infection based on Scoring’ model (PARIS score), based on the relatively lower lymphocyte, eosinophil, neutrophil and basophil counts seen in most COVID-19 patients.¹⁴ A scoring system that awarded two points for lymphopenia, and one point for any of the other cytopenias, reached a positive predictive value of 93% and a negative predictive value of 98% (AUC of 0.918) in a validation cohort of 261 COVID-19 patients and 144 controls. We investigated the diagnostic performance of this score in similar study populations as used for evaluating the MECOR score, that is, in a population with suspicion of or confirmed respiratory illness, and in a broader population including infectious and non-infectious illness. **We find AUCs of, respectively, 0.70 and 0.74, demonstrating superiority of this model compared to the MECOR score and all single hemocytometric parameters** (see Figure 8).

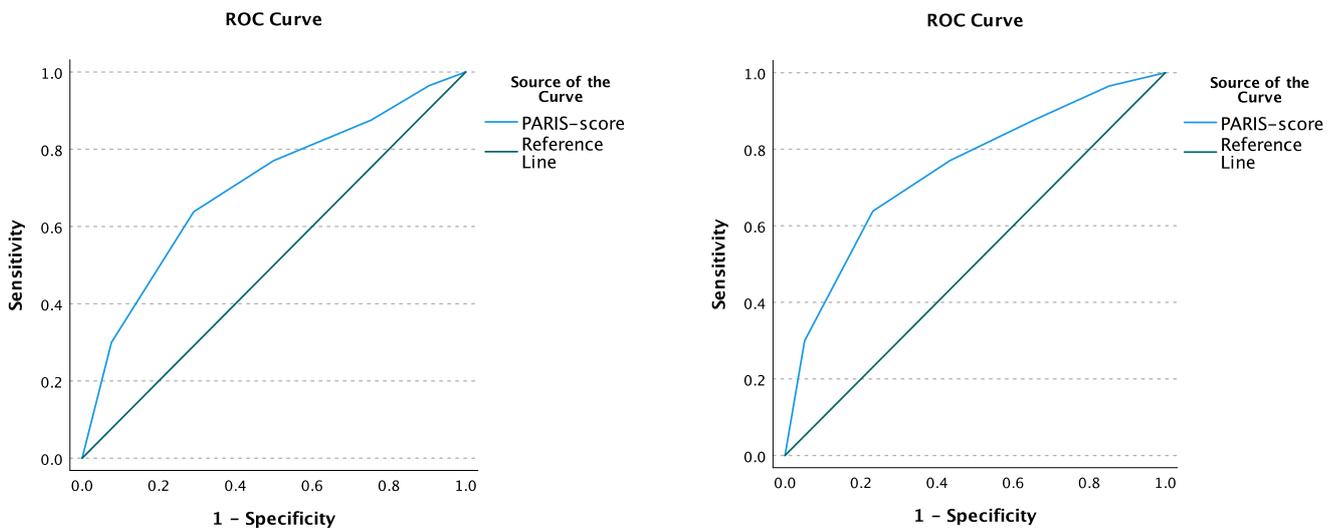


Figure 8. *Left panel:* ROC Curve of the PARIS score for prediction of COVID-19 positive PCR (n = 257) compared to 653 controls with respiratory illness (AUC 0.70). *Right panel:* ROC Curve of the PARIS score for differentiating COVID-19 from other infectious and non-infectious control cohorts (n = 1261) (AUC 0.74).

The ‘sandglass’ pattern on the WDF scattergram

Osman et al. report that recognition of a ‘sandglass’ pattern on the WDF scattergram (a cluster of lymphocytes in the highest-fluorescence region, but not in the intermediate fluorescence region) has a sensitivity of 85.9% and specificity of 83.5% for predicting SARS-CoV-2 infection as determined by PCR and/or chest CT (see Figure 9). This test was validated in a cohort of 85 COVID-19 patients and 85 patients with influenza, EBV, *Mycoplasma pneumoniae* and parvovirus infections.

This method, however, is difficult to standardize and to scale. No other groups have validated this finding in an independent cohort. On reviewing WDF scatterplots of COVID-19 patients, we find that, often, the distribution of lymphocyte dots cannot unambiguously be identified as a ‘sandglass’ pattern, rather than representing a contiguous lymphocyte cluster.

In order to more objectively investigate diagnostic value of lymphocyte distribution on the WDF scattergram, we used image analysis software to quantify the number of dots in high and intermediate fluorescence regions as a ratio, that, when elevated, suggests the presence of a greater number of lymphocytes in higher fluorescence regions than in lower regions. Three zones were distinguished on the WDF scattergram; a high-fluorescence zone, an intermediate fluorescence zone and a low fluorescence zone (see Figure 10). As to determine an optimal threshold for separating both regions, we varied the threshold from the upper limit of the scattergram (0 dots) to 150 dots down the upper limit of the scattergram (see Appendix E). All lymphocytes below 150 dots from the upper limit were at all times excluded from the ratio. This region was considered the ‘low fluorescence region’. Using this approach, we identify various thresholds that yield ratios of which the AUC surpasses that of the PARIS score. Using a threshold placed at 75 dots from the upper limit of the WDF scattergram, we find an of 0.78 for differentiating COVID-19 from all control patients (Figure 10, right panel, red line). Using other thresholds (100 and 125 dots) results in slightly lower AUCs (Figure 10, right panel, green and purple lines). Considering the total number of dots in the intermediate and high fluorescence

region, we find an AUC of 0.46 (Figure 10, right panel, blue line). This suggests that the distribution of lymphocytes, rather than the total lymphocyte count in upper fluorescence regions, is of diagnostic value.

Although it is hardly feasible to implement this process in routine practice, this finding demonstrates that alterations in lymphocyte subsets have some specificity for COVID-19, as suggested by the study of Osman et al. Algorithms that examine the distribution of lymphocytes on the WDF could be implemented in the Sysmex IPU, and could be combined with other parameters (such as cytopenias) to enhance specificity for COVID-19.

A potential weakness of our study is that we did not obtain screenshots of all COVID-19 patients (screenshots in 204 of 257 patients) and control patients (screenshots in 978 of 1261 controls). However, selection of these samples was random, so that these samples are taken to be representative of the complete COVID-19 and control cohorts.

We have not yet validated this finding in an independent cohort.

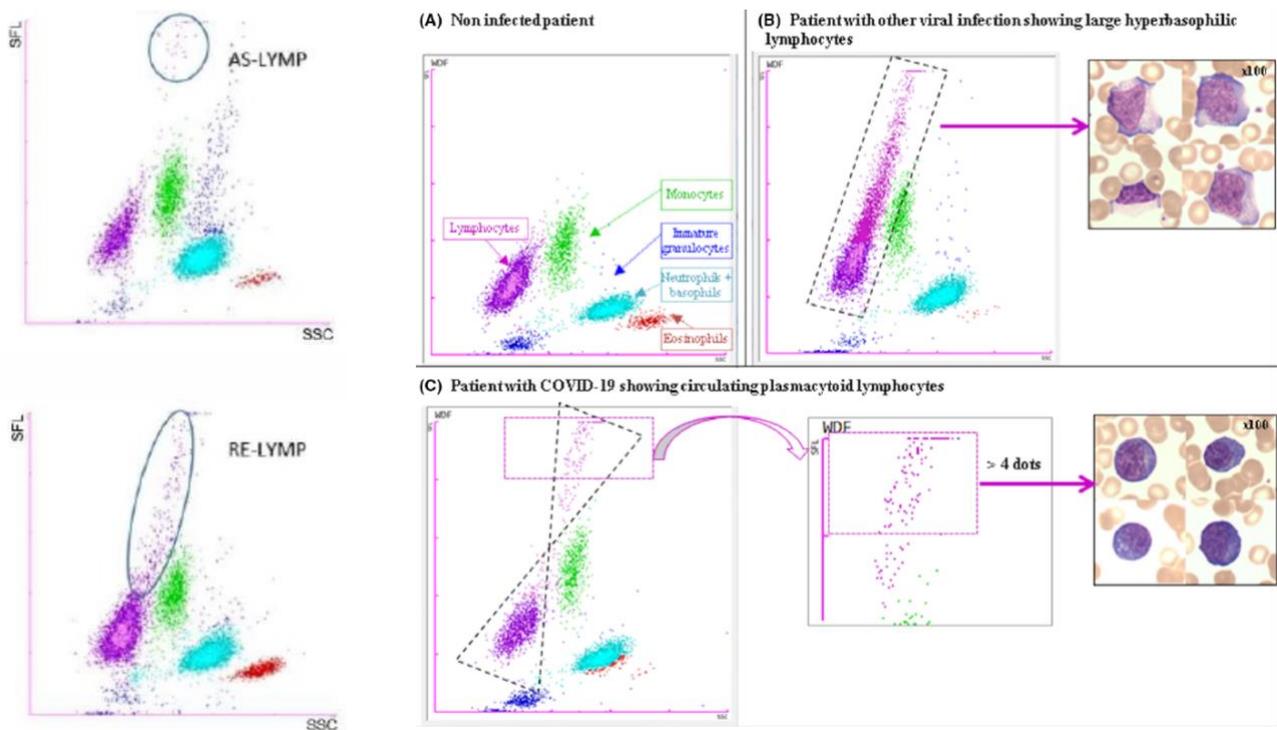


Figure 9. Left panel: the position of the AS-LYMP and RE-LYMP cell counting gates on the WDF scattergram. Image credit: <https://www.sysmex.nl/academy/knowledge-centre/parameter/re-lympas-lymp.html>. Right panel: the ‘sand-glass’ pattern in COVID-19 patients. Image credit: Osman et al.³

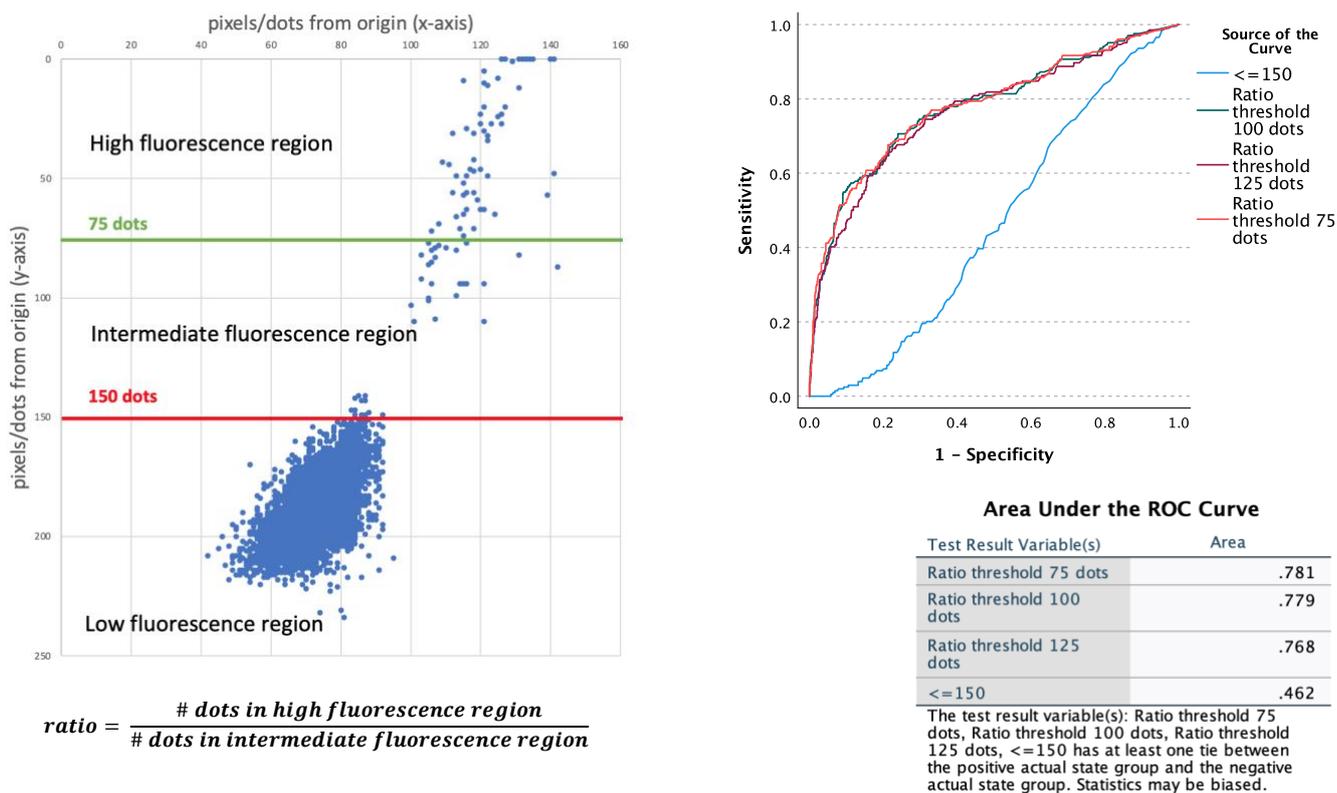


Figure 10. *Left panel.* Defining a high fluorescence, intermediate fluorescence and low fluorescence region on the WDF scattergram. *Right panel.* Setting the threshold for differentiation of high and intermediate fluorescence regions at 75 dots, the ratio of dots in the high fluorescence and intermediate fluorescence region has an AUC of 0.78 for differentiating 204 COVID-19 patients from 978 controls.

2) What analytical factors affect hemocytometric parameters in COVID-19?

Analytical cutoff values

As mentioned above, lymphocyte counts are affected by disease severity in both COVID-19 and in other viral illnesses. This, in part, explains the differences in rates of lymphopenia seen between studies, and therefore, the varying sensitivity of lymphopenia for predicting a diagnosis of COVID-19.

An evident explanation for these discrepancies is the use of different analytical cut offs for defining lymphopenia. In a meta-analysis of lymphopenia in COVID-19 patients, cutoffs for absolute lymphopenia that have been used include $\leq 1.0 \times 10^3$ lymphocytes (ly)/ μL , $\leq 1.1 \times 10^3$ ly/ μL , $\leq 1.2 \times 10^3$ ly/ μL , $\leq 1.5 \times 10^3$ ly/ μL .⁴

In Figure 11 (left panel) we demonstrate the impact of using variable cutoffs on rates of lymphopenia. In the same study population, lymphopenia rates may vary from 54.9% to 76.3%, depending on the analytical cutoff that is used. Using cutoff values in routine clinical use, **the rate of lymphopenia may therefore vary to up to 20 percentage points in the same population.** In an ICU-admitted COVID-19 population, this difference reaches approximately 35 percentage points (data not shown).

For eosinopenia, likewise, cutoffs have ranged from 0/ μL ,^{7,15} 10/ μL ,⁸ 15/ μL ,¹⁶ 20/ μL ,¹⁷⁻²⁰ 40/ μL and 50/ μL ,⁹ 45/ μL ,²¹ to 100/ μL .²² In our study population of 257 COVID-19 patients, **this translates to differences in eosinopenia rates of up to 35 percentage points,** employing commonly used cutoffs for eosinopenia (see Fig. 11, right panel).

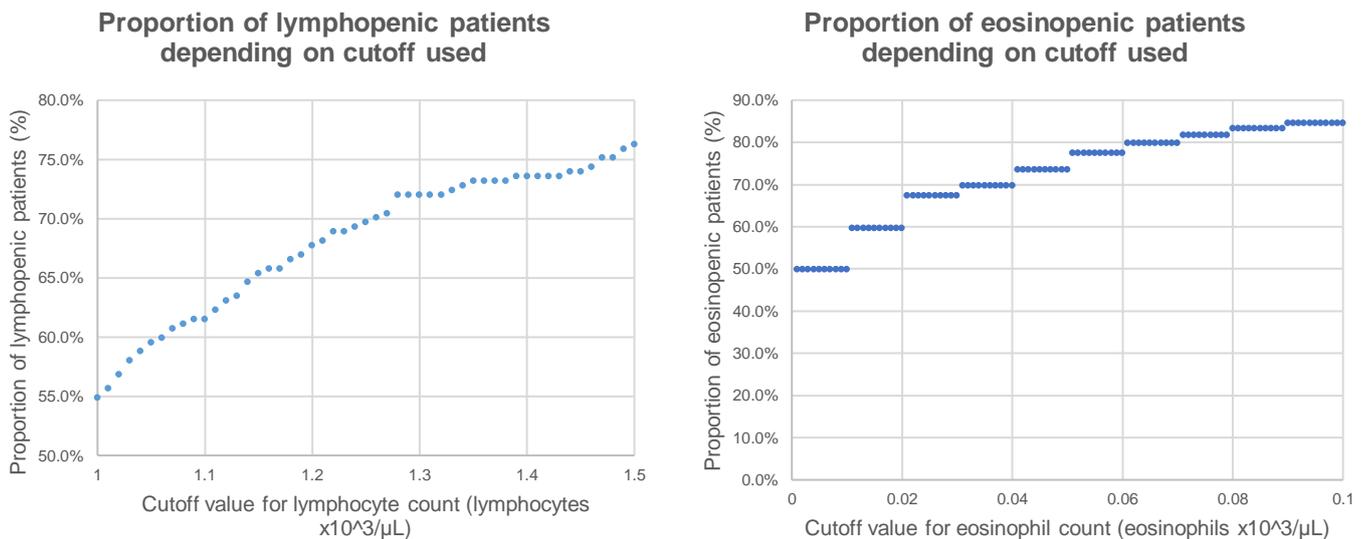


Figure 11. Impact of analytical cutoff value used on the proportion of lymphopenia (left panel) and eosinopenia (right panel) in a cohort of 257 COVID-19 patients.

Variables between flow cytometry and microscopy

Secondly, we propose that variables in the different methods used in hematologic cell counting affects lymphocyte counts. In many modern laboratories, routine peripheral blood cell counts are obtained using a two-step algorithm. A differential white blood cell count is usually performed using fluorescence flow cytometry in a first step. Based on light scattering characteristics and/or numerical abnormalities of different cell populations, samples may be ‘flagged’ for microscopic review in a second step.

Many modern laboratory hematology platforms offer a module capable of automated peripheral blood smear preparation, automated microphotography and automated cytomorphological pre-classification of peripheral blood cells, in ‘flagged’ samples. Depending on the type of abnormality that triggered microscopic revision, both the flow cytometrical lymphocyte count or the manual lymphocyte count may be regarded as a more correct value. If adequate discrimination between cell populations is obtained by flow cytometry (as is evidenced by clearly distinct cell clusters on flow cytometry scattergrams), the flow cytometrical count may be preferred to a manual one, given that it is based on a much greater total cell count. If cell clusters are not distinct on the scattergram, or when cells are not classifiable using flow cytometrical characteristics, the manual count may be preferred.

This two-tier workflow incorporates multiple variables into WBC differentiation that may cause discrepancies between lymphocyte counts in different studies. These variables include the criteria used for flagging of samples for manual counting, technical variables in smear preparation (such as the angle and speed of the spreader blade), variables in smear microphotography (the number and the smear density of photographed fields), the microscopist or operator’s skill in differentiating white blood cells, and, finally, the decision on whether the flow cytometrical or microscopic count is deemed more trustworthy and should be reported. Laboratories in resource-constrained settings may rely completely on manual microscopic differentiation, which might further widen the variability in lymphocyte counts compared to automatized laboratories.

In order to investigate the impact of some of these variables, we first examined the differences in lymphocyte counts obtained with the flow cytometrical method (using the Sysmex XN module), and the microscopic method (using the Sysmex DI-60 module) as paired measurements on the same sample. Both measurements are only available for samples that, based on flow cytometrical abnormalities, have been selected for microscopic review.

For CMV/EBV and COVID-19 cohorts, lymphocyte counts are on average more broadly distributed and higher than a non-infectious control population ($n = 131$) when measured by flow cytometry, than by microscopy (see Figure 12, left panel). The mean difference between flow cytometry and microscopy is significantly different between CMV/EBV smears and non-infectious controls ($p = 0.004$) but only approaches statistical significance when comparing COVID-19 patients and non-infectious controls ($p = 0.07$). Upon review of White blood cell Differential (WDF) scattergrams of patients with in these cohorts, we noticed that gating errors of the lymphocyte cluster (that could explain overestimation of lymphocyte counts by flow cytometry) are rare. Therefore, the cause of the difference between both methods, as it exists in CMV/EBV cohorts, and, possibly, in COVID-19 patients, is unclear. Both infections have been associated with the occurrence of reactive enlarged lymphocytes in the literature (see literature study below). Reactive (that is, enlarged) lymphocytes, we speculate, may be more fragile and could be more susceptible to lysis during smear preparation, causing lower intact lymphocyte counts by microscopy.

Notably, the proportions of samples selected for microscopic review differ significantly between different cohorts (see Figure 11, right panel). 53.7% of samples from COVID-19 patients are flagged for review; this is below the proportion of samples of, for example, sepsis patients (73.7%) and RSV infections (58.0%), but higher than in influenza-infected (49.4%) and other, largely non-

infectious controls (25.7%). The difference in rates of microscopic review of samples is therefore another variable that may increase the impact of the analytical variability that already exists between flow cytometry and microscopy for lymphocyte counts.

Examining the reasons for microscopic review of samples ('flags'), we find no increased proportion of flags related to lymphocyte abnormalities in the COVID-19 cohort, compared to other cohorts (see Fig. 13). The most common flag in COVID-19 patients is the 'WBC Abnormal Scattergram' flag, which can be triggered by a broad range of abnormalities regarding the total leukocyte count or leukocyte differential counting.

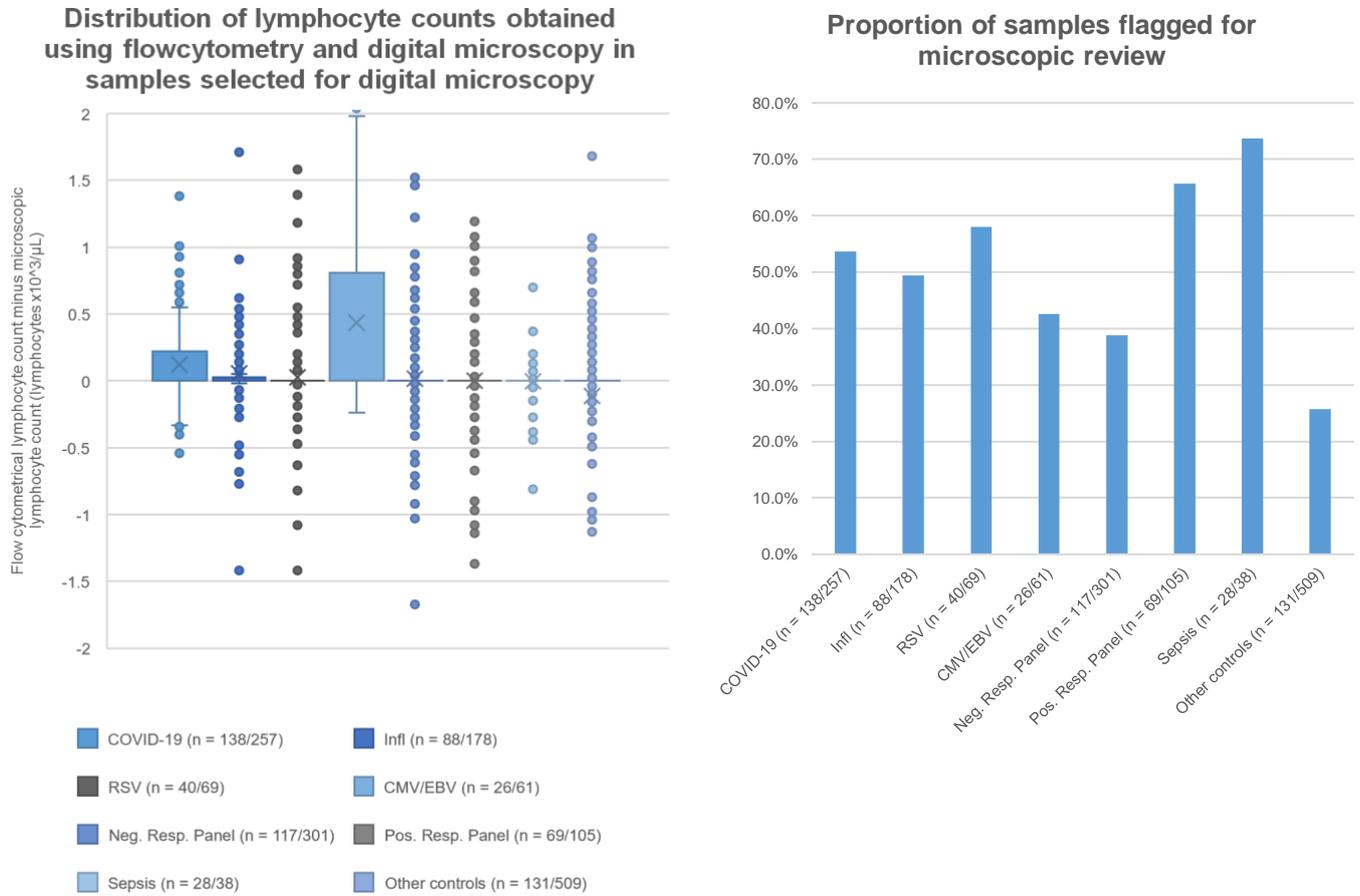


Figure 12. *Left panel:* distribution of the differences between lymphocyte counts obtained by flow cytometry (Sysmex XN10/XN20) and microscopy (Sysmex DI-60) as paired measurements on the same samples in COVID-19 and cohorts. *Right panel:* the proportion of samples per cohort selected for microscopic review.

The proportion of samples per cohort with a given flag for leukocyte abnormalities

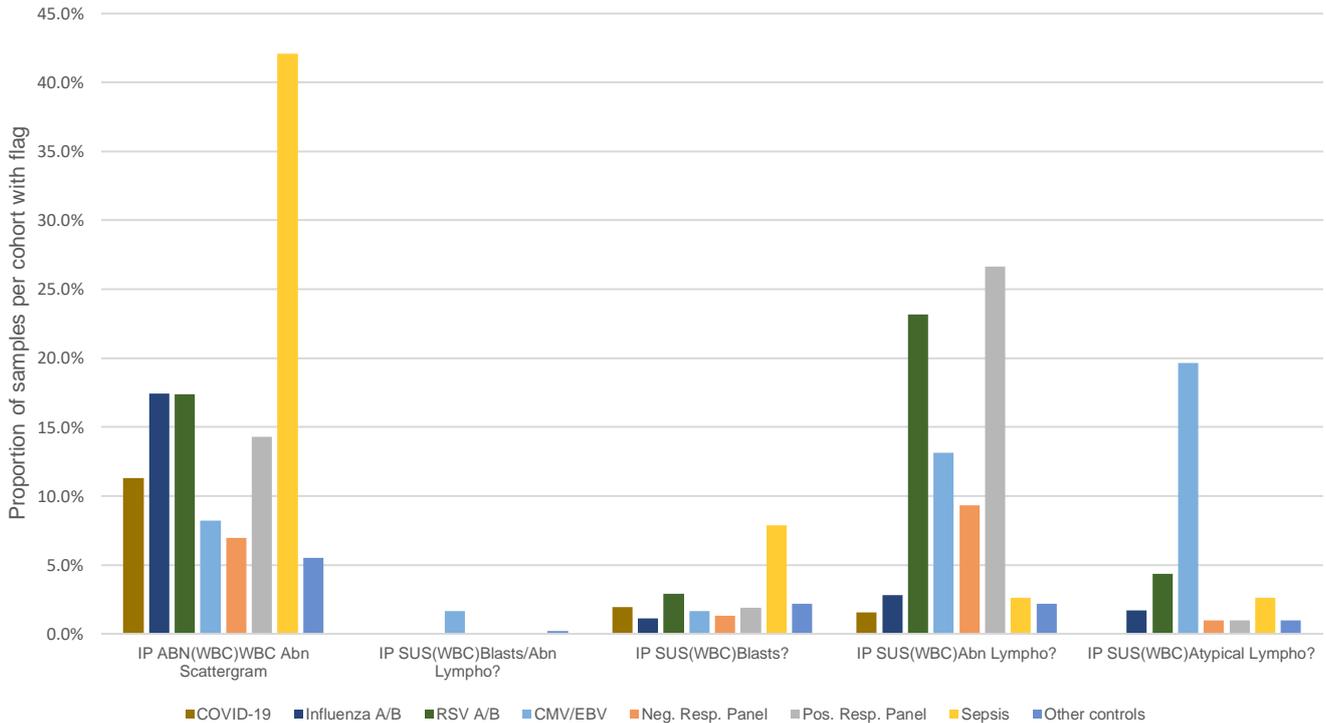


Figure 13. The proportion of flags for leukocyte abnormalities in COVID-19 and control cohorts.

Technical issues

Several studies report on the use of experimental or newly validated flow cytometrical parameters for COVID-19 diagnosis. On the Sysmex XN platform, this includes the Reactive Lymphocyte count (RE-LYMP) and the Antibody-Secreting Lymphocyte count (AS-LYMP). Known technical issues with these new parameters are not being taken into account in several of these studies. First, in a potentially significant proportion of samples, no values can be calculated for AS-LYMP and RE-LYMP. These are called ‘vote out’ results, and can occur due to detection of abnormalities suggestive of the presence of malignant cells (for example, the so-called ‘Blasts/Abnormal lymphocytes’ flag). Studying these parameters only in patients where these values are available, does not yield a representative image of the occurrence of AS-LYMP cells in a general COVID-19 population. Second, due to shortcomings in the algorithm for identification of a lymphocyte cluster in the highest fluorescence region, AS-LYMP could be falsely reported 0, even when of a significant number of lymphocytes were present in that region (personal communication, Sysmex Benelux). These shortcomings have been addressed in a software update released during the course of 2020 (IPU version 22.12).

In our study population, we noticed high rates of ‘vote out’ results for both parameters, particularly for the cohort with a positive respiratory PCR panel (non-COVID-19, non-RSV, non-influenza) and in RSV-infected patients (up to 28.6% and 26.1% of samples, respectively) (see Fig. 14). The distribution of the occurrence of vote out results in our different study cohorts closely mirrors the occurrence of the Abnormal lymphocyte flag in those cohorts (see Fig. 13).

In COVID-19 vote out results occur in only 3.5% of samples, corresponding with the relatively rare occurrence of the Abnormal lymphocyte flag.

The proportion of samples for AS-LYMP and RE-LYMP with vote-out results in COVID-19 and control cohorts

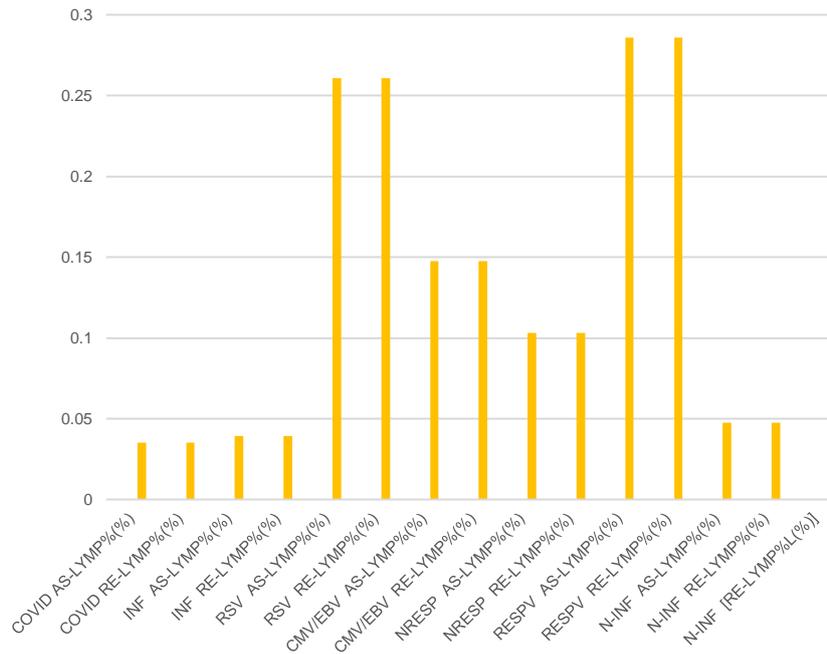


Figure 14. The proportion of 'vote out' results for AS-LYMP and RE-LYMP in COVID-19 and control cohorts.

COMMENTS

In a literature study and retrospective cohort study we identify and evaluate hemocytometric markers for diagnosis of COVID-19. Among models described in the literature, we find that the PARIS score, a composite hemocytometric score based on lymphopenia, neutropenia, eosinopenia and basopenia, performs best, albeit with a mediocre AUC of the ROC-curve of 0.74. We investigate the diagnostic value of patterns of lymphocyte distribution on the WDF scattergram and find a maximal AUC of 0.78 for a ratio of lymphocyte counts in the highest fluorescence region compared to counts in the intermediate fluorescence region. This implies that lymphocyte distribution on the WDF scattergram may have some diagnostic value in differentiating COVID-19 from other illnesses. In our large and diverse control cohort, we find that diagnostic performance of hemocytometric parameters is, generally, less than reported in the literature. This is in agreement with an earlier systematic review that describes poor selection of control cohorts and model overfitting in many studies of lab-based prediction models.¹ We conclude that, in times of improved availability of rapid RT-PCR assays and the introduction of well-performing antigen assays, hemocytometric parameters play no significant role in diagnosis of patients with possible SARS-CoV-2 infection. Clustering characteristics of lymphocytes in the upper fluorescence regions of the Sysmex XN WDF scattergram exhibit some specificity for COVID-19, and possible diagnostic use requires further investigation.

TO DO/ACTIONS

- 1) Examine the prognostic value of hemocytometric markers in COVID-19 using survival/mortality data.
- 2) Examine temporal trends in hematological alterations in COVID-19

Appendix A: literature study on hemocytometric changes in COVID-19

Lymphopenia

Studies early in the pandemic reported marked lymphopenia in a significant proportion of COVID-19 patients. Chen et al. describe absolute lymphopenia in 35% of 99 patients²³ and Huang et al. report absolute lymphopenia in 63% of 41 patients. Later studies describe low lymphocyte counts as a predictor of poor outcome, that is, predicting onset of *acute respiratory distress syndrome* (ARDS), intensive-care admission and mortality.²⁴ Nevertheless, a remarkable variation has been noted in the occurrence and degree of lymphopenia in different studies. In a review of studies up to February 2020, Lippi et al. report the occurrence of lymphopenia in a range from 35% up to 75% in studies with adult COVID-19 patients.²⁵ (Figure 14)

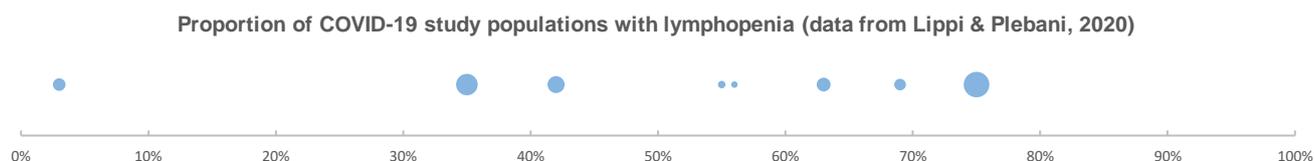


Figure 14. Proportion of COVID-19 study populations with lymphopenia (data from Lippi & Plebani, 2020). Dot size implies relative study population size.

Disease severity of the study population is a factor that may affect the proportion of lymphopenic patients. In a meta-analysis of lymphopenia in severe COVID-19, Huang and Pranata summarized studies that report lymphocyte counts in cohorts with favorable and unfavorable outcome (poor outcome being death, intensive care admission, development of ARDS, or 'severe' clinical course).²⁴ They showed that patients with poor outcome have significantly lower median lymphocyte counts. The proportion of lymphopenic patients was higher in cohorts with poor outcomes compared to matched cohorts with favorable outcome. Therefore, lymphopenia-based diagnostic models may exhibit limited sensitivity in a study population with mild illness.

Reactive lymphocytes, antibody-secreting lymphocytes, highly-fluorescent lymphocytes

COVID-19 has been pointed out as a cause of reactive lymphocytes in the peripheral blood smear.²⁶ A Singapore study reported reactive lymphocytes in 16 of 23 COVID-19 patients (69.6%).²⁷ Literature on the occurrence of reactive lymphocytes consists mostly of single case reports or small case series.^{26,28,29} Hampering study of this phenomenon is the lack of standardized criteria for defining a 'reactive' appearance of lymphocytes.

As an alternative to morphological assessment of reactive lymphocytes in blood smears, several hematology analyzers offer flow cytometric parameters that quantify various subsets of these cells. On Sysmex XN analyzers (Sysmex Corporation, Kobe, Japan), the highly-fluorescent lymphocyte count (HFLC) and antibody-secreting lymphocyte count (AS-LYMP) quantify a distinct population of within the lymphocyte cluster with the highest fluorescence signals on the white blood cell differential (WDF) scattergram. The AS-LYMP count is dynamically gated on the WDF scattergram, whereas the HFLC is determined using a static cell counting window. The presence of cells in this region correlates with circulating T-cell independent plasma cells or activated B cells.³⁰ The reactive lymphocyte count (RE-LYMP) quantifies a broader population of lymphocytes with higher-than-normal fluorescence (see Figure 9, section 1). These parameters may be expressed both as absolute counts, or as proportions of the total leukocyte count (e.g. AS-LYMP%), or of the lymphocyte count (e.g. AS-LYMP%/L).

Apart from COVID-19, use of these parameters for hematological diagnosis is mostly experimental, with a few reports on a potential role on differentiating viral infections from bacterial infections.^{31,32} In COVID-19 patients, reports on RE-LYMP, HFLC or AS-LYMP counts are sparse. Martens et al. report that a higher proportion of lymphocytes show above-normal fluorescence (as shown by an increased RE-LYMP) in COVID-19 patients compared to negative controls, and more so in those with cytokine-storm syndrome, compared to mild illness.⁵ A higher proportion of these 'reactive lymphocytes' are in the highest fluorescence region, as witnessed by the increased HFLC and AS-LYMP counts. Agreeing with these findings, Yun et al. report significantly higher levels of highly-fluorescent lymphocytes (as a percentage of the total leukocyte count; HFLC%) in 32 COVID-19 patients, compared to 2337 negative patients.³³ Conversely, comparing 20 COVID-19 patients with 20 patients with other viral infections (mostly with mononucleosis), Rutkowska et al. report a lower median RE-LYMP count in SARS-CoV-2 infection.⁶ Interestingly, Osman et al. report that recognition of a 'sand glass' pattern on the WDF scattergram (a cluster of lymphocytes in the highest-fluorescence region, but not in the intermediate fluorescence region) has a sensitivity of 85.9% and specificity of 83.5% for predicting SARS-CoV-2 infection as determined by PCR and/or chest CT (see Figure 9, section 1). This test was validated in a cohort of 85 COVID-19 patients and 85 patients with influenza, EBV, *Mycoplasma pneumoniae* and parvovirus infections. This method, however, is difficult to standardize and to scale. No other groups have validated this finding in an independent cohort.

Data on the clinical correlate of reactive lymphocytes in the peripheral blood is conflicting. One study reports parameters of disease severity and outcome in a cohort of 18 COVID-19 patients with reactive lymphocytes and 18 without.³⁴ The cohort without reactive lymphocytes exhibited longer hospitalization duration, higher rates of mechanical ventilation and of mortality. Other laboratory abnormalities associated with poor outcome (such as thrombopenia and lymphopenia) were more severely

disturbed in the group without reactive lymphocytes. A better survival was seen in the cohort with activated lymphocytes, presumably because these represent an effective immune response against the virus. However, no formal statistical analysis of the differences between cohorts was performed, and the cohorts were not controlled for confounders such as age. Flow immunocytometrical analysis of 13 of the included patients with reactive lymphocytes and of 4 without, suggested that the reactive lymphocytes consisted of activated T lymphocytes.

Boulanger et al., in a study of 2,301 COVID-19 patients, investigate the prognostic significance of the presence of plasma cells in the peripheral smear.³⁵ In patients with severe disease, the presence of plasmacytoid cells was an independent predictor of improved survival, potentially reflecting establishment of a successful immune response against the virus. However, similarly to the previous study, no standardized morphological criteria or quantitative cutoff values were provided for defining the presence of 'plasma cells'. Although plasma cells are defined as antibody-secreting B-lymphocytes, morphologically these are known to fall within a spectrum of reactive-looking lymphocytes, often resembling so-called Downey type III reactive lymphocytes. Based on morphological appearance only, reactive-looking lymphocytes are difficult to classify as pertaining to the B- or T-lymphocyte lineage.

Contrary to findings of morphological studies, Yip et al. report an increased antibody-secreting lymphocyte count (AS-LYMP) in 20 severely-ill COVID-19 patients compared to 56 patients with mild illness. This is in agreement with Martens et al., who also report higher values for RE-LYMP, AS-LYMP and HFLC in COVID-19 patients with cytokine storm syndrome. Linssen et al. incorporate increased AS-LYMP%/L as a predictor of severe disease in their composite hemocytometric prognostic score, based on a multicentric study of 366 critically-ill and 557 non-critically ill COVID-19 patients.³⁶ Wang et al., to the contrary, report a significantly higher HFLC% in 131 moderately-ill patients compared to 30 patients with severe disease.³⁷

Likely, these discrepant findings indicate that there is not a single category of 'reactive lymphocytes' that consistently may be linked to favourable or unfavourable outcome. In COVID-19, a successful immune response is known to depend on a delicate balance of pro- and anti-inflammatory factors. 'Crude' methods such as peripheral blood morphology or flowcytometrical measurement of lymphocyte fluorescence levels, may have insufficient resolution to differentiate prognostically significant subsets of activated lymphocytes. It is likely that different analytical methods may be biased towards detection of different subsets of activated or plasmacytoid lymphocytes. Studies that simultaneously assess immunological markers, fluorescence characteristics and peripheral blood morphology could clarify the relationship between these parameters. Moreover, different endpoints are used for defining unfavourable outcome. Therefore findings for different endpoints may not be directly comparable. Some of the aforementioned studies report development of severe disease, and others mortality as an endpoint for assessing the prognostic role of reactive lymphocytes. The prognostic significance of reactive lymphocytes in peripheral blood therefore remains incompletely defined.

Finally, analytical problems may contribute to discrepant findings in the literature. A known technical issue regarding the AS-LYMP parameter (in IPU version 22.08) is a potential false underestimation of cell counts in the highest-fluorescence region, particularly when more cells are present in the intermediate fluorescence region (personal communication, Sysmex Corporation Benelux). This issue seems not to be accounted for in publications reporting on the use of this parameter.^{5,36,38,39} In newer IPU versions (starting from version 22.12) the algorithm for calculation of AS-LYMP has been improved (personal communication, Sysmex Corporation Benelux).

Eosinopenia

Eosinopenia first came to attention when it was recognized that patients with atopic conditions are underrepresented in COVID-19 cohorts. This is unexpected, since atopic illnesses predispose to viral-induced asthma exacerbations (typically caused by common respiratory pathogens such as rhinovirus, RSV and influenza). Early studies observed an inverse relationship between eosinophil counts and disease outcome, which sparked interest in the use of eosinopenia as a feature with possible diagnostic and prognostic value in COVID-19 patients.⁴⁰

As a diagnostic marker, reported values for the area under the curve (AUC) of receiver operating characteristics curves (ROC) include 0.717,¹⁹ 0.74,¹⁶ 0.763,⁷ 0.84,²¹ 0.85,⁸ 0.97⁹ with sensitivities of 48.15%,⁷ 68.2%¹⁶, 74.7%,¹⁹ 80.68%⁹, 89.5%⁸, 94%²¹ and specificities of 63%,²¹ 68.7%,¹⁹ 75.0%,¹⁶ 78.1,⁸ 98.88%,⁷ 100%.⁹ Cutoffs used for defining eosinopenia have ranged from 0/ μ L,^{7,15} 10/ μ L⁸, 15/ μ L¹⁶, 20/ μ L¹⁷⁻²⁰, 40/ μ L and 50/ μ L⁹, 45/ μ L²¹ to 100/ μ L²².

Several authors note the limited specificity of eosinopenia for COVID-19, if used for differentiating COVID-19 from other infectious or inflammatory illnesses of diverse etiology.⁴¹ In 50 patients with influenza infection and 50 COVID-19 patients, Tanni et al. show a wide divergence in eosinopenia rates between the cohorts (16% versus 60%). However, the cohorts were not matched for disease severity, which may confound the apparent association between COVID-19 and eosinopenia.¹⁰ Xie et al., demonstrate eosinopenia (\leq 20/ μ L) in 5 of 12 COVID-19 (41.7%) patients and only in 2 of 12 influenza patients (16.7%). Both cohorts had similar median CRP values. In the control cohort of 176 other types of pneumonia (etiology not further specified), eosinopenia occurred in 22.7% of patients. This group reports an overall specificity of 75.0%. A weakness in many studies is the lack of a well described control cohort. Therefore, the presence of significant systemic inflammation may confound the specificity of eosinopenia for COVID-19.

An early meta-analysis questioned the prognostic value of eosinopenia.⁴² Other groups have described a modest association between the degree of eosinopenia and disease severity, noting high rates of heterogeneity between studies.⁴³ Le Borgne et al. describe eosinopenia (defined as the absence of detectable eosinophils) as a predictor for disease severity during initial Emergency department management, but not for overall mortality.⁴⁴ In a multivariate analysis by Yan et al., worsening eosinopenia over the course of an infection indicates a significantly higher chance of mortality.⁴⁵ Huang et al. describe eosinophil counts below 20/ μ L as a predictor for ICU transfer. Cazzaniga et al. report higher rates of intensive respiratory treatment and mortality in a cohort with undetectable eosinophils. Glickman et al. describe a significant association between mortality and eosinopenia in 1004 White and 1152 Hispanic patients, but not in 1141 Black or 202 Asian patients.⁴⁶

In addition to demographic factors and different cutoffs used for defining eosinopenia, analytical factors may also impede comparability between studies. Lippi et al. remark that eosinophil counts are subject to large interindividual biologic variation, as high as 76%. Analytical goals for quality control of analyzers are set as high as 26%, which exceeds the reported difference between eosinophil counts in cohorts with mild and severe illness in some studies.⁴³ In addition, the accurate differentiation of eosinophils from neutrophils by flowcytometry is notoriously difficult in certain samples with hypergranulated neutrophils. This may further contribute to measurement uncertainty. Microscopic differentiation of white blood cells is considered a reference method for eosinophil counting, although this method also has inherent flaws: usually 100-200 white blood cells are differentiated, and the proportion of eosinophils and the total white blood cell count is used to calculate an absolute eosinophil count. However, low eosinophil counts are inherently susceptible to a potentially significant sampling error (e.g. dependent by the part of the smear that is examined). The error rate in the manual differentiation translates to widely varying absolute counts. Certain inter-laboratory variables (for instance, whether smudge cells that resemble eosinophils are counted as eosinophils or not) may further add to variability. These variables may explain the wide range of diagnostic performances reported for eosinopenia as a diagnostic and prognostic marker.

Monocyte count and lymphocyte-to-monocyte ratio (LMR)

The monocyte count in peripheral blood has been relatively less well explored in COVID-19 patients.⁴⁷ Monocyte counts have inconsistently been described to be both elevated, decreased or in the normal range in COVID-19 patients.^{11,47} In a cohort study of 166 severe and 266 non-severe cases of COVID-19, median absolute monocyte counts were identical and within normal ranges in both cohorts. There was a slightly lower relative monocyte count in the severe group, that might result from the higher absolute neutrophil counts in that cohort.⁴⁸ Another study demonstrates significantly lower absolute monocyte counts in COVID-19 patients with cytokine storm syndrome.⁵ Conversely, Mertoglu et al. demonstrate that a larger subset of patients in ICU have monocytosis than those on general wards; monocytopenia is rare in both cohorts.⁴⁹ As a predictive test for a diagnosis of COVID-19, monocytosis displays low sensitivity in four pooled studies but moderate specificity (summary sensitivity of 13% with specificity 73%). In two of these studies, monocytopenia had a sensitivity of 1% and 2% and a specificity of 94% and 97%, in a cohort of patients with suspicion of COVID-19 and/or community-acquired pneumonia.⁴⁷ These divergent findings may reflect differing proportions of critically-ill patients or perhaps inter-laboratory differences.

Monocytosis and lymphopenia were used during the H1N1 pandemic for presumptive diagnosis of influenza.⁵⁰ For differentiating COVID-19 from influenza, Wang et al. report a high specificity of a predictive composite index based on age, relative lymphocyte count and absolute monocyte count.⁵¹ The median absolute monocyte count in influenza patients is twice that in COVID-19 patients (0.6 and 0.3 $\times 10^9/\mu$ L), similarly to what other groups describe;⁵² both values are within normal ranges. The study population consisted of consecutive patients at the Emergency department who had tested positive for either of these viruses; no data was reported on disease severity or admission rate. Different disease severity could have confounded these findings. Additionally, this index was not validated on an independent cohort, which might have resulted in overfitting of the model and lesser performance in other cohorts. Song et al. find a significantly higher proportion of influenza patients with monocytosis (33/48; 68.75%), than in bacterial pneumonia (34.78%; 16/46) and in COVID-19 (14.58%; 7/48). There are higher rates of lymphopenia in influenza (45.83%; 22/48) than in COVID-19 (29.17%; 14/48) or bacterial pneumonia (6.52%; 3/46). Consequently, they report a low lymphocyte-monocyte ratio (< 2.74) as a marker that is relatively specific to influenza infection. The LMR had an AUC of 0.895 for distinguishing influenza from COVID-19. Similarly to the study of Wang et al., these cohorts were not controlled for disease severity; rather, the influenza cohort contained a larger proportion of patients with high CRP values. Therefore, as monocytosis and lymphopenia may be more pronounced in severe disease than in mild disease in COVID-19, the reported specificity may be nullified when considering patients severely ill with either COVID-19 or influenza.

The lymphocyte-monocyte ratio has been explored in some studies as a parameter with prognostic value. A meta-analysis of mainly Chinese studies up to May 2020, reports a significantly higher MLR in severe COVID-19 patients.⁵³ Other studies show similar results: Kalabin et al. report higher survival in a cohort with increased LMR; an association not seen for platelet-lymphocyte ratio or neutrophil-lymphocyte ratio.⁵⁴ Ramos-Penafiel et al. report an AUC of 0.628 and 0.619 for predicting mortality and mechanical ventilation respectively (of the monocyte-to-lymphocyte ratio).⁵⁵ Mertoglu, Asan, and Biamonte et al. report a significantly lower lymphocyte-to-monocyte ratio in a cohort of ICU-admitted or deceased COVID-19 patients compared to non-ICU or surviving COVID-19 patients.^{49,56,57}

Several automated hematology analyzers offer experimental parameters that reflect qualitative abnormalities of the monocyte population. On Beckman Coulter analyzers, the monocyte distribution width (MDW) mainly reflects distribution of the size of monocytes.⁵⁸ It was first used as an aid in distinguishing sepsis from less severe infections or non-infectious conditions.⁵⁹ A meta-analysis in December 2020 found three eligible studies and concluded that MDW is higher at hospital admission in subjects with

active SARS-CoV-2 infection than in those without.⁵⁸ Some weaknesses of included studies included small study population (9 COVID-19 patients in one study)⁶⁰ and the exclusion of bacterial infections in the control cohort of another.⁶¹ This may undermine the reported diagnostic performance of this parameter, especially the specificity of this parameter for differentiating COVID-19 from other infectious illnesses. A similar parameter on Sysmex analyzers is the reactive monocyte count (RE-MONO); which quantifies a larger-sized monocyte subpopulation. Literature on this parameter is relatively scarce. One large study reports a significantly higher reactive monocyte count in COVID-19 patients versus controls, and in COVID-19 patients with cytokine storm syndrome versus those with milder disease.⁵ Another study uses the proportion of reactive monocytes over all monocytes (RE-MONO/M%) as part of a 10-parameter hemocytometric model that predicts the need for ventilatory support at admission.³⁶

Neutrophils and immature granulocytes

A large study reports lower median neutrophil counts in 553 COVID-19 patients versus 587 controls (7.7 versus 5.7 x10⁹/L). The cohort of COVID-19 patients with cytokine storm syndrome had higher median neutrophil counts than that with patients with mild disease (6.2 versus 5.5 x10⁹/L), but lower counts than the non-COVID-19 controls.

Many viral infections tend to cause suppressed neutrophil counts, which may be attribute to a wide variety of mechanisms. Influenza is a notable example.⁶² A systematic review including four studies from early 2020, demonstrates that neutropenia has a good specificity for a diagnosis of COVID-19 (median specificity 93%), while the sensitivity is low (10%).⁴⁷ The study population consisted of patients with febrile or respiratory illness. However, the number of confirmed diagnoses with respiratory pathogens other than SARS-CoV-2 was not reported, neither were the cohorts controlled for disease severity, which might act as a confounder.

As a diagnostic test, neutrophilia displays inferior sensitivity and specificity compared to neutropenia.⁴⁷ This is unsurprising since neutrophilia is a non-specific finding in diverse conditions of physiological stress. However, the occurrence of neutrophilia in SARS-CoV-2 infection may have prognostic value. A meta-analysis of 22 studies reported that, in addition to lymphopenia, neutrophilia is strongly associated with increased odds of development of severe disease and of mortality when present at admission. Neutrophilia and lymphopenia have been combined as a ratio (the neutrophil-lymphocyte or lymphocyte-neutrophil ratio; NLR or LNR), that, above or below a certain threshold, appears to be a consistent predictor of unfavorable outcome.¹¹ This ratio has long been employed as a generic inflammatory marker and predictor of poor outcome in both cardiovascular disease, malignancies and chronic obstructive pulmonary disease.^{63,64} Two meta-analyses report a significant association between a higher NLR and poor outcome in COVID-19.⁶⁵ However, employing mainly univariate analysis, these studies are vulnerable to confounders, such as comorbidity, which might affect outcome and NLR independent of SARS-CoV-2 infection. A more recent meta-analysis of 34 multivariate studies concluded that elevated NLR remains an independent predictor of poor outcome, albeit noting that a few individual studies do not support this conclusion.⁶⁵ Smaller studies have examined NLR as a diagnostic tool for differentiating COVID-19 from influenza and other respiratory infections (influenza infection exhibiting higher NLR), with mediocre performance (AUC 0,6727).^{12,60} In a study of 153 COVID-19 patients and 117 patients with other viral and bacterial infections, the NLR has an AUC of 0.939 for differentiating non-COVID-19 viral infections from bacterial infections and COVID-19 (the latter two which exhibit increased neutrophil counts).⁶⁶

A few studies have looked at markers of neutrophil activation or at markers that quantify the immature fraction of granulocytes in COVID-19. The Delta Neutrophil Index (DNI) is a parameter on the Advia hematology analyzers (Siemens Healthineers, Erlangen, Germany) that represents the ratio of immature granulocytes over the total granulocyte count. In a study of 388 patients receiving intensive care (of which 124 died), the delta neutrophil index had an AUC of 0.718 for predicting mortality.⁶⁷ Others report higher DNI values in a severely ill cohort of both influenza and COVID-19 compared to healthy controls.⁶⁸ On the Sysmex XN-10 platform, Martens et al. report a significantly higher side fluorescence (SFL) signal in a more monotonous population of neutrophils in COVID-19 positive patients, which suggests the presence of an activated or immature granulocytic population. The immature granulocyte count, is significantly higher in patients with cytokine storm syndrome than in patients with mild disease.⁵

Blood platelets

Thrombocytes, as part of the coagulation system, have received attention as key players in the pathogenesis of thromboembolism in COVID-19. Thromboembolism is a well-recognized complication in severe SARS-CoV-2 infection. Platelet activation and immune-mediated clearance, platelet consumption and sequestration, platelet destruction and reduced platelet synthesis have all been implicated in the development of thrombocytopenia, that is often concurrently seen in COVID-19.⁶⁹ Mild thrombocytopenia is found in 5 to 41.7% of SARS-CoV-2 infections and it occurs more often in severe disease. However, even in severe infections, thrombocytopenia is often mild (counts between 100,000 to 150,000 platelets per μ L are common). Severe thrombocytopenia is rare in COVID-19.⁶⁹

As a predictor for a diagnosis of COVID-19, thrombocytopenia displays low sensitivity (a median of 19% sensitivity), but reasonable specificity (median 88%) in four pooled studies.⁴⁷ However, as is the case for hematologic disturbances in COVID-19, thrombocytopenia is a non-specific finding that is common in diverse viral infections.⁷⁰ Thrombocytopenia is part of prognostic models in infectious and non-infectious critical illness (such as the sequential organ failure assessment score (SOFA)). Recent

meta-analyses find that thrombocytopenia is an independent predictor of poor outcome in COVID-19.^{71–73} Some earlier studies, however, were inconclusive.⁷⁴ It has been proposed that daily determination of platelet counts aids in assessment of prognosis and the course of infection.⁷⁵

Similarly to NLR, the platelet-to-lymphocyte ratio (PLR) was known before the pandemic as a nonspecific marker of systemic inflammation. Its prognostic value in COVID-19 seems less well established than that of the neutrophil-to-lymphocyte ratio (NLR). Studies comparing both parameters demonstrate inferior prognostic performance of the PLR or no prognostic value altogether (AUCs of 0.535, 0.59, 0.505, 0.703, 0.677 for several endpoints of unfavourable outcome).^{53,55,76–78}

The mean platelet volume (MPV) is a parameter that may be obtained using impedance methods on a wide range of hematology analyzers. The platelet distribution width (PDW) reflects the amount of variation in platelet size. Studies in inflammatory conditions of various etiologies mostly show increases in the MPV, which presumably is the result of an increased proportion of immature, larger platelets due to accelerated, cytokine-driven thrombopoiesis. In some viral and parasitic infections (such as RSV), a decrease in MPV was noted.⁷⁹ Several studies suggest a decrease in MPV in adult COVID-19 patients. A study of 54 COVID-19 patients and 43 influenza patients, found below-normal MPV values in both infections, and significantly lower MPV values in COVID-19 compared to influenza.⁸⁰ A concomitant increase in PDW (in COVID-19) in this study, suggests an increased variance in platelet sizes, with a net mean shift towards smaller platelet size. In a pediatric population, to the contrary, Gumus et al. find that in 55 SARS-CoV-2-positive patients the MPV is significantly higher than in a non-infectious cohort of 60 patients.⁸¹ Koç et al. describe a significantly lower MPV in pregnant patients with COVID-19, versus non-infectious pregnant patients.⁸²

Some studies point toward an increased MPV in severe COVID-19. Güçlü et al. describe an increased MPV and PDW values in non-surviving COVID-19 patients versus survivors.⁸³ Gowda et al. find that increased PDW, but not MPV, is associated with mortality in COVID-19.⁸⁴ Ozenen et al. demonstrate no differing MPV in children with a confirmed COVID-19 diagnosis versus a negative control cohort, but a higher MPV in hospitalized children than in outpatients.⁸⁵ Zhang et al. describe both a significantly higher MPV and PDW in severe COVID-19 versus more mild disease and compared to healthy and COVID-19-negative matched controls.⁸⁶ These divergent findings from mostly small-sized studies suggest that alterations in these parameters deserve further study. Not unimportantly, pre-analytical factors are known to affect measurements of platelet size significantly. In vitro measured platelet size is known to increase rapidly with time between blood draw and analysis (due to EDTA-induced platelet-swelling).

The immature platelet fraction (IPF) is relatively new parameter obtained on Sysmex XN analyzers, using a dedicated ‘PLT-F’ channel, employing a fluorescence colorant with enhanced specificity for blood platelets. It quantifies immature platelets by detection of their larger size and higher nucleic acid content. Its main application is the detection of active thrombopoiesis in differentiating aplastic causes of thrombopenia from platelet destruction or consumption. It was used as a predictor for serious adverse cardiac events, given that larger and highly fluorescent platelets are more thrombogenic.⁸⁷ In this context, the parameter has received some attention as a possible indicator of thrombotic risk in COVID-19 patients. Cohen et al. describe higher IPF counts in COVID-19 patients compared to negative controls; with similar IPF counts in a cohort admitted with acute myocardial infarction.⁸⁸ Given the influence of non-specific inflammation on platelet parameters, it is yet unclear whether IPF independently predicts the risk of thrombosis.

Basophils

Literature on alterations in basophil counts in COVID-19 is limited and inconclusive. Linssen et al., in 923 COVID-19 patients find identical median basophil counts in patients requiring intensive care and those who don't.³⁶ Martens et al., to the contrary, describe significantly lower basophil counts in patients with cytokine storm syndrome compared to those with mild illness, and compared to COVID-19-negative patients.⁵ Tordjman et al. describe lower basophil counts in COVID-19 patients versus negative controls, and include it in their diagnostic PARIS score.¹⁴ Occurring in low numbers in the peripheral blood, as eosinophil counts, basophil counts are also susceptible to similar analytical issues.

Red blood cell parameters

In contrast to several leukocyte parameters, most erythrocyte parameters are intrinsically more stable during acute disease, limiting their diagnostic utility in COVID-19. Linssen et al use different parameters of hemoglobinization (hemoglobin, Delta He, HYPO-He) and the presence of nucleated red blood cells, to predict the risk for development of ventilator-dependency.³⁶ In their role in determining prognosis of infected patients, they may act as surrogate markers for age or comorbidity, rather than being independent predictors of disease severity. Few studies have investigated alterations in red blood cell parameters as independent predictors of SARS-CoV-2 infection or of disease severity.

Composite hemocytometric parameters

The diagnostic accuracy of characteristic hematological alterations in COVID-19 may be enhanced by combining several individual parameters in composite models. Various diagnostic or prognostic scores have been devised incorporating both clinical, imaging-based and laboratory findings.¹ Hemocytometry-based scores, however, are particularly attractive, as a complete blood

count and leukocyte differential are readily available in almost all medical centers, are rapidly obtained and are inexpensive.

Tordjman et al. propose the so-called 'Pre-test probability for SARS-CoV-2 Infection based on Scoring' model (PARIS score), based on the relatively lower absolute lymphocyte, eosinophil, neutrophil and basophil counts seen in most COVID-19 patients.¹⁴ A scoring system that awarded two points for lymphopenia, and one point for any of the other cytopenias, reached a positive predictive value of 93% and a negative predictive value of 98% (AUC of 0.918) in a validation cohort of 261 COVID-19 patients and 144 controls. The sensitivity and specificity of the highest-probability score (score 4 or 5) was 79% and 90% respectively. As a potential weakness of this model, its performance was not reported in settings with lower COVID-19 prevalence, which might affect the reported positive predictive value. In addition, the composition of the COVID-19-negative control cohort was not clearly described, leaving some uncertainty about the specificity of this model in patient populations with diverse infectious and non-infectious illness.

Sambataro et al. describe the 'Model for Early Covid-19 Recognition' (MECOR) score, specifically for differentiating SARS-CoV-2 infection from other causes of community-acquired pneumonia (CAP).⁸⁹ The total leukocyte count, the lymphocyte, monocyte, neutrophil and platelet counts are combined in an elaborate formula, so to yield a parameter that has an AUC of 0.84 in a validation cohort of 135 COVID-19 patients and 115 patients with non-COVID-19 community-acquired pneumonia. Low counts for the named parameters suggest SARS-CoV-2 as a cause of pneumonia, rather than another pathogen. A MECOR score ≥ -0.13 displayed 84% sensitivity, 72% specificity and a positive predictive value of 78% and a negative predictive value of 79%. As a limitation of this model, no validation was performed in a non-infectious control cohort, limiting its use as a general-purpose screening tool in Emergency Departments. In addition, the etiology of pneumonia in the control cohort was not specified, potentially affecting the model's performance in healthcare settings with varying proportions of viral versus bacterial etiologies of CAP (such as at different times of influenza season).

In a large multicentric study, Linssen et al combine parameters on the Sysmex XN platform in a prognostic model that, at admission, predicts the probability of ventilation need or mortality within a two-week timeframe.³⁶ The study includes 366 critically ill and 557 COVID-19 patients with milder disease. The ten parameters used in the model are: the ratio of immature granulocytes over lymphocytes, the neutrophil-lymphocyte ratio, the proportion of reactive monocytes as part of all monocytes (RE-MONO/M%), the number antibody-secreting lymphocytes as part of the total lymphocyte count (AS-LYMP/L%), the amount of dehemoglobinized erythrocytes and reticulocytes as reflected in the Delta-He and HYPO-He parameters, hemoglobin, the nucleated red blood cell count, the platelet count, and the immature platelet fraction (IPF). The model yields a score between 0 and 28. The AUC of the model is 0.753 at admission and 0.875 at admission day 3. Sensitivity for predicting development of critical illness was 62.3% at day 0 of admission, 87.0% at day 3 and 93% at day 6. Specificity values were not reported. A limitation to generalized use of this model is its reliance on non-routine parameters such as IPF, that are not available on all XN-analyzers, and, even if available, are not routinely determined in all patients.

Appendix B: data table

	COVID-19 (n =257)		Influenza A/B (n =178)		RSV A/B (n =69)		CMV and EBV (n =61)	Negative Respiratory PCR panel (n =301)		Positive Respiratory PCR panel (non-RSV/Influenza) (n =105)		ED, not admitted (n =103)	ICU admission, various etiologies (n =100)	Internal medicine ward admission (n =51)	Geriatric ward admission (n =52)	Oncology-hematology ward admission (n =101)	Sepsis (n =38)	Surgical ward admission (n =102)
	ICU (n = 41/257; 16%)	Non-ICU (n = 216/257; 84%)	ICU (n = 16/178; 9%)	Non-ICU (n = 162/178; 91%)	ICU (n = 3/69; 4,3%)	Non-ICU (n = 66/69; 95,7%)	Non-ICU (n = 61/61; 100%)	ICU (n = 40/301; 13,3%)	Non-ICU (n = 261/301; 86,7%)	ICU (n = 7/105; 6,7%)	Non-ICU (n = 98/105; 93,3%)							
Age distribution; No. (%)																		
≤ 15 years	0 (0%)	1 (0,4%)	0 (0%)	62 (34,8%)	0 (0%)	44 (63,8%)	13 (21,3%)	0 (0%)	53 (17,6%)	0 (0%)	77 (73,3%)	11 (10,7%)	1 (1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
16-29 years	1 (0,4%)	9 (3,5%)	0 (0%)	20 (11,2%)	0 (0%)	5 (7,2%)	23 (37,7%)	4 (1,3%)	13 (4,3%)	0 (0%)	2 (1,9%)	22 (21,4%)	6 (6%)	2 (3,9%)	0 (0%)	4 (4%)	2 (5,3%)	5 (4,9%)
30-49 years	3 (1,2%)	28 (10,9%)	3 (1,7%)	24 (13,5%)	0 (0%)	1 (1,4%)	16 (26,2%)	6 (2%)	23 (7,6%)	0 (0%)	4 (3,8%)	27 (26,2%)	11 (11%)	7 (13,7%)	0 (0%)	9 (8,9%)	0 (0%)	13 (12,7%)
50-69 years	19 (7,4%)	63 (24,5%)	5 (2,8%)	20 (11,2%)	2 (2,9%)	6 (8,7%)	5 (8,2%)	15 (5%)	80 (26,6%)	3 (2,9%)	4 (3,8%)	31 (30,1%)	37 (37%)	16 (31,4%)	0 (0%)	32 (31,7%)	12 (31,6%)	27 (26,5%)
≥ 70 years	18 (7%)	115 (44,7%)	8 (4,5%)	36 (20,2%)	1 (1,4%)	10 (14,5%)	4 (6,6%)	15 (5%)	92 (30,6%)	4 (3,8%)	11 (10,5%)	12 (11,7%)	45 (45%)	26 (51%)	52 (100%)	24 (63,2%)	57 (55,9%)	
Sex																		
Female/male ratio	0,46	0,83	0,33	0,93	0,50	1,13	2,39	0,54	1,02	0,40	1,04	1,45	0,52	0,82	1,74	1,15	1,24	2,09
Median age (years)	68	71	70	29	64	2	28	66	61	74	2	43	67	70	84	72	76	74
Mortality rate	19,51%	17,59%	ND	ND	ND	Nd	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
General laboratory abnormalities																		
Median CRP (mg/L)	150	44	68	31	46	27,5	11	120	36,5	120	22	4,6	9,75	6,4	19	31	115	8,9
Median LDH (U/L)	415	260	250	210	290	210	210	330	220	210	250	180	240	200	200	230	230	200
Median Ferritin (µg/L)	1400	560	290	240	570	135	74	450	290	105,5	195	81	410	240	180	410	290	270
PT% (%)	92,5	95	85,5	86,5	61	67	96	77	93	64	85,5	99	97	99	87	86	80	93
APTT (s)	33,5	34	37,9	32,9	35,5	34,8	31,65	32,55	31,85	35,55	32,5	29,8	30,45	32,2	31,6	32,5	29,2	29
Median D-Dimer (mg/L)	0,93	0,71	5,36	0,53	ND	ND	0,44	19,10	0,63	1,34	1,60	0,28	1,67	0,65	0,59	0,71	2,12	4,05
Median PLT (×10 ³ /µL)	193	194,5	179	210,5	159	304	245	233,5	251	182	300	252	231	252	220,5	215	192	251
Cytological abnormalities																		
Median Ly abs (×10 ³ /µL)	0,62	0,965	0,53	0,86	0,72	2,505	2,68	1,26	1,30	1,07	3,13	2,01	1,46	1,94	1,12	0,83	0,55	1,41
Mean Ly abs (×10 ³ /µL)	0,79	1,11	0,70	1,18	0,57	3,10	3,36	1,35	1,91	2,01	3,57	2,11	1,82	1,91	2,02	3,36	0,74	1,64
%Lymphopenia (XN + DI-60)	90,2%	73,6%	93,8%	76,5%	100,0%	30,3%	14,8%	67,5%	55,6%	-	28,6%	29,1%	52,0%	35,3%	69,2%	77,2%	86,8%	52,9%
%DI-60 samples	68,29%	50,9%	56,25%	48,8%	66,67%	57,6%	42,6%	32,50%	39,8%	-	66,3%	12,6%	31,0%	11,8%	25,0%	44,6%	73,7%	22,5%
Median eosinophils abs (×10 ³ /µL)	0	0,01	0,01	0,01	0,00	0,02	0,08	0,05	0,10	0,10	0,04	0,12	0,07	0,14	0,04	0,04	0,00	0,10
Mean eosinophils abs (×10 ³ /µL)	0,02	0,05	0,06	0,05	0,01	0,08	0,14	0,11	0,17	0,23	0,14	0,18	0,14	0,17	0,08	0,21	0,02	0,13
Median monocytes abs (×10 ³ /µL)	0,29	0,47	0,58	0,61	0,99	0,815	0,56	0,59	0,64	0,93	0,81	0,66	0,73	0,84	0,93	0,71	0,55	0,82
Mean monocytes abs (×10 ³ /µL)	0,38	0,51	0,57	0,72	0,80	0,83	0,61	0,74	0,75	1,28	0,99	1,24	1,32	2,03	1,32	1,96	1,00	1,37

Appendix C: Materials and Methods

This concerned a single center retrospective cohort study of 257 COVID-19 patients and 1261 control patients.

All data were extracted from the Laboratory Information System (LIS) and the Information Processing Unit (IPU) of the Sysmex XN-9100 and Sysmex XN-1500 (IPU version 22.08) on two campuses of Jessa Ziekenhuis, Hasselt. Data were collated and analyzed using Microsoft Excel (v. 16.49) (Microsoft Corporation, Redmond, WA, USA), IBM SPSS (version 27) (IBM, Armonk, NY, USA), and Python 3.8 (Python Foundation, Wilmington, DE, USA). ImageJ (version 1.53) was used for feature extraction from WDF scatterplots.⁹⁰

The COVID-19 cohort consisted of 257 consecutive patients that tested positive for SARS-CoV-2 RNA using a custom RT-PCR assay. COVID-19-positive patients followed at our center with a hematological malignancy were excluded from the analysis. Laboratory test results were obtained from blood samples taken on the day of a positive RT-PCR result. Patients were stratified according to ICU admission. ICU admission was assumed when blood samples were received from an intensive care ward within three days before to three days after that patient testing positive for COVID-19.

The control cohort consisted of 1261 control patients that were consecutively included in the period from September 2019 to March 2020.

178 patients with influenza A or B infection were included. Influenza infection was diagnosed using one of two RT-PCR assays: GeneXpert Xpert Flu/RSV PCR assays (Cepheid, Sunnyvale, CA, USA) or the ARIES Flu A/B & RSV Assay (Luminex Corporation, Austin, TX, USA). ICU-admitted patients were selected using identical criteria as mentioned for COVID-19.

69 patients with RSV A or B infection were included. RSV infection was diagnosed using one of two RT-PCR assays: GeneXpert Xpert Flu/RSV PCR assays (Cepheid, Sunnyvale, CA, USA) or the ARIES Flu A/B & RSV Assay (Luminex Corporation, Austin, TX, USA). ICU-admitted patients were selected using identical criteria as mentioned for COVID-19.

61 patients with acute CMV or EBV infection were included. Acute CMV infection was diagnosed using CMV IgM titers (cutoff for positivity ≥ 0.70). Acute EBV infection was diagnosed based on VCA IgM index ≥ 1.0 . Serological tests were performed on an Architect i2000SR analyzer (Abbott Laboratories, Chicago, IL, USA). No patients with acute CMV or EBV infection were admitted to ICU.

406 patients that underwent testing with a respiratory multiplex PCR panel were included. Use of this panel was assumed to imply clinical suspicion of respiratory illness. The panel targets 18 respiratory pathogens. Of 406 included patients, 301 tested negative for all 18 targets. 105 patients tested positive for any of the 18 respiratory pathogens: adenovirus (n = 12), bocavirus (n = 10), *Bordetella pertussis* (n = 1), *Bordetella parapertussis* (n = 0), *Bordetella holmesii* (n = 0), *Chlamydomphila pneumoniae* (n = 0), Corona virus 229E (n = 0), Coronavirus HKU1 (n = 6), Coronavirus NL63 (n = 4), Coronavirus OC43 (n = 5), Enterovirus (n = 11), Human metapneumovirus (n = 4), Legionella pneumophila (n = 0), *Mycoplasma pneumoniae* (n = 6), Parainfluenza virus 1 (n = 7), Parainfluenza virus 2 (n = 0), Parainfluenza virus 3 (n = 1), Parainfluenza virus 4 (n = 3), Rhinovirus (n = 45).

Among patients that did not receive testing for influenza, RSV, CMV/EBV or testing with a respiratory PCR panel, we selected a population of 509 patients for whom a full blood count was available at the day of presentation at the emergency department.

We stratified these patients according to the medical ward where subsequent follow-up blood samples were sent from. This cohort included 103 patients who were not admitted (no follow up blood samples received), 100 ICU-admitted patients (non-COVID-19-, non-influenza-, non-RSV-infected patients who did not receive respiratory PCR panel testing), 51 patients who were admitted to various internal medicine wards (neurology, cardiology, gastroenterology), 52 patients that were admitted to a geriatric ward, 101 patients admitted to oncological or hematological wards, and 102 patients that were admitted to surgical wards. In addition, we included 38 random patients with at least one positive blood culture with a significant bacterial pathogen (that is *Enterobacterales*, *enterococci*, or *S. aureus*) at the day of admission. These are referred to as 'bacteremic' patients or patients with 'sepsis', although no clinical parameters were available to differentiate between the two conditions.

Biochemistry tests were performed on a Cobas 6000 system (Roche, Basel, Switzerland). Coagulation tests were performed on ACL Top 550 analyzers (Werfen, Barcelona, Spain).

Appendix D: HFLC for differentiating viral from bacterial illness

We noticed a trend of increased HFLC counts in COVID-19 and influenza compared to patients with bacterial sepsis. HFLC has been explored in earlier studies in the differential diagnosis of viral and bacterial illness. To investigate its use in the differential diagnosis of patients severely ill with either viral or bacterial infection, we performed an ROC analysis in 57 patients admitted to ICU with either COVID-19 (n = 41) or influenza (n = 16), compared to 38 patients with bacteremia with a significant pathogen. We find AUCs of 0.83 and 0.79 for HFLC% and the absolute HFLC count (HFLC#) for prediction of viral pneumonia (see Fig. 15). This suggests that HFLC may play a role in differentiating severe viral from severe bacterial illness. A weakness of this comparison in the present study are the limited number of included patients, and the fact that no formal comparison was made of disease severity between cohorts.

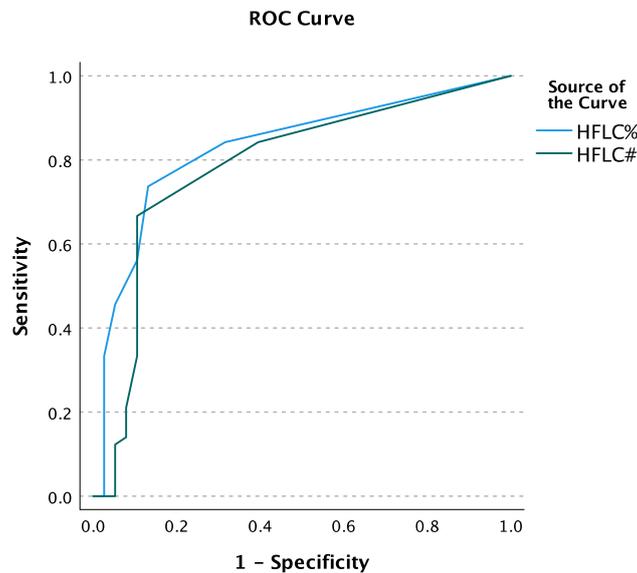


Figure 15. ROC analysis of HFLC% and HFLC absolute count (HFLC#) in 57 patients admitted to ICU with either COVID-19 (n = 41) or influenza (n = 16), compared to 38 patients with bacteremia with a significant pathogen. AUC of HFLC% is 0.83; AUC of HFLC# is 0.79.

Appendix E: Methodology for analysis of WDF scattergrams

Screenshots were obtained in tiff format from the WDF scattergram on the Graph tab of the IPU software from 204 COVID-19 patients and 978 controls. In an automatized process in the ImageJ software, the images were cropped (Fig. 16 a), binning was applied (to assign each dot to a single pixel, Fig. 15, b), color thresholding was applied to select dots belonging to the lymphocyte cluster (c), and the coordinates of dots were exported in a comma-separated file (csv) (Figure 15, d). This method is insensitive to overlaid dots. This is considered irrelevant given the low number of lymphocytes in the upper region of the WDF scattergram, and their largely even distribution.

Using the Python programming language for automatized processing of csv-files, the number of dots was calculated for each file in the high fluorescence region. This number was divided by the number of dots in the intermediate fluorescence regions. The threshold to delineate higher and lower fluorescence zones was varied between 0 and 150 pixels/dots in increments of 5 pixels. Lymphocytes in the lowest fluorescence region of the WDF plot (those below 150 pixels/dots from the origin) were excluded. For each sample and for each threshold a ratio was calculated, yielding 30 ratios per sample. ROC analysis of the ratios was performed in SPSS version 27.

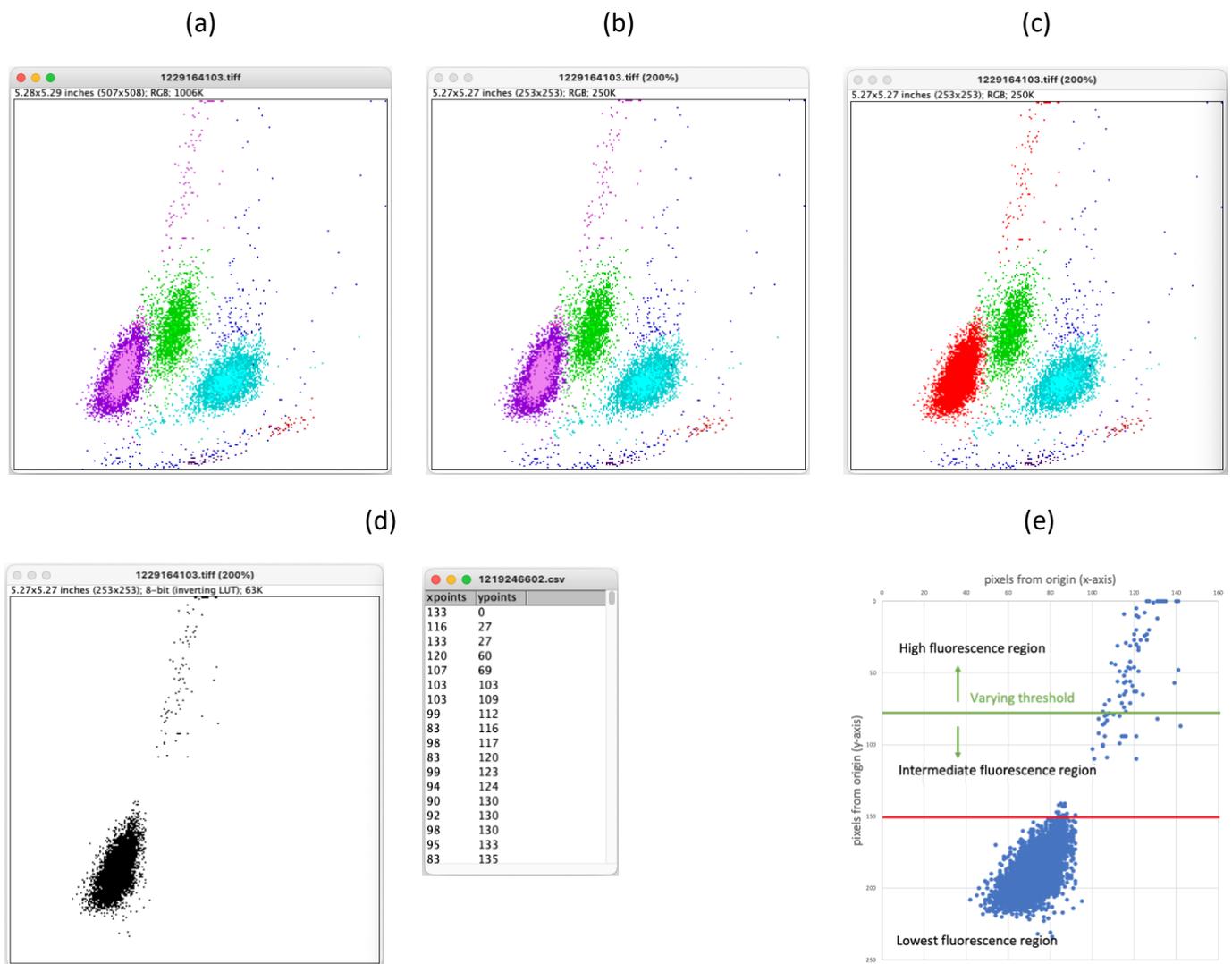


Figure 16. Steps for obtaining dot coordinates in ImageJ (panels a-d). Delineating high and lower fluorescence regions (panel e).