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Hemocytometric characteristics of COVID-19 patients with and without cytokine storm syndrome on the sysmex XN-10 hematology analyzer

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Abstract

Objectives: COVID-19 is an ongoing global pandemic. There is an urgent need for identification and understanding of clinical and laboratory parameters related to progression towards a severe and fatal form of this illness, often preceded by a so-called cytokine-storm syndrome (CSS). Therefore, we explored the hemocytometric characteristics of COVID-19 patients in relation to the deteriorating clinical condition CSS, using the Sysmex XN-10 hematology analyzer.

Methods: From March 1st till May 16th, 2020, all patients admitted to our hospital with respiratory complaints and suspected for COVID-19 were included (n=1,140 of whom n=533 COVID-19 positive). The hemocytometric parameters of immunocompetent cells in peripheral blood (neutrophils [NE], lymphocytes [LY] and monocytes [MO]) obtained upon admission to the emergency department (ED) of COVID-19 positive patients were compared with those of the COVID-19 negative ones. Moreover, patients with CSS (n=169) were compared with COVID-19 positive patients without CSS, as well as with COVID-19 negative ones.

Results: In addition to a significant reduction in leukocytes, thrombocytes and absolute neutrophils, it appeared that lymphocytes-forward scatter (LY-FSC), and reactive lymphocytes (RE-LYMPHO)/leukocytes were higher in COVID-19-positive than negative patients. At the moment of presentation, COVID-19 positive patients with CSS had different neutrophils-side fluorescence (NE-SFL), neutrophils-forward scatter (NE-FSC), LY-FSC, RE-LYMPHO/lymphocytes, antibody-synthesizing (AS)-LYMPHOs, high fluorescence lymphocytes (HFLC), MO-SSC, MO-SFL, and Reactive (RE)-MONOs. Finally, absolute eosinophils, basophils, lymphocytes, monocytes and MO-FSC were lower in patients with CSS.

Conclusions: Hemocytometric parameters indicative of changes in immunocompetent peripheral blood cells and measured at admission to the ED were associated with COVID-19 with and without CSS.

Keywords: cell population data; COVID-19; cytokine storm syndrome; hemocytometry; SARS-CoV-2.

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Introduction

COVID-19 is a still ongoing global pandemic. Although the majority of SARS-CoV-2-infected individuals may have no

or mild symptoms, some patient groups, in particular, older patients and those with chronic underlying conditions may have a worse outcome [1]. These patients often have a clinical constellation including respiratory failure, cytokine storm syndrome (CSS) and eventually death [2].

CSS is an uncontrolled increase of the inflammatory response, usually occurring after 7–10 days of relatively mild, stable disease [3, 4]. It may be characterized by excessive stimulation of cytokine release, and activation of macrophages, complement-, coagulation- and fibrinolytic pathways [5]. Whether this phenomenon represents a natural reaction to an increase in viral load or a misbalance between pro-inflammatory and anti-inflammatory responses is still not clear. Regardless of the underlying mechanism, CSS is clearly associated with increased mortality [6]. Early identification and treatment of CSS is of utmost importance because it can lead to a reduction in mortality [7].

The question remains on who will develop CSS, when and to what extent. A better characterization of the peripheral blood immune cells involved may increase our understanding of the immune response underlying the presence of CSS and thereby possibly also improve the selection of patients who may benefit from immunomodulatory therapy [7].

A complete blood count (CBC) is the most commonly performed hematological laboratory test worldwide and most routine laboratories are equipped with, often high-throughput, hematology analyzers (so-called hemocytometers). In this respect, leukocytosis, neutrophilia, and lymphopenia have been reported in COVID-19 positive patients [8, 9]. However, some of these routine hematology analyzers further differentiate the subsets of leukocytes involved, based on scatter- and fluorescence properties (size and cell volume [FSC], internal structure [SSC] and DNA/RNA content [SFL]), reported as research parameters in the cell population data (CPD). Although there are some short reports published about the individual CPD parameters in COVID-19 [10–13], these studies in general examined small patient populations, lacked a control group and only studied a selected set of leukocyte parameters that can be measured nowadays.

Therefore, we examined associations of both routine and research hemocytometric parameters with COVID-19 and CSS in particular in a retrospective cohort of patients who presented to the emergency department (ED) with respiratory symptoms and were hospitalized with either COVID-19 (without and with CSS) or turned out to be COVID-19 negative.

Materials and methods

Patients

All patients admitted to Zuyderland Medical Center (ZMC) suspected for COVID-19 were retrospectively registered in the ZuyderLand COVId-19 regiStry (ELVIS). Demographic data and data on clinical signs and symptoms at presentation were collected from the electronic medical record (Figure 1).

In order to avoid exhaustion of the hospital care system, the ZMC had agreed upfront with local general practitioners and nursing home physicians to not refer (suspected) patients with COVID-19 to the hospital for diagnosis and supportive care if severe pre-existing clinical frailty was present, life expectancy was obviously limited or severe comorbidity in combination with COVID-19 was expected to have a very unfavorable outcome.

For the present retrospective cross-sectional study, patients who were admitted to the ED and hospitalized with respiratory complaints suspected for COVID-19 from March 1st, 2020 until May 16th, 2020 were included. Patients without respiratory complaints were excluded to avoid inclusion of patients who had low clinical suspicion of COVID-19 but were routinely tested. In addition, patients were required to have complete data on hemocytometric parameters, and no history of hematologic malignancy or suspicion of hematologic malignancy based on hemocytometry as this may result in misclassification of hemocytometry parameters (see Figure 1).

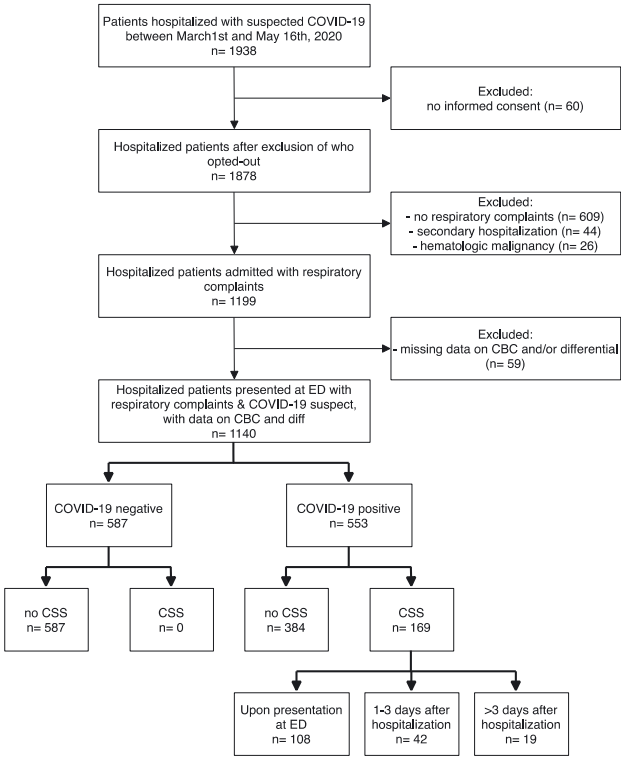

Figure 1: Enrollment and inclusion of patients in this study. CBC, complete blood count; CSS, cytokine storm syndrome.

COVID-19 and CSS

The diagnosis of COVID-19 was based on the presence of clinical signs and symptoms suggestive of COVID-19 combined with a positive real-time polymerase chain-reaction (RT-PCR) for SARS-CoV-2 on naso-pharyngeal and orolaryngeal swab samples (proven COVID-19), or a chest computed tomography (CT) result of COVID-19 CT classification (CORADS) four or five in case of negative RT-PCR (highly suspected COVID-19). When the RT-PCR assay was negative in a COVID-19 suspected patient, the test was repeated (with a maximum of three tests). Patients not fulfilling these criteria were classified as COVID-19 negative.

CSS was defined if the following criteria were fulfilled [7]: patients had to show clinical deterioration, oxygen saturation at rest ≤ 94% (ambient air) or tachypnea (>30/min). In addition, patients had to meet at least two out of the following three biomarker criteria: high CRP (>100 mg/L), high serum-ferritin (>900 µg/L at one occasion, or two-fold increase of the level at admission within 48 h) and high D-Dimer level (>1,500 µg/L). When the presence of a bacterial infection was strongly suspected or confirmed, patients were not considered to have a COVID-19 associated CSS.

Two physicians (CD and CMC) independently verified the presence of CSS in the patient's electronic record and cases of disagreement were decided by consensus with a third physician (SR), without knowledge of the clinical course and outcome.

Clinical chemistry for establishing the diagnosis CSS

The clinical chemistry parameters CRP and ferritin were obtained on routine Cobas analyzers (Roche Diagnostics, Basel, Switzerland), and D-Dimers on a CS2500 coagulation analyzer (Sysmex Corp., Kobe, Japan).

Hemocytometry at time of presentation at ED

A CBC was assessed on the day of presentation at the ED by hemocytometric analyses of an EDTA-anticoagulated peripheral blood sample with the XN-10 analyzer (Sysmex, Kobe, Japan). All samples were analyzed within 2 h after venipuncture. Inter-instrument agreement and internal quality control is performed by daily measurement of a CBC of a random collected patient peripheral blood sample on all hemocytometers (in total five machines, distributed over two locations), and compared with the results of these measurements of preceding daily measurements (in a range of 365 days). Next to this, two commercially available control samples (XN-Check, level 1 and 2; Sysmex) were measured daily and send to a digital webservice of Sysmex (xQC, Sysmex). The results of both internal control methods must fulfill predefined performance indicators. The analyzer utilizes fluorescence flow cytometry for the leukocyte differential count, allowing for enhanced subset differentiation based on size (forward scatter; FSC), internal structure/granularity (side scatter; SSC) and DNA/RNA content (fluorescence expression; SFL).

The white blood cell (WDF) channel of this analyzer specifically discriminates leukocytes using flow cytometry. Analysis includes the labeling of blood cells with a fluorescent dye after perforation of the cell membrane with specific lysis buffer. Mean values and standard deviations are recorded for each leukocyte subpopulation (neutrophils, monocytes, lymphocytes, etc.). Parameters related to dispersion of values around the mean are expressed according to distribution width (e.g. for SSC, SFL and FSC); this represents the range of the distribution of the major population, excluding outliers with amplitude of less than 20% of the peak of the distribution curve. These parameters, the so-called cell population data (CPD) values, are stable for 6 h after blood drawn (own validation study, data not shown) and reported in arbitrary units of light scattering (channels [ch]). In Figure 2 an explanation is shown of most of the parameters used in this study, as well as striking examples of WDF results in the different patient groups. A summary and explanation of all hemocytometric parameters used in this study can be found in Table 1.

In addition, we have investigated several hemocytometric ratios (neutrophil/lymphocyte- [N/L], platelets/lymphocyte- as well as lymphocyte/monocyte ratio) as described in the recent literature [11, 12, 14].

Ethical considerations

The medical ethics committee of ZMC (Zuyderland METC Zuyd, registration nr METCZ20200057) approved this study. All patients in the ELVIS-registry received written information about the registry as well as an opt-out form in case they did not want to participate.

Statistics

Characteristics of the study population stratified according to patient group (i.e., COVID-19 negatives, COVID-19 without CSS, COVID-19 with CSS) were presented as means with standard deviation (SD), medians with 25th and 75th percentiles, and counts with percentages, as appropriate.

Associations of the individual hemocytometric parameters as independent variables and patient group as dependent variable were evaluated with multivariable multinomial logistic regression analyses. For primary analyses, hemocytometry parameters were split at the median, or the first higher level if ties prohibited this.

Odds ratio (ORs) represent the odds of having 'COVID-19 without CSS' or 'COVID-19 with CSS' compared to being in the control group. In addition, the OR for having 'COVID-19 with CSS' as compared with 'COVID-19 without CSS' was presented. For hemocytometry parameters that showed higher levels in COVID-19, above-median levels were compared with below median-levels. In contrast, for parameters that showed lower levels in COVID-19, below-median levels were compared with above-median levels. Regression models were adjusted for the following potential confounders: age, sex, obesity, diabetes (with and without complications), hypertension, cardiovascular disease (cardiovascular disease, heart failure, cerebrovascular disease, and/or peripheral vascular disease), chronic kidney disease, chronic obstructive pulmonary disease, asthma, malignant neoplasm, autoimmune disorder, immunodeficiency or immunocompromised state, and liver disease (mild and moderate to severe liver disease). These variables were selected based on their putative effects on both COVID-19 outcome and distribution of hemocytometric parameters.

We performed additional analyses to assess the robustness of the results. First, multinomial logistic regression analyses were repeated

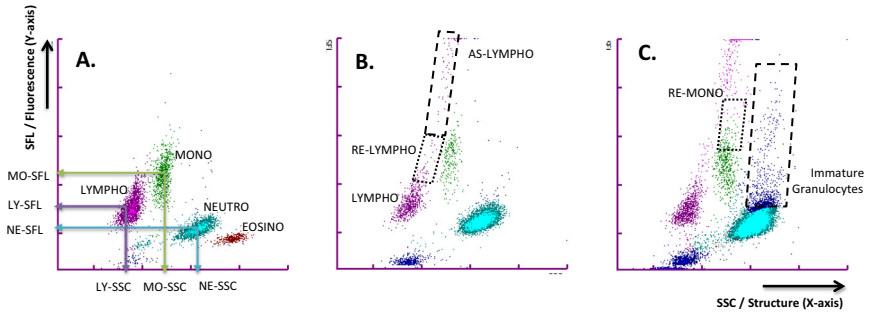

Figure 2: Routine and research parameters from hemocytometric plot. Examples of typical hemocytometric graphs of the different patient groups, including definition of several distinct hemocytometric parameters.
(A) COVID-19 negative patient. The different cell populations as well as their side scatter- (X-axis) and fluorescence-intensities (Y-axis) are explained. (B) COVID-19 positive patient without CSS. This panel shows the area where the reactive lymphocytes (RE-LYMPHO; dotted box) and antibody-synthesizing lymphocytes (AS-LYMPHO; dashed box) are measured. (C) COVID-19 positive patient with CSS. This panel shows the areas that are used for the measurement of the reactive monocytes (RE-MONO; dotted box) and immature granulocytes (dashed box).

with the hemocytometric parameters categorized into quartiles instead of dichotomized at the median. For this purpose, ties were put in random order to prevent biased quartiles in hemocytometric parameters that contained large counts of zeros (e.g., AS-Lympho). The first quartile was used as the reference. Second, analyses were repeated with 'COVID-19 with CSS' stratified by timing of CSS diagnosis (i.e., diagnosis at admission, 1–3 days following admission, and >3 days following admission). Third, analyses were repeated after exclusion of patients whose diagnosis of COVID-19 was based on imaging only (negative PCR).

Analyses were performed with R (version 3.6.3) [15] and with RStudio (version 1.2.5033) [16] combined with the packages tidyverse, readxl, lubridate, ggpubr, knitr, gt, gtsummary, labeled, nnet, and broom. Boxplots were constructed with Prism 8 (version 8.2.1; GraphPad Software, San Diego, CA, USA).

Results

Patient demographics and clinical characteristics

In the present study, 1,140 patients hospitalized with respiratory complaints and possible COVID-19 were included (Figure 1). Of these 1,140 patients, 49% were diagnosed with COVID-19, of whom 169 patients (31%) developed CSS.

Demographic and clinical characteristics are shown in Table 2 and Supplementary Table S1. COVID-19 positive patients who developed CSS were more often male (81%) as compared to those without CSS (54%) or without COVID-19 (55%).

In general, comorbidities were distributed equally between COVID-19 positive/CSS negative patients and COVID-19 negative ones. However, the COVID-19 positive/CSS positive group had in general less comorbidities but were more often obese.

Hemocytometric laboratory results by presentation at ED

COVID-19 positive patients showed distinct reduced absolute counts for total leukocytes as well as for neutrophils, total lymphocytes and monocytes as compared to the COVID-19 negative patients. The neutrophils in COVID-19 positive patients were significantly smaller in size compared to COVID-19 negative patients (NE-FSC; odds ratio [OR] 0.72 [95% CI 0.55–0.94]) and showed an increased fluorescence signal (NE-SFL; OR 1.66 [1.27–2.17])). The dispersion of this signal was smaller in patients with COVID-19 (NE-SFL width; OR 0.56 [0.42–0.73]) (Table 3; Supplementary Table 2).

In addition to lower absolute counts, lymphocytes of patients positive for COVID-19 were slightly but significantly larger in size (LY-FSC; OR 2.21 [1.68–2.90]). The fractions of reactive lymphocytes within the lymphocyte population (RE-LYMPHO/lympho; OR 2.64 [1.99–3.51]), the concentration of antibody-synthesizing (AS-LYMPHO; OR 11.55 [7.87–16.96]) as well as high fluorescence lymphocytes (HFLC; (absolute: OR 9.18 [6.60–12.78], and relative: OR 12.46 [8.88–17.47]) were significantly increased in patients with COVID-19.

These patients had decreased absolute counts of monocytes (OR 0.33 [0.25–0.43]). The reactive monocytes (absolute (OR 2.02 [1.54–2.65]) and relative (OR 3.43 [2.59–4.56]), as well as expressed as fraction within the monocyte population (OR 3.49 [2.63–4.62]) were significantly increased in COVID-19 positive patients. Furthermore, cell properties changed; there was a significant increase in granularity/internal structure (MO-SSC; OR 3.72 [2.81–4.94]) as well as in permeability of the cell membrane, resulting in higher MO-SFL levels (OR 2.38 [1.81–3.13]).

Table 1: Routine and research hemocytometric analyzer parameters used in this study.

Parameters	Alternative names	Parameter description	Unit
CBC			
WBC	LEUKO	White blood cell count (#)	$\times 10^9$/L
PLT	THROMBO	Absolute number of thrombocytes	$\times 10^9$/L
Granulocytes			
NEUT	NE	Neutrophil count (#) or percentage (%)	$\times 10^9$/L or %
NE-SSC	NE-X; NEUT-GI	Neutrophil granularity index (reactivity of neutrophils (cytoplasmic granulation)	Ch
NE-SFL	NE-Y; NEUT-RI	Neutrophil reactivity index (reactivity of neutrophils (metabolic activity)	Ch
NE-FSC	NE-Z	Size or volume of neutrophils	Ch
NE-SSC (width)	NE-WX	Dispersion of the NE-SSC signal of the neutrophils	Ch
NE-SFL (width)	NE-WY	Dispersion of the NE-SFL signal of the neutrophils	Ch
NE-FSC (width)	NE-WZ	Dispersion of the NE-FSC signal of the neutrophils	Ch
BASO		Basophilic count (#) or percentage (%)	$\times 10^9$/L or %
EO		Eosinophilic count (#) or percentage (%)	$\times 10^9$/L or %
IG		Immature granulocyte count (#) or percentage (%)	$\times 10^9$/L or %
Monocytes			
MONO	MO	Monocyte count (#) or percentage (%)	$\times 10^9$/L or %
MO-SSC	MO-X	Monocyte nucleus irregularity, cytoplasmic granulation and/or vacuolization	Ch
MO-SFL	MO-Y	Monocyte metabolic activity and/or permeability of cell membrane	Ch
MO-FSC	MO-Z	Size or volume of monocytes	Ch
RE-MONO		Numbers of monocytes with a side fluorescence signal >150 channels, representing activated monocytes	$\times 10^9$/L or %
RE-MONO/L		Activated monocytes as fraction of the leukocytes	%
RE-MONO/M		Activated monocytes as fraction of the monocytes	%
Lymphocytes			
LYMPHO	LY	Lymphocyte count (#) or percentage (%)	$\times 10^9$/L or %
LY-SSC	LY-X	Lymphocyte nucleus irregularity, cytoplasmic granulation and/or vacuolization	Ch
LY-SFL	LY-Y	Lymphocyte metabolic activity and/or permeability of the cell membrane	Ch
LY-FSC	LY-Z	Size or volume of lymphocytes	Ch
AS-LYMPHO		Antibody synthesizing lymphocyte count (#) or percentage (%)	$\times 10^9$/L or %
AS-LYMPHO/L		Antibody synthesizing lymphocytes as fraction of lymphocytes	%
AS-LYMPHO/Le		Antibody synthesizing lymphocytes as fraction of the leukocytes	%
RE-LYMPHO		Reactive lymphocyte count (#) or percentage (%)	$\times 10^9$/L or %
RE-LYMPHO/L		Reactive lymphocytes as fraction of lymphocytes	%
RE-LYMPHO/Le		Reactive lymphocytes as fraction of the leukocytes	%
HFLC		High fluorescence lymphocyte count (#) or percentage (%)	$\times 10^9$/L or %
Red blood cells			
HB	HGB	Hemoglobin concentration	g/L
ERY	RBC	Absolute number of erythrocytes	$\times 10^{12}$/L
NRBC		Nucleated red blood cells	$\times 10^9$/L

PLT, platelets; Ch, channel unity.

When analyzing COVID-19 positive patients that developed CSS, the absolute counts of lymphocytes (OR 0.41 [0.26–0.62]), eosinophils (OR 0.34 [0.20–0.57]), basophils (OR 0.39 [0.20–0.74]) and monocytes (OR 0.52 [0.33–0.79]) were markedly lower when compared to COVID-19 positive patients without CSS. Furthermore, RE-LYMPHO/lympho (OR 2.44 [1.55–3.84]), AS-LYMPHOs (absolute and relative: OR 3.91 [2.50–6.12]), HFLCs (absolute: OR 2.84 [1.77–4.56], and relative: OR 3.41 [1.99–5.87]), RE-MONOs (absolute: OR 2.57 [1.67–3.93], and relative: OR 3.03 [1.89–4.86], as well as the fraction of RE-MONOs within the monocyte population: OR 3.20 [1.99–5.14]) increased significantly when compared to COVID-19 positive/CSS negative patients.

Besides these quantitative differences, there were also significant qualitative differences. The neutrophils showed a smaller size (NE-FSC; OR 0.45 [0.30–0.67]), as compared to COVID-19 positive patients without CSS. Finally, the monocytes showed an increased granularity (MO-SSC; OR 2.83 [1.80–4.46]) and higher permeability of the cell membrane (MO-SFL; OR 1.91 [1.27–2.88]).

Concerning the different hemocytometric ratio's, the Thrombo/lymphocyte ratio was significantly higher in COVID-19 positive vs. -negative patients (OR 1.65 [1.26–2.16]).

Table 2: Baseline demographic, clinical and biochemical characteristics.

Characteristics	Overall, n=1,140	Control, n=587	COVID-19 without CSS, n=384	COVID-19 with CSS, n=169
Patient characteristics				
Age, years	71 (60–80)	71 (58–80)	73 (62–81)	68 (59–75)
Gender, male	665 (58.3%)	321 (54.7%)	208 (54.2%)	136 (80.5%)
Obesity	271 (23.8%)	118 (20.1%)	102 (26.6%)	51 (30.2%)
Diabetes	261 (22.9%)	129 (22.0%)	101 (26.3%)	31 (18.3%)
Hypertension	418 (36.7%)	209 (35.6%)	162 (42.2%)	47 (27.8%)
Cardiovascular disease	460 (40.4%)	256 (43.6%)	159 (41.4%)	45 (26.6%)
Chronic kidney disease	115 (10.1%)	59 (10.1%)	47 (12.2%)	9 (5.3%)
Chronic obstructive pulmonary disease	261 (22.9%)	167 (28.4%)	76 (19.8%)	18 (10.7%)
Asthma	119 (10.4%)	56 (9.5%)	52 (13.5%)	11 (6.5%)
Malignant neoplasm	67 (5.9%)	48 (8.2%)	15 (3.9%)	4 (2.4%)
Autoimmune disorder	96 (8.4%)	42 (7.2%)	34 (8.9%)	20 (11.8%)
Immunodeficiency or immunocompromised	13 (1.1%)	10 (1.7%)	3 (0.8%)	0 (0.0%)
Liver disease	32 (2.8%)	25 (4.3%)	5 (1.3%)	2 (1.2%)
Clinical signs at emergency department				
Temperature, °C[a]	37.6 (1.2}	37.3 (1.1}	37.9 (1.1}	38.3 (1.1}
Respiratory rate, breaths/min[a]	21 (18–26)	20 (17–24)	22 (18–26)	25 (20–30)
Oxygen saturation (%) at room air[a]	95 (92–97)	96 (94–98)	94 (91–96)	90 (80–94)
Oxygen saturation (%) with oxygen supply[a]	95 (93–97)	95 (93–97)	95 (94–97)	94 (92–96)
Oxygen supply, L/min[a]	2 (0–3)	0 (0–2)	2 (0–3)	4 (2–12)
Chemistry and coagulation at emergency department				
CRP, mg/L[a]	53 (12–122)	22 (4–95)	63 (31–109)	120 (82–185)
Ferritin, µg/L[a]	390 (158–851)	209 (101–453)	484 (218–846)	1,203 (839–1,994)
D dimers, mg/L[a]	1,110 (555–2,335)	1,195 (568–2,514)	930 (526–1,751)	1,253 (637–2,633)
Diagnosis				
Diagnosis at discharge				
Proven COVID-19	452 (39.6%)	0 (0.0%)	292 (76.0%)	160 (94.7%)
Highly suspected COVID-19	101 (8.9%)	0 (0.0%)	92 (24.0%)	9 (5.3%)
Other	587 (51.5%)	587 (100.0%)	0 (0.0%)	0 (0.0%)
Timing of CSS diagnosis (days from admission)	NA	NA	NA	0 (0–2)
Timing of CSS diagnosis				
Day 0	NA	NA	NA	108 (63.9%)
Day 1–3	NA	NA	NA	42 (24.9%)
>3 days	NA	NA	NA	19 (11.2%)

Data are presented as mean (SD), median [25th–75th percentile], count (percentage). ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; CRP, C-reactive protein; CSS, cytokine release syndrome; NA, not applicable. [a]Missing in (overall/control/COVID-19 without CSS/COVID-19 with CSS): n=18/13/4/1 for temperature; n=83/55/21/7 for respiratory rate; n=455/197/151/107 for oxygen saturation at room air; n=494/323/151/20 for oxygen saturation with oxygen supply; n=76/44/29/3 for oxygen supply; n=106/60/38/8 for ferritin; n=1/1/0/0 for CRP; n=411/180/166/65 for D-dimers.

The neutro/lympho ratio was significantly higher in the group of COVID-19 positive patients that developed CSS vs. the CSS negative patients (OR 1.81 [1.22–2.68]).

Additional analyses

First, when the multinomial logistic regression analyses were repeated with the hemocytometry parameters categorized into quartiles instead of dichotomized, results were largely similar. In addition, these analyses suggested a dose-response relationship with most associations becoming stronger at higher levels of hemocytometry parameters (data not shown). Second, when CSS was stratified according to timing of diagnosis (Day 0, Day 1–3, >3 days), most associations were present irrespective of timing of diagnosis (Supplementary Table S3; statistical analyses not shown). Third, after exclusion of patients in whom COVID-19 diagnosis was based on imaging only (negative PCR) associations were similar or became somewhat stronger, in particular for COVID-19 without CSS (data not shown).

Table 3: Hemocytometric laboratory results of the different patient groups at presentation at the ED.

Characteristics	Overall, n=1,140	Control, n=587	COVID-19 without CSS, n=384	COVID-19 with CSS, n=169
CBC				
Hemoglobin, g/L	135.4 (120.9–146.6)	133.7 (119.2–146.6)	133.7 (120.9–146.6)	140.2 (133.7–151.5)
Erythrocytes, $\times10^{12}$/L	4.5 (4.0–4.9)	4.4 (4.0–4.9)	4.5 (4.0–4.9)	4.7 (4.3–5.0)
Thrombocytes, $\times10^{9}$/L	233 (178–298)	246 (188–318)	220 (171–287)	214 (166–267)
Leukocytes, $\times10^{9}$/L	8.7 (6.4–12.2)	10.2 (7.9–14.2)	7.3 (5.4–10.1)	7.5 (5.4–9.4)
Granulocytes				
Neutrophils, $\times10^{9}$/L	6.7 (4.6–9.8)	7.7 (5.4–11.2)	5.5 (4.0–8.0)	6.2 (4.0–8.2)
NE-SSC, ch	153 (5)	154 (5)	153 (5)	152 (5)
NE-SFL, ch	46 (4)	46 (5)	46 (4)	47 (3)
NE-FSC, ch	86 (5)	86 (5)	85 (4)	84 (4)
NE-SSC, width, ch	319 (307–330)	317 (306–327)	320 (307–334)	323 (312–333)
NE-SFL, width, ch	646 (618–691)	655 (624–700)	641 (613–683)	634 (608–675)
NE-FSC, width, ch	656 (625–690)	657 (628–688)	650 (618–690)	666 (630–698)
Eosinophils, $\times10^{9}$/L	0.02 (0.00–0.11)	0.08 (0.02–0.17)	0.01 (0.00–0.04)	0.00 (0.00–0.01)
Basophils, $\times10^{9}$/L	0.03 (0.01–0.05)	0.04 (0.02–0.06)	0.02 (0.01–0.03)	0.01 (0.01–0.02)
IG#, $\times10^{9}$/L	0.04 (0.03–0.09)	0.05 (0.03–0.10)	0.04 (0.02–0.07)	0.04 (0.03–0.07)
IG%, %	0.50 (0.40–0.80)	0.50 (0.30–0.80)	0.50 (0.40–0.80)	0.60 (0.40–0.90)
Lymphocytes				
Lymphocytes, 10^{9}/L	1.0 (0.7–1.6)	1.2 (0.8–1.8)	0.9 (0.7–1.4)	0.8 (0.6–1.0)
LY-SSC, ch	81 (3)	81 (3)	81 (3)	81 (3)
LY-SFL, ch	70 (5)	70 (5)	70 (4)	69 (5)
LY-FSC, ch	57 (2)	57 (2)	58 (2)	58 (2)
LY-SSC, width, ch	481 (441–533)	472 (436–517)	489 (443–537)	514 (456–579)
LY-SFL, width, ch	848 (769–932)	878 (804–964)	818 (748–895)	797 (713–897)
LY-FSC, width, ch	522 (483–559)	515 (480–547)	527 (483–565)	550 (503–588)
RE-LYMPHO#, $\times10^{6}$/L[a]	70 (40–110)	60 (30–110)	70 (40–110)	70 (50–110)
RE-LYMPHO% (% lymphocytes)[a]	6.50 (3.85–10.40)	5.10 (3.00–8.90)	7.45 (4.90–11.00)	9.35 (6.60–13.28)
RE-LYMPHO% (% leukocytes)[a]	0.80 (0.40–1.35)	0.60 (0.30–1.20)	0.90 (0.57–1.50)	1.00 (0.60–1.60)
AS-LYMPHO#, $\times10^{6}$/L	0 (0–20)	0 (0–0)	0 (0–40)	40 (20–60)
AS-LYMPHO% (% lymphocytes)	0.00 (0.00–3.03)	0.00 (0.00–0.00)	0.00 (0.00–4.44)	4.69 (2.00–9.18)
AS-LYMPHO% (% leukocytes)	0.00 (0.00–0.33)	0.00 (0.00–0.00)	0.00 (0.00–0.58)	0.54 (0.18–0.88)
HFLC#, $\times10^{6}$/L	10 (0–30)	0 (0–10)	20 (10–40)	40 (20–60)
HFLC% (% lymphocytes)	0.91 (0.00–3.40)	0.00 (0.00–0.83)	2.37 (0.68–4.66)	4.69 (2.50–9.18)
HFLC% (% leukocytes)	0.10 (0.00–0.40)	0.00 (0.00–0.10)	0.30 (0.10–0.60)	0.50 (0.30–0.90)
Monocytes				
Monocytes, $\times10^{9}$/L	0.6 (0.4–0.9)	0.8 (0.6–1.0)	0.6 (0.4–0.8)	0.4 (0.3–0.6)
MO-SSC, ch	122 (3)	120 (3)	122 (3)	123 (2)
MO-SFL, ch	112 (11)	110 (10)	113 (11)	117 (10)
MO-FSC, ch	65 (3)	66 (3)	65 (3)	65 (3)
MO-SSC, width, ch	256 (238–272)	259 (244–276)	252 (233–268)	247 (232–268)
MO-SFL, width, ch	726 (658–797)	723 (659–785)	738 (662–810)	700 (623–804)
MO-FSC, width, ch	575 (532–624)	566 (525–609)	576 (535–628)	604 (556–656)
RE-MONO#, $\times10^{6}$/L	4 (2–9)	3 (1–7)	5 (2–10)	8 (4–13)
RE-MONO% (% monocytes)	0.65 (0.27–1.53)	0.42 (0.17–0.83)	0.85 (0.41–1.75)	1.82 (0.91–3.45)
RE-MONO% (% leucocytes)	0.05 (0.02–0.11)	0.03 (0.01–0.06)	0.07 (0.03–0.13)	0.10 (0.06–0.18)
Ratio's				
Neutro/lympho	6.4 (3.6–11.2)	6.2 (3.4–12.1)	6.0 (3.5–10.0)	7.4 (4.7–11.7)
Thrombo/lympho	219.8 (145.2–328.7)	193.7 (126.3–300.8)	234.4 (158.5–343.4)	264.3 (201.2–381.5)
Lympho/mono	1.7 (1.1–2.6)	1.7 (1.0–2.6)	1.7 (1.1–2.7)	1.8 (1.3–2.9)

Data are presented as mean (SD), median [25th–75th percentile], count (percentage). CSS, cytokine storm syndrome; Hb, hemoglobin; NE, neutrophils; SSC, side scatter; SFL, side fluorescence; FSC, forward scatter; IG, immature granulocytes; #, absolute counts; LY, lymphocytes; RE, reactive; AS, antibody synthesizing; HFLC, high fluorescence lymphocytes; MO, monocytes. [a]Missing in (overall/control/COVID-19 without CSS/COVID-19 with CSS): n=49/14/16/19 for RE-LYMPHO#; n=49/14/16/19 for RE-LYMPHO% (% lymphocytes), n=49/14/16/19 for RE-LYMPHO% (%leukocytes), n=7/5/1/1 for RE-MONO#; n=7/5/1/1 for RE-MONO% (%monocytes); n=7/5/1/1 for RE-MONO% (%leukocytes).

Discussion

In this study on hemocytometric characteristics in COVID-19, patients with COVID-19 showed both quantitative and qualitative differences in leukocyte populations as compared with patients who were hospitalized with respiratory complaints but had no diagnosis of COVID-19. These differences were even more striking in case the COVID-19 infection was complicated by CSS. Our results are consistent with a dysregulated immune response in, particularly, patients with severe COVID-19.

An efficient immune system is the basis of control and eradication of infections, and uncontrollable reactions are likely leading to immunopathogenesis. The immunopathology of severe COVID-19 may, therefore, be the result of an excessive dysregulated immune response, while humoral immunity is thought to be essential in controlling the persistent phase of infection [3].

Granulocytes

Neutrophils of patients with COVID-19 showed a (somewhat) increased uptake of fluorescent dye (expressed as NE-SFL), indicating higher cell membrane permeability. Also, a smaller width of the NE-SFL signal (NE-SFL [width]) in the WDF scattergram indicated less heterogeneity in the neutrophil population in COVID-19 patients. These results correspond to activation or immaturity of these cells (i.e., activated neutrophils or band cells) [17, 18]. In patients with CSS, an increase in relative counts of immature granulocytes (IG, representing metamyelocytes, myelocytes, and promyelocytes) supported the latter. Further, neutrophils in patients with COVID-19 were smaller (size expressed as a decrease in forward scatter light signal [NE-FSC]), particularly in those with CSS.

Eosinophils are potent proinflammatory cells that are capable of developing different antiviral mechanisms and may even participate in the initiation of the adaptive T-lymphocyte response [4]. Absolute counts of eosinophils and basophils in healthy individuals are normally very low. In this study, eosinophils and basophils were lower, and even absent, in patients with COVID-19 infection. Again, this was more pronounced in patients who had or developed CSS. In agreement with the latter, a recent study showed that the absolute eosinophil count in peripheral blood was reduced in almost all patients who died from COVID-19 [19]. The mechanism underlying eosino- and basopenia is unclear and likely multifactorial. It could be either a sign of host exhaustion due to clearance of invaded COVID-19 or the primary risk factor for severe and invasive infection with this virus [20]. Interestingly, increasing eosinophil counts might predict clinical improvement in COVID-19 [21].

Monocytes

Monocytes, forming the population of tissue macrophages, are a group of innate immune cells that function as antigen-presenting cells to lymphocytes, and respond to microbial threats by producing inflammatory molecules that eliminate pathogens and promote tissue repair [22]. It may be speculated that the lower absolute counts of total monocytes observed in patients with COVID-19 may be explained by exhaustion due to extravasation and migration of these monocytes to the affected tissues [23]. Increased counts of infiltrating macrophages into lung tissue have indeed been identified in autopsy and animal models, and may be responsible for fueling inflammation [24, 25]. Notwithstanding quantitative differences, monocytes of COVID-19 positive patients had higher granularity (MO-SSC), partly due to an increased presence of vacuoli and granulae in their cytoplasm (personal microscopic observation), and higher permeability of their cell membrane (MO-SFL). It can be hypothesized that this reflects the activated state of these monocytes [26]. Indeed, the concentration of reactive monocytes was higher in patients with COVID-19, particularly in those with CSS. This agrees with previously reported associations of activation of proinflammatory monocytes with disease severity in COVID-19, especially in the elderly upon early diagnosis [26–28].

Lymphocytes

It is known from MERS-CoV and SARS-CoV infections that the host will react with a T-cell mediated immune response [3]. T-cells, and particularly CD8+ T-cells play an important role in the combat against viral pathogens and risk of overwhelming inflammation [29]. Post-mortem examination of lung tissue of patients with COVID-19 showed perivascular and patchy lymphocyte-plasmacytoid infiltrations [30]. In addition, exhaustion of especially CD8+ T-cells was observed in severely affected patients, leading to a reduction in their cellular immune response to

SARS-CoV-2 [31, 32]. As CD8+ T-cells also produce IL-5, which contributes to eosinophil proliferation and activation in peripheral blood, their exhaustion may reduce eosinophils as well [33–35].

The associations of lower absolute counts of lymphocytes with COVID-19 as well as the presence of CSS in affected patients agree with previously reported associations of lymphopenia with COVID-19 and disease severity [36–39]. As T-lymphocytes normally form the major part of the lymphocytes (~70%) [40], this lymphopenia may be explained by T-cell depletion and consumption caused by COVID-19 [41, 42].

Despite a decrease in lymphocyte count, an increase in certain subpopulations of lymphocytes was observed in COVID-19. Indeed, reactive lymphocytes (RE-LYMPHO), antibody-synthesizing lymphocytes (AS-LYMPHO) and high fluorescence lymphocyte cells (HFLC) were higher as compared with controls. Additionally, these subsets constituted an even higher proportion of total lymphocytes in patients with CSS. These findings agree with previously reported increases in RE-LYMPHO and AS-LYMPHO during the course of disease, particularly in the second week of illness [13], which may be explained by seroconversion in the second week [43].

Indeed, the so-called high fluorescence cells represent lymphoplasmacytoid B cells and plasma cells [13, 44–46]. These lymphocytes are responsible for the adaptive humoral immune response and in earlier studies it was shown that a week after symptom onset, antibody responses to the spike proteins (S protein) of the coronavirus can be found [47, 48]. Linssen et al. [44] showed in an earlier study, using the XE2100 hemocytometer, that the so-called AS-LYMPHOs corresponds to the antibody-synthesizing lymphocytes. In the last decade, the measurement techniques of the new generation XN-10 analyzers have been improved (using a combination of new hardware, software algorithms and reagents). At the moment there is no published validation study that the XN-hemocytometer measures the same fraction of antibody-synthesizing lymphocytes as the XE2100.

Strengths and limitations

Key strengths of our study are its large sample size, attempted inclusion of all patients who were hospitalized and had symptoms suspected for COVID-19 to improve generalizability and reduce risk of selection bias, extensive hemocytometric testing and availability of COVID-19 negative hospital controls. The latter reduced the risk of control selection bias as well.

Nevertheless, this study also has some limitations. First, despite extensive efforts to achieve inclusion of all eligible patients, selection and information bias due to incomplete medical records cannot be fully excluded in this retrospective study. In addition, agreements with local GPs to refrain from admission of patients with severe pre-existing clinical frailty may have biased results. Second, the criteria used to define CSS were based on expert-opinion. This may have resulted in misclassification of CSS status in some patients [7]. Nevertheless, the high mortality observed in patients with CSS based on these criteria stresses their relevance. Third, this concerns a monocentric study carried out using hemocytometers of a specified supplier, Sysmex. Although most other suppliers of hematology analyzers provide parameters related to those mentioned in this study (Table 1), transferability of our results should be carefully validated before application on another analytical platform. Finally, this study was conducted in a period with a low prevalence of, for example, influenza or other viral/bacterial infections. Further research should examine whether the above-mentioned findings are unique to COVID-19.

Conclusions

In this study it was shown that especially the presence of antibody-synthesizing lymphocytes (AS-LYMPHO), higher permeability of monocytes in general (MO-SFL), and an increase in reactive monocytes (RE-MONO) were associated with COVID-19 and CSS in particular. This may reflect a derailed interaction between SARS-CoV-2 and the host immune system (innate as well as adaptive). In addition, our data suggest that hemocytometric patterns at the time of admission to hospital could provide a clue for the development of CSS during hospitalization.

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Competing interests: Remy Martens, Arjan van Adrichem, Nadine Mattheij, Calvin Brouwer, Jasper Broerse, Daan van Twist, César Magro-Checa, and Math Leers have nothing to disclose. Christel M.P. van Dongen reports personal fees from Novartis, personal fees from Roche, outside the submitted work. Rémy L.M. Mostard reports personal fees from Boehringer Ingelheim, personal fees from Roche, personal fees from Galapagos, outside the submitted work. Sofia Ramiro reports personal fees from AbbVie, personal fees from Eli Lilly, grants and personal fees from MSD, personal fees from Novartis, personal fees from UCB, personal fees from Sanofi, outside the submitted work. Robert Landewé reports personal fees from AbbVie, personal fees from BMS, personal fees from Galapagos, personal fees from Gilead, personal fees from Jansen, personal fees from Novartis, personal fees from Pfizer, personal fees from Roche, personal fees from UCB, outside the submitted work; and Owner and director of Rheumatology Consultancy BV, a company that provides consultancy and read-services for clinical trials. Finally, Jo Linssen and Claudia Wienefoet, two permanent employees of Sysmex, helped us with retrieving the raw measurement data from our hematology analyzers, and converted it into readable CSV-files.

Informed consent: Informed consent was obtained from all individuals included in this study.

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