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T-cell immune response predicts the risk of critical SARS-Cov2 infection in hospitalized COVID-19 patients

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\textbf{A R T I C L E   I N F O}

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\textbf{A B S T R A C T}

\textbf{Introduction:} This study aimed to identify markers of disease worsening in patients hospitalized for SARS-Cov2 infection.

\textbf{Patients and methods:} Patients hospitalized for severe recent-onset (<1 week) SARS-Cov2 infection were prospectively included. The percentage of T-cell subsets and plasma IL-6 at admission (before any steroid therapy) were compared between patients who progressed to a critical infection and those who did not.

\textbf{Results:} Thirty-seven patients (18 men, 19 women) were included; 11 (30\%) progressed to critical infection. At admission, the critical infection patients were older ($P = 0.021$), had higher creatinine levels ($P = 0.003$), and decreased percentages of circulating B cells ($P = 0.04$), T cells ($P = 0.009$), and CD4+ T cells ($P = 0.004$) than those with a favorable course. Among T cell subsets, there was no significant difference between the two groups except for the percentage of Th17 cells, which was two-fold higher in patients who progressed to critical infection ($P = 0.028$). Plasma IL-6 at admission was also higher in this group ($P = 0.018$). In multivariate analysis, the percentage of circulating Th17 cells at admission was the only variable associated with higher risk of progression to critical SARS-Cov2 infection ($P = 0.021$).

\textbf{Conclusion:} This study suggests that an elevated percentage of Th17 cells in patients hospitalized for SARS-Cov2 infection is associated with an increased risk of progression to critical disease. If these data are confirmed in a larger study, this marker could be used to better target the population of patients in whom tocilizumab could decrease the risk of progression to critical COVID-19.

1. Introduction

The SARS-CoV2 infection pandemic that began in December 2019 has so far infected 525 million people and caused at least 6.285 million deaths around the world. In France, 28.5 million people were infected and 144,809 died between the start of the pandemic and May 2022 [1]. Since its emergence, the management of SARS-CoV2 infection has improved, first with the use of dexamethasone and more recently with tocilizumab, a monoclonal antibody directed against soluble and membranous receptors of IL-6, has emerged as a potential therapy to improve the prognosis of patients with severe SARS-CoV-2 infection.

Although the precise pathophysiology of this infection is not fully understood, studies have shown that SARS-CoV-2 infection causes a cytokine storm with significant IL-6 release [2]. Based on these data, tocilizumab, a monoclonal antibody directed against soluble and membrane receptors of IL-6, has emerged as a potential therapy to improve the prognosis of patients with severe SARS-CoV-2 infection. Since then, numerous clinical studies have been conducted to evaluate the efficacy of this drug, and there is a growing body of evidence on the efficacy of this treatment [3]. When infected patients are hospitalized,

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they currently receive supportive care, oxygen therapy and dexamethasone. If the disease worsens, tocilizumab is added to better control the cytokine storm, based on recent randomized controlled trials [4,5]. However, clinicians do not have a reliable marker to predict the risk of disease worsening. Therefore, they currently use this treatment according to the clinical condition of patients or predictive scores such as NEWS2 [6], which in some cases come too late to avoid worsening and transfer to intensive care. If we were able to better identify patients at high risk of progression to critical disease, tocilizumab therapy could be initiated earlier and possibly reduce the need for mechanical ventilation [5]. Along this line, a well-documented clinical case reported by Xu et al. [7] described the T-cell immune response of a 50-year-old patient who died of a severe form of COVID-19. Total CD4 and CD8 T cells were decreased in the blood, but their phenotypic study revealed signs of cell hyperactivation, characterized by a high proportion of HLA-DR+ and CCR6+ Th17 cells. Based on the premise that IL-6 is a key cytokine in Th17 differentiation [8] and that tocilizumab decreases the Th17 response [9], we hypothesized that studying the T-cell immune response in patients hospitalized with SARS-Cov-2 infection could predict the risk of worsening. Therefore, in a cohort of patients hospitalized with SARS-Cov2 infection, we assessed and compared T-cell response between patients in whom the infection progressed to a critical form and those who remained stable.

2. Methods

2.1. Studied population and definitions

Patients admitted to the Dijon University Hospital for the management of severe, RT-PCR proven SARS-CoV-2 infection less than one week from onset were prospectively included in this study after giving written informed consent. The study was approved by the Institutional Review Board of the Dijon University Hospital. Patients under 18 years, pregnant women and those who were unable to give consent were not included in this study.

Blood samples were obtained at inclusion and before starting corticosteroid treatment in order to isolate fresh peripheral blood mononuclear cells (PBMC) for flow cytometry analyses (FACS).

Demographical, clinical, biological, radiological and outcome data were prospectively recorded on paper case report forms. The NEWS2 score was calculated at inclusion as previously described [6]. Critical infection was defined as transfer to intensive care or death, whatever occurred first.

2.2. Cell preparation, antibodies and flow cytometry analysis

All analyses were performed on fresh PBMCs obtained by Ficoll gradient centrifugation. Membrane staining was performed in 10^6 PBMCs. Cells were fixed and permeabilized (Fixation Permeabilization buffer, ThermoFisher scientific, ebioscience, France) before intracellular staining. Intracellular cytokine staining was performed starting from 10^6 PBMCs that were cultured in 24-well plates in 1 mL of RPMI 1640 (Bio Plug; BD Bioscience). After 4 h, cells were harvested and stained with anti-CD3 BV510, anti-CD4 PerCP vio700 and anti-CD8 PE Cy7. After fixation and permeabilization, intracellular staining with anti-IFN-γ APC, anti-IL-17 PE and anti-IL-4 FITC was performed. Data were acquired on an Attune cytometer and analyzed with FlowJo® v10 software. Antibodies are listed in Supplementary Table 1.

2.3. Cytokine assays

Plasma IL-6 was measured by Simple Plex Technology (immunomasays in a microfluidic Simple Plex cartridge). Each sample was diluted at 1:2 in a provided dilution buffer and run in triplicate through a microfluidic channel that binds IL-6 on an Ella automated system (Bio-Technie), following the manufacturer’s instructions.

2.4. Statistics

Data are expressed as numbers (%) for categorical variables and medians (interquartile range [IQR]) for continuous variables. Chi-2 or Fisher’s exact tests were used to compare categorical variables and Mann Whitney U tests to compare continuous variables, as appropriate.

A binary logistic regression with backward selection of variables (exit threshold: P<0.1) was used to identify variables associated with severe disease. Candidate variables were all non-redundant variables with P ≤ 0.2 in the univariate analysis. Statistical significance was set at P<0.05 (two-tailed). IBM SPSS statistics was used for analyses.

A ROC curve was calculated to study sensitivity and specificity of Th17 percentage in predicting critical COVID-19. Kaplan-Meier curves were plotted to study survival, and differences between groups were calculated using the log-rank test.

Statistical analyses were performed with IBM SPSS v21 and Graph-Pad Prism v9.

3. Results

From September 2020 to December 2020, 40 patients hospitalized at the Dijon University Hospital for severe SARS-CoV2 infection diagnosed by a positive RT-PCR less than one week previously and for whom no limitation of care was envisaged were included. Three were excluded from the final analysis because biological sampling could not be performed (n = 2) or because of lack of data (n = 1). Finally, 37 patients (18 males, 19 females) were included in the final analysis (age 81.7 (70.3–87.5) years, BMI 25.7 (23.7–29) kg/m², hypertension in 54%, diabetes in 24%, dyslipidemia in 35%, smoking in 24%, ischemic heart disease in 8%, cerebrovascular disease in 11%). The duration of follow-up was 10 (8–15) days. Median (IQR) NEWS2 score was at 5 (3–6) points
at inclusion. Detailed characteristics of the studied population are summarized in Table 1.

Among the 37 patients, 11 (30%) progressed to a critical SARS-Cov2 infection, including 4 transfers to ICU and 8 deaths. Patient data obtained at hospital admission were compared between those who progressed to critical infection and those who did not. Gating strategy for flow cytometry analysis is reported in Fig. 1 and the main results are summarized in Table 2. Notably, patients who progressed to critical SARS-Cov2 infection were older, with higher creatinine levels, and decreased percentages of circulating B cells, T cells, and CD4+ T cells among total lymphocytes at inclusion. The NEWS-2 score was not statistically different between groups: 5 (4–6) vs. 4.5 (3–5) (P = 0.168). Within T-cell subsets, patients whose disease progressed to a critical form had twice as many circulating Th17 cells at hospital admission:

Fig. 1. Gating strategy for flow cytometry analysis. First, we gated on monocytes or lymphocytes in a FSC/SSC window. CD14 and CD16 expressions were studied in monocytes after gating on total CD14+ cells: classical monocytes (CD14\textsuperscript{hi}CD16\textsuperscript{hi}), non-classical monocytes (CD14\textsuperscript{lo}CD16\textsuperscript{hi}) and intermediate monocytes (CD14\textsuperscript{lo}CD16\textsuperscript{lo}). Among total lymphocytes, we quantified B-cells (CD19\textsuperscript{+}) and CD4 and CD8 T cells (CD3\textsuperscript{+}CD8\textsuperscript{+} and CD3\textsuperscript{+}CD4\textsuperscript{+}). Naive (CD45RA\textsuperscript{+}) and memory (CD45RA\textsuperscript{−}) were measured in each T-cell fraction. Cytotoxic CD8 T cells were defined as CD3\textsuperscript{+}CD8\textsuperscript{+} granzymeB\textsuperscript{+} perforin\textsuperscript{+} cells. CD63\textsuperscript{+} identified cytotoxic cells that had degranulated among total CD8 cells. The expression of intracellular cytokines identified: Th1 (CD3\textsuperscript{+}CD4\textsuperscript{+}IFN-\textgamma\textsuperscript{+}), Th2 (CD3\textsuperscript{+}CD4\textsuperscript{+}IL-4\textsuperscript{+}), Th17 (CD3\textsuperscript{+}CD4\textsuperscript{+}IL-17\textsuperscript{+}), Tc1 (CD3\textsuperscript{+}CD8\textsuperscript{+}IFN-\textgamma\textsuperscript{+}), Tc2 (CD3\textsuperscript{+}CD8\textsuperscript{+}IL-4\textsuperscript{+}) and Tc17 (CD3\textsuperscript{+}CD8\textsuperscript{+}IL-17\textsuperscript{+}) cells. Treg were defined as CD4\textsuperscript{+}CD25\textsuperscript{hi}FoxP3\textsuperscript{+} cells and distinguished between activated Treg (CD45RA\textsuperscript{−} FoxP3\textsuperscript{hi}) and resting Treg (CD45RA\textsuperscript{+} FoxP3\textsuperscript{lo}).
Comparison of patients with critical and non-critical SARS-CoV2 infection (univariate analysis).

Table 2

|                      | Non-critical (n = 26) | Critical (n = 11) | P     |
|----------------------|-----------------------|-------------------|-------|
| Age (years), median (IQR) | 79.6 (70.3–84.4) | 87.7 (80.4–92.0) | 0.021 |
| BMI (kg/m²), median (IQR) | 25.5 (23.0–28.7) | 27.3 (23.8–30.2) | 0.494 |
| Past medical history, n (%) |                      |                   |       |
| Hypertension          | 12 (46)              | 8 (73)            | 0.138 |
| Diabetes              | 7 (27)               | 2 (18)            | 0.695 |
| Dyslipidemia          | 7 (27)               | 6 (55)            | 0.143 |
| Tobacco use           | 6 (23)               | 3 (27)            | 1.00  |
| Ischemic cardiopathy  | 1 (4)                | 2 (18)            | 0.205 |
| Stroke                | 1 (4)                | 3 (27)            | 0.070 |
| COPD                  | 1 (4)                | 0 (0)             | 1.00  |
| Asthma                | 4 (15)               | 0 (0)             | 0.296 |
| Neoplasia             | 2 (8)                | 3 (27)            | 0.144 |
| NEWS2 score, median (IQR) | 4.5 (3–5)        | 5 (4–6)           | 0.168 |
| Biology, median (IQR) |                      |                   |       |
| Creatininemia (µmol/L) | 68.5 (58.0–87.0) | 116.0 (84.0–147.0) | 0.003 |
| Hemoglobin (g/dL)     | 11.8 (11.0–13.4) | 13.4 (11.7–14.6) | 0.058 |
| CRP (mg/L)            | 18.5 (8.3–89.3) | 58.0 (34.3–93.0) | 0.17  |
| IL-6 (pg/mL)          | 13.1 (6.1–33.2) | 39.0 (31.4–48.5) | 0.018 |
| Leukocytes (G/L)      | 6.4 (3.5–8.9) | 7.0 (5.1–10.4) | 0.308 |
| Neutrophils (G/L)     | 4.4 (2.5–7.0) | 5.2 (2.7–9.0) | 0.52  |
| Lymphocytes (G/L)     | 0.8 (0.6–1.4) | 0.6 (0.3–0.9) | 0.086 |
| CD3⁺ (in total Lc)    | 40.6 (29.6–50.7) | 25.7 (19.5–31.0) | 0.009 |
| CD4⁺ (in total Lc)    | 32.7 (22.5–44.9) | 21.3 (11.9–26.0) | 0.004 |
| naïve CD4 T cells (CD4⁺CD45RA⁺) (%) | 68.3 (53.1–75.4) | 68.8 (31.9–79.5) | 0.958 |
| memory CD4 T cells (CD4⁺CD45RA⁻) (%) | 31.8 (24.6–46.9) | 31.3 (20.5–68.2) | 0.958 |
| Th1 cells (CD4⁺IFN-γ⁺) | 3.86 (2.72–5.75) | 3.89 (0.65–6.27) | 0.535 |
| Th2 (CD4⁺IL-4⁺)       | 1.70 (1.00–3.09) | 2.43 (1.12–3.31) | 0.458 |
| Th17 cells (CD4⁺IL-17⁺) | 0.23 (0.17–0.36) | 0.44 (0.28–0.57) | 0.028 |
| Treg (CD4⁺CD25⁺FoxP3⁺⁺) | 2.77 (1.83–4.20) | 3.29 (2.74–5.25) | 0.105 |
| activated Treg (CD4⁺CD45RA⁺FoxP3⁺⁺) | 0.98 (0.34–2.21) | 2.59 (0.80–4.37) | 0.064 |
| resting Treg (CD4⁺CD45RA⁻FoxP3⁺⁺) | 0.25 (0.14–0.41) | 0.38 (0.13–0.46) | 0.434 |
| CD3⁺CD8⁺ (in total Lc) | 3.2 (1.2–5.5) | 2.7 (0.9–9.7) | 0.958 |
| naïve CD8 T cells (CD8⁺CD45RA⁺) | 88.1 (77.1–93.8) | 86.8 (74.2–90.5) | 0.454 |
| memory CD8 T cells (CD8⁺CD45RA⁻) | 11.9 (6.3–22.9) | 13.2 (9.5–28.8) | 0.454 |
| CD6⁺CD63 | 25.9 (16.4–42.4) | 28.3 (9.1–32.3) | 0.577 |
| cytotoxic CD8 T cells (CD8⁺perforin⁺granymeMβ⁺) | 6.38 (3.53–8.97) | 5.23 (3.31–9.77) | 0.577 |
| Tc1 (CD8⁺IFN-γ⁺) | 24.70 (16.94–32.94) | 17.90 (13.06–27.65) | 0.193 |
| Tc17 (CD8⁺IL-17⁺) | 0.12 (0.00–0.42) | 0.16 (0.01–0.19) | 0.862 |
| CD19⁺ (in total Lc) | 12.2 (6.6–22) | 7.0 (1.4–10.9) | 0.04  |
| Monocytes (G/L)       | 0.4 (0.2–0.6) | 0.37 (0.17–0.67) | 0.787 |
| CD14⁺HLA-DR | 11.6 (7.8–18.5) | 7.7 (5.2–20.3) | 0.287 |
| CD14⁺CD16 | 74.8 (65.7–82.7) | 77.4 (66.8–84.4) | 0.524 |
| CD14⁺CD16low | 23.7 (14.9–29.2) | 17.3 (11.6–27.7) | 0.305 |
| CD14lowCD16high | 2.8 (1.4–5.2) | 2.4 (0.9–8.0) | 0.658 |

BMI: body mass index; COPD: chronic obstructive pulmonary disease.

0.44 vs. 0.23% of total CD4⁺ T cells (P = 0.028) (Fig. 2B). By contrast, we did not find any difference between the two groups in CD8 T-cell and monocyte subpopulations. Plasma IL-6 was also higher in patients who progressed to critical infection: 39.0 vs. 13.1 pg/mL (P = 0.018) (Table 2 and Fig. 2A).

In multivariate analysis (binary logistic regression including the following variables: age, serum creatinine, hemoglobin, CD4 T cells, Th17, activated Treg, B cells and plasma IL-6), the only variable associated with progression to critical SARS-CoV2 infection was the percentage of circulating Th17 cells among total CD4 T cells at baseline (P = 0.021) (sup Table 2).

The ROC curve evaluating the sensitivity and specificity of the percentage of Th17 cells to distinguish COVID-19 patients who progressed to critical infection showed good discrimination performance (AUC = 0.754; 95% confidence interval [CI]: 0.564–0.945; P = 0.022). It identified the threshold of 0.435% of Th17 cells as having the best performance (Se = 60.0% [95% CI: 26.2–87.8]; Sp= 91.3% [72.0–99.0]) (Fig. 2C). This threshold was tested in survival analysis, and showed that patients with a percentage of Th17 lymphocytes >0.435% of total CD4 T cells at admission had lower survival (Fig. 2D).

4. Discussion

Epidemiological studies have previously shown that patients with chronic medical conditions (hypertension, obesity, diabetes, cardiovascular diseases, chronic respiratory diseases) or higher NEWS2 score were more likely to develop critical COVID-19 [6,10,11]. Probably because of the small number of patients enrolled, these factors were not found to be significantly associated with progression to critical Covid-19 in the present study. However, we observed that the percentage of Th17 lymphocytes was higher at baseline in patients who subsequently progressed to critical COVID-19. The fact that a significant result was obtained despite the small number of included patients indicates that this marker could be valuable for predicting of the risk of progression to critical COVID-19 in hospitalized patients with SARS Cov-2 infection. Th17 cells are a distinct CD4 T cell subset firstly described in 2005 and characterized by their ability to produce IL-17 [12,13]. In humans, several cytokines such as IL-6, IL-1β, IL-21 and TGF-β are involved in the differentiation of naive T cells into Th17 cells [14]. Moreover, exposure to IL-23 is essential for expansion of Th17 cells, and triggers their pathogenicity. Th17 cells secrete numerous cytokines, mainly IL-17A, IL-17F, IL-21, IL-22, GM-CSF, IL-10 and IFN-γ [15]. Th17 cells have both a protective and a pathogenic role. In one hand, their involvement has been reported in the pathophysiology of many autoimmune and
Inflammatory diseases. In particular, Th17 cells can rapidly initiate an inflammatory response dominated by neutrophils because most parenchymal cells express IL-17 receptors and, exposed to IL-17, produce several chemokines, mainly CXCL8, that recruit neutrophils. Therefore, immune response mediated by Th17 cells, which is important at epithelial and mucosal surfaces, can cause severe damage [14]. On the other hand, Th17 cells are important for clearing infections. It has been shown in the hyper-IgE syndrome, a disease characterized by a Th17 deficiency related to a mutation of STAT3 that is one of the main transcription factors of the Th17 lineage. Patients with this disease have recurrent Candida albicans and Staphylococcus aureus infections in the skin and lungs but no severe viral infections [16]. Thus, Th17 lymphocytes do not appear to play a major role in the defense against viral infections. It is therefore likely that their excess in Covid-19 is more the consequence and the cause of an excessive inflammatory response than beneficial to viral clearance.

A study conducted in Wuhan, China, between January and March 2020 assessed T-cell response and serum cytokine assays in a population of 1018 hospitalized COVID-19 patients. After adjusting for major comorbidities, the two factors independently associated with risk of death were serum IL-6 >20 pg/mL and circulating CD8 T-cell count <165/mm$^3$ at enrollment [17]. However, this study did not analyze T-cell polarization as we did here. Considering the major role of IL-6 in the polarization of T cells towards the Th17 pathway, we can consider that our results are in agreement with this study. Along this line, another study found that the levels of IL-6 and IL-10 were significantly higher in severe COVID-19 patients and that the percentages of circulating Th17 cells, which were higher in COVID-19 patients compared to healthy controls, tended to be increased in severe COVID-19 patients compared to non-severe COVID-19 patients, but without reaching significance [18].

Corticosteroid therapy is now the cornerstone treatment for patients with severe COVID-19 [4]. When clinicians are concerned that the disease is worsening, the addition of tocilizumab therapy to block IL-6 signaling can be considered. However, the major recent studies [5,19, 20] that evaluated tocilizumab for the treatment of severe COVID-19 reported that tocilizumab did not improve clinical condition or mortality, raising doubts about the therapeutic effectiveness of this treatment. The first study demonstrated that a 14-day course of tocilizumab was not able to prevent mechanical ventilation or death in a randomized, placebo-controlled trial that enrolled 243 patients with SARS-CoV-2 infection [20]. In the study performed by Salama et al., including 389 patients with COVID-19 pneumonia that were randomized between receive standard care plus one or two doses of either tocilizumab (8 mg per kilogram of body weight intravenously, n = 249 patients) or placebo (n = 128 patients), tocilizumab reduced the likelihood of progression to the composite outcome of mechanical ventilation or death, but it did not improve survival [5]. In the COVACTA study including 438 patients (294 in the tocilizumab group and 144 in the placebo group), tocilizumab did not reduce mortality after 28 days of follow-up [19]. Finally a large meta-analysis analyzing a total of 10,930 patients included in 27 trials showed that administration of IL-6 antagonists in patients hospitalized for COVID-19 led to lower 28-day all-cause mortality compared to usual care (odds ratio [95% CI] = 0.86 [0.79–0.95]) [21]. While pathophysiology studies support the use of therapeutic strategies targeting IL-6 or its receptor, the results of
suggest a modest effect of treatments targeting IL-6, which may result from heterogeneity in the activation of IL-6 signaling among patients. Based on the results of our study and on the fact that IL-6 is the key cytokine in Th17 differentiation [8], it could be hypothesized that monitoring the percentage of Th17 cells at admission in patients hospitalized for COVID-19 could help to identify the patients in whom treatment with drugs targeting IL-6 or its receptor may be more effective in preventing critical disease. Thereby, the therapeutic effect of tocilizumab could be explained by the faculty of this treatment to inhibit Th17 polarization in patients with a high percentage of Th17 without altering the Th1 immune response, which is essential for viral clearance [9].

In routine practice, the use of this biomarker can be problematic due to its complexity and the time required to obtain it. More specifically, the evaluation of the percentage of circulating Th17 lymphocytes among total T lymphocytes requires analysis by flow cytometry with prior activation of PBMC by PMA and ionomycin for 4 h. Thus, the result is generally not available in less than 6 h, which can be a limiting factor in emergency situations. However, it has been shown that the percentage of Th17 lymphocytes correlates with the percentage of CD4⁺CCR6⁺CD161⁺ T lymphocytes, which can be rapidly assessed in routine hematology laboratories [22,23]. Unfortunately, we did not measure this subpopulation here, but this question warrants further study.

In conclusion, this study suggests that an increase in the percentage of Th17 lymphocytes in patients hospitalized with SARS-CoV2 infection is indicative of the risk of progression to critical disease. Considering the current challenge of identifying the population of COVID-19 patients who would most likely benefit from early tocilizumab therapy, our findings warrant confirmation in a larger number of patients.

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Contributorship statement

MS and BB were the principal investigators and take primary responsibility for the paper. MS, BN, AG, HG, MB, LP, TR, HD, PM, SA, BB recruited the patients. MC, CC, SF and DL performed the laboratory work for this study. MS and HD did the statistical analyses. MS and BB coordinated the research. MS and BB drafted the manuscript. MS and BB contributed to data interpretation.

Declaration of Competing Interest

Maxime SAMSON: Roche-Chugai (invitation to congresses, remuneration for symposium and consulting payments), Abbvie (consulting payments), Novartis (consulting payments), Vifor Pharma (invitation to congresses, remuneration for symposium and consulting payments) and Boehringer Ingelheim (invitation to congresses, consulting payments).

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ejim.2022.06.001.

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