ORIGINAL ARTICLE

Characteristics and Predictive Value of T-lymphocyte Subset Absolute Counts in Patients with COVID-19-associated Acute Respiratory Failure: A Retrospective Study

Sonali Vadi[1], Ashwini Pednekar[2], Durga Suthar[3], Neha Sanwalka[4], Kiran Ghodke[5], Nikhil Rabade[6]

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Abstract

Background: Of the factors influencing severity and outcomes following coronavirus disease-2019 (COVID-19), cellular immune response has a strong impact. The spectrum of response varies from over-activation to hypo-functioning. The severe infection leads to reduction in numbers and dysfunction of T-lymphocytes/subsets.

Patients and methods: This retrospective, single-center study aimed to analyze the expression of T-lymphocyte/subsets by flow cytometry and inflammation-related biomarker, serum ferritin in real-time polymerase chain reaction (RT-PCR) positive patients. According to oxygen requirements, patients were stratified into nonsevere (room air, nasal prongs, and face mask) and severe [nonrebreather mask (NRBM), noninvasive ventilation (NIV), high-flow nasal oxygen (HFNO), and invasive mechanical ventilation (IMV)] subgroups for analysis. Patients were classified into survivors and nonsurvivors. Mann–Whitney *U* test was used to analyze differences in T-lymphocyte and subset values when classified according to gender, the severity of COVID, outcome, and prevalence of diabetes mellitus (DM). Cross tabulations were computed for categorical data and compared using Fisher's exact test. Spearman correlation was used to analyze the correlation of T-lymphocyte and subset values with age or serum ferritin levels. $p < 0.05$ values were considered to be statistically significant.

Results: A total of 379 patients were analyzed. Significantly higher percentage of patients with DM were aged ≥61 years in both nonsevere and severe COVID groups. A significant negative correlation of CD3+, CD4+, and CD8+ was found with age. CD3+ and CD4+ absolute counts were significantly higher in females as compared to males. Patients with severe COVID had significantly lesser total lymphocyte (%), CD3+, CD4+, and CD8+ counts as compared to those with nonsevere COVID ($p < 0.05$). T-lymphocyte subsets were reduced in patients with severe disease. A significant negative correlation of total lymphocyte (%), CD3+, CD4+, and CD8+ counts was found with serum ferritin levels.

Conclusions: T-lymphocyte/subset trends are an independent risk factor for clinical prognosis. Monitoring may help in intervening in patients with disease progression.

Keywords: Acute respiratory failure, Coronavirus disease-2019, Disease severity, Severe acute respiratory syndrome CoV-2 infection, T-lymphocyte subsets

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Highlights

- Total lymphocytes and their subsets were below the lower limit of normal in the severe subgroups.
- Hyperglycemia-induced dysfunctional adaptive immune response could have led to severe infection despite an increase in the T-cell subsets.
- Increased serum ferritin levels were negatively correlated with T-lymphocytes/subsets.

Introduction

Effective immune response to SARS CoV-2 infection depends upon activation and function of T cells. Helper T cells (CD4+) regulate the function of CD8+ cells, and cytotoxic T cells (CD8+) eliminate viruses by secreting interferons, perforin, and granzyme,[1] a reduction of which influences severity and mortality following SARS CoV-2 infection. T-cell lymphopenia is a consistently reported feature following severe acute respiratory syndrome coronavirus (SARS CoV), Middle East respiratory syndrome (MERS) coronavirus, and other ribonucleic acid (RNA) virus infections. Cell death, increased cytokines and chemokines, co-inhibitory molecules, inhibition of lymphopoiesis, lymphocyte trafficking, metabolic disorders, and glucocorticoids are some mechanisms leading to immune cell depletion.[2] Whether severe disease leads to lymphopenia

[1–3]Department of Intensive Care Medicine, Kokilaben Dhirubhai Ambani Hospital & Medical Research Institute, Mumbai, Maharashtra, India

[4]Department of Nutrition and Biostatistics, NutriCanvas, Mumbai, Maharashtra, India

[5,6]Department of Hematology, Kokilaben Dhirubhai Ambani Hospital & Medical Research Institute, India

Corresponding Author: Sonali Vadi, Department of Intensive Care Medicine, Kokilaben Dhirubhai Ambani Hospital & Medical Research Institute, Mumbai, Maharashtra, India, Phone: +91 2242696969, e-mail: sonalivadi@hotmail.com

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or lymphopenia leads to disease progression remains to be established. Yet lymphopenia is linked to disease severity in patients with COVID-19[3–5] and DM.[6]

Baseline reductions in lymphocyte counts are higher in patients with severe disease, reflecting it to be a predictor of disease severity.[7]

Of the various immune facets influencing the progression of infection, we studied T-lymphocyte subsets aiming to understand their predictive role in patients with COVID-19 associated acute respiratory failure (C-ARF), especially those with DM.

Patients and Methods

Study Design and Population

A single-center retrospective study involving patients from 23 March, 2020 to 20 November, 2021 was conducted at a tertiary care center in Mumbai. Patients aged >18 years of age with SARS CoV-2 as confirmed by quantitative RT-PCR on nasopharyngeal swab in the COVID intensive care unit (ICU) with acute respiratory failure were included. Patients who were coincidentally RT-PCR positive without any evidence of severe acute respiratory infection and those with duplicate records were excluded. These included direct admissions, patients transferred from the COVID ward, or referred to the hospital for further management. All patients received corticosteroids.

The study was approved by Institutional Ethics Committee (047/2021), and written informed consent was waived.

Study Outcomes

The endpoint of the study was to demonstrate whether T-lymphocytes/subsets have a predictive role for severity in patients with C-ARF. We looked at the differences in T-lymphocyte subsets in patients with and without DM. The correlation between absolute lymphocyte counts and serum ferritin levels was also assessed.

Data Collection

Demographic data (age, gender), comorbidity (history of DM), laboratory data (white blood cell count, T-lymphocyte subsets – absolute CD3+, absolute CD4+, absolute CD8+, % lymphocyte count, worst serum ferritin, HbA1c), maximum oxygen required, and outcomes were collected. Sample acquisition time was following COVID ICU admission. All blood tests were performed in the hospital's central laboratory adhering to the standard procedures. The authors guarded the validity and completeness of the data.

Patient Classification

According to oxygen requirements, patients were divided into those on room air, nasal prongs, face/venturi mask, NRBM, NIV, HFNO, and IMV. These were further stratified into nonsevere (room air, nasal prongs, and face mask) and severe (NRBM, NIV, HFNO, and IMV) subgroups for analysis. Asymptomatic patients were those who were co-incidentally RT-PCR swab positive but required ICU care.

Patients were classified into survivors—discharged in a stable condition or nonsurvivors—died during the hospital stay, or were discharged against medical advice in an unstable condition.

Study Definitions

Clinically patients in the nonsevere subgroup were those who presented with mild or moderate symptoms. Patients in the severe subgroup clinically had respiratory rate >30/minute, SpO_2 <93% on room air, arterial oxygen tension/inspiratory oxygen fraction (PaO_2/FiO_2) <300 mm Hg, or critically ill requiring invasive ventilation, in circulatory failure, or required organ support.

Flow Cytometry

We studied CD4-CD8 enumeration using the 10-color Navios EX Flow cytometer, Beckman Coulter. Whole blood collected in EDTA anticoagulant tubes was used for the test. Samples were prepared by stain-lyse-wash protocol, and the reverse pipette technique was used. The single-platform method that uses bead-based technology to obtain absolute lymphocyte count was used. The differential count was performed on samples prior to staining to ensure that the total lymphocyte counts were within linearity ranges.

Protocol

About 100 µl of the sample was stained using 10 µl of tetra cocktail antibody (CD45-FITC/CD4-RD1/CD8-ECD/CD3-PC5) and mixed gently using a vortex. Staining incubation was carried out for 20 minutes in dark at room temperature (20–25°C). About 500 µl OptiLyse lysing solution (Beckman Coulter) was used for lysing followed by 10 minutes of incubation in dark at room temperature (20–25°C). After incubation, lysing process was stopped using 500 µl IsoFlow (Beckman Coulter), followed by the immediate addition of 100 µl of Flow Count Fluorospheres (Beckman Coulter) using the reverse pipette technique. Tubes were vortexed and acquired immediately.

Analysis

The absolute number (cell/µl) of positive cells in the sample was determined by comparing cellular events to bead counts. Gating and calculations were automated, and hence the gate impurity issues were taken care of. Table 1 shows normal ranges of T-lymphocyte subsets in healthy adults.

Statistical Analysis

Data were analyzed using SPSS version 25 for Windows (version 25, 2017, IBM Corporation, Armonk, New York, United States). Normality of continuous data was assessed using Shapiro–Wilk test. Data were presented as median [inter quartile range (IQR)] or frequency (%). Mann–Whitney *U* test was used to analyze differences in T-lymphocyte and subset values when classified according to gender, the severity of COVID, and prevalence of DM. Mann–Whitney *U* test was also used to analyze the difference in HbA1C levels between severely infected DM and nonseverely infected DM. Cross tabulation was computed for categorical variables and compared using Fisher's exact test. Spearman correlation was used to analyze the correlation of T-lymphocyte and subset values with age, serum ferritin, or HbA1c levels. $p < 0.05$ values were considered to be statistically significant.

The study followed the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline.

Table 1: Normal ranges of T-lymphocyte subsets in healthy adults

Cell type	*Unit*	*Biological reference range/interval*
WBC count	10^3/µl	4–11
CD3+ (T-lymphocyte) absolute count	/µl	537–2,939
CD4+ (T-helper cells) absolute count	/µl	321–2,124
CD8+ (T-cytotoxic cells) absolute count	/µl	46–1,346

Results

Baseline Characteristics

Of 430 patients who had their T-lymphocyte counts/subsets tested, 30 patients who were coincidentally COVID-19 positive and 21 patients with duplicate records were excluded. In all, 379 patients were analyzed. Table 2 gives baseline characteristics and T-lymphocyte count/subsets of all the study participants.

A significantly higher percentage of patients with DM were aged ≥61 years in both nonsevere and severe COVID groups (p <0.05) (Table 3).

Correlation of T-lymphocyte/Subsets to Age

A significant negative correlation of CD3+, CD4+, and CD8+ was found with age (p <0.05) (Table 4).

Correlation of T-lymphocyte/Subsets to Gender

CD3+ and CD4+ absolute counts were significantly higher in females as compared with males (p <0.05) (Figs 1A to C).

Correlation of T-lymphocyte/Subsets to Disease Severity

Patients with severe COVID had significantly lesser total lymphocyte (%), CD3+, CD4+, and CD8+ counts as compared to those with nonsevere COVID (p <0.05) (Figs 2A to C). White blood cell (WBC) count was significantly lower in patients with nonsevere COVID as compared to patients with severe COVID (p <0.05). No significant difference in HbA1c levels was observed between severely (median: 8%) and nonseverely (median: 7.6%) infected DM patients (p >0.05).

Table 2: Baseline characteristics and T-lymphocyte/subsets in study cohort

	Frequency (n = 379)	*Percentage*
Age		
<50 years	70	18.5
51–60 years	106	28
61–70 years	100	26.4
>71 years	103	27.2
Gender		
Male	266	70.2
Female	113	29.8
Diabetes mellitus		
Yes	160	42.2
No	219	57.8
Severity of illness		
Nonsevere	129	34
Severe	250	66
	Median	*Interquartile range (IQR)*
T-lymphocyte and subsets		
WBC (10^3/µl)	10.7	8.02
Lymphocyte (%)	4.8	10.7
CD3+	383.4	396.2
CD4+	223.2	274.4
CD8+	135	155.9

Comparison of T-lymphocyte/Subsets in Patients with and without DM

Total lymphocyte (%) and CD3+ count were significantly lower in non-DM patients as compared with DM patients (p <0.05) (Figs 3A to C).

Correlation of T-lymphocyte/Subsets with HbA1c and Serum Ferritin

A significant negative correlation of total lymphocyte (%), CD3+, CD4+, and CD8+ counts was found with ferritin levels (p <0.05) (Table 5). Serum ferritin levels and WBC count were positively correlated (p <0.05). Lymphocyte (%) and CD8+ counts were significantly positively correlated with HbA1c levels (p <0.05).

Correlation of T-lymphocyte/Subsets to Outcomes

Though the percentage of survivors varied slightly when classified according to gender or age, this difference was not significant (Table 6). On the other hand, higher percentage of participants in the severe group did not survive as compared to nonsevere group (p <0.05).

Discussion

We noted a dysregulated immune system. SARS-CoV-2 affects T cells leading to an impaired immune system following COVID-19 infection. Male predominance in the incidence of COVID-19 and increased incidence in patients aged >50 years was noted. An efficient immune response is necessary as a defense against viral infection, dysregulation of which results in severe disease and also affects patient survival. Whether this gender difference in prevalence and outcomes is because of sex hormones or X-chromosome-encoded immune genes[8] remains to be determined. Aging affects T-lymphocyte repertoire diversity,[9] and can lead to T-lymphocyte senescence[10] causing ineffective response to infection. We observed that total lymphocytes and its subsets were below the lower limit of normal in the severe subgroups. Increased serum ferritin levels were negatively correlated with T-lymphocytes/subsets. This could be secondary to cytokine storm.

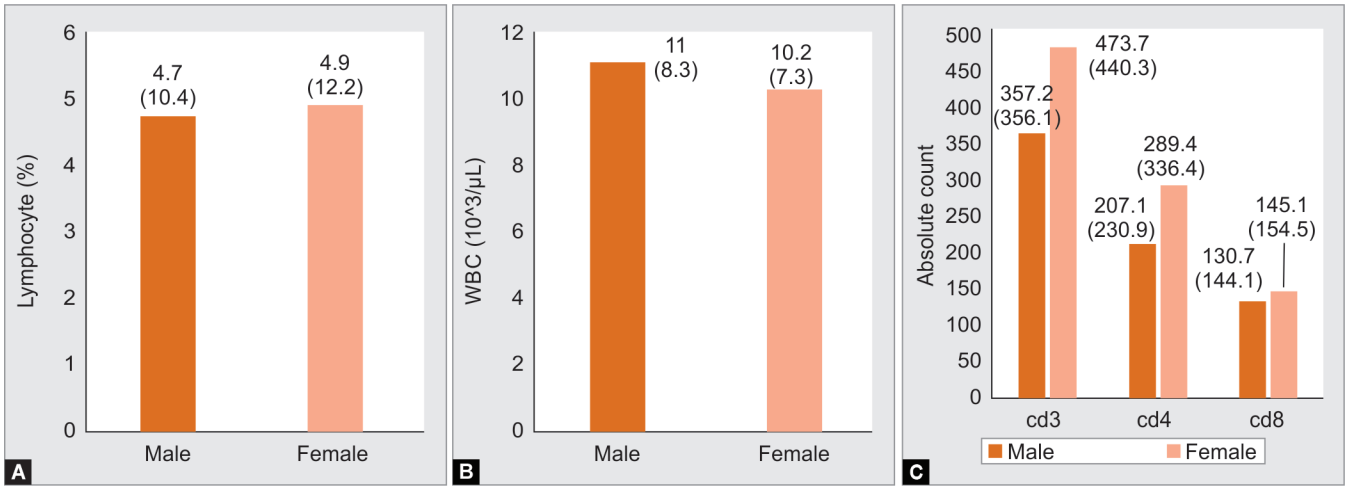
Patients with DM had more severe infections. Increased T-lymphocyte subsets in patients with DM were noted. The

Table 4: Correlation of T-lymphocyte/subsets with age

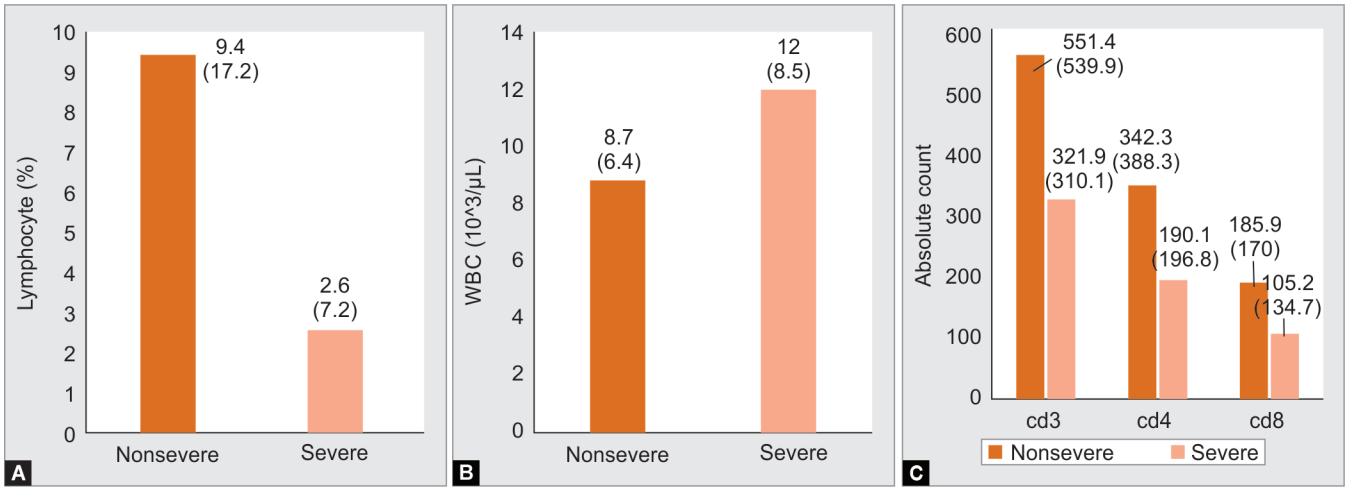
	Lymphocyte (%)	*CD3+*	*CD4+*	*CD8+*
Spearman's Rho value	–0.043	–0.132	–0.093	–0.175
p-value	0.403	0.010	0.071	0.001

Table 3: Relationship between COVID severity, DM, and age

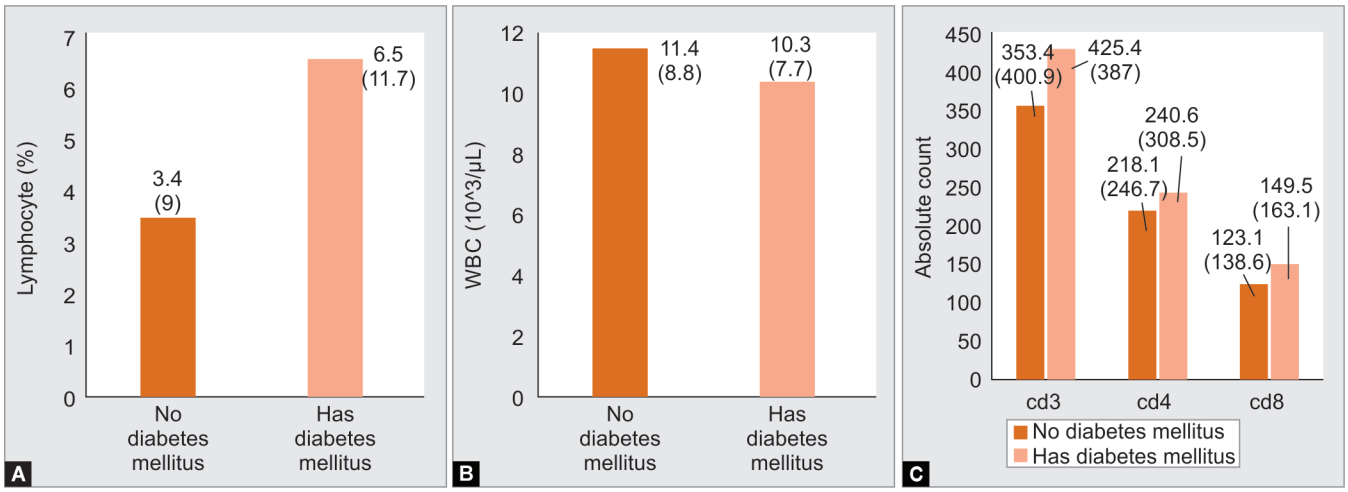
	Non-severe		*Severe*	
	No diabetes mellitus (n = 65)	*Has diabetes mellitus (n = 64)*	*No diabetes mellitus (n = 154)*	*Has diabetes mellitus (n = 96)*
Age ≤60 years	36 (55.4)	24 (37.5)	80 (51.9)	36 (37.5)
Age ≥61 years	29 (44.6)	40 (62.5)	74 (48.1)	60 (62.5)
p-value	0.042		0.026	



Figs 1A to C: T-lymphocyte, WBC, and absolute counts of subsets when classified according to gender. Data presented as median (IQR)



Figs 2A to C: T-lymphocytes, WBC, and absolute counts of subsets when classified according to the severity of COVID. Data presented as median (IQR)



Figs 3A to C: T-lymphocytes, WBC, and absolute counts of subsets when classified according to DM. Data presented as median (IQR)

Table 5: Correlation of T-lymphocytes/subsets with serum ferritin and HbA1c

	WBC (10^3/μl)	*Lymphocyte (%)*	*CD3+ (absolute)*	*CD4+ (absolute)*	*CD8+ (absolute)*
Correlation with serum ferritin levels					
Spearman's Rho value	0.213	–0.238	–0.253	–0.248	–0.211
p value	0.001	0.001	0.001	0.001	0.003
Correlation with HbA1c levels					
Spearman's Rho value	–0.076	0.174	0.114	0.056	0.172
p-value	0.293	0.015	0.116	0.443	0.017

Table 6: Overall disease outcomes

	No diabetes mellitus				*Has diabetes mellitus*			
	Nonsevere		*Severe*		*Nonsevere*		*Severe*	
Age	≤60 (*n* = 36)	≥61 (*n* = 29)	≤60 (*n* = 80)	≥61 (*n* = 74)	≤60 (*n* = 24)	≥61 (*n* = 40)	≤60 (*n* = 36)	≥61 (*n* = 60)
Survivor	36 (100)	27 (93.1)	46 (56.5)	35 (47.3)	23 (95.8)	38 (95)	29 (80.6)	38 (63.3)
Nonsurvivor	–	2 (6.9)	34 (42.5)	39 (52.7)	1 (4.2)	2 (5)	7 (19.4)	22 (36.7)
	Males (n = 48)	*Females (n = 17)*	*Males (n = 111)*	*Females (n = 43)*	*Males (n = 42)*	*Females (n = 22)*	*Males (n = 65)*	*Females (n = 31)*
Survivor	46 (95.8)	17 (100)	59 (53.2)	22 (51.2)	40 (95.2)	21 (95.5)	44 (67.7)	23 (74.2)
Nonsurvivor	2 (4.2)	–	52 (46.8)	21 (48.8)	2 (4.8)	1 (4.5)	21 (32.3)	8 (25.8)

Data presented as frequency (%)

spectrum of T-lymphocyte response following COVID-19 infection varies from inadequate to dysfunctional to excessive. As described in patients with chronic viral infection and malignancy, T-cell exhaustion or dysfunction leads to poor effector function.[11] Hyperglycemia-induced dysfunctional adaptive immune response could have led to severe infection despite an increase in the T-cell subsets.

Our study has certain limitations. This was a retrospective, single-center study with findings that cannot be generalized to other geographical regions. We did not note the timing of immunological analysis in relation to duration of illness. The strength of immune response can be correlated with the stage of infection, an important information in interpreting the responses of T-lymphocyte subsets. T-lymphocyte counts/subsets have been reported to remain low for several weeks post-symptom onset in severe cases[12] with a gradual improvement in those who tend to recover. This lays the significance of these immune cells as an indicator for the severity of disease even during advanced course of illness. Trends of T-lymphocyte subsets were not monitored. Hence, we could not assess the dynamics of cellular immune response during the illness. Notwithstanding, the sample size included during the peak of both waves is sizeable. Specifically, we appreciated a more dysregulated immune in COVID-19 patients with DM influencing patient survival.

Keeping in mind the alterability in treatment options till date, our results merit added substantiation prior to generalizing findings to patients of diverse geographical locations as well as special COVID-19 cohorts such as the obstetric population.

Conclusion

Future studies understanding the dynamics of cellular immune responses can have potential therapeutic implications as also studying the rate of recovery following the infection.

Orcid

Sonali Vadi https://orcid.org/0000-0002-7341-2407
Ashwini Pednekar https://orcid.org/0000-0003-3917-7498
Durga Suthar https://orcid.org/0000-0002-9533-1069
Neha Sanwalka https://orcid.org/0000-0003-3428-3144
Kiran Ghodke https://orcid.org/0000-0002-0810-2349
Nikhil Rabade https://orcid.org/0000-0003-4074-4920

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