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Original Article

Increased risk of *Pneumocystis jirovecii* colonization in rheumatoid arthritis patients on biologics and Janus kinase inhibitor

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Abstract *Background:* The prevalence of *Pneumocystis jirovecii* (PJ) pneumonia among rheumatic patients is rising. PJ colonization serves as a reservoir for transmission and precedes the development of PJ pneumonia. We aim to clarify the association of PJ colonization in patients of rheumatoid arthritis (RA) treated with biologics or Janus kinase inhibitors (JAKi).
Methods: A prospective cohort study was performed from March 2021 to July 2022 in the rheumatology outpatient department of National Cheng Kung University Hospital. We obtained oral-wash samples from asymptomatic RA patients treated with biologic disease-modifying antirheumatic drugs (bDMARDs) and JAKi. A real-time quantitative polymerase chain reaction assay focusing on the mitochondrial large subunit ribosomal ribonucleic acid gene of PJ was applied to detect colonization.
Results: One hundred and ten RA patients were enrolled. Adjusted odds ratios (ORs) of PJ colonization were 6.40 (95% CI 1.34-30.57, p-value =0.02) in patients receiving bDMARDs or JAKi. Specifically, in patients treated with bDMARDs the adjusted OR was 8.08 (95% CI 1.57-41.51, p-value=0.012), and a trend toward developing PJ colonization was further identified in patients receiving JAKi (adjusted OR: 4.79, 95% CI 0.89-25.91, p=0.069). Among patients treated with bDMARDs or JAKi, medication duration >3 years and age >60 y/o are risk factors for PJ colonization.
Conclusion: RA patients on bDMARDs or JAK inhibitors have an approximately 6-fold higher risk of developing P. jirovecii colonization. Patients treated with bDMARDs had an 8-fold higher risk of *P. jirovecii* colonization. Risk factors of PJ colonization are medication duration >3 years and age > 60 y/o.

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Introduction

Rheumatoid arthritis (RA) is characterized by chronic erosive arthritis leading to irreversible deformity if untreated. Since the early twenty-first century, biologic disease-modifying antirheumatic drugs (bDMARD), including TNF-α inhibitor, IL-6 inhibitor, CTLA4 inhibitor, and anti-CD20 monoclonal antibody, as well as Janus kinase (JAK) inhibitor, have been administered to RA patients to achieve disease remission, but with accompanying increases in susceptibility to infection.[1]

Pneumocystis jirovecii pneumonia (PJP) is a lethal threat in immunocompromised patients.[2] The incidence of PJP among HIV populations has decreased since the introduction of highly active antiretroviral therapy, while in the same time period the occurrence of PJP has risen significantly in patients with connective tissue disease (CTD),[3,4] which may be attributed to the advancement of immunosuppressive agents. Compared to HIV-PJP, patients with CTD-PJP have more fulminant clinical courses and devastating outcomes, including severe respiratory insufficiency, intensive care unit hospitalization, and even death.[5—8] Previous studies have described higher incidence of PJP in RA patients treated with TNF-α inhibitor[9—11] and rituximab.[12] Since there is no established consensus of PJP prophylaxis for RA patients on immunosuppressants, early identification of potential PJP cases among this population is crucial.

Individuals with *P. jirovecii* colonization serve as a reservoir for transmission and for subsequent development of PJP.[13,14] A cohort study in the UK reported the odds of PJP compared to *P. jirovecii* colonization were 12 times greater for those ever exposed to immunosuppressive agents (OR 12.1; 95 % CI 1.94, ∞).[15] Therefore, more attention should be paid to *P. jirovecii* colonized patients treated with immunosuppressive agents due to this elevated risk of developing subsequent PJP. Published studies reported the prevalence and risk factors of *P. jirovecii* colonization in rheumatic patients using various conventional synthetic DMARDs (csDMARDs) and infliximab.[16—20] These study results were either limited by very small sample sizes[16—18] or by a heterogenous disease population.[18—20] No other study has explored the association between bDMARD or JAK inhibitor therapy and *P. jirovecii* colonization. We aim to clarify the risk of *P. jirovecii* colonization in RA patients treated with DMARDs in a more comprehensive range of different forms.

Methods

Eligible participants

This prospective cohort study was performed from March 2021 to July 2022 in the rheumatology outpatient department of National Cheng Kung University Medical Center. RA patients $\geq$20 years old undergoing treatment with DMARDs including csDMARD, bDMARD, or JAK inhibitor were included; to increase statistical power, we recruited at least 30 patients in each of these 3 treatment groups. National Cheng Kung University Medical Center prescribes csDMARDs including hydroxychloroquine, methotrexate, sulfasalazine, and leflunomide; bDMARDs including TNF-α inhibitor (etanercept, adalimumab, golimumab), IL-6 inhibitor (tocilizumab), CTLA-4 inhibitor (abatacept), and anti-CD20 Ab (rituximab); and JAK inhibitors including tofacitinib, baricitinib, and upadacitinib. Prescribing a specific DMARD treatment is based on an assessment of the RA patient's characteristics, disease activity, and comorbidities. Therefore, random assignment was not conducted in this study.

All patients signed informed consent prior to participation in our study. We excluded patients with acute respiratory symptoms, a diagnosis of active cancer or HIV. This study was approved by the National Cheng Kung University Hospital institutional review board (approval number: A-BR-109-057).

Data collection

Demographic characteristics were recorded, including age, sex, and immunocompromised status associated with *P. jirovecii* colonization. Also recorded were the patient's current medications, including steroids, csDMARDs, bDMARDs and JAK inhibitors. Total white blood count and lymphocyte count were collected for each patient at the time of their entry to the study. Patients on bDMARDs or JAK inhibitors were further divided into five groups according to mechanism of action, including: TNF inhibitor group (etanercept, adalimumab, golimumab), JAK inhibitor group (tofacitinib, baricitinib, upadacitinib), CTLA-4 inhibitor group (abatacept), IL-6 inhibitor group (tocilizumab), and anti-CD20 Ab group (rituximab). Patients under the treatment of hydroxychloroquine, methotrexate, sulfasalazine, or leflunomide were classified as the csDMARD group. For patients treated with tofacitinib, baricitinib, or upadacitinib, they were categorized as the JAK inhibitor group.

Sampling and definition of *P. jirovecii* colonization and pneumonia

Each participant received a clinical examination during their visit to our clinic. Demographic variables, underlying comorbidities, and current related medication were recorded using a standardized form. Oral-wash samples were obtained by having the participants gargle 20 mL of sterile saline for 1 min. Samples were centrifuged at 2900$\times g$ for 5 min and kept frozen at -20 °C until DNA

extraction. DNA was extracted from the oral-wash samples using the NucliSens kit (Organon Teknika), as reported elsewhere.[21,22]

P. jirovecii colonization was defined when a person met the following conditions: 1) there were no respiratory symptoms 2) there was no evidence of pneumonia patch on chest X-ray 3) the oral wash specimen contained detectable *P. jirovecii* DNA identified using nested polymerase chain reaction (PCR) in 2 independent analyses, or a successful quantification by targeting mitochondrial large subunit ribosomal RNA (mtLSU-rRNA) using quantitative PCR.

P. jirovecii pneumonia was defined by 1) patient presenting with respiratory symptoms or 2) chest X-ray showing pneumonia patch 3) the oral wash specimen containing detectable *P. jirovecii* DNA using nested PCR in 2 independent analyses, and with successful quantification by targeting mtLSU-rRNA using quantitative PCR.

DNA extraction and polymerase chain reaction

We extracted genomic DNA from oral wash fluid (QIAamp DNA kit; QIAGEN, Hilden Germany), collecting a variable DNA yield ranging from 3 to 12 mg. A 2-step protocol was administered for the *P. jirovecii* internal transcribed spacer nested PCR, the detailed procedure being described elsewhere.[23] Several preventive measures were applied to avoid PCR contamination, including pre- and post-PCR reactions being performed in separate rooms with individual sets of micropipettes. The PCR mixture and extraction steps were performed in 2 different laminar flow cabinets. In addition, each sample was amplified twice, with a negative control using distilled water included in each PCR step.

Quantitative PCR assay

A real-time quantitative PCR assay focusing on the mtLSU-rRNA gene of *P. jirovecii* was applied, as described by previous studies.[24,25] We operated thermocycling and fluorescence detection on a PCR system (7500 PCR system, Applied Biosystems) by using the TaqMan Gene Expression Master Mix (Applied Biosystems). Plasmid suspensions were prepared as standards for quantification and positive controls by cloning the mtLSU-rRNA insert into the plasmid vector pGEM-T (pGEM-T Easy Vector System II; Promega). Each PCR step consisted of 10 serial 10-fold dilutions of the plasmid suspension, with a range of $7.0 \times 10\text{-}2$ to $7.0 \times 10\text{-}7$ copies/mL of extracted DNA, and 2 negative controls.

We used plasmid dilutions to create a calibration curve giving the correlation between cycle threshold values and the number of copies per microliter of extracted DNA. The detection limit was estimated at 7 copies/mL of extracted DNA, correlating to a cycle threshold value of 43. All oral wash samples, plasmid dilutions, and negative controls were performed in duplicate, with 1 tube containing an internal positive control (TaqMan Exogenous Internal Positive Control Reagents; Applied Biosystems) to detect PCR inhibitors.[24]

Statistical analysis

Continuous variables were expressed as medians with ranges and compared using the Kruskal—Wallis test. Categorical variables were expressed as a percentage and compared using Fisher's exact test. Demographic characteristics were compared between different treatment groups. Further, we performed a subgroup analysis of RA patients treated with bDMARD or JAK inhibitor to identify risk factors for colonization. A multivariate logistic regression analysis was performed using the variables with a P value < 0.05 from the univariate analysis of RA patients. $P < 0.05$ was defined as statistically significant. All analyses were conducted by SAS 9.4 statistical software (SAS Institute Inc., Cary, NC, USA).

Results

One hundred and ten RA patients were included during March 2021 to July 2022, and median age was 56.11 years old. All patients are Taiwanese and the majority were female (83.6%). All participants satisfied 2010 American College of Rheumatology/The European League Against Rheumatism classification criteria of RA. To reach sufficient statistical power, we included over 30 patients in each group. There were 31 patients treated with csDMARDs, 48 patients treated with bDMARDs, and 31 patients treated with JAK inhibitors. *P. jirovecii* colonization rates in the csDMARD group and bDMARD/JAKi group were respectively 6.45% and 31.6%. Patients on bDMARD or JAKi were further divided into five groups according to different mechanism of action, including TNFi group (etanercept, adalimumab, golimumab), JAKi group (tofacitinib, baricitinib, upadacitinib), CTLA-4i group (abatacept), IL-6i group (tocilizumab), and anti-CD20 Ab group (rituximab). On comparing the variables of age, sex, related comorbidities, lymphopenia (lymphocyte <1000 per microliter), and steroid use in the six treatment groups, statistically significant differences ($p = 0.001$) are present for patients with any of the following 8 comorbidities, including non-tuberculosis mycobacterium (NTM), type 2 diabetes mellitus (T2DM), tuberculosis (TB), chronic obstructive pulmonary disease (COPD), interstitial lung disease (ILD), chronic kidney disease (CKD), liver cirrhosis, and malignancy history (Table 1).

P. colonization rates were respectively 6.45%, 29.17%, 28.57%, 60%, 60%, and 25.81% in the six groups csDMARD, TNFi, CTLA-4i, IL-6i, anti-CD20 Ab, and JAKi (Table 1 and Fig. 1). No patient developed PJP during the study period. In the multivariate model, after adjusting for age, sex, related comorbidity, and steroid use, patients treated with bDMARDs or JAK inhibitors have a 6-fold higher risk of *P. jirovecii* colonization compared to those treated with csDMARDs (adjusted OR: 6.40, 95% CI 1.34—30.57). Further, we divided bDMARDs according to mechanism of action. Patients currently treated with TNF-α inhibitors, tocilizumab, and rituximab are at a significantly increased risk of *P. jirovecii* colonization, their adjusted odds ratios being 6.84 (95% CI 1.14—41.03, $p = 0.035$), 12.47 (95% CI 1.07—145.17,

Table 1 Characteristics of patients with RA on DMARDs with different mechanism of action.

Variables	csDMARD (N = 31)	TNFi (N = 24)	ABA (N = 14)	TCZ (N = 5)	RTX (N = 5)	JAKi (N = 31)	p-value[a]
	n (%)	n (%)	n (%)	n (%)	n (%)	n (%)	
Age							
≤ 60	21 (67.74)	11 (45.83)	7 (50.00)	1 (20.00)	2 (40.00)	19 (61.29)	0.275
> 60	10 (32.26)	13 (54.17)	7 (50.00)	4 (80.00)	3 (60.00)	12 (38.71)	
Median (IQR)	56.0 (39.0, 61.0)	61.5 (47.5, 68.0)	58.0 (41.0, 71.0)	64.0 (62.0, 67.0)	77.0 (55.0, 84.0)	58.0 (44.0, 65.0)	0.218
Male	6 (19.35)	4 (16.67)	3 (21.43)	0 (0.00)	1 (20.00)	4 (12.90)	0.918
Related Comorbidity							
NTM	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (20.00)	0 (0.00)	0.091
T2DM	1 (3.23)	3 (12.50)	0 (0.00)	0 (0.00)	3 (60.00)	1 (3.23)	0.006
TB	0 (0.00)	4 (16.67)	3 (21.43)	0 (0.00)	0 (0.00)	4 (12.90)	0.102
COPD	0 (0.00)	1 (4.17)	0 (0.00)	0 (0.00)	1 (20.00)	1 (3.23)	0.254
ILD	0 (0.00)	0 (0.00)	0 (0.00)	0 (0.00)	1 (20.00)	0 (0.00)	0.091
CKD	0 (0.00)	5 (20.83)	2 (14.29)	1 (20.00)	2 (40.00)	0 (0.00)	0.001
Cirrhosis	0 (0.00)	1 (4.17)	0 (0.00)	0 (0.00)	0 (0.00)	1 (3.23)	0.840
Malignancy	1 (3.23)	0 (0.00)	0 (0.00)	0 (0.00)	1 (20.00)	0 (0.00)	0.190
Any comorbidity	2 (6.45)	11 (45.83)	5 (35.71)	1 (20.00)	4 (80.00)	8 (25.81)	0.001
Lymphopenia (lymphocyte< 1000 per microliter)							
No	28 (96.55)	23 (100.00)	12 (85.71)	4 (80.00)	4 (80.00)	25 (83.33)	0.079
Yes	1 (3.45)	0 (0.00)	2 (14.29)	1 (20.00)	1 (20.00)	5 (16.67)	
Median (IQR)	2075.0 (1546.0, 3690.0)	1752.0 (1522.0, 2867.0)	1886.5 (1594.0, 2383.0)	1193.0 (1106.0, 1340.0)	1572.0 (1320.0, 1626.0)	1698.5 (1082.0, 2303.0)	0.102
Steroid use							
No	18 (58.06)	17 (70.83)	9 (64.29)	5 (100.00)	5 (100.00)	20 (64.52)	0.322
Yes	13 (41.94)	7 (29.17)	5 (35.71)	0 (0.00)	0 (0.00)	11 (35.48)	
Treatment duration (mo)							
≤ 36	N/A	9 (37.50)	8 (57.14)	4 (80.00)	2 (40.00)	27 (87.10)	0.001
>36	N/A	15 (62.50)	6 (42.86)	1 (20.00)	3 (60.00)	4 (12.90)	
PJ PCR positive	2 (6.45)	7 (29.17)	4 (28.57)	3 (60.00)	3 (60.00)	8 (25.81)	0.014

[a] Fisher's exact test for categorical data and Kruskal–Wallis test for continuous data.
csDMARD: conventional synthetic disease-modifying anti-rheumatic drug; TNFi: tumor-necrosis factor inhibitor; JAKi: Janus kinase inhibitor; TCZ: tocilizumab; ABA: abatacept; RTX: rituximab; NTM: non-tuberculosis mycobacterium; T2DM: type 2 diabetes mellitus; TB: tuberculosis; COPD: chronic obstructive pulmonary disease; ILD: interstitial lung disease; CKD: chronic kidney disease; mo: month. IQR: interquartile range; LYM: lymphocyte; PJ: *Pneumocystis jirovecii*; PCR: polymerase chain reaction.

p = 0.044), 41.79 (95% CI 3.12–558.98, p = 0.005), respectively. Those currently treated with CTLA-4 inhibitor demonstrate a trend toward developing *P. jirovecii* colonization compared to patients on csDMARDs, with adjusted odds ratios of 6.62 (95% CI 0.97–45.30, p = 0.054). Acknowledging the limited number of participants in certain treatment groups, we merged all bDMARDs together, revealing that compared to patients on csDMARDs, patients treated with bDMARDs had an 8-fold higher risk of *P. jirovecii* colonization (adjusted OR: 8.08, 95% CI 1.57-41.51, p = 0.012). A tendency toward *P. jirovecii* colonization was also identified in those receiving JAK inhibitors (adjusted OR: 4.79, 95% CI 0.89-25.91, p = 0.069) (Table 2). We calculated the post-power of CTLA-4i (ABA) and JAKi groups as 0.83, and 0.92 respectively.

In addition, we analyzed the risk factors of *P. jirovecii* colonization among RA patients on bDMARDs or JAK inhibitors (Table 3). Further multivariate regression model analysis showed that among RA patients treated with bDMARDs or JAK inhibitors, age >60 y/o (adjusted OR 3.53, 95% CI 1.06–11.82, p = 0.040) and a bDMARD or JAK inhibitor treatment duration of >3 years (adjusted OR 5.00, 95% CI 1.60–15.64, p = 0.006) were significantly associated with *P. jirovecii* colonization (Table 4).

Discussion

Biologic DMARDs and JAK inhibitors have been used worldwide to treat RA patients with inadequate response to csDMARDs.[1] In comparison to RA patients treated with csDMARDs, we found that those treated with bDMARDs or JAK inhibitors are approximately 6 times more likely to develop *P. jirovecii* colonization. Of the bDMARDs we assessed, patients on TNF-α inhibitors, tocilizumab and rituximab were exposed to nearly 7-fold, 12-fold, and 41-fold increases, respectively, in the risk of *P. jirovecii*

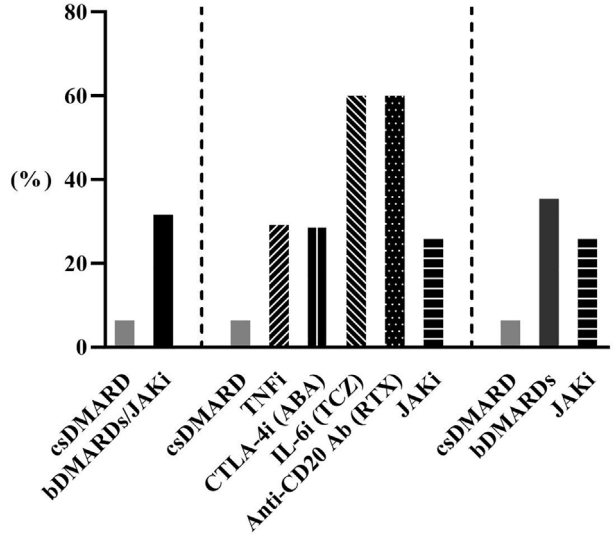

Fig. 1. *P. jirovecii* colonization rates for RA patients in different groups.

Table 2 Risk of *Pneumocystis jirovecii* colonization in RA patients on csDMARDs, bDMARDs, or JAKi.

	Crude OR (95 % CI)	p-value	Adjusted OR (95 % CI)	p-value
Treatment Group				
csDMARD	Ref.		Ref.	
bDMARD/ JAKi	6.71 (1.48 −30.37)	0.013	6.40 (1.34 −30.57)	0.020
Treatment Group				
csDMARD	Ref.		Ref.	
TNFi	5.97 (1.11 −32.09)	0.037	6.84 (1.14 −41.03)	0.035
CTLA-4i (ABA)	5.80 (0.92 −36.64)	0.062	6.62 (0.97 −45.30)	0.054
IL-6i (TCZ)	21.75 (2.20 −215.26)	0.009	12.47 (1.07 −145.17)	0.044
Anti-CD20 Ab (RTX)	21.75 (2.20 −215.26)	0.009	41.79 (3.12 −558.98)	0.005
JAKi	5.04 (0.98 −26.09)	0.054	4.93 (0.91 −26.84)	0.065
Treatment Group				
csDMARD	Ref.		Ref.	
bDMARD	7.95 (1.69 −37.47)	0.009	8.08 (1.57 −41.51)	0.012
JAKi	5.04 (0.98 −26.09)	0.054	4.79 (0.89 −25.91)	0.069

Adjusted variables: age, sex, steroid use, and any comorbidity.
csDMARD: conventional synthetic disease-modifying anti-rheumatic drug; bDMARD: biologic disease-modifying anti-rheumatic drug.
JAKi: Janus kinase inhibitor; TNFi: tumor-necrosis factor inhibitor; IL-6i: interleukin-6 inhibitor; TCZ: tocilizumab.
CTLA-4i: cytotoxic T-lymphocyte-associated antigen 4 inhibitor.
ABA: abatacept; Anti-CD20 Ab: anti-CD20 monoclonal antibody; RTX: rituximab.

Table 3 Pneumocystis jirovecii colonization status of RA patients on bDMARD or JAK inhibitor.

Variables	Positive (N = 25)	Negative (N = 54)	p-value
	n (%)	n (%)	
Age			
≤ 60	10 (40.00)	30 (55.56)	0.296
> 60	15 (60.00)	24 (44.44)	
Median (IQR)	61.0 (52.0, 68.0)	58.0 (43.0, 69.0)	0.378
Male	1 (4.00)	11 (20.37)	0.091
Related Comorbidity			
NTM	0 (0.00)	1 (1.85)	1.000
T2DM	4 (16.00)	3 (5.56)	0.199
TB	1 (4.00)	10 (18.52)	0.159
COPD	2 (8.00)	1 (1.85)	0.234
ILD	1 (4.00)	0 (0.00)	0.317
CKD	3 (12.00)	7 (12.96)	1.000
Cirrhosis	0 (0.00)	2 (3.70)	1.000
Malignancy	0 (0.00)	1 (1.85)	1.000
Patients with any comorbidity	7 (28.00)	22 (40.74)	0.400
Steroid			
No	19 (76.00)	37 (68.52)	0.679
Yes	6 (24.00)	17 (31.48)	
Lymphopenia (lymphocyte <1000 per microliter)			
No	21 (84.00)	47 (90.38)	0.461
Yes	4 (16.00)	5 (9.62)	
Medication Duration (mo)			
≤ 36m	11 (44.00)	39 (72.22)	0.030
> 36m	14 (56.00)	15 (27.78)	
Median (IQR)	1539.0 (1121.0, 1926.0)	1810.5 (1408.5, 2502.0)	0.224

bDMARD: biologic disease-modifying anti-rheumatic drug; JAKi: Janus kinase.
NTM: non-tuberculosis mycobacterium; T2DM: type 2 diabetes mellitus; TB: tuberculosis; COPD: chronic obstructive pulmonary disease; ILD: interstitial lung disease; CKD: chronic kidney disease mo: month.

colonization, when compared to RA patients on csDMARDs. However, the odds ratio may be unstable due to the small number of events. Recognizing the limited number of participants in certain bDMARD groups, we merged all bDMARDs together for further analysis. The results still showed that in comparison with patients on csDMARDs, patients with RA treated with bDMARDs had significantly higher risk of *P. jirovecii* colonization.

Two previous studies have investigated *P. jirovecii* colonization among TNF-α inhibitor users.[19,20] Fritzsche et al. reported higher prevalence of *P. jirovecii* colonization with TNF-α inhibitors than with only csDMARDs in rheumatic patients.[19] Wissmann et al. discovered infliximab treatment duration was significantly associated with *P. jirovecii* colonization among patients with inflammatory arthropathies.[20] Post-marketing surveillance studies of infliximab, etanercept, and adalimumab therapies among RA patients in Japan reported PJP incidence rates of 0.4%, 0.2%, and 0.3%, separately.[9–11] However, no study

Table 4 Risk factors of *Pneumocystis jirovecii* colonization in RA patients on bDMARD/JAKi.

Group (bDMARD/JAKi) (N = 79)	Crude OR (95 % CI)	p-value	Adjusted OR (95 % CI)	p-value
Age				
≤ 60	Ref.		Ref.	
>60	1.88 (0.72–4.91)	0.201	3.53 (1.06–11.82)	0.040
Gender				
Female	Ref.		Ref.	
Male	0.16 (0.02–1.34)	0.091	0.08 (0.01–0.79)	0.031
Steroid				
No	Ref.		Ref.	
Yes	0.69 (0.23–2.03)	0.497	0.92 (0.25–3.32)	0.895
Patients with any comorbidity				
No	Ref.		Ref.	
Yes	0.57 (0.20–1.58)	0.278	0.30 (0.08–1.08)	0.066
Treatment duration (mo)				
≤ 36 m	Ref.		Ref.	
>36 m	3.31 (1.23–8.90)	0.018	5.00 (1.60–15.64)	0.006

bDMARD: biologic disease-modifying anti-rheumatic drug; JAKi: Janus kinase inhibitor; mo: month.

addressed the prevalence of *P. jirovecii* colonization with other classes of bDMARD and JAK inhibitor. Our study is the first to identify that in RA patients, not only TNF-α inhibitors but also IL-6 inhibitor and anti-CD20 Ab are associated with increased risk of *P. jirovecii* colonization.

Our study result suggests that TNF-α, IL-6, and B lymphocyte may play roles in immune responses toward *P. jirovecii* colonization, which echoes previous research.[26–29] Patil et al., using an HIV-infected monkey model, revealed elevated levels of TNF-α in bronchoalveolar lavage had persisted for weeks to months before respiratory symptoms of PJP appeared.[26] Another study suggested that COPD patients colonized with pneumocystis had elevated serum interleukin-6 (IL-6) and TNF-α in peripheral blood.[27] Therefore, using TNF-α and IL-6 inhibitors may interfere with immune system activation against *P. jirovecii*, leading to a higher colonization rate. Evidence indicates that both anti-pneumocystis antibody production and activation of CD4+T cells are important protective components of the B cell against pneumocystis infection,[28,29] which can be observed indirectly in clinical studies revealing PJP after B cell-depletion therapy.[30–32] These findings indirectly support our result that certain cytokine inhibitors ameliorate the systemic immune response toward *P. jirovecii*, thus potentially increasing the risk of *P. jirovii* colonization.

We also found that among RA patients treated with bDMARDs or JAK inhibitors, medication duration >3 years, and age >60 y/o are risk factors of *P. jirovecii* colonization. Our study mirrored the published results.[19,20] Fritzsche et al. found that age >60 years was significantly associated with *P. jirovecii* colonization in rheumatic patients on current steroid therapy.[19] Wissmann et al. examined oropharyngeal washes collected from 125 patients with RA and spondyloarthropathy, reporting that a duration of infliximab treatment >3 years markedly increased the risk of *P. jirovecii* colonization.[20]

Steroid use does not show as increasing risk of *P. jirovecii* colonization in our study, which may be explained by evidently fewer steroid users and lower daily steroid usage of our participants when compared to other studies.[18,33] With 58% of their study population exposed to steroids, Mekinian et al. reported a significant influence of steroid use on *P. jirovecii* colonization in rheumatic patients on higher daily steroid doses (25 mg per day).[18] A further study found significant correlation between recent one-week corticosteroid use and *P. jirovecii* colonization among ICU patients, with daily steroid doses ranging from 8 to 336 mg per day (average accumulative dose of 86.22 mg during 7 days).[33] In our study, fewer than one-third of participants were exposed to steroids; the median daily steroid dose among steroid users was 6.22 mg. The impact of low-dose steroid therapy on *P. jirovecii* colonization is likely to be small. Besides, since daily steroid of >20 mg for more than 1 month is reported as a risk factor of PJP,[34] lower concurrent steroid dosage may also explain why no incident PJP case appeared in our study during the study period, which is in concordance with previous literature.[18–20]

In contrast to previous studies focusing on PJP, our study did not reveal a link between lymphopenia and *P. jirovecii* colonization. However, our study result is consistent with previous study results.[16–20] The different lymphocytic response toward *P. jirovecii* colonization and PJP may indicate different underlying immune mechanisms, requiring further study for elucidation.

Our study underlines the importance of early identification of *P. jirovecii* colonization among patients with RA treated with certain bDMARDs. In a previous study using the Taiwan National Health Insurance Research Database, Hsu et al. reported that patients with autoimmune rheumatic disease (ARD) had a significantly higher incidence of PJP than the comparisons (0.18% vs. 0.001%, $p < 0.0001$). The risk of PJP is increased in patients with RA (IR 1.46, IRR 6.19, 95% CI 4.31–8.89).[35] Despite the relatively low incidence of PJP in ARD, the clinical course of PJP in patients with RA is often fulminant and life-threatening.[7] The current study highlights the critical importance of early identification of *P. jirovecii* colonization for at-risk patients receiving treatment for RA.

There are several strengths in our study. First, we used quantitative real-time PCR targeting mitochondrial multicopy genes in respiratory specimens to detect *P. jirovecii* colonization, a method which has shown great sensitivity[21,36,37] and good accuracy in non-invasive respiratory specimens such as oral washes.[38,39] Second, we restricted our study population to rheumatoid arthritis patients to improve comparability. Third, by adjusting for age, sex, steroid use, and related comorbidities, we limited the impact of confounding variables.

There are some limitations to the current study. In the COVID-19 pandemic, concern about spreading the virus through airborne droplets while obtaining our saliva specimens had a slight influence on our sample size. The significance of links between certain bDMARDs and *P. jirovecii* colonization should be interpreted with caution due to the

relatively small number of events. Another limitation is that since the csDMARD group contains patients with combination treatment regimens, including hydroxychloroquine, methotrexate, sulfasalazine, and leflunomide, it is difficult to evaluate the effect of individual csDMARDs on *P. jirovecii* colonization. Third, since the incidence of PJP in patients with RA is rare,[40] no PJP cases occurred during the study, which may be also attributable to a lower proportion of steroid users, as well as a lower concurrent daily steroid dosage among our participants, indicating that these patients may be less vulnerable to PJP infection. Fourth, randomization of participants into different DMARD treatment groups was not conducted in our study, which may lead to selection bias. Further investigation with a larger sample size and longer follow-up may be warranted to explore the long-term implications of *P. jirovecii* colonization in this population, as well as the correlation between *P. jirovecii* colonization and PJP.

Through advanced diagnostic tools and strict methodology, our study found that for RA patients treated with bDMARDs or JAK inhibitors there is an almost 6-fold increase in the risk of *P. jirovecii* colonization, compared to patients on csDMARDs. Notably, patients treated with bDMARDs had an 8-fold higher risk of *P. jirovecii* colonization. Risk factors of *P. jirovecii* colonization are age more than 60 y/o and medication duration more than 3 years. These findings enable improved awareness of *P. jirovecii* colonization impacting RA patients treated with bDMARDs or JAK inhibitors.

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CRediT authorship contribution statement

Ya-Chun Huang: Writing — original draft, Methodology, Investigation, Formal analysis, Data curation. **Nan-Yao Lee:** Methodology, Funding acquisition, Data curation, Conceptualization. **Meng-Yu Weng:** Writing — review & editing, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization.

Declaration of competing interest

All authors have no conflict of interest to declare.

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