筑　波　大　学

博士（医学）学位論文

Clinical research on the preventive efficacy of sulfasalazine against *Pneumocystis jirovecii* pneumonia in patients with rheumatoid arthritis

(関節リウマチ患者におけるスルファサラジンのニューモシスチス・イロベチイ肺炎予防効果に関する臨床研究)

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Abbreviations and acronyms

PJP	*Pneumocystis jirovecii* pneumonia
RA	rheumatoid arthritis
SSZ	sulfasalazine
PCR	polymerase chain reaction
TNF	tumor necrosis factor
DMARD	disease-modifying anti-rheumatic drug
HIV	human immunodeficiency virus
BAL	bronchoalveolar lavage
BDG	1,3-β-D-glucan
TMP/SMX	trimethoprim/sulfamethoxazole
OR	odds ratio
CI	confidence interval
MTX	methotrexate
bDMARD	biological disease-modifying antirheumatic drug
IRIS	immune reconstitution inflammatory syndrome
NinJa	the National Database of Rheumatic Diseases by iR-net in Japan
GC	glucocorticoid
TAC	tacrolimus
AOR	adjusted odds ratio
CLR	conditional logistic regression

Summary

Pneumocystis jirovecii pneumonia (PJP) is an opportunistic infection caused by the fungus *Pneumocystis jirovecii*. With the increase in the use of immunosuppressive drugs, PJP is increasingly being reported in patients with rheumatoid arthritis (RA). Since PJP can be life-threatening, the prevention of the infection is an important challenge in clinical practice in RA. Sulfasalazine (SSZ) is one of the traditional disease-modifying anti-rheumatic drugs. Recently, an experimental study suggested the preventive efficacy of SSZ against PJP. However, so far, there has been just one clinical study with a small sample size regarding this topic. In this thesis, I investigated the prophylactic effect of SSZ by the two types of case-control studies.

Summary of study 1

***Objective*:** To evaluate the effect of sulfasalazine (SSZ) on the presence of *Pneumocystis jirovecii* (*P. jirovecii*) in the lungs of rheumatoid arthritis (RA) patients.

***Methods*:** I retrospectively studied episodes of suspected *P. jirovecii* pneumonia (PJP) which were examined for *P. jirovecii* with polymerase chain reaction (PCR). I employed a test-negative design case-control study; the cases were episodes of suspected PJP that were positive for PCR, and the controls were episodes of suspected PJP that were negative

for PCR. The odds ratio for the positive PCR result associated with SSZ use was estimated by Firth's logistic regression.

***Results*:** Between 2003 and 2017, 36 cases and 83 controls were identified. While none of the cases received SSZ before the episode, 18 of the controls received the drug. In the primary analysis involving all the episodes, SSZ use was negatively associated with PCR positivity (adjusted odds ratio, 0.087; confidence interval, <0.001-0.789). The sensitivity analysis, excluding those who received PJP prophylaxis, showed the same association as the primary analysis (adjusted odds ratio 0.085, 95% CI <0.001-0.790).

***Conclusion*:** This study demonstrated that SSZ use is associated with the absence of *P. jirovecii* in the lung, suggesting the preventive efficacy of the drug against PJP.

Summary of study 2

***Objectives*:** To evaluate the prophylactic effect of sulfasalazine against *Pneumocystis jirovecii* pneumonia (PJP) among rheumatoid arthritis (RA) patients.

***Methods*:** I used a nationwide Japanese multicenter RA database to extract data from 2005 to 2014. To identify PJP cases, I selected patients hospitalized for PJP and verified their diagnosis. Two control groups, one unmatched and the other matched for age, sex, glucocorticoid, methotrexate, and tacrolimus dosage, and the use (and type, if used) of

biological disease-modifying antirheumatic drug were selected by incidence-density sampling. The odds ratios for PJP associated with sulfasalazine use and other clinical factors were estimated by exact and standard conditional logistic regression.

***Results*:** From 18,668 participants, 60 cases, 356 unmatched controls, and 337 matched controls were selected. None of the cases received sulfasalazine before PJP onset. A comparison of the cases with the unmatched controls showed that sulfasalazine use carried a decreased risk of PJP (adjusted odds ratio 0.18, 95% confidence interval 0.00–0.92). A comparison of the cases and matched controls also showed that sulfasalazine use had a decreased risk of PJP (0.08, 0.00–0.36). In an analysis of the cases and unmatched controls who did not receive sulfasalazine, an increased risk of PJP was associated with lung disease (3.88, 1.89–7.95) and the use of glucocorticoid (5.71, 2.68–12.19), methotrexate (5.25, 2.01–13.74), and tumor necrosis factor inhibitors (2.32, 1.10–4.93).

***Conclusions*:** The results of this nested case-control study demonstrated the preventive effect of sulfasalazine against PJP. The results await confirmation by future prospective studies.

Chapter 1

Overall background

Pneumocystis jirovecii pneumonia (PJP) is an opportunistic infection caused by the fungus *Pneumocystis jirovecii* and often affects patients infected with the human immunodeficiency virus. With the increase in the use of immunosuppressive drugs, especially methotrexate and biological agents, PJP is increasingly being reported in patients with rheumatoid arthritis (RA) [1]. Since PJP can be life-threatening, the prevention of the infection is an important issue in clinical practice in RA.

Trimethoprim/sulfamethoxazole has been used as first-line prophylactic agent against PJP because of its proven efficacy. Sulfasalazine (SSZ) is one of the traditional disease-modifying anti-rheumatic drugs and has the anti-inflammatory and antibiotic properties. Recently, an experimental study suggested the preventive efficacy of SSZ against PJP [2]. If the efficacy is demonstrated in patients with RA, it has important clinical implications. However, so far, there has been just one clinical study with a small sample size regarding this topic [3]. In this thesis, I investigated the prophylactic effect of SSZ by the two types of case-control studies.

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Chapter 2

Effect of sulfasalazine use on the presence of *Pneumocystis* organisms in the lung among patients with rheumatoid arthritis: A test-negative design case–control study with PCR tests

(This is the pre-peer reviewed version of the following article: Effect of sulfasalazine use on the presence of *Pneumocystis* organisms in the lung among patients with rheumatoid arthritis: A test-negative design case–control study with PCR tests. Takahiro Nunokawa, Naoto Yokogawa, Kota Shimada and Shoji Sugii. Mod Rheumatol. 2018 May 3:1-5, which has been published in final form at https://doi.org/10.1080/14397595.2018.1465647. Underlined parts were added to the pre-peer reviewed version)

Introduction

Pneumocystis jirovecii pneumonia (PJP) is an opportunistic lung infection caused by *Pneumocystis jirovecii* (*P. jirovecii*) and can be life-threatening. With the increasing use of immunosuppressive drugs, particularly tumor necrosis factor (TNF) inhibitors, PJP has been reported in patients with rheumatoid arthritis (RA) [1]. Sulfasalazine (SSZ), a traditional, synthetic, disease-modifying anti-rheumatic drug (DMARDs), is a conjugate

of the anti-inflammatory 5-aminosalicylic acid and the antibacterial sulfapyridine [2]. One experimental animal study reported that SSZ enhances *Pneumocystis* clearance without exacerbating inflammation [3]. However, so far, there has been just one, small, case-control study on the topic [4]. Although this study indicated the preventive potential of SSZ against PJP, further investigation is needed because of its small sample size of ten cases. The case-control study is a practical and efficient design when dealing with a rare complication like PJP. However, a misclassification of the outcome status can happen in a case-control study in which the outcome is PJP development because making a definitive diagnosis of PJP by microscopic detection of the pathogenic organism in patients not infected with human immunodeficiency virus (HIV) is difficult due to the small organism burden [5]. Therefore, I conducted a case-control study in which the outcome was the presence of *P. jirovecii* in the lung instead of PJP development. Furthermore, I employed the test-negative design including only subjects tested with polymerase chain reaction (PCR) to confirm the outcome status of all subjects without misclassification [6,7].

Method

Study design and participants

This retrospective case-control study was conducted at Tokyo Metropolitan Tama Medical Center in Japan. The study period was from July 2003 to January 2017. All episodes of suspected PJP among patients with RA, whose respiratory specimens were tested with PCR for *P. jirovecii* in any department of the hospital, were enrolled, including patients referred to the hospital for acute symptoms. I used the test-negative design to evaluate the association between SSZ use and PCR positivity; the cases were all episodes of suspected PJP that had a positive PCR result, and the controls were all episodes of suspected PJP that had a negative PCR result. In an individual with multiple episodes of suspected PJP for which PCR was performed, each episode was counted individually.

This study was conducted in accordance with the Declaration of Helsinki and was approved by the ethical committee of Tokyo Metropolitan Tama Medical Center.

Data collection

The demographic data and clinical information were collected from medical charts retrospectively. The enrolled patients were categorized into two age groups: <65 and ≥65 years. For the detection of *P. jirovecii*, respiratory specimens, either of induced sputum or

bronchoalveolar lavage (BAL) or both, were examined with qualitative PCR analysis at SRL Inc. (Tokyo, Japan), and a patient was considered to be positive for *P. jirovecii* if at least one PCR of these specimens was positive. These specimens were also microscopically analyzed with Grocott stain. Serum 1,3-β-D-glucan (BDG) was measured by Fungitec G test MK or MK Π (Seikagaku, Tokyo, Japan) using their cut-off value. Since the remote use of medications for RA and PJP prophylaxis was considered not to be associated with PCR positivity, the medications prescribed at the last visit before the onset of PJP were collected. Trimethoprim/sulfamethoxazole (TMP/SMX), dapsone, atovaquone, pentamidine, and pyrimethamine were considered to be prophylactic drugs against PJP. Data on comorbidities, including cases of diabetes mellitus requiring treatment with medication, liver cirrhosis, and malignancies, were collected. A malignancy without any recurrence for more than five years after the completion of treatment was excluded. Estimated glomerular filtration rate was calculated using a formula developed by the Japanese Society of Nephrology and was categorized as <60 or ≥60 ml per min/1.73 m^2 [8]. The HIV status of the patients was not collected.

Statistical analysis

I determined the difference between the cases and controls using Fisher's exact test

for categorical variables and Student's t test for continuous variables. A p-value <0.05 was considered significant. To estimate the unadjusted and adjusted odds ratios (ORs) and the corresponding 95% confidence intervals (CIs) for the association between SSZ use and the PCR positivity, I used a logistic regression model. On the basis of prior knowledge, the possible confounders were identified as age group, lung diseases, malignancies, the use of PJP prophylaxis, types of respiratory specimen tested with PCR (induced sputum, BAL, or both), and the use of corticosteroid, methotrexate, tacrolimus, biological DMARDs (TNF inhibitors, tocilizumab, and abatacept), and immunosuppressants (cyclophosphamide, cyclosporine A, and azathioprine) [9–19]. Among these variables, those yielding p-values <0.2 in univariate analysis were included in the adjusted model of the primary analysis. Coefficients in the logistic regression model were estimated using Firth's penalized likelihood method instead of the maximum likelihood method since the issue of quasi-complete separation arose in this data set [20]. Specifically, PJP did not develop in any of the RA patients receiving SSZ or PJP prophylactics. A sensitivity analysis was performed, limiting the episodes to those without PJP prophylaxis. In the sensitivity analysis, unadjusted and adjusted ORs were estimated using the same statistical method as the primary analysis, and the adjusted model included the covariates used in the primary analysis except the use of PJP prophylaxis. All analyses

were done with R (R Foundation for Statistical Computing, Vienna, Austria).

Results

During the study period, 119 episodes of suspected PJP in 95 patients with RA were tested with PCR. Of all the episodes, 36 (30.3%) had positive and 83 (69.7%) had negative PCR results and were included in my analyses as the cases and the controls, respectively. There were no missing data in any of the variables. The clinical characteristics of the cases and the controls are summarized in Table 1. There were no significant differences in sex, age, disease duration or the prevalence of comorbidities. While none of the cases received any prophylactic drugs against PJP, 16 of the controls received one drug (14: TMP/SMX, 1: atovaquone, and 1: pentamidine). The diagnosis of PJP was made in 31 (86.1%) cases, but in only eight (9.6%) of the controls. The cases presented with hypoxia, fever, and dyspnea and needed O2 supplementation more frequently than the controls. PCR was performed for the BAL samples in 30 cases (83.3%) and 47 controls (56.6%). Interstitial infiltrate on chest imaging and positive BDG results were more frequent in the cases than in the controls (100.0% vs 68.7% and 77.8% vs 34.9%, respectively). All the cases and 48 (57.8%) of the controls received antimicrobial treatment for PJP. Mortality was similar in the two groups (22.2% vs 19.5%).

The RA medications given to the two groups are shown in Table 2. While none of the cases was treated with SSZ, 18 of the controls were treated with SSZ. Methotrexate was used significantly more often for the cases than for the controls (75.0% vs 48.2%). The use and dosage of other RA medications were similar between the two groups.

Based on univariate analysis, the use of PJP prophylaxis, types of respiratory specimen, and the use of corticosteroid, methotrexate, and tumor necrosis factor inhibitors were introduced into the adjusted model of the primary analysis, and the same variables except the use of PJP prophylaxis were introduced into adjusted model of the sensitivity analysis. The primary analysis showed that SSZ use was negatively associated with PCR positivity in both the unadjusted model (OR 0.049, 95% CI <0.001-0.374) and adjusted model (OR 0.087, CI <0.001-0.789). The sensitivity analysis involving only those who did not receive PJP prophylaxis (all the cases and 67 controls) showed the same association as in the primary analysis in both the unadjusted model (OR 0.051, 95% CI <0.001-0.400) and adjusted model (OR 0.085, 95% CI <0.001-0.790).

As for the predictor variables other than SSZ, in the primary analysis with the adjusted model, the use of PJP prophylaxis (OR 0.055, <0.001-0.489) and corticosteroid (OR 3.572, <1.288-11.313) showed a negative and positive association with PCR positivity, respectively, whereas types of respiratory specimen (OR 1.615, <0.875-3.052) and the

use of methotrexate (OR 1.575, 0.529-4.939) and tumor necrosis factor inhibitors (OR 1.393, 0.438-4.479) were not associated with PCR positivity. The result did not substantially differ in the sensitivity analysis.

Discussion

I hypothesized that SSZ use was associated with the absence of *P. jirovecii* in the lung and tested this hypothesis with a test-negative design case-control study using PCR. In both the primary and sensitivity analysis, SSZ use was associated with a negative PCR result. This finding corroborates the result of a previous case-control study by Mizushina *et al.,* which indicated that SSZ has a preventive effect against PJP [4].

Person-to-person airborne transmission is thought to be the most likely route of PJP transmission based on previous studies [21–23], and Mori *et al.* reported an asymptomatic carrier state of *P. jirovecii* and the subsequent development of PJP among RA patients receiving low-dose MTX therapy [24]. Wang *et al.* reported in their experimental study using a mouse model of PJP-related immune reconstitution inflammatory syndrome that SSZ enhances *Pneumocystis* clearance from the lung without inducing inflammation by accelerating the $CD4^+$ T cell-dependent alveolar macrophage phagocytic response and by promoting a TH2-polarized cytokine environment, which alters the macrophage

phenotype [3]. These findings suggest that SSZ may prevent RA patients exposed to *P. jirovecii* from pooling the organisms in their lungs and subsequently developing PJP.

The gold standard of diagnosis of PJP is the microscopic detection of the organisms in respiratory specimens [25]. However, in patients not infected with HIV, the sensitivity of the microscopic analysis is low due to a low organism burden [5]. Previous case-control studies of PJP among RA patients used diagnostic criteria consisting of the presence of either a positive PCR or an increased BDG level, clinical manifestations and radiological findings consistent with PJP, and response to standard treatment for PJP [12,26,27]. However, since it is difficult to validate the diagnostic criteria due to the lack of a reference standard, it is possible that PJP diagnoses based on these criteria may have led to the misclassification of the outcome status. Although the specificity of PCR for PJP is not high because the test cannot distinguish the disease from *Pneumocystis* colonization, the test is the most appropriate for examining the presence of the organisms in the lungs of individuals not infected with HIV regardless of whether the condition is *Pneumocystis* colonization or PJP [28]. In view of the previous reports of PJP development following colonization [24,29], my findings of the negative correlation between SSZ use and the presence of *P. jirovecii* in the lung can be seen as corroborating the prophylactic effectiveness of SSZ against PJP.

Considering that PJP is a rare complication among RA patients and that the conditions for SSZ use change with time in a RA patient, the cohort study is impractical for my purposes. The case-control study can circumvent these problems. However, without confirmation of laboratory test results for the controls, the traditional case-control study may result in misclassification of the outcome status [6]. Therefore, I employed the test-negative design for my case-control study to reduce the risk of misclassification of the outcome status by enrolling only patients with a PCR result [7].

My study has several limitations. Since it was a single institution study, my subjects cannot be considered as representative of RA patients in Japan. Furthermore, the small sample size of my study may have caused the quasi-separation of my data requiring special statistical methods. In the logistic regression analysis of data with quasi-separation, coefficients cannot be appropriately calculated by maximum likelihood estimation, the standard method [30]. Therefore, I used Firth's penalized likelihood method which allows the coefficient to be correctly estimated [20]. Further investigation with a larger sample size involving other populations is needed to verify the generalizability of my findings and to confirm my results using more straightforward analyses.

In conclusion, my test-negative design case-control study using PCR tests revealed that SSZ use is associated with the absence of *P. jirovecii* in the lung of RA patients,

suggesting a preventive efficacy of the drug against PJP.

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Tables

Table 1. Characteristics of the cases and controls

	Cases (n=36)	Controls (n=83)	P value
Sex, male	8 (22.2)	25 (30.1)	0.504
Age, year	69.2 ± 7.6	72.2 ± 11.8	0.167
Age ≥65 years	25 (69.4)	72 (86.7)	0.038
Disease duration, year	13.7 ± 14.0	11.6 ± 13.7	0.450
Comorbidities			
Lung diseases	19 (52.8)	51 (61.4)	0.421
Malignancies	1 (2.8)	6 (7.2)	0.673
Liver cirrhosis	0 (0.0)	1 (1.2)	1.000
Diabetes mellitus	7 (19.4)	20 (24.1)	0.641
eGFR <60 mL/min/1.73 m^2	13 (36.1)	29 (34.9)	1.000
PJP prophylaxis use	0 (0.0)	16 (19.3)	0.003
Clinical diagnosis of the episode			<0.001
PJP	31 (86.1)	8 (9.6)	
Possible PJP	3 (8.3)	19 (22.9)	
Non-PJP	2 (5.6)	56 (67.5)	
Hypoxia[a]	29 (85.3)	49 (62.8)	0.024
O_2 supplementation	34 (94.4)	64 (77.1)	0.034
Fever (≥37.5°C)	27 (75.0)	41 (51.2)	0.024
Dyspnea	32 (88.9)	56 (67.5)	0.022
Cough	24 (66.7)	48 (59.3)	0.538
Type of specimen examined with PCR			0.011
Sputum only	6 (16.7)	36 (43.4)	
Bronchoalveolar lavage fluid only	19 (52.8)	34 (41.0)	
Both	11 (30.6)	13 (15.7)	
Interstitial infiltrate on chest imaging	36 (100.0)	57 (68.7)	<0.001
1,3-β-D-glucan positive	28 (77.8)	29 (34.9)	<0.001
Antimicrobial treatment for PJP	36 (100.0)	48 (57.8)	<0.001
Outcome of the episode, death	8 (22.2)	17 (19.5)	0.805

[a]Hypoxia was defined as PaO2 less than 60 mmHg or SpO2 less than 90% in ambient air.

Values are the mean ± SD or the number (%).

eGFR, estimate glomerular filtration rate; PCR, polymerase chain reaction; PJP, *Pneumocystis jirovecii* pneumonia

Table 2. Treatment for rheumatoid arthritis in the cases and controls

	Cases (n=36)	Controls (n=83)	P value
Sulfasalazine use	0 (0.0)	18 (21.7)	0.001
Sulfasalazine dose (mg/day)	0.0 ± 0.0	210.8 ± 414.1	0.003
Corticosteroid use	30 (83.3)	56 (67.5)	0.117
Corticosteroid dose (mg/day)	4.39 ± 3.44	5.65 ± 7.29	0.323
Methotrexate use	27 (75.0)	40 (48.2)	0.009
Methotrexate dose (mg/week)	5.97 ± 4.20	6.23 ± 22.01	0.944
Tacrolimus use	4 (11.1)	9 (10.8)	1.000
Tacrolimus dose (mg/day)	0.15 ± 0.49	0.19 ± 0.60	0.727
Bucillamine use	6 (16.7)	10 (12.0)	0.562
Iguratimod use	1 (2.8)	5 (6.0)	0.666
Leflunomide use	0 (0.0)	2 (2.4)	1.000
Tofacitinib use	0 (0.0)	0 (0.0)	NA
Tumor necrosis factor inhibitor use	8 (22.2)	10 (12.0)	0.171
Tocilizumab use	0 (0.0)	2 (2.4)	1.000
Abatacept use	0 (0.0)	0 (0.0)	NA
Other DMARD use	2 (5.6)	1 (1.2)	0.217
Immunosuppressive drug use	1 (2.8)	8 (9.6)	0.274

Values represent the mean ± SD or the number (%).

DMARD, disease modified anti-rheumatic-drug; NA, not applicable

Table 3. Sulfasalazine use and positive PCR results

	Primary analysis	**Sensitivity analysis**
	Odds ratio (95% CI)	Odds ratio (95% CI)
Unadjusted	0.049 (<0.001-0.374)	0.051 (<0.001-0.400)
Adjusted	0.087 (<0.001-0.789)[a]	0.085 (<0.001-0.790)[b]

[a]Adjusted for age group, use of prophylaxis against *Pneumocystis jirovecii* pneumonia, types of respiratory specimen, use of corticosteroid, methotrexate, and tumor necrosis factor inhibitors.

[b]Adjusted for age group, types of respiratory specimen, use of corticosteroid, methotrexate, and tumor necrosis factor inhibitors.

CI, confidence interval

Chapter 3

Prophylactic effect of sulfasalazine against *Pneumocystis* pneumonia in patients with rheumatoid arthritis: A nested case-control study

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Introduction

Pneumocystis jirovecii pneumonia (PJP) mainly occurs in immunocompromised individuals and has also been reported among rheumatoid arthritis (RA) patients, especially those receiving low-dose methotrexate (MTX) [1,2] and biological (b) disease-modifying antirheumatic drugs (DMARDs) [3–8]. PJP incidence among RA patients is low but when it does occur, it can be fatal. Regardless of the underlying disease or condition, trimethoprim/sulfamethoxazole (TMP/SMX) is recommended as first-line prophylaxis for PJP based on its proven efficacy [9,10]. Sulfasalazine (SSZ) is a traditional DMARD and consists of anti-inflammatory 5-aminosalicylic acid and anti-bacterial sulfapyridine. Recently, an experimental study using mice with PJP-related immune reconstitution inflammatory syndrome (IRIS) reported that SSZ enhanced fungal clearance from the lungs [11]. Furthermore, in a case-control study of PJP and SSZ among

RA patients treated with low-dose MTX, no PJP cases received SSZ before PJP onset [12]. Although this finding suggested a possible preventive effect of SSZ against PJP, it was not conclusive because only ten cases were studied. The present study aimed to examine the hypothesis that SSZ lowers PJP incidence among RA patients. To this end, I conducted a nested case-control study with unmatched and matched control groups using a nationwide, Japanese, multicenter RA database.

Methods

Data smyce and study design

I obtained data from the National Database of Rheumatic Diseases by iR-net in Japan (NinJa), a dynamic cohort which commenced in 2002 and is one of the largest registries of RA patients in Japan [13]. Patients fulfilling the standard classification criteria for RA [14,15] were continually added to the cohort, and their clinical data have been accumulated yearly. Hospitalizations in a given year and their reasons, and the RA medications prescribed at a single visit arbitrarily chosen by a physician from all the visits in the given year (referred to as the data point of the year) were registered. However, part of the requisite information for my study, including PJP prophylaxis and co-morbidities, was not recorded in NinJa. Furthermore, the follow-up period of patients registered in

NinJa varied as did the status of the use of SSZ and other medications over time. Therefore, I adopted the nested case-control design with incidence-density sampling. I sampled both the unmatched and matched controls for the same cases and analyzed them separately (henceforth the unmatched and matched study, respectively) because previous reports on PJP risk among RA patients are limited to case reports [1,2,16], post-marketing surveillance [5–8], and case-control studies of patients receiving tumor necrosis factor (TNF) inhibitors [17–19]. I used the data from 2005 to 2014 because hospitalizations for PJP began to be registered after 2005. I excluded patients from hospitals which withdrew from NinJa before 2014. This study was conducted in compliance with the Declaration of Helsinki principles and was approved by the local ethics committee or institutional review board at each participating site.

Case-control selection and additional data collection

Initially, patients hospitalized for PJP during the study period were identified from NinJa, and then diagnostic information on PJP and data on PJP prophylaxis and co-morbidities at the last visit before the hospitalization were collected retrospectively by reviewing their medical charts (Fig.1). Regarding RA medications, I also retrospectively obtained the data from the last outpatient visit prior to hospitalization instead of using the

data from NinJa due to the arbitrary time point of the data in this registry. Since the diagnoses were made at each participating hospital without standardized diagnostic criteria, I verified the diagnostic information and considered as cases those meeting the following criteria based on a modification of the definitions from previous studies of PJP in RA patients [17–19]: (1) detection of *P. jirovecii* in respiratory specimens [by microscopy with staining or polymerase chain reaction (PCR)] and/or increased level of serum 1,3-β-D-glucan(BDG); (2) clinical manifestations (pyrexia, dry cough, or dyspnea); (3) diffuse interstitial infiltrate on chest imaging; (4) absence of PJP prophylaxis, including TMP/SMX, dapsone, atovaquone, pentamidine, and pyrimethamine. An increased level of BDG was defined as a level above the commercial cut-off value according to any of following assays: Fungitec G test MK or MK Π; Seikagaku, Tokyo, Japan, Wako β-glucan test; Wako Pure Chemical Industries, Tokyo, Japan, or β-glucan test MARUHA; Maruha Nichiro Corporation, Tokyo Japan. The absence of PJP prophylaxis was added to my definition because those receiving the prophylaxis were unlikely to develop PJP, especially when receiving TMP/SMX, a highly effective prophylactic [9,10]. I did not include hypoxemia or response to the standard treatment for PJP in my definition as previous studies have done because the severity of PJP varies widely; while the clinical awareness about PJP among non-human

immunodeficiency virus (HIV)-infected individuals has made the diagnosis of this condition before the development of respiratory failure more common [20,21], the mortality rate of non-HIV-uninfected individuals with PJP in several reports was high [22–24].

Next, I identified a risk set for each case from NinJa. The risk set consisted of individuals who had been a member of the cohort in the year in which PJP developed in the case (the index year) but had not themselves experienced PJP onset until the end of the same index year. From the risk set, six individuals were randomly selected for the unmatched group. Six other individuals were selected for the matched group by matching with the case for age, sex, glucocorticoid (GC), MTX, and tacrolimus (TAC) dosage, and the use (and type, if used) of bDMARD (TNF inhibitors, abatacept, or tocilizumab) at the data point of the index year recorded in NinJa (Fig.1). I chose these medications as possible confounders based on previous reports of PJP among RA patients [1,2, 5–8, 16–19] and among patients with other conditions [20,25]. Although co-morbidities were possible confounders, they could not be included as matching variables because they were not recorded in NinJa. Data on PJP prophylaxis and the co-morbidities at the data point of the index year for the selected individuals were collected retrospectively by reviewing their medical charts (Fig.1). Further, those who had received PJP prophylaxis were

excluded from the control group. A control could later become a case and be selected as a control for different cases. A subject could also be selected as both unmatched and matched controls for the same case. Matching was done using "optmatch" in R 3.3.2 (R Foundation for Statistical Computing, Vienna, Austria) [26].

Predictor variables

In addition to matching variables, the two co-morbidities of lung disease and malignancy were possible confounders [17,19,20,25]. All analyses used data obtained at the last outpatient visit before hospitalization for the cases or the data obtained at the data point of the index year for the controls (Fig.1). Age was categorized into two groups: <65 and ≥65years. A malignancy with no recurrence for more than five years after the completion of treatment was excluded. Information on HIV status was not collected from the cases or controls.

Statistical analysis

Categorical variables between the cases and controls were compared using the Mantel-Haenszel test with the exact method based on the following strata: the index year in the unmatched study and matched set in the matched study. Continuous variables were

compared using two-way analysis of variance with the outcome status and the strata as factors. A *p*-value of <0.05 denoted statistical significance. The balance of matching variables between the cases and matched controls was assessed using the standardized difference.

In both the unmatched and matched studies, the adjusted odds ratios (AORs) and corresponding confidence intervals (CIs) for PJP associated with SSZ use were estimated using exact conditional logistic regression (CLR) with the same stratifications as described above because the issue of quasi-complete separation arose in this dataset [27–29]. Specifically, PJP did not develop in any of the RA patients receiving SSZ. In the unmatched study, the odds ratio was adjusted for age group, sex, lung disease, malignancy, the use of GC, MTX, and TAC, and the use (and type, if used) of bDMARD. In the matched study, the odds ratio was adjusted for lung disease and malignancy.

To estimate the PJP risk associated with the variables used in matching, the standard CLR relying on the maximum likelihood estimation was performed in the unmatched study and included the following variables: age group, sex, lung disease, malignancy, the use of GC, MTX, and TAC, and the use (and type, if used) of bDMARD. The analysis was limited to those who did not receive SSZ to remove the effect of SSZ on the risk of PJP.

The exact CLR was performed using PROC LOGISTIC with EXACT and STRATA statements of SAS V.9.4 (SAS Institute, Cary, North Carolina, USA), and the other analyses were performed using R 3.3.2.

Sensitivity analysis

For sensitivity analysis, I limited the subjects to cases with hypoxemia and response to standard treatment for PJP and the corresponding unmatched and matched controls when calculating the AORs for SSZ using the exact CLR.

Results

Study population

Fig.2 shows the flow diagram of the study. In the smyce population of 18,668 subjects with 64,335 subject-years of observation from 37 hospitals, a total of 73 subjects hospitalized for PJP were identified. Seven subjects were excluded due to the lack of detailed diagnostic information on PJP, and six were excluded for not fulfilling the definition of a case. In two of the former and in all the latter, the information on SSZ use before hospitalization was available: none of the subjects had received SSZ. The remaining 60 subjects from 23 hospitals were included as cases. For each control group,

360 subjects were sampled. Those who received PJP prophylaxis were excluded. The remaining 356 subjects from 30 hospitals in the unmatched group and 337 subjects from 35 hospitals in the matched group were included in my analysis. A subject was selected as a non-matched control and a matched control simultaneously for the different cases.

Clinical characteristics of the study participants

The clinical and diagnostic information regarding the PJP episodes of the cases is summarized in Table1. *P. jirovecii* was not identified in any of 21 cases whose respiratory specimens were microscopically examined. The BDG assay and PCR of the respiratory specimens were performed in all and in 37 cases, showing a positivity of 95.0% and 67.6%, respectively. All cases received standard treatment for PJP. Of these, 55 (91.7%) survived and 5 (8.3%) died.

Table2 shows the clinical characteristics of the cases and the two control groups divided into those that were used for matching and those that were not. There were no missing data in any of the variables. In the comparison of the matching variables in the unmatched study, older age, the use of GC, MTX, and TNF inhibitors, and a higher dosage of GC, MTX, and TAC were significantly associated with PJP. Judging from the standardized differences, the matching variables were well-balanced in the matched study

except for the dosage of GC. Although the standardized difference in the dosage of GC was high at 0.179, the difference in the frequency of GC use was sufficiently low at 0.077. Regarding the comparison of the non-matching variables, the cases had lung diseases more frequently than the controls both in the unmatched and matched study (41.7 % vs 15.7% and 20.2%), but there was no significant difference in the existence of malignancies. While none of the cases received SSZ, 17.4% of the unmatched controls and 12.8% of the matched controls received the drug. In Japan, the maximum approved dosage of SSZ is 1.0 g/day. Therefore, most of the doses were less than or equal to 1.0 g/day both in the unmatched controls (11 with0.5 g/day and 51 with 1 g/day) and in the matched controls (fmy with 0.5 g/day,38 with 1 g/day, and one with 1.5 g/day).

Effect of SSZ use on the risk of PJP

In the analysis of the unmatched study using the exact CLR, SSZ use had a decreased risk of PJP (AOR 0.18, 95% CI 0.00–0.92) after adjusting for age group, sex, lung disease, malignancy, the use of GC, MTX, and TAC, and the use (and type, if used) of bDMARD. In the analysis of the matched study using the exact CLR, SSZ use also had a decreased risk of PJP (AOR 0.08, 95% CI 0.00–0.36) after adjusting for lung disease and malignancy (Table3).

Risks for PJP

The risks for PJP were estimated with the standard CLR analysis of the cases and 294 unmatched controls not receiving SSZ. The adjusted model included age group, sex, lung disease, malignancy, the use of GC, MTX, and TAC, and the use (and type, if used) of bDMARD. Lung disease (AOR 3.88, 95% CI 1.89–7.95) and the use of GC (AOR 5.71, 95% CI 2.68–12.19), MTX (AOR 5.25, 95% CI 2.01–13.74), and TNF inhibitors (AOR 2.32, 95% CI 1.10–4.93) significantly increased the risk of PJP, and age ≥65years (AOR 1.83, 95% CI 0.94–3.56) and TAC use (AOR 2.22, 95% CI 0.86–5.71) tended to increase the risk of PJP. Sex, malignancy, and the use of tocilizumab and abatacept were not associated with the risk of PJP.

Sensitivity analyses

When only cases with hypoxemia and a response to standard treatment for PJP (44 cases) and the corresponding controls (261 unmatched and 250 matched controls) were included in an analysis with the exact CLR, the AORs for SSZ use did not substantially change either in the unmatched (AOR 0.13, 95% CI 0.00–0.78) or the matched study (AOR 0.10, 95% CI 0.00–0.48).

Discussion

In both the unmatched and matched studies in the current nested case-control analysis, SSZ use reduced PJP risk. In the unmatched study, the presence of lung disease and the use of GC, MTX, and TNF inhibitors were significant risks for PJP. Age ≥65years and TAC use tended to elevate the risk. Based on this fact, my selection of matching variables was deemed appropriate. These results supported my hypothesis that SSZ has a prophylactic effect against PJP and agreed with the results of the previous single-center case-control study by Mizushina et al., which used a smaller sample size than mys [12].

Sulfapyridine was developed as antibiotics in the 1930′s and was combined with 5-aminosalicylic acid to synthesize an antirheumatic agent with both anti-microbial and anti-inflammatory properties because infection was believed to cause RA at that time [30,31]. There has been no research on the effect of sulfapyridine on PJP. However, one possible explanation for the prophylactic property of SSZ against PJP found in my study is the structural commonalities between sulfapyridine and PJP prophylactics. For example, both TMP/SMX and sulfapyridine belong to the class of the antimicrobial sulfonamides [32]. Furthermore, dapsone, an effective PJP prophylactic for patients who cannot tolerate TMP/SMX, is not a sulfonamide but is structurally related to sulfapyridine [33,34].

Previous studies revealed that PJP in non-HIV-infected individuals produces more intense lung inflammation than in HIV patients despite the low organism burden [35,36]. In their experimental study using a mouse model of PJP-related IRIS, Wang et al. reported that SSZ enhances *Pneumocystis* clearance without intensifying inflammation by accelerating CD4+ T cell-dependent alveolar macrophage phagocytosis and by promoting TH-2 polarized cytokine environment leading to alternative macrophage activation [11]. From these findings, SSZ can be seen as a reasonable choice for PJP prophylaxis in RA patients.

My study has several limitations. My results derived from a Japanese cohort. Therefore, further investigation involving other population is needed to verify the generalizability. A possible misclassification of outcomes is also an important consideration. For the case definition of PJP in my study, I modified the definitions used in previous studies of PJP in RA patients. The original definition as well as my modified version involved using PCR and BDG assays as diagnostic tests [17–19], but neither of the definitions has been validated. Furthermore, in my study, while all the cases were tested with the BDG assay, 23 of 60 cases (38.3%) were not examined with PCR probably because the test is not covered by Japanese national health insurance. Since BDG values can be high during infections caused by fungi such as Candida and Aspergillus spp., this

test is not specific for PJP. However, a recent meta-analysis evaluating the diagnostic accuracy of BDG detection for PJP among those without invasive fungal infections showed moderately high specificity at 86.3% [37]. Therefore, my case definition can be considered reliable because the clinical manifestations and imaging findings compatible with PJP lower the possibility of other fungal infections. Furthermore, all the cases in the sensitivity analyses responded to standard treatment for PJP, making the possibility of other fungal infections still lower, and the results did not show any significant changes from those of the primary analyses.

Another limitation is the quasi-complete separation between PJP and SSZ use due to the absence of SSZ users among the cases. In this situation, a coefficient of the predictor variable in the logistic regression becomes inappropriately infinite by the maximum likelihood estimation [38]. There are two ways of dealing with this problem: Firth's logistic regression and exact logistic regression [28,39], and I chose the latter because only this method was applicable for matched datasets in currently available statistical software to the best of my knowledge. This method is similar to Fisher's exact test in that it is based on exact conditional inferences and can provide a finite estimate of the coefficient even when analyzing data with quasi-complete separation [40].

It is unlikely that methodological flaws in the study design led to the underestimation of SSZ use in the PJP cases and the quasi-complete separation of my data. First, measurement errors in SSZ use were unlikely because I obtained the information from medical records and clearly specified the time point of the measurement. Second, since my study used a large RA cohort without any inclusion or exclusion criteria and thus can be considered representative of RA patients in Japan, there is almost no possibility of my cases being unrepresentative in terms of SSZ use. Finally, although some of my cases may have had TMP/SMX allergy and their physicians might have avoided prescribing SSZ due to possible cross-reactivity between SMX and SSZ, which was demonstrated only in a small in-vitro study [41], I believe that this possible unmeasured confounder was adequately controlled by the matching. Particularly since the matched controls had a PJP risk comparable to that of the cases and did not receive TMP/SMX, the possibility of TMP/SMX allergy was deemed not to be substantially different between the cases and matched controls. Future studies with a larger number of cases will doubtless confirm my results with more straightforward analyses.

One strength of my study is that the data derived from one of the largest RA databases in Japan, allowing us to estimate the precise effect of SSZ on PJP risk by matching the important confounders.

The prophylactic efficacy of SSZ against PJP demonstrated by my study can have several implications for clinical practice in RA, for example, by promoting the use of SSZ for patients with a high risk for PJP or by discmyaging the administration of PJP prophylactics to patients already receiving SSZ. My results provide justification for future prospective studies.

In conclusion, in this nested case-control study based on a nationwide, Japanese, multicenter RA database, I was able to demonstrate the preventive effect of SSZ against PJP. My promising results should be confirmed by prospective studies.

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Tables and Figures

Table 1. Clinical characteristics and diagnostic test results of PJP cases

Clinical characteristics	
Pyrexia	55/60 (91.7)
Dry cough	42/50 (84.0)
Dyspnea	38/57 (66.7)
Hypoxemia	49/60 (81.7)
Response to standard treatment for PJP	55/60 (91.7)
PJP prophylaxis	0/60 (0.0)
Diagnostic tests	
Microscopic detection of *P. jirovecii*	0/21 (0.0)
Increased serum beta-D glucan	57/60 (95.0)
Positive PCR of respiratory specimens for *P. jirovecii*,	25/37 (67.6)
Diffuse interstitial infiltrate on chest imaging	60/60 (100.0)

PJP, *Pneumocystis jirovecii* pneumonia; *P. jirovecii*, *Pneumocystis jirovecii*; PCR, polymerase chain reaction.

Values represent the number of patients who were positive/number of patients for whom data on the clinical manifestations were available or who were examined with diagnostic tests (%).

Table 2. Clinical characteristics of the cases and the two control groups

		Unmatched study		Matched study		
	Cases (n=60)	Controls (n=356)	p Value	Controls (n=337)	p Value	Standerdized difference[a]
Matching variables						
Age, year	68.0 ± 11.4	63.5 ± 12.9	0.011	67.5 ± 11.1	–	0.045
Age ≥65 years	40 (66.7)	176 (49.4)	0.017	223 (66.2)	–	0.010
Sex, male	18 (30.0)	78 (21.9)	0.185	95 (28.2)	–	0.040
GC use	48 (80.0)	150 (42.1)	<0.001	259 (76.9)	–	0.077
GC dose, mg/day[b]	4.15 ± 3.74	1.71 ± 2.62	<0.001	3.55 ± 2.90	–	0.179
MTX use	53 (88.3)	222 (62.4)	<0.001	296 (87.8)	–	0.015
MTX dose, mg/week	7.87 ± 3.99	4.79 ± 4.33	<0.001	7.65 ± 3.94	–	0.055
TAC use	10 (16.7)	33 (9.3)	0.106	55 (16.3)	–	0.009
TAC dose, mg/day	0.33 ± 0.80	0.15 ± 0.51	0.019	0.32 ± 0.77	–	0.020
TNF inhibitor use[c]	19 (31.7)	54 (15.2)	0.003	106 (31.5)	–	0.005
Tocilizumab use	1 (1.7)	11 (3.1)	1.000	6 (1.8)	–	0.009
Abatacept use	3 (5.0)	8 (2.2)	0.202	15 (4.5)	–	0.026
Non-matching variables						
Disease duration, year	14.0 ± 12.2	12.4 ± 11.1	0.328	13.5 ± 11.0	0.776	–
Lung disease	25 (41.7)	56 (15.7)	<0.001	68 (20.2)	<0.001	–
Malignancy	1 (1.7)	19 (5.3)	0.325	11 (3.3)	0.701	–
SSZ use	0 (0.0)	62 (17.4)	<0.001	43 (12.8)	0.001	–
SSZ dose, mg/day	0 ± 0	159 ± 355	<0.001	123 ± 328	<0.001	–
Bucillamine use	7 (11.7)	51 (14.3)	0.687	26 (7.7)	0.444	–
Leflunomide use	0 (0.0)	5 (1.4)	1.000	0 (0.0)	NA	–
Tofacitinib use	0 (0.0)	2 (0.6)	1.000	0 (0.0)	NA	–
Use of other DMARD[d]	4 (6.7)	11 (3.1)	0.248	7 (2.1)	0.070	–
Immunosupressant use[e]	0 (0.0)	0 (0.0)	NA	0 (0.0)	NA	–

DMARD, disease-modifying antirheumatic drug; GC, glucocorticoids; MTX, methotrexate; NA, not applicable; SD, standard deviation; SSZ, sulfasalazine; TAC, tacrolimus; TNF, tumor necrosis factor.

Each control group was compared with the cases. Values are the mean ± SD or the number (%).

[a] Absolute values.

[b] Prednisolone equivalent.

[c] Adalimumab, certolizumab pegol, etanercept, golimumab, and infliximab are included.

[d] Mizoribine, iguratimod, actarit, D-penicillamine, auranofin, and lobenzarit are included.

[e] Azathioprine, cyclophosphamide, and cyclosporine A are included.

Table 3. The use of sulfasalazine and the risk of PJP

Variables	Unmatched study[a]			Matched study[b]		
	Adjusted OR	95% CI		Adjusted OR	95% CI	
		Lower	Upper		Lower	Upper
Sulfasalazine	0.18	0.00	0.92	0.08	0.00	0.36

CI, confidence interval; OR, odds ratio.

[a] Adjusted for age group, sex, lung disease, malignancy, use of glucocorticoid, methotrexate, and tacrolimus, and use (and type, if used) of biological disease-modifying antirheumatic drug (tumor necrosis factor inhibitor, abatacept, or tocilizumab).

[b] Adjusted for lung disease and malignancy.

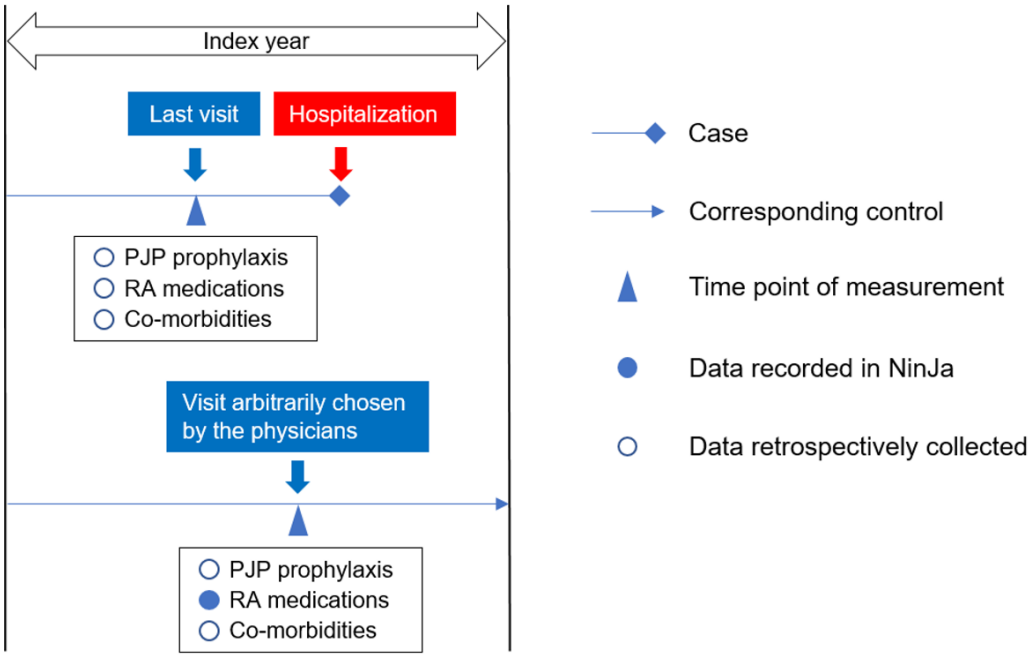

Figure 1. Time points in the assessment of *Pneumocystis jirovecii* pneumonia (PJP) prophylaxis, rheumatoid arthritis (RA) medications, and co-morbidities in a case and its corresponding control. NinJa = National Database of Rheumatic Diseases by iR-net in Japan

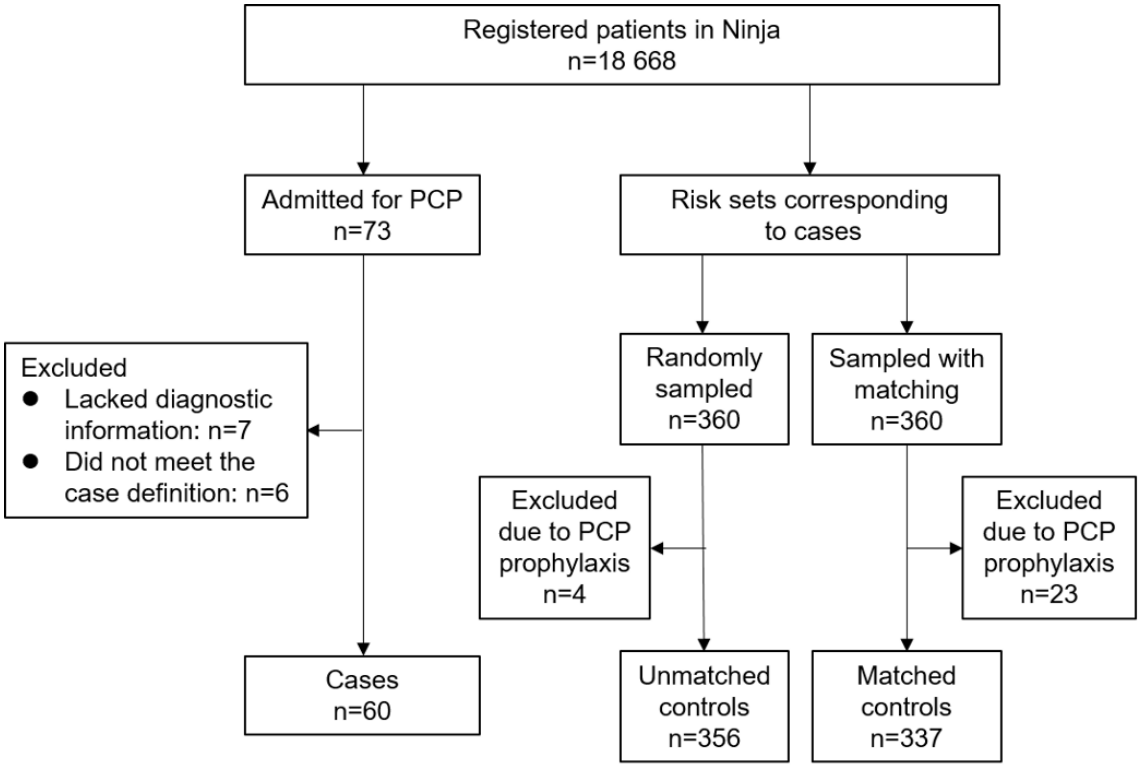

Figure 2. Study flow diagram. NinJa = National Database of Rheumatic Diseases by iR-net in Japan; PJP = *Pneumocystis jirovecii* pneumonia.

Chapter 4.

Overall discussion and conclusion

In this thesis, the prophylactic efficacy of sulfasalazine (SSZ) against *Pneumocystis jirovecii* pneumonia (PJP) suggested by an experimental study [1] is investigated. Since the cohort study is not feasible due to the low incidence of PJP among patients with rheumatoid arthritis (RA), I chose the case-control study. Although the gold standard for establishing PJP diagnosis is microscopic detection of *Pneumocystis* organisms in respiratory specimens, it is known that *Pneumocystis* organisms are rarely microscopically detected in respiratory specimens from PJP developed in patients with not infected with human immunodeficiency virus [2]. Therefore, special consideration for the outcome status determination were made in my studies.

In Chapter 2, a test-negative design case-control study has been described. In this study, the outcome was the presence of *P. jirovecii* in the lung determined by polymerase chain reaction (PCR) instead of PJP development because of the concern regarding the possible misclassification of outcome status derived from the difficulty in making the definite diagnosis of PJP developed in patients with RA. In the test-negative design case-control study, all subjects receive the test for the disease of interest, which still lowers the possibility of the outcome status misclassification [3]. This study demonstrated that SSZ

use was negatively associated with the presence of *P. jirovecii* in the lung. This study does not directly assess the association between SSZ use and PJP development because PCR does not distinguish the colonization of *Pneumocystis* organisms from PJP. However, considering the development of PJP after an asymptomatic carrier state of the organisms has been reported in patients with RA, my study suggests that SSZ use suppresses PJP incidence [4].

In Chapter 3, a nested case-control study using incidence density sampling has been described. Although a previous case-control study suggested the prophylactic effect of SSZ against PJP, its sample size was too small (ten cases) to be conclusive [5]. I utilized one of the largest RA cohort in Japan, the National Database of Rheumatic Diseases by iR-net in Japan (NinJa) [6]. By using the cohort as a study base, I sampled more cases and controls than the previous study with matching important confounders. The main difficulty encountered in this study was confirming the outcome status because the definite diagnosis of PJP with microscopic examinations was difficult to make in PJP developed in patients with RA. Therefore, I increased the reliability of the outcome classification by using the case definition composed of diagnostic test results and clinical characteristics. In more than half of the cases, the diagnosis was made based on the increased level of serum 1,3-β-D-glucan (BDG). Although BDG is a common cell wall

constituent of most fungi and not specific for PJP, the assay has proved to be useful for the diagnosis of PJP when the clinical manifestations and imaging findings compatible with PJP lower the possibility of other fungal infections [7]. In this study, SSZ users showed the reduced risk of PJP.

The results of both of the case-control studies conducted by us indicated the preventive efficacy of SSZ against PJP. The fact that they enrolled different RA populations and setting different outcomes increased the generalizability of their results. My results have important implications in clinical practice of RA treatment. For instance, SSZ use is advisable for patients with a high risk for PJP, and PJP prophylactics are discmyaged in patients already receiving SSZ.

In both of the case-control studies, the issue of quasi-complete separation arose because none of the cases used SSZ, which precluded the estimation using the maximum likelihood method in the logistic regression [8]. I dealt with the issue by means of Firth's logistic regression and exact logistic regression [9,10], which are the currently available solutions for this statistical problem. Measurement errors in SSZ use were unlikely because the information was obtained from medical records.

In NinJa, which is one of the largest RA cohort in Japan and was used as a study base in the nested case-control study described in chapter 3, the usage rate of SSZ did not

substantially change at approximately 17% throughout the study period (16,6% in 2005 and 17.3% in 2014). In the nested case-control study, both of the unmatched and matched controls were selected from the members of the cohort in the year in which PJP developed in the case. Therefore, the observation year of the both control groups was matched with the cases. In the test-negative design case-control study, the observation year was not matched between the cases and controls and was not included in the adjusted model of the multivariate analysis. Although there is a possibility that the observation year was a confounder of the study, on the basis of the steady usage rate observed in NinJa, it was not deemed to have large effects on the study results.

In the study of mouse models of PJP by Bhagwat et al, revealed that innate immunity with alternative activation of macrophage is required in the protection against *Pneumocystis* infection [12]. Furthermore, in their experimental study using mice with PJP-related immune reconstitution inflammatory syndrome, Wang et al. reported that SSZ enhances *Pneumocystis* clearance without intensifying inflammation by accelerating CD4+ T cell-dependent alveolar macrophage phagocytosis and by promoting TH-2 polarized cytokine environment leading to alternative macrophage activation [1]. These findings could explain the underlying mechanism of the prophylactic effect of SSZ against PJP shown in my studies.

In my studies, the effect of the predictor variables other than SSZ on the development of PJP or PCR positivity was estimated. In the nested case-control study, the use of corticosteroid, methotrexate and tumor necrosis factor inhibitors significantly increased the risk of PJP. In the test-negative design case-control study, the use of corticosteroid was significantly associated with PCR positivity, but the use of methotrexate and tumor necrosis factor inhibitors was not associated the positivity. This inconsistency may come from the difference in the outcome setting between the two studies or the insufficient sample size of the test-negative design case-control study to find the associations.

In conclusion, clinical research using case-control studies in this thesis revealed the efficacy of SSZ as a prophylactic measure against PJP. This would contribute to the safety of tight control treatment strategies for RA using immunosuppressive drugs. My results should be confirmed by the prospective cohort

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