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ORIGINAL RESEARCH

Serum p-Glycoprotein and Monomeric C-Reactive Protein are Elevated in Takayasu Arteritis

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Video abstract

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Purpose: Existing biomarkers including C-reactive protein (CRP) do not adequately distinguish active and inactive TAK. We compared serum p-glycoprotein (p-gp)/Multidrug Resistance Protein 1 (MDR1), monomeric CRP (mCRP), CRP, and mCRP:CRP ra tio in Takayasu arteritis (TAK) and healthy controls and their relationship with disease activity.

Patients and Methods: Serum p-gp mCRP (ELISA) and CRP (nephelometry) were compared between consecutive adults with TAK (>18 years) enrolled from a prospective cohort (n = 92) and healthy controls (n = 29), and between active vs inactive TAK (n = 46 each). In a subset of active immunosuppressive-naïve TAK (n = 29), correlation was assessed between serum p-gp and p-gp expression on circulating T helper lymphocyte populations: overall (CD4+), Th17 (CD4+IL-17+), Th17.1 (CD4+IL-17+IFN-γ+) lymphocytes [normalized to Tregs (CD4+CD25+FoxP3+)]. Changes in serum p-gp, mCRP, CRP, and mCRP:CRP were compared before and after immunosuppression (n = 29). Data was represented using median (Q1-Q3). Receiver operating characteristics (ROC) curves were generated for TAK vs controls, and active vs inactive TAK with serum p-gp, mCRP, CRP, and mCRP:CRP. Multivariable-adjusted linear regression was used to predict active disease with serum p-gp, mCRP, CRP, or mCRP:CRP.

Results: Serum p-gp (11.19 vs 8.05 ng/mL), mCRP (1.61 vs 1.25 µg/L), and CRP (5.40 vs 2.1 mg/L) were elevated in TAK vs controls ($p < 0.05$ for all). CRP was higher and mCRP:CRP ratio was lower in active vs inactive TAK ($p < 0.001$). ROC curves identified moderate prediction for active disease with CRP and inactive disease with serum p-gp (area under ROC curve 0.705 and 0.392, respectively). Multivariable-adjusted linear regression confirmed association of CRP with active disease ($p = 0.009$) and serum p-gp with inactive disease ($p = 0.041$). In treatment-naïve TAK, serum p-gp negatively correlated with p-gp+Th17.1 lymphocytes (Spearman's rho=−0.39, $p = 0.046$). CRP and serum p-gp were significantly lowered following immunosuppressive therapy in treatment-naïve TAK ($p < 0.05$).

Conclusion: Serum p-gp and mCRP are elevated in TAK. Serum p-gp is associated with inactive disease.

Keywords: Takayasu arteritis, MDR1 protein, C-reactive protein, large vessel vasculitis, aortoarteritis, disease activity

Introduction

Takayasu arteritis (TAK) is a rare large vessel vasculitis (LVV) commoner in Asian countries than elsewhere.[1–3] TAK is associated with a greater risk of mortality despite predominantly affecting young female adults.[4] The pathology of TAK involves arteritis of the aorta and its major branches, resulting in fibrosis and arterial stenosis.[5] The arterial wall injury in

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patients with TAK involves the activation of both innate and adaptive immune cells.[5] T lymphocytes in particular have been implicated in the pathogenesis of TAK. Increased circulating Th1 lymphocytes (which secrete interferon-gamma – IFN-γ) and Th17 lymphocytes (which secrete interleukin 17 – IL17) have been observed in patients with TAK as well as in the counterpart LVV of Giant Cell Arteritis (GCA).[6–8] However, unlike GCA, the Th17 lymphocytes from TAK are not responsive to corticosteroids.[6,8]

Recently, Th17.1 lymphocytes, a subset of Th17 lymphocytes that secrete both IFN- γ and IL-17 (sharing the phenotypic characteristics of both Th1 and Th17 lymphocytes), have been found to be increased in patients with TAK than in healthy controls and are associated with active disease.[9] The Th17.1 lymphocyte population also expresses the drug efflux protein p-glycoprotein (p-gp) or Multidrug Resistance Protein 1 (MDR1) which impart upon it the property of corticosteroid resistance.[10–12] CD4+ T lymphocytes expressing p-gp have been found to be elevated in patients with TAK.[13] The expression of p-gp on lymphocytes or T lymphocyte populations has been associated with treatment refractoriness in other immune-mediated inflammatory diseases such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE).[14–17] The expression of p-gp on peripheral blood lymphocytes is also associated with disease activity in patients with SLE.[16] The assessment of p-gp expression on lymphocyte populations by flow cytometry requires considerable technical expertise. A few studies have evaluated whether circulating p-gp in the peripheral blood might reflect disease activity in inflammatory diseases. A cross-sectional study of 151 patients with RA reported higher levels of serum p-gp assessed using enzyme-linked immunosorbent assay (ELISA) in patients with RA than in healthy control subjects. Serum levels of p-gp were associated with disease activity of RA, and increased serum p-gp was associated with 2.6 times greater odds of treatment-refractory RA.[18] Another study reported higher levels of serum p-gp in 93 patients with SLE when compared with healthy controls. Serum p-gp was associated with disease activity scores in SLE even after multivariable adjustment.[19] Therefore, it can be hypothesized that serum p-gp might reflect disease activity in patients with TAK and might reflect the expression of p-gp on circulating T lymphocyte populations (Th1, Th17, Th17.1).

Overall, the assessment of disease activity in patients with TAK is challenging.[20] Sites of arterial pathology in TAK are inaccessible except during open surgical procedures, which are rarely undertaken for revascularization or aneurysm repair.[3,20] The erythrocyte sedimentation rate reflects the levels of circulating fibrinogen which is an acute-phase reactant; therefore, ESR indirectly reflects the acute-phase response. However, ESR and the classical acute phase reactant C-reactive protein (CRP) do not accurately reflect TAK disease activity.[3,20] A classical study from the National Institutes of Health, United States of America, revealed ongoing active arterial inflammation on arterial biopsies in 4/9 patients undergoing arterial wall surgery despite clinically quiescent disease and normal ESR. From this cohort, only three-fourths of those with active disease had an elevated ESR. Conversely, more than half of patients with inactive disease had high ESR.[21] Another study from France reported persistent arterial wall inflammation in 42% of patients undergoing arterial wall repair despite clinically inactive disease.[22] Studies using 18-F fluorodeoxyglucose (18-F FDG) positron emission tomography (PET) to assess the disease activity of TAK have reported similar values of ESR and CRP in patients with TAK with active or inactive TAK as indicated by arterial wall uptake of 18-F FDG with no observed correlation between arterial segments with 18-F FDG uptake or maximum 18-F FDG uptake with ESR or CRP.[23] The pentamer CRP dissociates in vivo into a monomeric form [monomeric CRP (mCRP), measurable using ELISA].[24–26] mCRP has recently been evaluated as a marker of inflammation in preclinical models of inflammatory arthritis.[24–26] Blocking mCRP has also been found to ameliorate joint inflammation and kidney inflammation in animal models of inflammatory arthritis and lupus, respectively, thereby suggesting the biological activity of this molecule.[27] Circulating levels of mCRP have been associated with disease activity in anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis (AAV).[28] mCRP has recently been identified to be a good marker of active disease in settings associated with low-grade inflammation such as in chronic obstructive pulmonary disease.[29] However, mCRP has not yet been evaluated in TAK.

In this context, we evaluated the differences in serum p-gp, mCRP, CRP, and the ratio of mCRP:CRP between patients with TAK and healthy controls, their ability to reflect the disease activity of TAK, differences in patients with respect to treatment, and the correlation of serum p-gp with p-gp expression on circulating T lymphocyte populations known to express p-gp.

Materials and Methods

Participant Recruitment and Ethics Approval

Consecutive patients with TAK were recruited from a longitudinal cohort of patients following up at the Department of Clinical Immunology and Rheumatology at the Sanjay Gandhi Postgraduate Institute of Medical Sciences (SGPGIMS), Lucknow (details of the cohort published elsewhere).[4,30–32] The study was approved by the Institute Ethics Committee of SGPGIMS (document submission number 2022–83-DM-EXP-48; date of approval 24th August 2022). The study complies with the Declaration of Helsinki regarding ethical considerations for research on human subjects.

Objectives

The primary objective of the study was to compare serum levels of p-glycoprotein, monomeric CRP, CRP, and the ratio of mCRP:CRP between patients with TAK and healthy controls in a cross-sectional study. Secondary objectives related to the comparison of serum p-glycoprotein monomeric CRP, CRP, and the ratio of mCRP:CRP between patients with TAK with active and inactive disease and between those on treatment with corticosteroids or other immunosuppressive therapies compared with those without. Other secondary objectives were the correlation between levels of serum p-glycoprotein with circulating p-gp-expressing CD4+ T lymphocytes, Th17 lymphocytes, p-gp-expressing Th17 lymphocytes, Th17.1 lymphocytes, and p-gp-expressing Th17.1 lymphocytes. Serial changes in serum p-glycoprotein, monomeric CRP, CRP, and the ratio of mCRP:CRP in a subset of patients with active TAK before and after immunosuppressive treatment were also assessed.

Inclusion and Exclusion Criteria

Adult patients with TAK (>18 years of age) who fulfilled either the 1990 American College of Rheumatology (ACR) classification criteria[33] or the 2012 Chapel Hill Consensus Conference definition[34] or the 2022 ACR-European Alliance of Associations for Rheumatology (EULAR) classification criteria[35] were included after seeking written informed consent. Healthy controls were similar in age and sex to the recruited patients without any known metabolic or autoimmune diseases. Individuals with other autoimmune diseases, other forms of vasculitis, or atherosclerosis were excluded. Patients belonging to vulnerable populations such as children and pregnant women or those who were unable to provide informed consent such as critically sick patients were excluded.

Disease Activity Assessment

Patients with TAK with both active or inactive disease were included. Active disease was defined by physician global assessment (PGA) as the gold standard, defined as the presence of clinical features suggestive of active TAK such as constitutional symptoms, carotidynia, new onset vascular features such as pulse loss or claudication with either elevated acute-phase reactants (ESR or CRP) or evidence of metabolically active disease on 18-F FDG PET. In addition, the Indian Takayasu Arteritis Clinical Activity Score (ITAS2010) and the Disease Extent Index for Takayasu Arteritis (DEI. TAK) were also scored for all the included patients as secondary measures of disease activity as they are imperfect measures of disease activity in TAK.[20]

Study Procedures

Patients with TAK were recruited consecutively for the cross-sectional arm of the study between September 2022 and October 2023. From these patients, 3 mL of serum was collected and frozen at −80 degrees C for analysis at the end of the study. A subset of the included patients who were immunosuppressive treatment-naïve with active disease were followed longitudinally. In these patients, the levels of serum p-glycoprotein with circulating p-gp-expressing CD4+ T lymphocytes, Th17 lymphocytes, p-gp-expressing Th17 lymphocytes, Th17.1 lymphocytes, and p-gp-expressing Th17.1 lymphocytes assessed by flow cytometry performed on 3 mL of whole blood collected in EDTA vials (detailed later). This same subset of treatment-naïve patients was followed up, and another serum sample was collected 2–6 months after treatment with immunosuppressive drugs (at which time the disease was inactive) to assess the serial

changes in serum p-glycoprotein, monomeric CRP, CRP, and the ratio of mCRP:CRP following immunosuppressive therapy. Figure 1 represents the workflow of the study.

Assessment of Serum p-Glycoprotein, C-Reactive Protein, Monomeric CRP, and the Ratio of Monomeric CRP:CRP

Serum p-glycoprotein and mCRP were assessed using commercially available ELISA kits [for human P-glycoprotein (MyBiosource.com, USA) and mCRP (PromedeusLab, Czech Republic)] as per manufacturer's protocol. ELISA micro-titer plates pre-coated with antibodies specific to human P-glycoprotein and mCRP were used. Standards or human samples were added to each well in the respective ELISA plates. Thereafter, biotinylated antibodies specific to p-gp or mCRP and avidin-horseradish peroxidase (HRP) conjugate were added. Non-adsorbed components were washed away. Then, the substrate solution was added to each plate. The enzyme-substrate reaction was terminated by a stop solution. The optical density was measured at a wavelength of 450 nm for each ELISA. The lower limit of detection for human p-gp was 0.31 ng/mL and for m-CRP was 1.25 µg/L. Where undetectable, the lower limit of detection was recorded for the purpose of analysis. CRP was measured using nephelometry (using the IMMAGE Immunochemistry highly sensitive CRP kit from Beckman Coulter, USA) which utilizes rabbit anti-CRP antibodies coated on latex particles for the test reaction. The ratio of mCRP to CRP was calculated after converting both to units per L (µg/L for mCRP and mg/L for CRP). As per the literature of the kits, the antibodies reactive to CRP and mCRP in the two different kits were specific for CRP and mCRP, respectively. Given the difference in the magnitude of values of mCRP (in µg/L) and CRP (in mg/L), any cross-reactivity of mCRP with CRP (or vice versa) was unlikely to be of significance.

Flow Cytometry for Cell Populations and p-Gp Function

Intracellular staining of T helper lymphocyte subsets was done after 6-hour incubation of whole blood in complete Roswell Park Memorial Institute (RPMI) culture media with activation by phorbol myristate acetate (PMA, 50ng/mL), ionomycin (1µg/mL) and monensin (2µM) in a 5% carbon dioxide incubator. Thereafter, red blood corpuscles (RBC) were lysed by using 1X RBC lysis buffer for 10 minutes at room temperature. The cells were incubated with

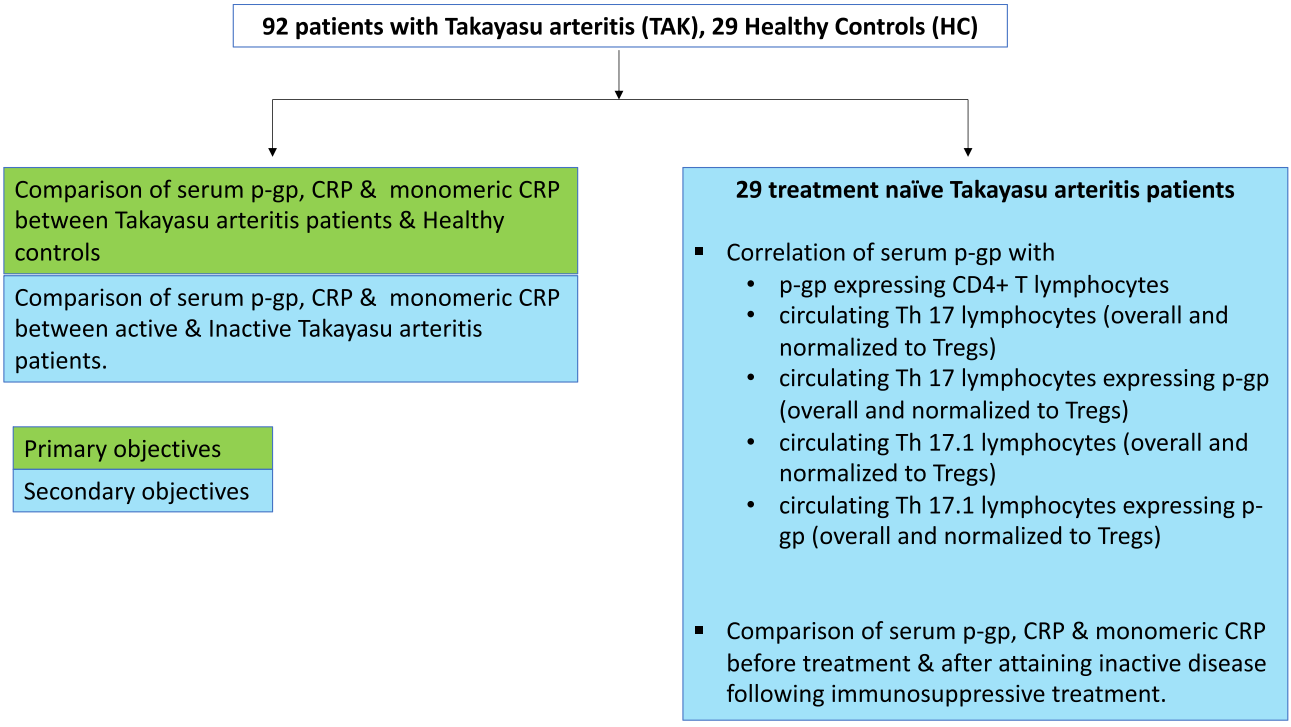

Figure 1 Workflow for the study.

anti-CD4 (BV510), anti-CD25 (PECy7), and anti-human P-glycoprotein (PE) for surface stain, and subsequently, cells were fixed and permeabilized by using Cytofix/Cytoperm W/Golgi Stop (554715, BD Biosciences). For intracellular T helper lymphocyte subset staining, the cells were incubated with anti-human IFN-γ (PE-Cy7), anti-human IL-17A (Alexa Fluor 488), and anti-human FoxP3 (Alexa Fluor 647). The cells were then resuspended in 500 µL of 1X phosphate buffer saline for acquisition on a BD FACS Canto II (BD BioSciences, USA) flow cytometer. The Th1 lymphocytes were identified as CD4+ IFN-γ+, Th17 lymphocytes as CD4+ IL-17A+, Th17.1 lymphocytes were as CD4+ IL17A+ IFN-γ+, p-gp expressing T lymphocytes as CD4+ p-gp+, Th17+p-gp expressing lymphocytes as CD4+Th17+ p-gp+ and Th17.1+p-gp expressing lymphocytes as CD4+ IL-17A+ IFN-γ+ p-gp +. For T-regulatory cells, CD25 (PECy7) was gated on the total CD4 T cells after that FoxP3 (Alexa Fluor 647) was gated on CD4+ CD25+ dual positive T cells. Figure 2 represents the gating strategies for the different cell populations studied. The populations of the Th17 and Th17.1 lymphocytes were normalized to regulatory T lymphocytes.

Functional assay for p-gp was performed on peripheral blood mononuclear cells (PBMCs) using a commercially available kit (EFLUXX ID® Green multidrug resistance assay kit, Catalog No. ENZ-51029-K100). The cells were pre-incubated in a warm RPMI 1640 medium (indicator free) after two washing steps to remove the remaining serum components and incubated for 5–10 minutes with or without MDR1 inhibitor (Verapamil). Thereafter, EFLUXX ID green dye (MDR1 substrate + detection dye) was added and further incubated for 30 min at 37° C. After this incubation, propidium iodide (PI) solution was added for monitoring cell viability. Fluorescence was measured using BD FACS Canto II (BD BioSciences, USA) flow cytometer. Multi-resistance Activity Factor (MAF) for p-gp was calculated using the formula: MAFMDR1 = 100 × (FMDR1 - F0)/ FMDR1 (FMDR1 = Mean fluorescence intensity of MDR1 with verapamil, F0 = Mean fluorescence intensity of stain control).[36]

Sample Size Calculation and Statistical Analysis

Since no information was available regarding serum p-gp levels in patients with TAK, the data for sample size calculation was extrapolated from another inflammatory condition viz. RA.[18] Using an online sample size calculator,[37] assuming a mean serum p-glycoprotein level of 158.70 ng/mL with a standard deviation of 182.71 ng/mL in patients with TAK and 30.56 ng/mL in healthy controls, with $\alpha = 0.05$ and $\beta = 0.20$, the proportion of patients with TAK to healthy controls 4:1, the estimated sample size was 82 patients with TAK and 21 healthy controls. Assuming a non-participation rate of 10%, the sample size was expanded to 90 patients with TAK and 25 healthy controls. In this study, we recruited 92 TAK patients and 29 healthy controls.

Quantitative demographic data and clinical characteristics were expressed as numbers with percentages. Statistical analyses were conducted using Prism 10 for macOS [version 10.3.0 (461), GraphPad Software LLC, USA] or STATA 16.1 I/C (StataCorp, USA). Mann Whitney *U*-test was used to compare unpaired data, whereas, the Wilcoxon matched-pairs signed rank test was used to compare before-after data. Categorical data were compared using the chi-squared test or Fisher's exact test (if any of the four cells contained a value less than 5). Pairwise correlations were assessed using the Spearman correlation coefficient. The ability of serum p-gp, mCRP, and CRP to distinguish patients with TAK versus healthy controls or to distinguish patients with active or inactive TAK was assessed using receiver operating characteristics (ROC) curves generated using the roctab command on STATA. Optimal cut-offs for the various parameters used to distinguish patients with TAK vs healthy controls or patients with TAK with active or inactive disease were determined using the *cutpt* command on STATA. At these cut-offs, the performance of the different ROC curves was compared using the Youden index [(sensitivity+specificity)-100]. Univariable logistic regression was used to calculate odds ratios (OR, with 95% confidence intervals) for TAK vs healthy controls and for active vs inactive TAK using these cut-offs. Multivariable-adjusted linear regression analyses were conducted to analyze the prediction of disease activity as per PGA, ITAS2010, or DEITAK with serum p-gp, mCRP, or CRP. The multivariable-adjusted models were considered adequately powered if there were at least eight events for each variable selected in the multivariable model.[38] p values <0.05 were considered statistically significant.

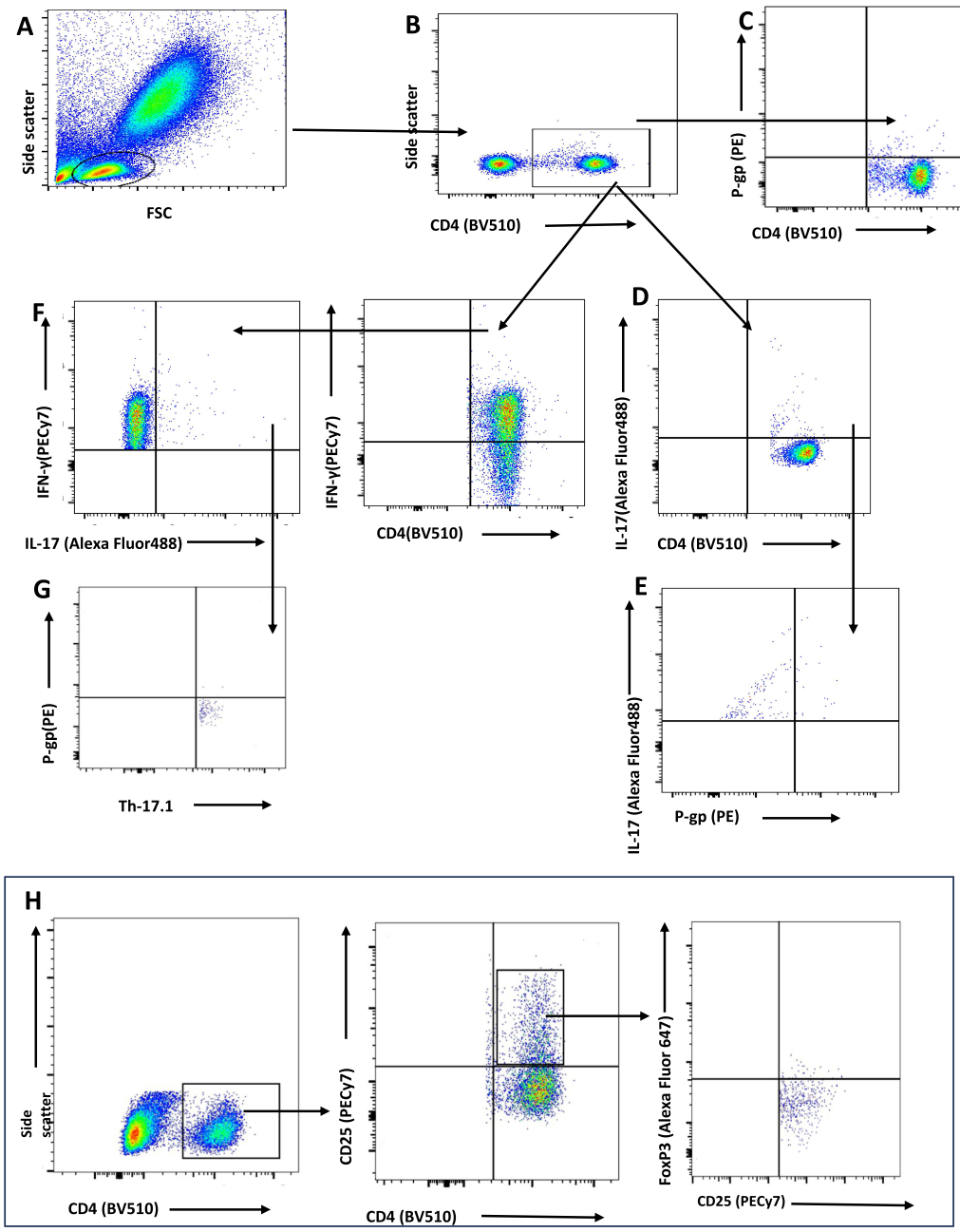

Figure 2 Representative flow cytometry plots for different T helper lymphocyte populations. The lymphocyte population was gated on a plot of forward vs side scatter of whole blood (**A**). Further, CD4+ T lymphocytes were gated on a plot of CD4 vs side scatter (**B**), and the population of CD4+ T lymphocytes expressing p-glycoprotein (p-gp) was identified (**C**). Th17 lymphocytes were identified as CD4+ T lymphocytes expressing IL-17 (**D**), and the sub-population of Th17 lymphocytes expressing p-gp was further gated (**E**). Th17.1 lymphocytes were identified as a sub-population of CD4+ T lymphocytes expressing both IFN-γ and IL-17 (**F**), and the sub-population of Th17.1 lymphocytes expressing p-gp was further gated (**G**). Regulatory T lymphocytes (Treg) were identified as CD4+ T lymphocytes expressing both CD25 and FoxP3 (**H**).

Results

Characteristics of the Study Participants

Ninety-two patients with TAK [median (IQR) age 31.5 (24.00–41.75) years, 63 females] and 29 healthy controls [median (IQR) age 33.00 (29.00–34.00) years (p-value for comparison with TAK 0.792), 20 females (p-value for chi-squared test vs TAK 0.961)] were recruited. All the healthy controls clinically had the absence of any chronic disease or comorbid conditions such as diabetes, hypertension, hypothyroidism, or history of malignancy. Forty-six patients had active disease at enrollment. Eighteen patients were on corticosteroids, whereas seventeen were on disease-modifying anti-rheumatic drugs (DMARDs) at

enrollment. The characteristics of the patients with TAK and their clinical features at presentation are detailed in Table 1. Twenty-nine patients with active disease at enrollment were immunosuppressive treatment-naïve. The levels of serum mCRP were weakly correlated with serum CRP (Spearman's rho 0.22, p = 0.03) in patients with TAK.

Comparison Between Patients with TAK and Healthy Controls

The serum levels of p-gp (p = 0.012), mCRP, and CRP (p < 0.001) but not mCRP:CRP were significantly higher in patients with TAK than in healthy controls (Table 2). ROC curves identified a moderate distinction between patients with TAK or healthy controls using either the serum levels of p-gp, mCRP, or CRP but not with the ratio of mCRP:CRP (Figure 3). At the optimal cut-offs for the ROC curves, serum levels of p-gp (OR 2.31), mCRP (OR 18), and CRP (OR

Table 1 Baseline Characteristics of the Patients with Takayasu Arteritis

Parameters	**TAK (n=92)**	**Healthy Controls (n=29)**
Age [median (Q1-Q3)]	31.5 (24.00–41.75)	33.00 (29.00–34.00)*
Female: Male	63:29	20:9
Clinical features at presentation (%)		
Constitutional features	42 (45.7)	-
Carotidynia	7 (7.6)	-
Syncope, dizziness, or vertigo	24 (26.1)	-
Transient ischemic attack or stroke	5 (5.4)	-
Vision blurring or loss	12 (13)	-
Inequality of pulse or blood pressure	52 (56.5)	-
Pulse loss	61 (66.3)	-
Vascular bruits	67 (72.8)	-
Claudication of upper limbs	27 (29.3)	-
Claudication of lower limbs	18 (19.6)	-
Hypertension	67 (72.8)	-
Aortic regurgitation	4 (4.3)	-
Impaired renal functions	10 (10.9)	-
Abdominal angina	3 (3.3)	-
Chest pain (angina or attributable to TAK)	9 (9.8)	-
Heart failure	3 (3.3)	-
Comorbidities		
Diabetes mellitus	3 (3.3)	0
Hypertension[#]	67 (72.8)	0
Hypothyroidism	5 (5.4)	0
Malignancy	1 (1.1)	0

(*Continued*)

Table 1 (Continued).

Parameters	TAK (n=92)	Healthy Controls (n=29)
Angiographic subtypes (%)		
Hata's I	13 (14)	-
Hata's IIA	4 (4.3)	-
Hata's IIB	10 (10.8)	-
Hata's III	6 (6.5)	-
Hata's IV	8 (8.6)	-
Hata's V	51 (55.4)	-
Active disease as per PGA [n(%)]	46 (50)	-
Disease activity scores [median (Q1-Q3)]		
ITAS2010	3 (0–8)	-
DEI.TAK	2 (0–8)	-
Acute phase reactants [median (Q1-Q3)]		
ESR (mm/hour)	38 (20–55)	-
CRP (mg/L)	5.40 (2.48–11.68)	-
Medications used [n(%)]		
Number of Patients on Glucocorticoids	18 (19.5)	-
Glucocorticoids dose (mg/day) mean (SD)	3.1 (1.25–5)	-
Number of Patients on DMARDs	17 (18.4)	-
Methotrexate	5/17 (29.4)	-
Tacrolimus	7/17 (41.2)	-
Azathioprine	2/17 (11.7)	-
Mycophenolate	3/17 (17.6)	-

Notes: *p=0.792 vs patients with TAK (Mann Whitney *U*-test. **p=0.961 vs patients with TAK (Chi squared test). #Hypertension is an important clinical feature of patients with TAK.

Abbreviations: CRP, C-reactive protein; DEI.TAK, Disease Extent Index in Takayasu Arteritis; DMARD, Disease-modifying anti-rheumatic drugs; ESR, Erythrocyte Sedimentation Rate; ITAS2010, Indian Takayasu Arteritis Clinical Disease Activity Score; PGA, Physician Global Assessment; SD, Standard deviation; TAK, Takayasu arteritis.

Table 2 Comparison of Serum p-Gp, mCRP, and CRP Between Patients with TAK and Healthy Controls

Parameters [Median (Q1 – Q3)]	TAK (n=92)	Healthy Controls (n=29)	p value
Serum p-gp (ng/mL)	11.19 (6.94–15.81)	8.05 (2.29–12.82)	**0.012**
mCRP (µg/L)	1.61 (1.25–4.57) (n=91)	1.25 (1.25–1.25)	**<0.001**
CRP (mg/L)	5.40 (2.48–11.68)	2.1 (0.60–5.50)	**<0.001**
mCRP:CRP	0.00041 (0.00019–0.00124)	0.00060 (0.00023–0.000208)	0.17

Note: Significant p values are highlighted in bold.

Abbreviations: CRP, C reactive protein; mCRP, monomeric CRP; p-gp, p-glycoprotein; TAK, Takayasu arteritis.

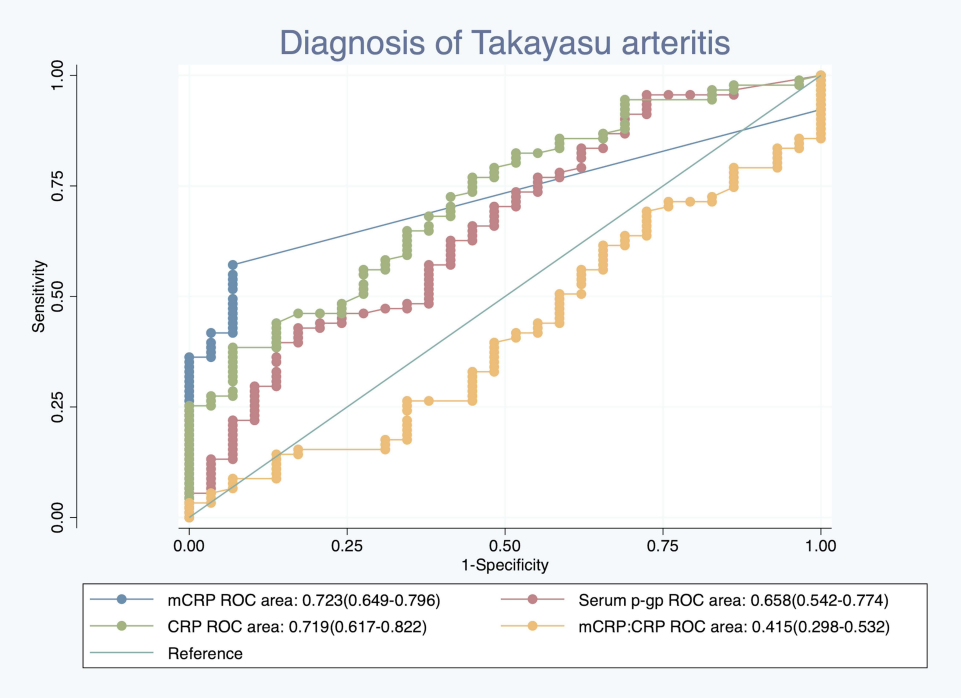

Figure 3 Receiver operating characteristics curve for the distinction between patients with TAK and healthy controls with serum p-glycoprotein (p-gp), C-reactive protein (CRP), monomeric CRP (mCRP) and mCRP:CRP ratio.

3.56) but not mCRP:CRP could reliably distinguish patients with TAK from healthy controls. mCRP had the best performance for this (Youden index 50) (Table 3).

Comparison Between Patients with TAK with Active or Inactive Disease

Serum levels of CRP were higher in patients with active TAK than in those with inactive disease ($p < 0.001$). The levels of serum p-gp were higher in patients with inactive disease and mCRP was higher in patients with active TAK than in active TAK; however, these differences were not statistically significant ($p > 0.05$). The ratio of mCRP:CRP was significantly higher in patients with TAK with inactive disease ($p = 0.021$) (Table 4). ROC curves identified a moderate prediction for active disease with CRP and for inactive disease with serum p-gp or mCRP:CRP (Figure 4). At the optimal cut-offs for the ROC curves, mCRP (OR 2.36) or CRP (OR 4.68) could reliably distinguish patients with TAK with active or inactive disease. CRP had the best performance for this (Youden index 35) (Table 5).

Table 3 Performance of the Cut-offs for Serum p-Gp, mCRP, CRP and mCRP:CRP to Distinguish Patients with TAK and Healthy Controls

Parameter	AUC Overall	Optimal Cut-off	Odds Ratio at Cut-off (With 95% CI	Sensitivity at Cut-off	Specificity at Cut-off	AUC at Cut-off	Youden Index at Cut-off
Serum p-gp (ng/mL)	0.658 (0.542–0.774)	9.615	2.31 (0.99–5.40)	62%	59%	0.60	21
mCRP (µg/L)	0.723 (0.649–0.796)	1.251	18 (4.04–80.28)	57%	93%	0.75	50
CRP (mg/L)	0.719 (0.617–0.822)	3.545	3.56 (1.48–8.57)	65%	66%	0.65	31
mCRP:CRP	0.415 (0.298–0.532)	0.00036	0.78 (0.33–1.84)	56%	38%	0.47	−6

Abbreviations: 95% CI, 95% confidence interval; AUC, Area under the receiver operating characteristic curve; CRP, C reactive protein; mCRP, monomeric CRP; p-gp, p-glycoprotein; TAK, Takayasu arteritis.

Table 4 Comparison Between Patients with TAK with Active and Inactive Disease at Study Enrolment

Parameters [Median (Q1 – Q3)]	Active TAK (n=46)	Inactive TAK (n=46)	p value
Serum p-gp (ng/mL)	9.73 (5.47–15.73)	13.58 (8.92–16.51)	0.055
mCRP (µg/L)	2.76 (1.25–6.30) (n=45)	1.25 (1.25–4.56)	0.113
CRP (mg/L)	8.13 (3.90–23.43)	3.86 (1.48–6.65)	**<0.001**
mCRP:CRP	0.00029 (0.00013–0.00081)	0.00047 (0.00027–0.00191)	**0.021**

Note: Significant p values are highlighted in bold.
Abbreviations: CRP, C reactive protein; mCRP, monomeric CRP; p-gp, p-glycoprotein; TAK, Takayasu arteritis.

As there were 46 patients with active disease, the linear regression models were adequately powered to identify associations between PGA, ITAS2010, or DEI.TAK with serum p-gp, mCRP, CRP, or ratio of mCRP:CRP. Multivariable-adjusted linear regression analyses identified CRP (p = 0.009) as a significant predictor of active disease and serum p-gp (p = 0.041) as a significant predictor of inactive disease as per the PGA. However, no significant associations were evident between ITAS2010 or DEI.TAK with serum p-gp, mCRP, CRP, or mCRP:CRP (Table 6).

Comparisons Between Patients with TAK in Relation to Corticosteroid or Immunosuppressive Therapy

CRP (p = 0.003) was significantly higher and the ratio of mCRP:CRP (p = 0.005) was lower in patients with TAK who were not on corticosteroids when compared with those on corticosteroids. However, serum p-gp and mCRP were similar between these two groups. No significant differences were observed in serum p-gp, mCRP, CRP, or mCRP:CRP between patients with TAK on DMARDs or not on DMARDs (Table 7).

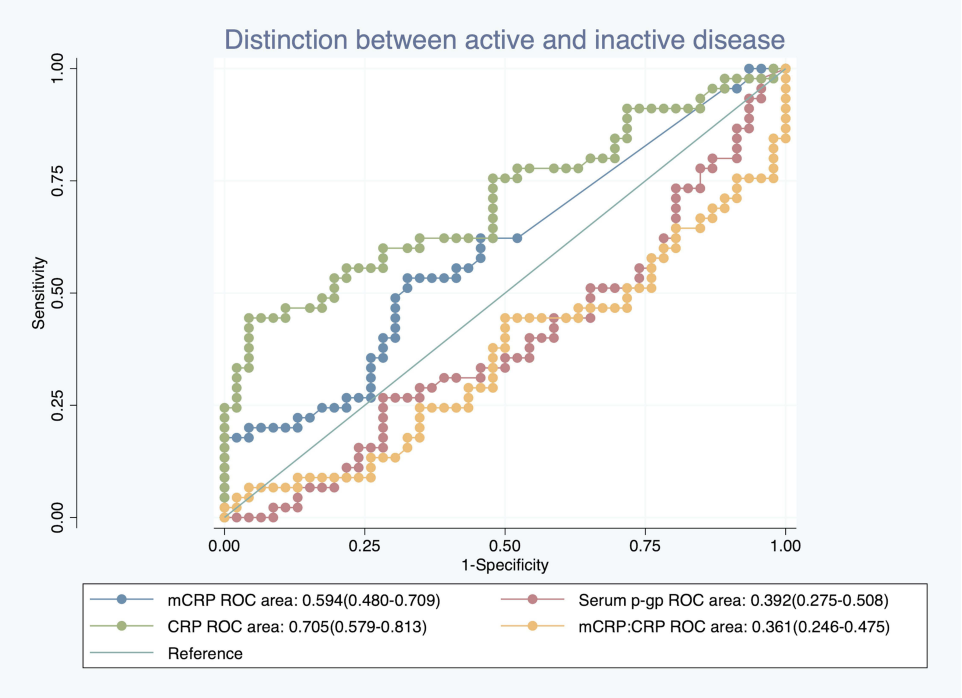

Figure 4 Receiver operating characteristics curve for the distinction between patients with TAK with active vs inactive disease with serum p-glycoprotein (p-gp), C-reactive protein (CRP), monomeric CRP (mCRP) and mCRP:CRP ratio.

Table 5 Performance of the Cut-offs for Serum p-Gp, mCRP, CRP and mCRP:CRP to Distinguish Patients with TAK with Active and Inactive Disease

Parameter	AUC Overall	Optimal Cut-off	Odds Ratio at Cut-off (With 95% CI	Sensitivity at Cut-off	Specificity at Cut-off	AUC at Cut-off	Youden Index at Cut-off
Serum p-gp (ng/mL)	0.392 (0.275–0.508)	15.679	0.90 (0.36–2.25)	26%	72%	0.49	−2
mCRP (µg/L)	0.594 (0.480–0.709)	2.358	2.36 (1.01–5.53)	53%	67%	0.60	20
CRP (mg/L)	0.705 (0.579–0.813)	7.240	4.68 (1.88–11.64)	57%	78%	0.67	35
mCRP:CRP	0.361 (0.246–0.475)	0.00044	0.80 (0.35–1.82)	44%	50%	0.47	−6

Abbreviations: 95% CI, 95% confidence interval; AUC, Area under the receiver operating characteristics curve; CRP, C reactive protein; mCRP, monomeric CRP; p-gp, p-glycoprotein; TAK, Takayasu arteritis.

Table 6 Multivariable Adjusted Linear Regression Models for the Prediction of Disease Activity in Patients with TAK Using Serum p-Gp, mCRP, CRP, and mCRP: CRP Ratio

Constituents of the Model	β Coefficient	95% Confidence Intervals	p value
Prediction of active disease reflected by the physician global assessment			
Serum p-gp	−0.018	−0.035 - −0.001	**0.041**
Monomeric CRP	0.012	−0.006–0.030	0.181
CRP	0.003	0.001–0.006	**0.009**
Prediction of active disease reflected by the physician global assessment			
Serum p-gp	−0.017	−0.035–0.001	0.061
Monomeric CRP:CRP	2.07	−12.37–16.52	0.776
Prediction of ITAS2010			
Serum p-gp	−0.053	−0.281–0.174	0.643
Monomeric CRP	−0.013	−0.251–0.225	0.912
CRP	0.028	−0.006–0.062	0.109
Prediction of ITAS2010			
Serum p-gp	−0.036	−0.260–0.188	0.752
Monomeric CRP:CRP	−85.59	−267.75–96.57	0.353
Prediction of DEITAK			
Serum p-gp	−0.056	−0.253–0.143	0.579
Monomeric CRP	−0.031	−0.240–0.175	0.759
CRP	0.026	−0.004–0.056	0.086

(*Continued*)

Table 6 (Continued).

Constituents of the Model	β Coefficient	95% Confidence Intervals	p value
Prediction of DEITAK			
Serum p-gp	−0.037	−0.233–0.158	0.707
Monomeric CRP:CRP	−89.64	−248.45–69.18	0.265

Note: Significant p values are highlighted in bold.
Abbreviations: CRP, C reactive protein; DEI.TAK, Disease Extent Index in Takayasu Arteritis; ITAS2010, Indian Takayasu Arteritis Clinical Disease Activity Score; mCRP, monomeric CRP; p-gp, p-glycoprotein; TAK, Takayasu arteritis.

Table 7 Comparison Between Patients with TAK in Relation to Corticosteroid or DMARD Use

Parameters [Median (Q1 – Q3)]	On Corticosteroids (n=18)	Not on Corticosteroids (n=74)	p value
Serum p-gp (ng/mL)	12.32 (9.69–17.06)	11.17 (5.90–15.73)	0.329
mCRP (ng/mL)	1.73 (1.25–8.52)	1.61 (1.25–4.29) (n=73)	0.741
CRP (mg/L)	2.55 (0.82–6.00)	6.07 (3.20–12.85)	**0.003**
mCRP:CRP	0.00105 (0.00042–0.00191)	0.00034 (0.00017–0.00096	**0.005**
	On DMARDs (N=17)	**Not on DMARDs (N=75)**	
Serum p-gp (ng/mL)	11.83 (9.68–15.77)	11.18 (5.63–15.84)	0.436
mCRP (ng/mL)	1.25 (1.25–9.24)	1.72 (1.25–4.15)	0.541
CRP (mg/L)	3.04 (0.90–9.30)	5.43 (2.90–12.20)	0.070
mCRP:CRP	0.00089 (0.00024–0.00194)	0.00039 (0.00019–0.00098)	0.119

Note: Significant p values are highlighted in bold.
Abbreviations: CRP, C reactive protein; DMARD, Disease-modifying anti-rheumatic drugs; mCRP, monomeric CRP; p-gp, p-glycoprotein; TAK, Takayasu arteritis.

Comparison Between Treatment-Naïve Patients with TAK Before and After Immunosuppressive Therapy

In the 29 patients with active TAK not on immunosuppressive treatment, follow-up samples were collected at a median of 3.30 (2.43–4.58) months after the initiation of immunosuppressive treatment (corticosteroids in all, mycophenolate in eight and tacrolimus in twenty patients). Serum p-gp ($p < 0.001$) and CRP ($p = 0.009$) were significantly lowered after immunosuppressive treatment. However, no significant differences were observed in the levels of serum mCRP or the ratio of mCRP:CRP (Figure 5). Since tacrolimus is a direct inhibitor of the expression and function of p-gp,[39] additional analyses were conducted excluding the twenty patients who had been treated with tacrolimus. In this subset, there were no significant differences between serum p-gp levels before and after immunosuppressive treatment (Figure 6).

Correlation Between Serum p-Gp and Circulating T Lymphocyte Populations

A significant negative correlation was observed between serum p-gp levels and the circulating population of Th17.1 lymphocytes expressing p-gp (Spearman's rho = −0.39, $p = 0.046$). None of the other observed correlations of serum p-gp with cell populations or with p-gp function on PBMCs were significant (Figure 7).

Discussion

The present study identified an elevation of serum p-gp, mCRP, and CRP in patients with TAK when compared with healthy controls. On univariable analyses, CRP was higher whereas mCRP:CRP ratio was lower in patients with active

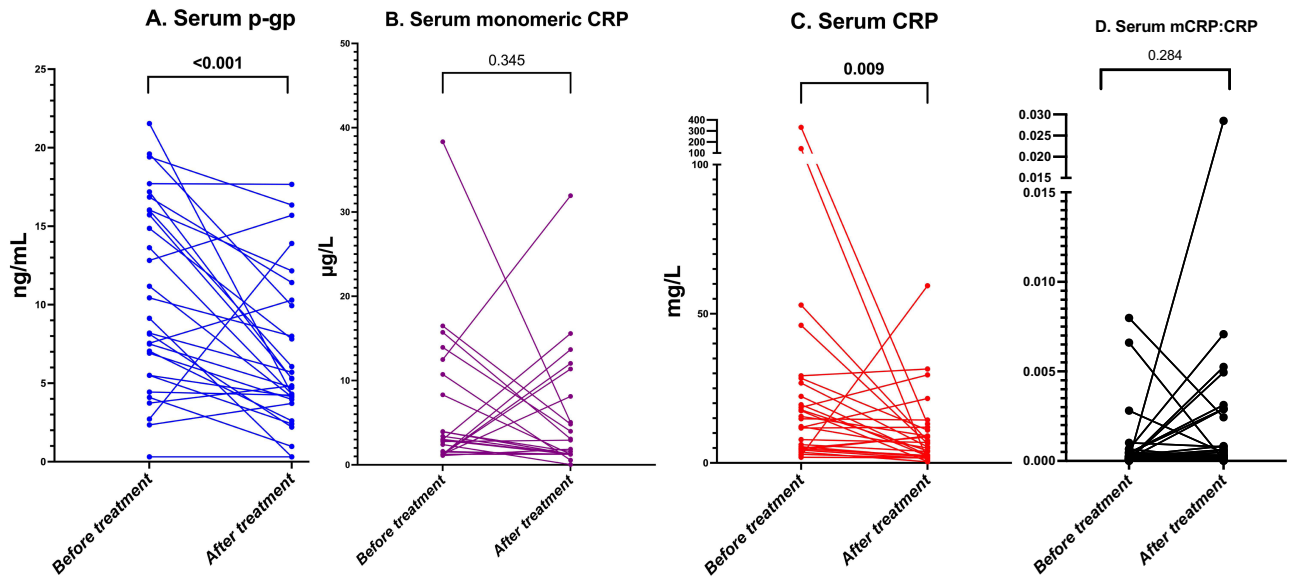

Figure 5 Comparison of serum p-glycoprotein (p-gp) (**A**), monomeric C-reactive protein (CRP) (**B**), CRP (**C**), and mCRP:CRP ratio (**D**) before and after immunosuppressive therapy in 29 patients with TAK with active disease who were treatment-naïve.

TAK than in those with inactive TAK. Serum p-gp levels were associated with inactive TAK and CRP was associated with active TAK after multivariable-adjusted regression analyses. The levels of serum p-gp and CRP were reduced following the initiation of immunosuppressive therapy in a subset of patients with immunosuppressive-naïve TAK. However, excluding those patients who were on tacrolimus which directly inhibits p-gp, no significant reduction in serum p-gp levels was observed after immunosuppressive treatment. A significant negative correlation of weak magnitude was observed between serum levels of p-gp and circulating Th17.1 lymphocytes expressing p-gp.

This study discovered for the first time that serum p-gp and monomeric CRP are elevated in patients with TAK when compared with healthy controls. Serum mCRP had the highest Youden index and odds ratio at the optimal cut-off (higher than CRP) to distinguish patients with TAK from healthy controls. Only two previous studies have assessed serum p-gp levels in patients with immune-mediated inflammatory diseases. Perez-Guerrero et al reported higher serum p-gp levels in patients with 151 patients with active RA than in 30 healthy controls (mean 158.70 vs 14.12 ng/mL).[18] Another study from the same group compared serum p-gp levels in 49 patients with active SLE, 44 patients with inactive SLE, and 43 healthy controls. They reported higher levels of serum p-gp in patients with active SLE than in healthy controls. However, serum p-gp was comparable between patients with inactive SLE and healthy controls.[19] The levels of serum p-gp in our patients with TAK were lesser than those observed in the studies in patients with RA and SLE. This might relate to different pathophysiological mechanisms operational in these diseases. Whereas humoral immunity and autoantibodies play a major role in driving RA and SLE, the pathology of TAK is predominantly driven by cell-mediated immunity with little contribution from autoantibodies. Arteritis, the dominant site of pathology in patients with TAK, is much less common in patients with SLE or RA.[5,40–42] Few studies have evaluated monomeric CRP in patients with immune-mediated inflammatory diseases. Karlsson et al reported lower levels of serum mCRP in patients with SLE than in those with AAV. In this study, serum mCRP levels were comparable in patients with SLE and healthy controls.[28] Wu et al reported higher levels of plasma mCRP in patients with AAV than in healthy controls.[43] Fujita et al reported higher plasma levels of mCRP in patients with Adult-onset Still's disease (AOSD), RA, or polymyalgia rheumatica than in healthy controls, with the highest levels observed in patients with AOSD.[44]

We observed that serum p-gp levels were higher in patients with inactive TAK than in those with active TAK. Analysis using ROC curves also suggested that serum p-gp levels were predictive of inactive TAK. This is contrary to what has been reported in the literature in other immune-mediated inflammatory diseases. From a previous study, serum p-gp was weakly correlated with the disease activity score assessed using 28 joints (r = 0.39), tender joint count (r = 0.37), swollen joint count (r = 0.22), or patient global assessment (r = 0.22) in patients with RA.[18] In patients with SLE,

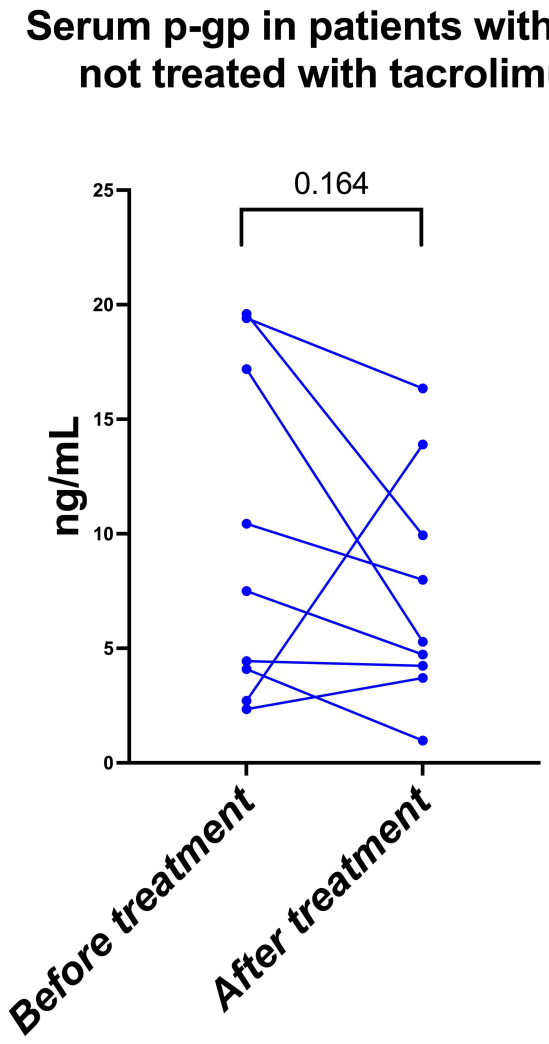

Figure 6 Comparison of serum p-glycoprotein (p-gp) in nine patients with TAK with active disease who were treatment-naïve and were not treated with the p-gp inhibitor tacrolimus.

serum p-gp was higher in patients with active than with inactive lupus, and weakly correlated with the SLE disease activity index (SLEDAI, r = 0.32). Serum p-gp also moderately correlated with the SLE damage index (r = 0.47).[19]

Upon serial follow-up, serum p-gp levels were significantly lowered in patients with TAK after treatment with immunosuppressive therapies. This appears contrary to the observation of higher serum p-gp levels in patients with inactive disease in the cross-sectional arm of the study. Several patients with TAK in the longitudinal arm of the study had been treated with the immunosuppressive agent tacrolimus, which is also a direct inhibitor of p-gp expression and function.[39] Sub-group analyses excluding those patients who had been treated with tacrolimus revealed no significant differences in serum p-gp levels before or after immunosuppressive therapy.

Akin to other studies, we observed higher levels of serum CRP in patients with active TAK than in those with inactive disease. CRP is a well-recognized marker of inflammation. However, CRP imperfectly reflects the disease activity of TAK. Incerti et al reported similar levels of serum CRP in patients with active or inactive TAK based on 18-F FDG PET.[23] A meta-analysis revealed that CRP only moderately reflected TAK disease activity assessed using PET.[45] Therefore, we evaluated mCRP to explore whether it might better reflect TAK disease activity. However, no significant differences were observed in the levels of mCRP in patients with active or inactive TAK. In the longitudinal arm of the study, no significant differences were observed in serum mCRP before (during active disease) or after immunosuppressive therapy (during inactive disease). Similar to this observation, Karlsson et al reported comparable levels of serum

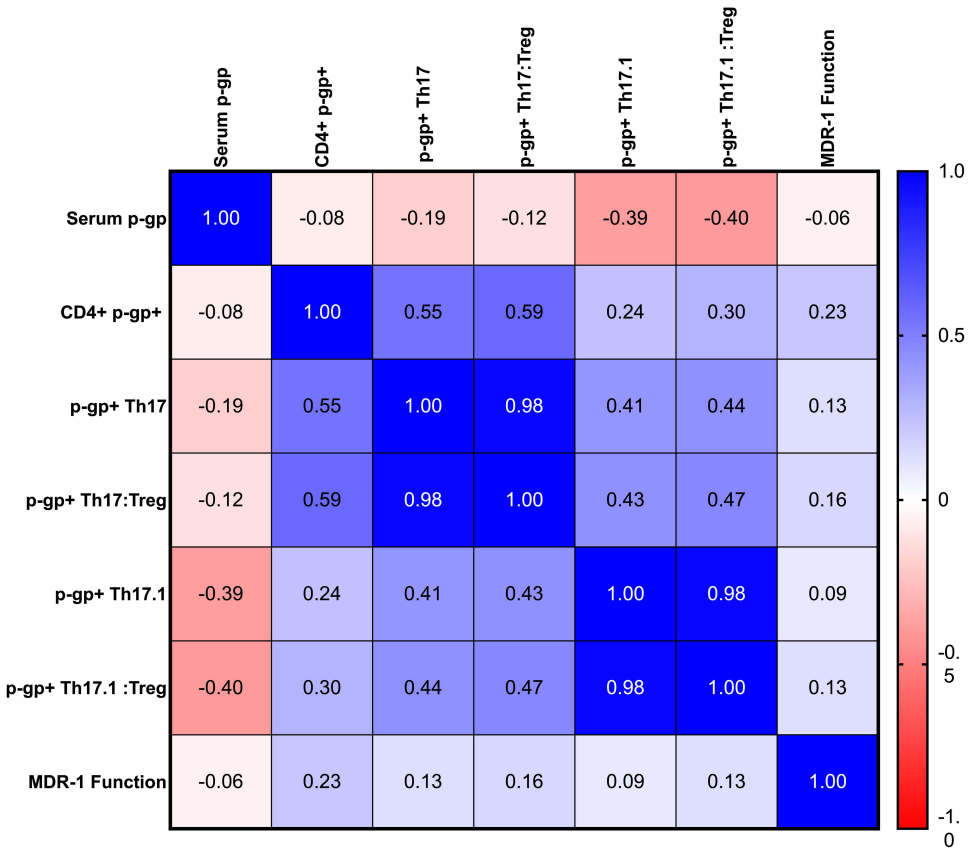

Figure 7 Spearman correlation coefficient (rho) for the correlation between serum p-glycoprotein (p-gp) circulating populations of CD4+ T lymphocytes expressing p-gp (CD4+ p-gp+), Th17 lymphocytes expressing p-gp (p-gp+ Th17) overall or normalized to regulatory T cells (Treg) (p-gp+ Th17:Treg), Th17.1 lymphocytes expressing p-gp (p-gp+ Th17.1) overall or normalized to regulatory T cells (Treg) (p-gp+ Th17.1:Treg), and the function of p-gp/Multidrug Resistance Protein 1 (MDR-1).

mCRP between patients with active or inactive SLE.[28] Wu et al reported a weak correlation of plasma CRP levels with disease activity in patients with AAV assessed using the Birmingham Vasculitis Activity Score (BVAS).[43]

We hypothesized that serum p-gp levels might reflect circulating populations of CD4+ T, Th17, and Th17.1 lymphocytes expressing p-gp. We identified a weak negative correlation between serum p-gp and Th17.1 lymphocytes expressing p-gp but not with the other populations of T lymphocytes. A previous study from our group revealed that elevation of the Th17.1 lymphocyte population was associated with active TAK.[9] This might explain the negative association of serum p-gp levels with TAK disease activity.

As expected, levels of serum CRP were higher in active TAK. However, the ratio of mCRP:CRP was higher in patients with inactive TAK. mCRP is thought to be the active form of CRP; therefore, this finding was surprising. A previous study compared CRP and mCRP:CRP ratio in patients with SLE and AAV and observed a similar difference in the direction of association between CRP and mCRP:CRP. Whereas CRP was lower in patients with SLE than in AAV, the ratio of mCRP:CRP was higher in SLE. In the same study, patients with inactive SLE or normal ESR also had a higher ratio of mCRP:CRP than those with active disease or elevated ESR.[28]

There were limitations to our study. Due to the absence of prior data on the levels of serum p-gp in patients with TAK, the sample size was calculated based on differences in serum p-gp in patients with RA and healthy controls.[18] Moreover, the levels of serum p-gp earlier reported in patients with RA were higher than those observed in patients with TAK in the present study. Therefore, it is possible that the study was underpowered. However, the sample size was adequate to demonstrate a difference in levels of serum p-gp between patients with TAK and healthy controls. Only a fifth of the included patients in our study were on corticosteroids or DMARDs in the cross-sectional arm of the study. However, we evaluated the levels of serum p-gp and mCRP before and after the initiation of immunosuppressive therapy in a subset of patients with active disease who were not on treatment. We have evaluated the association of serum p-gp with T helper

lymphocyte populations which have been previously implicated in disease activity and treatment refractoriness in immune-mediated inflammatory diseases. Various other innate and adaptive immune cells also express p-gp; therefore, serum p-gp levels might reflect immune activation distinct from T lymphocyte activation alone.[46] The cross-sectional nature of our study did not permit the assessment of the relationship between serum p-gp and mCRP with the long-term prognosis of TAK. The strengths of our study were that it included a large number of patients with TAK, which is a rare disease. Previous studies on serum p-gp and mCRP in rheumatic diseases have evaluated these markers in a cross-sectional manner, whereas, we evaluated the changes in these markers before and after treatment.

Conclusion

Serum p-gp and mCRP are elevated in patients with TAK than in healthy controls. Serum levels of p-gp are associated with inactive TAK and negatively correlated with p-gp expression on circulating Th17.1 lymphocytes. No association with TAK disease activity was evident with mCRP. Future studies are required to validate the observation of elevated serum p-gp as a marker of inactive TAK.

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Disclosure

The authors report no conflicts of interest in this work.

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