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ORIGINAL ARTICLE

Rheumatoid arthritis (RA) disease activity predicts function of ABCB1 (P-gp) and ABCG2 (BCRP1) drug-efflux transporters

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Abstract

Background: *P-gp and BCRP1 are transporter proteins that may confer drug resistance.* **Objective:** *To compare P-gp and BCRP1 function in rheumatoid arthritis patients with active and inactive disease and to define their relation with disease activity.* **Methods:** *We included 17 active patients paired (age, gender, disease duration) to 17 inactive patients. All had baseline evaluations and 27 had additional six-month follow-up. P-gp and BCRP1 functional activity was measured in peripheral mononuclear cells by flow cytometry. Percentage of lymphocytes able to extrude substrates for P-gp and BCRP1 were recorded in the presence/absence of selective inhibitors. Informed consent was obtained. Descriptive statistics and linear regression model were applied.* **Results:** *Active patients had higher efflux function of both transporters than inactive patients: median (25-75 IQR) P-gp of 7.1% (1.4-29.3) vs. 1.6% (0.7-3.5), p = 0.02 and BCRP1 of 6.2% (1.3-22.4) vs. 1.3% (0.7-2), p = 0.007. At baseline, disease activity was the only predictor of both transporter functions. At follow-up, changes in disease activity correlated with shift in P-gp (r = 0.35, p = 0.07) and BCRP1 (r = 0.33, p=0.09) function.* **Conclusions:** *Patients with active rheumatoid arthritis had a higher efflux function of P-gp and BCRP1 compared to inactive patients. The behavior of P-gp and BCRP1 appeared to be conditioned by disease activity.* (Gac Med Mex. 2016;152:662-74)

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Introduction

The phenomenon of multidrug resistance was initially described in cancer. It is characterized by the extrusion of substances (contextually drugs) from inside the cell to the extracellular space by means of transporters, which act through energy-dependent channels; as a consequence, intracellular drug concentration is decreased and, hence, its efficiency[1]. The first transporter to be described was the permeability glycoprotein (P-gp), encoded by the *MDR1* gene[2], currently identified with the generic name ATP-binding cassette (ABC) *B1* (ABCB1). Other of the most widely studied transporters is ABCG2 or breast cancer resistant protein 1 (BCRP1)[3]. Both work as energy-dependent pumps with variable specificity that is physiologically related to hormone secretion and bacterial toxins

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expulsion from cells. Increased function of both transporters results in drug resistance. Drugs, same as toxins, can be transporter substrates, which prevents intracellular levels from becoming sufficient to exert their therapeutic action. In recent years, the knowledge generated on their role in the response to oncologic therapy has expanded to other drugs such as antiviral[4] and immunosuppressant drugs[5].

Rheumatoid arthritis (RA) is a chronic autoimmune disease characterized by inflammation of the synovial membrane of joints, localized preferably in the joints of hands and feet[6]. It is distributed globally, with preference for the female gender and it has a wide variety of extra-articular manifestations. Early, aggressive treatment aimed at reaching remission with disease-modifying anti-rheumatic drugs (DMARDs) favorably impacts on patient outcomes[7-9]. It should be noted that, in the Latin American population, the disease has distinctive characteristics of prevalence[10], demographics, clinical presentation and treatment response[11].

The concept of early (or recent-onset) arthritis appeared approximately two decades ago and includes terms corresponding to other entities, such as undifferentiated arthritis (which can progress to RA or not), RA early phases and even other entities[12]. Differentiation of each one of them is fundamental due to the functional implications for patients. In the RA clinical context, remission has been seen more frequently achieved by those who start DMARD treatment with less than 4 months of symptoms' duration than by those who start treatment later[13]. Similarly, radiographic disease progression has been shown to be more effectively delayed in those in whom treatment is implemented before 3 months than in those in whom it is started after 12 months[14]. Given that the erosion rate is higher within the two first years after diagnosis[15,16], early establishment of adequate diagnosis and treatment is currently more than a recommendation, a health necessity. This has been possible thanks to novel diagnostic tools, with the classification criteria proposed by the American College of Rheumatology and the European League Against Rheumatism in 2010 standing out[17]. In addition, the clinical settings where patient diagnosis, treatment and follow-up are optimized are the "early (rheumatoid) arthritis clinics".

RA economic impact is considerable[18], since the disease affects adults in the fourth decade of life and produces work incapacity[19], treatment with DMARDs is prolonged and patients require frequent modifications of the employed treatments. In the most recent update of the Mexican Guidelines for the management of RA[20],

more than 15 DMARDs are included; since usually they are used in combination, treatment options are numerous; however, more than 50% of patients do not respond adequately to standard treatment[21], which implies worse clinical outcomes, use of potentially more toxic combinations and increased costs. Therefore there is the need to provide the patients with more efficacious and rational (and early) treatment, which requires further investigating the lack of response to treatment. An attractive hypothesis is that patients with active RA have or acquire resistance to DMARDs.

In the RA clinical setting, there are few studies that have looked into the relationship between the activity of transporters and the response to the treatment with DMARDs and/or corticosteroids, and the results have been ambiguous. In studies with cross-over design, an increase in P-gp activity has been demonstrated in peripheral blood lymphocytes of patients with active RA[22], of patients with active RA previously treated with prednisolone[23] and in synovial fluid lymphocytes of patients previously treated with DMARDs[24]. However, another study group showed an increase in drug extrusion in peripheral blood lymphocytes of heavy treatment-experienced patients and, notwithstanding, a full response to corticosteroids (in vitro)[25]. With regard to methotrexate, studies have been equally conflicting[26,27], although one of the few studies with longitudinal design demonstrated that the expression of the multi-drug resistance protein (MRP1) involved in methotrexate extrusion was unexpectedly down-regulated six months after having started treatment with this drug[27]. Finally, inverse activity of two transporters has been described in patients with RA and acquired resistance to sulfasalazine, in such a way that the response to different DMARDs can be increased and decreased in a single patient depending on the employed treatment[28-30].

Since a considerable proportion of treated RA patients persist with important activity, the concept of resistance as a consequence of DMARDs extrusion owing to an increase in the function of certain transporters is a stimulating working hypothesis in the setting of an early RA clinic, where rigorous patient follow-up is carried out.

The purposes of the present work were:

– To determine and compare P-gp and BCRP1 transporters activity in RA patients with disease activity and RA patients without disease activity (or in remission).
– To investigate associations between corticosteroids and/or DMARDs doses and P-gp and BCRP1 transporters activity.

– To investigate the impact of RA on P-gp and BCRP1 transporters functional activity.

Material and methods

Study population: Early Arthritis Clinic

Patients were selected from the Early Arthritis Clinic of the *Instituto Nacional de Ciencias Médicas y Nutrición Salvador Zubirán* (INCMNSZ). Briefly, the clinic was opened in February 2004 with the purpose to early identify and treat patients with suspected RA and symptoms of less than 12 months' duration. It is constituted by a multidisciplinary team (rheumatologist, nurse and social worker) that carries out an exhaustive patient follow-up, which includes RA assessment (swollen and tender joint count, acute phase reactants, physician assessment and patient-reported outcomes), progression evaluation (hand and feet X-rays, with annual periodicity), assessment of response and treatment adherence, and assessment for the presence of comorbidities and eventual complications and/or adverse events. Patient follow-up is every 6 or 8 weeks for the first 2 years, and subsequently every 3, 4 or 6 months, depending on each patient's clinical status. Treatment includes the use of DMARDs and it is aimed at achieving remission. In addition to the performance of healthcare activities, the clinic was designed for the development of research on RA in our country, and it has updated databases available with information related to clinical, serologic, radiologic and functional patient outcomes throughout follow-up, as well as a blood and genetic and biological material bank.

To date, 187 patients have been recruited, out of whom 160 are on active follow-up, two have died, 7 have a diagnosis other than RA and 18 have been lost to follow-up (more than 1 year without attending the clinic). The clinic has more than 10 years' experience and, therefore, 4% of patients have less than one year of evolution (recent admission), 30% from 1 to 5 years' follow-up and 66% more than 5 years' evolution[31-40].

Study design and definition of variables

This is a case-control study nested within the cohort of patients with early RA.

Any patient with RA according to two sets of international classification criteria[17,41], who had a disease activity score in 28 joints (DAS28) ≥ 3.2 at the moment of inclusion to the study[42] in spite of receiving therapeutic, stable (at least 2 months) doses of DMARDs, was considered as a case.

Any patient with RA meeting the same above-mentioned criteria except for the one regarding disease activity at the moment of study enrollment was considered as a control; controls were defined as being in remission, i.e., DAS28 < 2.6.

Each case was matched with its respective control (1:1) according to the following variables: age (± 10 years), gender and disease evolution time or follow-up time at the clinic (± 5 years).

The disease activity (DA) level was defined according to the DAS28 value in 4 categories: high, moderate disease activity, low and remission[42].

Patient assessment and sample collection

The below-described assessments were carried out at the moment the patients were enrolled in the study and at 6 months' follow-up. The patient-inclusion strategy was first by identifying cases and, in a time no longer than one week, adequately matched controls were included.

Clinical evaluation included:
– A brief interview to corroborate socio-demographic data, investigating for DA symptoms, treatment and treatment adherence confirmation and defining the presence of comorbidities.
– Swollen and tender joint identification and count (28 possible locations instead of sites)[42].
– Determination of serum acute phase reactants (globular sedimentation rate and C-reactive protein [CRP]).
– Application of pain and general disease status visual analogue scales to the patient, as well as Spanish-validated self-assessment questionnaires to define activity, fatigue, pain, disability and quality of life[35].
– DAS28 calculation.
– 5 ml peripheral blood extraction for P-gp and BCRP1 transporters functional activity measurement by flow cytometry.

Sample processing

All samples were processed in a time no longer than 1 h after extraction.

Peripheral blood (anticoagulated with 2% of ethylenediaminetetraacetic acid [EDTA]) mononuclear cells were isolated by centrifugation gradient with Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). After two washings, the mononuclear cells were counted, adjusted to 3×10^6/ml and three 1×10^6 aliquots were

made. P-gp activity was measured by incubating the cells in the presence of 60 µl of 400 µM daunorubicin (a P-gp fluorescent substrate; Sigma-Aldrich, Saint Louis, MO) for 1 h under the following conditions; 1×10^6 at 4 °C (as extrusion negative control), 1×10^6 at 37 °C (to enable extrusion) and 1×10^6 at 37 °C with 5 µl of 50 mM verapamil (a P-gp inhibitor; Sigma-Aldrich, Saint Louis, MO). To measure BCRP1 activity, 20 µl of 500 µM mitoxantrone (a fluorescent BCRP1 substrate; Sigma-Aldrich, Saint Louis, MO) were added to 3×10^6 cells, and 75 µl of 10 µM KO143 (a BCRP1-specific inhibitor; Sigma-Aldrich, Saint Louis, MO) were used as inhibitor, with incubation under the same previously-described conditions. Subsequently, the samples were washed with cold phosphate buffered saline (PBS) and centrifuged for 10 min at 1,600 rpm. The supernatant was removed, and the cells were resuspended in cold PBS and maintained on ice until their analysis. Flow cytometry was performed in a FACS Canto II equipment (BD Biosciences, San Jose, CA). Daunorubicin fluorescence was analyzed with a neon-helium laser at 488 nm, and mitoxantrone fluorescence with an argon laser at 633 nm. In all cases, 20,000 events were acquired and the analytical program BD FACSDiva was used (Figures 1 and 2).

Each transporter's functional activity was expressed as the percentage of lymphocytes that expelled the substrate (daunorubicin for P-gp and mitoxantrone for BCRP1). P-gp and BCRP1 transporters resistance was defined when the extrusion percentage was higher than 1.67% of lymphocytes. This cutoff point was established based on both transporters' activity determined in 25 healthy subjects (mean + 2 standard deviations [SD]).

Ethical considerations

The clinic was approved by the INCMNSZ Institutional Committee of Biomedical Research in Humans, as well as the present project (Reference: 1128). All patients signed an informed consent document agreeing to undergo the assessments required by the clinic, to provide blood samples, to have their medical records reviewed and for data obtained in this study to be published.

Statistical analysis

The sample size was calculated based on the study's primary hypothesis, i.e., patients with RA in remission display lower percentage of P-gp and BCRP1 transporters functional activity than patients with DA, taking into account an estimated difference for extrusion percentage averages by flow cytometry. A difference of 30 percentage points was estimated between both groups, with a power of 80% and an alpha value of 0.025 owing to the presence of two primary hypotheses (due to the analysis of two transporters). Therefore, a total of 9 patients per group were required (18 in total). However, in an attempt to normalize the results' distribution and considering the multivariate analysis with the treatment variables, by convenience we decided to include a total of 40 patients (20 per group).

Statistical analysis strategy

For the data descriptive analysis, relative frequencies and central and dispersion indices adequate to the form of distribution and measurement scale were used. For the comparison of continuous variables, Student's t-test and Mann-Whitney's U-test or Wilcoxon's signed-rank sum test were used according to the variables' distribution. To find out the relationship degree between DA and medication isolated or cumulative doses over the last year with the transporters' activity measurement, Spearman's Rho was used. Finally, the multivariate analysis to establish the association of DA with the value of both transporters' activity (each one independently) adjusted for the received medications' doses, was carried out using a multiple linear regression model including those drugs that showed significant association for each transporter.

A two-tailed p-value ≤ 0.05 was assumed as being significant. The statistical program SPSS, version 20, was used.

Results

In the present work, the preliminary results obtained after the analysis of the first 17 case-control pairs are reported, with a total of 34 patients with RA, out of which 27 had a follow-up assessment at 6 months.

General characteristics of the study population

The most relevant characteristics of the population at the moment of enrollment in the study are presented in table 1. Most subjects were females (94.1%) at the fourth decade of life (mean age ± SD: 41.6 ± 10.8 years), with complete basic education (mean years of education ± SD: 10.6 ± 4.2); in general, most patients (at clinic admission) were rheumatoid factor (RF)-positive (85.3%)
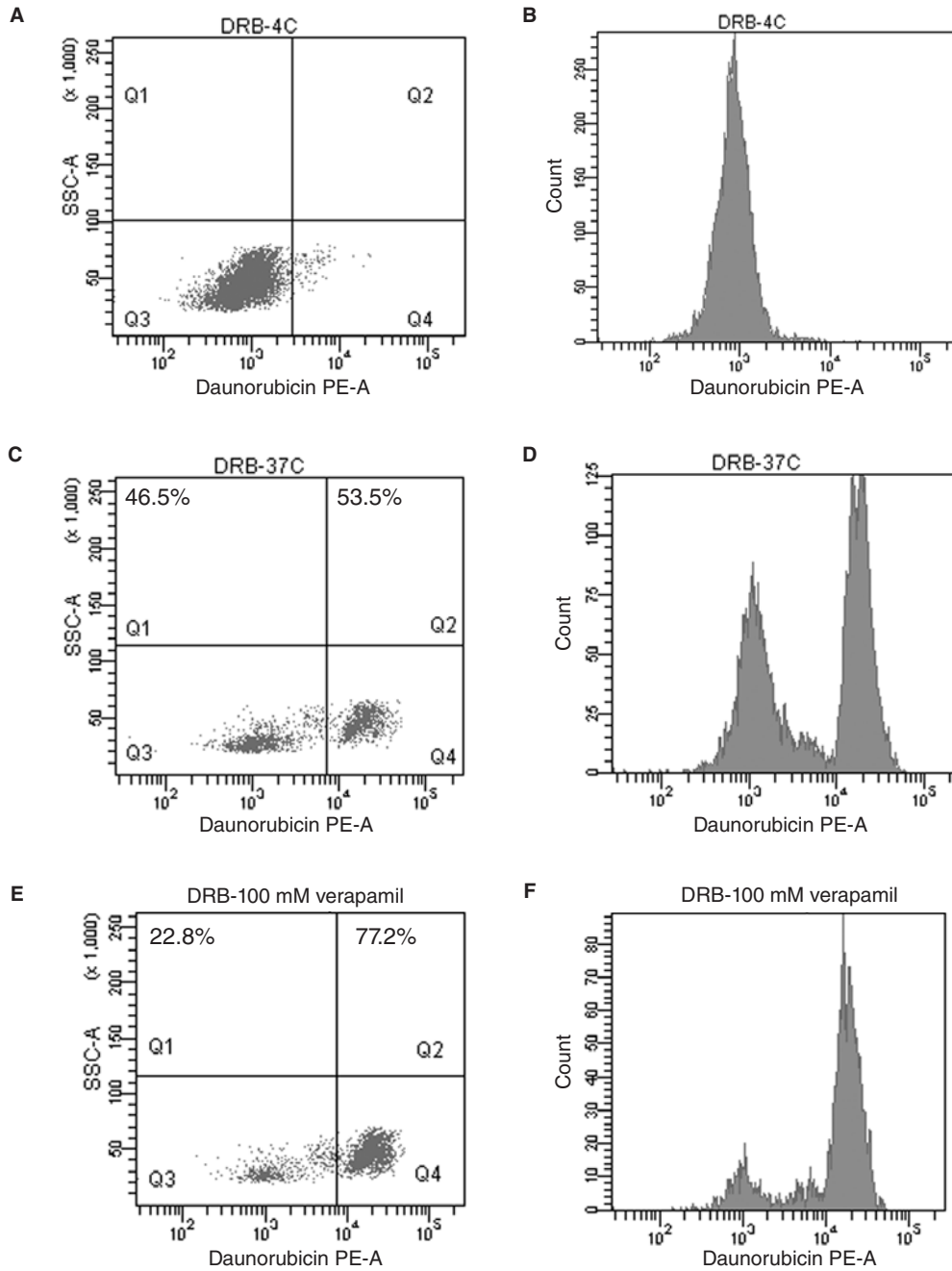
Figure 1. *P-gp functional activity in RA patients' lymphocytes. The figure shows a representative experiment of daunorubicin-expelling lymphocytes in a patient with RA and active disease. **A** and **B**: cytogram and histogram of lymphocytes incubated at 4 °C in the presence of daunorubicin. **C** and **D**: cytogram and histogram of lymphocytes incubated at 37 °C in the presence of daunorubicin. Both non-expelling (higher fluorescence intensity at right [53.5%]) and expelling lymphocytes (lower fluorescence intensity at left [46.5%]) are clearly evident. **E** and **F**: cytogram and histogram of lymphocytes incubated at 37 °C in the presence of daunorubicin and verapamil (P-gp substrates competitive inhibitor). In vitro inhibition is clearly appreciated when histograms **D** and **F** are compared. The cutoff value for positivity was established as 2 SD above the mean of the control group (1.67% of daunorubicin-expelling lymphocytes).*
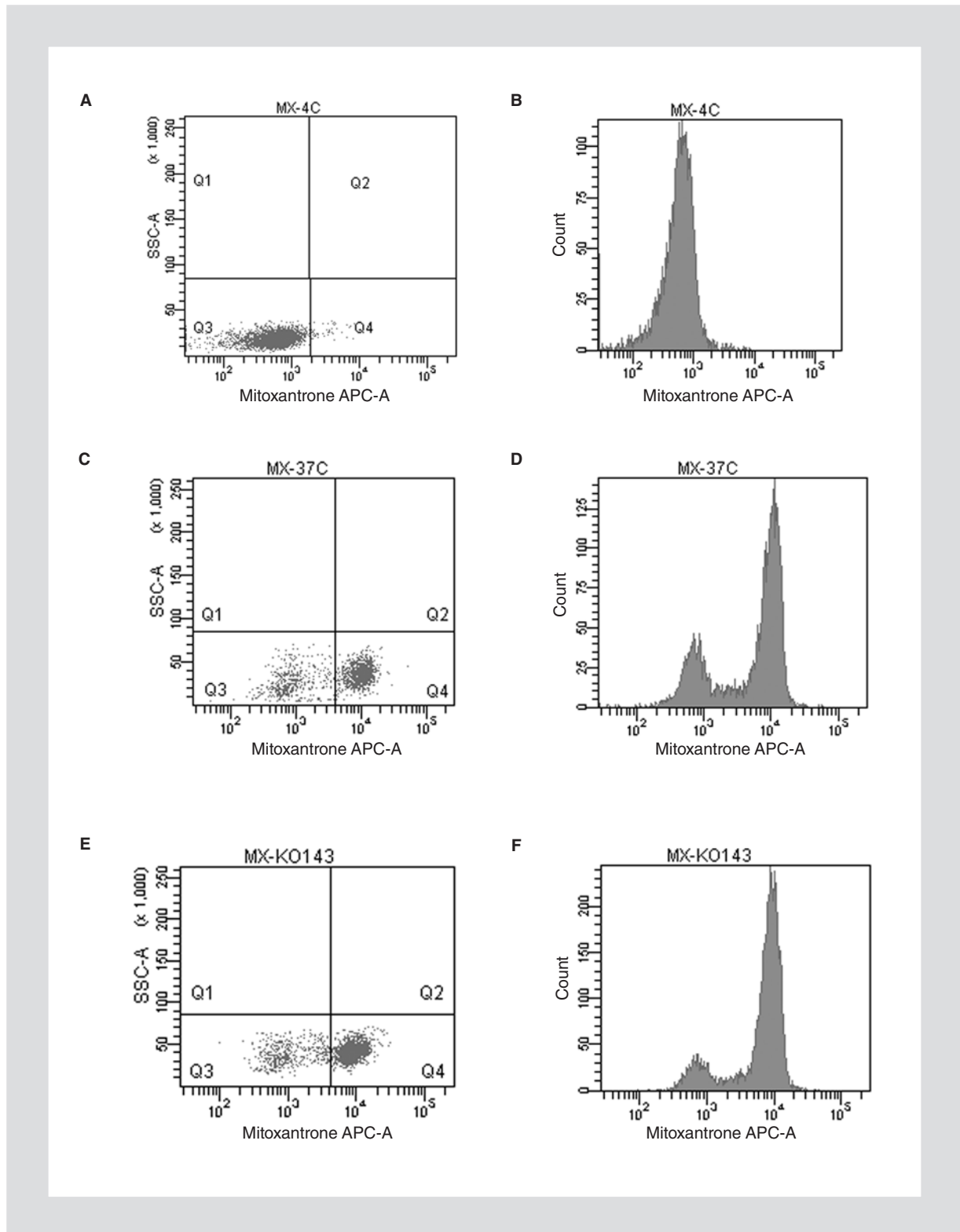
Figure 2. *BCRP1 functional activity in RA patients' lymphocytes. The figure shows a representative experiment of mitoxantrone-expelling lymphocytes in a patient with RA and active disease. **A** and **B**: cytogram and histogram of lymphocytes incubated at 4 °C in the presence of mitoxantrone. **C** and **D**: cytogram and histogram of lymphocytes incubated at 37 °C in the presence of mitoxantrone. Both non-expelling (higher fluorescence intensity at right [66.7%]) and expelling lymphocytes (lower fluorescence intensity at left [33.3%]) are clearly evident. **E** and **F**: cytogram and histogram of lymphocytes incubated at 37 °C in the presence of mitoxantrone and KO143 (BCRP1 substrates competitive inhibitor). In vitro inhibition is clearly appreciated when histograms **D** and **F** are compared. The cutoff value for positivity was established as 2 SD above the mean of the control group (1.67% of mitoxantrone-expelling lymphocytes).*

Table 1. Patient general characteristics at study enrollment and comparison between patients with active disease (cases) and in remission (controls)

	Total population (n = 34)	Patients with active RA (n = 17)	Patients with RA in remission (n = 17)	p
Demographic characteristics				
Number of female patients (%)	32 (94.1)	16 (94.1)	16 (94.1)	1
Age, years, at study enrollment*	41.6 ± 10.8	43.5 ± 12.1	39.6 ± 9.1	0.30
Number of smoker patients (%) at study enrollment	4 (11.8)	2 (11.8)	2 (11.8)	1
Completed years of education at clinic admission*	10.6 ± 4.2	9.5 ± 4.7	11.6 ± 3.4	0.15
RA specific characteristics				
RA follow-up time, years, at study enrollment*	6.3 ± 3.5	6.6 ± 3.9	6 ± 3.2	0.61
DAS28 at study enrollment*	3 ± 2.1	4.8 ± 1.3	1.2 ± 0.6	≤ 0.001
CRP at study enrollment (mg/dl)*	0.9 ± 1.1	1.6 ± 1.2	0.2 ± 0.2	≤ 0.001
Number of patients (%) with erosive disease at study enrollment	17 (50)	10 (58.8)	7 (41.2)	0.49
Number of patients (%) with RF+ at clinic admission	29 (85.3)	15 (88.2)	14 (84.4)	1
Number of patients (%) with CCP antibodies at clinic admission	33 (97.5)	16 (94.1)	17 (100)	1
Comorbidities at study enrollment				
Number of patients (%) with ≥ 1 comorbidity	28 (82.4)	14 (82.4)	14 (82.4)	1
Charlson score*	1.1 ± 0.4	1.1 ± 0.5	1.1 ± 0.2	0.66
Treatment at study enrollment				
Number of patients (%) on corticosteroids	27 (79.4)	13 (76.5)	14 (82.4)	1
Corticosteroid dose/patient (mg/day)*†	8.1 ± 4.2	10.1 ± 5	6.3 ± 2.1	0.03
Cumulative corticosteroid dose/patient in previous year*	3134.4 ± 1625.2	4041.2 ± 1848.7	2348.5 ± 859.5	0.004
Number of DMARDs/patient*	1.7 ± 0.9	1.7 ± 0.9	1.8 ± 0.8	0.85

*Mean ± SD.
†Equivalent to prednisone mg/day, only in those who were receiving prednisone.

and anti-cyclic citrullinated peptide (CCP) antibody-positive (97.5%); in addition, they had a mean ± SD time of follow-up of 6.3 ± 3.5 years and at least one comorbidity (82.4%). All patients were receiving some DMARD, with the number (mean ± SD) of DMARDs/ patient being 1.7 ± 0.9; in addition, 27 patients (79.4%) were also receiving oral corticosteroids and daily average dose (± SD) equivalent to prednisone was 8.1 mg (± 4.2).

There were no differences in matching variables between the cases (patients with active disease) and the controls (Table 1). Compared with controls, the cases had higher disease activity, with a DAS28 (mean ± SD) at study enrollment of 4.8 ± 1.3 versus 1.2 ± 0.6 (p ≤ 0.001), and higher serologic activity, with a CRP concentration (mean ± SD) of 1.6 ± 1.2 versus 0.2 ± 0.2 mg/dl (p ≤ 0.001). Cases had also higher corticosteroid daily doses (mean ± SD) at study enrollment and higher cumulative doses in the previous year than controls: 10.1 ± 5 versus 6.3 ± 2.1 mg/day in prednisone equivalent (p = 0.03) and 4041.2 ± 1848.7 versus 2348.5 ±

859.5 mg (p = 0.004), respectively. The use of DMARDs was similar in both groups.

Transporters' activity at study inclusion

Table 2 and figure 3 summarize relevant results. Patients with DA (DAS28 ≥ 3.2) showed P-gp and BCRP1 transporters' resistance more frequently than patients in remission (DAS28 < 2.6): 12 patients (70.6%) versus 8 (47.1%) (p = 0.3) and 12 patients (70.6%) versus 6 (35.3%) (p = 0.08); the percentage of each transporter functional activity (median, interquartile range) was higher in patients with DAS28 ≥ 3.2 than in patients in remission: 7.1% (1.4-29.3) versus 1.6% (0.7-3.5) (p = 0.02) and 6.2% (1.3-22.4) versus 1.3% (0.7-2) (p = 0.007). Similar results were obtained when the subgroup of patients with RA and resistance to P-gp (n = 12) and BCRP1 (n = 12) was analyzed (Table 2 and Fig. 3).

Finally, both transporters, P-gp and BCRP1, were significantly correlated with DA, as evaluated with

Table 2. Number of patients (%) with active RA (cases) and in remission (controls) with P-gp and BCRP1 resistance and the respective functional activity percentages of both transporters

|	Patients with active disease (DAS28 ≥ 3.2) (n = 17)	Patients in remission (DAS28 < 2.6) (n = 17)	p
P-gp			
Number of patients (%) with P-gp resistance	12 (70.6)	8 (47.1)	0.30
Median (Q25-Q75) functional activity % in all patients	7.1 (1.4-29.3)	1.6 (0.7-3.5)	0.02
Median (Q25-Q75) functional activity % in patients with P-gp resistance (n = 12)	19.7 (6.9-38.4)	3.5 (2.9-9.1)	0.01
BCRP1			
Number of patients (%) with BCRP1 resistance	12 (70.6)	6 (35.3)	0.08
Median (Q25-Q75) functional activity % in all patients	6.2 (1.3-22.4)	1.3 (0.7-2)	0.007
Median (Q25-Q75) functional activity % in patients with BCRP1 resistance (n = 12)	11.4 (4.2-32.8)	2.49 (2-4)	0.02
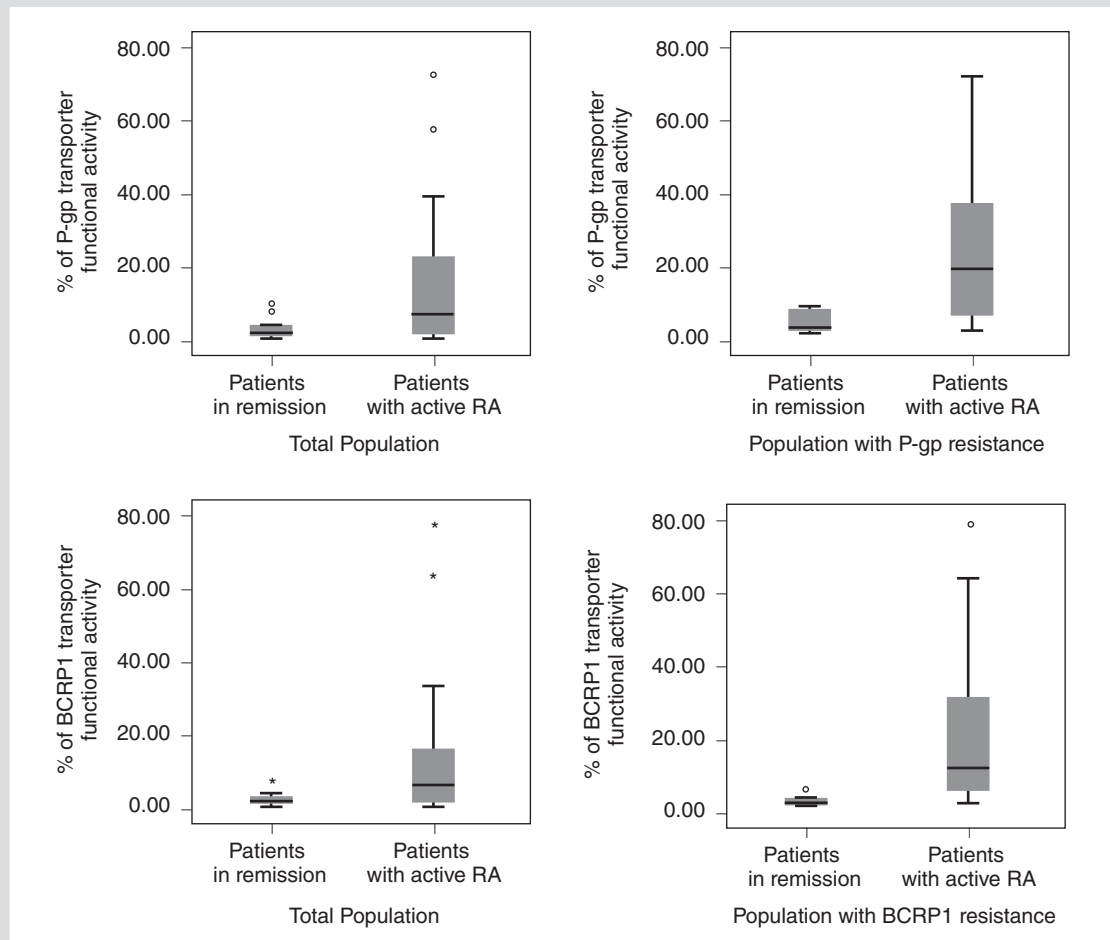
Figure 3. *P-gp and BCRP1 percentage functional activity in patients with RA (cases) and patients in remission (controls). The figure shows the percentage of P-gp (upper panels) and BCRP1 (lower panels) functional activity in patients with active disease and patients in remission. Left panels show the analysis in the entire population, and right panels, in the population of patients with RA and resistance to transporters. Each box represents the median (horizontal bar in bold), the 25th and 75th percentiles (lower and upper limits of the box) and minimum and maximum (protruding lines).*
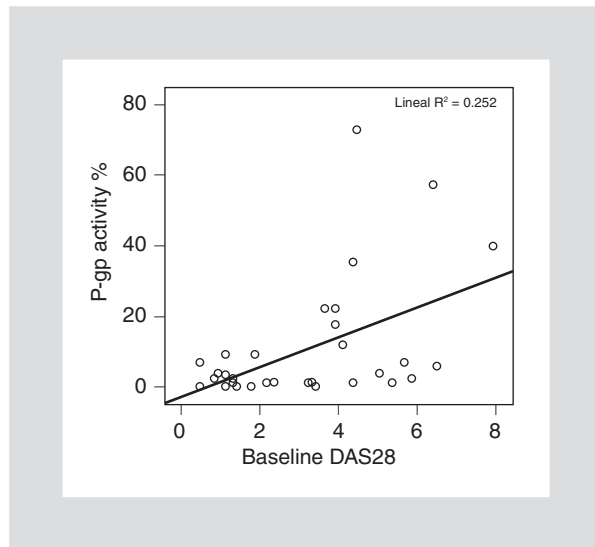
Figure 4. *Correlation between P-gp functional activity and DAS28-assesed DA. The figure shows the correlation between P-gp functional activity (ordinate line) and DA according to DAS28 (abscissa line).*
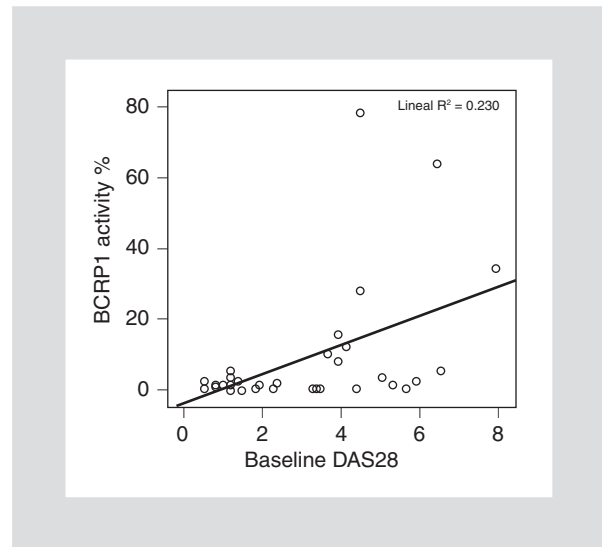
Figure 5. *Correlation between BCRP1 functional activity and DAS28-assesed DA. The figure shows the correlation between BCRP1 functional activity (ordinate line) and DA according to DAS28 (abscissa line).*

DAS28: Rho of 0.45 (p = 0.008) and 0.52 (p = 0.002), respectively (Figures 4 and 5).

P-gp and BCRP1 functional activity correlation with drug doses

Drug doses at study enrollment and cumulative doses in the year previous to enrollment were determined for each patient; analyzed medications were corticosteroids (prednisone equivalent dose), methotrexate, chloroquine (or hydrochloroquine), azulfidine, leflunomide (one patient) and azathioprine (one patient). The results of the more commonly indicated drugs in the entire population at study enrollment are summarized in table 3. There were no differences in medication doses (specific or cumulative) between patients with transporter resistance and patients without resistance. In addition, there was no correlation between each transporter (P-gp and BCRP1) functional activity and the doses of all different medications (data not shown).

Impact of DA on transporters' functional activity

The linear regression model with the baseline data of all 34 patients showed that DAS28 was the only predictor of P-gp (beta coefficient: 0.50; 95% confidence interval [CI]: 1.6-6.7; p = 0.002; R^2 = 0.23) and

BCRP1 functional activity (beta coefficient: 0.48; 95% CI: 1.4-6.8; p = 0.004; R^2 = 0.21.

At the moment the data here presented were analyzed, 27 patients had completed six months of follow-up: 4 of them (14.8%) showed an increased level of activity according to DAS28, 13 (48.1%) maintained the same level and 10 (37.1%) showed a decreased level of DA. At six months, the number of patients with resistance of both transporters was increased, although the increase was lower in patients with DA improvement (Table 4); similarly, the magnitude of the increase in transporters' functional activity was lower in patients with clinical improvement (Table 4; p with no statistical significance).

Finally, the DAS28 differences (between baseline and six-month assessments) were correlated with the differences in both transporters' functional activity, with moderate correlation and significant tendency being found: r = 0.35 (p = 0.07) for P-gp and r = 0.33 (p = 0.09) for BCRP1 (Fig. 6).

Discussion

There is limited information on the participation of the P-gp and BCRP1 transporters in autoimmune rheumatic diseases. In patients who require anti-inflammatory drugs and/or DMARDs administration, an increase in the function of these transporters induced by immune system cells might be associated with insufficient

Table 3. Treatment at study enrollment and cumulative in the entire population of patients with RA; includes most commonly indicated corticosteroids and DMARDs

	RA population (n = 34)
Number of patients (%) with corticosteroids	27 (79.4)
Prednisone/patient (mg/day)*	7.5 (5-10)
Previous year cumulative prednisone milligrams/patient*	2807.5 (1880.6-3773.8)
Number of patients (%) with methotrexate	30 (88.2)
Methotrexate (mg/week), median (min-max)*	25 (23.8-25)
Previous year cumulative methotrexate milligrams/patient*	1303.6 (930.5-1303.6)
Number of patients (%) with chloroquine	1 (35.3)
Chloroquine/patient (mg/day)*	150 (150-150)
Previous year cumulative chloroquine milligrams/patient*	39.700 (26.700-54.862.5)
Number of patients (%) with azulfidine	13 (38.2)
Azulfidine/patient (g/day)*	2 (2-3)
Previous year cumulative azulfidine grams/patient*	493 (295.6-714.8)

*Median (Q25-Q75): data obtained from the subpopulation taking that drug.

Table 4. Transporter resistance and functional activity at six months' follow-up according to changes in patients DA level

Disease behavior at 6 months	DAS28 at 6 months*	Number of patients (%) with resistance	p	Functional activity % at 6 months*	p	Number of patients (%) increasing functional activity	p	Functional activity % increase*	p
P-gp transporter									
Patients who get worse, (n = 4)	3 (2.7-3)	3 (75)	0.4	9 (0-22)	0.5	3 (75)	0.6	3.7 (3.7-21.6)	0.9
Patients who maintain the same level of DA activity, (n = 13)	2 (1-4)	12 (92)	0.4	5 (2.8-23)	0.5	10 (77)	0.6	4.4 (1.3-16.1)	0.9
Patients who improve, (n = 10)	2.4 (1.8-3)	7 (70)	0.4	2.5 (1.2-21)	0.5	5 (50)	0.6	2.7 (0.6-37.7)	0.9
BCRP1 transporter									
Patients who get worse, (n = 4)	3 (2.7-3)	3 (75)	0.4	12 (0-20)	0.8	3 (75)	0.4	13.9 (7.3-19.7)	0.7
Patients who maintain the same level of DA activity, (n = 13)	2 (1-4)	12 (92)	0.4	4 (2.7-28)	0.8	11 (85)	0.4	3.1 (1.5-31.9)	0.7
Patients who improve, (n = 10)	2.4 (1.8-3)	7 (70)	0.4	3 (1-19.8)	0.8	6 (60)	0.4	6.2 (2-27)	0.7

*Median (Q25-75).
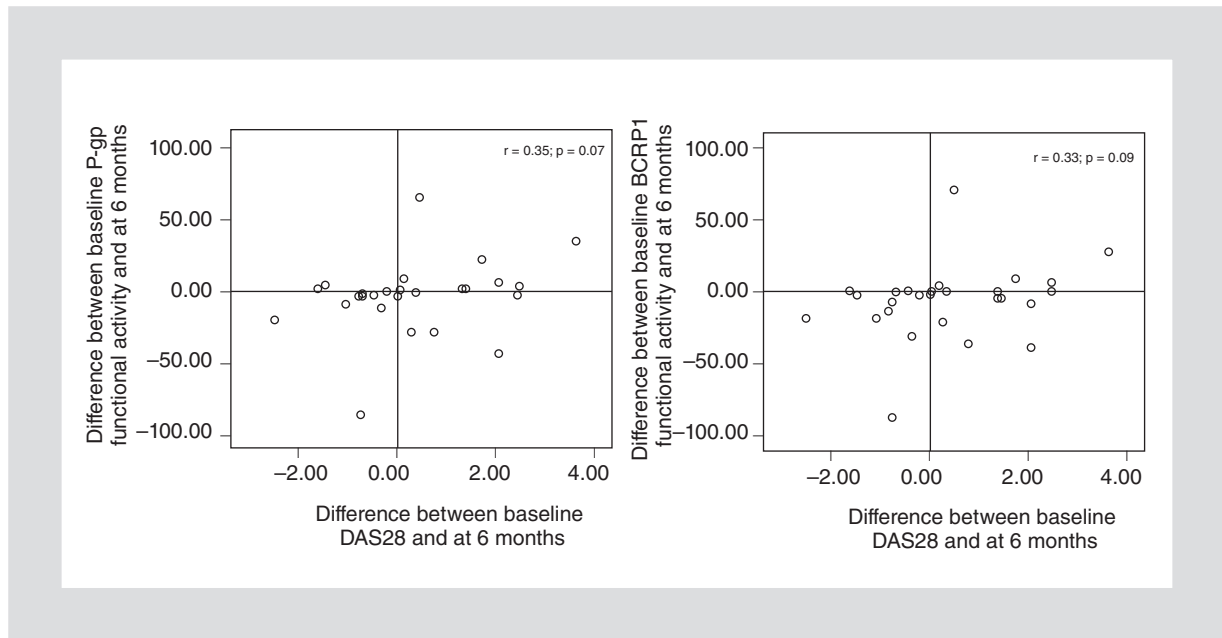
Figure 6. *Correlation between DAS28 and transporter functional activity changes (at six months' follow-up). The figure represents the correlation between DAS28 changes (abscissa line) and P-gp (left panel) and BCRP1 (right panel) transporters' functional activity changes. Each panel is divided by a vertical line, which indicates there is no DAS28 variation, and a horizontal line indicating there is no transporter functional activity variation. Each point represents one patient. The points located at the right of the vertical line represent patients with DA increase (and vice versa). The points located above the horizontal line represent patients with transporter functional activity increase.*

therapeutic effect. In RA, response to therapy plays a determinant role in patient prognosis. Moreover, currently there is no precise formula or drug combination that have been shown to be universally effective. There are numerous reasons that can be invoked, although one of them is based on the idiosyncratic response of patients to the doses, combinations and periods of different therapeutic treatments, in the conceptual framework of a treatment that, even today, is considered to be life-long. Among the most frequently used drugs, prednisone, antimalarials (chloroquine and hydroxychloroquine), methotrexate, leflunomide and sulfazalazine stand out, which are P-gp (the former two) and BCRP1 transporter substrates (the latter three).

In the present work, patients with DA were also observed to have P-gp and BCRP1 transporters higher activity, as well as higher resistance conferred by them. In addition, a significant correlation became evident between both transporters and DA. Finally, disease activity, as assessed through a composite validated index, was the only predictor of P-gp and BCRP1 functional activity in baseline samples, with a similar trend being found when 6-month follow-up samples were included. In this regard, it should be highlighted that one of both transporters physiological functions is

cell detoxification; therefore, the possibility of both proteins' hyperfunctionality being the result of the algebraic sum of therapeutic schemes with multiple agents and inflammation mediators active secretion, but not necessarily a disease-inherent defect, cannot be ruled out. Thus, many cytokines such as TNF-γ, interleukin (IL) 2, IL-12 and interferon (IFN)-γ are known to be substrates of transporters of the ABC family, including P-gp and BCRP1[43]. Interestingly, in vitro blockage of both transporters with chemosensitizers such as verapamil and KO143 has been shown to be able to inhibit both resistance to some DMARDs and secretion of pro-inflammatory cytokines, particularly TNF-α, which plays a key role in RA pathophysiology[44].

One of the strengths of the present work is the clinical setting where it was developed. The study population included patients with RA of short evolution time, and with an active and standardized follow-up. The vast majority of patients had antibodies (RF and anti-CCP), which confer serologic and prognostic homogeneity to the population, since both determine worst disease outcomes, such as higher clinical activity and radiologic progression[45,46]. On the other hand, patient follow-up was rigorous, since activity and response international indices, validated in our population, were regularly applied. With regard to the treatment, it was

recorded with an also standardized instrument, and was corroborated with an adherence evaluation; therefore, accurately determining the real accumulated treatment and investigating its possible association with both transporters' functional activity was possible. In this regard, it should be noted that, although it is true that included patients had a short mean follow-up of six years, from the point of view of accumulated medication, the dose was considerable. The use of some drugs has been described to induce transporter expression[47]; however, whether this is determined by time of use, cumulative doses, combination of employed treatments or by the sum of all the above is not known. Although we failed to find an association between accumulated medication dose in the previous year and transporter activity, this might have to do with our population's characteristics at study enrollment, since it was treatment-experienced. Ideally, transporter functional activity should be measured in treatment-naive patients (highly unlikely because of the type of institution we work in) or soon after having started therapy. Such a study is currently underway at the early RA clinic.

Another point to be highlighted as one of the strengths of this work is the procedure employed to detect the transporters' functional activity by measuring the efflux of P-gp and BCRP1-dependent fluorescent compounds, namely daunorubicin, rodamine 123 and/or mitoxantrone. With these substances, measuring each cell's individual capacity to expel drugs that are substrates of said transporters is possible[48]. This way, percentages as low as 3% of either daunorubicin or mitoxantrone-expelling cells could be responsible for producing a state of resistance to drugs related to these transporters (or reflect disease activity or exacerbation). Interestingly, there are several inhibitors, in particular of P-gp, that are able to revert the resistance phenotype[49]; although these have been employed in neoplasms, we might argue a therapeutic benefit (whenever added to the employed treatments) as optimizers of DMARDs pharmacological action (provided they are substrates of the transporters here described).

In sum, our results show that patients with active RA display P-gp and BCRP1 transporters higher functional activity in comparison with patients in remission. DA is positively correlated with transporter activity both at specific time points and at six months' follow-up. To our knowledge, this is the first study in the literature to concomitantly assess P-gp and BCRP1 transporters function in RA. In spite of our our data being preliminary, it would appear to be conclusive that, in patients with RA, P-gp and BCRP1 behavior is determined by disease activity.

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