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# MDR-1 and MRP-1 activity in peripheral blood leukocytes of rheumatoid arthritis patients

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## Abstract

**Background:** Rheumatoid Arthritis is a chronic disease leading to decreased quality of life with a rather variable response rate to Disease Modifying Anti Rheumatic Drugs. Methotrexate (MTX) is the gold standard therapy in Rheumatoid Arthritis. The Multidrug resistance Related Protein and Multi Drug Resistance protein 1, also called P-glycoprotein-170 transporters can alter the intracellular concentration of different drugs. Methotrexate is an MRP1 substrate and thus the functional activity of MRP1 might have a clinical impact on the efficiency of the Methotrexate-therapy in Rheumatoid Arthritis.

**Methods:** We have compared the functional Multidrug Activity Factors (MAF) of the MDR1 and MRP1 transporters of Peripheral Blood Leukocytes of 59 Rheumatoid Arthritis patients with various response rate to MTX-therapy (MTX-responder, MTX-resistant and MTX-intolerant RA-groups) and 47 non-RA controls in six different leukocyte subpopulations (neutrophil leukocytes, monocytes, lymphocytes, CD4+, CD8+ and CD19+ cells). There was a decreased MAF of RA patients compared to non- Rheumatoid Arthritis patients and healthy controls in the leukocyte subpopulations. There was a significant difference between the MAF values of the MTX-responder and MTX intolerant groups. But we have not found significant differences between the MAF values of the MTX-responder and MTX-resistant Rheumatoid Arthritis -groups.

**Results:** Our results suggest that MDR1 and MRP1 functional activity does not seem to affect the response rate to MTX-therapy of Rheumatoid Arthritis-patients, but it might be useful in predicting MTX-side effects. We have demonstrated the decreased functional MDR-activity on almost 60 Rheumatoid Arthritis patients, which can be interpreted as a sign of the immune-suppressive effect of the MTX-treatment.

**Keywords:** ABC-transporter, Disease modifying anti rheumatic drugs-DMARDs, Methotrexate, MultiDrug resistance, Rheumatoid arthritis

## Background

Rheumatoid arthritis (RA) is a chronic inflammatory disease of small articulations often leading to functional impairments and seriously affecting the quality of life [1]. Currently methotrexate (MTX) is the gold standard therapy of the disease modifying antirheumatic drugs (DMARDs), however the response rate is rather variable

and many patients are completely resistant to MTX therapy [2, 3]. It has been previously reported, that the Multidrug Resistance Protein 1 (MDR1) and the Multi-drug resistance Related Protein 1 (MRP1) transporters - responsible for chemo-resistance of tumor cells - may also have pathophysiological role in different chronic diseases, including rheumatoid arthritis [4, 5].

ATP Binding Cassette (ABC) -transporters are ATP-driven pumps of the cell membranes present on many cell surfaces, but especially on barriers, bile-transporters, kidney-tubules, enterocytes, blood-brain barriers and inflammatory cells. These transporters extrude xenobiotic substances including various drugs via active transport [6]. According to their wide substrate

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specificity, these transporters may interfere with the absorption and distribution of many therapeutic agents and could influence their serum concentration which in turn can alter the efficiency of different treatments.

The MRP1-transporter substrate MTX is the most widely used DMARD. Therefore, the elevated MDR1/MRP1 expression in the synovium of RA-patients suggested prognostic role to these proteins [3, 7–10]. On the other hand, many studies have reported, that expression levels of MDR1 and MRP1 proteins do not correlate with the functional activity of these proteins predicting the superiority of functional determination of transport activities over expression data [11–13].

The clinical relevance and usefulness of calcein-AM based functional multidrug-assay has been demonstrated in acute myeloid leukemia [14]. We have optimized this assay for the determination of functional MDR1- and MRP1-activity of different peripheral blood leukocyte subtypes of RA-patients to study their possible role in the response to DMARD-therapy.

## Results

Our results suggest that MDR1 and MRP1 functional activity does not seem to affect the response rate to MTX-therapy of Rheumatoid Arthritis-patients, but it might be useful in predicting MTX-side effects. We have demonstrated the decreased functional MDR-activity on almost 60 Rheumatoid Arthritis patients, which can be interpreted as a sign of the immune-suppressive effect of the MTX-treatment. First, we have compared the  $MAF_{Total}$ ,  $MAF_{MDR1}$  and  $MAF_{MRP1}$  of all six leukocyte subgroups of the five different patient groups to define if there is any significant difference between any of those. We did not find significant differences in the MAF-values of the RA-patients according to their responder-rate to MTX-therapy. Because most of the patients were taking other drugs as well, we have collected the data of medicine-intake with doses and analyzed the potential effect of these on each MAF value. After adjusting the model with the significant drug-effects on the MAF-values, we have compared each RA and non-RA groups pairwise with each other (Table 1).

With the adjusted model, the  $MAF_{MDR1}$  of the granulocytes was significantly higher in the MTX-responder group than the MTX-resistant group. Another finding was that the MTX-intolerant RA-group had significantly higher  $MAF_{MRP1}$  than the MTX-responders in the granulocyte subpopulation (Fig. 1). Unfortunately, the granulocyte representation was sometimes low in our samples and not all blood samples contained enough granulocytes to be subject of precise MAF determination.

With a potential clinical application, another interesting finding was the difference in MAF values in the granulocyte, monocyte and CD19+ cells between the MTX-responder and the MTX-intolerant groups. The

$MAF_{MRP1}$  of granulocytes in the MTX-intolerant group was significantly higher than that of MTX-responder RA-patients, and the  $MAF_{MDR1}$  of MTX-intolerant group was significantly higher than that of MTX-responder RA-patients in the granulocyte population. In contrast, we have found significantly lower  $MAF_{MDR1}$  of the CD19+ cells of RA-patients intolerant to MTX-therapy than those of MTX-responders.

According to our results, the most prominent difference was found between the different RA-groups and the two control groups in almost all leukocyte subgroups. Granulocytes and CD19+ cells had higher  $MAF_{MRP1}$  in the RA-groups with the adjusted model, while the other leukocyte groups have higher  $MAF_{Total}$  and  $MAF_{MDR1}$  and lower  $MAF_{MRP1}$ . The differences become more pronounced in the following sequence: monocyte, lymphocyte, CD4+ cells and CD8+ cells (Fig. 1).

## Discussion

The first report on the potential role of MDR-proteins in RA found significantly higher MDR1 (P-glycoprotein) expression in steroid-treated RA-patients independently from other factors [15]. Later, in a small study by Yudoh et al. increased expression of P-Glycoprotein on Th1-cells correlated with drug resistance was observed after two month of therapy with bucillamin or sulfasalazine [16]. As DMARD therapy moved forwards, the MRP1-substrate MTX and the role of the MRP1 proteins have emerged in defining the response to MTX-therapy [17].

Despite our original hypothesis on the effect of functional MDR-activity on the response rate to MTX-therapy, in this study we were not able to demonstrate significant differences in the functional MDR-activities of MTX-responder and MTX-resistant RA patients, except for the higher  $MAF_{MDR1}$  of granulocytes of MTX-responders than MTX-resistants. However, we found another significant difference in the MAF values of the granulocytes: the MTX-intolerant RA-group had significantly higher  $MAF_{MRP1}$  than the MTX-responder RA-group. This difference may be useful in identifying the MTX-intolerant patients, who cannot benefit from MTX-therapy.

The  $MAF_{MRP1}$  of granulocytes and the  $MAF_{MDR1}$  of monocytes was significantly higher in the MTX-intolerant patients than in MTX-responders, while they had significantly lower  $MAF_{MDR1}$  in the CD19+ population. The combination of these three results could be clinically relevant in determining the MTX intolerant patient population.

In clinical setting: if a patient needs dose escalation of MTX and has side effects probably related to MTX, a scheduled MDR-functional assessment finding the upper mentioned triad of granulocytic  $MAF_{MRP1}$  and monocytic, CD19+ lymphocytic  $MAF_{MDR1}$  values might help in deciding to turn off ineffective dose-escalation of MTX. Titrating MTX-dosage in RA-patients is quite

**Table 1** Shows the significant differences between different patient groups with (**adjusted p**) and without (**p**) the adjustment of the medicine effects on the different MAF values

| Cell type    | MAF type | Group 1         | Mean  | Group 2         | Mean  | p             | Adjusted p    | After adjustment |
|--------------|----------|-----------------|-------|-----------------|-------|---------------|---------------|------------------|
| Granulocytes | Total    | MTX-intolerant  | 15    | nonRA control   | 11    | <b>0,0068</b> | <b>0,0026</b> | significant      |
| Granulocytes | Total    | MTX-responder   | 14    | nonRA control   | 11    | <b>0,0097</b> | 0,2005        | non significant  |
| Granulocytes | MRP1     | MTX-intolerant  | 5     | MTX-responder   | 2,5   | 0,3664        | <b>0,0276</b> | significant      |
| Granulocytes | MRP1     | MTX-intolerant  | 5     | nonRA control   | -2,5  | 0,0897        | <b>0,0472</b> | significant      |
| Granulocytes | MDR1     | MTX-resistant   | 11    | MTX-responder   | 10    | 0,2475        | <b>0,0284</b> | significant      |
| Monocytes    | Total    | MTX-responder   | 7     | nonRA control   | 12    | <b>0,0214</b> | 0,1114        | non significant  |
| Monocytes    | Total    | MTX-responder   | 7     | Healthy control | 15,98 | <b>0,0023</b> | 0,6893        | non significant  |
| Monocytes    | Total    | MTX-intolerant  | 9     | Healthy control | 15,98 | <b>0,0266</b> | 0,9483        | non significant  |
| Monocytes    | Total    | MTX-resistant   | 7,5   | Healthy control | 15,98 | <b>0,0075</b> | 0,7462        | non significant  |
| Monocytes    | MDR1     | MTX-resistant   | 7     | Healthy control | 11,99 | <b>0,0044</b> | 0,6792        | non significant  |
| Monocytes    | MDR1     | MTX-resistant   | 7     | nonRA control   | 12,5  | <b>0,0245</b> | 0,4869        | non significant  |
| Monocytes    | MDR1     | MTX-intolerant  | 7     | Healthy control | 11,99 | <b>0,0071</b> | 0,3590        | non significant  |
| Monocytes    | MDR1     | MTX-responder   | 5,5   | MTX-intolerant  | 7     | 0,8766        | <b>0,0276</b> | significant      |
| Monocytes    | MDR1     | MTX-intolerant  | 7     | nonRA control   | 12,5  | <b>0,0443</b> | <b>0,0472</b> | significant      |
| Lymphocytes  | Total    | Healthy control | 34,29 | MTX-resistant   | 27,5  | <b>0,0023</b> | 0,0504        | trend            |
| Lymphocytes  | Total    | Healthy control | 34,29 | MTX-intolerant  | 26    | <b>0,0014</b> | <b>0,0121</b> | significant      |
| Lymphocytes  | Total    | Healthy control | 34,29 | nonRA control   | 29    | <b>0,0359</b> | 0,0537        | trend            |
| Lymphocytes  | Total    | Healthy control | 34,29 | MTX-responder   | 26    | <b>0,0011</b> | <b>0,0084</b> | significant      |
| Lymphocytes  | MRP1     | nonRA control   | 0     | MTX-responder   | 6,5   | 0,0842        | 0,0510        | trend            |
| Lymphocytes  | MRP1     | nonRA control   | 0     | MTX-intolerant  | 7     | 0,0532        | <b>0,0139</b> | significant      |
| Lymphocytes  | MRP1     | nonRA control   | 0     | MTX-resistant   | 4     | 0,0689        | <b>0,0022</b> | significant      |
| Lymphocytes  | MRP1     | nonRA control   | 0     | Healthy control | 10,55 | <b>0,0023</b> | <b>0,0014</b> | significant      |
| Lymphocytes  | MDR1     | nonRA control   | 32    | Healthy control | 21,73 | <b>0,0129</b> | 0,0847        | trend            |
| Lymphocytes  | MDR1     | nonRA control   | 32    | MTX-responder   | 22,5  | <b>0,0164</b> | <b>0,0084</b> | significant      |
| Lymphocytes  | MDR1     | nonRA control   | 32    | MTX-resistant   | 21,5  | <b>0,003</b>  | <b>0,0008</b> | significant      |
| Lymphocytes  | MDR1     | nonRA control   | 32    | MTX-intolerant  | 21    | <b>0,002</b>  | <b>0,0007</b> | significant      |
| CD4+ cells   | Total    | nonRA control   | 23,5  | MTX-resistant   | 20    | <b>0,0156</b> | <b>0,0270</b> | significant      |
| CD4+ cells   | Total    | Healthy control | 26,81 | MTX-responder   | 21,5  | <b>0,036</b>  | 0,2212        | non significant  |
| CD4+ cells   | Total    | nonRA control   | 23,5  | MTX-intolerant  | 21    | <b>0,0211</b> | <b>0,0064</b> | significant      |
| CD4+ cells   | Total    | Healthy control | 26,81 | MTX-resistant   | 20    | <b>0,0174</b> | <b>0,0278</b> | significant      |
| CD4+ cells   | Total    | Healthy control | 26,81 | MTX-intolerant  | 21    | <b>0,0032</b> | <b>0,0081</b> | significant      |
| CD4+ cells   | MRP1     | nonRA control   | -1    | Healthy control | 7,8   | <b>0,0118</b> | <b>0,0103</b> | significant      |
| CD4+ cells   | MRP1     | Healthy control | 7,8   | MTX-resistant   | 2     | 0,076         | <b>0,0492</b> | significant      |
| CD4+ cells   | MRP1     | nonRA control   | -1    | MTX-intolerant  | 4     | <b>0,0121</b> | 0,0950        | trend            |
| CD4+ cells   | MDR1     | nonRA control   | 27,5  | MTX-intolerant  | 16    | <b>0,0004</b> | <b>0,0015</b> | significant      |
| CD4+ cells   | MDR1     | nonRA control   | 27,5  | MTX-resistant   | 17,5  | <b>0,0046</b> | <b>0,0154</b> | significant      |
| CD4+ cells   | MDR1     | nonRA control   | 27,5  | MTX-responder   | 17    | <b>0,0364</b> | 0,0698        | trend            |
| CD4+ cells   | MDR1     | nonRA control   | 27,5  | Healthy control | 17,7  | <b>0,0108</b> | <b>0,0448</b> | significant      |
| CD8+ cells   | Total    | nonRA control   | 35    | MTX-intolerant  | 31    | <b>0,0262</b> | 0,0585        | trend            |
| CD8+ cells   | Total    | nonRA control   | 35    | MTX-resistant   | 31    | <b>0,0078</b> | <b>0,0046</b> | significant      |
| CD8+ cells   | Total    | nonRA control   | 35    | MTX-responder   | 29    | <b>0,0008</b> | <b>0,0001</b> | significant      |
| CD8+ cells   | Total    | Healthy control | 40,15 | MTX-intolerant  | 31    | <b>0,0036</b> | <b>0,0120</b> | significant      |
| CD8+ cells   | Total    | Healthy control | 40,15 | MTX-resistant   | 31    | <b>0,0004</b> | <b>0,0032</b> | significant      |

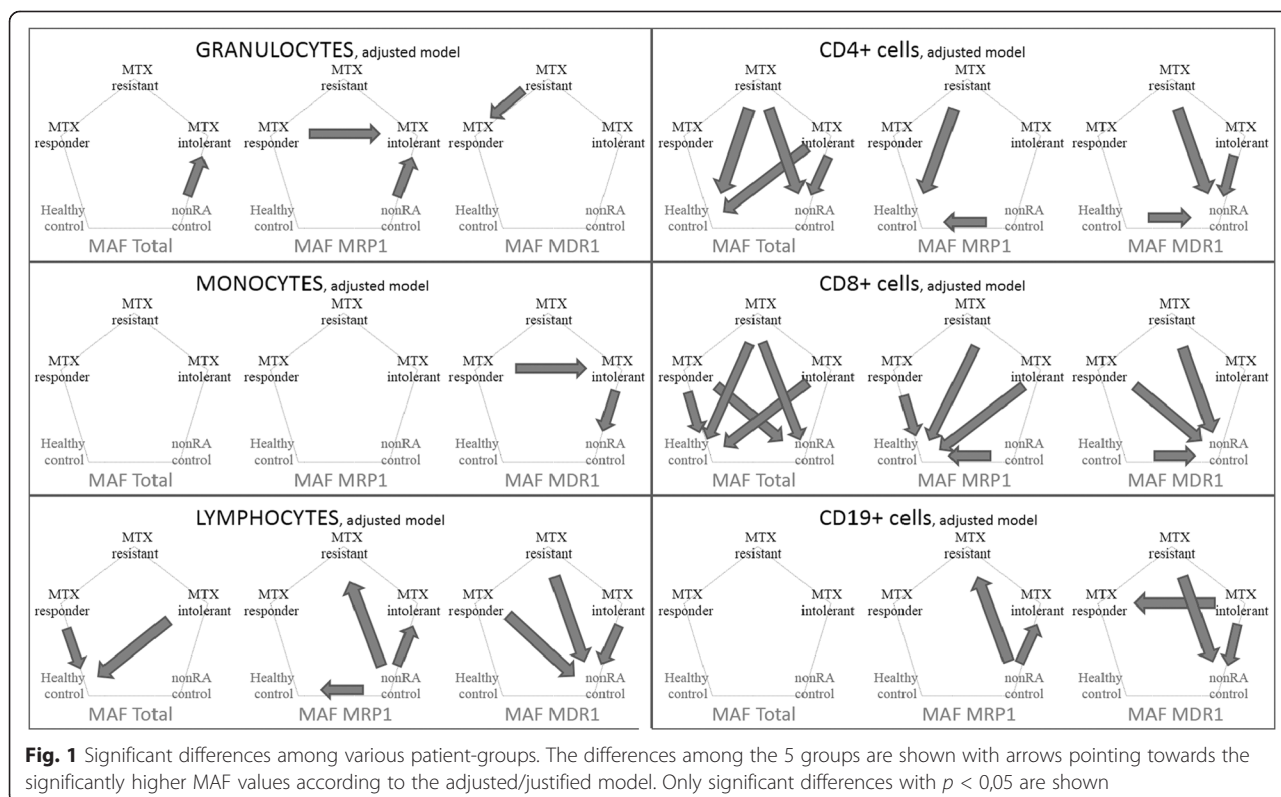
**Table 1** Shows the significant differences between different patient groups with (**adjusted p**) and without (**p**) the adjustment of the medicine effects on the different MAF values (*Continued*)

|             |       |                 |       |                 |       |               |               |                 |
|-------------|-------|-----------------|-------|-----------------|-------|---------------|---------------|-----------------|
| CD8+ cells  | Total | Healthy control | 40,15 | MTX-responder   | 29    | <b>0,0002</b> | <b>0,0003</b> | significant     |
| CD8+ cells  | Total | MTX-responder   | 29    | MTX-intolerant  | 31    | 0,1309        | 0,0601        | trend           |
| CD8+ cells  | MRP1  | Healthy control | 14,35 | MTX-intolerant  | 5     | <b>0,0003</b> | <b>0,0004</b> | significant     |
| CD8+ cells  | MRP1  | Healthy control | 14,35 | MTX-resistant   | 6     | <b>0,002</b>  | <b>0,0017</b> | significant     |
| CD8+ cells  | MRP1  | Healthy control | 14,35 | MTX-responder   | 6,5   | <b>0,0069</b> | <b>0,0031</b> | significant     |
| CD8+ cells  | MRP1  | Healthy control | 14,35 | nonRA control   | 0     | <b>0,0053</b> | <b>0,0131</b> | significant     |
| CD8+ cells  | MDR1  | nonRA control   | 37,5  | MTX-intolerant  | 28    | <b>0,0302</b> | 0,0634        | trend           |
| CD8+ cells  | MDR1  | nonRA control   | 37,5  | MTX-resistant   | 24,5  | <b>0,0071</b> | <b>0,0014</b> | significant     |
| CD8+ cells  | MDR1  | nonRA control   | 37,5  | MTX-responder   | 21,5  | <b>0,0057</b> | <b>0,0012</b> | significant     |
| CD8+ cells  | MDR1  | nonRA control   | 37,5  | Healthy control | 21,93 | <b>0,0089</b> | <b>0,0324</b> | significant     |
| CD19+ cells | Total | nonRA control   | 20,5  | MTX-resistant   | 16,5  | <b>0,0435</b> | 0,6173        | non significant |
| CD19+ cells | MRP1  | nonRA control   | 0     | MTX-intolerant  | 5     | <b>0,0426</b> | <b>0,0057</b> | significant     |
| CD19+ cells | MRP1  | nonRA control   | 0     | MTX-resistant   | 3,5   | 0,3154        | <b>0,0180</b> | significant     |
| CD19+ cells | MDR1  | nonRA control   | 22,5  | MTX-intolerant  | 14    | <b>0,0175</b> | <b>0,0491</b> | significant     |
| CD19+ cells | MDR1  | nonRA control   | 22,5  | MTX-resistant   | 15    | <b>0,0109</b> | <b>0,0000</b> | significant     |
| CD19+ cells | MDR1  | MTX-responder   | 17    | MTX-intolerant  | 14    | 0,4548        | <b>0,0058</b> | significant     |

Significant *p*-values are highlighted in **bold**

laborious, so disclosing patients who will not benefit from dose elevation and thus avoiding the harmful side effects of MTX-treatment might have clinical relevance in the future. Although, further studies are still needed for the clinical implementation/validation of these findings.

There are only few previous studies on RA patients investigating the role of MDR-transporters in MTX-response. Wolf et al. determined the functional activity of Reduced Folate Carrier (RFC), which transports MTX into the cells, and MRP1, which effluxes MTX out of the



cells. They logically anticipated that RFC+ and MRP-patients should have better response to MTX-therapy, but instead, they observed the opposite result, since these patients had the worse response rate, while the lack or presence of both proteins was linked to significantly better therapeutic outcome [18]. In another study, they determined the mRNA-levels of FolylPolyGlutamyl Synthetase (FPGS) enzyme, which accumulates MTX into the cells, and they also found the contradictory result, such that FPGS mRNA expression is an independent predictive factor associated with poor response to MTX-therapy in RA patients [19]. Considering the rather complex metabolic and effector mechanism of MTX, more studies are needed to evaluate the effects of the various functional activities on MTX-response [20].

Our most relevant finding was the major difference between the RA and control groups. In general, the significant differences were the decreased  $MAF_{Total}$  and  $MAF_{MDR1}$  and higher  $MAF_{MRP1}$  in the RA patients compared to the control-patients calculated with or without adjustment of the effects of medicine-intake. This difference was most prominent in case of the CD8+ cells. These cells had the highest MAF-values, which is in agreement with the literature [21, 22]. Interestingly, although MTX is an MRP1 substrate, the most important and significant differences were found in  $MAF_{Total}$  and  $MAF_{MDR1}$ . Hider et al. described the down regulation of MRP1 protein in PBLs by immunocytochemistry of early RA-patients after six months of MTX-therapy. They studied the MRP1 expression of 18 RA patients and 14 control persons, but they also couldn't find any difference between the MRP1 levels of the responder and non-responder RA-patients [23]. In our study we found similar results, but we used the dynamic data of functional activity determination of MDR1 and MRP1-proteins instead of the static representation of MRP1 proteins. This, according to the earlier data, may give better insight to the significance of the MDR-proteins on treatment. Furthermore, comparing 3 RA-groups of altogether 59 RA-patients and two control groups of altogether 47 persons, we found multiple significant differences between those groups. Our results demonstrate that MTX-treatment inhibits total MDR and MDR1 functional activity in RA-patients.

## Conclusions

In conclusion, by comparing the MAF values of six leukocyte subpopulations among 3 RA-patient and 2 control groups, we have found significant difference in case of granulocytes between the MTX-responder and MTX-resistant RA-group. Our results have shown multiple differences in MAF values of the MTX-responder and MTX-intolerant RA-groups, and therefore, determining MAF in RA-patients might be useful in predicting MTX-intolerance. Titrating MTX-dosage in RA-patients

is quite laborious, so disclosing patients who will not benefit from dose elevation and thus avoiding the harmful side effects of MTX-treatment may have clinical relevance in the future. Our most relevant finding was, however, the decreased functional MDR1- and MRP1-activity in MTX treated RA-patients, which might be interpreted as part of the immunosuppression.

## Methods

### Patient samples

RA patients were categorized by their DAS28 improvement rate into MTX-responder ( $n = 18$ ) and MTX-resistant ( $n = 20$ ) groups. An additional MTX-intolerant group was also selected as RA-patients who terminated MTX-therapy ( $n = 21$ ) due to intolerable side effects. The two control groups were composed as either hospitalized (due to other diseases or traumas) patients without RA ( $n = 20$ ) as well as healthy, young volunteers ( $n = 27$ ). Altogether, 106 samples were grouped in three RA-groups ( $n = 59$ ) and two control groups ( $n = 47$ ). All peripheral blood samples were collected with the approval of the national and local ethical committee (ETT TUKEB: Scientific and Research Ethics Committee of the Medical Research Council. Ministry of Health, Medical Research Council, Budapest, H-1051, Arany János u. 6–8, Hungary.) and all patients were involved after signing their written agreement. Blood samples were taken in 5 ml BD Vacutainer tubes with EDTA (**BD #367861**) and stored at 4 °C until transported and processed within 6 h after sampling. PBLs were isolated using HISTOPAQUE 1077 (Sigma-Aldrich, 10771).

### Calcein assay

The original calcein assay was obtained from the Multi-DrugQuant Assay™ (Product No. 5 599880 083012, SOLVO Biotechnology Inc. Budaörs, Hungary, <http://www.solvobiotech.com/>). MDQuant Assay™ was used to determine the functional activity of MDR1 and MRP1 transporters in MAF (Multidrug Activity Factor), which is a dimensionless and standardized value calculated by the mathematical formula:  $MAF_{Total} = 100 \times (F_{Verapamil} - F_{HBSS}) / F_{Verapamil}$ ;  $MAF_{MRP1} = 100 \times (F_{MK571} - F_{HBSS}) / F_{Verapamil}$ ;  $MAF_{MDR1} = MAF_{Total} - MAF_{MRP1}$  where F stands for the average mean calcein-fluorescence values of the parallel samples. Functional MDR Activity Factor (MAF) was determined in six PBL populations (granulocytes, monocytes, lymphocytes, CD4+, CD8+, CD19+ cells).

### Immunocytochemistry

Immunostaining of the different PBL-subpopulations was performed at room temperature by incubating the blood cells in 100  $\mu$ l HBSS containing either 1  $\mu$ g of mouse anti-IgG1 antibody for isotype control (X0931, Dako) or 6  $\mu$ l anti-CD3,4,8 (CD3: C7225, CD4: C24865,

CD8: C9494, Dako) or 12ul of anti-CD19 (C24825, Dako). The immunolabeling was visualized by incubation with 1  $\mu$ g secondary Cy5-conjugated goat anti-mouse IgG antibody (115-175-003, Jackson Immuno Research).

#### Viability staining

Non-viable cells were labeled by incubation in 200  $\mu$ L HBSS containing 1  $\mu$ g/ml propidium iodide (PI; 287075, Sigma-Aldrich,) for 30 min at room temperature.

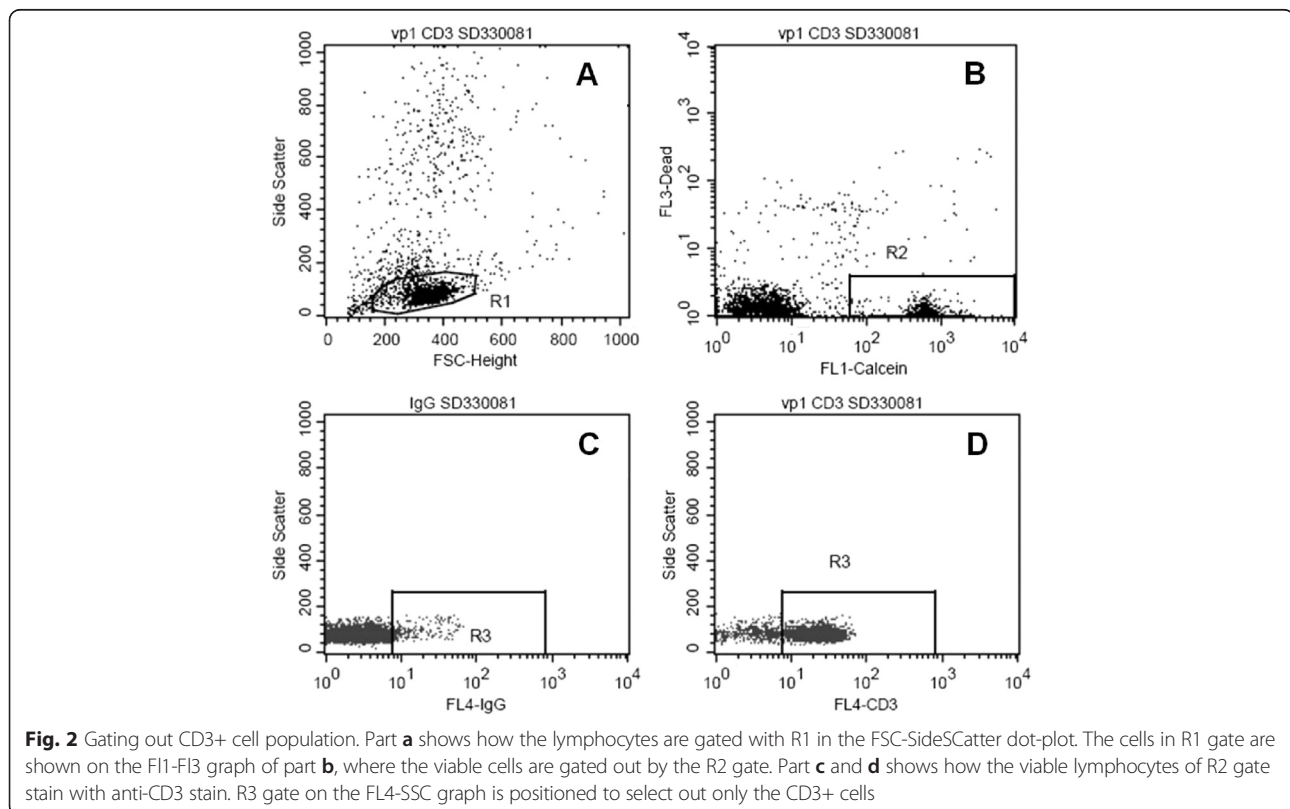
#### Determination of MAF-values of different white blood cell subpopulations

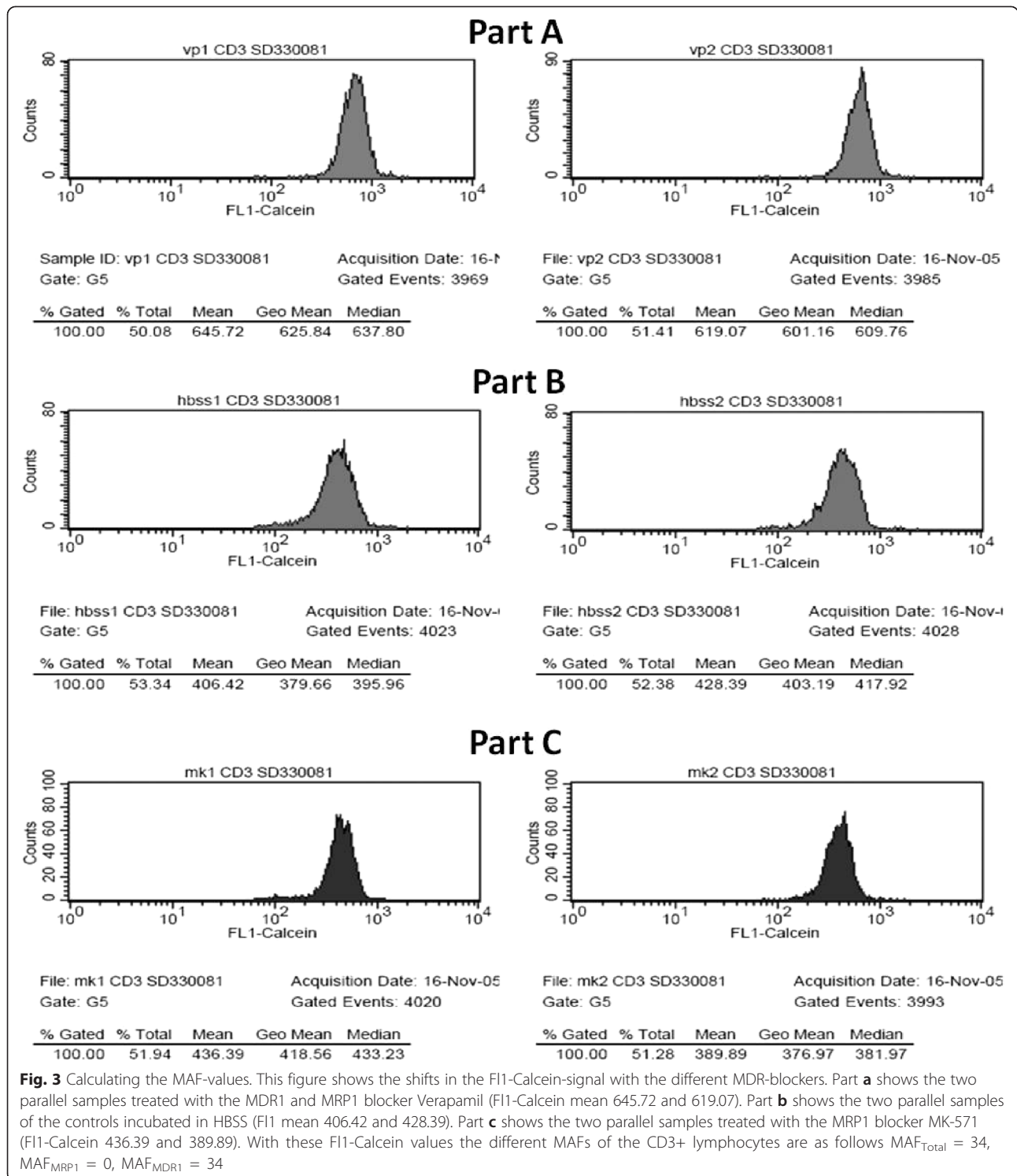
Measurements were performed on a FacsCalibur (Becton Dickinson, Franklin Lakes, NJ, USA) flow cytometer. The viable cells were selected by the positive staining with Calcein and lack of PI staining. Granulocytes, monocytes and lymphocytes were gated on the FSC-SSC scatterplot. The CD4, CD8 and CD19 positive subpopulations were gated based on their positivity with the corresponding antibody detected at FL4 against the non-specific binding detected with IgG1 isotype control. (Gating algorithm is shown in Fig. 2.) The shifts in the calcein signal of the leukocyte subpopulations were determined with different MDR-blockers (verapamil and MK-571) compared to HBSS control.

$MAF_{Total}$ ,  $MAF_{MDR1}$  and  $MAF_{MRP1}$  was calculated on each subpopulations (Fig. 3).

#### Statistical methods

MAF measurements were modeled using analysis of covariance (ANCOVA) with residual error stratified in patient groups to allow for unequal variances. Models were fitted using the R statistical system (R Development Core Team 2007, ISBN 3-900051-07-0), with the GLS (generalized least squares) procedure of the NLME package (R Core team, 2007, R-package version 3.1-86). Models were compared by using maximum likelihood (ML) fits, and Wald-type significance tests were performed using restricted maximum likelihood (REML) estimates. Differences were considered statistically significant below the level of  $p = 0.05$ . The following predictors were considered during model selection: patient group (5 levels), sample storage duration (continuous), drug doses of methotrexate, delagil, arava, medrol (continuous), administration of the same drugs (yes/no), administration of NSAIDs (yes/no). We have selected the best model using the Akaike Information Criterion (AIC) (Akaike 1974: A new look at the statistical model identification. IEEE Transactions on Automatic Control 19 (6): 716–723.) All subsets regression was not computationally feasible due to the large number of predictors, so forward and backward





stepwise variable selection algorithms were applied. When the stepwise algorithm yielded a model where the same drug was included both as a continuous and binary predictor, we checked if the coefficients corresponded to a monotonous dose-effect relationship. In case of non-monotonicity we have considered the two

parameters for the same drug as overfitting, dropped the model and the stepwise algorithm was restarted twice, first omitting the binary dose variant, second omitting the continuous one. When the stepwise algorithms led to different models, the one with lowest AIC value was selected.

### Abbreviations

ABC-proteins: ATP-binding cassette proteins; AIC: Akaike information criterion; Calcein-AM: calcein acetoxymethyl ester; DMARDs: Disease modifying anti-rheumatic drugs; EDTA: Ethylene diamine tetraacetic acid; FPGS: Folylpolyglutamyl synthetase; FSC-SSC: Forward Scatter – Side Scatter dot-plot; HBSS: Hank's balanced salt solution; MAF: Multidrug activity factor; MDR: Multidrug resistance; MDR1: MultiDrug resistance protein 1, also called P-glycoprotein-170; MRP1: Multidrug resistance related protein 1; MDQ Assay: MultiDrug quant assay<sup>TM</sup>; MTX: Methotrexate; NSAIDs: Non-steroid anti-inflammatory drugs; PBLs: Peripheral blood leukocytes; PI: Propidium iodide; RA: Rheumatoid arthritis; RFC: Reduced folate carrier.

### Competing interests

There is no conflict of interest.

### Authors' contributions

AL carried out the flow cytometric studies, participated in the preparation of the manuscript. TM performed the statistical analysis and drafted the manuscript. JG provided the samples, participated in the design of the study. RS and IP conceived of the study, and participated in its design and coordination and helped to draft the manuscript. All authors read and approved the final manuscript.

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