RHEUMATOLOGY

Original article

Effects of methotrexate on the expression of the translational isoforms of glucocorticoid receptors α and β: correlation with methotrexate efficacy in rheumatoid arthritis patients

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Abstract

Objectives. To test the effect of MTX on the expression of glucocorticoid receptor (GR) α and β isoforms AB, C and D in peripheral blood mononuclear cells (PBMCs) in culture, from newly diagnosed RA patients and to evaluate whether the test results correlate with patients' subsequent response to MTX treatment.

Methods. Twenty patients with early active RA were enrolled. Patients who had previously received any DMARD or cytotoxic agent, or who had received CSs in the 6 months before enrolment were excluded. PBMCs from all patients were obtained and cultured in the presence and absence of MTX (10^{-4}, 10^{-6} and 10^{-8} M). The expression of GR isoforms was evaluated by western blot. After blood samples were taken, patients entered a 24-week study receiving MTX, diclofenac and prednisone (10 mg/day). At Week 24, the ACR core set of disease activity measures was calculated and a correlation between the MTX effect on patients' PBMC GR expression *in vitro* and the ACR response was evaluated.

Results. MTX 10^{-6} M in the culture medium induced the expression of the PBMC isoform AB of GRα ($P = 0.009$). Other GR isoforms were unaffected. The magnitude of the induced expression correlated with the ACR response to treatment at Week 24 of therapy ($r = 0.92$, $P = 0.00003$).

Conclusion. MTX *in vitro* induces greater expression of GRαAB isoform in PBMC from RA patients who later respond to MTX treatment than in non-responding patients. This may have clinical applications for predicting MTX efficacy in RA patients.

Key words: Autoimmunity, Rheumatoid arthritis, Inflammation, Glucocorticoid receptor, Methotrexate, Glucocorticoid receptor α, Glucocorticoid receptor β.

Introduction

RA is a chronic autoimmune disease that primarily affects the joints. The aetiology of RA remains obscure. MTX and glucocorticoids (GCs) have been widely used to treat RA [1–5]. GC actions are mediated by an intracellular GC receptor (GR) [6–8]. Although the GR is the product of a single gene, several isoforms have been described [9–11].

GRα is the classic GR mediator of the hormone response. Under certain conditions, an alternative splicing variant termed GRβ has a dominant negative effect over GRα [12–16]. Recently, it has been demonstrated that the GRα transcript gives rise to several protein isoforms termed A (94 kDa), B (91 kDa), C (82 kDa) and D (54 kDa) [17, 18]. These isoforms are the product of alternative translation initiation and their expression levels differ among different tissues. Functional analyses have shown that they have different transcriptional activities and that they can regulate a set of common genes and unique sets of genes in the same cell [18, 19].

Since GRβ is identical to GRα up to amino acid 724, it would be expected that GRβ transcript generates the same protein isoforms as a product of different translation initiation; however, this had not previously been studied.

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Neither was it known if the expression of the translation isoforms can be regulated differentially or if the ratio of expression among them stays constant in different situations.

Recently, we showed that MTX induces the expression of GRα, while leaving the expression of GRβ unchanged or decreased in lymphocytes' cell lines and in peripheral blood mononuclear cells (PBMCs) from healthy individuals. We evaluated this effect in AB isoforms of GRα and β, but we did not measure the effect on the other translation isoforms. We also demonstrated that the GC sensitivity of the cells increased upon MTX treatment. Therefore, this novel MTX effect may be an action mechanism in the treatment of autoimmune diseases such as RA [20].

At the start of RA treatment, it is not known which patients will respond to MTX. Treatment typically begins with an initial weekly dose of MTX, which is then increased in stages if the patient does not respond. If there is still no improvement after a maximum weekly dose has been reached, then MTX treatment is stopped. This usually takes months and implies that patients who do not respond to MTX are unnecessarily exposed to a potentially toxic drug and lose time in starting an effective drug treatment in pathology where the existence of a window of opportunity for therapy has been proposed [21]. It would, therefore, be useful in clinical practice to be able to predict efficacy of MTX treatment in RA patients.

Since the MTX effect on GRα expression is a potential action mechanism in the treatment of autoimmune diseases, it is possible that this effect, tested *in vitro*, could predict *in vivo* MTX efficacy for individual RA patients. However, the effect of MTX on cell lines or normal PBMCs might not necessarily be the same as the effect on cells from patients with autoimmune/inflammatory diseases. Also, even if MTX induces the same effect on PBMCs from RA patients *in vitro*, this might not reflect the *in vivo* drug efficacy. Therefore, we evaluated the effect of low-dose MTX on GRα translation initiation isoforms AB, C and D expression on PBMCs from newly diagnosed RA patients. We also tested the MTX effect on the expression of GRβ isoforms. Finally, we evaluated whether the effect of this *in vitro* test correlates with the MTX response to treatment, defined as ACR20, ACR50 and ACR70 responses at Week 24 of therapy.

Patients and methods

Patients

Twenty consecutive patients attending the outpatient clinics of the University of Chile's Clinical Hospital who met the eligibility criteria and gave informed consent were enrolled in the study. Demographic details are shown in Table 1. Entry requirements included meeting the ACR (formerly the ARA) criteria for RA [22]. Patients were required to be at least 18 years old with disease duration of <2 years. They had to have an active disease, defined by the presence of at least three of the following four criteria: $\geqslant 6$ tender joints, $\geqslant 3$ swollen joints, Westergren

TABLE 1 Demographics and baseline clinical characteristics of patients

Age, years	45 (26–61)
Women/men	16/4
Disease duration, months	8 (2–18)
Tender joint count	14 (8–22)
Swollen joint count	10 (5–16)
ESR, mm/h	33 (15–68)

Values are expressed as median (range), except for women/men.

ESR of $\geqslant 28$ mm/h and morning stiffness $\geqslant 60$ min in duration.

Patients who had ever received any previous DMARD or cytotoxic agent, or who had received any form of CS in the 6 months before the enrolment, were not eligible. NSAIDs were allowed. Patients suffering from a major comorbidity, including other autoimmune or chronic inflammatory diseases and disorders of the hypothalamic-pituitary-adrenal (HPA) axis as well as patients having a contraindication for the use of MTX, diclofenac or prednisone were also excluded.

The study was approved by the ethics committee of the Faculty of Medicine of the University of Chile. After informed consent was given, a 30-ml whole-peripheral venous blood sample was obtained from all patients for testing of the *in vitro* MTX effect on GR expression. After blood samples were taken, patients entered a 24-week study receiving MTX (initial dose 10 mg/week), diclofenac (100 mg/day) and prednisone (10 mg/day). Patients were evaluated monthly by the supervising rheumatologist who had been instructed to increase the MTX dose up to a maximum of 20 mg/week, depending on the degree of clinical response and/or adverse events. Folic acid supplementation was not allowed. The ACR core set of disease activity measures [23] was calculated at the end of the study (Week 24). The correlation between the MTX effect on patients' PBMC GR expression *in vitro*, performed before the start of treatment, and the ACR response was evaluated. All clinical assessments were performed blinded to the test results of the MTX effect on patients' PBMC GR expression *in vitro*.

PBMC isolation

Thirty millilitres of whole-peripheral venous blood samples from all patients were collected in tubes with EDTA 0.5 M, pH 8. PBMCs were isolated immediately by density gradient centrifugation (Histopaque, Sigma Diagnostic, St Louis, MO, USA).

In vitro test of MTX effect on GR expression

The PBMCs, obtained from all patients before treatment, were incubated for 36 h in the presence or absence of MTX (10^{-4}, 10^{-6} and 10^{-8} M). The protein expression of GR isoforms α and β were then evaluated by western blot.

Western blot

Cells were harvested after specific treatments, and proteins were extracted as described previously [24]. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) was performed under reducing conditions on 10% polyacrylamide gels, using $40\,\mu g$ of proteins. The resolved proteins were transferred onto nitrocellulose sheets, and reacted overnight at $4°C$ with specific polyclonal rabbit anti-GRα $(1:200)$ [GR (8P-20): sc-1002 Santa Cruz Biotechnology, Inc.] or β $(1:500)$ (PA3-514 Affinity Bioreagents, Inc., now called Thermo Scientific Pierce antibodies) antibodies, and 1 h at room temperature with mouse monoclonal anti-β actin $(1:40\,000)$ antibodies (Santa Cruz Biotechnology, Inc). GRα and GRβ antibodies are directed to the C-terminus of each protein and can recognize all translational isoforms A, B, C1–C3 and D1–D3. Secondary biotinilated polyclonal antibodies against rabbit or mouse IgG (DAKO) were used for GRs and β actin detection, respectively. Immunoreactivity was detected by using enhanced chemiluminescence (ECL, Amersham Bioscience). Since the expression of the different translational GR isoforms were different in magnitude within each patient's sample, we adjusted the blot developing conditions using several films until a set of bands appropriate for quantification was obtained. Densitometry and quantification of western blot bands was performed by using National Institutes of Health Image J software. The expression of each GC isoform was normalized to β actin expression.

Statistical analysis

The overall effect of the different MTX concentrations on the expression on each GR isoforms (AB, C and D) was assessed by means of the Kruskal–Wallis ANOVA. The GRα and GRβ isoforms were analysed separately. When a statistically significant overall difference was detected, paired comparisons between the baseline *vs* the GR isoform expression at each MTX concentration was further assessed by means of the Wilcoxon matched pairs test. The direction and strength of the relationship between the number/rate of patients who achieved or did not achieve the ACR20, ACR50, ACR70 response criteria, and the magnitude of the expression of the GC variants AB, C, D for each of the GRα and GRβ isoforms, was analysed by means of the Spearman's rank correlation test.

Results

Baseline characteristics of the patients

The baseline demographic and clinical characteristics of the patients are summarized in Table 1. Mean age was 45 years (range 26–61 years), mean duration of the disease was 8 months (range 2–18 months) and the mean percentage of RF-positive patients was 70%. All RA patients at the entry of the study showed a high level of disease activity as assessed by visual assessment scale of pain (mean 52.5, range 32–90), number of tender joints (mean 14, range 8–22) and number of swollen joints (mean 10, range 5–16).

MTX induces the expression of the GRα AB isoform in cultured PBMCs from RA patients

To determine the effect of MTX on GRα expression from RA patients' cells *in vitro*, PBMCs from newly diagnosed RA patients were obtained before treatment. The PBMCs were incubated for 36 h with different concentrations of MTX and then the GRα protein expression was evaluated by western blot using an antibody specific for the GRα isoform, which can recognize all translational isoforms of GRα. Recently, it has been demonstrated that the GRα transcript gives rise to eight protein isoforms termed A (94 kDa), B (91 kDa), C (app 82 kDa), which includes the isoforms C1–C3, and D (app 54 kDa), which includes the isoforms D1–D3 [17, 18] These isoforms are the product of alternative translation initiation and their expression levels differ among tissues [25]. Figure 1 shows that it was possible to recognize bands at the predicted molecular weights for these translational isoforms. In most cases, the predominantly expressed isoform was AB, but in some cases the expression of isoform C was higher. We then evaluated the effect of MTX on isoforms AB, C and D. For seven patients, the band at the predicted molecular weight for isoform D of the GRα was too faint for proper

FIG. 1 Western blot analysis of GRα and GRβ isoforms of PBMCs from RA patients. PBMCs from early active RA patients were obtained before treatment and cultured in the presence or absence of MTX. Following a 48-h culture, the cells were harvested. GRα and GRβ isoforms AB, C and D protein expression was measured by western blot analysis. Representative western blot analysis of GRα, GRβ and β actin levels is shown. C: control—absence of MTX in culture medium.
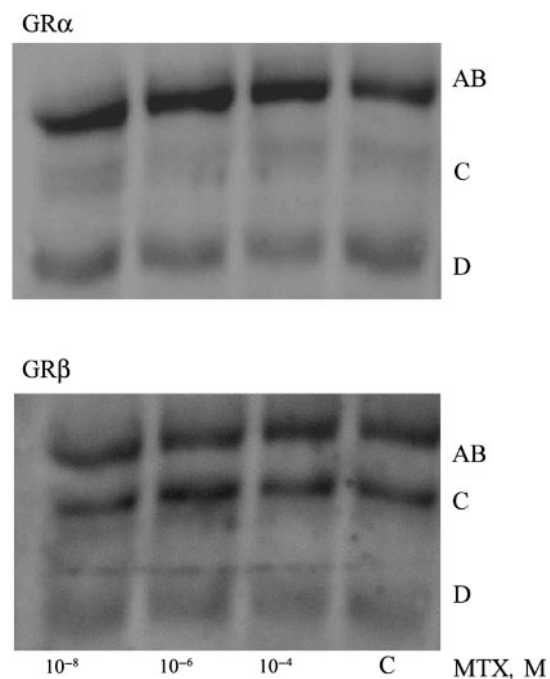
evaluation. Therefore, we analysed the GRα D isoform on the remaining 13 patients. As shown in Fig. 2, when cells were treated with MTX at 10^{-6} M, which corresponds to plasma concentrations obtained with MTX doses used in the treatment of autoimmune diseases [26], there was an increase in GRα AB isoforms (38.25% median increase, $P = 0.009$). The expression of GRα C and D isoforms was not changed by MTX treatment.

MTX does not change the expression of GRβ isoforms in cultured PBMCs from RA patients

Since translation initiation isoforms of GRα are due to different start codons in the N terminus of the protein and given that GRα and β splice variants are identical until amino acid 727, it would be expected that GRβ mRNA would give rise to the same translation initiation isoforms as those described for GRα. However, this had not previously been studied. Figure 1 shows that the western blot analysis for GRβ, in PBMCs from our patients, showed bands at the predicted molecular weight of GRβ isoforms generated through translational mechanisms (AB, C and D). This finding is not a definitive proof of the existence of C and D isoforms, but we consider it strongly suggestive. Therefore, the effect of MTX was evaluated separately on GRβ AB, and on the bands at the expected molecular weights of the GRβ C and D isoforms, which in this article will be named as such. However, it is important to note that the bands in the western blot analysis of

GRβ isoforms in some patients were very faint, making an accurate densitometric analysis of them impossible. The number of cases available for analysis of GRβ AB, C and D isoforms were 12, 17 and 7, respectively. As shown in Fig. 2, MTX did not significantly change the expression of any GRβ isoforms at the tested concentrations in PBMCs from these RA patients *in vitro*.

Treatment response correlates with *in vitro* MTX effect on GRα isoform AB expression

At Week 24, 75% of patients achieved an ACR20 response, 50% of patients achieved an ACR50 response and 43.7% of patients achieved an ACR70 response. Twenty per cent of patients failed to achieve an ACR20 response at Week 24. Our *in vitro* assays showed that the MTX effect on the expression of GRα had high interindividual variability. While the cells of some patients showed little or no response to MTX (Fig. 3A), other patients' cells responded with significant increase in GRα AB expression in the presence of MTX in the culture medium (Fig. 3B). Therefore, we evaluated whether the different responses to the *in vitro* MTX effect on GR isoform expression could be related to the different patient's response to treatment. Our results show that higher ACR responses correlated with greater *in vitro* induction of GRα AB isoforms at 10^{-4} and 10^{-6} M MTX concentrations ($r = 0.84$, $P = 0.009$ and $r = 0.92$, $P = 0.00003$ for AB isoform, MTX 10^{-4} and 10^{-6} M, respectively). Our results

Fig. 2 Effect of MTX on GRα and GRβ protein expression from PBMCs from RA patients. The quantification of the western blot analysis of translational isoforms of GRα and GRβ of PBMCs from 20 RA patients cultured in the presence or absence of MTX is shown. The values were normalized to β actin expression and are expressed as percentage values of the control (GR expression from PBMCs, cultured in the absence of MTX). Data are presented as median value, 25–75% (box) and min–max (vertical line). *$P < 0.05$. C: control—absence of MTX in culture medium.
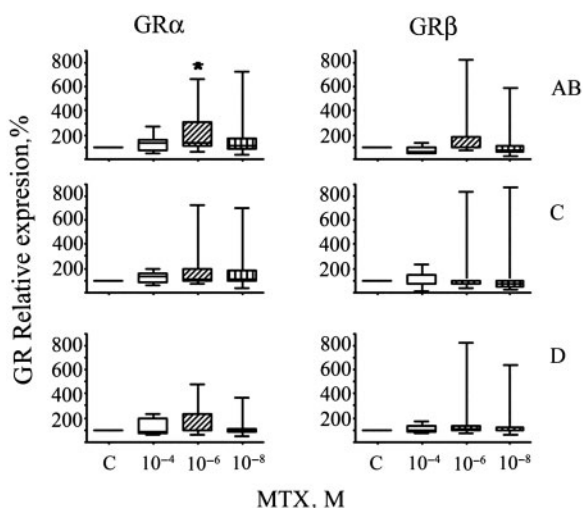
Fig. 3 Effect of MTX on the expression of GRα AB in RA patients with good or bad response to MTX treatment. The *in vitro* effect of MTX on the expression of GRα and GRβ was evaluated on PBMCs from RA patients before treatment. The patients were then treated for 24 weeks as described in the 'Patients and methods' section. After 24 weeks of treatment we classified as bad responders those patients who failed to achieve a response ACR50 at Week 24, and good responders those who achieved a response ACR50 or more at Week 24. MTX induced a significant increase in GRα AB expression in the good responders but not the bad responders. Examples of the *in vitro* effect of MTX on GRα AB expression in one bad responder (**A**) and one good responder (**B**) are shown. C: control—absence of MTX in culture medium.
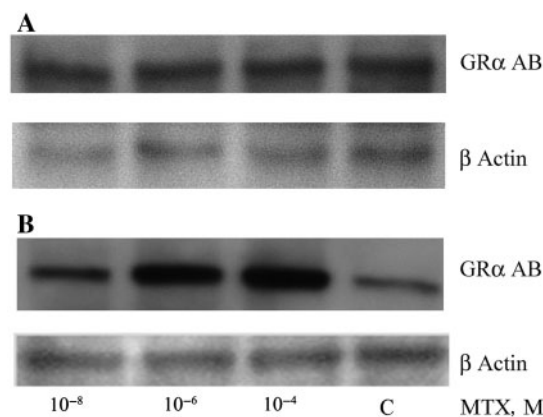
did not find a correlation between the MTX effect on GRα C or D isoforms or on any GRβ isoform and the ACR response at Week 24 of treatment. To further explore if pre-treatment evaluation of the *in vitro* MTX effect on GR isoforms expression could differentiate between patients who would have a bad response to the drug from those who would respond well, we evaluated whether the effect of MTX on GR isoform expression was different between these groups. For this purpose, we designated the patients as either bad or good responders to MTX. Patients with an ACR response of <50 at Week 24 were defined as bad responders. Those with an ACR response $\geqslant 50$ were good responders. As shown in Fig. 4, the MTX effect on GRα isoforms AB was significantly higher in patients with a good response to treatment compared with the bad responders ($P = 0.02$ for MTX 10^{-4} M, and $P = 0.001$ for MTX 10^{-6} M). The MTX effect on GRα isoforms C and D and on any of the GRβ isoforms was not significantly different between the groups (Fig. 4).

Discussion

RA is a disabling disease, affecting ∼1% of the population. MTX is the first-line therapy for the treatment of RA. Recent clinical trials have also demonstrated that early combination therapy including MTX and GCs are superior to monotherapy with DMARDs and improve prognosis in RA [27]. Despite their wide use, the action mechanisms of MTX in the therapy of autoimmune/inflammatory diseases are not completely understood [28, 29].

We recently demonstrated that MTX induces the expression of GRα *in vitro*, leaving the expression of GRβ unchanged or decreased in lymphocytes cell lines and PBMCs from healthy individuals [20]. We also demonstrated that upon MTX treatment the cells' GC sensitivity increased [20]. Since GCs have powerful anti-inflammatory and immunosupresor effects, this MTX effect could be another action mechanism in the treatment of autoimmune diseases such as RA.

In recent decades, the emphasis on RA treatment and their goals have changed. It is now accepted that early and more aggressive interventions with DMARDs are necessary to prevent disability and irreversible damage [27, 30, 31]. However, adverse events and efficacy of DMARDs differ considerably between individuals and there has not been a good way to predict individual responses to therapy to date.

Therefore, we hypothesized that the *in vitro* MTX effect on GR isoforms from patients' PBMCs could predict the individual response to therapy. To explore this hypothesis, we evaluated the effect of MTX *in vitro* on GRα and GRβ expressions on PBMCs from active RA patients before treatment (Table 1). The patients then entered a 24-week study receiving MTX, diclofenac and prednisone. Patients were evaluated monthly by the supervising rheumatologist who had been instructed to increase the MTX dose, depending on the degree of clinical response and/or adverse events. At Week 24, we evaluated whether the pre-treatment *in vitro* MTX effect correlated with the individual ACR response to treatment.

Fig. 4 Effect of MTX on the expression of GRα and GRβ in RA patients with a good or bad response to MTX treatment. The *in vitro* effect of MTX on the expression of GRα and GRβ was evaluated on PBMCs from RA patients before treatment. The patients were then treated for 24 weeks as described in the 'Patients and methods' section. After 24 weeks of treatment we classified as bad responders (BRP) those patients who failed to achieve a response ACR50 at Week 24, and good responders (GRP) those who achieved a response ACR 50 or more at Week 24. The quantification of the MTX *in vitro* effect on GR isoforms in both groups of patients is shown. Data are represented as median value, 25–75% (box) and min–max (vertical line). *$P < 0.05$.
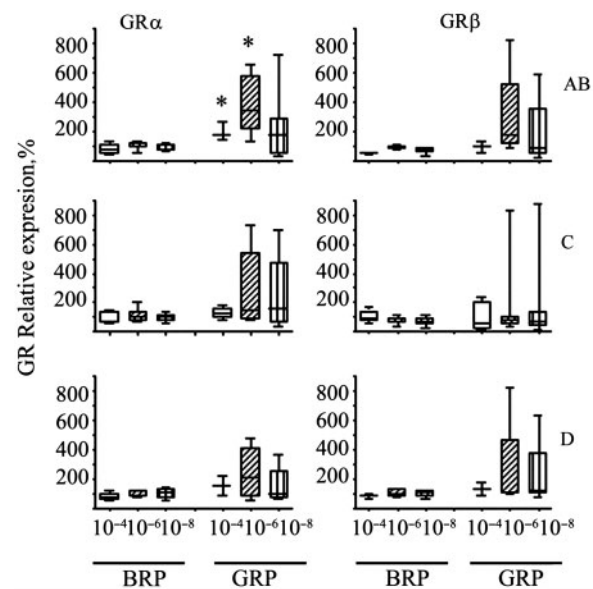
In recent years, much interest has been generated by the description of eight GRα isoforms, termed A (94 kDa), B (91 kDa), C (app 82 kDa), which includes the isoforms C1–C3, and D (app 54 kDa), which includes the isoforms D1–D3 [17, 18]. These isoforms are the product of alternative translation initiation and their expression levels differ among tissues. Functional analyses have shown that they have different transcriptional activities and that they can regulate a set of common genes and also unique sets of genes in the same cell [18]. Our previous report on the MTX effect on GRα and GRβ evaluated only the isoforms AB of those receptors [20]. Furthermore, previous experiments evaluating GR have focused on the AB isoforms of GR and there is very little information on the other translation initiation isoforms. In the case of GRβ, even though these isoforms were expected to be expressed, they had not been demonstrated.

Therefore, in our study we evaluated the effect of MTX on the transcriptional isoforms of GRα. As expected, the GRβ western blot also demonstrated bands at the predicted molecular weight for all GR translation isoforms (Fig. 1). Therefore, we also evaluated the effect of MTX

on these bands, which we termed GRβ AB, C and D and their correlation with the ACR response to treatment at the end of the study. Our results showed that MTX at 10^{-6} M increased the expression of GRα AB isoform, leaving unchanged other GRα isoforms and all GRβ isoforms (Fig. 2). After 24 weeks of standard treatment, patients were classified as non-responders or as achieving ACR20, ACR50 or ACR70 responses. A positive correlation was found between the magnitude of MTX effect on GRα AB isoform at 10^{-4} and 10^{-6} M and the ACR response. To further evaluate whether the *in vitro* MTX effect could discriminate between good responders and bad responders to treatment, we classified the patients into two categories: (i) the bad responders, defined as those who failed to achieve an ACR50 response; and (ii) the good responders, who achieved at least an ACR50 response. The MTX effect on GRα AB at the above concentrations was significantly different between the groups (Fig. 4). We did not find a correlation between the MTX effect on other GRα or GRβ isoforms and treatment response in this study. However, possible effects of a smaller magnitude on the other isoforms not captured in our study cannot be ruled out. Even if correlations between the *in vitro* MTX effect on other GR isoforms and later response to treatment were to be found, these effects would be more difficult to evaluate due to the technical difficulties entailed by the proteins' low expression, and therefore they would be unlikely to be suitable for a test with clinical applications. On the other hand, the isoform AB of GRα in the western blot analysis produced a prominent band in all patients studied. Therefore, our results support a potential role, which will need further evaluation, of a pre-treatment test of *in vitro* MTX effect at 10^{-4} and 10^{-6} M on GRα AB expression as a predictor of the *in vivo* MTX efficacy in RA patients.

Rheumatology key messages

- MTX induced the expression of the PBMC GRα AB isoform.
- The magnitude of MTX-induced GRα AB expression correlates with RA patients' subsequent response to treatment.

Acknowledgements

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