The influence of methotrexate on the gene expression of the pro-inflammatory cytokine IL-12A in the therapy of rheumatoid arthritis

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Abstract

Objective

Methotrexate (MTX) is a cornerstone in the treatment of rheumatoid arthritis (RA). Among its anti-proliferative activity, the anti-inflammatory mechanisms of MTX seem to play a major role in the treatment of RA. MTX reduces the production of pro-inflammatory cytokines such as interleukin (IL)-1, IL-2, IL-6 and interferon (INF)-γ, while the gene expression of anti-inflammatory Th2 cytokines like IL-4 and IL-10 is increased - altogether resulting in the anti-inflammatory effect. As little is known about the impact of MTX on other cytokines involved in the pathogenesis of RA, the present trial investigated the effect of MTX on IL-12A and IL-18 gene expression by peripheral blood mononuclear cells (PBMCs). For comparison, the effect on IL-6 and tumour necrosis factor (TNF) was analysed.

Methods

Using real-time PCR, mRNA concentrations of pro-inflammatory cytokines were determined in PBMCs from 17 patients before and during MTX therapy. Furthermore, gene expression was correlated with clinical and pharmacokinetic parameters such as methotrexate polyglutamate concentrations (Spearman's correlation coefficient). To eliminate concomitant corticosteroids as confounding factor, a subgroup analysis for methotrexate without corticosteroids was performed in 6 patients.

Results

MTX statistically significantly reduced the mRNA expression of IL-12A by PBMCs in rheumatoid arthritis patients (Wilcoxon-test for paired samples, $p \leq 0.046$). Consistent with other reports, IL-6 was reduced under MTX treatment. Although the combination of MTX and corticosteroids significantly reduced the gene expression of IL-18, this key molecule was unaffected by MTX without corticosteroids. Our results were further supported by a negative correlation of methotrexate polyglutamate concentrations and the mRNA expression of the pro-inflammatory cytokines IL-6 and IL-12A.

Conclusion

We describe a novel effect of MTX reducing the gene expression of IL-12A independently of corticosteroid application in patients. This impact was further enhanced by a reduction of IL-12A-producing lymphocytes and neutrophils under MTX treatment. These results expand the understanding of the mechanism of action of the most widely used drug in RA.

Key words

methotrexate, rheumatoid arthritis, cytokines, gene expression, IL-12A

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Introduction

Because of its positive benefit-risk-ratio, low dose methotrexate (MTX), a disease-modifying anti-rheumatic drug (DMARD), is a cornerstone in the treatment of rheumatoid arthritis (RA). Among its anti-proliferative activity on immune cells mediated by inhibition of dihydrofolate reductase and folate dependent reactions, the anti-inflammatory mechanisms of methotrexate seem to play a major role in the treatment of RA (1).

Hyperplasia of the synovial membrane, increased vascularity and an infiltrate of inflammatory cells, primarily $CD4^+$-T-helper cells, are typical symptoms of RA (2).

Interleukins (IL) – a group of cytokines – are mainly synthesised by $CD4^+$-T-helper lymphocytes, monocytes, macrophages and endothelial cells and have important functions in regulating the immune system. They promote the development and differentiation of T-, B-, and haematopoietic cells. The chronic autoimmune response, which is characteristic of rheumatoid arthritis, is mediated by $CD4^+$ T-cells. It was shown, that Th1-cells produce pro-inflammatory cytokines such as IL-2, IFN-γ and IL-12, activate macrophages and mediate cellular immunity. In contrast, Th2-cells secrete anti-inflammatory cytokines such as IL-4, IL-5 and IL-10 and down-modulate macrophage activation. Therefore, imbalances in Th1/Th2-cytokines are associated with the pathogenesis of autoimmune-diseases (3). Further data suggest that the common mechanism in the rheumatoid nodule is a Th1 autoimmune response, presented by the expression of IFN-γ, IL-1ß, tumour necrosis factor (TNF) together with IL-12, IL-18 and IL-15 (4).

In previous studies it was observed, that MTX treatment reduces the production of pro-inflammatory monocytic/macrophagic cytokines such as IL-1 and IL-6. In addition, pro-inflammatory Th1 cytokines (*i.e.* IL-2, IFN-γ) are reduced, while the gene expression of anti-inflammatory Th2 cytokines like IL-4 and IL-10 is increased, altogether resulting in an anti-inflammatory effect (1, 5). In particular, the polyglutamated derivates of methotrexate (MTX-PGs) inhibit various folate dependent enzymes and the 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, contributing to the anti-proliferative and anti-inflammatory properties of MTX. Because of these functions, the concentration of MTX-PGs in erythrocytes is supposed to correlate with clinical efficacy (6, 7).

More detailed information on the role of methotrexate in rheumatic diseases is provided in the following supplement: *Clin Exp Rheumatol* 2010; 28(5), Suppl. 61.

As little is known about the impact of MTX on other cytokines involved in the pathogenesis of RA, the present study was initiated to expand the knowledge about the molecular effects of MTX, in particular to investigate the influence of MTX on the gene expression of IL-12A and IL-18 by peripheral blood mononuclear cells (PBMCs). These pro-inflammatory cytokines were chosen because of their pleiotropic role in the pathogenesis of RA.

IL-12A is a potent Th1-inducing cytokine in immune diseases, is mainly produced by antigen-presenting cells and has an important role in immunoregulation. The cytokine is involved in Th1-cell proliferation and maturation, T-cell and NK-cell cytotoxicity as well as B-cell activation and promotes antibody-induced joint inflammation by suppressing transforming growth factor (TGF)-ß (8, 9).

IL-18, a member of the IL-1 cytokine superfamily, is expressed at sites of chronic inflammation and is amongst others found in macrophages, dendritic cells, Kupffer cells, keratinocytes, osteoblasts and synovial fibroblasts. It was shown, that IL-18 levels correlate with disease activity of RA. Interaction with IL-12 results in an up-regulation of IL-18R on naive T-cells, Th1-cells and B-cells. In contrast, IL-4 causes a down-regulation of the receptor complex. Functionally, IL-18 induces T- and NK-cell maturation, cytokine production (TNF, GM-CSF, IFN-γ and IL-6) as well as cytotoxicity. Further, cell adhesion molecules such as ICAM-1 and VCAM-1 are up-regulated on endothelial cells and synovial fibroblasts. In the context of articular inflammation,

these effects result in a pro-inflammatory effect of IL-18 (10-14).

We evaluated the influence of pharmacological treatment with MTX on the immune response of patients with rheumatoid arthritis by real-time polymerase chain reaction (RT-PCR). Previous studies have shown that the mRNA expression of TNF is hardly affected (15) but plasma levels are diminished (16) and that serum and mRNA levels of IL-6 are reduced by MTX (17-19). Nevertheless, we analysed the mRNA expression under MTX to correlate the results with clinical and pharmacokinetic parameters. Because the circulating intracellular levels of MTXPGs are supposed to correlate with clinical efficacy (20), a correlation analysis between erythrocyte methotrexate polyglutamate concentrations and PBMC gene expression of modulated cytokines was performed.

In this investigation, we show that pro-inflammatory cytokines are modulated by the use of MTX with and without concomitant corticosteroids in patients with rheumatoid arthritis.

Materials and methods

Patients and study protocol

After obtaining approval from the local ethics committee (Ethikkommission der Stadt Wien) and informed consent from each patient in writing, the randomised, double-blind, controlled clinical trial was performed in accordance with Good Clinical Practice guidelines and ethical principles that have their origin in the Declaration of Helsinki.

MTX-naïve and newly diagnosed RA patients who fulfilled the American College of Rheumatology criteria for rheumatoid arthritis were enrolled at the Medical Department for Rheumatology at the Kaiser-Franz-Josef Hospital in Vienna between July 2008 and July 2009.

Patients with a former MTX use, pulmonary or infectious (HIV, hepatitis B and C) diseases, low disease activity (DAS-28 ≤3.2) or contraindications for MTX were excluded from participation.

The present study was originally designed to compare the pharmacokinetics as well as the clinical response of two different MTX starting doses. Using a stratified randomisation, patients were assigned to start either with 15 mg or 25 mg MTX, administered orally. This route of administration was chosen because in Austria MTX is generally given as an oral therapy. To get a 100% reference level for bioavailability, a subcutaneous dose of 25 mg MTX was administered to all patients at week 5. A weekly oral dose of 25 mg MTX was maintained until week 16. Concomitant therapy with non-steroidal anti-inflammatory drugs (NSAIDs – diclofencac, ibuprofen, celecoxib and naproxen) and corticosteroids (prednisolone ≤10 mg per day) was allowed because of ethical reasons and to obviate disease progression. Corticosteroid therapy was started shortly before or even together with MTX to bridge the time until response to MTX was achieved. If justified, the prednisolone dose was reduced according to the patient's disease status. Combination therapy with other DMARDs was not permitted during the study. To prevent food-drug-interactions, patients were instructed to take MTX in the fasting state. In addition, 5 mg of folic acid were administered two days after MTX intake to increase the tolerability of methotrexate. Overall, patients were observed for 16 weeks.

Clinical assessment

Disease activity was assessed using the Disease Activity Score in 28 joints (DAS-28 4v, ESR – Erythrocyte sedimentation rate), the swollen joint count (SJC), the tender joint count (TJC), the duration of morning stiffness (min), Patient's global assessment (PGA), Evaluator's global assessment (EGA) and a modified version of the Health Assessment Questionnaire (HAQ). The intensity of pain and fatigue was measured using a visual analogue scale (VAS, 0–100 mm).

To avoid inter-observer variability, joint counts were performed by a single trained person. Demographic and clinical data were recorded using standardised case record forms. In addition, patients received a questionnaire to self-monitor a potential improvement in disease activity and possible adverse events.

Laboratory assessment

Laboratory measurement included a complete blood cell count and liver and kidney function tests. In addition, inflammatory parameters like the erythrocyte sedimentation rate (ESR) and the C-reactive protein (CRP) as well as rheumatoid factors were determined.

Gene expression analysis

Messenger ribonucleic acid (mRNA) concentrations of TNF, IL-6, IL-12A and IL-18 were determined in PBMCs from 17 patients before and during methotrexate therapy. EDTA whole blood samples were collected immediately before starting MTX therapy and at week 5 (before MTX as well as 4 and 168 hours after MTX intake), because at this point all patients received a subcutaneous dose of 25 mg MTX. Samples with a volume of 6 ml were stored up to four hours at 4°C until further processing. After a 20-minute centrifugation step (500 x g at 4°C; density gradient centrifugation on Biocoll®, Biochrom AG, Germany) to separate the buffy coat (leukocytes and platelets) from red blood cells and plasma, leukocytes were washed twice with 14 ml phosphate-buffered saline (PBS)-solution and centrifuged for 10 min at 200 x g (4°C). For real-time PCR, total RNA was isolated from PBMCs using Tri Reagent®, a mixture of guanidine thiocyanate and phenol in a mono-phase solution, according to the manufacturer's instructions (Sigma-Aldrich). Prior to PCR, aliquots of 30 ng RNA were reverse transcribed with Multiscribe Reverse Transcription TaqMan® Kit (Applied Biosystems) by the use of random primers (Invitrogen). To check cDNA synthesis, a PCR for amplification of the ß-actin gene (housekeeping gene) was performed. Aliquots of 0.89 ng cDNA were amplified in the presence of TaqMan® Universal PCR Master Mix (Applied Biosystems), gene specific TaqMan® probes (Hs00174128_m1 (TNF), Hs99999032_m1 (IL-6), Hs00168405_m1 (IL-12A), Hs99999040_m1 (IL-18), all from Applied Biosystems) and water. Gene specific PCR products were measured using the ABI Prism 7700 Sequence Detection System (Applied Biosystems).

Measurement of MTX polyglutamates
Erythrocyte methotrexate polyglutamates (MTXPG1-7) were analysed by a high-pressure liquid chromatography (HPLC) method using post-column photooxidation followed by fluorimetric detection, as previously described by Dervieux and co-workers (6). Studies were performed on an Agilent 1100 HPLC Chemstation system consisting of a binary pump, an autoinjector, a fluorimetric detector and a system controller. For post-column photo-oxidation, a photochemical reactor unit equipped with a 254 nm low-pressure mercury UV-lamp and a 1/16-inch (o.d.) teflon tubing (0.25 mm i.d.) (Aura Industries, New York, USA) was implemented between the analytical column and the fluorimetric detector (correlation coefficients ≥0.996, limit of detection: 0.37 nM for MTXPG1, 0.52 nM for MTXPG2, 2 nM for MTXPG3, 5 nM for MTXPG4, 13 nM for MTXPG5, 28 nM for MTXPG6 and 48 nM for MTXPG7).

Results

Demographics
Seventeen patients participated in gene expression analysis. Nine patients received a standard starting dose of 15 mg and eight patients immediately started with 25 mg MTX. The median age was 56 years. 68% of patients were females and 32% were male. Among the study population, 42% were rheumatoid factor positive. At study entry, rheumatoid arthritis patients had on average 5.9 swollen joints, 8.9 tender joints and scored 52.4 by VAS for joint pain and 44.5 by VAS for fatigue. Mean DAS-28 4v (ESR) was 4.7 and mean HAQ-Score was 1.5.
No statistically significant differences in baseline demographic and clinical characteristics were observed between the two patient groups receiving different dosing schemes (Mann-Whitney-U-test for independent samples, p=0.05).

Methotrexate and corticosteroids
Descriptive statistics. Under the combined action of MTX (mean dosage: 22 mg/week) and prednisolone (mean dosage: 6.5 mg/day), statistically significant changes in mRNA gene expression were observed for IL-6 and IL-12A. mRNA expression of IL-18 was less influenced, but was significantly reduced at week 6. The gene expression of TNF was hardly influenced. Using the $2^{-\Delta\Delta Ct}$ method (21), relative changes in gene expression were calculated. The baseline sample taken before starting MTX therapy was used as calibrator with the baseline level set to 1 (Table I). Because of a high variance, the intra- and inter-subject variability has to be considered for data interpretation.
Correlation analysis of gene expression and pharmacokinetics. At week 5, a negative correlation of erythrocyte methotrexate polyglutamate C_{max}-levels with TNF, IL-6 and IL-12A was found using the Spearman's correlation coefficient – implicating that higher concentrations of these metabolites correlate with lower levels of pro-inflammatory cytokines. Interestingly, a higher gene expression of IL-18 was linked to higher concentrations of MTXPGs (Table II, week 5 mean values).
Correlation analysis of gene expression and clinical parameters. A positive correlation of TNF and monocytes (%) was observed (+0.503, p=0.040) after week 5, meaning that a decreased TNF gene expression was accompanied by lower levels of monocytes.
Gene expression of IL-12A positively correlated with CRP (mg/l) (+0.647, p=0.005) and neutrophils (/nl) (+0.517, p=0.034), both useful markers in rheumatology.
IL-18 revealed a strong involvement with inflammatory parameters, resulting in significant positive correlations of IL-18 gene expression with ESR (mm/h) (+0.631, p=0.007), leukocytes (/nl) (+0.645, p=0.005), CRP (mg/

Table I. Descriptive statistics of real-time PCR parameters presenting relative changes in gene expression using the $2^{-\Delta\Delta Ct}$ method (MTX in combination with corticosteroids), Wilcoxon-test for paired samples, significance level 0.05.

Parameter	Mean (± SD)	Minimum	Maximum	Significance
TNF				
Week 5 Pre-value	0.91 (± 0.33)	0.29	1.37	0.193
Week 5 4 hours	1.09 (± 0.44)	0.59	2.39	0.717
Week 6 Pre-value	0.97 (± 0.49)	0.32	2.00	0.605
IL-6				
Week 5 Pre-value	0.37 (± 0.63)	0.00	1.91	0.026
Week 5 4 hours	0.36 (± 0.64)	0.00	1.88	0.017
Week 6 Pre-value	0.17 (± 0.23)	0.00	0.60	0.007
IL-12A				
Week 5 Pre-value	0.69 (± 0.29)	0.39	1.51	0.003
Week 5 4 hours	0.85 (± 0.53)	0.29	2.21	0.076
Week 6 Pre-value	0.71 (± 0.40)	0.23	1.63	0.019
IL-18				
Week 5 Pre-value	0.92 (± 0.26)	0.42	1.29	0.309
Week 5 4 hours	0.85 (± 0.38)	0.31	1.92	0.061
Week 6 Pre-value	0.81 (± 0.29)	0.37	1.48	0.025

Baseline level set to 1.

Table II. Correlation analysis of cytokine gene expression and pharmacokinetic parameters using Spearman's correlation coefficient (MTX in combination with corticosteroids).

Parameter	Correlation partner	Correlation	Significance
TNF			
Week 5 Mean	MTXPG1 C_{max} (nM) Week 5	- 0.500	0.041
IL-6			
Week 5 Mean	MTXPG1 C_{max} (nM) Week 5	- 0.534	0.027
IL-12A			
Week 5 Mean	MTXPG1 C_{max} (nM) Week 5	- 0.549	0.022
IL-18			
Week 5 Mean	MTXPG2 AUC (nM*h) Week 5	+ 0.607	0.010

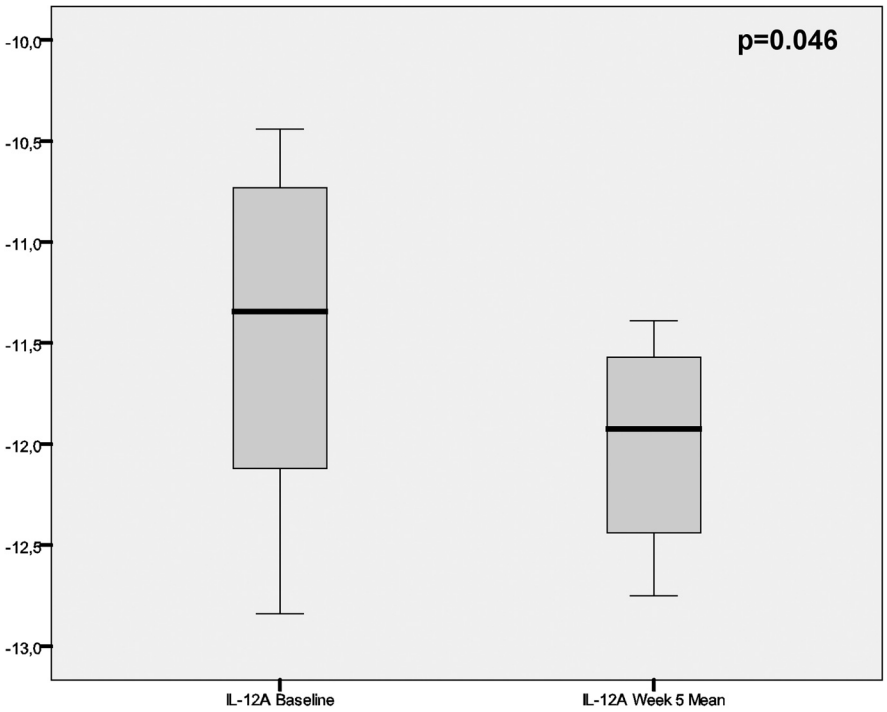

Fig. 1. Box-and-Whisker plot IL-12A (comparison baseline and week 5 mean) (MTX without corticosteroids).
Values given as the median with 25th and 75th percentiles; min, max.

Table III. Descriptive statistics of real-time PCR parameters presenting relative changes in gene expression using the $2^{-\Delta\Delta Ct}$ method (MTX without corticosteroids), Wilcoxon-test for paired samples, significance level 0.05.

Parameter	Mean (± SD)	Minimum	Maximum	Significance
TNF				
Week 5 Pre-value	1.07 (± 0.29)	0.68	1.37	0.600
Week 5 4 hours	1.33 (± 0.54)	0.87	2.39	0.080
Week 6 Pre-value	1.08 (± 0.63)	0.50	2.00	0.917
IL-6				
Week 5 Pre-value	0.12 (± 0.24)	0.00	0.48	0.059
Week 5 4 hours	0.47 (± 0.94)	0.00	1.88	0.131
Week 6 Pre-value	0.24 (± 0.29)	0.00	0.60	0.066
IL-12A				
Week 5 Pre-value	0.68 (± 0.23)	0.40	0.94	0.028
Week 5 4 hours	0.67 (± 0.25)	0.40	1.06	0.046
Week 6 Pre-value	0.98 (± 0.53)	0.23	1.63	0.917
IL-18				
Week 5 Pre-value	0.94 (± 0.33)	0.42	1.27	0.917
Week 5 4 hours	0.81 (± 0.56)	0.31	1.92	0.344
Week 6 Pre-value	0.77 (± 0.39)	0.37	1.48	0.249

Baseline level set to 1.

l) (+0.669, p=0.003) and ANC (/nl) (+0.672, p=0.003) at week 5.

Methotrexate without corticosteroids
Descriptive statistics. When excluding concomitant use of corticosteroids as potential confounding factor, MTX had no influence on IL-18 (p=0.173) in the remaining six patients (4 women, 2 men) not receiving corticosteroids, but statistically significantly reduced the gene expression of IL-6 (week 5 pre-value: p=0.028, week 5 mean: p=0.046) and IL-12A (week 5 pre-value: p=0.028, week 5 mean: p=0.046, Fig. 1).

Relative changes in gene expression using the $2^{-\Delta\Delta Ct}$ method are shown in Table III. The baseline sample taken before starting MTX therapy was used as calibrator.
Correlation analysis of gene expression and pharmacokinetics. To analyse the relationship between erythrocyte methotrexate polyglutamate levels and the gene expression of IL-6 and IL-12A, a correlation analysis calculating the Spearman's coefficient was performed. The observation that MTX modulates the gene expression of IL-6 and IL-12A was confirmed by a negative correlation of these pro-inflammatory cytokines with pharmacokinetic parameters. In week 5, a reduced gene expression of IL-12A went along with higher C_{max} and AUC levels of MTXPG3 (both -0.829, p=0.042). Furthermore, a negative correlation of IL-6 and the mean concentration of MTXPG3 in week 5 was observed (-0.886, p=0.019). Together, this supports the concept that MTX alone and its metabolites MTXPGs reduced the mRNA expression of the pro-inflammatory cytokines IL-6 and IL-12A.
Correlation analysis of gene expression and clinical parameters. Additionally, the results that MTX reduced the gene expression of IL-6 and IL-12A were supported by negative correlations of these pro-inflammatory cytokines and inflammatory parameters in patients not receiving concomitant corticosteroids. Lower levels of monocytes (%) were associated with a lower gene expression of IL-6 (+0.812, p=0.050) in week 5. Moreover, lower levels of neutrophils (/nl) (+0.829, p=0.042) and lymphocytes (%) (+0.886, p=0.019, Fig. 2) went along with a reduction in IL-12A gene expression.

Discussion

Using RT-PCR we demonstrated for the first time, that MTX without corticosteroids significantly reduced the mRNA gene expression of IL-12A in PBMCs of rheumatoid arthritis patients. In agreement with other studies (1, 17-19), it was confirmed that IL-6 mRNA expression decreased under low-dose methotrexate treatment.
Previous investigations have shown that corticosteroids inhibit the produc-

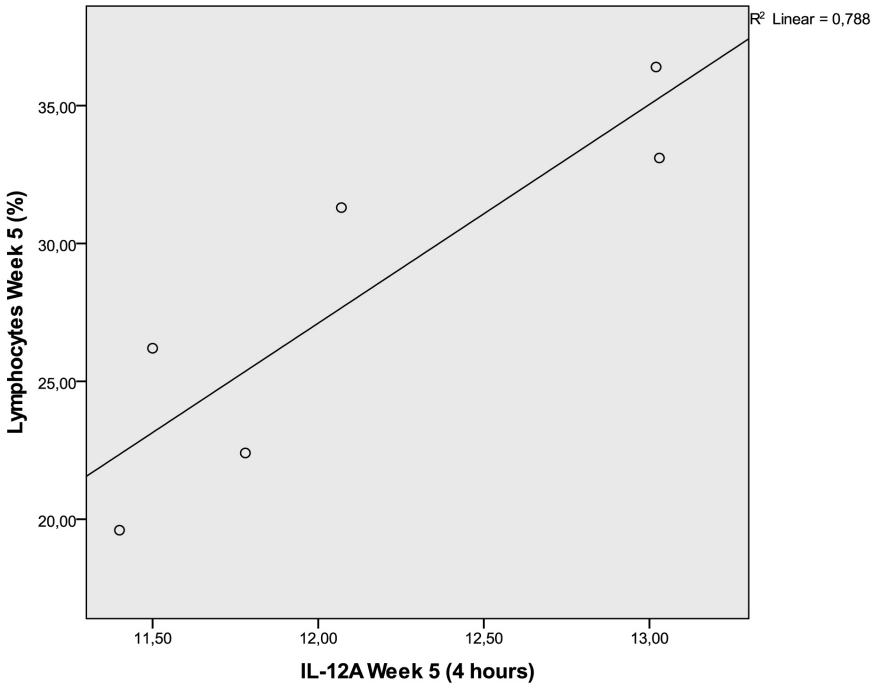

Fig. 2. Scatter-plot: correlation of lymphocytes (Week 5, %) and gene expression of IL-12-A (Week 5, 4 hours ΔCt) (MTX without corticosteroids).

tion of IL-12A in monocytes (22). Due to ethical reasons in the present study patients were allowed to take concomitant corticosteroids to prevent disease progression. To eliminate this confounding factor, we performed a subgroup analysis for study participants taking only methotrexate without concomitant prednisolone. Even in this small group of six patients mRNA expression of IL-12A was markedly reduced ($p \leq 0.046$), reflecting the impact of MTX on this pro-inflammatory cytokine involved in the pathogenesis of rheumatoid arthritis. These results are further supported by a significant negative correlation of IL-12A gene expression with the concentration of erythrocyte MTXPGs ($p \leq 0.042$), in addition to a positive correlation with inflammatory parameters such as neutrophils and lymphocytes ($p \leq 0.042$). Although this effect is of limited value due to the small number of patients treated without corticosteroids, our observations substantiate the efficacy of methotrexate in altering the inflammatory environment. Furthermore, these results strengthen the finding that MTX modulates the immune status towards Th2 dominance, amongst others by decreasing the IL-12 receptor (23). The measurement of MTXPGs in order to correlate their concentration with the mRNA concentrations of the pro-inflammatory cytokines additionally secures the causal relationship between MTX treatment and reduction of cytokines.

Although the gene expression of IL-18 inclined to be reduced under combination therapy of MTX with corticosteroids, this key mediator of Th1 polarisation and macrophage activation (10) was unaffected by MTX without corticosteroids. The observed decreased IL-18 expression in the presence of corticosteroids is inconsistent with the results of Möller and co-workers, who found that IL-18 mRNA levels in PBMCs positively correlated with administered steroid doses in RA, implicating that prednisolone induces IL-18 expression in mononuclear cells (24). Though the hypothesis of Möller would be consistent with the positive correlation between IL-18 mRNA expression and MTXPGs in the present study, it would be contradicting the anti-inflammatory properties of MTXPGs (1). Therefore, the effect of anti-inflammatory agents such as corticosteroids and MTX on IL-18 expression remains to be clarified in future investigations.

Limitations of our study are the small sample size and that participants were allowed a concomitant therapy with NSAIDs so that an independent effect of NSAIDs on IL-12A cannot be fully excluded. However, to our knowledge, there is no published information available about the influence of NSAIDs on the gene expression of IL-12A. Further investigations are needed to study this issue and also to verify the present results in a larger population of rheumatoid arthritis patients.

Despite the small sample size, the observation that the gene expression of IL-12A was reduced by MTX in patients expands the understanding of the mechanism of action of the most widely used drug in rheumatoid arthritis.

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