

## **Methotrexate modulates the kinetics of adenosine in humans *in vivo***

Niels P Riksen<sup>1,2</sup>, MD  
Pilar Barrera<sup>3</sup>, MD, PhD  
Petra HH van den Broek<sup>1</sup>  
Piet LCM van Riel<sup>3</sup>, MD, PhD  
Paul Smits<sup>1,2</sup>, MD, PhD  
Gerard A Rongen<sup>1,2</sup>, MD, PhD.

<sup>1</sup>Departments of Pharmacology-Toxicology, <sup>2</sup>Internal Medicine, and <sup>3</sup>Rheumatology, Radboud University Nijmegen Medical Center, PO Box 9101, 6500 HB, the Netherlands.

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### **Corresponding author**

Niels P. Riksen, MD  
Department of Pharmacology-Toxicology 149  
Radboud University Nijmegen Medical Center  
Geert Grooteplein 21, 6525 EZ Nijmegen, The Netherlands  
PO Box 9101, 6500 HB Nijmegen, The Netherlands

E-mail: N.Riksen@aig.umcn.nl

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## Abstract

**Objective** Animal studies suggest that the anti-inflammatory effect of methotrexate (MTX) is mediated by increased adenosine concentrations. We aimed to assess the effect of MTX on the vasodilator effects of adenosine and the nucleoside uptake inhibitor dipyridamole in humans *in vivo* as a marker for changes in adenosine kinetics.

**Methods** Ten patients with active arthritis were treated with MTX (15 mg/week). Measurements were performed before and after 12 weeks of therapy. At these timepoints, activity of adenosine deaminase was measured in isolated lymphocytes, and forearm blood flow (FBF) was determined by venous occlusion plethysmography during administration of adenosine and dipyridamole into the brachial artery.

**Results** The  $V_{\max}$  of adenosine deaminase in lymphocytes was reduced by MTX therapy ( $P<0.05$ ). MTX significantly enhanced vasodilator response to adenosine (0.5 and 1.5  $\mu\text{g}/\text{min}$  per dl of forearm tissue; FBF ratio increased from  $1.2\pm 0.2$  to  $1.4\pm 0.2$  and  $2.2\pm 0.2$  ml/dl/min before and from  $1.3\pm 0.1$  to  $1.8\pm 0.2$  and  $3.2\pm 0.5$  during MTX therapy, mean  $\pm$  SE;  $P<0.05$ ). Also, dipyridamole-induced vasodilation (30 and 100  $\mu\text{g}/\text{min}/\text{dl}$ ) was enhanced by MTX (FBF ratio increased from  $1.2\pm 0.2$  to  $1.5\pm 0.3$  and  $1.8\pm 0.2$  before and from  $1.3\pm 0.1$  to  $1.8\pm 0.2$  and  $2.4\pm 0.4$  during MTX therapy;  $P<0.05$ ).

**Conclusions** MTX therapy inhibits deamination of adenosine and potentiates adenosine-induced vasodilation. Also dipyridamole-induced vasodilation is enhanced by MTX treatment, suggesting an increased extracellular formation of adenosine. This is the first study that shows that MTX therapy affects adenosine kinetics in humans *in vivo*. These effects probably contribute to the therapeutic efficacy of MTX.

**Key Words:** Methotrexate, Adenosine, Blood flow.

## Introduction

Because of its favourable efficacy and toxicity profile, methotrexate (MTX) is often the first disease modifying antirheumatic drug (DMARD) prescribed in patients with rheumatoid arthritis [1]. The mechanism of action of MTX remains a subject of controversy, although it is generally accepted that this is different from the anti-proliferative effect of high-dose MTX therapy in the treatment of neoplasms [2;3]. In the last decade, animal studies have provided several lines of evidence that the anti-inflammatory effect of MTX is mediated by adenosine receptor stimulation [2].

Adenosine is a purine-nucleoside that is formed both intra- and extracellularly by degradation of adenosine mono-phosphate (AMP) (figure 1) [4]. Degradation of adenosine by the enzymes adenosine deaminase and adenosine kinase, however, is confined to the intracellular compartment. As such, facilitated transport of adenosine over the cellular membrane is mainly directed from outside the cell inwards [5]. There are four types of G-protein-coupled adenosine receptor, which are designated A<sub>1</sub>, A<sub>2A</sub>, A<sub>2B</sub> and A<sub>3</sub> receptors [4]. Stimulation of the A<sub>2A</sub> receptor potently inhibits inflammation [6], and induces vasodilation [7].

In humans, evidence for a role of adenosine is either indirect [8;9] or controversial [10-14]. This is mainly due to methodological shortcomings and the highly complicated determination of the adenosine concentration [15].

In the present study we aimed to assess whether adenosine receptor stimulation is indeed increased by MTX therapy in humans *in vivo*. We avoided the methodological problems of measuring adenosine concentrations by determining the vasodilator response to adenosine and dipyridamole, reflecting alterations in degradation and formation of adenosine, respectively. Dipyridamole is a potent inhibitor of the equilibrative nucleoside transporter (figure 1)[16], and consequently increases extracellular adenosine, and induces vasodilation, at a rate proportional to extracellular formation of adenosine. Finally, we also determined the activity of adenosine deaminase in lymphocytes and erythrocytes to explore the mechanism of the altered adenosine kinetics by MTX.

## Patients and Methods

### Subjects

Adult outpatients with active arthritis in whom MTX therapy was indicated according to their treating rheumatologist were asked to participate. Concomitant use of other DMARD's, non-steroidal anti-inflammatory drugs (NSAID's) or corticosteroids was not allowed to be changed from one month before therapy initiation until the end of the study. Exclusion criteria were pregnancy, breast-feeding, asthma, alcohol abuse (> 20 units/week), elevated liver enzymes (alanine aminotransferase - ALT - >3 times upper limit), renal insufficiency (estimated clearance <50 ml/min), thrombocytopenia (<120×10<sup>9</sup>/l) or leukocytopenia (<3.5 ×10<sup>9</sup>/l). Patients were not allowed in the study if they had been treated previously with MTX, or if they were treated currently with sulfasalazine, dipyridamole, folic acid or folinic acid. Folic acid supplementation was not given in the 12-week treatment period to avoid any possible interference with our measurements.

The study protocol was approved by the Institutional Review Board of the Radboud University Nijmegen Medical Center and the investigation conforms with the principles outlined in the Declaration of Helsinki. Ten patients agreed to participate and signed written informed consent before participation. Seven patients were diagnosed with rheumatoid arthritis, two patients with psoriatic arthritis and one patient with an unspecified oligoarthritis. Other baseline characteristics are shown in table 1.

**Table 1:** Baseline characteristics of the patients.

Parameter	Mean ± SD
Sex (m/f)	5 / 5
Age (years)	53.2 ± 12.0
Disease Activity Score (DAS)	4.0 ± 1.1
Body Mass Index (kg/m <sup>2</sup> )	24.7 ± 5.4
Creatinine (µmol/l)	88.0 ± 56.4
Cholesterol (mmol/l)	5.4 ± 0.6
Plasma glucose (mmol/l)	5.3 ± 0.5

### Experimental Protocol

All patients started oral therapy with MTX at a dose of 15 mg per week during the study period. Vasodilator effects of adenosine and dipyridamole were assessed before initiation of therapy, and 12 weeks after therapy initiation, 2 hours after the intake of the weekly MTX dose. All experiments were performed in the morning in a temperature-controlled laboratory (23 °C). Participants were asked to abstain from caffeine containing products for at least 24 hours before each experiment because caffeine is an effective adenosine receptor antagonist [17]. NSAID's were discontinued for at least 24 hours before each experiment to avoid any influence of cyclooxygenase inhibition on vascular function.

On these visits the disease activity score (DAS) [18] was obtained by a rheumatologist (PB), and blood was drawn for determination of ALT, alkaline phosphatase (AF), creatinine, total blood count, C-reactive protein (CRP), erythrocyte sedimentation rate (ESR), total plasma homocysteine and the activity of the adenosine deaminase enzyme in erythrocytes and lymphocytes.

After local anesthesia (xylocaine 2%) the brachial artery of the non-dominant arm was cannulated with a 20-gauge catheter (Angiocath, Deseret Medical, UT, USA) for intra-arterial drug administration (automatic syringe infusion pump, type STC-521, Terumo Corporation,

Tokyo, Japan) and blood pressure recording (Hewlett Packard GmbH, Böblingen, Germany). Forearm blood flow was registered simultaneously on both arms by venous occlusion plethysmography using mercury-in-silastic strain gauges (Hokanson EC4, Hokanson, Inc., Washington, USA) as previously described [19]. Each drug dose was infused for 5 minutes and forearm blood flow was recorded three times per minute during the last 2 minutes of each infusion. Before each drug infusion, a wrist cuff was inflated to 200 mmHg to exclude the hand circulation from the measurements.

Baseline forearm blood flow was assessed during infusion of saline into the brachial artery. Subsequently adenosine was infused in a dose of 0.5 and 1.5  $\mu\text{g}/\text{min}$  per dl of forearm tissue. After 30 minutes of wash-out baseline forearm blood flow was measured again during infusion of saline and finally, dipyridamole was infused in a dose of 30 and 100  $\mu\text{g}/\text{min}/\text{dl}$ .

#### *Analytical methods*

Total plasma homocysteine was determined using reversed-phase HPLC as previously described [20]. For the determination of adenosine deaminase activity, erythrocytes were isolated from freshly-drawn blood by centrifugation, washed two times in saline and resuspended in MOPS buffer (10 mM MOPS, 0.9% NaCl, pH 7.4) to obtain a 20% (vol/vol) solution. Subsequently, the erythrocytes were lysed by adding 6 volumes of cold distilled water for 5 minutes. After centrifugation for 10 minutes at 4 °C the supernatant was stored at -70 °C until analysis. Lymphocytes were isolated by Ficoll-Paque centrifugation and were lysed with M-PER<sup>®</sup> Mammalian protein extraction reagent in the presence of Halt<sup>™</sup> protease inhibitor cocktail and EDTA solution. After incubation for 10 minutes (22 °C), and centrifugation for 15 minutes, the supernatant was stored at -70 °C until analysis.

For determination of adenosine deaminase activity, adenosine was added to lysate in a final concentration of 0, 25, 50, 100, 200 and 300  $\mu\text{mol}/\text{l}$  at 37 °C. Each 200  $\mu\text{l}$  of incubation mixture contained 25  $\mu\text{l}$  cellular lysate and 50 mM Tris-HCl (pH 7.4). After 15 minutes the reaction was stopped by the addition of 50  $\mu\text{l}$  1.5 M  $\text{HClO}_4$  followed by centrifugation (3 minutes). Subsequently 125  $\mu\text{l}$  of the supernatant was mixed with 125  $\mu\text{l}$  trioctylamine in chloroform, and after centrifugation (3 minutes) 50  $\mu\text{l}$  of the neutralized upper layer was used for HPLC analysis of inosine and hypoxanthine with UV-detection.

#### *Drugs and solutions*

Solutions of adenosine (Adenocor, Sanofi-Synthelabo, Maassluis, the Netherlands) and dipyridamole (Persantin, Boehringer Ingelheim, Espana S.A., Spain) were freshly prepared before each experiment with saline as solvent.

#### *Data analysis*

All data are shown as mean  $\pm$  SD unless stated otherwise. For each patient,  $V_{\text{max}}$  and  $K_{\text{m}}$  values of adenosine deaminase were calculated according to Michaelis-Menten kinetics (GraphPad Prism 4 for Windows). Activity was related to the total protein content in lymphocytes and to the protein content of the membranous fraction in erythrocytes, as determined by the Lowry-assay. The effect of MTX therapy on laboratory values and disease activity scores was calculated using a Wilcoxon signed ranks test as not all variables showed a Gaussian distribution.

Forearm blood flow was measured in both arms simultaneously and the ratio of the forearm blood flow in the experimental arm to the control arm (FBF ratio) was calculated to adjust for random changes unrelated to the local stimulus [21]. The effect of MTX therapy on the vasodilator response to adenosine and dipyridamole was calculated using analysis of variance for repeated measures (SPSS for windows, release 12.0.1).

## Results

One patient was excluded from analysis because MTX therapy had to be discontinued as a result of a rise in ALT activity during the study. Disease activity score was not significantly decreased by MTX (from  $4.0 \pm 1.1$  to  $3.2 \pm 2.0$ ,  $n=9$ ,  $P>0.1$ ). Plasma concentrations of CRP and Hb tended to decrease during MTX therapy (table 2,  $P=0.1$ ). ALT activity and total plasma homocysteine concentration were significantly increased by MTX therapy (table 2). Other biochemical or hemodynamic parameters did not change significantly.

**Table 2:** Laboratory values and hemodynamic parameters.

Parameter	Before methotrexate	During methotrexate
ALT (U/l)	$20 \pm 11$	$45 \pm 24^*$
AF (U/l)	$77 \pm 17$	$75 \pm 23$
Creatinine ( $\mu\text{mol/l}$ )	$88 \pm 56$	$83 \pm 46$
CRP (mg/l)	$24 \pm 48$	$10 \pm 15$
ESR (mm/h)	$15 \pm 18$	$16 \pm 22$
Hb (mmol/l)	$8.1 \pm 0.8$	$7.7 \pm 1.1$
Leucocytes ( $\times 10^9/l$ )	$7.8 \pm 2.8$	$7.1 \pm 2.4$
Thrombocytes ( $\times 10^9/l$ )	$253 \pm 92$	$278 \pm 88$
SBP (mmHg)	$142 \pm 20$	$139 \pm 18$
DBP (mmHg)	$70 \pm 7$	$70 \pm 9$
Heart rate (bpm)	$65 \pm 12$	$66 \pm 13$
Homocysteine ( $\mu\text{mol/l}$ )	$10.6 \pm 4.1$	$14.9 \pm 4.9^*$

\* $P<0.01$  for the effect of methotrexate.

In lymphocytes, but not in erythrocytes, the  $V_{\text{max}}$  value of the enzyme adenosine deaminase was significantly decreased by MTX therapy, whereas  $K_m$  values were not significantly affected (table 3).

**Table 3:** adenosine deaminase activity.  $V_{\text{max}}$  values expressed as nmol/min/mg protein;  $K_m$  values expressed as  $\mu\text{mol/l}$ .

Parameter	Before methotrexate	After methotrexate
Lymphocytes		
$V_{\text{max}}$	$20.7 \pm 5.6$	$16.2 \pm 6.0^*$
$K_m$	$40.7 \pm 2.9$	$38.3 \pm 3.6$
Erythrocytes		
$V_{\text{max}}$	$100.9 \pm 31.7$	$111.3 \pm 42.3$
$K_m$	$43.9 \pm 6.9$	$42.6 \pm 5.5$

\* $P<0.05$  for the effect of methotrexate.  $P>0.1$  for other parameters.

Baseline forearm blood flow in the experimental arm was similar on both occasions ( $2.9 \pm 0.9$  ml/min/dl before and  $2.9 \pm 0.8$  during MTX therapy,  $P>0.1$ ). Also on both occasions, forearm blood flow completely returned towards baseline levels after the 30-minutes period between the adenosine and dipyridamole infusions and MTX therapy did not significantly affect FBF in the control arm (data not shown). MTX therapy resulted in a significant enhancement of both adenosine-induced vasodilation and dipyridamole-induced vasodilation ( $P<0.05$ ; figure 2).

## Discussion

In the present study, we demonstrated in humans *in vivo* that MTX therapy inhibits adenosine deaminase. Also, adenosine-induced forearm vasodilation was significantly potentiated by MTX. This could be due to the decreased intracellular deamination of adenosine, since it was previously shown that adenosine deamination rather than transport of adenosine over the cellular membrane is rate-limiting for the overall catabolism of adenosine [22]. We also showed that dipyridamole-induced vasodilation is potentiated by MTX therapy. This observation indicates that, besides inhibition of intracellular degradation, also extracellular formation of endogenous adenosine is increased, which is compatible with previous *in vitro* findings [23]. In conclusion, we demonstrated for the first time in humans *in vivo* that therapy with MTX potentiates adenosine A<sub>2A</sub> receptor mediated effects.

The effect of MTX on the metabolism of adenosine was first described by Cronstein *et al ex vivo* and in animal experiments [24]. They showed that in isolated human fibroblasts and endothelial cells pretreatment with MTX increases extracellular adenosine. Recently, it was demonstrated that in a rat model of adjuvant arthritis adenosine receptor antagonists completely abolish the anti-inflammatory effects of MTX [25]. Moreover, in adenosine A<sub>2A</sub> and A<sub>3</sub> receptor knock-out mice, MTX therapy does no longer induce any anti-inflammatory effects [26].

Human *in vivo* data on the potential role of adenosine in the mechanism of action of MTX are scarce, controversial and indirect. Two recent reports suggest that a high intake of caffeinated products diminishes the anti-rheumatic effect of MTX [9]. As caffeine is an effective adenosine receptor antagonist already at concentrations reached after regular coffee consumption [27], this is consistent with a role for adenosine receptor stimulation in the mechanism of action of MTX. Interventional studies which determined adenosine concentration before and after administration of MTX provided controversial results and most studies showed negative results [10-14]. An important reason for these inconsistent and mostly negative results is the duration of MTX therapy. It is necessary to administer MTX for several days to weeks to permit polyglutamation and intracellular accumulation of AICAR, which is required for enhanced adenosine formation [26]. In contrast, in most previous studies, adenosine concentrations were determined after at most one day [11-13] or a few days [10]. Also, reliable measurement of adenosine is highly cumbersome as its halflife is less than one second and the endothelium constitutes an active metabolic barrier for adenosine, resulting in a functional compartmentalization of adenosine [15;28]. In the present study, we administered MTX for 12 weeks and bypassed the methodological difficulties of adenosine determination by using adenosine- and dipyridamole-induced vasodilation as a reflection of adenosine degradation and formation, respectively.

The mechanism by which MTX affects the kinetics and dynamics of adenosine has partially been elucidated by previous animal studies. Most probably, MTX interferes with the *de novo* purine synthesis pathway in cells [3;29]. Polyglutamates of MTX, the long-lived intracellular metabolites of MTX, are potent competitive inhibitors of the enzyme 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase, resulting in the intracellular accumulation of AICAR [29] (Figure 1). In turn, it has been shown *in vitro* that AICARiboside inhibits catalytic activity of adenosine deaminase [29;30], and AICARibotide inhibits AMP deaminase [29]. Recently, Morabito *et al* showed that the anti-inflammatory effects of MTX could be abolished by inhibition of ecto-5'-nucleotidase [23], emphasizing the importance of extracellular conversion of AMP to adenosine. It was suggested that inhibition

of AMP deaminase promotes release of adenine nucleotides, by an as yet unidentified mechanism, which are converted by extracellular 5'-nucleotidase to adenosine (figure 1).

Our study provides additional mechanistic insight which links up nicely with the abovementioned previous findings. It was previously shown in humans that MTX therapy decreases the activity of adenosine deaminase. However, effects of MTX on the  $V_{max}$  and  $K_m$  values of this enzyme were not assessed [31]. The present study showed that MTX decreases the  $V_{max}$  but not the  $K_m$  of adenosine deaminase in lymphocytes. Unfortunately, from these results it cannot be concluded whether this observed change is due to direct non-competitive enzyme inhibition or decreased enzyme levels, or whether this reflects changes in lymphocyte subpopulations, which could differ in their adenosine deaminase activity [32]. To provide further evidence that MTX modulates adenosine metabolism by interfering with *de novo* purine synthesis, we also determined activity of adenosine deaminase in erythrocytes, a cell type that lacks the capacity for *de novo* purine synthesis [33]. Indeed, in these cells we observed no change of activity of adenosine deaminase during MTX therapy. Unfortunately, we were not able to assess enzymatic activity of other enzymes involved in purine metabolism, such as AMP deaminase and ecto-5'-nucleotidase. It has to be realized that erythrocytes are responsible for the bulk of breakdown of circulating adenosine and that therefore adenosine deamination in lymphocytes probably only plays a minor role in the observed increased adenosine-induced vasodilation in our study. However, endothelial cells and vascular smooth muscle cells contribute significantly to adenosine metabolism [34]. We postulate that in these cells, adenosine deaminase activity is also reduced, resulting in an increased adenosine concentration able to stimulate adenosine receptors on these cells. In contrast to the breakdown of circulating adenosine, lymphocytes might play an important role in the regulation of adenosine concentration in areas of inflammation. Therefore, the decreased adenosine deaminase activity in lymphocytes could contribute to the anti-inflammatory effect of MTX.

Additional mechanistic information could be obtained from the *in vivo* part of this study. As dipyridamole prevents cellular uptake of endogenous adenosine, it increases the extracellular concentration of adenosine at a rate which is proportional to extracellular formation of adenosine. We have demonstrated previously that dipyridamole-induced vasodilation in humans is indeed due to inhibition of cellular adenosine uptake: dipyridamole potentiates the vasodilator response to adenosine [35], dipyridamole increases the adenosine concentration in the forearm venous effluent during administration of adenosine into the brachial artery [36], and dipyridamole-induced vasodilation (100  $\mu\text{g}/\text{min}/\text{dl}$ ) is inhibited by the adenosine receptor antagonist theophylline [37]. The present study shows that dipyridamole-induced vasodilation is significantly enhanced during MTX therapy. This finding translates the previous *in vitro* finding of increased extracellular dephosphorylation of AMP during therapy with MTX [23] to the human *in vivo* situation.

The present study revealed that MTX therapy potentiates the vasodilator effect of adenosine. It has to be realised that adenosine receptor stimulation not only inhibits inflammation and induces vasodilation, but that it initiates various cardiovascular effects, such as negative inotropic and chronotropic cardiac effects, presynaptic inhibition of sympathetic neurotransmitter release, inhibition of vascular smooth muscle cell proliferation, and inhibition of thrombocyte aggregation [7]. Also, adenosine receptor stimulation renders the myocardium more resistant to ischaemia and reperfusion injury [38]. Taken together, these effects have the potential to protect the heart during ischaemia and prevent or slow down the process of atherosclerosis. Patients with rheumatoid arthritis have a higher incidence of

cardiovascular disease than the general population [39;40]. Interestingly, a recent study suggested that the beneficial effect of MTX on cardiovascular mortality is superior to that of other antirheumatic drugs [41], although another study did not find this benefit [42]. When considering the abovementioned cardiovascular effects of adenosine, one could easily appreciate that increased adenosine receptor stimulation could be responsible for this beneficial cardiovascular effect of MTX. Indeed, it was shown previously in canine hearts that MTX limits myocardial infarct size via adenosine-dependent mechanisms [43].

Finally, potential limitations of the present study need to be discussed. It has to be realized that, due to the use of a clinical study population in need of immediate treatment, the design of the present study was open-labeled and non-randomized. In healthy volunteers a more sophisticated design would have been possible, but in our opinion it is unethical to administer MTX to healthy volunteers for a long time. We assured that the use of other (anti-inflammatory) drugs was kept constant during the study period, in order to prevent confounding by other drugs. Finally, plasma homocysteine concentration needs to be considered as determinant of adenosine-induced vasodilation. MTX therapy increases plasma homocysteine concentration by interfering with folate-dependent remethylation of homocysteine [44]. Any increase in plasma homocysteine, in turn, could stimulate synthesis of S-adenosylhomocysteine at the expense of free adenosine, as we previously described [45;46]. Also, hyperhomocysteinemia induces endothelial dysfunction [47]. Fortunately, these potential effects of homocysteine on vascular reactivity are opposite to our present findings, and therefore our conclusion would even be more convincing when we would have been able to correct for the rise in homocysteine.

Our study adds important human *in vivo* data to the growing body of evidence that adenosine is an important mediator of the therapeutic efficacy of MTX in patients with rheumatoid arthritis. These insights provide potential alternative targets for pharmacological intervention in these patients, such as adenosine uptake inhibition. Dipyridamole, either alone or as additive to MTX, also increases extracellular endogenous adenosine and as such would also be expected to suppress inflammation in this patient group. To our knowledge, this potential anti-inflammatory effect of dipyridamole has never been systematically studied in a clinical population.

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## Figure Legends

### Figure 1

Simplified representation of the effect of methotrexate on adenosine metabolism. Polyglutamated methotrexate inhibits AICAR transformylase, resulting in the intracellular accumulation of AICAR, which inhibits adenosine deaminase and AMP deaminase. Consequently, irreversible degradation of adenosine to inosine is inhibited as well as the conversion of AMP in IMP. Subsequently, AMP is extracellularly converted to adenosine by the ecto-5'-nucleotidase.

AICAR: 5-aminoimidazole-4-carboxamide ribonucleotide; ENT: equilibrative nucleoside transporter; FAICAR: 10-formyl AICAR; FGAR: 10-formyl GAR; GAR: glycinamide ribonucleotide; IMP: inosine mono-phosphate; 5'-NT: 5'-nucleotidase;

### Figure 2

Forearm vasodilation (mean  $\pm$  SE) induced by the infusion of adenosine (a) and dipyridamole (b) into the brachial artery before initiation of MTX therapy (filled squares) and after 12 weeks of therapy (open squares). *P*-values denote the results of analysis of variance for repeated measures.

## Reference List

- (1) Guidelines for the management of rheumatoid arthritis: 2002 Update. *Arthritis Rheum* 2002;**46**:328-46.
- (2) **Cronstein BN**. Low-dose methotrexate: a mainstay in the treatment of rheumatoid arthritis. *Pharmacol Rev* 2005;**57**:163-72.
- (3) **Cutolo M**, Sulli A, Pizzorni C, Seriola B, Straub RH. Anti-inflammatory mechanisms of methotrexate in rheumatoid arthritis. *Ann Rheum Dis* 2001;**60**:729-35.
- (4) **Fredholm BB**, IJzerman AP, Jacobson KA, Klotz KN, Linden J. International Union of Pharmacology. XXV. Nomenclature and classification of adenosine receptors. *Pharmacol Rev* 2001;**53**:527-52.
- (5) **Deussen A**, Stappert M, Schafer S, Kelm M. Quantification of extracellular and intracellular adenosine production: understanding the transmembranous concentration gradient. *Circulation* 1999;**99**:2041-7.
- (6) **Hasko G**, Cronstein BN. Adenosine: an endogenous regulator of innate immunity. *Trends Immunol* 2004;**25**:33-9.
- (7) **Rongen GA**, Floras JS, Lenders JW, Thien T, Smits P. Cardiovascular pharmacology of purines. *Clin Sci (Lond)* 1997;**92**:13-24.
- (8) **Silke C**, Murphy MS, Buckley T, Busted S, Molloy MG, Phelan M. The effects of caffeine ingestion on the efficacy of methotrexate. *Rheumatology (Oxford)* 2001;**40**:34. [Abstract]
- (9) **Nesher G**, Mates M, Zevin S. Effect of caffeine consumption on efficacy of methotrexate in rheumatoid arthritis. *Arthritis Rheum* 2003;**48**:571-2.
- (10) **Smolenska Z**, Kaznowska Z, Zarowny D, Simmonds HA, Smolenski RT. Effect of methotrexate on blood purine and pyrimidine levels in patients with rheumatoid arthritis. *Rheumatology* 1999;**38**:997-1002.
- (11) **Laghi-Pasini F**, Capecchi PL, Di Perri T. Adenosine plasma levels after low dose methotrexate administration. *J Rheumatol* 1997;**24**:2492-3.
- (12) **Egan LJ**, Sandborn WJ, Mays DC, Tremaine WJ, Lipsky JJ. Plasma and rectal adenosine in inflammatory bowel disease: effect of methotrexate. *Inflamm Bowel Dis* 1999;**5**:167-73.
- (13) **Baggott JE**, Morgan SL, Sams WM, Linden J. Urinary adenosine and aminoimidazolecarboxamide excretion in methotrexate-treated patients with psoriasis. *Arch Dermatol* 1999;**135**:813-7.
- (14) **Dolezalova P**, Krijt J, Chladek J, Nemcova D, Hoza J. Adenosine and methotrexate polyglutamate concentrations in patients with juvenile arthritis. *Rheumatology* 2005;**44**:74-9.
- (15) **Moser GH**, Schrader J, Deussen A. Turnover of adenosine in plasma of human and dog blood. *Am J Physiol* 1989;**256**:C799-C806.
- (16) **Riksen NP**, Oyen WJG, Ramakers BP, van den Broek PHH, Engbersen R, Boerman OC et al. Oral therapy with dipyridamole limits ischemia-reperfusion injury in humans. *Clin Pharmacol Ther* 2005;**78**:52-9.
- (17) **Smits P**, Lenders JW, Thien T. Caffeine and theophylline attenuate adenosine-induced vasodilation in humans. *Clin Pharmacol Ther* 1990;**48**:410-8.
- (18) **Prevo ML**, 't Hof MA, Kuper HH, van Leeuwen MA, van de Putte LB, van Riel PL. Modified disease activity scores that include twenty-eight-joint counts. Development and validation in a prospective longitudinal study of patients with rheumatoid arthritis. *Arthritis Rheum* 1995;**38**:44-8.

- (19) **Rongen GA**, Ginneken E, Thien T, Lutterman JA, Smits P. Preserved vasodilator response to adenosine in insulin-dependent diabetes mellitus. *Eur J Clin Invest* 1996;**26**:192-8.
- (20) **te Poele-Pothoff MT**, van den Berg M, Franken DG, Boers GH, Jakobs C, de Kroon IF et al. Three different methods for the determination of total homocysteine in plasma. *Ann Clin Biochem* 1995;**32**:218-20.
- (21) **Petrie JR**, Ueda S, Morris AD, Murray LS, Elliott HL, Connell JM. How reproducible is bilateral forearm plethysmography? *Br J Clin Pharmacol* 1998;**45**:131-9.
- (22) **Snoeck E**, Ver DK, Jacqmin P, Van Belle H, Dupont AG, Van Peer A et al. Physiological red blood cell kinetic model to explain the apparent discrepancy between adenosine breakdown inhibition and nucleoside transporter occupancy of draflazine. *J Pharmacol Exp Ther* 1998;**286**:142-9.
- (23) **Morabito L**, Montesinos MC, Schreiber DM, Balter L, Thompson LF, Resta R et al. Methotrexate and sulfasalazine promote adenosine release by a mechanism that requires ecto-5'-nucleotidase-mediated conversion of adenine nucleotides. *J Clin Invest* 1998;**101**:295-300.
- (24) **Cronstein BN**, Eberle MA, Gruber HE, Levin RI. Methotrexate inhibits neutrophil function by stimulating adenosine release from connective tissue cells. *Proc Natl Acad Sci U S A* 1991;**88**:2441-5.
- (25) **Montesinos MC**, Yap JS, Desai A, Posadas I, McCrary CT, Cronstein BN. Reversal of the antiinflammatory effects of methotrexate by the nonselective adenosine receptor antagonists theophylline and caffeine: evidence that the antiinflammatory effects of methotrexate are mediated via multiple adenosine receptors in rat adjuvant arthritis. *Arthritis Rheum* 2000;**43**:656-63.
- (26) **Montesinos MC**, Desai A, Delano D, Chen JF, Fink JS, Jacobson MA et al. Adenosine A2A or A3 receptors are required for inhibition of inflammation by methotrexate and its analog MX-68. *Arthritis Rheum* 2003;**48**:240-7.
- (27) **Fredholm BB**, Battig K, Holmen J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. *Pharmacol Rev* 1999;**51**:83-133.
- (28) **Nees S**, Herzog V, Becker BF, Bock M, Des RC, Gerlach E. The coronary endothelium: a highly active metabolic barrier for adenosine. *Basic Res Cardiol* 1985;**80**:515-29.
- (29) **Baggott JE**, Vaughn WH, Hudson BB. Inhibition of 5-aminoimidazole-4-carboxamide ribotide transformylase, adenosine deaminase and 5'-adenylate deaminase by polyglutamates of methotrexate and oxidized folates and by 5-aminoimidazole-4-carboxamide riboside and ribotide. *Biochem J* 1986;**236**:193-200.
- (30) **Allegra CJ**, Drake JC, Jolivet J, Chabner BA. Inhibition of phosphoribosylaminoimidazolecarboxamide transformylase by methotrexate and dihydrofolic acid polyglutamates. *Proc Natl Acad Sci U S A* 1985;**82**:4881-5.
- (31) **van Ede AE**, Laan RF, De Abreu RA, Stegeman AB, van de Putte LB. Purine enzymes in patients with rheumatoid arthritis treated with methotrexate. *Ann Rheum Dis* 2002;**61**:1060-4.
- (32) **Massaia M**, Ma DD, Sylwestrowicz TA, Tidman N, Price G, Janossy G et al. Enzymes of purine metabolism in human peripheral lymphocyte subpopulations. *Clin Exp Immunol* 1982;**50**:148-54.
- (33) **Fontenelle LJ**, Henderson JF. An enzymatic basis for the inability of erythrocytes to synthesize purine ribonucleotides de novo. *Biochim Biophys Acta* 1969;**177**:175-6.
- (34) **Mattig S**, Deussen A. Significance of adenosine metabolism of coronary smooth muscle cells. *Am J Physiol Heart Circ Physiol* 2001;**280**:H117-H124.

- (35) **van Ginneken EE**, Rongen GA, Russel FG, Smits P. Diadenosine pentaphosphate vasodilates the forearm vascular bed: inhibition by theophylline and augmentation by dipyridamole. *Clin Pharmacol Ther* 2002;**71**:448-56.
- (36) **Riksen NP**, van Ginneken EE, Van den Broek PH, Smits P, Rongen GA. In vivo evidence against a role for adenosine in the exercise pressor reflex in humans. *J Appl Physiol* 2005;**99**:522-7.
- (37) **Bijlstra P**, van Ginneken EE, Huls M, Van Dijk R, Smits P, Rongen GA. Glyburide inhibits dipyridamole-induced forearm vasodilation but not adenosine-induced forearm vasodilation. *Clin Pharmacol Ther* 2004;**75**:147-56.
- (38) **Headrick JP**, Hack B, Ashton KJ. Acute adenosinergic cardioprotection in ischemic-reperfused hearts. *Am J Physiol Heart Circ Physiol* 2003;**285**:H1797-H1818.
- (39) **Turesson C**, Jarenros A, Jacobsson L. Increased incidence of cardiovascular disease in patients with rheumatoid arthritis: results from a community based study. *Ann Rheum Dis* 2004;**63**:952-5.
- (40) **Solomon DH**, Karlson EW, Rimm EB, Cannuscio CC, Mandl LA, Manson JE et al. Cardiovascular morbidity and mortality in women diagnosed with rheumatoid arthritis. *Circulation* 2003;**107**:1303-7.
- (41) **Choi HK**, Hernan MA, Seeger JD, Robins JM, Wolfe F. Methotrexate and mortality in patients with rheumatoid arthritis: a prospective study. *Lancet* 2002;**359**:1173-7.
- (42) **Landewe RB**, van den Borne BE, Breedveld FC, Dijkmans BA. Methotrexate effects in patients with rheumatoid arthritis with cardiovascular comorbidity. *Lancet* 2000;**355**:1616-7.
- (43) **Asanuma H**, Sanada S, Ogai A, Minamino T, Takashima S, Asakura M et al. Methotrexate and MX-68, a new derivative of methotrexate, limit infarct size via adenosine-dependent mechanisms in canine hearts. *J Cardiovasc Pharmacol* 2004;**43**:574-9.
- (44) **van Ede AE**, Laan RF, Blom HJ, Boers GH, Haagsma CJ, Thomas CM et al. Homocysteine and folate status in methotrexate-treated patients with rheumatoid arthritis. *Rheumatology (Oxford)* 2002;**41**:658-65.
- (45) **Riksen NP**, Rongen GA, Blom HJ, Russel FGM, Boers GHJ, Smits P. Potential role for adenosine in the pathogenesis of the vascular complications of hyperhomocysteinemia. *Cardiovasc Res* 2003;**59**:271-6.
- (46) **Riksen NP**, Rongen GA, Boers GHJ, Blom HJ, van den Broek PHH, Smits P. Enhanced cellular adenosine uptake limits adenosine receptor stimulation in patients with hyperhomocysteinemia. *Arterioscler Thromb Vasc Biol* 2005;**25**:109-14.
- (47) **Bellamy MF**, McDowell IF, Ramsey MW, Brownlee M, Bones C, Newcombe RG et al. Hyperhomocysteinemia after an oral methionine load acutely impairs endothelial function in healthy adults. *Circulation* 1998;**98**:1848-52.

Figure 1

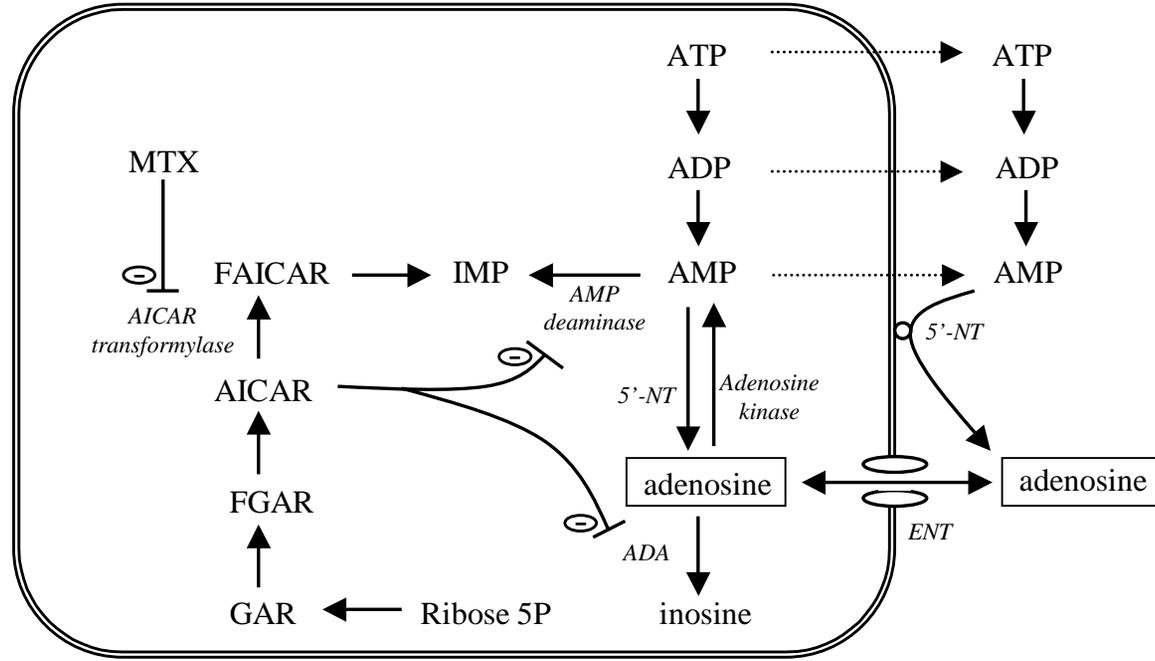


Figure 2

