

# Methotrexate did not improve endothelial function in rheumatoid arthritis: a study in rats with adjuvant-induced arthritis

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## Abstract

### Objective

Rheumatoid arthritis is associated with an increased cardiovascular risk, secondary to endothelial dysfunction. There is accumulating evidence that methotrexate reduces cardiovascular risk in rheumatoid arthritis, but the mechanisms involved are still unknown. In this study, we aimed to determine the effect of methotrexate on endothelial function and traditional cardiovascular risk factors in the adjuvant-induced arthritis (AIA) rat model.

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### Methods

On the first signs of arthritis, methotrexate (1 mg/kg/week, s.c.) or saline (Vehicle) was administered to AIA for 3 weeks. Endothelial function was studied in aortic rings relaxed with acetylcholine in the presence or not of inhibitors of nitric oxide synthase, cyclooxygenase-2, arginase, EDHF and superoxide anions production. Arthritis and radiological scores, blood pressure and blood levels of cytokines, triglycerides, cholesterol, homocysteine and BMP-4 were measured.

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### Results

Although methotrexate significantly reduced the arthritis score, it had no effect on Ach-induced relaxation. As regards mechanisms, methotrexate increased nitric oxide synthase activity and reduced the superoxide anions production but did not change arginase, cyclooxygenase-2 and EDHF pathways. Methotrexate did not change the radiological score or blood pressure, lipid, glucose and homocysteine levels. By contrast, methotrexate significantly reduced plasma IL-1 $\beta$  and TNF- $\alpha$  levels and increased serum BMP-4 level.

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### Conclusion

Despite a reduction of clinical and biological inflammation, methotrexate did not improve endothelial function in AIA rats. Overall data suggest that mechanisms other than the ED reduction are likely involved, and remain to be elucidated to better understand the cardiovascular benefits of methotrexate in rheumatoid arthritis.

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### Key words

adjuvant-induced arthritis, endothelial dysfunction, methotrexate, mechanisms

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## Introduction

Rheumatoid arthritis (RA), one of the most common chronic autoimmune diseases, is associated with a 2-fold increased mortality (1). One of the leading causes of excess mortality in RA patients is cardiovascular (CV) diseases due to accelerated atherogenesis (2). The risk of incident CVD is increased by 50% in RA patients, even in the early stage of the disease (3). Therefore, the reduction of increased morbidity and mortality from CV diseases associated with RA is currently acknowledged as a major goal of the global strategy for management of RA patients (4). The reasons explaining the excessive CV risk are not fully understood. Although traditional CV risk factors account for some of this risk, systemic inflammation, dysregulated immunity, iatrogenic factors or RA-dependent factors are likely to play a role (5, 6). Evidence from clinical studies showed that endothelial dysfunction (ED), the “*sine qua non*” condition for atherosclerosis appearance, is the key promoter of CVD in RA (7) and as such is a seminal target for reducing CV risk in RA (8). Methotrexate (MTX), a dihydrofolate reductase inhibitor, has been used as a first-line Disease-modifying antirheumatic drug (DMARDs) in the treatment of RA for decades. Although initial retrospective cohort study found that patients with known CVD had a higher risk of death after a MTX treatment (9), a recent meta-analysis suggested that MTX decreased the overall CV risk in RA (1, 10-12). Surprisingly, although a better understanding of the effect of drugs on the CV disease in RA will allow a fine-tuned use of different DMARDs in order to obtain optimal control of CV risk, the mechanisms by which MTX may reduce the CV risk in RA is still indefinite (3). Regarding the effect of MTX on ED specifically, the available data in the literature are sparse and controversial. In a recent cohort of 33 RA patients, ED was found in RA patients treated with DMARDs including MTX as compared to control subjects (13). Likewise, Hansel et al. found that MTX-treated RA patients had a reduced endothelial function as compared to control subjects

(39). Another study reported no change in the endothelial function in RA patients after a short-term treatment with DMARDs including MTX (14). By contrast other studies reported that MTX improved the endothelial function in RA patients (15, 16). A recent study reported that MTX enhanced the microvascular endothelial function but that the effect was more pronounced at 6 weeks than at 6 months of treatment (17). Because these clinical studies in RA patients have a lot of confounding factors, animal models of RA are useful to specifically study the impact of one medication on the endothelial function. Previous studies identified the presence of aortic ED in the widely-used model of adjuvant-induced arthritis (AIA) in rats (18), due to decreased EDHF availability, increased arginase and cyclo-oxygenase-2 (COX-2) activity as well excessive superoxide anions ( $O_2^{\cdot-}$ ) production (19), with a prominent role of the cross-talk between endothelial COX-2/Nitric oxide Synthase/Arginase/ $O_2^{\cdot-}$  pathways (20). By using the same model, the benefits of etanercept or high dose of glucocorticoids for improving the endothelial function were recently demonstrated (21).

In the present study, the effect of MTX on the endothelial function was studied on isolated aortic rings on day 33 after arthritis induction in AIA rats (*i.e.* at a time when ED had been previously observed in this model (18)), and the mechanisms involved were dissected. The effect of MTX on the disease severity and systemic inflammation was assessed by measurement of arthritis and radiographic scores and plasma IL-1 $\beta$  and TNF- $\alpha$ , respectively. We also measured the blood pressure, heart rate, blood glucose, serum lipid and plasma homocysteine levels to assess whether the treatment modified CV risk factors, as well as serum BMP-4 levels (bone morphogenetic protein 4) to understand the link between the bone biology and the endothelial function after MTX therapy.

## Methods

### Animals

Six weeks old male Lewis rats (n=30) were purchased from Janvier (Le Gen-

Competing interests: none declared.

est Saint Isle, France). Animals were kept under a 12h-12h light-dark cycle and allowed free access to food and water. The experimental procedures were approved by the local committee for ethics in animal experimentation n°2015-001-CD-5PR of Franche-Comté University (Besançon, France), and complied with the “Animal Research: Reporting In Vivo Experiments” ARRIVE guidelines.

#### *Induction and clinical evaluation of the arthritis model*

Adjuvant arthritis was induced by a single intradermal injection at the base of the tail of 120 µL of 1 mg of heat-killed *Mycobacterium butyricum* (Difco, Detroit, MI) suspended in 0.1 mL of mineral oil (Freund's incomplete adjuvant (Difco, Detroit, MI)). The AIA model is characterised by a rapid onset and progression of a robust and easily measurable polyarthritis, characterised by severe erythema and diffuse soft tissue swelling with complete ankyloses and malformations in the paws, reduced locomotor activity, frequently associated to ears and tail inflammation, weight loss, anorexia and diarrhea. Rats were daily weighed and monitored for clinical signs of arthritis. The scoring system was employed as follows (22): arthritis of one finger scores 0.1, weak and moderate arthritis of one big joint (ankle or wrist) scores 0.5 and, intense arthritis of one big joint scores 1. Tarsus and ankle were considered as the same joint. Sum of joints scores of 4 limbs leads to an arthritis score of maximum 6 for each rat.

#### *Analysis of radiographic parameters*

Radiographs of hind paws were performed with a Block Matching Algorithm High Resolution Digital X Ray (40mV, 10mA) – D3A Medical Systems (Orleans, France). A score of 0-20 was determined for each paw using a grading scale modified by Ackerman *et al.* (23). This score takes into account: the soft tissue swelling; the osteoporosis; the loss of cartilage; the bone erosion; and the heterotopic ossification. This score used the scale: 0 (normal); 1 (weak); 2 (mild); 3 (moderate); 4 (severe) abnormalities in the tissue for

each of the five characteristics previously described. The maximum score for each rat is 40.

#### *Drug treatment*

On the day of the first inflammatory symptoms (*i.e.* at day 11-12 post-immunisation), AIA rats were randomised in two groups. One group received MTX (Sigma-Aldrich, ref: M9929) at a dose of 1 mg/kg/week (s.c.) (“MTX”, n=15) for 3 weeks (“sub-chronic” treatment). Another group received phosphate buffer saline at 1 mL/kg/week (s.c.) for 3 weeks (“Vehicle”, n=15). The dose chosen corresponds to a dose that resulted in a significant reduction in arthritis severity in the AIA model (24, 25).

#### *Tissue collection, blood pressure and heart rate measurements*

Twenty-one days after the treatment initiation, rats were anaesthetised with pentobarbital (60 mg/kg, i.p.). Arterial systolic (SBP), diastolic (DBP), and heart rate were measured after cannulation of the left carotid artery and connection of the catheter to a pressure recorder system (Easy Graf, Gould, USA) under rectal temperature control. Blood was then withdrawn from the abdominal aorta and centrifuged to obtain serum and plasma, divided into aliquots and stored at -80°C until analysis. Thoracic aortas were removed and immediately used for vascular reactivity studies.

#### *Vascular reactivity*

At the end of the treatment period, thoracic aorta was excised, cleaned of connective tissue, and cut into rings of ~2 mm in length. Rings were suspended in Krebs solution (mmoles/litre: NaCl 118, KCl 4.65, CaCl<sub>2</sub> 2.5, KH<sub>2</sub>PO<sub>4</sub> 1.18, NaHCO<sub>3</sub> 24.9, MgSO<sub>4</sub> 1.18, glucose 12, pH 7.4), maintained at 37°C and continuously aerated with 95% O<sub>2</sub>, 5% CO<sub>2</sub> for isometric tension recording in organ chambers, as previously described (26). To test viability, rings were contracted by concentrated KCl Krebs solution (100 mmoles/litre). In some rings, the endothelium was mechanically removed. The completeness of the endothelial denudation

was confirmed by the absence of relaxation to the endothelium-dependent agonist acetylcholine (ACh, 10<sup>-6</sup> moles/litre). After a 90-min-equilibration period under a resting tension of 2g, to determine whether MTX improved the endothelial function, rings with intact endothelium were constricted with phenylephrine (PE, 10<sup>-6</sup> moles/litre), and the endothelium-dependent relaxation to ACh (10<sup>-11</sup>-10<sup>-4</sup> moles/litre) was compared between MTX and the vehicle group. To investigate the contribution of NOS, O<sub>2</sub><sup>•-</sup>, EDHF, arginase, and COX-2, rings were previously incubated for 1 hour with the non-selective NOS inhibitor N<sub>w</sub>-nitro-L-arginine methyl ester (L-NAME, 10<sup>-4</sup> moles/litre), the superoxide dismutase mimetic (SOD) Tempol (10<sup>-4</sup> moles/litre), the Ca<sup>2+</sup>-dependent K<sup>+</sup> channel inhibitors apamin (10<sup>-7</sup> moles/litre) and charybdotoxin (10<sup>-7</sup> moles/litre), and for 30 minutes with the arginase inhibitor N<sub>w</sub>-hydroxy-nor-L-arginine (nor-NOHA, 10<sup>-4</sup> moles/litre) and the selective COX-2 inhibitor (NS-398, 10<sup>-5</sup> moles/litre) respectively. Endothelium-denuded rings were used to determine the vasoconstrictive response to norepinephrine (NE, 10<sup>-11</sup>-10<sup>-4</sup> moles/litre) and the vasorelaxant response to the NO-donor sodium nitroprusside (SNP, 10<sup>-11</sup>-10<sup>-4</sup> moles/litre) after preconstruction with PE 10<sup>-6</sup> moles/litre.

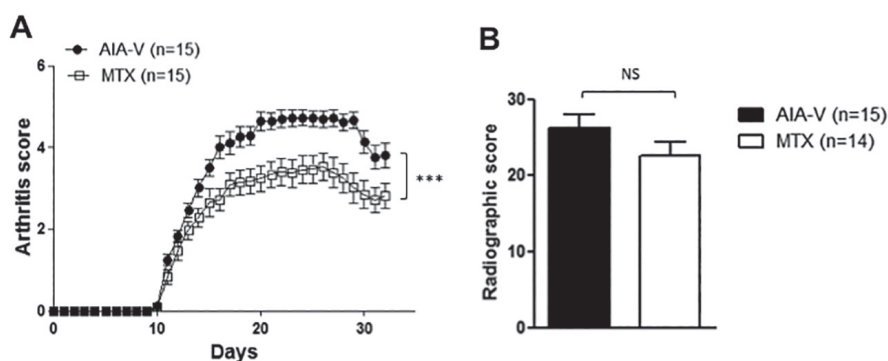
#### *Blood measurements*

Total cholesterol and triglycerides were measured in serum (Vista, Siemens, USA) and glucose was measured in blood by using glycometer (GlucoMen, Menarini diagnostics, Italy). Plasma levels of pro-inflammatory cytokines (interleukin 1β (IL-1β) and Tumor Necrosis Factor α (TNF-α)) were measured by using Milliplex magnetic bead panel kits (eBioscience, Vienne, Austria) that were analysed using a Luminex MAGPIX system (Luminex Corporation; Houston, TX) and Milliplex Analyst software (Millipore; St. Charles, MO). Plasma levels of homocysteine (HCy) and serum levels of bone morphogenetic protein 4 (BMP-4) were measured by using ELISA kits (Cusabio, Hubei, China). The limits of

**Table I.** Effect of MTX on physiological and biological parameters.

	Vehicle	MTX
Body weight (g)	222 ± 4	227 ± 4
SBP (mm Hg)	107 ± 6	110 ± 13
DBP (mm Hg)	72 ± 5	70 ± 10
Heart rate (bpm)	261 ± 12	273 ± 18
Total cholesterol (g/L)	0.99 ± 0.03	0.89 ± 0.05
Triglycerides (g/L)	0.47 ± 0.04	0.48 ± 0.04
Blood glucose (g/L)	1.11 ± 0.04	1.09 ± 0.04
TNF-α (pg/mL)	41.5 ± 5.2	22.3 ± 2.1**
IL-1β (pg/mL)	102.5 ± 14.5	48.5 ± 4.9**
BMP-4 (pg/mL)	5.19 ± 0.69	8.11 ± 1.46*
Homocysteine (nmol/mL)	1.67 ± 0.25	1.20 ± 0.10

All parameters were measured in AIA rats at day 33 post-immunisation after 21 days of treatment (s.c) with saline (vehicle) or methotrexate (1 mg/kg/week). SBP: systolic blood pressure, DBP: diastolic blood pressure. Values are expressed as means ± SEM (n=8 to 15 rats per group), \*p<0.05, \*\*p<0.01,



**Fig. 1.** Evolution of arthritis in vehicle and methotrexate-treated rats. Arthritis scores were plotted over time after AIA induction in rats treated with methotrexate (MTX) and compared to saline-treated rats (AIA-V) (A). Radiographic scores were measured at the end of the treatment period (21 days after the onset of arthritis) (B). Values are the mean ± SEM (n=14-15 rats/group). \*\*\*p<0.001, NS: not significant.

detection provided by the manufacturer for IL-1β, TNF-α, BMP-4 and Hcy were 13, 3.78 and 3.9 pg/mL, and 0.78 nmol/mL, respectively.

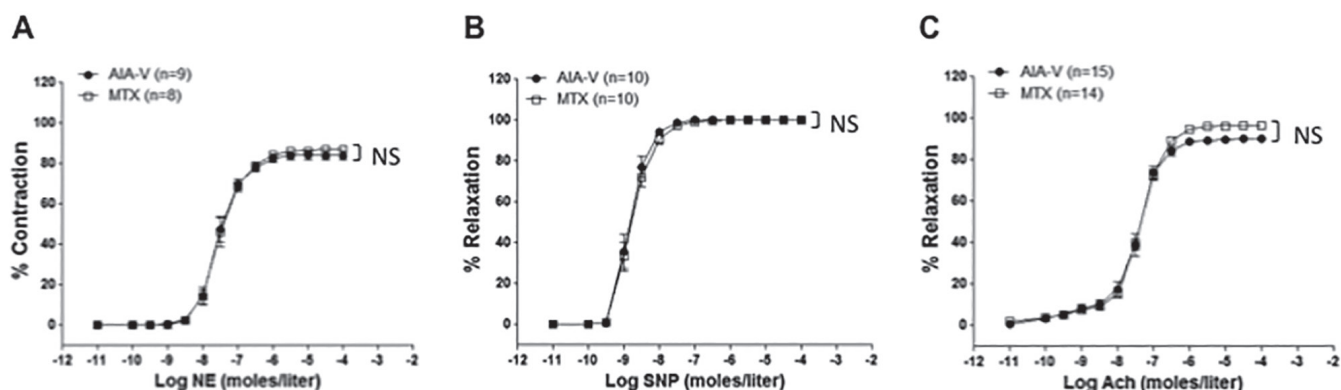
**Data and statistical analysis**  
Values are presented as means ± SEM. Data were analysed using GraphPad Prism software (v. 5.3). Contractile re-

sponses to NE were expressed as the percentage of the maximum response to KCl 100 mmoles/litre. Relaxant responses to SNP and Ach were expressed as the percentage of relaxation of the contractile response to PE 10<sup>-6</sup> moles/litre. Concentration-responses curves to Ach, SNP and NE in vehicle and MTX were compared by 2-way analysis of variance (ANOVA) for repeated measures. In each group, concentration-response curves to Ach with or without a specific inhibitor were compared by 2-way ANOVA for repeated measures. Comparison between two values was assessed by unpaired Student *t*-test or a Mann-Whitney *U*-test when data were not normally distributed. The analysis of the relationship between two parameters was determined by using linear regression analysis, and a Spearman's correlation coefficient was calculated between these variables. p<0.05 was considered statistically significant.

**Results**

*Effects of MTX on the clinical and biological parameters*

MTX did not influence body weights as compared to the vehicle (222±4 vs. 227±2, p=0.371, Table I). MTX significantly reduced arthritis score (Fig. 1A) but did not change radiologic score (Fig. 1B). SBP, DBP, heart rate, glycaemia, lipid and Hcy levels were unchanged by MTX as compared to the Vehicle (Table I). Conversely MTX significantly reduced plasma IL-1β (-53%



**Fig. 2.** Vascular reactivity to vasoconstrictors and vasodilators in AIA treated with methotrexate. Experiments were performed on thoracic aortic rings harvested at the end of the treatment period (21 days after the onset of arthritis) from AIA rats with saline (Vehicle) or with methotrexate (MTX, 1 mg/kg/week). NE induced-contraction was performed on endothelium-denuded aortic rings (A). Concentration-response curves of SNP were obtained on endothelium-denuded aortic rings precontracted with PE 10<sup>-6</sup> moles/litre (B). Ach induced-relaxation was studied on endothelium-intact aortic rings precontracted with PE 10<sup>-6</sup> moles/litre (C). Values are the mean ± SEM (n=8-15 aortic rings from 15 rats per group). NS: not significant; NE: norepinephrine; SNP: sodium nitroprusside.



vs. Vehicle,  $p < 0.05$ ) and TNF- $\alpha$  levels (-46% vs. Vehicle,  $p < 0.01$ ) (Table I). Serum BMP-4 levels were below the detection limit in 33% of the samples from the Vehicle and MTX groups. Of interest, BMP-4 levels were significantly increased after the MTX treatment (+56% vs. Vehicle,  $p < 0.05$ , Table I).

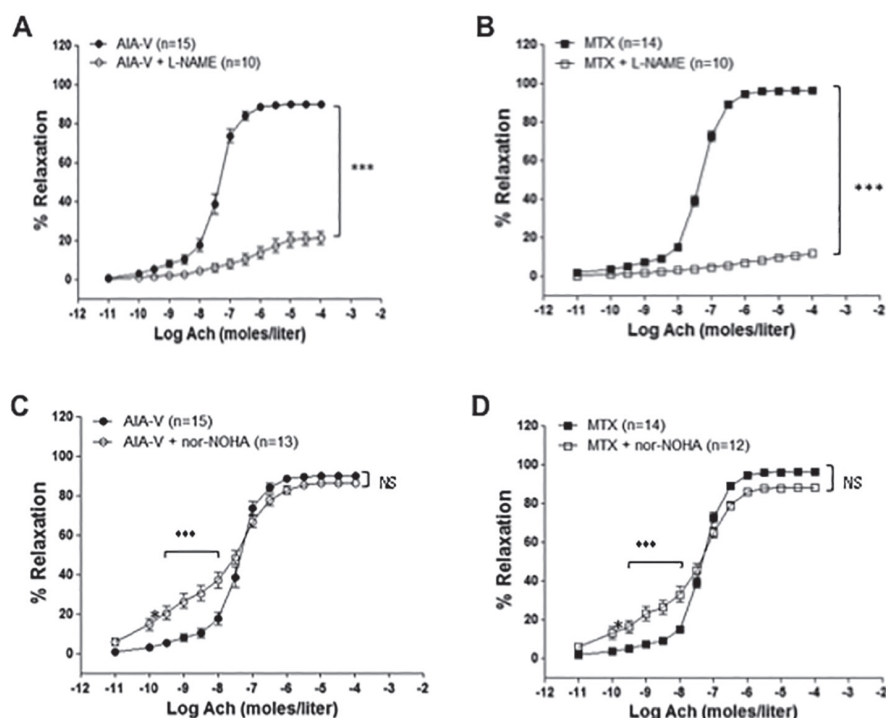
#### MTX did not change the endothelium-independent responses to the relaxing and constricting agents

To ascertain that the effect of MTX on the endothelial function was not influenced by the impaired response of VSMCs to the vasoconstrictive stimulus or to the relaxant effect of NO, the effect of MTX on NE-induced vasoconstriction and on SNP-induced vasodilation was determined on endothelium-denuded aortic rings. Results demonstrated that the response to the NE (Fig. 2A) or to NO-donor SNP (Fig. 2B) showed no difference between MTX and Vehicle rats.

#### MTX did not improve the endothelial function in AIA while changing some endothelial pathways

To determine whether MTX modified the endothelial function, the concentration-response curves to Ach, an endothelium-dependent relaxant agonist, were compared between MTX and vehicle-treated AIA rats. As shown in Fig. 2C, concentration-effects curves of Ach were not significantly different between MTX- and vehicle-treated rats.

As MTX was previously found to improve (27) or deteriorate (27) endothelial relaxing pathways, we tested the hypothesis that MTX induced contradictory effects on endothelial biology. Actually, despite its lack of effect on the endothelial function, certain endothelial pathways were modified by MTX but not all. Incubation of rings with L-NAME significantly blunted Ach-associated relaxation in both vehicle (Fig. 3A) and MTX group (Fig. 3B), but the effect of L-NAME was greater in MTX compared to the vehicle (% reduction of  $E_{max}$ :  $89 \pm 2$  vs.  $75 \pm 3$  in vehicle,  $p = 0.001$ ) thus indicating that NOS activity was enhanced by MTX. As arginase and NOS compete for the same substrate, L-arginine, to determine whether



**Fig. 3.** Effects of methotrexate on NOS/arginase pathways. Experiments were performed on thoracic aortic rings harvested at the end of the treatment period (21 days after the onset of arthritis) from AIA rats with saline (Vehicle) or with methotrexate (MTX, 1 mg/kg/week). Cumulative concentration-response curves of Ach were obtained after incubation or not with L-NAME at  $10^{-4}$  moles/litre (A, B), and nor-NOHA at  $10^{-4}$  moles/litre (C, D). Values from the concentration-response curves are the mean  $\pm$  SEM ( $n = 10$ -15 rings from 15 rats per group). \*\*\* $p < 0.001$ , NS: not significant.

increased NOS activity was secondary to decreased arginase activity, rings were incubated with the arginase inhibitor nor-NOHA. As shown in Fig. 3C and D, the effect of nor-NOHA was not different between the vehicle- and MTX-treated rats, showing that MTX did not change the arginase activity. In Vehicle AIA, as a reflection of increased  $O_2^{\circ}$  production, Tempol significantly improved Ach-induced vasorelaxation (Fig. 4A). The treatment of AIA with MTX abolished the impact of Tempol on Ach-induced relaxation (Fig. 4B). As regards COX-2 activity, in agreement with the excessive deleterious activation of COX-2 in vessels from AIA rats (20), the selective COX-2 inhibitor NS-398 significantly enhanced Ach-induced relaxation in aortas from vehicle-treated animals (Fig. 4C). In MTX-treated rats, NS-398 still enhanced the Ach-induced vasorelaxation showing that the COX-2 activity had not been reduced by the treatment (Fig. 4D). A deficit in EDHF production was present in Vehicle AIA since apamin and charybdotoxin did not reduce Ach-induced vasodilation (Fig.

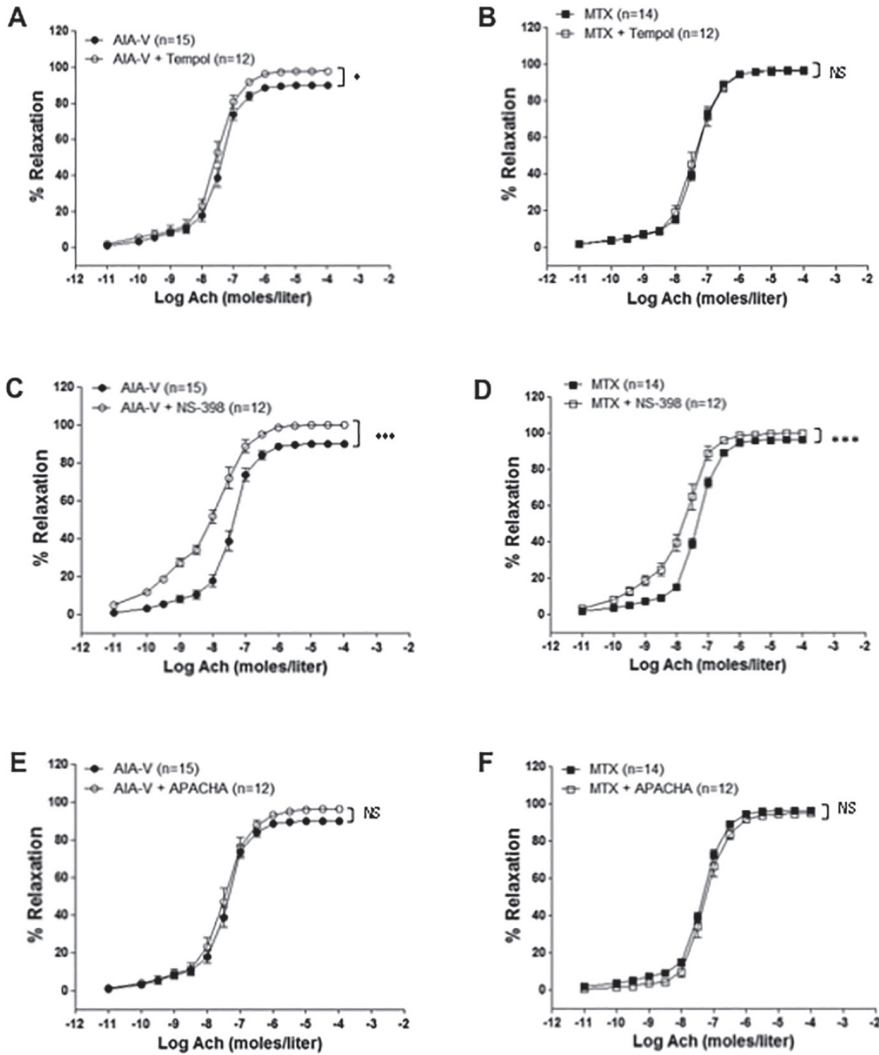
4E). MTX did not improve the blunted EDHF production as it did not change the effect of apamin and charybdotoxin (Fig. 4F). In summary, MTX increased the NOS activity and decreased the  $O_2^{\circ}$  production but had no effect on the vascular EDHF production, COX-2 and arginase activity.

To discard the possibility that the dose of MTX used in the present study (1 mg/kg/week) was sub-optimal for inducing an effect on the endothelial function, the Ach-induced relaxation was further investigated in an additional group of AIA rats ( $n = 15$ ) treated with MTX at 2 mg/kg/week (s.c.). As shown in Figure 5, this higher dose significantly reduced the arthritis score (Fig. 5A), but did not change the relaxation induced by Ach as compared to the vehicle-treated rats (Fig. 5B).

Of note, no correlation was found between arthritis score and  $E_{max}$  of Ach ( $r = 0.308$ ,  $p = 0.068$ ) (all AIA rats).

#### Discussion

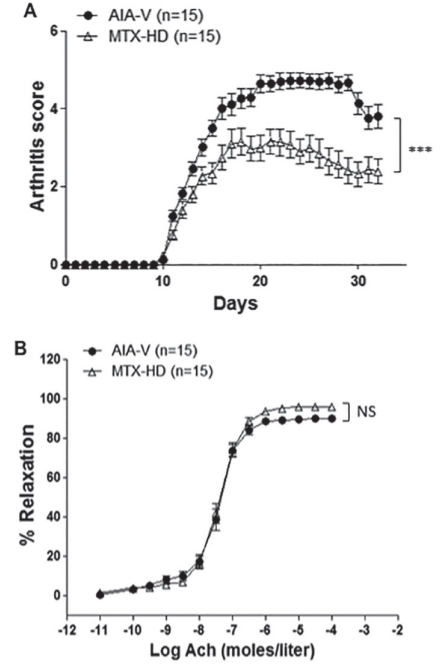
Although the endothelial dysfunction is acknowledged as the early vascular



**Fig. 4.** Effects of methotrexate on COX-2 pathway, superoxide anions and EDHF productions. Experiments were performed on thoracic aortic rings harvested at the end of the treatment period (21 days after the onset of arthritis) from AIA rats with saline (Vehicle) or with methotrexate (MTX, 1 mg/kg/week). Cumulative concentration-response curves of Ach were obtained after incubation or not with Tempol at  $10^{-4}$  moles/litre (A, B), NS-398 at  $10^{-5}$  moles/litre (C, D), and apamin and charybdotoxin at  $10^{-7}$  moles/litre each (E, F). Values from the concentration-response curves are the mean  $\pm$  SEM (n=12-15 rings from 15 rats per group). \* $p$ <0.05, \*\*\* $p$ <0.001, NS: not significant.

event leading to atherogenesis and in turn increased risk of CV morbidity and mortality in RA patients (8, 29), only a few studies were designed to identify the therapeutic options able to reduce this vascular impairment. MTX, the cornerstone of RA treatment, was suggested to reduce CV risk in RA but whether its potential benefits rely on positive effects on the vascular function is currently unknown. Because clinical studies led to inconsistent results, the use of animal models of arthritis is a benchmark choice to address this question as they offer the opportunity to avoid the numerous confounding factors present in clinical studies. By

using the AIA model, the present study revealed that a curative treatment with MTX failed to improve the endothelial function in AIA. Of note, MTX did not *deteriorate* the endothelial function *in vivo*, whereas MTX used at concentrations corresponding to serum concentrations in RA was previously reported *in vitro* to decrease the endothelial cell proliferation and viability (28) and to promote their apoptosis (30). Moreover MTX administered to rats with no pathological condition at 0.35 mg/kg/day for 2 weeks was able to induce ED (31). The study of the effect of MTX on the major endothelial pathways revealed that MTX induced some chang-



**Fig. 5.** Effect of high dose of methotrexate on arthritis evolution and vascular reactivity to acetylcholine. Arthritis scores were plotted over time after AIA induction in rats treated with methotrexate high-dose (MTX-HD, 2 mg/kg/week) and compared to saline-treated rats (AIA-V) (A). At the end of the treatment, concentration-response curve of Ach were obtained on endothelium-intact aortic rings precontracted with PE  $10^{-6}$  moles/litre (B). Values are the mean  $\pm$  SEM (n=15 aortic rings from 15 rats per group). \*\*\* $p$ <0.001, NS: not significant.

es in impaired vascular pathways, but only partly: it decreased  $O_2^{\circ}$  production and enhanced NOS activity but did not normalise neither COX-2 or arginase activities nor EDHF production. From a therapeutic perspective, our data suggest that a combination of actions on endothelial pathways is required to improve ED in arthritis, as previously suggested in studies in the AIA model reporting that the beneficial effects of etanercept (21) or high dose of prednisolone (32) on ED were underpinned by combined effects on NOS, arginase, COX-2 and  $O_2^{\circ}$  pathways. Conversely, a low dose of prednisolone that increased NOS activity and downregulated  $O_2^{\circ}$  pathways without changing arginase and COX-2 activities failed to improve the endothelial function (32), as observed in the present study with MTX. From a pathophysiological perspective overall data highlight a seminal role of the COX-2/arginase pathways in RA-associated ED.

Even though the reasons for premature CVD in RA are not fully clarified, inflammation is suspected to play a seminal role (33, 34). Consistent with this, cytokines such as IL-6 are strongly correlated with endothelium activation markers and reduction of its concentration attenuates atherogenesis in active RA (35, 36). Thus, one possible explanation for the lack of effect of MTX on ED was that the reduction of inflammation was insufficient. Our data revealed that it was not the case as MTX significantly reduced arthritis severity as well circulating pro-inflammatory cytokines. These results are consistent with the previously reported lack of association between the cumulative inflammation and the endothelial function in RA patients (37–39). Likewise, a recent meta-analysis indicated that despite a decrease in biological inflammation, anti-TNF- $\alpha$  therapy induced no changes in ED (40). More recently, in line with our results, a 6-month-treatment with MTX in patients with inflammatory rheumatic diseases was found to decrease disease activity, CRP and ESR, but to have no effect on pentraxin 3, a systemic marker of the endothelial dysfunction (41). These data also resonate with those of Hansel *et al.* (42) showing that young RA patients with a low disease activity, treated by MTX, suffered from ED. Actually overall results do not argue against a contribution of inflammation to ED but rather highlight the complexity of the relationship between these two parameters and argue against the use of “conventional” circulating markers of inflammation to predict the endothelial function in RA. Besides inflammation, traditional CV risk factors also contribute, albeit partly, to ED in RA (43). Among them, hyperhomocysteinaemia (HyperHCy), an independent CV risk factor, has been associated with atherosclerotic vascular diseases and ischaemic heart attacks due to HCy-induced ED (44). As hyperHCy is a possible side effect of MTX due to its mode of action, we tested the hypothesis that MTX-induced HyperHCy may explain the lack of effect of MTX on ED. The results showed that MTX did not change HCy levels. As regards other traditional CV risk factors

that are known to contribute to the excess of CV risk in RA (45), our data revealed that MTX changed neither blood pressure, glycaemia nor cholesterol and triglycerides levels, thus excluding these parameters as culprits for the lack of effect of MTX on ED.

An interesting result of our study is that MTX decreased clinical arthritis but had no effect on joint damage as attested by the lack of difference of radiological score between the vehicle- and MTX-treated AIA groups. The most likely hypothesis is the short duration of MTX treatment in our conditions (3 weeks). Given that a burgeoning literature suggests a link between bone metabolism alterations and ED in RA (46–48), and as BMP-4 was reported to induce vascular inflammation, ED and atherosclerosis (49), we explored the effect of MTX on circulating BMP-4 levels. Our results revealed that MTX significantly enhanced serum BMP-4 levels in AIA. Even though BMP-4 has never been measured in the serum of RA patients, this result is in line with the finding that MTX, contrary to the other DMARDs, increased BMP gene expression in peripheral blood mononuclear cells in RA patients (50). Thus, further studies are now needed to determine whether this impairment might explain the lack of effect of MTX on ED.

In conclusion, the present study demonstrated that a treatment with MTX did not change endothelial function in arthritis. Importantly, and contrary to data obtained in “non inflammatory” conditions (28, 31), MTX did not worsen the endothelial function. Our data do not undermine the benefits of MTX on CVR in RA but indicate that mechanisms other than the reduction in ED are likely involved, such as the effects on the lipoprotein function (51) or the decreased prothrombotic propensity (52). From a therapeutic perspective, they suggest that MTX-treated patients might benefit from add-on endothelium-protective therapies to help normalising the CV risk of RA patients against the general population.

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