Study Title

Methotrexate, blood pressure and arterial function in patients with rheumatoid arthritis

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Study protocol

Four groups of patients will be studied:

1. Newly diagnosed RA patients started on subcutaneous MTX and studied at baseline, 1 and 6 months (n=31). Patients will be initially prescribed 10 mg subcutaneous MTX once a week. Then, the dose will be adjusted based on the response by 5 mg increments up to 25 mg a week. If required, use of steroids, NSAIDs, and/or other DMARDs is allowed during the study period.
2. Newly diagnosed RA patients started on another DMARD (sulfasalazine) and studied at baseline, 1 and 6 months (n=31). Patients will be initially prescribed 500 mg sulfasalazine daily. Then, the daily dose will be increased by 500 mg each week to a maximum of 2 g daily after 4 weeks. According to clinical response, the maintenance dose will be 2-3 g daily. If required, use of steroids, NSAIDs, and/or other DMARDs (except MTX), is allowed during the study period.
3. RA patients on chronic treatment (>1 year) with oral MTX (with or without issues with tolerability, e.g. gastrointestinal side effects) at baseline and switched to subcutaneous MTX at the same dose (studied at baseline, 1 and 6 months, n=31).
4. RA patients on chronic treatment (>1 year) with other synthetic DMARDs at baseline and continued on the same DMARDs, and their dose, at 1 and 6 months (studied at baseline, 1 and 6 months, n=31).

Groups 1 and 2: randomization to subcutaneous MTX vs. sulfasalazine will be open to both patients and rheumatologists, facilitating monitoring of response and potential side effects. However, they will be asked not to disclose their treatment to the research team.

Groups 3 and 4: this is an open observational study where group 3 patients switch their oral MTX to subcutaneous MTX, and group 4 patients continue their treatment with the same DMARDs throughout the study duration.

Study population and eligibility criteria: Adult RA patients, diagnosed according to established criteria (2010 ACR/EULAR), will be recruited from rheumatology clinics at Flinders Medical Centre (Southern Adelaide Local Health Network). Demographic and clinical characteristics and genetic polymorphisms will be assessed at baseline in newly diagnosed RA patients commencing either subcutaneous MTX (group 1) or sulfasalazine (group 2), and in RA patients already on long-term (>1 year) treatment with either oral MTX (group 3) or other synthetic DMARDs (group 4). Group 1 and group 2 (newly diagnosed RA patients) will be randomized to either subcutaneous MTX (group 1) or sulfasalazine (group 2, age- and sex-matched) in a 1:1 ratio.

Inclusion criteria

- Patient with rheumatoid arthritis according to EULAR/ACR 2010 criteria.
- Age ≥18 years.
- Written informed consent, dated and signed before initiating any study-related procedure.

Exclusion criteria

- Contraindication to MTX or sulfasalazine.
- Patient who cannot be followed during 6 months.

- Active alcohol or substance abuse within the last 12 months.
- Participation in a clinical trial within 3 months prior to the start of the study.
- Body mass index >35 Kg/m^2.
- Secondary causes of hypertension.
- Grade 2 (moderate) or 3 (severe) hypertension: clinic blood pressure >160/100 mm Hg.
- Resistant hypertension: clinical blood pressure ≥140/90 mm Hg despite concurrent use of three antihypertensive agents of different classes, one of which is a diuretic.
- Clinical systolic blood pressure <100 mm Hg or history of symptomatic orthostatic hypotension.
- Cardiovascular event, procedure, or hospitalization for unstable angina with the last 6 months.
- Atrial fibrillation.
- Heart failure.
- Treatment with nitrates.
- Estimated glomerular filtration rate (eGFR) <45 mL/min.
- Diagnosis of polycystic kidney disease.
- Glomerulonephritis treated with or likely to be treated with immunosuppressant drugs
- Uncontrolled diabetes with HbA1c >9.0% (>75 mmol/mol).
- Uncontrolled dyslipidaemia with total serum cholesterol >7.5 mmol/L or triglycerides >5.6 mmol/L.
- Clinical diagnosis of dementia, treatment with medications for dementia or, in the opinion of the study staff, the participant is cognitively unable to follow the protocol.
- Other medical, psychiatric, or behavioural factors that in the judgment of the study staff may interfere with study participation.
- Cancer diagnosed and treated within the past 2 years that, in the judgment of the study staff, would compromise a participant's ability to comply with the protocol and complete the study.
- Any organ transplant.
- Pregnancy, currently trying to become pregnant, or of child bearing potential and not using birth control.
- Significant illness within 2 weeks of study start.
- Patients with an unstable active medical condition that could impair evaluation of study results.

Withdrawal criteria

- Treatment-related toxicity.
- Voluntary withdrawal from study by participant.
- Any other intercurrent medical condition or circumstance that precludes completion of the study.

Clinical, demographic and laboratory variables: Data collected during each study visit will include medical history, age, gender, weight, height, RA duration, DAS28, Stanford Health Assessment Questionnaire, current medications and lifestyle assessment (diet and exercise). Type and dose of medications and the following laboratory data will be collected during each assessment visit (baseline, 1 and 6 months): renal function, erythrocyte sedimentation rate, and C-reactive protein. Adverse events and adverse drug reactions will also be recorded.

Blood pressure and arterial function: BP, AIx (arterial wave reflection) and PWV (arterial stiffness) will be assessed at baseline, 1 and 6 months. Clinic BP will be measured in the

morning (between 9.00am and 11.00am) in a quiet, appropriate environment at room temperature, according to current guidelines, using an automated BP monitor (UA-767PC, A&D Medical) clinically validated against the British Hypertension Society protocols. Prior to BP measurement, patients will abstain from alcohol for ≥12-hrs, and from tobacco and caffeine for ≥4-hrs. Patients will also be required to fast for ≥6-hrs. The arm used for BP measurement will be free of constricting clothing to avoid impediment of the cuff. The cuff will be wrapped snugly around the upper arm with the centre of the cuff bladder positioned over the brachial artery and the lower border of the cuff approximately 2 cm above the elbow bend. The cuff will be placed at heart level by supporting the arm. Patients will sit (with legs not crossed) and relax for at least 5 min, and then three BP measurements will be taken at 2-min intervals. The average of the last two BP measurements will be calculated and used in the analyses.

Central and 24-hr BP will be assessed using PWA (SphygmoCor, AtCor Medical, West Ryde, NSW) and Mobil-O-Graph technology (I.E.M., Stolberg, Germany) available within our group. Prior to PWA, patients will abstain from alcohol for ≥12-hrs, and from tobacco and caffeine for ≥4-hrs. Patients will be required to fast for ≥6-hrs. A small probe will be placed on the radial artery, and AIx and central BP measurements will take approximately 10 min. The Mobil-O-Graph will provide a 24-h assessment of peripheral and central BP, and PWV. \

MTX-polyglutamates PGs (MTX-PGs) and 5-MTHF: RBCs MTX-PGs concentrations will be measured at each assessment visit with a validated LC-MS/MS assay developed at the School of Pharmacy and Medical Sciences, University of South Australia. Whole blood samples will be centrifuged, plasma removed and cells are washed two times with PBS. Packed cells are then stored at -80 °C prior to analysis. Stable isotope internal standard is added to 0.25mL packed RBC and proteins are precipitated with perchloric acid. MTX-PGs are purified by solid phase extraction (Strata-X Strong Cation Exchange) prior to LC separation via HILIC LC (ZIC-HILIC Column, Merck Millipore) and analysis on a Shimazdu 8060 triple quadrupole MS/MS. This assay quantifies the five MTX-PGs commonly seen in RA patients and has a limit of detection of <0.2 nmol/L packed RBCs. Whole blood and plasma 5-MHTF will be measured at each assessment visit according to a method previously published. 50µL of whole blood or plasma is diluted with 50µL of 5% ascorbic acid solution containing 10mM mercaptoethanol and 10µL of internal standard (stable isotope labelled 5-MHTF) and incubated at 37 °C for 60 min. 60µL of 10% trichloroacetic acid is added, the mixture vortexed and the clear supernatant removed. 5-MTHF is separated via reverse phased LC using a Waters Acquity BEH C18 column (100X2.1mm), and detected with a Shimadzu 8060 Triple Quadrupole MS/MS.

Genetic polymorphisms: Pharmacogenetic variability will focus on single-nucleotide polymorphisms (SNPs) shown to be important in the effect of MTX in RA, or demonstrating a theoretical association with the cardiovascular effects of MTX: ABCB1 3435C>T (rs1045642), ABCC2 1249G>A (rs2272397), ABCG2 421C>A (rs2231142), AMPD1 34C>T (rs17602729), ATIC 347C>G (rs2372536), MTHFR 677C>T (rs1801133), MTHFR 1298 A>C (rs1801131), MTR 2756 A>G (rs1805087), MTRR 66A>G (rs1801394), SHMT 1420C>T (rs1979277), SLC19A1 80G>A (rs1051266), GGH 354G>T (rs719235) and GGH 16T>C (rs1800909). DNA will be extracted from whole blood samples and SNPs will be determined via validated Taqman™ SNP genotyping assays, performed according to manufacturer instructions. In addition, the number of 28 base-pair repeats in the promoter region of thymidylate synthase will be determined by PCR.

Adenosine: Plasma adenosine concentrations will be measured using our Waters Aquity UPLC coupled with quadrupole-time-of-flight (qToF) mass spectrometer in MS-MS mode (CIA and AI Elliot), according to a method previously reported. Samples that are chilled and plasma rapidly removed from RBCs following centrifugation are stable when stored at -80 °C, through 3 freeze thaw cycles, and stored at room temperature up to 24-hr. Consequently, a protocol for rapid separation of plasma from whole blood will be adopted. To achieve the required selectivity adenosine will be extracted from plasma through protein precipitation with trichloroacetic acid followed by solid phase extraction on a Varian SPEC SCX cartridge. Furthermore, chromatographic separation on a UPLC column (Gemini NX C18, 100 x 2 mm, 3 μm) prior to MS-MS analysis will enhance assay selectivity. Isolation of adenosine from the biological matrix by SPE and UPLC removes interferences that reduce signal through ion suppression and minimises the non-specific background noise. This results in a high sensitivity assay with the ability to discriminate small, yet potentially meaningful changes in adenosine concentrations.

Asymmetric dimethylarginine (ADMA): eNOS activity will be indirectly assessed by measuring plasma ADMA, an endogenous eNOS inhibitor, at baseline, 1 and 6 months. ADMA will be measured using a validated LC-MS method on our Waters Aquity UPLC and qToF Premier mass spectrometer. Stable isotope internal standard is added to 50 μL of patient plasma followed by protein precipitation with acidified solvent. After centrifugation the supernatant layer is decanted and evaporated to dryness under vacuum. The residue is redissolved in mobile phase then analysed by reverse phase chromatography as previously published by CIA.

Statistical methods

Statistical analysis will be performed using linear mixed models. In addition to providing estimated treatment effects, this analysis will also provide estimates of the individual variability in the longitudinal changes in BP and arterial function in each of the 4 groups over time. Due to the partly observational nature of the study (group 4), models will also adjust for known potential confounders, including age, gender, co-morbidities, RA duration, 5-MTHF, drugs potentially affecting BP and their doses, DAS28, biochemical parameters and inflammatory markers. Models with and without adjustment for BP will also be assessed to test the direct and indirect effects of MTX on markers of arterial function. Similarly, we will also use mediation analysis to assess the potential direct and indirect effects of MTX-PGs, adenosine concentrations and genetic polymorphisms on the associations between MTX, BP and markers of arterial function. We will perform these analyses using Mplus software (StatModel version 7.4), which provides appropriate bootstrapping routines for estimating the non-parametric 95% CIs of indirect effects.