We investigated the effect of single-nucleotide polymorphisms (SNPs) spanning 10 methotrexate (MTX) pathway genes, namely AMPD1, ATIC, DHFR, FPGS, GGH, ITPA, MTHFD1, SHMT1, SLC19A1 (RFC) and TYMS on the outcome of MTX treatment in a UK rheumatoid arthritis (RA) patient cohort. Tagging SNPs were selected and genotyping was performed in 309 patients with predefined outcomes to MTX treatment. Of the 129 SNPs tested, 11 associations were detected with efficacy (P-trend ≤0.05) including four SNPs in the ATIC gene (rs12995526, rs3821353, rs7563206 and rs16853834), six SNPs in the SLC19A1 gene region (rs11702425, rs2838956, rs7499, rs2274808, rs9977268 and rs7279445) and a single SNP within the GGH gene (rs12681874). Five SNPs were significantly associated with adverse events; three in the DHFR gene (rs12517451, rs10072026, and rs1643657) and two of borderline significance in the FPGS gene. The results suggest that genetic variations in several key MTX pathway genes may influence response to MTX in the RA patients. Further studies will be required to validate these findings and if confirmed these results could contribute towards a better understanding of and ability to predict MTX response in RA.

The Pharmacogenomics Journal (2013) 13, 227–234; doi:10.1038/tpj.2012.7; published online 27 March 2012

Keywords: ATIC; methotrexate; pharmacogenetics; polymorphism; RFC; rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic disabling disease, requiring long-term treatment. Drug therapy is a key component of the treatment pathway and disease-modifying anti-rheumatic drugs (DMARDs) provide the mainstay of therapy, with mounting evidence suggesting that earlier treatment with DMARDs offers benefits in the longer term.1,2 There are several DMARDs available, but in clinical practice methotrexate (MTX) is increasingly recognised as the anchor drug for the treatment of RA.3–6 This is because of substantial clinical experience, established efficacy, superior continuation rates, affordability and the fact that treatment with MTX not only reduces disease activity in the short term, but can also delay or stabilise the development of bone erosions in some patients over the longer term.7,8 Nevertheless, there is still significant variability in patient responses to treatment, with an estimated one-third of patients failing to respond to MTX either due to lack of efficacy or adverse events (AEs).9–13 As a result of this inter-patient variability in response and the fact that no predictive tests are available, routine blood and liver function testing are required in clinical practice that can be costly and inconvenient for the
patients. For these reasons, MTX represents an interesting target for pharmacogenetic testing, to identify response predictors that could maximise response and minimise toxicity.

Treatment response is a complex multi-factorial trait with various contributing factors, including individual patient factors (age, sex, ethnicity, co-morbidities), disease specific factors (disease duration, severity, activity) and genetic factors. A complex interplay of several genes encoding proteins involved in drug uptake and disposal, absorption, retention, distribution, metabolism and interaction with cellular targets can influence drug actions and thus these genes represent logical targets for pharmacogenetic testing. The actual mechanism of action of low-dose MTX, used in the treatment of RA, is still not fully understood, but it is thought that the anti-inflammatory effects, mediated by adenosine release, are more important than the anti-proliferative effects. In order to enter the cell, MTX is internalised by the reduced folate carrier (SLC19A1/RFC), with impaired transport correlated with MTX resistance. Once internalised, MTX requires intracellular polyglutamation, controlled by the polyglutamation-deconjugation cycle that is instigated by the enzymes γ-folypolyglutamate synthetase (FPGS) and glutamyl hydrolase (GGH), respectively. It is these active MTX-polyglutamates that determine MTX functional status and have an important role in directly suppressing various enzymes, such as dihydrofolate reductase (DHFR), thymidylate synthase (TYMS) and 5-aminomimidazole 4-carboximidine ribonucleotide (AICAR) transformylase (ATIC), and have an indirect effect on methylenetetrahydrofolate reductase.

The association of polymorphisms in these genes with MTX response have been described by various groups, although many of these studies have tested isolated polymorphisms within a few genes relevant to MTX metabolism. Our aim was to examine several important MTX pathway genes to determine whether single-nucleotide polymorphism (SNP) markers, selected to comprehensively cover the genes, were associated with MTX treatment response outcomes in a well-characterised group of patients with established RA.

Patients and methods

Study subjects and outcomes

Details of the patients included in this study and the methods by which they were recruited are outlined elsewhere. In brief, all subjects were considered eligible for inclusion if they had taken MTX monotherapy for RA for at least 3 months, aged over 18 years, were of white Caucasian ethnic origin and were classified as having RA according to the ACR 1987 criteria. The patient cohort was recruited retrospectively from two hospitals: The University Hospital of North Staffordshire (UHNS) and Central Manchester NHS Foundation Trust (CMFT). Patients were identified either via an electronic database (UHNS) or case note review (CMFT) (Table 1). Eligible patients had to fulfil one of three defined outcomes to MTX: (i) good responder (physician statement of good response plus a stable dose of MTX for at least 6 months, with an ESR <20 and/or normal CRP); (ii) inefficacy failure (physician statement of inefficacy plus failure to reduce ESR and/or CRP by at least 20% with MTX therapy for at least 3 months at a minimum dose of 15 mg per week) or (iii) AE failure (AEs had to be persistent or serious and lead to treatment cessation: verified by medical record review. Furthermore, the AEs had to resolve on treatment cessation and, in the case of GI AEs, recur after MTX re-challenge). Individuals that did not meet one of these defined outcomes were not included in the study.

Ethical approval for the study was obtained from the North Staffordshire LREC (Ref 03/20) and the Central Manchester

| Number patients (%) | Responders | IE failure | AE failure |
|---------------------|------------|------------|------------|
| Age at diagnosis in years median (range) | 147 (48) | 101 (33) | 61 (19) |
| Gender: female (%) | 103 (70) | 77 (76.2) | 50 (82) |
| Age at MTX start in years median (range) | 57.4 (49.6–64.6) | 52.8 (46.3–59.3) | 52.2 (46.5–60.7) |
| RF +ve status (%) | 99 (67.4) | 76 (75.3) | 45 (73) |
| Erosions (%) | 115 (78) | 93 (92) | 52 (87) |

Abbreviations: AE, adverse event; DMARD, disease modifying anti-rheumatic drug; IE, inefficacy; MTX, methotrexate; RF, rheumatoid factor; SE, shared epitope.

Table 1 Patients demographics and baseline characteristics

| Copies SE (%) | Responders | IE failure | AE failure |
|---------------|------------|------------|------------|
| 0             | 27 (19) | 15 (15) | 13 (22) |
| 1             | 66 (47) | 40 (41) | 24 (41) |
| 2             | 48 (34) | 43 (44) | 22 (37) |

| No previous DMARDs median (range) | Responders | IE failure | AE failure |
|----------------------------------|------------|------------|------------|
| MTX first DMARD (%) | 2 (0–3) | 2 (1–3) | 2 (2–4) |
|                      | 40 (27.2) | 21 (18.1) | 7 (11.5) |

Abbreviations: AE, adverse event; DMARD, disease modifying anti-rheumatic drug; IE, inefficacy; MTX, methotrexate; RF, rheumatoid factor; SE, shared epitope.

\[ n = 297 \text{ with information.}\]

\[ n = 301 \text{ with information.}\]
LREC (Ref 03/CM/315), and written consent was obtained from subjects according to the Declaration of Helsinki.

Selection of SNPs and genotyping
Ten candidate genes were selected for study on the basis of putative involvement in the MTX metabolic pathway and previous evidence from the literature. They included genes involved in MTX cellular influx (SLC19A/RFC), polyglutamation (GGH, FPGS), folate pathway (DHFR, SHMT1, MTHFD1), purine synthesis (ATIC), pyrimidine synthesis (TYMS), adenosine pathway (AMPD1, ATIC) and ITPA (Supplementary Figure 1).

For each gene SNPs were selected based on a pair-wise tagging SNP approach, supplemented with other commonly investigated SNPs from the literature. Marker coverage of each gene was extended to include the 10-kb upstream and downstream flanking region. Tag SNPs for each gene were selected from the CEPH/CEU Hapmap dataset (release 22) (http://www.hapmap.org) and this downloaded SNP data was then filtered through the Haploview software (http://www.broad.mit.edu/haploview/) and pair-wise tagging SNPs ($r^2$ cut off >0.8 and MAF >5%) were selected for genotyping. In addition to the tag SNPs identified, we also included additional SNPs in each gene in case of SNP failure, seven duplicate SNPs for quality control purposes and one 28-base pair variable-number tandem repeat located in the 5'UTR of the TYMS gene.

Genotyping
SNP genotyping was performed using the Sequenom iPLEX MASS ARRAY platform according to the manufacturers' instructions (Sequenom, San Diego, CA, USA, http://www.sequenom.com). Genotyping for the variable-number tandem repeat was performed in a 5-m reaction volume, using primer sequences as described by Zhang et al. Amplicons were electrophoresed through a 3% agarose gel and visualised with ethidium bromide staining.

Quality control procedures before analysis were used such that 80% sample and polymorphism genotyping success rate was required, and any samples and polymorphisms failing to meet this threshold were removed from further analysis.

Statistical analysis
Each polymorphism was tested for association with MTX efficacy and AEs. Genotype and allele frequencies were compared between the groups and analysed as a nested case–control study with the responders as the referent. Genotype and allele frequencies were estimated from the samples, and compared between the groups and analysed as a nested case–control study with the responders as the referent.

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Table 1 shows the clinical characteristics of the subjects in the two study groups. The median age at MTX onset varied between 54.2 years in the efficacy group and 54.1 years in the inefficacy group. There were no significant differences in sex, disease duration, disease activity or current or previous use of MTX between the two groups.

Statistical analysis
Each polymorphism was tested for association with MTX efficacy and AEs. Genotype and allele frequencies were compared between the groups and analysed as a nested case–control study with the responders as the referent. Genotype and allele frequencies were estimated from the samples, and compared between the groups and analysed as a nested case–control study with the responders as the referent.

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determine whether the associations were independent of each other. The significantly associated markers were conditioned against the effect of the most significant marker in the gene, but there was no evidence of independence demonstrated in this sample (data not shown). Similarly, haplotype analysis did not reveal haplotypic effects (data not shown).

Association with MTX toxicity

With regard to MTX-related toxicity, two SNPs (rs10072026 and rs1643657) within the \(DHFR\) gene region were associated with a reduced risk of MTX-related AEs (OR 0.43, 95% CI 0.19–0.99; OR 0.60, 95% CI 0.39–0.99) and another (rs12517451) showed evidence of association with an increased risk of AEs (OR 1.68, 95% CI 1.03–2.75). In addition, two SNPs in high LD (\(r^2 = 0.96\)) within the \(FPGS\) gene showed borderline evidence of association with an increased risk of AEs (Table 3). Both SNPs in the \(FPGS\) gene (rs1054774 and rs44511422) were highly significant under a recessive model of inheritance (OR 3.03, 95% CI 1.14–7.99 and OR 3.60, 95% CI 1.39–9.33, respectively) (Table 3).

Replication of previous pharmacogenetic results

Twelve of the 145 SNPs were included in order to validate previously reported associations from the literature (Table 4). Of these, one SNP in the \(DHFR\) gene (rs1650697) for which a proxy SNP (rs12517451) (\(r^2 = 1.0\)) was genotyped provided evidence of association with AEs (OR 1.68, 95% CI 1.03–2.75) and two SNPs in the \(FPGS\) gene (rs1544105 and rs10106) for which proxies were genotyped showed borderline evidence of association (trend \(P = 0.06\)) with an increased risk of AEs, with a further increased risk with carriage of two copies of the minor allele under a recessive model (Table 4). No significant associations were revealed with any of the other previously reported SNPs (Table 4).

Discussion

The ability to individually tailor MTX treatment to meet individual patient’s needs remains an important goal and would be valuable if applied in clinical practice. Our study has identified 16 SNPs, some novel and others replicating previous findings, in five key MTX metabolic pathway genes, which show evidence for association with MTX efficacy or AEs in this cohort of RA patients. No significant associations or replications of previous associations of SNPs within the genes: \(SHMT1\), \(MTHFD1\), \(AMPD1\), \(ITPA\), and \(TYMS\) with MTX efficacy or AEs were found.

There have been a number of studies conducted to determine the MTX response in RA patients, with the majority adopting a candidate gene approach, genotyping isolated SNPs within the gene and testing for association. Our study also focused on candidate genes in the MTX metabolic pathway, partly because this approach has proven successful for a number of other common treatments. One of the best examples is the identification of polymorphisms within the genes: \(G6PD\), \(MTHFR\), \(AMPD\), \(ITPA\), and \(TYMS\) as important in determining the response to MTX treatment.

Abbreviations: CI, confidence interval; IE, inefficacy failure to MTX; MAF, minor allele frequency; R, responder; SNP, single-nucleotide polymorphism; 1_1, major allele homozygote; 1_2, heterozygote; 2_2, minor allele homozygote.

Table 2 SNPs significantly associated with MTX efficacy (\(P_{\text{trend}} \leq 0.05\))

| Gene   | SNP             | Base pairs | SNP position | MAF (%) | Genotype frequencies (%) | Trend P | Allelic OR (95% CI) |
|--------|-----------------|------------|--------------|---------|--------------------------|---------|-------------------|
|        |                 |            |              |         | R 1_1 1_2 2_2 IE 1_1 1_2 2_2 |         |                   |
|        |                 |            |              |         |                          |         |                   |
| ATIC   | rs7563206       | C/T        | Intron       | 42.4    | 45 (32.4) 70 (50.4) 24 (17.3) 20 (17%) | 0.01    | 1.60 (1.10–2.33)  |
|        | rs3821353       | G/T        | Intron       | 24.4    | 83 (59.7) 44 (31.7) 12 (8.6) 67 (71.3) 27 (28.7) 0 (0.0) | 0.009   | 0.51 (0.31–0.84)  |
|        | rs12995526      | C/T        | Exonic 5'UTR | 42.4    | 45 (34.1) 62 (47.0) 25 (18.9) 20 (22.0) 42 (46.2) 29 (31.9) | 0.01    | 1.65 (1.13–2.42)  |
|        | rs1863834       | C/T        | Exonic 5'UTR | 13.9    | 96 (73.8) 32 (24.6) 2 (1.5) 68 (72.8) 27 (31.4) 5 (5.8) | 0.04    | 1.70 (1.02–2.82)  |
| GGH    | rs2681874       | C/T        | Intron       | 16.9    | 104 (78.2) 25 (18.8) 4 (3.0) 77 (86.5) 9 (10.1) 3 (3.4) | 0.04    | 0.54 (0.30–1.00)  |
| SLC19A1 | rs1702425b     | T/C        | Exon         | 26.9    | 75 (53.2) 56 (39.7) 10 (7.1) 34 (35.1) 45 (47.5) 16 (16.5) | 0.001   | 1.86 (1.26–2.74)  |
|        | rs2838956       | A/G        | Exonic 5'UTR | 38.3    | 52 (36.9) 70 (49.6) 19 (13.5) 24 (24.7) 54 (55.7) 19 (19.6) | 0.04    | 1.45 (1.00–2.10)  |
|        | rs274808b       | C/T        | Intron       | 21.8    | 88 (62.9) 43 (30.7) 9 (6.4) 45 (46.4) 40 (41.2) 12 (12.4) | 0.009   | 1.76 (1.17–2.67)  |
|        | rs9977268b      | C/T        | Intron       | 18.9    | 95 (67.9) 37 (26.4) 8 (5.7) 52 (54.2) 34 (35.4) 10 (10.4) | 0.02    | 1.67 (1.08–2.58)  |
|        | rs279445b       | C/T        | Intron       | 45.0    | 43 (30.5) 69 (48.9) 29 (20.6) 18 (18.8) 52 (54.2) 26 (27.1) | 0.05    | 1.44 (0.99–2.08)  |

Abbreviations: CI, confidence interval; IE, inefficacy failure to MTX; MAF, minor allele frequency; R, responder; SNP, single-nucleotide polymorphism; 1_1, major allele homozygote; 1_2, heterozygote; 2_2, minor allele homozygote.

aOn the basis of carriage of the minor (rare) allele.
bIn an overlapping gene COL18A1.
in the vitamin K (VKORC1) and cytochrome p450 (CYP2C9) genes, which influence response to warfarin where findings have since been validated in independent studies.27–30 Our study had an additional strength that, rather than testing single SNPs in specific genes, we systematically screened selected MTX pathway genes ensuring gene coverage following quality control measures exceeded 85% when compared with the HapMap data. In this way, we can confidently exclude association with a number of genes in our cohort for effect sizes > 1.5. This was a retrospective study and patients were recruited to the study based on phenotypes defined using the data available in the patients notes. We set out to define phenotypes for both inefficacy and AEs in order to minimise variation and maximise the power to detect significant genetic effects. Furthermore, all of the patients were recruited within a well-defined geographical area and comprised an ethnically homogenous patient population.

Despite these strengths, our study has several limitations: first, incomplete knowledge of the MTX metabolic pathway means that we may have failed to screen some important genes and that although we have found several SNPs to be associated with either efficacy or AEs in this study, it is likely that combinations of risk SNPs will be more predictive of response to MTX than individual SNP effects, as shown in previous studies.31–33 It would be interesting to look at combinations of risk SNPs because, due to the limited sample size, the fact that corrective procedures may unnecessarily reduce power and a proportion of our positive findings do support those from previous studies suggesting that the effects seen may be true. For example, we report borderline association with a SNP (rs1544105) within the FPGS gene with MTX AEs. Interestingly a SNP in high LD with our associated SNP, namely rs1544105, has been associated with response in a previous study.34,35 Also, a SNP mapping to the DHFR gene (rs1650697) and perfectly correlated ($r^2 = 1$) with rs12517451, which we report to be associated with AEs, specifically liver AEs (data not shown), was associated with the occurrence of hepatitis in RA patients in an independent study.36 Finally, a SNP mapping to the ATIC gene and tested in this RA cohort for the first time has been reported to be associated with MTX in a juvenile idiopathic arthritis cohort as described later. In terms of MTX efficacy, several studies have investigated the role of the SLC19A1 (RFC) gene, focusing on the RFC 80A/C non-synonymous SNP (rs1051266), which results in a substitution of arginine to histidine at codon 27 in the first transmembrane domain of the RFC protein.37–41 Our results did not replicate previous findings of association with this particular SNP in

Table 3: SNPs significantly associated with MTX related AE ($P$ trend ≤ 0.05)

| Gene | SNP | Base pairs | SNP position | MAF (%) | Genotype frequencies (%) | Trend P | Allelic OR (95% CI)* |
|------|-----|------------|--------------|---------|--------------------------|---------|---------------------|
|      |     |            |              |         | R: Responders | AE: Adverse events |         |                     |
|      |     |            |              |         | 1,1   | 1,2   | 2,2 | 1,1   | 1,2   | 2,2 |                     |
| DHFR | rs12517451 | C/T | Intron | 20.0 | 29.7 | 91 (65.0) | 42 (30.0) | 7 (5.0) | 32 (54.2) | 19 (32.2) | 8 (13.6) | 0.04 | 1.68 (1.03–2.75) |
|      | rs1643657 | A/G | Intron | 31.0 | 21.2 | 66 (48.2) | 57 (41.6) | 14 (10.2) | 36 (61.0) | 21 (35.6) | 2 (3.4) | 0.04 | 0.60 (0.39–0.99) |
|      | rs10072026 | T/C | Exonic 3’UTR | 12.7 | 5.9 | 107 (75.9) | 32 (22.7) | 2 (1.4) | 53 (89.8) | 5 (8.5) | 1 (1.7) | 0.04 | 0.43 (0.19–0.99) |
| FPGS | rs1054774 | A/C | S’ gene | 39.6 | 49.0 | 48 (35.8) | 67 (50.0) | 19 (14.2) | 16 (30.8) | 21 (40.4) | 15 (28.8) | 0.06 | 1.52 (0.98–2.34) |
|      | rs4451422 | T/A | Exonic 5’UTR | 40.0 | 49.0 | 46 (34.3) | 69 (51.5) | 19 (14.2) | 13 (28.8) | 23 (44.2) | 14 (26.9) | 0.06 | 1.49 (0.97–2.30) |

Abbreviations: AE, adverse events to methotrexate; CI, confidence interval; MAF, minor allele frequency; R, responders; SNP, single-nucleotide polymorphism; 1_1, major allele homozygote; 1_2, heterozygote; 2_2, minor allele homozygote.

On the basis of carriage of the minor (rare) allele.

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### Table 4 Results for SNPs previously found to show evidence of association with MTX response

| Gene (ref) | SNP from literature | Reported association | Position | Proxy SNP typed | MAF R | AE | IE | Trend P | R vs AE | R vs IE | Trend P | R vs AE | R vs IE | Trend P | R vs AE | R vs IE |
|-----------|---------------------|----------------------|----------|----------------|-------|----|----|---------|---------|--------|---------|---------|---------|--------|---------|--------|---------|
| ITPA      | rs1127354           | Efficacy             | 94 C/A   | 9.6 9.3 8.8    | 0.93  | 0.97 | 0.47–2.02 | 0.76  | 0.90  | 0.48–1.71 |
| DMR       | rs1650967           | AE/efficacy          | –473 G/A | 20.0 29.7 23.7 | 0.94  | 1.68 | 1.03–2.75 | 0.35  | 1.24  | 0.80–1.93 |
| DHFR      | rs11545078          | Efficacy             | 452 C/T  | 10.7 11.9 8.6  | 0.74  | 1.12 | 0.57–2.20 | 0.49  | 0.80  | 0.42–1.50 |
| AMPD1     | rs17602729          | Efficacy             | 34 C/T   | 13.2 19.6 12.1 | 0.13  | 1.61 | 0.88–2.94 | 0.72  | 0.91  | 0.51–1.61 |
| MTHFD1    | rs17850560          | AE/efficacy          | 1958 G/A | 46.8 50.8 49.0 | 0.44  | 1.18 | 0.75–1.81 | 0.64  | 1.09  | 0.76–1.56 |
| SLC19A1   | rs1051266           | AE/efficacy          | 80 G/A   | 41.4 42.0 47.8 | 0.70  | 1.02 | 0.66–1.59 | 0.17  | 1.29  | 0.89–1.89 |
| FPGS      | rs1544105           | Efficacy             | 1994 A/G | 39.9 49.0 39.9 | 0.06  | 1.52 | 0.98–2.34 | 0.48  | 0.88  | 0.60–1.29 |
| TYMS      | rs10106             | AE/efficacy          | N/A      | 45.0 46.5 47.9 | 0.80  | 1.06 | 0.69–1.64 | 0.53  | 1.12  | 0.78–1.62 |
| SHMT1     | rs1979277           | AE/efficacy          | 1420 C/T | 31.9 29.7 35.6 | 0.67  | 0.89 | 0.56–1.43 | 0.41  | 1.17  | 0.80–1.73 |

Abbreviations: AE, Adverse event failure; CI, confidence interval; IE, Inefficacy failure; MAF, minor allele frequency; MTX, methotrexate; OR, odds ratio; R, responders; SNP, single-nucleotide polymorphism; VNTR, variable-number tandem repeat.

*On the basis of carriage of the minor allele.* Emboldened SNPs are significant or approaching statistical significance.

**2R2R genotype.**

Emboded SNPs are significant or approaching statistical significance.

### Discussion

In summary, results from this study replicate some previous findings reported in the literature and at the same time we report associations between several MTX pathway genes and either efficacy or AEs in MTX-treated RA patients. In particular, there is growing evidence to support the role of the ATIC gene in the response to MTX treatment. Many of the SNPs reported in the literature have been associated with MTX response in different cohorts, suggesting that this gene may have a role in determining efficacy. The magnitude of effect of these SNPs varies across different studies, with some patients responding to MTX while others do not.

The ATIC gene is highly polymorphic in humans and shows strong LD across the gene encoding adenosine receptors and response to MTX. The ATIC gene contains several SNPs that are associated with MTX response in different cohorts, with a single causal variant suggested to explain the association with this SNP and MTX response. In keeping with the results from some studies, 34,36,39,42 we have found several other SNPs in the ATIC gene that are significantly associated with MTX response. These SNPs are located in non-coding regions of the gene and therefore may have a role in determining efficacy. The fact that these SNPs show significant association with MTX response suggests that they may have a role in the biology of MTX treatment. Further study is required to determine the actual causal variant responsible for these associations.

**Abbreviations:**
- AE: Adverse event failure
- CI: Confidence interval
- IE: Inefficacy failure
- MAF: Minor allele frequency
- MTX: Methotrexate
- OR: Odds ratio
- R: Responders
- SNP: Single-nucleotide polymorphism
- VNTR: Variable-number tandem repeat
Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

We thank the Arthritis Research UK for their support (grant reference no 17552). SH, PM, INB, AB, WT are funded by the Arthritis Research UK and SAO's salary is funded by Pfizer. We acknowledge the NIHR Manchester Biomedical Research Centre for their support.

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