The Pharmacogenomics Journal (2018) 18:528–538
https://doi.org/10.1038/s41397-018-0025-5

CONSENSUS STATEMENT

Genome-wide association study of response to methotrexate in early rheumatoid arthritis patients

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Received: 5 May 2017 / Revised: 10 October 2017 / Accepted: 9 February 2018 / Published online: 25 May 2018

Abstract

Methotrexate (MTX) monotherapy is a common first treatment for rheumatoid arthritis (RA), but many patients do not respond adequately. In order to identify genetic predictors of response, we have combined data from two consortia to carry out a genome-wide study of response to MTX in 1424 early RA patients of European ancestry. Clinical endpoints were change from baseline to 6 months after starting treatment in swollen 28-joint count, tender 28-joint count, C-reactive protein and the overall 3-component disease activity score (DAS28). No single nucleotide polymorphism (SNP) reached genome-wide statistical significance for any outcome measure. The strongest evidence for association was with rs168201 in *NRG3* ($p = 10^{-7}$ for change in DAS28). Some support was also seen for association with *ZMIZ1*, previously highlighted in a study of response to MTX in juvenile idiopathic arthritis. Follow-up in two smaller cohorts of 429 and 177 RA patients did not support these findings, although these cohorts were more heterogeneous.

Introduction

Rheumatoid arthritis (RA) is a common autoimmune inflammatory arthritis, with a significant effect on health and wellbeing. The first choice of treatment for RA patients for many years was monotherapy with the conventional synthetic disease-modifying anti-rheumatic drug (csDMARD) methotrexate (MTX), with or without glucocorticoids [1]. However, a substantial proportion of patients do not respond adequately to treatment with MTX monotherapy [2] at which point treatment may be escalated to combination csDMARDs or biological DMARDs (bDMARDs). Unfortunately, by this stage irreversible joint damage may have occurred [3]. This has led to the introduction of the treat-to-target approach, which advocates frequent clinical review and more rapid escalation to combination csDMARDs or bDMARDs in order to achieve remission or low disease activity, with good evidence that these are achieved more rapidly [4, 5]. Irrespective of the treatment protocol, MTX is the cornerstone of therapy in RA and is often continued in patients receiving bDMARDs [6].

It would be of both clinical and economic benefit to be able to target initial treatment, so that patients unlikely to respond to MTX monotherapy could be offered alternative therapies at an earlier stage. Genetic variants do not change over time and are easily measured; identifying genetic

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Please see Appendix I.

Please see Appendix II.

See Supplementary Information.

Electronic supplementary material The online version of this article (https://doi.org/10.1038/s41397-018-0025-5) contains supplementary material, which is available to authorized users.

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predictors of response to treatment could therefore be of great clinical utility if sufficiently predictive.

A number of investigations of genetic predictors of response to MTX in RA patients have been published, but these have largely been candidate gene studies based on genes involved in MTX metabolism, including the folate pathway, and almost all have had small sample sizes [7–10]. A few genetic variants have been nominally associated with response in more than one study (rs2372536 in *ATIC*, reviewed in Plant et al. [11]) or in a meta-analysis (rs1051266 in *SLC19A1 (RFC1)* [12]), but results are inconsistent across studies and not close to reaching genome-wide significance levels. Senapati et al. [13] carried out the first genome-wide association (GWA) analysis of response to MTX monotherapy, in 457 RA patients from North India, comparing good and poor responders; they found two loci reaching significance levels of less than 10^{-5}, but nothing reached genome-wide significance. Cobb et al. [14] carried out a GWA study of response to MTX in 759 European patients with juvenile idiopathic arthritis (JIA), identifying 14 regions reaching a significance level of less than 10^{-5}.

The clinical importance of this question motivated the formation of the international Pharmacogenomics of Methotrexate in RA (PAMERA) consortium and the UK MAximizing Therapeutic Utility in RA (MATURA) consortium (www.matura.whri.qmul.ac.uk), which has the wider remit of using blood-based biomarkers and pathobiology to inform the stratification of all stages of RA treatment [15]. In this study, we have combined data from these two consortia to report the first published GWA study of response to MTX monotherapy in RA patients of European ethnicity, based on a much larger sample size than previously reported studies.

Subjects and methods

All component studies were approved by a Research Ethics Committee (see Supplementary Information) and all patients provided written informed consent for genetic studies.

Study population

The study was split into two phases. The first phase comprised RA cases treated with MTX monotherapy, obtained from three observational UK studies (the Yorkshire Early Arthritis Register (YEAR) [16], the Manchester Rheumatoid Arthritis Medication Study (RAMS) [17] and the Leeds Inflammatory Arthritis disease CONtinuum registry (IACON) [18]), four UK-led clinical trials (Infliximab as inDuction therapy in Early rheumatoid Arthritis (IDEA) [19], Etanercept and Methotrexate in Patients to Induce Remission in Early arthritis trial (EMPIRE) [20], Combination Anti-Rheumatic Drugs in Early Rheumatoid Arthritis (CARDERA-1) [21] and Effect of anakinra as combination therapy: second UK combination therapy in early rheumatoid arthritis trials (CARDERA-2) [22]), a US-led trial (Treatment of Early Aggressive Rheumatoid arthritis (TEAR) [23]), a Swedish trial (SWEdish FarmacOTherapy (SWEFOT) [24]) and a Dutch observational study (Synoviomics [25]). Although 1952 patients on MTX monotherapy were genotyped, a maximum of 1424 were available for analysis after exclusions for high glucocorticoid use, missing clinical data or quality control (Supplementary Table 1). The second phase was used for follow-up and consisted of two datasets: Phase 2a cases were obtained from the Scottish Early Rheumatoid Arthritis (SERA) study [26], and Phase 2b cases were from the MTX control arms of three international clinical trials programs (a 3-trial program from the Actemra versus Methotrexate double-Blind Investigative Trial In mONotherapy (AMBITION) study [27], a 3-trial program involving MabThera/Rituximab [28–30] and a 4-trial program involving Ocrelizumab [31]).

For inclusion in Phase 1 of the study, individuals were required to have a consultant diagnosis of RA, a maximum of 12-month disease duration prior to starting MTX and to have started treatment with MTX monotherapy; those starting with any additional csDMARDs, bDMARDs or high dose oral glucocorticoid (relevant to the CARDERA studies) were excluded from analysis. The study was restricted to patients of self-reported European ancestry, further validated through single nucleotide polymorphism (SNP) genotyping. Clinical measurements collected included swollen 28-joint count (SJC28), tender 28-joint count (TJC28) and C-reactive protein (CRP, in mg/L), or erythrocyte sedimentation rate (ESR), if CRP was not available. Measurements of these were taken at baseline (up to 6 weeks before the individual's MTX start date) and again at follow-up (6 months from the individual's MTX start date, or 3 months if either this was not available or the patient started any additional DMARD between 3 and 6 months). Three component disease activity scores DAS28CRP3 (calculated as $1.10[0.56(\sqrt{\text{TJC28}}) + 0.28(\sqrt{\text{SJC28}}) + 0.36(\log_e(\text{CRP} + 1))] + 1.15$) and DAS28ESR3 (calculated as $1.08[0.56(\sqrt{\text{TJC28}}) + 0.28(\sqrt{\text{SJC28}}) + 0.70(\log_e(\text{ESR}))] + 0.16$) were calculated for each individual at baseline and follow-up.

For Phase 2 of the study, there was some relaxation of the entry criteria. For Phase 2a, cases were not required to have started treatment on MTX monotherapy, and additional csDMARDs were permitted in combination with MTX. For Phase 2b, cases were all treated with MTX monotherapy, but were not required to have disease duration of less than 12 months.

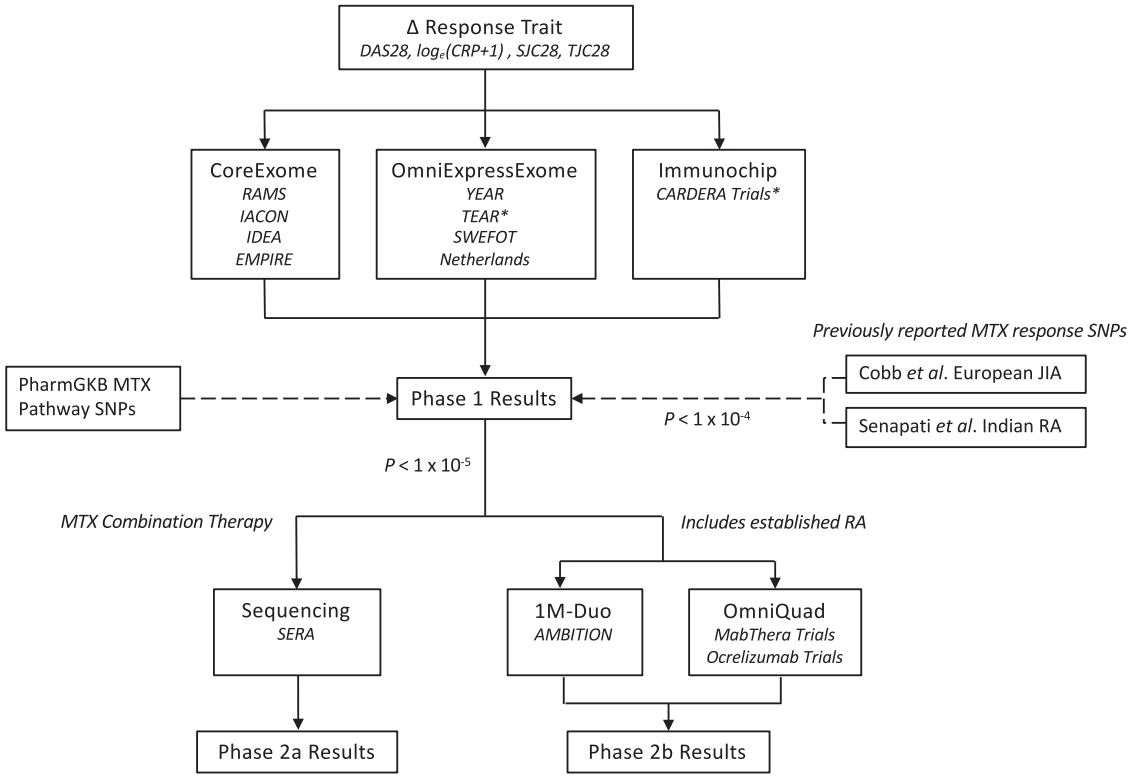

Fig. 1 Study design. Study names are given in italics. (*) indicates ESR was used in the DAS28 calculation for that study instead of CRP

Further information on the contributing studies is given in Supplementary Information.

Genotyping

Samples were genotyped using five different Illumina arrays (Supplementary Table 1), including (for one study) Immunochip, which has less than genome-wide coverage. Quality control on samples and SNPs was performed separately for each of the genotype arrays. Samples were excluded for any of the following reasons: (i) a call rate of <98% (of the total number of SNPs on the chip); (ii) evidence of non-European origin from principal components analysis using EIGENSTRAT [32] after combining with HapMap and in-house European samples; (iii) sex as ascertained by genotyping not matching reported gender; (iv) evidence of first degree relationship or identity with another sample (in which case the sample with the lower call rate of the pair was excluded). SNPs were excluded for any of the following reasons: (i) Hardy–Weinberg equilibrium p-value $<10^{-6}$; (ii) call rate <98%; (iii) minor allele frequency (MAF) <0.01. Imputation was conducted using IMPUTEv2 [33] with the 1000 Genomes haplotypes Phase 3 integrated variant set as reference. Genotypes were phased within IMPUTEv2 and imputed in a 2-Mb window (non-CoreExome genotyped samples) or pre-phased using SHAPEITv2-r837 [34] and imputed in a 7-Mb window (CoreExome genotyped samples). The MCMC options used in IMPUTEv2 for all imputations were ($k = 80$, iter = 30, burnin = 10) with effective size of population set as Ne = 20,000. Following imputation, only SNPs that had an INFO score >0.8 were retained for analysis.

Ultra-low coverage whole-genome sequencing at a mean depth of 0.5× was available for the SERA participants. The raw sequencing reads were aligned to the HG19 reference genome using the Torrent 538 Mapping Alignment Program for Ion Torrent™ Data (TMAP) software. Imputation to the 1000 Genomes Phase 3 reference panel was performed using the GeneImp software [35]. Similarly to the genotype data, relatedness was evaluated based on a subset of the imputed genotypes thinned for linkage disequilibrium (LD) using PLINK [36], and samples were excluded on evidence of a first-degree relationship with another sample.

Statistical analyses

In Phase 1 of the study, four separate genome-wide analyses were conducted for change in outcome from baseline to follow-up (follow-up measure minus baseline measure) of DAS28 (either DAS28CRP3 or DAS28ESR3 dependent on study), SJC28, TJC28 and $\log_e(\mathrm{CRP} + 1)$; CRP measures were log-transformed because the distribution of measures

Table 1 Summary of the demographics and clinical characteristics for the patients included in the analysis for Phase 1 and 2

Study	Sample origin	*N*	Age (years)		Female		RF positive		Mean duration <1 year	MTX starting dose (mg)		Baseline DAS28	
			Mean	(SD)	*N*	(%)	*N*	(%)		Min	Max	Mean	(SD)
Phase 1													
YEAR	UK	343	58.3	(13.1)	239	(70)	243	(73)	Yes	5	22.5	5.0	(1.2)
TEAR[a]	US	117	49.8	(12.0)	91	(66)	118	(86)	Yes	2.5	10	5.6	(1.0)
SWEFOT	Sweden	325	53.9	(14.0)	233	(71)	225	(69)	Yes	10	10	5.1	(0.9)
Netherlands	Netherlands	38	54.7	(12.2)	26	(68)	22	(58)	Yes	7.5	20	4.6	(1.2)
RAMS	UK	274	56.8	(14.1)	190	(69)	158	(58)	Yes	2.5	25	4.4	(1.2)
IDEA	UK	29	54.6	(12.0)	24	(69)	24	(71)	Yes	10	15	4.9	(1.3)
IACON	UK	128	59.5	(13.5)	91	(66)	78	(59)	Yes	5	25	4.3	(1.3)
EMPIRE	UK	22	53.1	(12.8)	17	(74)	14	(61)	Yes	10	10	4.2	(0.9)
CARDERA Trials[a]	UK	148	54.4	(12.9)	108	(67)	101	(68)	Yes	7.5	7.5	5.8	(1.1)
Phase 2a													
SERA	UK	429	58.4	(13.2)	279	(65)	169[b]	(65)	No[c]	5	30	4.8	(1.2)
Phase 2b													
AMBITION	International	85	52.5	(13.8)	69	(74)	62	(67)	No	7.5	7.5	6.0	(0.8)
MabThera Trials	International	32	49.6	(12.5)	23	(72)	29	(91)	No	7.5	7.5	5.7	(0.9)
Ocrelizumab Trials	International	60	51.7	(11.1)	42	(70)	55	(92)	No	7.5	7.5	6.0	(0.9)

DAS28 disease activity score in 28 joints (3 component CRP version), *RF* rheumatoid factor, *SD* standard deviation

[a]CRP not available, DAS28 calculated using ESR

[b]Data available for 262 samples

[c]Median symptom duration 172 days

was highly positively skewed. Imputed genotypes were analyzed as expected genotype counts based on the posterior probabilities (gene dosage) using linear regression implemented in SNPTEST2 [37], assuming an additive genetic model, with baseline measure included as a covariate. As outliers from count data may have a large influence on the fitted linear regression models for rarer SNPs, the results presented in this study are restricted to SNPs with a MAF >0.05. Analyses were performed separately for each of the three groups of studies measured on the same genotype array (see Fig. 1), and meta-analysis was then conducted across groups using PLINK. A fixed effects model was used for the meta-analysis unless there was evidence of heterogeneity between the study arrays ($I^2 > 31\%$) [38], in which case a random-effects model was used. To account for potential population stratification in the OmniExpressExome samples from the USA, UK, Sweden and the Netherlands, the first two principal components were included as covariates in the regression model. The analysis of the most significant SNPs was repeated including the first 5 principal components and had little effect on the results. All outcomes were mean-centred and scaled to have a variance of 1 within each group before the linear regression. Since ESR measures were used in place of CRP measures for the CARDERA and TEAR samples, the scaling for DAS28 was carried out separately for these studies.

All regions that included an SNP with a p-value $< 10^{-5}$ and additional SNPs within 200 kb reaching $p < 5 \times 10^{-5}$ were followed up in Phase 2. All SNPs within the region with a p-value < 0.001 were tested using the same regression models as in Phase 1. Two separate genotype arrays were used for the Phase 2b samples (Fig. 1), and data from the two arrays were meta-analyzed using a fixed effects model.

Follow-up of candidate genes and previous studies

Candidate genes were identified based on their putative role in MTX metabolism and mechanism of action. MTX is a structural analogue of folic acid and interferes with the folate cycle through competitive inhibition of dihydrofolate reductase, which is important for the effective metabolism of biologically active folate cofactors. These are required for the generation of methionine from homocysteine and for the synthesis of purine and pyrimidine nucleosides. Within cells, MTX is rapidly converted to γ-glutamyl polyglutamates that inhibit enzymes crucial for de novo synthesis of nucleotide precursor metabolites and ultimately inhibition of enzymes involved in adenosine metabolism. Relevant pathways, including drug transporters, were identified in the Pharmacogenomics Knowledgebase PharmGKB, using the search term “methotrexate”. The

Table 2 Summary of the most highly associated regions at $p < 1 \times 10^{-5}$ identified in Phase 1 for each of the 4 traits analyzed genome-wide

						Phase 1			Phase 2a		Phase 2b		
Trait	Marker	Chr	Position	Effect allele	Allele frequency	β[a] (95% CI)	p-Value	I^2 (%)	β[a] (95% CI)	p-Value	β[a] (95% CI)	p-Value	Genes[b]
DAS28	rs2372751	3	81124821	C	0.49	0.17 (0.10, 0.24)	2.8×10^{-6}	0	−0.02 (−0.14, 0.10)	0.77	0.13 (−0.08, 0.34)	0.23	*LINC02027*
	rs58840038	5	125773164	T	0.31	−0.17 (−0.24, −0.09)	8.0×10^{-6}	0	−0.04 (−0.16, 0.09)	0.57	0.03 (−0.16, 0.23)	0.73	*GRAMD3*
	rs6903359	6	21986214	C	0.13	−0.23 (−0.34, −0.13)	7.4×10^{-6}	0	0.07 (−0.12, 0.27)	0.46	0.10 (−0.17, 0.38)	0.45	*CASC15*
	rs168201	10	84610081	G	0.48	0.18 (0.12, 0.25)	9.8×10^{-8}	0	−0.10 (−0.21, 0.02)	0.091	0.08 (−0.10, 0.26)	0.38	*NRG3*
	rs57816977	18	4012466	C	0.18	−0.22 (−0.31, −0.12)	5.3×10^{-6}	0	0.12 (−0.03, 0.27)	0.10	0.00 (−0.24, 0.25)	1.00	*DLGAP1*
$\log_e$(CRP + 1)	rs79244342	6	54660244	G	0.09	−0.26 (−0.37, −0.15)	3.3×10^{-6}	0	−0.05 (−0.24, 0.13)	0.57	0.02 (−0.34, 0.37)	0.93	*KRASP1*
	rs1889339	9	96053928	G	0.18	0.19 (0.11, 0.27)	5.7×10^{-6}	0	−0.06 (−0.20, 0.07)	0.38	0.17 (−0.05, 0.39)	0.14	*WNK2*
SJC28	rs35179427	1	95670888	A	0.08	0.21 (0.12, 0.30)	7.8×10^{-6}	0	−0.05 (−0.20, 0.09)	0.49	−0.25 (−0.54, 0.04)	0.10	*RWDD3 TMEM56-RWDD3*
	rs1453301	2	138080695	A	0.16	0.15 (0.09, 0.22)	7.1×10^{-6}	0	−0.05 (−0.16, 0.06)	0.39	−0.03 (−0.22, 0.17)	0.79	*THSD7B*
	rs77458347	4	109896081	T	0.14	0.18 (0.10, 0.26)	6.7×10^{-6}	0	−0.02 (−0.14, 0.10)	0.77	0.18 (−0.10, 0.47)	0.21	*COL25A1*
	rs144940912	4	155092290	T	0.07	0.26 (0.15, 0.36)	1.4×10^{-6}	16	0.11 (−0.05, 0.27)	0.20	0.11 (−0.32, 0.53)	0.62	
	rs12663189	6	162730663	C	0.43	0.12 (0.07, 0.17)	2.0×10^{-6}	0	−0.01 (−0.09, 0.08)	0.87	0.04 (−0.12, 0.20)	0.64	*PARK2*
	rs314637	7	4421893	A	0.22	0.15 (0.08, 0.21)	2.9×10^{-6}	13	0.08 (−0.02, 0.18)	0.11	0.19 (−0.01, 0.38)	0.06	
	rs113798271	7	145059089	G	0.21	0.14 (0.08, 0.21)	9.4×10^{-6}	29	0.02 (−0.08, 0.12)	0.67	0.02 (−0.16, 0.21)	0.81	
	rs1175813	19	49737486	G	0.11	0.20 (0.12, 0.29)	1.4×10^{-6}	0	−0.15 (−0.27, −0.03)	0.012	0.00 (−0.24, 0.25)	0.98	
	rs1042579	20	23028724	A	0.23	0.15 (0.08, 0.21)	6.9×10^{-6}	0	0.02 (−0.09, 0.12)	0.76	0.24 (0.04, 0.44)	0.020	*THBD*
	rs2836915	21	40509189	T	0.31	−0.13 (−0.18, −0.07)	4.5×10^{-6}	0	−0.05 (−0.14, 0.04)	0.26	−0.06 (−0.24, 0.12)	0.51	
TJC28	rs10058818	5	56808696	G	0.21	0.17 (0.10, 0.25)	2.4×10^{-6}	8	0.06 (−0.06, 0.17)	0.31	−0.10 (−0.32, 0.12)	0.37	*LINCR-0003*
	rs10515242	5	95982675	C	0.06	0.28 (0.16, 0.40)	4.8×10^{-6}	0	0.17 (−0.01, 0.35)	0.069	−0.08 (−0.42, 0.26)	0.64	
	rs2026708	6	21986895	G	0.14	−0.22 (−0.31, −0.13)	1.9×10^{-6}	0	0.02 (−0.14, 0.19)	0.78	0.06 (−0.19, 0.30)	0.65	*CASC15*
	rs2776898	6	37541111	T	0.35	0.16 (0.10, 0.23)	9.9×10^{-7}	0	−0.11 (−0.21, −0.01)	0.027	0.14 (−0.03, 0.32)	0.11	
	rs114461403	7	16547268	T	0.33	0.15 (0.08, 0.21)	7.5×10^{-6}	0	0.00 (−0.11, 0.10)	0.98	−0.12 (−0.30, 0.07)	0.21	
	rs28442057	15	55110958	T	0.34	0.14 (0.08, 0.21)	7.9×10^{-6}	0	−0.01 (−0.11, 0.09)	0.82	0.04 (−0.15, 0.22)	0.68	
	rs12446816	16	14092341	G	0.38	0.15 (0.09, 0.21)	5.1×10^{-6}	0	−0.01 (−0.11, 0.08)	0.77	0.01 (−0.16, 0.17)	0.95	
	rs9910936	17	42949168	T	0.28	0.15 (0.09, 0.22)	6.7×10^{-6}	0	0.05 (−0.06, 0.16)	0.38	0.08 (−0.11, 0.28)	0.40	*CCDC103 EFTUD2 GFAP KIF18B*

DAS28 disease activity score in 28 joints, *CRP* C-reactive protein, *SJC28* swollen joint count 28, *TJC* tender joint count 28, *Chr* chromosome, β beta, *CI* confidence interval, I^2 heterogeneity statistic

[a]Per-allele for change in outcome in number of standard deviations, positive values correspond to worse response

[b]SNP or supporting SNPs with $p < 5 \times 10^{-5}$ located within the gene

resultant pathways were "Antimetabolite Pathway" and "Methotrexate Pathway (Cancer Cell) Pharmacokinetics". Non-redundant genes listed under these headings were included as candidates. In addition, adenosine receptor genes listed in a recent MTX pathway PharmGKB [39] summary were included in the analysis (Supplementary Table 2). All SNPs found within the genes were tested in Phase 1 for each of the four traits.

Two previously reported GWA studies of response to MTX were followed up using the results of our overall DAS28 analysis. The first was a study of 457 Indian RA patients [13] with an outcome classified as good response (a decrease in the DAS28ESR3 score by 1.2 and the final DAS28ESR3 ≤3.2 for at least 6 months after MTX monotherapy) or poor response (duration of illness not exceeding 5 years and active disease (DAS28ESR3 ≥5.1) despite at least 3 months of therapy with MTX). The second was a study of 374 European JIA patients [14] categorized as responders according to the American College of Rheumatology paediatric 30, 50 and 70 improvement criteria or as non-responders. All SNPs with $p < 0.0001$ in the discovery phase of these two studies were followed up in our Phase 1 results for DAS28 outcome. Any such SNP which additionally reached $p < 0.01$ in Phase 1 was followed up in Phase 2.

Further details of methods used in the interpretation of results can be found in Supplementary Information.

Results

A total of 1424 RA cases passed quality control for Phase 1 (Supplementary Table 1), with the maximum number available for analysis dependent on the trait: 1392 for DAS28, 1424 for SJC28/TJC28 and 1133 for CRP. The numbers were lower for areas of the genome not covered by Immunochip (1244 and 1276 for DAS28 and SJC28/TJC28, respectively, with number for CRP unchanged). Demographic and clinical characteristics are presented in Table 1 and Supplementary Table 3. Starting doses of MTX ranged from 2.5 to 25 mg, and highest mean baseline disease activity was observed in the patients from the CARDERA trials.

No SNP reached genome-wide statistical significance ($p < 5 \times 10^{-8}$) for any of the four outcomes. The most significant region, around the gene Neuregulin 3 (*NRG3*) on chromosome 10, reached 9.8×10^{-8} for change in DAS28 (Table 2, Fig. 2, Supplementary Figs. 1, 2). The SNP rs168201 in this region was also the SNP with the most consistent evidence of association over the four outcomes; rs168201 is an intronic variant in *NRG3*, having $p = 9.8 \times 10^{-8}$ for DAS28 and $p < 0.01$ for all 3 of the components SJC28, TJC28 and CRP (Fig. 3).

In total, 25 regions were identified harbouring SNPs with suggestive evidence for association with MTX response ($p < 10^{-5}$ for at least one SNP, and additional supporting SNPs within 200 kb reaching $p < 5 \times 10^{-5}$). Only two regions reached this level of significance for change in CRP (around the genes *KRASP1* and *WNK2*). The analysis of SJC28 identified the most regions reaching $p < 10^{-5}$, including regions encompassing the genes *RWDD3*, *PARK2*, *COL25A1*, *THSD7B* and *THBD*. The most significant region for TJC28 was at an intergenic region on chromosome 6 (Table 2).

There were 429 RA cases available for analysis in Phase 2a, of whom only 154 (36%) were on MTX monotherapy, the remainder being on MTX in combination with one or more of prednisolone (116), sulphasalazine (155), hydroxychloroquine sulphate (175) and leflunomide (9). For Phase 2b, 177 cases were available, with 99 (53%) of these having disease duration greater than 12 months; mean baseline disease activity was higher than for Phase 1 cases (Table 1, Supplementary Table 3). All SNPs with $p < 0.001$ in the 25 regions were followed up, but no SNP reached a level of $p < 0.001$ in either Phase 2a or 2b (Table 2, Supplementary Tables 4-7).

Details of the genes tested from the MTX metabolism candidate gene selection can be found in Supplementary Table 2. The number of SNPs tested for each gene ranged from 7 (*FPGS*) to 643 (*ADK*) with 3168 tested in total (Supplementary Fig. 3). The most significant SNP was rs7996393 for both DAS28 and TJC28, found in the gene *AMPD1* ($p = 0.0008$ and $p = 0.0011$, respectively). For CRP, the most significant SNP was rs4148160 ($p = 0.0078$) in *ABCG2*, and for SJC28 the most significant SNP was rs2236224 ($p = 0.0064$) in *MTHFD1*. The genes selected included two SNPs nominally associated in more than one previous study. In *ATIC*, rs2372536 was not associated with DAS28, CRP or SJC28 ($p = 0.11$, $p = 0.20$ and $p = 0.83$, respectively) but was nominally associated with TJC28 (beta (95% CI) = 0.06 (0.00, 0.13), $p = 0.042$). In *SLC19A1*, rs1051266 was not associated with DAS28, CRP, SJC28 or TJC28 ($p = 0.76$, $p = 0.46$, $p = 0.24$ and $p = 0.42$, respectively).

We used the results from the analysis of DAS28 to follow up all SNPs with $p < 10^{-4}$ in either of two previous GWA studies of response to MTX, of Indian RA patients and European JIA patients (Supplementary Table 8 and 9). No SNP from the Indian RA study showed evidence of association at $p < 0.01$ in our results when taking direction of effect into account. One region of association at the *ZMIZ1* gene in the JIA study showed some evidence of association in our results (rs703979: $p = 7.7 \times 10^{-4}$ in the JIA study, $p = 1.1 \times 10^{-4}$ for DAS28 in this study, and rs703970: $p = 8.3 \times 10^{-5}$ in the JIA study, $p = 1.8 \times 10^{-4}$ for DAS28 in this study) with corresponding directions of

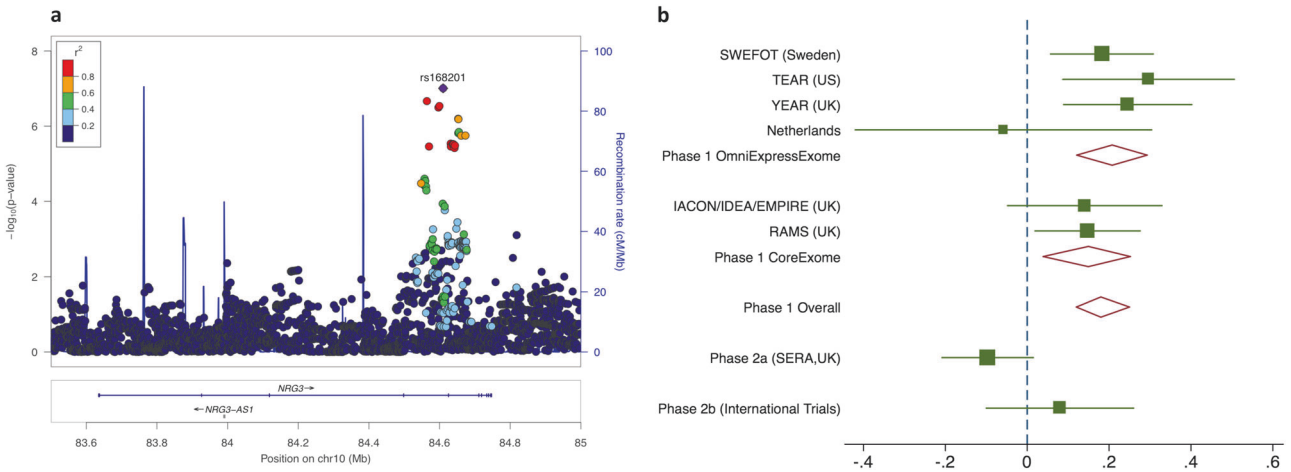

Fig. 2 Results for the SNP (rs168201) reaching the highest level of significance from the linear regression of change in DAS28 in phase 1 of the study. **a** $-\log_{10}$ *p*-values from Phase 1 for the SNPs surrounding rs168201 on chromosome 10. **b** Forest plot of estimated per allele betas for the effect of rs168201 on change in DAS28 in number of standard deviations by study phase

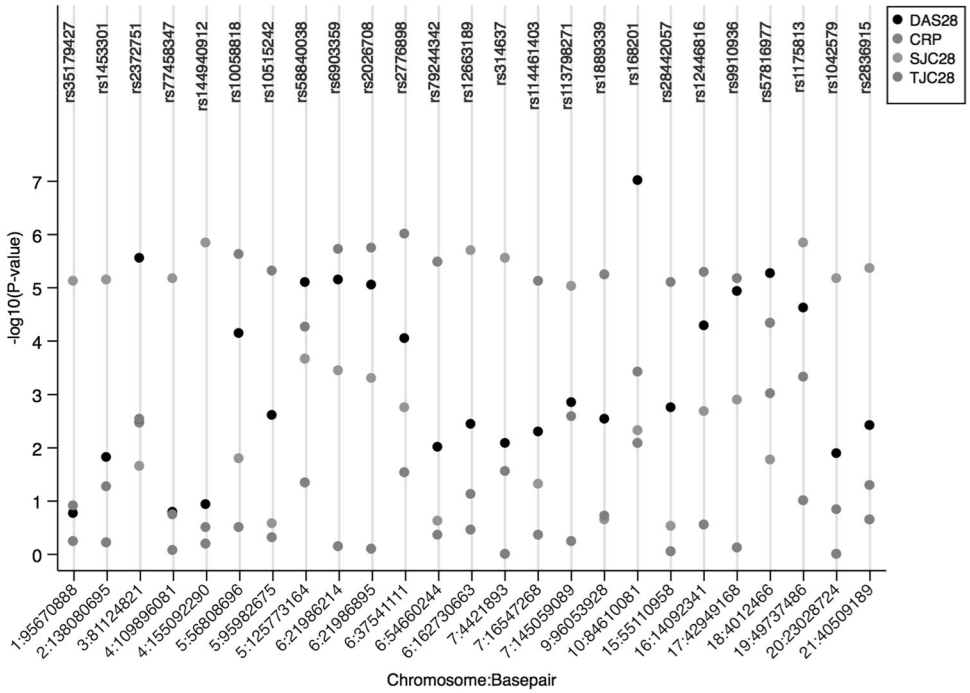

Fig. 3 Results in all traits for the lead SNP of the 25 most highly associated regions identified in Phase 1 in DAS28, CRP, SJC28 or TJC

effect. When using Fisher's method for combining *p*-values across the JIA study and this study, this gives 1.4×10^{-6} for rs703979 and 2.8×10^{-7} for rs703970. These two SNPs are in almost complete LD ($r^2 = 0.98$ for the OmniExpressExome samples).

Discussion

This study is by far the largest GWA study of response to MTX in RA patients reported to date. Although no SNP showed association with response at a genome-wide level, several of the findings are of potential interest.

The SNP showing the most significant association with DAS28 (or any outcome) is rs168201, an intronic variant in *NRG3*. In an early GWA study [40], rs10509440 in *NRG3* was reported to show some evidence of association with RA susceptibility ($p = 6 \times 10^{-5}$), with weak supportive evidence from a neighbouring SNP, rs12358407, in a follow-up study ($p = 0.003$) [41]. These findings have however not been replicated in more recent larger GWA studies. SNPs in the *SFTPD* gene close to *NRG3* have been associated with RA susceptibility at a genome-wide significance level in Asian but not European populations (rs726288) [42]; these SNPs are not in LD with the previously reported *NRG3* SNPs.

NRG3 encodes a cell–cell signalling protein (NRG3), which is a ligand for the ERBB4 (HER4) receptor tyrosine kinase [43]. There is some evidence linking NRG3 signalling to MTX response. Knockdown of the NRG3 receptor, ERBB4 (HER4), in osteosarcoma cell lines increased sensitivity to MTX (60% increase in apoptosis) but not other chemotherapies [44]. In a peripheral blood monocyte study of 32 RA patients treated with anakinra and MTX, *NRG3* is one of the seven genes in a gene signature predicting response, being upregulated in therapeutic non-responders [45]. High resolution (5 kb) Hi-C data in the GM12878 B lymphoblastoid cell line (see Supplementary Information) shows that rs168201 interacts most significantly with the *NRG3* promoter, underlining it as a candidate. From promoter capture Hi-C data in a GM12878 B cell line [46], it is seen that a DNA fragment ~100 kb from the LD block around rs168201 shows some evidence of interaction (CHiCAGO score = 5.41) [47] with the promoter of *MAT1A* (around 2.5 Mb upstream of rs168201), encoding methionine *S*-adenosyltransferase (MAT), a key enzyme in the transmethylation cycle. However, we were unable to demonstrate that the associated variants were in LD with known eQTL in *NRG3* or *MAT1A*, nor splice QTL in NRG3. This gene is of interest because low-dose MTX has been shown to inhibit its expression and activity [48].

Previously reported findings and candidate genes from the MTX metabolism pathway were followed up in this study, but little evidence was seen for their association with response. A number of these genes showed weak chromatin interactions in GM12878 cells with SNPs from the top 25 regions (e.g. *ADK* with rs703987, a SNP associated with change in DAS28). These interactions are listed in Supplementary Table 10.

The strongest support from candidate gene analyses was for SNPs in *ZMIZ1*, one of three genes highlighted in a GWA study of response to MTX in JIA patients [14]. This gene is involved in transcription factor regulation and has been associated with several autoimmune diseases, including psoriasis, Crohn's disease [49] and multiple sclerosis (MS) [50]. Fewings et al. have recently shown that expression of *ZMIZ1* varies in response to vitamin D and to certain MS therapies and may indicate a type of immune dysregulation potentially related to therapeutic response [51].

The top 25 loci were also annotated using two approaches —high-resolution long-range chromatin interaction and correlating regional genetic scores with genetic scores for other traits (see Supplementary Information and Supplementary Table 11). A few of the candidates from the Hi-C analysis have been indicated in inflammation biology, e.g. *NINJ1* and *RWDD3* [52, 53]. The intergenic region on chromosome 6 most strongly associated with change in TJC28 interacts with the *CCDC167* promoter in GM12878 B lymphoblastoid cells, a gene which is highly expressed in the immune system. In addition, rs9910936, also associated with change in TJC28, is close to several genes, but this analysis shows that the most relevant gene is likely to be *EFTUD2*, since the SNP is an expression-quantitative-trait locus for this gene and also shows chromatin interaction. Interestingly, some of the SNPs in the *NRG3* region of association interact with the *ZMIZ1* promoter, but the interaction is much weaker than with the *NRG3* promoter, so this is not shown as a candidate in Supplementary Table 11.

Progress is slow in identifying genetic predictors of treatment response, and a recent collaborative effort to identify genetic predictors of response to anti-TNF was not able to significantly improve predictive performance relative to standard clinical traits [54]. Although this is by far the largest study of MTX response to date, it is still small compared with current genome-wide studies of disease susceptibility. The study had 86% power to detect a genetic variant explaining 3% of the variance in outcome at a genome-wide significance level, but a sample size of at least 4000 patients would be needed to have over 80% power to detect a variant explaining only 1% of the variance. The challenge for pharmacogenetics is to assemble large cohorts of sufficiently homogeneous patients. Efforts were made in this study to minimize heterogeneity by using strict entry criteria. Inevitably, there was heterogeneity in the rate of escalation of MTX dose, however, most studies aimed for a dose between 15–20 mg within the first six weeks. Outcome measures are difficult to measure consistently, and there was a need to scale DAS28-CRP and DAS28-ESR separately, since only the latter measure was collected for the CARDERA and TEAR cohorts. Although overall measures such as DAS28 are important clinically, the analysis was also carried out on its individual components, since some of these are more heritable [55] and more objective than others. For clinical interest, analyses of "responders" (any response versus no response, and good response versus no response, according to EULAR criteria [56]) were also carried out (Supplementary Tables 12 and 13). Results were similar to those from the analysis of DAS28 but generally less highly significant.

This analysis suggests that no individual common variants are likely to be sufficiently predictive of response to be of clinical utility, but with larger studies it may be possible to create genetic risk scores, which, in combination with other factors, can be of use to target initial treatment. Larger sample sizes are needed, together with follow-up of the potentially interesting findings reported here.

Acknowledgements We thank all the patients who have contributed to this research, clinical staff who supported patient recruitment and laboratory staff who undertook sample processing. We thank the Medical Research Council (MRC) and Arthritis Research UK (ARUK) for their joint funding of PEAC and MATURA (grant codes 36661 and MR/K015346/1 and 20670 & 20022 (Experimental Arthritis Treatment Centre), respectively). The RAMS cohort was part funded by ARUK (grant code 20385) and the National Institute for Health

Research (NIHR) Manchester Musculoskeletal Biomedical Research Unit (BRU). The YEAR and IACON studies were part funded by program grants from ARUK (grant codes 18475 and 18387), the NIHR Leeds Musculoskeletal BRU and Diagnostic Evaluation Co-operative, the British Medical Association (Doris Hillier Award) and the Ann Wilks Charitable Foundation. The IDEA study was supported by a research grant from Investigator-Initiated Studies Program of Merck Sharp & Dohme Limited. The opinions expressed in this paper are those of the authors and do not necessarily represent those of Merck Sharp & Dohme Limited. Pfizer provided study drug and unrestricted grant funding for the EMPIRE study. The authors had the sole responsibility for data analysis and manuscript preparation. ARUK paid for the genotyping of CARDERA-1 and 2 (grant reference 19739). The SERA cohort was funded by Pfizer and the Scottish Government (ETM40), and the SERA genomic analysis was funded by the Stratified Medicine Scotland Innovation Centre (SMS-IC007). Research in the Newcastle University Musculoskeletal Research Group is supported by the National Institute for Health Research Newcastle Biomedical Research Centre based at Newcastle Hospitals NHS Foundation Trust and Newcastle University. The views expressed are those of the author(s) and not necessarily those of the NHS, the NIHR or the Department of Health. I.C.S. and ST held Academic Clinical Lectureships funded by the NIHR. This article presents independent research partly funded by the NIHR. The views expressed are those of the authors and not necessarily those of the NHS, the NIHR or the Department of Health. The funders had no role in the study design, data collection and analysis, data interpretation, the writing of the manuscript or the decision to submit the manuscript for publication. B.M. holds an MRC eMedLab Medical Bioinformatics Career Development Fellowship, funded from award MR/L016311/1. Part of this project was enabled through access to the MRC eMedLab Medical Bioinformatics infrastructure (grant code MR/L016311/1) and the MRC Leeds Medical Bioinformatics infrastructure (grant code MR/L01629X/1). PAMERA was supported by the US NIH Pharmacogenomics Research Network (PGRN) funded by NIGMS (U19 GM61388) and the RIKEN Center for Integrative Medical Sciences. It was funded in part by the Biobank Japan Project, funded by the Ministry of Education, Culture, Sports, Science and Technology of Japan. Acquisition and analysis of genetic and treatment response data from the TEAR Trial were supported in part by NIH R01 AR052658 (SLB, Jr., PI) Predictors of Treatment Response in Early Aggressive RA. The Synoviomics study was supported by the Dutch Arthritis Foundation (grant NR06/1/303).

Compliance with ethical standards

Conflict of interest Paul P Tak is an employee and shareholder of GlaxoSmithKline; GSK has not been involved in this study. Jianmei Wang is an employee of Roche Products and Felix Agakov is an employee of Pharmatics Ltd., UK. Dr. Weinshilboum is a co-founder and stockholder in OneOme LLC, a Pharmacogenomics Decision Support Company. Paul Emery has undertaken clinical trials and provided expert advice to Pfizer, MSD, Abbvie, BMS, UCB, Roche, Novartis, Samsung, Sandoz and Lilly. The authors declare no conflict of interest with the content contained in this manuscript.

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