

LONDON  
SCHOOL of  
HYGIENE  
& TROPICAL  
MEDICINE



## LSHTM Research Online

Cobb, J; Cule, E; Moncrieffe, H; Hinks, A; Ursu, S; Patrick, F; Kassoumeri, L; Flynn, E; Bulatović, M; Wulffraat, N; +102 more... van Zelst, B; de Jonge, R; Bohm, M; Dolezalova, P; Hirani, S; Newman, S; Whitworth, P; Southwood, TR; De Iorio, M; Childhood Arthritis Response to Medication Study (CHARMS); Childhood Arthritis Prospective Study (CAPS); BSPAR study group; Wedderburn, LR; Thomson, W; , COLLABORATORS; Baildam, E; Bishop, N; Brown, L; Buckley, J; Chieng, A; Carrasco, R; Cobb, J; Cook, L; Davidson, J; Duggan, A; Eltringham, M; Foster, H; Friel, E; Friswell, M; Gardner-Medwin, J; Gilbert, P; Gould, V; Hadfield, K; Hyrich, K; Jones, J; Lal, S; Lay, M; Lloyd, G; Lloyd, O; Lydon, C; Makengo, N; McGovern, A; Meijer, A; Mills-Wierda, N; Moorcroft, T; Price, V; Qiao, L; Riding, K; Sim, J; Southwood, T; Thomson, W; Todd, M; Tremble, S; Venter, K; Wade, D; Ward, P; Watson, S; Webster, G; Wedderburn, LR; Zelenovic, J; Buerkle, K; Cobb, J; Cotter, C; Etheridge, A; Gilbert, P; Hinks, A; Hirani, S; Kassoumeri, L; Lal, S; Melville, L; Moncrieffe, H; Mulligan, K; Newman, S; Patrick, F; Southwood, T; Thomson, W; Ursu, S; Wedderburn, LR; Whitworth, P; Woo, P; Abinum, M; Bell, A; Craft, AW; Crawley, E; David, J; Foster, H; Gardener-Medwin, J; Griffin, J; Hall, A; Hal, M; Herrick, AL; Hollingworth, P; Holt, L; Jones, S; Pountain, G; Ryder, C; Southwood, T; Stewart, I; Venning, H; Wedderburn, LR; Woo, P; Wyatt, S; (2014) Genome-wide data reveal novel genes for methotrexate response in a large cohort of juvenile idiopathic arthritis cases. *The pharmacogenomics journal*, 14 (4). pp. 356-64. ISSN 1470-269X DOI: <https://doi.org/10.1038/tpj.2014.3>

Downloaded from: <http://researchonline.lshtm.ac.uk/2901244/>

DOI: <https://doi.org/10.1038/tpj.2014.3>

### Usage Guidelines:

Please refer to usage guidelines at <https://researchonline.lshtm.ac.uk/policies.html> or alternatively contact [researchonline@lshtm.ac.uk](mailto:researchonline@lshtm.ac.uk).

Available under license: <http://creativecommons.org/licenses/by-nc-nd/2.5/>

<https://researchonline.lshtm.ac.uk>

Published in final edited form as:

*Pharmacogenomics J.* 2014 August ; 14(4): 356–364. doi:10.1038/tpj.2014.3.

## Genome-Wide Data reveals Novel Genes for Methotrexate Response in a Large Cohort of Juvenile Idiopathic Arthritis Cases

Joanna Cobb, PhD<sup>1,^,#</sup>, Erika Cule, PhD<sup>2,^</sup>, Halima Moncrieffe, PhD<sup>3,^</sup>, Anne Hinks, PhD<sup>1</sup>, Simona Ursu, PhD<sup>3</sup>, Fiona Patrick, BSc<sup>3</sup>, Laura Kassoumeri, BSc<sup>3</sup>, Edward Flynn, MSc<sup>1</sup>, Maja Bulatovi, MD<sup>4</sup>, Nico Wulffraat, MD PhD<sup>4</sup>, Bertrand van Zelst, BSc<sup>5</sup>, Robert de Jonge, PhD<sup>5</sup>, Marek Bohm, PhD<sup>6</sup>, Pavla Dolezalova, PhD<sup>6</sup>, Shashi Hirani, PhD<sup>7</sup>, Stanton Newman, D.Phil<sup>7</sup>, Pamela Whitworth<sup>8</sup>, Taunton R Southwood, MD<sup>8</sup>, Childhood Arthritis Response to Medication Study (CHARMS), Childhood Arthritis Prospective Study (CAPS), BSPAR study group, Maria De Iorio, PhD<sup>9</sup>, Lucy R Wedderburn, MD PhD<sup>3,10,\*</sup>, and Wendy Thomson, PhD<sup>1,\*</sup>

<sup>1</sup>Arthritis Research UK Epidemiology Unit and NIHR Manchester Musculoskeletal Biomedical Research Unit, Central Manchester University Hospitals National Health Service Foundation Trust, Manchester Academic Health Science Centre, The University of Manchester, UK

<sup>2</sup>Department of Epidemiology and Biostatistics, Imperial College London, London, UK

<sup>3</sup>Rheumatology Unit, UCL Institute of Child Health, 30 Guilford Street, London, UK <sup>4</sup>Department of Paediatric Immunology, University Medical Centre Utrecht, Wilhelmina Children's Hospital, Utrecht, The Netherlands <sup>5</sup>Department of Clinical Chemistry, Erasmus University Medical Centre,

Users may view, print, copy, and download text and data-mine the content in such documents, for the purposes of academic research, subject always to the full Conditions of use:[http://www.nature.com/authors/editorial\\_policies/license.html#terms](http://www.nature.com/authors/editorial_policies/license.html#terms)

**#Corresponding Author:** Dr Joanna Cobb, Arthritis Research UK Epidemiology Unit, The University of Manchester, Oxford Road, Manchester, M13 9PT, UK, joanna.cobb@manchester.ac.uk, Tel: +44 161 275 1669; Fax: +44 161 275 5043.

<sup>^</sup>These authors contributed equally

<sup>\*</sup>These authors made equal contribution as senior authors

### **British Society of Paediatric and Adolescent Rheumatology (BSPAR) study group:**

Mario Abinum, A. Bell, Alan W. Craft, Esther Crawley, Joel David, Helen Foster, Janet Gardener-Medwin, Jane Griffin, A. Hall, M. Hal, Ariane L. Herrick, P. Hollingworth, Lennox Holt, Stan Jones, Gillian Pountain, Clive Ryder, Tauny Southwood, I. Stewart, Helen Venning, Lucy R. Wedderburn, Patricia Woo, Sue Wyatt.

### **Childhood arthritis prospective study (CAPS):**

Eileen Baidam, Nick Bishop, Lynsey Brown, Joanne Buckley, Alice Chieng, Roberto Carrasco, Joanna Cobb, Lucy Cook, Joyce Davidson, Annette Duggan, Michael Eltringham, Helen Foster, Elizabeth Friel, Mark Friswell, Janet Gardner-Medwin, Paul Gilbert, Vikki Gould, Kelly Hadfield, Kimme Hyrich, Julie Jones, Sham Lal, Mark Lay, Gabrielle Lloyd, Olivia Lloyd, Carol Lydon, Natasha Makengo, Ann McGovern, Alexandra Meijer, Nicola Mills-Wierda, Theresa Moorcroft, Vicki Price, Liang Qiao, Kay Riding, Jane Sim, Tauny Southwood, Wendy Thomson, Maureen Todd, Susan Tremble, Katharine Venter, Debbie Wade, Peter Ward, Sharon Watson, Gwen Webster, Lucy R Wedderburn, Jadranka Zelenovic

### **Sparks-Childhood Arthritis Response to Medication Study (CHARMS):**

Katrin Buerkle, Joanna Cobb, Catherine Cotter, Angela Etheridge, Paul Gilbert, Anne Hinks, Shashi Hirani, Laura Kassoumeri, Sham Lal, Laura Melville, Halima Moncrieffe, Kathleen Mulligan, Stanton Newman, Fiona Patrick, Tauny Southwood, Wendy Thomson, Simona Ursu, Lucy R Wedderburn, Pamela Whitworth, Patricia Woo.

### **CONTRIBUTOR STATEMENT**

WT, LRW, M de I, HM, EC, and JC led the study. LRW established the CHARMS study cohort. LW, SU, HM, FP, LK, MB, NW, MB, PD, PW, and TRS collected samples and data. JC, EC, HM, LW and WT wrote the manuscript. JC and EF performed the genotyping. JC, EC, M de I performed the statistical analysis. All other authors contributed primarily to the patient ascertainment and/or data and sample collection and preparation. All authors reviewed the final manuscript.

### **COMPETING INTERESTS**

NW received an unrestricted grant from MEDAC Germany. PD received a research grant from Novartis. The other authors declare no competing interests.

Rotterdam, The Netherlands <sup>6</sup>First Faculty of Medicine and General Faculty Hospital, Charles University in Prague, Praha, Czech Republic <sup>7</sup>Centre for Health Services Research, School of Health Sciences, City University London, London, UK <sup>8</sup>Institute of Child Health, Birmingham Children's Hospital, Birmingham, UK <sup>9</sup>Department of Statistical Sciences, University College London, Gower Street, London, UK <sup>10</sup>Arthritis Research UK Centre for Adolescent Rheumatology, UCL Institute of Child Health, 30 Guilford Street, London, UK

## Abstract

Clinical response to methotrexate (MTX) treatment for children with juvenile idiopathic arthritis (JIA) displays considerable heterogeneity. Currently, there are no reliable predictors to identify non-responders: earlier identification could lead to targeted treatment. We genotyped 759 JIA cases from the UK, Netherlands and Czech Republic. Clinical variables were measured at baseline and 6 months after start of treatment. In Phase I analysis samples were analysed for association with MTX response using ordinal regression of ACR-pedi categories and linear regression of change in clinical variables, and identified 31 genetic regions ( $P < 0.001$ ). Phase II analysis increased SNP density in the most strongly associated regions, identifying 14 regions ( $P < 1 \times 10^{-5}$ ): three contain genes of particular biological interest (*ZMIZ1*, *TGIF1* and *CFTR*). These data suggest a role for novel pathways in MTX response and further investigations within associated regions will help reach our goal of predicting response to MTX in JIA.

## Keywords

methotrexate; juvenile idiopathic arthritis; pharmacogenetics; response

## INTRODUCTION

Juvenile Idiopathic Arthritis (JIA) is a heterogeneous condition with variable outcome and considerable ongoing disease burden [1]. Studies indicate that functional disability and complications due to JIA are still common in many teenagers and young people with JIA, and that the effects of early, uncontrolled inflammation may cause irreversible damage to joints and other tissues [2-4]. Thus improving long-term outcomes of children with JIA remains a critical challenge. Recent studies in JIA have indicated that early control of joint inflammation correlates with much improved outcomes, suggesting an early 'window of opportunity' when disease control can translate to profound long-term benefit [5]. It is known that not all children respond equally well to any given therapy. Despite increasing availability of new therapeutic options for treating inflammation in JIA, clinicians have no validated tools to help predict likelihood of good response to a particular drug. Therefore the current treatment strategy is to offer disease-modifying drugs in a sequential approach, with choices typically driven more by cost or safety profile than by scientific evidence.

The first line disease-modifying agent for JIA is methotrexate (MTX). Although MTX has proven efficacy in randomised trials and a good long-term safety record, response to MTX displays considerable heterogeneity in JIA with a significant 'non-response' rate of 35% or more of cases [6]. Additionally, consensus concerning level of 'response' that is considered

acceptable has shifted, with a target of complete control of inflammation now being advocated [7]. These developments combined with ever increasing availability of newer biologic treatments, provide further imperative for the discovery of biomarkers to aid the identification of children who require early aggressive therapy, compared to those who can achieve clinically inactive disease on MTX alone.

Response to treatment is thought to be a complex trait involving multiple genetic variants and environmental factors [8]. However, to date genetic studies of MTX response have been limited for both JIA and rheumatoid arthritis (RA) and have only utilised a candidate gene approach, focussing largely on genes affecting MTX transport and metabolism, enzymes influenced by MTX and adenosine pathways [9-16], recently reviewed in [17].

Given that the mechanisms of action of MTX in JIA are poorly understood, candidate gene studies may miss key pathways of mechanistic importance. Pharmacogenetic studies in other diseases have shown that genes other than those directly involved in known drug pathways often play key roles in variation of drug response [18]. In order to capture the genetic component more comprehensively, we brought together an International Consortium of investigators (the CHARMS-JIA GWAS International Consortium), and employed a genome wide approach to study response to MTX in a large cohort of children with JIA.

## METHODS

### Study Population

A cohort of children was recruited for the SPARKS-CHARM (CHildhood Arthritis Response to Medication) Study which has the overall aim to improve understanding of the variability in response to treatment observed in children with JIA and ultimately define a multifactorial model of response outcomes [14], and through the CHARMS-JIA GWAS International Consortium. All cases fulfilled the International League of Associations for Rheumatology (ILAR) criteria for JIA and were about to start new MTX treatment for active arthritis [14]. The study had full ethical approval and was fully compliant with the Declaration of Helsinki; parents provided fully informed consent, and patients provided age-appropriate assent. Samples and data were collected using the same inclusion and exclusion criteria at Great Ormond Street Hospital London, Birmingham Children's Hospital, Department of Paediatrics and Adolescent Medicine Charles University Prague, Wilhelmina Children's Hospital and University Medical Centre Utrecht, and also as part of the Childhood Arthritis Prospective Study (CAPS), a prospective inception cohort study of JIA cases from five centres across the UK [19].

A total of 759 individuals were included from all subtypes of JIA, classified according to ILAR criteria [20]. Demographic and clinical data were collected at baseline (up to 4 weeks before beginning MTX treatment) and again after 6 months (median 6.2 months, range 4–8 months) of MTX treatment. MTX was given orally or subcutaneously at 10–15 mg/m<sup>2</sup> per week (median 11.3 mg/m<sup>2</sup> per week). No other DMARDS were taken concurrently. Steroid treatment was recorded if administered at any point between baseline and follow-up. 370 children received at least one form of steroid medication: this comprised oral prednisolone in 204 children, pulsed iv methylprednisolone was taken by 99 children, while intra-articular

joint injections were given to 204 children. Despite patients coming from different regions in Europe, the indications and protocols for use of MTX in JIA were the same across centres.

Clinical data included the six core set variables; erythrocyte sedimentation rate (ESR), childhood health assessment questionnaire (CHAQ) 0-3 [21], active joint count (AJC), limited joint count (LJC), physician's global assessment on a visual analogue scale (PhysVAS) 0-10cm, and the parent/patient global assessment (ParVAS) 0-10cm. As this is an observational study, missing data differed for each core set variable. These variables were used to categorise patients according to the American College of Rheumatology paediatric (ACR-pedi) 30, 50, and 70 improvement criteria, or as non-responders [22]. Note that all children who reach ACR-pedi70 automatically also reach ACR-pedi30 and 50, while those who achieve ACR-pedi50 also achieve ACR-pedi30. In order not to count any child more than once, we defined the level of response for each child by the highest level of response achieved (ACR-pedi 30, 50 or 70).

### Genotyping

Samples were genotyped using the Illumina HumanOmniExpress Infinium array, according to Illumina's protocols in Manchester, UK. The default Illumina clustering algorithm (GenTrain2.0) was used to cluster SNPs in the software package GenomeStudio. SNPs were excluded if they had a call rate <98% and a cluster separation score of <0.4. Samples were then excluded for call rate <98%, incompatible recorded and genotype inferred gender, duplicates and evidence of identity by descent, or those with outlying heterozygosity. Combining the samples with data from HapMap Phase 3 individuals, principal component (PC) analysis was performed using Eigensoft v4.2 to identify extreme ethnic outliers [23;24]. PC analysis was performed on a subset of SNPs with minor allele frequency (MAF) >0.05, selected by removing SNPs in known regions of high linkage disequilibrium (LD) [25] and further pruned for LD between markers. Samples failing to cluster with European HapMap individuals were visually identified and removed. SNPs were excluded from the analysis if they had a MAF<0.05 and failed the Hardy-Weinberg equilibrium test ( $P < 0.001$ ). To assess our dataset for potential systematic over-inflation due to stratification, the genomic control inflation factor ( $\lambda_{GC}$ ) was calculated using the same SNP subset as used in the PC analysis. Cluster plots were visually inspected for the most associated SNPs to confirm genotyping quality.

### Statistical Analysis

Data were available on a number of potential confounding variables: gender, sample collection centre, presence or absence of concurrent steroid treatment, age at treatment baseline, time to treatment, duration of treatment and ILAR subtype (grouped into three categories: (1) oligoarthritis: persistent and extended, (2) polyarthritis: RF negative and positive, (3) psoriatic, enthesitis related, systemic and unclassifiable arthritis) (9). Each of these potential confounders were assessed for association with each core set variable. Moreover, presence of population stratification was checked using the first five PCs of the genotype dataset.

Statistical analysis was performed using Plink v1.07 [26] and R v2.15 (<http://www.r-project.org>), and plots were generated using R and LocusZoom [27]. SNPs were coded by minor allele count as 0, 1, 2. MTX response was defined using the ACR-pedi criteria with four categories: non-responders (reference category), ACR-pedi30, ACR-pedi50, ACR-pedi70, and association between genotype and MTX response was analysed using ordinal regression. Similar to other genetic studies of drug response measured by composite disease scores [18], we hypothesise that attempting to identify the underlying genetic basis of MTX response may be usefully performed by analysing each of the core set variables individually, since it is likely that the genetic basis of each of these is different, with varying contributions to MTX response. As the core set variables are not entirely independent from one another or the ACR-pedi status, the multiple testing burden is not as great as if we performed multiple tests on independent outcomes. Core set variables (ESR, CHAQ, AJC, LJC, PhysVAS, ParVAS) were recoded as change between baseline and follow-up, and linear regression was used to assess the strength of association for each.

We conducted our analysis in two phases (I and II) with the same individuals in each analysis phase. We utilised a low stringency of significance in Phase I of the analysis in order to maximise discovery of loci for more detailed investigation in Phase II, where a more stringent significance threshold was set. In Phase I of the analysis, results from the ordinal regression of ACR-pedi categories and six linear regressions of the core set variables (ESR, CHAQ, AJC, LJC, PhysVAS, ParVAS), totalling seven analyses, were used to identify genomic regions of interest for further investigation. The significance threshold selected ( $P < 0.001$ ) allows for greater emphasis on power than reducing type I error to enable hypothesis generation, an approach taken previously [18].

Regions were then selected for further analysis (Phase 2) by searching for clusters of associated SNPs ( $P < 0.001$  in at least two of the seven analyses) and extending out to include all SNPs within the annotated gene (based on the Illumina HumanOmniExpress gene annotation file, hg19). This resulted in regions of interest of varying sizes (range 0.02kb-12.8mb, average 1359kb). The aim of Phase II was to refine these regions. This was performed using SNP imputation to increase the density of SNP coverage in those regions. SHAPEIT v1 was used to pre-phase genotypes and SNPs were imputed against the 1000 Genomes Project reference panel (approximately 37 million SNPs) using IMPUTE2 [28;29]. Imputed SNP genotypes reaching the probability threshold 0.9 were included in the follow-up re-analysis, which focused on only these imputed regions using the same samples as Phase I and performed ordinal and linear regressions as described in Phase I. Regions containing at least one SNP in Phase II with association  $P < 1 \times 10^{-5}$  are the focus of the results presented here.

### Power Calculation

Study power was estimated at the two significance thresholds used in Phase I ( $P < 0.001$ ) and Phase II ( $P < 1 \times 10^{-5}$ ) of the analyses, over the range of sample sizes available, and assuming the variance explained by the additive effect of the SNP tested ranged from 0.01-0.1 under an additive genetic model.

## Functional Annotation

In order to gain a better understanding of the potential biological impact of our results, the most highly associated SNPs identified in Phase II (as well as SNPs in high linkage disequilibrium ( $r^2 > 0.8$ )) were queried using the web tool Assimilator (<http://assimilator.mhs.manchester.ac.uk/cgi-bin/assimilator.pl>) [30]. This facilitates collation of functional annotations from the publically available ENCODE and UCSC Genome Browser databases. Using the advanced search options available, output was focussed on whether any of the Phase II associated SNPs have shown evidence of transcription factor binding sites, evidence for open chromatin suggesting regions of active gene expression, and epigenetic marks which may be affected by drug treatment [31].

## RESULTS

Following stringent quality control, 694 JIA cases were available for analysis (Supplementary Table 1A and 1B) comprising individuals from all ILAR subtypes (Table 1). 31% of children were non-responders (less than ACR-pedi30 response). Among responders, categorised by their highest level of response achieved, 8.6% of children reached ACR-pedi30, 14.6% ACR-pedi50 and 45.8% ACR-pedi70 response. Samples clustered together in the PC analysis (Supplementary Figure 1) and therefore were analysed together. Following the SNP quality control steps and removal of low frequency variants ( $MAF < 0.05$ ), 586,062 SNPs were included in the Phase I analyses (Supplementary Table 1). Quantile-quantile plots and inflation factors showed no systematic inflation of  $P$  values (Supplementary Figure 2), and power was estimated to range from 10-100% across the analysed sample sizes for various effect sizes (Supplementary Figure 3).

None of the potential confounding variables tested (gender, ILAR JIA subtype, centre, age at treatment baseline, duration of treatment, time to treatment, steroid treatment or PCs generated to identify ethnic outliers) were associated with all six individual core set variables (data not shown); therefore to reduce loss of analysis power no adjustments were made to the linear or ordinal regressions.

In the hypothesis generating Phase I of the analysis, using both ACR-pedi and the individual core set variables, 31 genetic regions encompassing 75 nearby genes achieved our defined level of significance ( $P < 0.001$  in at least 2 of the 7 analyses), Table 2 and Supplementary Table 2. This included several notable associations such as genes related to TGFbeta signaling (*ZMIZ1*: zinc finger MIZ-type containing 1, *TGIF1*: TGFbeta-induced factor homeobox 1) and a member of the multi-drug resistance subfamily of the ATP-binding cassette transporter proteins (*CFTR*: cystic fibrosis transmembrane conductance regulator). Overall in Phase I, the most significant was a variant within an intron of the calcium channel *CACNA1I* (voltage-dependent calcium channel T type alpha 1I subunit) in the analysis of active joint count (AJC) (rs136855, region 31,  $\beta$  coefficient = 2.71,  $P = 9.18 \times 10^{-8}$ , see Supplementary Table 2). Two regions showed strong evidence with 13 SNPs in each associated at  $P < 1 \times 10^{-4}$  across several analyses (Region 12, *CFTR-CTTNBP2*: ParVAS, LJC, CHAQ; Region 20, *ZMIZ1*: ACR-pedi, ParVAS, ESR, CHAQ LJC, AJC; Figure 1). Using the 31 significant genetic regions found in the discovery phase of the analysis, the next analysis performed was to narrow down the genetic region of interest.

Phase II of analysis involved imputation of SNPs within the 31 regions identified in Phase I to refine the association signals by increasing SNP density. After imputation, using the increased Phase II significance threshold of  $P < 1 \times 10^{-5}$  this analysis identified 14 of the initial 31 genetic regions as the most strongly associated with response to MTX (Table 3, Supplementary Figure 4). Overlapping associations of SNPs were revealed for AJC and LJC in several genetic regions (regions 16, 17, 23 and 28). In one of these regions (region 23, chromosome 11 intergenic between *ANGPTL5-KIAA1377*) the top associated SNP was the same (rs11225055), and in the other three regions the most significant associated SNPs for the AJC and LJC analyses were in very high LD ( $r^2 > 0.97$ ). In all four regions showing association with ACR-pedi (regions 2, 12, 20 and 24), the ParVAS and/or PhysVAS scores were also associated.

Functional annotations for the most highly associated Phase II SNPs from Table 3 (plus SNPs in high LD ( $r^2 > 0.8$ ) with these lead SNPs) were assigned using Assimilator software [30]. The results presented in Supplementary Table 3 suggest the regions identified in Phase II of the analysis contain evidence of many markers of regulation and highlight many possible functional mechanisms. Certain regions were less fully covered by current databases, for example region 14 containing the gene *CSMD1*. Others including region 30 containing the gene *CYTH4* (cytohezin 4), have more evidence for regulatory activity, including multiple SNPs showing evidence of acting as an expression quantitative trait loci (eQTL) [32].

## DISCUSSION

Recent developments in treatments and management of childhood arthritis have led to increased expectations from clinicians, parents and patients for complete control of disease and consequent reduction of long-term adverse health outcomes [4;5;33]. The first step in JIA treatment, in parallel with joint injections, is typically administration of MTX: however it is clear that a proportion of patients treated with MTX will fail to respond adequately. Given recent recommendations for early aggressive treatment, it is important that MTX treatment is targeted to those children most likely to respond well [34]. Increasing our understanding of the influence of genetic variants in MTX response could assist clinicians to choose the best treatment options for their patients and identify patients who need more aggressive treatments. Performing large-scale genetic studies searching for variants contributing to MTX response has great appeal, but has proved challenging due to the relative rarity of JIA and lack of well co-ordinated international efforts. With this in mind, the CHARMS-JIA GWAS International Consortium facilitated the collection of carefully phenotyped response to medication data, and DNA samples from children with JIA treated with MTX for their arthritis, enabling the largest genetic analysis of MTX response in JIA to date. In Phase I of the analysis a total of 31 regions were identified as associated with response to MTX at  $P < 0.001$ . To narrow down these genetic regions, additional SNPs were imputed in Phase II of the analysis, with the results in 14 regions satisfying a more stringent cut-off of  $P < 1 \times 10^{-5}$ . The most strongly associated locus was *CACNA1I* which encodes the alpha chain of a low voltage-activated calcium channel that has been implicated in calcium signalling in neurons and may have other roles that have yet to be characterised. Other notable associated genes include *CFTR*, *ZMIZ1* and *TGIF1*. Although the genes identified



from this analysis need replication in independent cohorts, they provide some plausible novel candidates for further investigations into MTX response.

One association of considerable interest is the cystic fibrosis transmembrane conductance regulator gene, *CFTR*. The peak association signal for this region is within the 3' end of the downstream *CTTNBP2* gene (Figure 1b); this is interesting since it is known that *CFTR* expression is regulated by complex structural looping involving this region of *CTTNBP2* [35]. *CFTR*, also known as *ABCC7*, is a member of the ATP-binding cassette transporter superfamily, specifically the multi-drug resistance subfamily. These proteins are known to be important to drug transport and elimination [36]. Interestingly, a gene within the same subfamily, *ABCC3*, which is known to be involved in MTX efflux has recently been shown to contain a SNP (rs4793665) associated with MTX response in a cohort of 287 Dutch JIA patients [10]. This finding led us to specifically review this gene within our results despite it not fulfilling the selection criteria for Phase I or II. We found that rs4793665 was not directly genotyped in this study nor were there SNPs within  $r^2 = 0.8$  on the chip; however there were 38 SNPs within the introns/exons of *ABCC3* genotyped, with 15 showing association in our cohort at  $P < 0.05$  with the MTX response outcomes analysed (except ESR). The most associated of these, rs4148411 within an intron of *ABCC3*, was found in the PhysVAS analysis ( $P = 7.55 \times 10^{-5}$ ) and is in low LD with the SNP identified by de Rotte and colleagues ( $r^2 = 0.02$ ), suggesting that comprehensive further investigation of this gene is warranted.

Interestingly our study identifies several genes related to TGFbeta signalling as being associated with response to MTX. *ZMLZI* has been identified in several GWAS of autoimmune diseases [37-40]. It is a member of the protein inhibitor of activated STAT family, is known to regulate several transcription factors (androgen receptor, Smad3/4, p53) and TGFbeta/SMAD signalling, and is induced by retinoic acid [41]. It is well established that TGFbeta/SMAD and retinoic acid have important roles in the balance between Th17 and Treg cells [42], which are known to impact directly upon JIA severity [43]. Therefore a possible role for this gene in response to treatment in JIA is of considerable interest. Corroborating this finding, another associated region contains TGFbeta-induced factor homeobox 1 (*TGFI1*), known to be an active transcriptional corepressor of SMAD2 and to modulate the down-regulation of aryl hydrocarbon receptor (AhR) [44;45]. Together these results suggest TGFbeta signalling is a strong biological candidate for a role in reducing disease activity with MTX treatment. These data are of particular interest, since they directly parallel our gene expression profiling studies, which identified TGFbeta signalling, TGFB-2 and the zinc finger protein ZEB1, which interacts with SMAD signalling proteins, as being involved in response to MTX in children with JIA [14].

Four genetic regions associated with ACR-pedi status also showed associations with either ParVAS or PhysVAS. In some research studies, using the ACR-pedi status can present difficulties due to missing data observed in long-term observational cohorts. This finding suggests the ACR-pedi, ParVAS and PhysVAS scores measure MTX response similarly, leading to the possibility that both ParVAS and PhysVAS could be used to measure response to treatment, when full clinical data are unavailable.

To our knowledge, this is the first report of a large-scale genetic association study of MTX response in inflammatory arthritis: despite several international efforts in GWAS studies of JIA and RA as a whole, no previous large-scale analysis of MTX response are available to date, perhaps in part due to the considerable challenges of collecting adequate numbers of cases with detailed response data as well as DNA. A study investigating interferon-beta treatment in MS (using 53 responders and 53 non-responders), found that of the best associations most were in glutamate and interferon receptors, a cell-cycle dependent protein, and guanosine triphosphatase-activating and zinc-finger proteins, all genes not known to be directly involved in the drug metabolism pathway [46]. A recent GWAS in 706 RA patients treated with tocilizumab, a biologic therapy targeting the interleukin (IL)-6 receptor, found eight putative loci associated with tocilizumab efficacy; however none were in known RA risk or IL-6 pathways [18]. Similarly, our results suggest that multiple genes determine response to MTX treatment in JIA, and not just those in known MTX pathways. In fact, none of the MTX pathway genes previously investigated in candidate gene studies in both JIA and RA met our selection criteria for Phase II. This is possibly due to the small sample size and lack of power in previous studies resulting in false positive associations, and may be additionally confounded by the power limitations of our study including the availability of ACR-pedi scores on only a subset of our cohort. Despite this, the lack of strong association in the MTX pathway genes is interesting, and suggests that novel pathways and mechanisms, hitherto not known, may be important to pursue in order to understand and fully elucidate the actions of MTX and the genes involved in success or failure of MTX treatment [47]. It also suggests that the previously developed MTX efficacy prediction models for both RA [16] and JIA [9] could be further enhanced or further optimised by incorporating additional genetic variants outside the MTX pathway genes.

Previous investigations of the genetics of MTX response in JIA have been small, often underpowered, studies taking a candidate gene approach focussing on genes in MTX drug pathways. In contrast, this study is large and comprehensively covers the genome, the first of its kind for JIA. We have identified several regions of interest, three of which show a remarkable degree of functional overlap with genes and pathways implicated by gene expression profiling and previous candidate gene studies. By analysing each clinical outcome variable individually we show their genetic contributions to MTX response may differ, although with interesting overlap in novel candidates including *TGIF1*, *ZMIZ1* and *CFTR*. Future targeted replication of the exciting novel regions identified is now required to confirm these findings. This study provides an excellent basis for the future development of genetic risk models for MTX response prediction.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

## ACKNOWLEDGEMENTS

The authors thank all the patients and their families for participating in this study and members of the CHARMS and CAPS study groups and the CHARMS-JIA GWAS International Consortium, for collecting samples and data. We are grateful to Prof Gudrun Moore for scientific advice in the planning of the study and Mr N Evans and Dr Susan Thompson for insightful comments on the study. The SPARKS-CHARMS study was funded by SPARKS UK (08ICH09) and the Big Lottery Fund UK (RG/1/010135231) and is supported by the UK National Institutes for

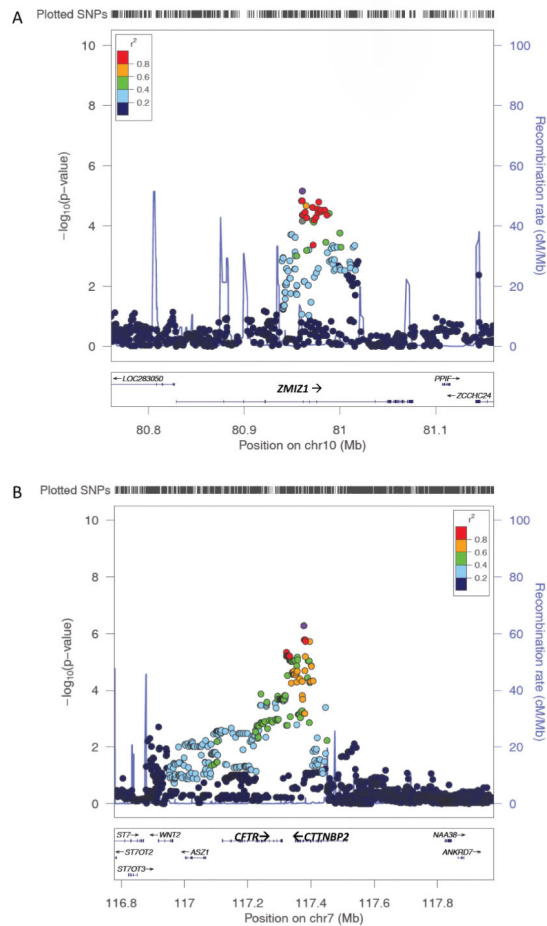
Heath research (NIHR) Medicines for Children Research Network. LW has support from the Great Ormond Street Hospital Children's Charity and Arthritis Research UK (20164) and the GOSH/UCL ICH NIHR Biomedical Research Centre. The CAPS study is funded by Arthritis Research UK (20542). PD has support from Charles University Grant Agency (GAUK 52608/2008). NW has support from Reumafonds (DAA 07-01-19). WT has support from Arthritis Research UK (17552). This report includes independent research supported by the National Institute for Health Research Biomedical Research Unit Funding Scheme. The views expressed in this publication are those of the authors and not necessarily those of the NHS, the National Institute for Health Research or the Department of Health.

## Reference List

- (1). Moorthy LN, Peterson MG, Hassett AL, Lehman TJ. Burden of childhood-onset arthritis. *Pediatr Rheumatol Online J*. 2010; 8:20. [PubMed: 20615240]
- (2). De K,I, Brinkman DM, Ferster A, Abinun M, Quartier P, Van Der NJ, et al. Autologous stem cell transplantation for refractory juvenile idiopathic arthritis: analysis of clinical effects, mortality, and transplant related morbidity. *Ann Rheum Dis*. Oct; 2004 63(10):1318–26. [PubMed: 15361393]
- (3). Magni-Manzoni S, Rossi F, Pistorio A, Temporini F, Viola S, Beluffi G, et al. Prognostic factors for radiographic progression, radiographic damage, and disability in juvenile idiopathic arthritis. *Arthritis Rheum*. Dec; 2003 48(12):3509–17. [PubMed: 14674002]
- (4). Nordal E, Zak M, Aalto K, Berntson L, Fasth A, Herlin T, et al. Ongoing disease activity and changing categories in a long-term nordic cohort study of juvenile idiopathic arthritis. *Arthritis Rheum*. Sep; 2011 63(9):2809–18. [PubMed: 21560116]
- (5). Wallace CA, Giannini EH, Spalding SJ, Hashkes PJ, O'Neil KM, Zeff AS, et al. Trial of early aggressive therapy in polyarticular juvenile idiopathic arthritis. *Arthritis Rheum*. Jun; 2012 64(6):2012–21. [PubMed: 22183975]
- (6). Ruperto N, Murray KJ, Gerloni V, Wulfraat N, de Oliveira SK, Falcini F, et al. A randomized trial of parenteral methotrexate comparing an intermediate dose with a higher dose in children with juvenile idiopathic arthritis who failed to respond to standard doses of methotrexate. *Arthritis Rheum*. Jul; 2004 50(7):2191–201. [PubMed: 15248217]
- (7). Wallace CA, Giannini EH, Huang B, Irtter L, Ruperto N. American College of Rheumatology provisional criteria for defining clinical inactive disease in select categories of juvenile idiopathic arthritis. *Arthritis Care Res (Hoboken)*. Jul; 2011 63(7):929–36. [PubMed: 21717596]
- (8). Ma Q, Lu AY. Pharmacogenetics, pharmacogenomics, and individualized medicine. *Pharmacol Rev*. Jun; 2011 63(2):437–59. [PubMed: 21436344]
- (9). Bulatovic M, Heijstek MW, Van Dijkhuizen EH, Wulfraat NM, Pluijm SM, de JR. Prediction of clinical non-response to methotrexate treatment in juvenile idiopathic arthritis. *Ann Rheum Dis*. May 10, 2012
- (10). de Rotte MC, Bulatovic M, Heijstek MW, Jansen G, Heil SG, van Schaik RH, et al. ABCB1 and ABCC3 gene polymorphisms are associated with first-year response to methotrexate in juvenile idiopathic arthritis. *J Rheumatol*. Oct; 2012 39(10):2032–40. [PubMed: 22859359]
- (11). Dervieux T, Wessels JA, Kremer JM, Padyukov L, Seddighzadeh M, Saevarsdottir S, et al. Patterns of interaction between genetic and nongenetic attributes and methotrexate efficacy in rheumatoid arthritis. *Pharmacogenet Genomics*. Jan; 2012 22(1):1–9. [PubMed: 22044941]
- (12). Hinks A, Moncrieffe H, Martin P, Ursu S, Lal S, Kassoumeri L, et al. Association of the 5-aminoimidazole-4-carboxamide ribonucleotide transformylase gene with response to methotrexate in juvenile idiopathic arthritis. *Ann Rheum Dis*. Aug; 2011 70(8):1395–400. [PubMed: 21515602]
- (13). Lee YC, Cui J, Costenbader KH, Shadick NA, Weinblatt ME, Karlson EW. Investigation of candidate polymorphisms and disease activity in rheumatoid arthritis patients on methotrexate. *Rheumatology (Oxford)*. Jun; 2009 48(6):613–7. [PubMed: 19193698]
- (14). Moncrieffe H, Hinks A, Ursu S, Kassoumeri L, Etheridge A, Hubank M, et al. Generation of novel pharmacogenomic candidates in response to methotrexate in juvenile idiopathic arthritis: correlation between gene expression and genotype. *Pharmacogenet Genomics*. Nov; 2010 20(11):665–76. [PubMed: 20827233]

- (15). Schmeling H, Biber D, Heins S, Horneff G. Influence of methylenetetrahydrofolate reductase polymorphisms on efficacy and toxicity of methotrexate in patients with juvenile idiopathic arthritis. *J Rheumatol. Sep*; 2005 32(9):1832–6. [PubMed: 16142884]
- (16). Wessels JA, van der Kooij SM, le CS, Kievit W, Barerra P, Allaart CF, et al. A clinical pharmacogenetic model to predict the efficacy of methotrexate monotherapy in recent-onset rheumatoid arthritis. *Arthritis Rheum. Jun*; 2007 56(6):1765–75. [PubMed: 17530705]
- (17). Malik F, Ranganathan P. Methotrexate pharmacogenetics in rheumatoid arthritis: a status report. *Pharmacogenomics. Feb*; 2013 14(3):305–14. [PubMed: 23394392]
- (18). Wang J, Bansal AT, Martin M, Germer S, Benayed R, Essioux L, et al. Genome-wide association analysis implicates the involvement of eight loci with response to tocilizumab for the treatment of rheumatoid arthritis. *Pharmacogenomics J. Jun*; 2013 13(3):235–41. [PubMed: 22491018]
- (19). Adib N, Hyrich K, Thornton J, Lunt M, Davidson J, Gardner-Medwin J, et al. Association between duration of symptoms and severity of disease at first presentation to paediatric rheumatology: results from the Childhood Arthritis Prospective Study. *Rheumatology (Oxford). Jul*; 2008 47(7):991–5. [PubMed: 18417527]
- (20). Petty RE, Southwood TR, Manners P, Baum J, Glass DN, Goldenberg J, et al. International League of Associations for Rheumatology classification of juvenile idiopathic arthritis: second revision, Edmonton, 2001. *J Rheumatol. Feb*; 2004 31(2):390–2. [PubMed: 14760812]
- (21). Nugent J, Ruperto N, Grainger J, Machado C, Sawhney S, Baildam E, et al. The British version of the Childhood Health Assessment Questionnaire (CHAQ) and the Child Health Questionnaire (CHQ). *Clin Exp Rheumatol. Jul*; 2001 19(4 Suppl 23):S163–S167. [PubMed: 11510323]
- (22). Giannini EH, Ruperto N, Ravelli A, Lovell DJ, Felson DT, Martini A. Preliminary definition of improvement in juvenile arthritis. *Arthritis Rheum. Jul*; 1997 40(7):1202–9. [PubMed: 9214419]
- (23). Patterson N, Price AL, Reich D. Population structure and eigenanalysis. *PLoS Genet. Dec.2006* 2(12):e190. [PubMed: 17194218]
- (24). Price AL, Patterson NJ, Plenge RM, Weinblatt ME, Shadick NA, Reich D. Principal components analysis corrects for stratification in genome-wide association studies. *Nat Genet. Aug*; 2006 38(8):904–9. [PubMed: 16862161]
- (25). Price AL, Weale ME, Patterson N, Myers SR, Need AC, Shianna KV, et al. Long-range LD can confound genome scans in admixed populations. *Am J Hum Genet. Jul*; 2008 83(1):132–5. [PubMed: 18606306]
- (26). Purcell S, Neale B, Todd-Brown K, Thomas L, Ferreira MA, Bender D, et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. *Am J Hum Genet. Sep*; 2007 81(3):559–75. [PubMed: 17701901]
- (27). Pruim RJ, Welch RP, Sanna S, Teslovich TM, Chines PS, Gliedt TP, et al. LocusZoom: regional visualization of genome-wide association scan results. *Bioinformatics. Sep 15*; 2010 26(18):2336–7. [PubMed: 20634204]
- (28). Delaneau O, Marchini J, Zagury JF. A linear complexity phasing method for thousands of genomes. *Nat Methods. Feb*; 2012 9(2):179–81. [PubMed: 22138821]
- (29). Howie BN, Donnelly P, Marchini J. A flexible and accurate genotype imputation method for the next generation of genome-wide association studies. *PLoS Genet. Jun.2009* 5(6):e1000529. [PubMed: 19543373]
- (30). Martin P, Barton A, Eyre S. ASSIMILATOR: a new tool to inform selection of associated genetic variants for functional studies. *Bioinformatics. Jan 1*; 2011 27(1):144–6. [PubMed: 21177990]
- (31). Ellis JA, Munro JE, Chavez RA, Gordon L, Joo JE, Akikusa JD, et al. Genome-scale case-control analysis of CD4+ T-cell DNA methylation in juvenile idiopathic arthritis reveals potential targets involved in disease. *Clin Epigenetics. 2012*; 4(1):20. [PubMed: 23148518]
- (32). Veyrieras JB, Kudaravalli S, Kim SY, Dermitzakis ET, Gilad Y, Stephens M, et al. High-resolution mapping of expression-QTLs yields insight into human gene regulation. *PLoS Genet. Oct.2008* 4(10):e1000214. [PubMed: 18846210]
- (33). Beresford MW. Juvenile idiopathic arthritis: new insights into classification, measures of outcome, and pharmacotherapy. *Paediatr Drugs. Jun 1*; 2011 13(3):161–73. [PubMed: 21500870]

- (34). Beukelman T, Patkar NM, Saag KG, Tolleson-Rinehart S, Cron RQ, DeWitt EM, et al. 2011 American College of Rheumatology recommendations for the treatment of juvenile idiopathic arthritis: initiation and safety monitoring of therapeutic agents for the treatment of arthritis and systemic features. *Arthritis Care Res (Hoboken)*. Apr; 2011 63(4):465–82. [PubMed: 21452260]
- (35). Ott CJ, Blackledge NP, Kerschner JL, Leir SH, Crawford GE, Cotton CU, et al. Intronic enhancers coordinate epithelial-specific looping of the active CFTR locus. *Proc Natl Acad Sci U S A*. Nov 24; 2009 106(47):19934–9. [PubMed: 19897727]
- (36). Conseil G, Deeley RG, Cole SP. Polymorphisms of MRP1 (ABCC1) and related ATP-dependent drug transporters. *Pharmacogenet Genomics*. Aug; 2005 15(8):523–33. [PubMed: 16006996]
- (37). Ellinghaus D, Ellinghaus E, Nair RP, Stuart PE, Esko T, Metspalu A, et al. Combined analysis of genome-wide association studies for Crohn disease and psoriasis identifies seven shared susceptibility loci. *Am J Hum Genet*. Apr 6; 2012 90(4):636–47. [PubMed: 22482804]
- (38). Franke A, McGovern DP, Barrett JC, Wang K, Radford-Smith GL, Ahmad T, et al. Genome-wide meta-analysis increases to 71 the number of confirmed Crohn's disease susceptibility loci. *Nat Genet*. Dec; 2010 42(12):1118–25. [PubMed: 21102463]
- (39). Imielinski M, Baldassano RN, Griffiths A, Russell RK, Annese V, Dubinsky M, et al. Common variants at five new loci associated with early-onset inflammatory bowel disease. *Nat Genet*. Dec; 2009 41(12):1335–40. [PubMed: 19915574]
- (40). Patsopoulos NA, Esposito F, Reischl J, Lehr S, Bauer D, Heubach J, et al. Genome-wide meta-analysis identifies novel multiple sclerosis susceptibility loci. *Ann Neurol*. Dec; 2011 70(6):897–912. [PubMed: 22190364]
- (41). Li X, Thyssen G, Beliakoff J, Sun Z. The novel PIAS-like protein hZimp10 enhances Smad transcriptional activity. *J Biol Chem*. Aug 18; 2006 281(33):23748–56. [PubMed: 16777850]
- (42). Mucida D, Park Y, Kim G, Turovskaya O, Scott I, Kronenberg M, et al. Reciprocal TH17 and regulatory T cell differentiation mediated by retinoic acid. *Science*. Jul 13; 2007 317(5835):256–60. [PubMed: 17569825]
- (43). Nistala K, Wedderburn LR. Th17 and regulatory T cells: rebalancing pro- and anti-inflammatory forces in autoimmune arthritis. *Rheumatology (Oxford)*. Jun; 2009 48(6):602–6. [PubMed: 19269955]
- (44). Pessah M, Prunier C, Marais J, Ferrand N, Mazars A, Lallemand F, et al. c-Jun interacts with the corepressor TG-interacting factor (TGIF) to suppress Smad2 transcriptional activity. *Proc Natl Acad Sci U S A*. May 22; 2001 98(11):6198–203. [PubMed: 11371641]
- (45). Wolff S, Harper PA, Wong JM, Mostert V, Wang Y, Abel J. Cell-specific regulation of human aryl hydrocarbon receptor expression by transforming growth factor-beta(1). *Mol Pharmacol*. Apr; 2001 59(4):716–24. [PubMed: 11259615]
- (46). Comabella M, Craig DW, Morcillo-Suarez C, Rio J, Navarro A, Fernandez M, et al. Genome-wide scan of 500,000 single-nucleotide polymorphisms among responders and nonresponders to interferon beta therapy in multiple sclerosis. *Arch Neurol*. Aug; 2009 66(8):972–8. [PubMed: 19667218]
- (47). Daly AK. Genome-wide association studies in pharmacogenomics. *Nat Rev Genet*. Apr; 2010 11(4):241–6. [PubMed: 20300088]



**Figure 1.**

A) Linear regression analysis of *ZMIZ1* (region 20) for the change in ESR with MTX treatment, with the top hit in this region rs2802369 coloured purple. Similar results were seen for the ParVAS, CHAQ, and ACR-pedi analyses of this region. B) Linear regression analysis of *CFTR-CTTNBP2* (region 12) for parent's global assessment (ParVAS), with the top hit in this region rs757278 coloured purple. Similar results were seen for the ACR-pedi and LJC analyses of this region. Coordinates are based on the NCBI36 assembly.

**Table 1**  
Demographics of CHARMS-JIA GWAS International Consortium individuals included in this analysis by sample centre/country.

Sample Centre/Country	N	Gender		Age at onset (median years (IQR))	Time to treatment from JIA onset (median months (IQR))	ILAR Subtype (N (% of total per centre))							Unclassifiable or unknown
		Female (%)	Male (%)			Persistent oligo	Extended oligo	RF- poly	RF+ poly	Systemic	Psoriatic	Enthesitis related	
UK	501	340 (67.9)	161 (32.1)	4.6 (2.1-8.7)	9.5 (4.1-28.1)	58 (11.6)	110 (22)	180 (34.1)	26 (7)	55 (11)	20 (4)	26 (5.2)	26 (5.2)
Prague	20	10 (50.0)	10 (50.0)	5.2 (2.4-9.9)	3.1 (0.9-6.8)	0 (0)	0 (0)	14 (70)	0 (0)	2 (10)	3 (15)	1 (5)	0 (0)
Utrecht	173	109 (63.0)	64 (37.0)	5.3 (2.7-10.0)	16.1 (5.8-50.4)	51 (29.5)	31 (17.9)	47 (27.2)	10 (5.8)	12 (6.9)	9 (5.2)	9 (5.2)	4 (2.3)
TOTAL	694	495 (66.1)	235 (33.9)	4.8 (2.1-9.0)	9.8 (4.2-30.2)	109	141	241	36	69	32	36	30

**Table 2**

Summary of the most highly associated regions identified in phase I of analysis, indicating the genes nearby each region and the total number of SNPs associated at  $P < 0.001$  and for which analysis. This formed the basis of the regions selected for SNP imputation in Phase II (some nearby regions were merged and others were expanded to comply with the minimum size requirements in IMPUTE2). Region coordinates based on the NCBI37 assembly. See Supplementary Table 2 for full results.

Region	Chr	Region start (bp)	Region end (bp)	Nearby genes	Number of SNPs in region (across all analyses)	Analyses with associated SNPs (at $P < 0.001$ )								
						ESR	CHAO	Phys VAS	Par VAS	AJC	LIC	ACR-pedi		
1	1	162918233	163039777	C1orf110, RGS4	7	x			x					x
2	1	165103951	165103971	LMX1A, PBX1	3			x		x				x
3	1	209121677	209420098	PLXNA2	13		x		x					x
4	3	115447624	115523299	GAP43, LSAMP	8			x			x			x
5	3	126099612	126175401	CCDC37, KLF15, ZXDC	13		x		x		x			x
6	3	144100011	145662790	C3orf58, PLOD2	15		x		x		x		x	
7	5	30284836	31185714	CDH6	10				x				x	
8	5	153846868	166676216	HAND1, SAP30L, LARPI, SGCD, KID43, HAVCR2, THG1L, ADRA1B, GABRG2, CCNG1, MAT2B, ODZ2	27		x		x		x		x	
9	7	52018700	53011628	POM121L12	14				x			x		
10	7	77663087	78947912	MAGI2	13		x						x	
11	7	94932904	94948028	PONI	25						x			x
12	7	117261293	117517293	CFTR, CTTNBP2, LSM8	20				x				x	
13	7	122057923	122520343	CADPS2	3					x				x
14	8	2520024	6082233	CSMD1	19		x		x		x		x	
15	8	76462383	77366608	HNF4G	6				x					x
16	8	82892518	85802488	RALYL, SNX16	19					x			x	
17	8	129703419	129884159	PVT1	15								x	
18	9	81265380	84141525	PSATI, TLE4, TLE1	5				x					x
19	10	29900664	30286263	KIAA1462, SVIL	16				x				x	
20	10	80872270	80999869	ZMIZ1	22				x				x	
21	10	130778452	131633463	MGMT, MKI67, EBF3	19					x				x
22	11	97748769	100456604	CNTN5, JRKL	10						x			x
23	11	101771433	101806639	ANGPTL5, KIAA1377	4								x	



Region	Chr	Region start (bp)	Region end (bp)	Nearby genes	Number of SNPs in region (across all analyses)	Analyses with associated SNPs (at $P < 0.001$ )								
						ESR	CHAQ	Phys VAS	Par VAS	AJC	LIC	ACR-pedi		
24	12	101843240	101856596	ARL1, SPIC	4	x					x			x
25	12	104048454	109147745	STAB2, CHST11, C12orf75, NUAK1, POLR3B, RFX4, BTBD11, CMKLR1, FICD, CORO1C, SSH1	20	x	x	x	x		x			x
26	13	101597058	101840684	NALCN, TMTC4	11	x								x
27	15	26373375	27462334	GABRB3, GABRG3	17			x	x		x			x
28	18	3388779	3405415	MYL12B, TGIF1	5						x			x
29	20	58951428	60326201	CDH4	10			x	x					x
30	22	37581485	37763862	CIQTNF6, CYTH4, RAC2, ELFN2	8	x					x			x
31	22	39395612	40083730	APOBEC3B, APOBEC3C, CACNA1I, SYNGRI	14		x	x	x		x			x

**Table 3**

Summary of most highly associated Phase II results (including imputed genotypes<sup>#</sup>). The total number of SNPs associated at  $P < 1 \times 10^{-5}$  and the most associated SNP for each analysis are listed (including the best result for core set variables also associated in this region that are  $P < 1 \times 10^{-4}$ ) for each of the 14 Phase II regions that contain at least one association at  $P < 1 \times 10^{-5}$ . Region numbers are carried over from Phase I analysis (Table 2). Region coordinates based on the NCBI37 assembly. N indicates number of successfully genotyped samples at each SNP.

Region	Total number of SNPs at $P < 1 \times 10^{-5}$	Best SNP	CHR	Position (bp)	Minor Allele	Analysis	N	BETA	SE	95% CI	P value	Genes within Region
2	6	rs60624478 <sup>#</sup> rs60950492 <sup>#</sup>	1	165107979 165110832	A G	PhysVAS ACR-pedi	413 369	1.17 -0.87	0.26 0.21	(0.67,1.67) (-1.29,-0.45)	$5.66 \times 10^{-6}$ $4.05 \times 10^{-5}$	LMX1A, PBX1
7	4	rs75104673 <sup>#</sup> rs12652364 <sup>#</sup>	5	29283857 30836079	C A	ESR PhysVAS	520 415	-16.89 -1.45	3.8 0.29	(-24.34,-9.44) (-2.02,-0.89)	$1.09 \times 10^{-5}$ $6.38 \times 10^{-7}$	CDH6
12	31	rs757278 <sup>#</sup> rs7800668 <sup>#</sup> rs115255079 <sup>#</sup>	7	117377645 117459043 117528054	T C C	ParVAS ACR-pedi LJC	364 373 587	1.32 -1.34 -2.68	0.26 0.3 0.67	(0.81,1.82) (-1.92,-0.76) (-3.99,-1.36)	$5.37 \times 10^{-7}$ $5.90 \times 10^{-6}$ $7.28 \times 10^{-5}$	CFTR, CTTNBP2
14	17	rs73185595 <sup>#</sup> rs4395908 <sup>#</sup> rs1512817	8	3116686 4025644 5644933	A C A	PhysVAS CHAQ AIC	415 367 620	0.84 -0.27 -1.88	0.2 0.05 0.45	(0.46,1.23) (-0.38,-0.16) (-2.77,-1)	$2.06 \times 10^{-5}$ $1.03 \times 10^{-6}$ $3.55 \times 10^{-5}$	CSMD1
16	20	rs10113213 <sup>#</sup> rs7839040 <sup>#</sup> rs139456570 <sup>#</sup>	8	82888608 82894306 82899934	T G C	AJC LJC PhysVAS	604 604 415	-1.99 -1.81 -0.92	0.43 0.38 0.22	(-2.83,-1.16) (-2.54,-1.07) (-1.34,-0.49)	$3.34 \times 10^{-6}$ $1.94 \times 10^{-6}$ $2.74 \times 10^{-5}$	SNX16
17	18	rs144444502 <sup>#</sup> rs10956445 <sup>#</sup> rs3829210	8	129773535 129787976 132054152	A C G	LJC AJC ParVAS	589 610 366	-2.27 -2.73 1.01	0.5 0.54 0.23	(-3.25,-1.29) (-3.79,-1.68) (0.55,1.47)	$6.44 \times 10^{-6}$ $4.82 \times 10^{-7}$ $2.07 \times 10^{-5}$	PVT1, ADCY8
20	4	rs10128264 <sup>#</sup> rs2802369 <sup>#</sup> rs942794 <sup>#</sup>	10	80959973 80960828 80965109	C C T	ParVAS ESR ACR-pedi	358 523 371	-1.18 -9.37 0.67	0.24 2.06 0.15	(-1.64,-0.71) (-13.41,-5.33) (0.37,0.96)	$1.07 \times 10^{-6}$ $6.93 \times 10^{-6}$ $1.06 \times 10^{-5}$	ZMIZ1

Region	Total number of SNPs at $p < 1 \times 10^{-5}$	Best SNP	CHR	Position (bp)	Minor Allele	Analysis	N	BETA	SE	95% CI	P value	Genes within Region
		rs7922505#	10	82946940	C	CHAQ	381	0.24	0.06	(0.12,0.35)	$9.41 \times 10^{-5}$	
23	1	rs11225055	11	101771433	C	AJC	620	-3.25	0.78	(-4.79,-1.71)	$3.94 \times 10^{-5}$	ANGPTL5, KIAA1377
		rs11225055	11	101771433	C	LJC	607	-3.49	0.72	(-4.9,-2.09)	$1.43 \times 10^{-6}$	
24	2	rs4536282	12	993498282	A	PhysVAS	415	0.97	0.23	(0.51,1.43)	$4.35 \times 10^{-5}$	
		rs66861122#	12	101383958	G	ParVAS	354	1.82	0.39	(1.05,2.6)	$5.33 \times 10^{-6}$	ANKS1B, ANO4, ARL1, SPIC
		rs59420447#	12	101842391	C	ACR-pedi	372	-0.75	0.18	(-1.10,-0.39)	$3.56 \times 10^{-5}$	
25	5	rs313315#	12	104965584	C	PhysVAS	410	-0.745	0.18	(-1.09,-0.4)	$3.21 \times 10^{-5}$	
		rs10507177	12	105008908	C	AJC	620	-1.88	0.44	(-2.74,-1.01)	$2.72 \times 10^{-5}$	CMKLR1
		rs11113349#	12	107879066	T	ESR	505	-9.66	2.18	(-13.94,-5.39)	$1.16 \times 10^{-5}$	
		rs11113818	12	108717929	T	ParVAS	366	1.16	0.25	(0.67,1.64)	$3.84 \times 10^{-6}$	
27	11	rs61996546#	15	26804131	C	PhysVAS	413	-0.78	0.16	(-1.11,-0.46)	$2.88 \times 10^{-6}$	GABRB3
		rs8030011	15	26818362	A	LJC	607	-2.48	0.56	(-3.59,-1.38)	$1.30 \times 10^{-5}$	
28	3	rs9954608	18	3388779	G	AJC	620	1.73	0.43	(0.89,2.56)	$5.69 \times 10^{-5}$	TGIF1
		rs6506122#	18	3394449	C	LJC	595	1.82	0.39	(1.05,2.59)	$4.70 \times 10^{-6}$	
30	1	rs229527	22	37581485	A	AJC	620	-1.70	0.42	(-2.53,-0.88)	$5.96 \times 10^{-5}$	
		rs10084630#	22	37679487	G	ParVAS	360	1.13	0.25	(0.64,1.62)	$9.09 \times 10^{-6}$	CYTH4
		rs739189	22	37753999	A	ESR	523	8.59	2.16	(4.35,12.83)	$8.23 \times 10^{-5}$	
31	11	rs2294369#	22	40075400	A	AJC	612	2.77	0.51	(1.77,3.77)	$8.59 \times 10^{-8}$	CACNA II