Integrative genomic analysis of methylphenidate response in attention-deficit/hyperactivity disorder

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Methylphenidate (MPH) is the most frequently used pharmacological treatment in children with attention-deficit/hyperactivity disorder (ADHD). However, a considerable interindividual variability exists in clinical outcome. Thus, we performed a genome-wide association study of MPH efficacy in 173 ADHD paediatric patients. Although no variant reached genome-wide significance, the set of genes containing single-nucleotide polymorphisms (SNPs) nominally associated with MPH response ($P < 0.05$) was significantly enriched for candidates previously studied in ADHD or treatment outcome. We prioritised the nominally significant SNPs by functional annotation and expression quantitative trait loci (eQTL) analysis in human brain, and we identified 33 SNPs tagging cis-eQTL in 32 different loci (referred to as eSNPs and eGenes, respectively). Pathway enrichment analyses revealed an over-representation of genes involved in nervous system development and function among the eGenes. Categories related to neurological diseases, psychological disorders and behaviour were also significantly enriched. We subsequently meta-analysed the association with clinical outcome for the 33 eSNPs across the discovery sample and an independent cohort of 189 ADHD adult patients (target sample) and we detected 15 suggestive signals. Following this comprehensive strategy, our results provide a better understanding of the molecular mechanisms implicated in MPH treatment effects and suggest promising candidates that may encourage future studies.

Attention-deficit/hyperactivity disorder (ADHD) is a neurodevelopmental disorder characterised by persistent and age-inappropriate symptoms of inattention, hyperactivity and/or impulsivity, which significantly impacts on academic, social, emotional and psychological functioning. With a worldwide prevalence ranging from 5.3 to 7.1% in school-age children and adolescents, ADHD is one of the most common childhood...
psychiatric conditions and causes high costs to the healthcare system and society. Although its aetiology is largely unknown, several family, twin and adoption studies reported heritability estimates around 76%, suggesting a strong genetic component in the pathogenesis of the disorder.

Among the wide variety of pharmacological options available in ADHD treatment, methylphenidate (MPH) is the first-line choice in paediatric patients, given its proved general efficacy in reducing ADHD symptoms and improving neuropsychological performance on executive functions. However, a considerable interindividual variability exists in clinical outcome, optimal dosage and duration of effect, which may reflect underlying genetic influences.

Most of the pharmacogenetic studies conducted so far in ADHD patients have focused on genes related to the catecholamine neurotransmission, with SLC6A3 and DRD4 being the most extensively investigated, since MPH is thought to exert its therapeutic effects through the inhibition of the dopamine and the norepinephrine transporters. Based on this putative mechanism of action, additional genes such as DRD2, DRD5, COMT, SLC6A2, ADRA2A, TPH2, SLC6A4, HTR1B, HTR2A and MAOA have been considered plausible candidates that may influence medication response. Nevertheless, a recent review on ADHD pharmacogenetics in childhood reported no consistent effects for dopaminergic and serotonergic signaling, and suggested neurodevelopmental genes as new promising targets.

Given that candidate gene-based investigations have not reached many compelling results, genome-wide association studies (GWAS) may represent an alternative, hypothesis-free approach to unravel the molecular mechanisms implicated in MPH treatment. To date, only one prior GWAS evaluated the efficacy of a MPH transdermal system in 187 children with ADHD. Although no genome-wide significant associations were found, the metabolotropic glutamate receptor 7 (GRM7) and two SNPs within the SLC6A2 gene showed potential involvement in MPH response. Using that sample, Mick et al. conducted a secondary GWAS of changes in blood pressure after MPH therapy and detected nominal evidence for genes functionally related to blood pressure regulation and other cardiovascular phenotypes, including a SNP in a K+-dependent Na+/Ca2+ exchanger (SLC24A3). Furthermore, despite the fact that GWAS have been useful to identify genetic risk loci for multiple complex conditions, yet the functional effects of the trait-associated variants and the underlying pathological mechanisms remain mainly elusive.

Based on the absence of clear conclusions regarding MPH response raised by previous genetic studies, we undertook a GWAS of MPH efficacy in 173 ADHD paediatric patients and, for the first time to our knowledge, we integrated data from functional annotation, expression quantitative trait loci (eQTL) and enrichment analyses to characterise the biological pathways associated with treatment response. Additionally, we performed a polygenic risk score analysis and a meta-analysis across the study sample and an independent population of 189 ADHD adult patients.

Materials and Methods

Discovery population. The study sample included 173 ADHD paediatric patients for whom MPH was prescribed. Subjects were required to satisfy full DSM-IV criteria for ADHD, be under 18 years of age, Spanish of Caucasian origin and have never received MPH treatment. Patients with an IQ below 70 or having pervasive developmental disorders were not eligible for the investigation. Additional exclusion criteria included schizophrenia or other psychotic disorders; adoption; sexual or physical abuse; birth weight <1.5 kg; any significant neurological or systemic disease that might explain ADHD symptoms; and clinical contra-indication to MPH. Comorbid oppositional defiant disorder, conduct disorder, depression and anxiety disorders were allowed unless determined to be the primary cause of ADHD symptomatology. The study was approved by the Ethics Committee of the Hospital Universitari Vall d’Hebron and all methods were performed in accordance with the relevant guidelines and regulations. Written informed consent was obtained from parents/caregivers.

Clinical assessment. Diagnoses of ADHD and comorbidities were established by child psychiatrists blind to patients’ genotypes through the Present and Lifetime version of the Kiddie Schedule for Affective Disorders and Schizophrenia for School-Age Children (K-SADS-PL). Furthermore, families were interviewed with the Clinical Global Impression-Severity scale (CGI-S). Additional information on clinical assessment is available elsewhere.

Pharmacological intervention. Patients were treated according to the program’s recommendations of initiating treatment with MPH at low to moderate dose and titrating to higher doses until no further clinical improvement or limiting adverse effects were observed. The mean daily dose of MPH prescribed was 1.06 mg/kg (SD = 0.28). Risperidone was the most frequent concomitant drug.

Treatment outcome. We considered the Clinical Global Impression-Improvement scale (CGI-I), which ranges from 1 (‘very much improved’) to 7 (‘very much worse’), as the primary outcome measure of treatment success. Those patients rated with a CGI-I score of two points or less after eight weeks of treatment were considered as responders, while the remaining were classified as non-responders.

Genome-wide association study. Genomic DNA was isolated from peripheral blood leukocytes by a salting out procedure. A total of 173 samples were genotyped on the Infinium PsychArray-24 BeadChip platform (Illumina, San Diego, CA, USA), which covers 588,628 markers, and processed at the Stanley Center for Psychiatric Research, Broad Institute of MIT and Harvard (Cambridge, MA, USA). Pre-imputation quality control and principal components analysis were implemented following the QC and PCA modules from the R package with the default settings. Genotype imputation was performed with the pre-phasing and imputation strategy using the EUR population of the 1,000 Genomes Project Phase 1 dataset as the reference panel. We assured the accuracy of the imputation...
data by filtering best-guess genotypes for an info score < 0.3. This resulted in a total of 11,051,824 markers eligible for association tests.

Before GWAS analysis, further quality control measures were applied using the PLINK software\textsuperscript{18}. Individuals exhibiting high rates of genotype missingness (> 98%) were removed, as well as SNPs with low call rate (< 0.99), MAF < 0.01 or failing Hardy-Weinberg equilibrium test (P < 1e-06).

Finally, 173 subjects and 3,566,199 variants were tested for association with MPH response through logistic regression under an additive model, which included those clinical variables (i.e., CGI-S baseline scores) and principal components (i.e., PC6) significantly associated with clinical outcome (P ≤ 0.05) as covariates.

Identification of candidate causal SNPs. Among the SNPs showing nominal association with treatment outcome (P < 0.05), we used the genome pipeline of SNPInfo (http://snpinfo.niehs.nih.gov/)\textsuperscript{19} to prioritise those that were more likely to affect protein sequence, transcriptional regulation, mRNA splicing or miRNA binding based on functional annotation. GenomePipe parameter values included: GWAS population = CEU; study population = CEU; flanking region = 200,000 bp; GWAS P-value < 0.05; LD threshold = 0.8; and MAF = 0.01 for all prediction methods. Additionally, we combined both the predicted conserved transcription factor-binding sites (TFBS) with the regulatory potential score (RP Score; available at http://genome.ucsc.edu) to improve predictions as suggested in several studies\textsuperscript{20-22}.

Cis-expression quantitative trait loci analysis. Cis-eQTL analysis was conducted on 193 neuropathologically normal cortical samples of adult humans from Myers et al.\textsuperscript{23}. Expression-genotype pairs were extracted after extending the genotyped data by imputation as previously described, and considering a 10 kb window around the untranslated regions. Rank-invariant normalised expression levels were log\textsubscript{2} transformed and transcripts detected in less than 75% of the samples were discarded from the study. Association tests were performed under a linear model with the MatrixEQTl R Package\textsuperscript{24}, including gender, age at death, cortical region, day of expression hybridisation, institute source of sample, post-mortem interval and transcripts detected rate in each sample as covariates.

Functional and pathway enrichment analysis. The biological functions and pathways related to genes containing at least one SNP nominally associated with both MPH response and human cortical expression levels (referred to as eSNPs) were assessed through the Ingenuity Pathway Analysis software (IPA) (Ingenuity Systems, Redwood City, CA, USA; www.ingenuity.com). IPA was also used to test for over-representation of genes previously studied in either ADHD or treatment outcome. Candidate genes for ADHD or MPH response were selected based on the gene list provided by the ADHDgene database (http://adhd.psych.ac.cn/index.do)\textsuperscript{25} and a comprehensive search for published reviews of ADHD genetic and pharmacogenetic studies\textsuperscript{11,12,26–31}. Thus, a total of 436 genes were considered (Supplementary Table S1). Fisher’s exact tests, with a Benjamini-Hochberg-adjusted P-value (P\textsubscript{T,0.1}) < 0.05 as significance threshold, were applied in all analyses. To achieve meaningful statistics and interpretation of the results, we restricted the enrichment analysis to those annotation terms that included ≥ 2 genes of our dataset.

Polygenic risk score analysis. We generated polygenic risk scores (PRS) based on the results of the present GWAS using the Polygenic Risk Score software (PRSice)\textsuperscript{32}. A logistic regression model was applied to test whether PRS at multiple stepwise P-value thresholds (i.e., P\textsubscript{T} < 1e-04, P\textsubscript{T} < 1e-03, P\textsubscript{T} < 0.05, P\textsubscript{T} < 0.1, P\textsubscript{T} < 0.2, P\textsubscript{T} < 0.3, P\textsubscript{T} < 0.4, and P\textsubscript{T} < 0.5) predicted treatment outcome in an independent sample of patients with ADHD (target population). The target population comprised 189 Brazilian adults from the Adult ADHD Outpatient Clinic of the Hospital de Clínicas de Porto Alegre, who underwent immediate-release MPH treatment. Diagnoses of ADHD and comorbidities, as well as inclusion/exclusion criteria, were achieved as previously described\textsuperscript{33}. The outcome measures of MPH treatment were the CGI-S scale, applied before medication and at least four weeks after its beginning, and the CGI-I scale. Drug response was defined following the criteria used in the discovery sample. Similarly, samples were genotyped and imputed using the same platform, imputation protocol and reference panel. The resulting dataset consisted of 7,304,149 SNPs with an info score > 0.6, a genotype call probability > 0.8 and a missing rate < 0.02.

Potential confounders were included as covariates in the PRS model if they were associated with MPH response (P ≤ 0.05) in the target sample (i.e., CGI-S baseline scores, use of concomitant medication and presence of phobia as comorbid condition), as well as the 10 first principal components to control for population stratification.

Meta-analysis. The eSNPs nominally associated with MPH response in the discovery sample were meta-analysed across the discovery and the target population used in the PRS analysis by the inverse-variance weighted method. The threshold for significance was set at P ≤ 1.52e-03 under the more conservative Bonferroni correction, taking into account 33 SNPs.

Data availability. The datasets generated and/or analysed during the current study are not publicly available due to ethics constraints but are available from the corresponding author on reasonable request.

Results

Genome-wide association study in the discovery population. Subjects were predominantly male (84.4%), with an average age at assessment of 9.59 (SD = 2.91) years (range 5–17). One hundred and thirty-one participants (75.7%) met DSM-IV criteria for ADHD-combined subtype, 37 (21.4%) had ADHD-inattentive subtype and 5 (2.9%) were diagnosed with ADHD-hyperactive-impulsive subtype. Comorbid disorders were present in a modest number of patients (22.5%), the main ones being disabilities in reading and writing (12.7%),
oppositional defiant disorder (5.8%) and tic disorders (1.7%). One hundred and forty-one subjects (81.5%) responded favourably to treatment according to the CGI-I scale, while 32 (18.5%) failed to show a clinical response to MPH. Responders and non-responders were comparable with regard to age, sex, ADHD subtype, comorbidity, use of concomitant medication, MPH dose and drug formulation (P > 0.05). There were significant differences, however, in the severity of symptoms as assessed by the CGI-S scale (P < 1e-03), with children resistant to MPH scoring higher at the baseline evaluation than children showing clinical improvement (Supplementary Table S2).

No variant reached genome-wide significance (P < 5e-08). However, the set of 4,709 genes containing SNPs nominally associated with MPH response (P < 0.05; Supplementary Table S3) was significantly enriched for candidates previously studied in ADHD or treatment outcome, with 199 out of 436 being present in this category (ratio = 0.46; P_{BH} = 1.56e-31).

Identification of candidate causal SNPs and cis-expression quantitative trait loci analysis. Considering these results, we prioritised the SNPs with P-values below 0.05 based on functional annotation and eQTL analysis rather than focusing on the top significant hits. Eight hundred and ninety-six independent markers were selected as candidate causal variants by functional annotation (Supplementary Table S4) and were subjected to further cis-eQTL analysis on a pre-existing dataset of 193 neuropathologically normal human cortical samples. After imputation and quality control, a total of 284 variants and 300 genes with detectable expression levels in at least 75% of the samples were available for 146 individuals. Of these, we identified 33 SNPs tagging cis-eQTL in 32 different loci (referred to as eGenes), with eight SNP-gene pairs surpassing the 0.05 false discovery rate (FDR) threshold: rs1230279-SP8B2, P_{FDR} = 1.13e-05; rs10611113-PTPRD2, P_{FDR} = 2.17e-04; rs2071421-ARSA, P_{FDR} = 7.26e-04; rs11553441-PDHB2, P_{FDR} = 7.26e-04; rs17297558-GGH, P_{FDR} = 0.013; rs9901673-SEN3P, P_{FDR} = 0.023; and rs17685420-PERP4, P_{FDR} = 0.041 (Table 1).

Functional and pathway enrichment analysis. The set of 32 eGenes included three candidates previously investigated in ADHD, namely ALDH1L1, CDH23 and CMTM8 (ratio = 0.007; P_{BH} = 0.023), and showed over-representation of genes implicated in abnormal morphology of molecular layer of cerebellum (P_{BH} = 0.012), abnormal morphology of white matter (P_{BH} = 0.012), morphology of axons (P_{BH} = 0.012), morphology and length of neurites (P_{BH} = 0.021 and P_{BH} = 0.022, respectively), coordination (P_{BH} = 0.022), and formation of hippocampus (P_{BH} = 0.033). Interestingly, categories related to neurological diseases, psychological disorders and behaviour were also significantly enriched, including learning deficit (P_{BH} = 0.012), hyperactive behaviour (P_{BH} = 0.015) and spatial learning (P_{BH} = 0.018) (Table 2).

Polygenic risk score analysis and meta-analysis using the target population. Finally, in order to assess the predictive value of our findings we first computed PRS derived from the present GWAS in an independent sample of ADHD adult patients for whom data on response to MPH were available. The demographic and clinical characteristics of the target population according to the response status are presented in Supplementary Table S5. Briefly, 85.2% of subjects (n = 161) were classified as responders, while 14.8% (n = 28) exhibited a reduced or lack of improvement. Responders and non-responders significantly differed with regard to CGI-S baseline scores, use of concomitant medication and presence of phobia as comorbid condition, and thus these additional risk factors were entered as covariates in the PRS model, as well as the 10 first principal components to control for population stratification. Since we did not detect significant results at any of the predefined P-value thresholds, we subsequently focused on the 33 eSNPs nominally associated with treatment outcome in the discovery sample and we increased statistical power by performing a meta-analysis across the discovery and the target population. Sixteen suggestive signals were identified (Table 3). Among them, 15 revealed the same direction of effect, with rs17685420 in the PERP4 gene being significant after Bonferroni correction (OR = 3.07 (1.76–5.35), P = 7.90e-05), followed by additional compelling markers such as rs2071421 within ARSA (OR = 2.63 (1.29–5.37), P = 7.71e-03), rs2886059 in ALDH1L1 (OR = 2.30 (1.14–4.66), P = 0.020), and rs17712523 in CDH23 (OR = 2.13 (1.07–4.24), P = 0.031).

Discussion

To our knowledge, this is the first study investigating the genetic basis of MPH response from an integrative perspective that combines GWAS data, functional annotation, eQTL in relevant tissues to ADHD and pathway enrichment analyses. Our results highlight genes related to nervous system development and function, neurological diseases and psychological disorders. Thus, this comprehensive strategy provides a better understanding of the molecular mechanisms implicated in MPH treatment effects and suggests promising candidates that may contribute to clinical outcome.

In our attempt to improve earlier genetic studies by bridging the gap between genotype and phenotype, we prioritised the nominally significant SNPs based on functional annotation and cis-eQTL analysis in human brain, and we identified three candidates previously investigated in ADHD: ALDH1L1, CDH23 and CMTM8. Of these, CMTM8 showed overlapping association between adult ADHD and bipolar disorder, and ALDH1L1, which yielded suggestive results in the present meta-analysis of MPH response, has been related to other neuropsychiatric conditions such as major depressive disorder or schizophrenia. In addition, the CDH23 harbour one of the top SNPs from a pooling-based GWAS of adult ADHD and reached nominal significance in our meta-analysis. CDH23 is a member of the cadherin superfamily that mediates calcium-dependent cell-cell adhesion. The activity of cadherins depends on their anchorage to the neuronal cytoskeleton through proteins termed catenins (e.g., CTNNA2), which in turn activate KALRN, a key regulator of calcium-dependent cell-cell adhesion. This study revealed that CDH23 is a promising candidate involved in MPH treatment effects.
of dendritic spine development and synaptic plasticity underlying learning and memory\(^4^0\). This is of particular interest since catenin-cadherin cell-adhesion complexes are important in cerebellar and hippocampal lamina-
\(^4^1\)tion and both CTNNA2 and KALRN have shown nominal associations with clinical outcome in our GWAS.

In this sense, Park et al.\(^4^1\) demonstrated that mice lacking the actin-binding domain of Ctnna2 (cdf-mutant mice) exhibited abnormal morphology of cerebellum and hippocampus. Moreover, the cdf-mutant mice showed an impaired control of the startle response and deficits in startle modulation have also been found in ADHD patients\(^4^2,4^3\). Therefore, cell-adhesion molecules and regulators of synaptic plasticity may play a role in MPH treatment effects, which is in agreement with data from genome-wide linkage and association studies pointing to cadherin 13 (CDH13) as one of the most consistent candidates implicated in ADHD pathophysiology. Specifically, CDH13 was detected in three independent GWAS\(^4^4,4^5\) and lies within the 16q22-16q24 region identified in a meta-analysis of seven ADHD linkage scans\(^4^5\). Furthermore, SNPs in this gene have been linked to deficits in verbal memory and hyperactive/impulsive symptoms in subjects with ADHD\(^4^6,4^7\), addiction vulnerability and drug dependence (e.g., methamphetamine, alcohol, and nicotine)\(^4^8,4^9\).

Pathway enrichment analysis provided further evidence for neuroplastic changes in response to MPH treatment, considering the over-representation of genes involved in morphology of neurons, neuroglia, white matter and brain regions relevant to ADHD (e.g., cerebellum, cerebral cortex, and hippocampus) that we found among cis-associated gene-SNP pairs with a nominal significant effect on methylphenidate response in the GWAS analysis. Note: SNP, single-nucleotide polymorphism; GWAS, genome-wide association study; Chr, gene chromosomal location; OR, odds ratio; CI, confidence interval; eQTL, expression quantitative trait loci. All relative to the human reference genome GRCh38 (NCBI Build 38). *Significance threshold for the False Discovery Rate (FDR) correction at P < 0.05.

**Table 1.** Cis-associated gene-SNP pairs with a nominal significant effect on methylphenidate response in the GWAS analysis. Note: SNP, single-nucleotide polymorphism; GWAS, genome-wide association study; Chr, gene chromosomal location; OR, odds ratio; CI, confidence interval; eQTL, expression quantitative trait loci. All relative to the human reference genome GRCh38 (NCBI Build 38). *Significance threshold for the False Discovery Rate (FDR) correction at P < 0.05.
| Categories                                                                 | Diseases or functions annotation                                      | Adjusted P-value (Benjamini-Hochberg) | Molecules                  |
|----------------------------------------------------------------------------|------------------------------------------------------------------------|---------------------------------------|-----------------------------|
| Nervous System Development and Function, Organismal Development            | abnormal morphology of molecular layer of cerebellum                   | 0.012                                 | ARSA, PURA                  |
| Nervous System Development and Function, Organismal Development, Tissue Morphology | abnormal morphology of white matter                                     | 0.012                                 | ARSA, PURA                  |
| Cellular Development, Embryonic Development, Organismal Development        | differentiation of neuronal progenitor cells                           | 0.012                                 | FXR2, HTT                   |
| Developmental Disorder, Neurological Disease                              | learning deficit                                                      | 0.012                                 | ARSA, HTT                   |
| Cell Morphology, Nervous System Development and Function, Organismal Development, Tissue Morphology | morphology of granule cells                                           | 0.012                                 | HTT, NFIB                   |
| Cell Morphology, Haematological System Development and Function, Nervous System Development and Function | morphology of microglia                                               | 0.012                                 | ARSA, HTT                   |
| Neurological Disease                                                       | gait disturbance                                                       | 0.012                                 | ARSA, HTT, PURA             |
| Cell Morphology, Nervous System Development and Function, Tissue Morphology | morphology of axons                                                   | 0.012                                 | ARSA, HTT, PURA             |
| Cell Morphology, Nervous System Development and Function                  | morphology of granule cells                                           | 0.012                                 | HTT, NFIB                   |
| Cell Morphology, Nervous System Development and Function, Organismal Development | morphology of brain cells                                             | 0.012                                 | ARSA, HTT, NFIB, PURA       |
| Cell Morphology, Nervous System Development and Function, Tissue Morphology | morphology of neurites                                                | 0.012                                 | ARSA, FARP2, HTT, PURA      |
| Cell Morphology, Nervous System Development and Function, Tissue Morphology | morphology of neurons                                                 | 0.012                                 | ARSA, CDH23, FARP2, HTT, NFIB, PURA |
| Neurological Disease                                                       | late-onset encephalopathy                                              | 0.014                                 | HTT                         |
| Psychological Disorders                                                    | hyperactive behaviour                                                 | 0.015                                 | ARSA, FXR2, HTT             |
| Neurological Disease                                                       | tremor                                                                 | 0.015                                 | ARSA, HTT, PURA             |
| Nervous System Development and Function, Organismal Development, Tissue Morphology | abnormal morphology of dentate gyrus                                   | 0.015                                 | NFIB, PURA                  |
| Cell Morphology, Nervous System Development and Function, Organismal Development, Tissue Morphology | abnormal morphology of Purkinje cells                                 | 0.015                                 | ARSA, PURA                  |
| Cell Death and Survival, Cellular Compromise, Neurological Disease, Organismal Injury and Abnormalities, Tissue Morphology | neurodegeneration of Purkinje cells                                   | 0.015                                 | ARSA, HTT                   |
| Nervous System Development and Function, Organismal Development           | abnormal morphology of telencephalon                                   | 0.015                                 | ARSA, HTT, NFIB             |
| Behaviour                                                                  | spatial learning                                                       | 0.018                                 | ARSA, FXR2, HTT             |
| Nervous System Development and Function, Organismal Development, Tissue Morphology | mass of brain                                                         | 0.019                                 | HTT, PURA                   |
| Cell Morphology, Cellular Function and Maintenance, Nervous System Development and Function, Tissue Morphology | length of neurites                                                    | 0.021                                 | FARP2, HTT                  |
| Organismal Injury and Abnormalities                                        | abnormality of head                                                   | 0.022                                 | HTT, NFIB                   |
| Nervous System Development and Function                                    | coordination                                                          | 0.022                                 | ARSA, FXR2, HTT             |
| Cellular Development                                                       | differentiation of stem cells                                          | 0.022                                 | FXR2, HTT, NFIB             |
| Developmental Disorder, Neurological Disease, Organismal Injury and Abnormalities | cerebral dysgenesis                                                   | 0.022                                 | NFIB, PURA                  |
| Nervous System Development and Function, Organismal Development, Tissue Morphology | morphology of cerebral cortex                                         | 0.023                                 | HTT, NFIB, PURA             |
| Organismal Development                                                     | size of head                                                           | 0.024                                 | HTT, NFIB, PURA             |
| Neurological Disease, Organismal Injury and Abnormalities                  | astrocytosis                                                          | 0.025                                 | ARSA, HTT                   |
| Cell Death and Survival, Cellular Compromise, Neurological Disease, Tissue Morphology | neurodegeneration of axons                                            | 0.026                                 | ARSA, HTT                   |
| Cellular Growth and Proliferation, Nervous System Development and Function, Organ Development | proliferation of brain cells                                          | 0.030                                 | HTT, PURA                   |
| Nervous System Development and Function, Organismal Development, Tissue Morphology | abnormal morphology of brain                                          | 0.030                                 | ARSA, HTT, NFIB, PURA       |
| Embryonic Development, Organismal Development, Tissue Development          | mesoderm development                                                  | 0.032                                 | CHURC1, HTT                 |
| Embryonic Development, Nervous System Development and Function, Organismal Development, Tissue Development | formation of hippocampus                                              | 0.033                                 | HTT, NFIB                   |
| Nervous System Development and Function, Organismal Development, Tissue Morphology | quantity of brain cells                                               | 0.042                                 | HTT, PURA                   |
| Nervous System Development and Function                                    | sensation                                                              | 0.047                                 | CDH23, FXR2, HTT             |

Table 2. Significantly enriched biological functions and diseases identified by Ingenuity Pathway Analysis within the eGenes associated with methylphenidate response. Note: eGenes, genes whose expression levels are associated with at least one genetic variant. *Significance threshold for the Benjamini-Hochberg correction at P < 0.05.
be normalised by MPH. In this sense, Rubia et al. demonstrated that MPH restores the aberrant activation and functional connectivity in attention, motivation and interference inhibition networks, as well as during error processing, thus improving neuropsychological performance of subjects with ADHD.

It should also be noted that 15 out of the 32 eGenes included in the pathway enrichment analysis harboured variants which provided preliminary evidence for an association with clinical outcome across the discovery and an independent sample. Our top hit from the meta-analysis, rs17685420, is located in the phosphatidylethanolamine N-methyltransferase binding protein 4 (PEBP4), a member of an evolutionary conserved family of proteins with pivotal biological functions such as cell proliferation and survival, stimulation of acetylcholine synthesis and inhibition of serine proteases. Given that serine proteases are implicated in many processes during development and tissue homeostasis (e.g., neuronal outgrowth, cell migration, and cell death), disturbances in their activity on the nervous system have been proposed as a possible pathological mechanism for neurological disorders. Indeed, Hohman et al. showed alterations after methamphetamine and morphine administration and also shown alterations after methamphetamine and morphine administration. Additional compelling results were detected for ARSA, SPSB2, CORO7 and PIGM. The ARSA gene encodes the arylsulfatase A, whose deficiency is characterised by decline in school performance, emergence of behavioural problems and neurologic symptoms, such as cerebellar ataxia, among others. The SPSB2 has been associated with borderline personality disorder in a genome-wide methylation analysis and CORO7, which has shown to be important in brain development, was identified as a novel candidate gene for emotionality by comparing the expression profile of two mouse lines

Table 3. Meta-analysis of the eSNPs nominally associated with methylphenidate response across the discovery and the target population. Note: eSNP, single-nucleotide polymorphism associated with cortical expression levels; Chr, gene chromosomal location; OR, odds ratio; CI, confidence interval. All relative to the human reference genome GRCh38 (NCBI Build 38). aSignificance threshold for Bonferroni correction at P < 1.52e-03.
with either high or low anxiety-related behaviour\(^6\). Finally, mutations in the PIGM gene, which encodes a protein involved in the synthesis of the glycosylphosphatidylinositol anchor, have been reported in individuals with severe neurological features, including seizures, muscular hypotonia and intellectual disability\(^6\).

Another interesting finding arising from our research is the significant enrichment for candidates previously related to ADHD or MPH response detected among the set of genes nominally associated with treatment outcome. It is worth mentioning that four of these candidates, namely CTNN2 (rs79067553, \(P = 3.51e-05\)), PARD3B (rs62172701, \(P = 3.28e-04\)), LRPIB (rs410870, \(P = 4.00e-04\)) and GRM7 (rs17047590, \(P = 6.36e-04\)), were significant at \(P < 1e-03\) in the present GWAS analysis. In particular, the metabotropic glutamate receptor 7 (GRM7), which is widely expressed in brain regions relevant to ADHD such as the cerebral cortex, the hippocampus and the cerebellum\(^5,20\), and has been associated with the disorder\(^11-21\), was also found among the top hits in a prior GWAS of MPH efficacy\(^13\), thus supporting the role of the glutaminergic system as a moderator of treatment outcome.

The main strengths of our design include the coverage of a considerably higher number of genetic variants in comparison with the study from Mick et al.\(^13\) (319,722 vs 3,566,199 markers), the use of an integrative approach that combines GWAS data with bioinformatic methods, and the follow up of our top signals in an independent cohort, which did increase the association of a number of markers located in loci with biologically plausible functions (PEBP4, ARSA, and SPSB2). Nevertheless, some limitations should also be considered when interpreting these results. Given the limited sample size, the present study might not be sufficiently powered to detect individual variants of modest effects and we did not identify any loci reaching the genome-wide threshold. This constraint, however, is heavily conditioned on the difficulty to find the required phenotype as shown by the sample size of the studies included in the last meta-analysis of candidate gene-based investigations on MPH response\(^4\). The small dimension of our paediatric sample could also explain the absence of significance of the PRS derived from the GWAS results in an independent population of ADHD adult patients. Alternatively, this discrepancy may be attributed to differences in the genetic background and the clinical heterogeneity (e.g., comorbidities, frequency of clinical subtypes, and sex ratio) of ADHD among children and adults, as suggested by most of the pharmacogenetic studies conducted in adult samples, which failed to replicate variants previously identified in children and adolescents\(^75\). Additional methodological aspects or distinct environmental influences between the discovery and the target population may also be responsible for the absence of association.

In conclusion, despite not reaching any genome-wide significant association, our results are consistent with previous findings and highlight genes related to morphological abnormalities in brain regions important for motor control, attention and memory, thus supporting the use of bioinformatic and biological evidence as a complement to GWAS data to disentangle the genetic basis of MPH response.

References

1. American Psychiatric Association. Diagnostic And Statistical Manual Of Mental Disorders, 5th Edition. (American Psychiatric Publishing, 2013).
2. Polanczyk, G. V., Willcutt, E. G., Salum, G. A., Kieling, C. & Rohde, L. A. ADHD prevalence estimates across three decades: an updated systematic review and meta-regression analysis. Int J Epidemiol. 43, 434–442 (2014).
3. Doshi, J. A. et al. Economic impact of childhood and adult attention-deficit/hyperactivity disorder in the United States. J Am Acad Child Adolesc Psychiatry. 51, 990–1002 e2 (2012).
4. Le, H. H. et al. Economic impact of childhood/adolescent ADHD in a European setting: the Netherlands as a reference case. Eur Child Adolesc Psychiatry. 23, 587–598 (2014).
5. Faraone, S. V. & Mick, E. Molecular genetics of attention deficit hyperactivity disorder. Psychiatr Clin North Am. 33, 159–180 (2010).
6. Blum, N. J., Jawad, A. F., Clarke, A. T. & Power, T. J. Effect of osmotic-release oral system methylphenidate on different domains of attention and executive functioning in children with attention-deficit-hyperactivity disorder. Dev Med Child Neurol. 53, 843–849 (2011).
7. Greenhill, L. et al. Guidelines and algorithms for the use of methylphenidate in children with Attention-Deficit/Hyperactivity Disorder. J Atten Disord. 6(Suppl 1), S89–100 (2002).
8. Charach, A., Ickowicz, A. & Schachar, R. Stimulant treatment over five years: adherence, effectiveness, and adverse effects. J Am Acad Child Adolesc Psychiatry. 43, 559–567 (2004).
9. Wolraich, M. L. & Doffing, M. A. Pharmacokinetetic considerations in the treatment of attention-deficit hyperactivity disorder with methylphenidate. CNS Drugs. 18, 243–250 (2004).
10. Wilens, T. E. Effects of methylphenidate on the catecholaminergic system in attention-deficit-hyperactivity disorder. J Clin Psychopharmacol. 28, 546–53 (2008).
11. Kieling, C., Genro, J. P., Hutz, M. H. & Rohde, L. A. A current update on ADHD pharmacogenomics. Pharmacogenomics. 11, 407–419 (2010).
12. Bruzel, E. M. et al. ADHD pharmacogenetics across the life cycle: New findings and perspectives. Am J Med Genet B Neuropsychiatr Genet. 165B, 263–282 (2014).
13. Mick, E., Neale, B., Middleton, F. A., McGough, J. J. & Faraone, S. V. Genome-wide association study of response to methylphenidate in 187 children with attention-deficit/hyperactivity disorder. Am J Med Genet B Neuropsychiatr Genet. 147B, 1412–1418 (2008).
14. Mick, E., McGough, J. J., Middleton, F. A., Neale, B. & Faraone, S. V. Genome-wide association study of blood pressure response to methylphenidate treatment of attention-deficit/hyperactivity disorder. Prog Neuropsychopharmacol Biol Psychiatry. 35, 466–472 (2011).
15. Pagerolos, M. et al. Pharmacogenetics of methylphenidate response and tolerability in attention-deficit/hyperactivity disorder. Pharmacogenomics J. 17, 98–104 (2017).
16. Guy, W. ECDEU Assessment Manual For Psychopharmacology, Revised. (US Department of Health, Education and Welfare, 1976).
17. Miller, S. A., Dykes, D. D. & Polesky, H. F. A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res. 16, 1215 (1988).
18. Purcell, S. et al. PLINK: a tool set for whole-genome association and population-based linkage analyses. Am J Hum Genet. 81, 559–575 (2007).
19. Xu, Z. & Taylor, J. A. SNPinfo: integrating GWAS and candidate gene information into functional SNP selection for genetic association studies. Nucleic Acids Res. 37, W600–605 (2009).
20. Eilutki, L. et al. Distinguishing regulatory DNA from neutral sites. Genome Res. 13, 64–72 (2003).
64. Wei, Q. H.

62. Maki, M.

60. George, A. J.

59. Hohman, T. J.

57. He, H.

56. Froehlich, T. E., McGough, J. J. & Stein, M. A. Progress and promise of attention-deficit hyperactivity disorder pharmacogenetics. CNS Drugs. 24, 99–117 (2010).

57. He, H.

54. Gao, Q., Liu, L., Qian, Q. & Wang, Y. Advances in molecular genetic studies of attention deficit hyperactivity disorder in China. Shanghai Arch Psychiatry. 26, 194–206 (2014).

53. Kasparek, T., Theiner, P. & Filova, A. Neurobiology of ADHD From Childhood to Adulthood: Findings of Imaging Methods. Curr Top Behav Neurosci. 28, 239–272 (2012).

52. Bonvicini, C., Farzone, S. V. & Scassellati, C. Attention-deficit hyperactivity disorder in adults: A systematic review and meta-analysis of genetic, pharmacogenetic and biochemical studies. Mol Psychiatry. 21, 872–884 (2016).

51. Cortese, S. The neurobiology and genetics of Attention-Deficit/Hyperactivity Disorder (ADHD): what every clinician should know. Eur Arch Psychiatry Clin Neurosci. 261, 205–211 (2011).

50. Castellanos, F. X. Involvement of hippocampal phosphatidylethanolamine-binding protein in morphine dependence and withdrawal. J Neurochem. 70, 1682–1689 (2001).

49. Treutlein, J. & Rietschel, M. Genome-wide association studies of alcohol dependence and substance use disorders. Hum Mol Genet. 22, R28–R27 (2013).
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Author Contributions
M.P., C.S.M., P.R. and I.G.M. participated in the DNA isolation and preparation of samples. M.P., C.S.M., P.R., M.S.A., I.G.M., B.S.S. and N.R.M. undertook the statistical analyses. V.E., E.C.S., M.C., M.M.V. and E.H.G. contributed to the clinical assessment and recruitment of patients. L.A.R., C.H.D.B., Prof. M.C. and J.A.R.Q. participated in the study design, clinical assessment and coordination of the clinical research. M.R. conceived the project, wrote the protocol and coordinated the study design and the statistical analyses. B.C., J.A.R.Q. and M.R. supervised the project and the manuscript preparation. All authors contributed to and have approved the final version.

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