Attention deficit hyperactivity disorder: genetic association study in a cohort of Spanish children

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Abstract

Background: Attention deficit hyperactivity disorder (ADHD) has a strong genetic component. The study is aimed to test the association of 34 polymorphisms with ADHD symptomatology considering the role of clinical subtypes and sex in a Spanish population.

Methods: A cohort of ADHD 290 patients and 340 controls aged 6–18 years were included in a case–control study, stratified by sex and ADHD subtype. Multivariate logistic regression was used to detect the combined effects of multiple variants.

Results: After correcting for multiple testing, we found several significant associations between the polymorphisms and ADHD (p value corrected ≤0.05): (1) SLC6A4 and LPHN3 were associated in the total population; (2) SLC6A2, SLC6A3, SLC6A4 and LPHN3 were associated in the combined subtype; and (3) LPHN3 was associated in the male sample. Multivariable logistic regression was used to estimate the influence of these variables for the total sample, combined and inattentive subtype, female and male sample, revealing that these factors contributed to 8.5, 14.6, 2.6, 16.5 and 8.5 % of the variance respectively.

Conclusions: We report evidence of the genetic contribution of common variants to the ADHD phenotype in four genes, with the LPHN3 gene playing a particularly important role. Future studies should investigate the contribution of genetic variants to the risk of ADHD considering their role in specific sex or subtype, as doing so may produce more predictable and robust models.

Keywords: Attention deficit hyperactivity disorder, ADHD, Association study, Case–control, LPHN3

Background

Attention deficit hyperactivity disorder (ADHD) is one of the most common neurodevelopmental disorders in young people, affecting 5.3 % of school-age children [1]. Also, approximately 65 % of children with ADHD continue to show symptoms in adulthood [2].

ADHD is a complex and heterogeneous disorder and its etiology remains unidentified to date [3]. Family, twin and adoption studies have shown that different genes play an important role in the etiology of ADHD, and the mean estimated heritability in childhood is 76 % [4], suggesting that ADHD is one of the psychiatric disorders with the most substantial genetic component.

Many association studies have investigated genetic susceptibility to ADHD. However, efforts to replicate these results have often been poor, yielding inconsistent results as demonstrated in meta-analysis of candidate gene studies [5], but also from linkage studies [6] and genome-wide association studies (GWAS) [7]. ADHD is a complex genetic disorder, in which environmental factors are involved and play a key role [7].

The aim of this study was to test whether previously reported common genetic variants (34 polymorphisms
in 18 genes) influence ADHD susceptibility in Spanish patients.

Based on the etiology of ADHD, we chose candidate genes that encode functionally relevant proteins involved in noradrenergic (SLC6A2, ADRA2A), dopaminergic (SLC6A3, DRD2, DRD4, COMT, DDC), and serotonergic (SLC6A4, HTR2A, HTR2C) neurotransmission. In addition, we evaluated other candidate genes frequently reported as being related with ADHD such as STS, FADS2 and SNAP25. Finally, significantly reported genes from GWAS studies such as CDH13, GFOD1, SLC6A9 and GRM7, and genes revealed in linkage as playing a role in ADHD susceptibility such as LPHN3 were included in the study (Table 1).

Methods

Patients and controls

A total of 320 Spanish ADHD patients of Caucasian ancestry and 344 healthy children and adolescents of the same nationality and ancestry were initially included in this case–control study. After a quality control procedure, 290 patients and 340 controls were included in the final analysis. ADHD patients were recruited and evaluated at Fundación Jiménez Díaz University Hospital, whereas the control sample was recruited at both the aforementioned hospital and primary and secondary schools. Exclusion criteria for the control sample included ADHD diagnosis or suspicion of symptomatology, and chronic illness. The sample (cases and controls) comprised subjects between the ages of 6 and 18 years. Even though we did not test for the structure in our cohort, a genome wide study of 800 subjects distributed throughout Spain discarded the presence of genetic stratification [8].

The study protocol was approved by the Research Ethics Committee of the IIS-Fundación Jiménez Díaz University Hospital. The study was conducted according to the tenets of 2008 declaration of Helsinki. Before enrollment, parents or legal guardians signed a written informed consent form after the study objectives and procedures had been explained.

Clinical assessment

Subjects were included in the study only after a diagnosis of ADHD was made by specialist clinicians according to the diagnostic and statistical manual of mental disorders, fourth edition, text revision (DSM-IV TR) [9]. Each diagnosis was checked by two clinical researchers. Where consensus could not be reached, cases were reviewed by an additional clinical researcher. The children were classified into the following ADHD subtypes: predominantly inattentive subtype, predominantly hyperactive/impulsive subtype and combined subtype.

All cases included underwent clinical assessment using the strengths and difficulties questionnaire (SDQ) for detecting psychological morbidity [10]. Severity of ADHD symptoms was based on the ADHD rating scale-IV (ADHD RS-IV) [11], whereas overall psychosocial functioning was assessed by means of the children’s global assessment scale (CGAS) and the clinical global impression scale (CGI) [12]. Information on obstetric complications, developmental features, medical and psychiatric history, family history, and treatment histories were obtained through maternal interview.

Exclusion criteria included other psychotic disorders (bipolar disorder or schizophrenia among others), pervasive developmental disorders, intelligence quotient (IQ) <70, and neurological damage.

DNA extraction and genotyping

Genomic DNA samples were obtained either from peripheral blood lymphocytes using an automatic DNA extractor (BioRobot EZI, Qiagen, Hilden, Germany) or from saliva using the Oragene DNA self-collection kit (DNA Genotek, Kanata, Ontario, Canada), according to the manufacturer’s recommendations. DNA concentration and sample quality were assessed spectrophotometrically (NanoDrop® ND-1000 Spectrophotometer, Wilmington DE, USA).

Candidate polymorphisms were selected based on their relevance as indicated in the literature on ADHD (Table 1).

All single nucleotide polymorphisms (SNPs) were typed using TaqMan Assays-on-Demand or pre-designed SNP genotyping assays following the manufacturer’s instructions (Applied Biosystems, Foster City, CA, USA). PCR and allelic discrimination assays were run using the LightCycler 480 System (Roche Diagnostics, Mannheim, Germany). The results were evaluated using LightCycler® 480 software, version 1.5 (Roche Diagnostics, Mannheim, Germany).

For each variable number tandem repeats (VNTR) polymorphism, subjects were categorized into three genotypes according to the risk allele previously described [5] as follows: SLC6A3 3’UTR VNTR (10/10, 10/-, -/-), SLC6A4 intron8 VNTR (6/6, 6/-, -/-), DRD4 promoter duplication VNTR (L/L, L/S, S/S), DRD4 exon3 VNTR (7/7, 7/-, -/-), SLC6A4 promoter VNTR (L/L, L/S, S/S), SLC6A4 intron2 VNTR (10/10, 10/-, -/-). Detection of VNTR polymorphisms was performed using fragment analysis. PCR products were visualized on an ABI Prism 3130xl DNA sequencer (Applied Biosystems Foster City, CA). The results were evaluated using the GeneMapper software, version 4.0 (Applied Biosystems, Foster City, CA). Primer sequences and conditions are available upon request.
Statistical analysis

For the case–control association study, Hardy–Weinberg equilibrium for all genetic variants was assessed only in the control population because deviation from HWE in cases sample might be an indication of association with the disorder; variants not in HWE (p value < 0.01) were excluded from the analysis.

A quality-control procedure was applied to the genotype data. The threshold applied in genotype call rates per sample and per polymorphism was 80%.

Logistic regression was used to examine the association of the genotype frequencies with the disorder. The effect of the genetic variant on outcome was adjusted by sex and age (covariates). To reduce genetic heterogeneity and to test if there were different genetic factors for the distinct ADHD subtypes, ADHD patients were subdivided into two main diagnostic groups, combined ADHD and inattentive ADHD. The hyperactive-impulsive ADHD subtype was not considered due to its small sample size. To examine differences between males and females, sex-stratified analyses were performed.

Logistic regression analysis was performed to analyze the five inheritance models (codominant, dominant, recessive, overdominant and log-additive) [13] using

| Gene | Description | Variant | Reference |
|------|-------------|---------|-----------|
| SLC6A2 | Norepinephrine transporter | rs28386840<sup>a</sup> | [19] |
|      |             | rs5569<sup>b</sup> | [5] |
| ADRA2A | Adrenergic receptor alpha 2A | rs1800544<sup>a</sup> | [5] |
|      |             | rs553668<sup>b</sup> | [5] |
| SLC6A3 | Dopamine transporter | rs250948<sup>b</sup> | [22] |
|      |             | rs2652511<sup>b</sup> | [22] |
|      |             | rs11564750<sup>a</sup> | [22] |
|      |             | 3′UTR VNTR<sup>b</sup> | [5] |
|      |             | Intron8 VNTR<sup>d</sup> | [5] |
| DRD2 | Dopamine receptor D2 | rs1800497<sup>a</sup> | [21] |
| DRD4 | Dopamine receptor D4 | rs3758653<sup>a</sup> | [20] |
|      |             | Exon3 VNTR<sup>c</sup> | [21] |
|      |             | Promoter duplication<sup>b</sup> | [21] |
| COMT | Catechol-O-methyltransferase | rs4680<sup>c</sup> | [5] |
|      |             | rs4818<sup>f</sup> | [5] |
| DDC | Dopa decarboxylase | rs6592961<sup>d</sup> | [51] |
| SLC6A4 | Serotonin transporter | Promoter VNTR<sup>b</sup> | [5] |
|      |             | Intron2 VNTR<sup>d</sup> | [5] |
| HTR2A | Serotonin-2A receptor | rs7322347<sup>d</sup> | [51] |
| HTR2C | Serotonin-2C receptor | rs6318<sup>c</sup> | [52] |
| SLC9A9 | Glycine transporter | rs9810857<sup>b</sup> | [53] |
| GRM7 | Glutamate receptor, metabotropic 7 | rs3792452<sup>d</sup> | [54] |
| SNAP25 | Synaptosomal-associated protein 25kDA | rs3746544<sup>a</sup> | [5] |
| CDH13 | Cadherin 13 | rs6565113<sup>d</sup> | [20] |
| GFCID1 | Glucose-fructose oxidoreductase domain containing 1 | rs552655<sup>d</sup> | [20] |
| STS | Steroid sulfatase | rs12861247<sup>c</sup> | [55] |
|      |             | rs17268988<sup>d</sup> | [55] |
| FADS2 | Fatty acid desaturase 2 | rs498793<sup>d</sup> | [31] |
| LPHN3 | Latrophilin 3 | rs1397548<sup>c</sup> | [17] |
|      |             | rs2305339<sup>d</sup> | [17] |
|      |             | rs6551655<sup>d</sup> | [17] |
|      |             | rs1866790<sup>d</sup> | [24] |
|      |             | rs6813183<sup>d</sup> | [24] |
|      |             | rs6858066<sup>d</sup> | [24] |

Position in the gene: * upstream gene variant, b promoter variant, c exon variant, d intron variant, e 3′UTR variant, f downstream gene variant.

Table 1 Description of the 34 polymorphisms analysed within 18 genes for ADHD
SNPstats software [14] and expressed as odds ratio (OR), 95% confidence interval (CI) and nominal significant differences (p value ≤ 0.05). If various inheritance models had significant results, we chose the one with the lowest Akaike information criteria (AIC value).

Genotypes frequencies of variants located on chromosome X (HTR2C and STS genes) were analyzed only in females.

The Benjamini and Hochberg false discovery rate method was performed for multiple testing corrections [15]. A p value threshold of 0.05 after correction was used to determine significance. Risk-prediction models to investigate the combined impact of multiple genetic variants were applied. For this purpose, polymorphisms with p values ≤ 0.25 were incorporated in a forward stepwise multivariate logistic regression analysis and expressed as the OR, 95% CI and p value.

The variability explained for each variable as measure of the effect size of the polymorphisms (defined by pseudo-r²) and the measure of model predictability (defined by AUC value) were calculated.

A post hoc analysis of statistical power was performed with the CaTS Power Calculator software [16] assuming an OR of 1.5, disorder prevalence of 5%, significance level of 0.05, and mean minor allele frequency (MAF) observed of 0.30. The statistical power calculated for the final sample included in this study (290 cases and 340 controls) was 89, 64, and 23% considering an additive, dominant and recessive model, respectively.

**Results**

A total of 320 patients and 344 controls were initially investigated. Thirty-four subjects were excluded because they showed genotype call rates <80%. Therefore, 290 patients and 340 controls were included in the final analysis. Per-marker genotype call rates were higher than 96% for all variants. The genotype distributions of all polymorphisms were consistent with HWE (p value > 0.01) in the control sample. The average age was 10.43 years (SD 2.9) for ADHD patients and 11.05 years (SD 3) for the controls. 80 and 66% of patients and controls were male, respectively. Clinical classification of the patients was the following: inattentive subtype (n = 102), hyperactive/impulsive subtype (n = 13) and combined subtype (n = 175). Demographic and clinical characteristics of the sample are reported in Table 2.

**Logistic regression results for single markers**

When the whole sample was considered (unstratified sample), logistic regression analysis for single markers, adjusted by sex and age, showed statistically significant results after correcting for multiple comparisons in two polymorphisms: *SLC6A4* promoter VNTR and *LPHN3* rs2305339 (Table 3; Additional file 1: Table S1).

In the combined ADHD subtype, association was statistically significant for *SLC6A2* rs28386840, *SLC6A3* rs11565750, *SLC6A4* promoter VNTR and *LPHN3* rs2305339. None of the individual comparisons was statistically significant after correcting for multiple comparisons in the inattentive subtype (Table 3 and Additional file 1: Table S1).

In the logistic regression analysis for single markers, adjusted by age, none of the individual comparisons was statistically significant after correcting for multiple comparisons in the female sample. In the male sample, only *LPHN3* rs2305339 remained statistically significant (Table 3 and Additional file 1: Table S1).

**Multivariate logistic regression results**

Figures 1 and 2 show multivariate logistic regression analyses for the total ADHD sample, subtype and sex stratification. The variables included in the model were ordered according to the amount of variance explained (r²).
In the total sample, eight polymorphisms located in seven genes were included in the regression equation: SLC6A4 promoter VNTR, LPHN3 (rs2305339, rs6551665), DRD4 exon3 VNTR, SNAP25 rs3746544, SLC6A3 rs11564750, SLC6A2 rs28386840 and FADS2 rs498793. The amount of the variance explained for the model was 8.5% and the AUC was 0.69 (Table 4).

In the case of the combined subtype, seven polymorphisms located in seven genes were included in the model: SLC6A4 promoter VNTR, SLC6A2 rs28386840, SLC6A3 rs11564750, LPHN3 rs2305339, DDC rs6592961, GRM7 rs3792453 and FADS2 rs498793. The amount of the variance explained was 14.6% and the AUC was 0.75. In the case of inattentive subtype, two polymorphisms were included in the model, LPHN3 rs6551665 and SNAP25 rs3746544. The amount of the variance explained for the model was 2.6% and the AUC was 0.60 (Table 4).

Five polymorphisms located in five genes (SLC6A3 rs11564750, SNAP25 rs3746544, LPHN3 rs6551665, DRD4 exon3 VNTR and SLC6A2 rs28386840) and seven polymorphisms located in five genes (LPHN3 rs2305339, rs6551665), SLC6A2 (rs28386840, rs53569), GRM7 rs3792452, SLC6A4 promoter VNTR and DRD4 exon3 VNTR) were included in the model for females and males, respectively. The amount of the variance explained for the sample including females was 16.5% and the AUC was 0.77. In the case of males, the amount of the variance explained was 8.5% and the AUC was 0.69 (Table 4).

### Discussion

This study aimed to both determine whether differential genetic variants may participate in distinct ADHD subtypes and also examine the sex-specific effects of this impact. Multivariate regression analyses of the effects of single genes were evaluated, but as ADHD is a complex polygenic disorder, the combined effect of multiple genes on the phenotype was also considered.

As seen in the logistic regression analysis for single markers, this study provides evidence of a strong association between the SLC6A4 gene and ADHD in the entire population; and between SLC6A2, SLC6A3 and SLC6A4 and ADHD in the combined subtype. Special attention should be given to the LPHN3 gene, since it was associated with the presence of ADHD in the entire population, the combined subtype and the male sample.

In order to clarify the genetic basis of ADHD, the effects of multiple risk factors were examined. In the entire sample, seven genes were included in the regression equation (SLC6A4, LPHN3, DRD4, SNAP25, SLC6A3, SLC6A2 and FADS2). The involvement of these genes in ADHD has been extensively studied [5, 17–23], some in Spanish populations [24, 25]. The contribution of each gene was modest, as expected for a complex genetic disorder (ranging...
from 0.4 to 1.6%). The model explained around 9% of the variance; 7% of this variance was due to genetic factors. A previous study, including 22 variants, found that 16% of the variance was due to genetic factors [26].

In the regression equation, two genes were included in the inattentive subtype (LPHN3 and SNAP25) and seven genes (SLC6A4, SLC6A2, SLC6A3, LPHN3, DDC, GRM7 and FADS2) in the combined subtype. A remarkable importance of the SLC6A4 gene was observed, accounting for 2.9% of the variance, above the usual threshold of 2% [27]. This study showed that the clinical subtypes analyzed share genetic risk factors (LPHN3), yet SNAP25 was associated with the inattentive subtype, whereas SLC6A4, SLC6A2, SLC6A3, DDC, GRM7 and FADS2 were implicated in the combined subtype. The presence of common as well as specific genetic variants for each...
The model showed a higher genetic loading for the variables analyzed in combined subtype (14.6 %) than in the inattentive subtype (2.6 %), finding consistent with the previously reported higher genetic loading in ADHD comorbid symptoms [37, 38]. It is important to note the importance of sex and age in the combined subtype (r² 5.9 %) but not in the inattentive sample. The model for the combined subtype seems to be more predictive than the inattentive subtype (AUC 0.75 and AUC 0.60, respectively) and better than the model used for the whole sample (AUC 0.69). This supports the idea that analyzing more homogeneous phenotypes facilitates the identification of genetic factors.

ADHD is known to have sex-based differences in severity and clinical course [39]. Herein, differences in genetic susceptibility between males and females were observed. In females, SLC6A3, SNAP25, LPHN3, DRD4 and SLC6A2 genes showed high r² values (range from 2 to 3.8 %). In males, LPHN3, SLC6A2, GRM7, SLC6A4, DRD4 and LPHN3 were included in the regression equation. The LPHN3 gene accounted for 3.4 % of ADHD variability. This analysis showed that genes such as DRD4, SLC6A2 and LPHN3 were associated in both sexes, with a stronger effect of SLC6A3 and SNAP25 in females (r² 3.8 and 3.3 % respectively) and a lesser effect of GRM7 and SLC6A4 in males (r² 0.8 %). The association between SLC6A4 and male sample was supported by previous studies [40], but not between SLC6A3 and female sample [41].

To the best of our knowledge, sex-based differences in the genetic risk for ADHD have not been previously reported in the SNAP25 and GRM7 genes. These results suggest the need to explore biological evidence of sexually dimorphic effects in such genes.

The percentage of variance explained in females was higher (16.5 %) than in males (8.5 %). The regression model for girls (AUC 0.77) seems to be more predictive than for boys (AUC 0.69). These results suggest that the set of variants analyzed has a higher genetic contribution for ADHD in girls than in boys. Females are less frequently affected because a more extreme genetic load is required for the liability threshold to be surpassed [37, 42]. Additionally, it has been reported that females referred to a clinic are more prone to exhibit other disruptive behaviors [43], although this seems to be a consequence of referral bias [44].

Our results add to extensive literature information about polymorphic variants in genes whose implication in ADHD is widely known through the pathophysiology as SLC6A2, SLC6A3, SLC6A4 and LPHN3. In some cases the polymorphism associated has a known functional implication, like rs28386840, a functional promoter variant of SLC6A2 gene. But there are also other polymorphisms associated with any biological meaning that could be in linkage disequilibrium with other unknown functional variants directly involved in genetic susceptibility to ADHD. These findings need to be further explored to improve the understanding of their implication with ADHD.

The conflicting genetic results show the difficulty of replicating across genetic association studies. Often there is important variation in the sample reported, particularly regarding the age, sex ratios and ethnic. Also, an accurate phenotype definition is crucial to obtain successful results in these studies. In our study a rigorously diagnostic criteria was applied. In addition, the specific effect of a gene could be different depending on the sets of genetic variants analyzed or the model of inheritance evaluated. In contrast with other studies, we evaluated genetic information under different models of inheritance without an a priori consideration of possible genetic effects. This makes it easier to detect genetic effects, since different genotypes of the same gene could be associated with different phenotypes.

The most important limitation of the study was the modest sample size. The statistical power decreased when the sample was subdivided according to ADHD subtype or sex stratification; thus, it is difficult to determine whether negative findings were due to low statistical power or to the absence of a true biological association. On the contrary, we only consider association that remain significant after multiple testing correction in the regression logistic of single markers so we avoid false positive (type I error) rates, giving us confident in the veracity of the results.

Conclusions
We report evidence of the genetic contribution of common variants to the ADHD phenotype in four genes, with the LPHN3 gene playing a particularly strong role.

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Table 4 Overview of multivariate logistic regression analysis

| Trait                  | Pseudo r² (%) | AUC (CI 95 %) | Genetic variants |
|------------------------|---------------|---------------|------------------|
| All sample             | 8.5           | 0.69 (0.65–0.73) | 8                |
| Combined subtype       | 14.6          | 0.75 (0.71–0.79) | 7                |
| Inattentive subtype    | 2.6           | 0.60 (0.54–0.67) | 2                |
| Female                 | 16.5          | 0.77 (0.66–0.84) | 5                |
| Male                   | 8.5           | 0.69 (0.64–0.74) | 7                |

AUC area under curve, CI confidence interval
The most predictable model described in this study was for females ($r^2 = 16.5\%$, AUC 0.77). As seen in this study, analysis of the contribution of multiple genes provides particularly useful insight for the effort to discover the genetic basis of polygenic disorders and multigene analysis had substantial advantages over the single-gene approach. However, the percentage of the variance in ADHD diagnosis explained remains low; hence, most of the genetic component in phenotypic variance remains unexplained when considering common variants. Additional studies including copy number variation [45, 46], exome sequencing studies [47] as well as gene–gene and gene-environment interactions [48, 49] could clarify the genetic contribution to ADHD.

Future studies should investigate the contribution of genetic variants to the risk of ADHD considering their role in specific sex or subtype in order to produce more predictable and robust models, enabling the development of an accurate diagnosis and hopefully improved treatment.

## Additional file

**Additional file 1: Table S1.** Significant results of logistic regression for single markers considering a nominal p value <0.05.

### Abbreviations

ADHD: attention deficit hyperactivity disorder; DSM-IV-TR: diagnostic and statistical manual of mental disorders, fourth edition, text revision; SDQ: strengths and difficulties questionnaire; ADHD-IV: ADHD rating scale-IV; CGAS: children’s global assessment scale; CGI: clinical global impression scale; SNPs: single nucleotide polymorphisms; VNTRs: variable number tandem repeats polymorphisms; HWE: Hardy–Weinberg equilibrium; CI: confidence interval; AIC: Akaike information criterion; AUC: area under the curve; MAF: minor allele frequency.

### Authors’ contributions

CIGS contributed to the design of the study, data collection process, data analysis, and wrote the first drafts of the manuscript. RSJ contributed to the design of the study and revision of the manuscript. VSI was involved in the recruitment of subjects, data collection, and revisions of the manuscript. PTR was involved in recruiting subjects, data collection, and revisions of the manuscript. PMJ contributed to the statistical analysis. FAS contributed to the revision of the manuscript. JC contributed to the design of the study, recruitment of subjects, data collection, and revisions of the manuscript. RDR contributed to the design of the study, data analysis, and revision of the manuscript. CA was involved in the design of the study, data analysis, intellectual content, and revisions of the manuscript. All authors read and approved the final manuscript.

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### Competing interests

The authors declare that they have no competing interests. The authors have no proprietary or commercial interest in any of the materials discussed in this article.

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