

## ORIGINAL ARTICLE

## Linkage and association analysis of ADHD endophenotypes in extended and multigenerational pedigrees from a genetic isolate

CA Mastronardi<sup>1,6</sup>, E Pillai<sup>1,6</sup>, DA Pineda<sup>2,6</sup>, AF Martinez<sup>3</sup>, F Lopera<sup>2</sup>, JI Velez<sup>1</sup>, JD Palacio<sup>2</sup>, H Patel<sup>1</sup>, S Easta<sup>4</sup>, MT Acosta<sup>3</sup>, FX Castellanos<sup>5</sup>, M Muenke<sup>3</sup> and M Arcos-Burgos<sup>1,2,3</sup>

Attention-deficit/hyperactivity disorder (ADHD) is a heritable, chronic, neurodevelopmental disorder with serious long-term repercussions. Despite being one of the most common cognitive disorders, the clinical diagnosis of ADHD is based on subjective assessments of perceived behaviors. Endophenotypes (neurobiological markers that cosegregate and are associated with an illness) are thought to provide a more powerful and objective framework for revealing the underlying neurobiology than syndromic psychiatric classification. Here, we present the results of applying genetic linkage and association analyses to neuropsychological endophenotypes using microsatellite and single nucleotide polymorphisms. We found several new genetic regions linked and/or associated with these endophenotypes, and others previously associated to ADHD, for example, loci harbored in the *LPHN3*, *FGF1*, *POLR2A*, *CHRNA4* and *ANKFY1* genes. These findings, when compared with those linked and/or associated to ADHD, suggest that these endophenotypes lie on shared pathways. The genetic information provided by this study offers a novel and complementary method of assessing the genetic causes underpinning the susceptibility to behavioral conditions and may offer new insights on the neurobiology of the disorder.

*Molecular Psychiatry* (2016) **21**, 1434–1440; doi:10.1038/mp.2015.172; published online 24 November 2015

## INTRODUCTION

Attention-deficit/hyperactivity disorder (ADHD) is one of the most common neurodevelopmental behavioral disorders, affecting ~5.3% of children and adolescents worldwide.<sup>1</sup> The etiology and pathophysiology of ADHD are still not completely defined, but twin, adoption and family-based studies indicate a strong genetic component, particularly because first- and second-degree relatives of patients with ADHD have markedly higher prevalence of the illness.<sup>2</sup> Multiple studies, based on twin concordance comparisons and complex segregation analyses of pedigrees, have shown that ADHD is highly heritable;<sup>2</sup> the additive variance of the phenotype attributed to genetic factors is approximately 76%.<sup>2</sup>

Though genetic factors have been broadly linked to the susceptibility to develop ADHD and some susceptibility genes have been identified, functional mutations harbored at these loci, such as the precise differences in base pairs, remain undefined.<sup>3</sup> Given that ADHD has a highly variable clinical manifestation with a complex syndromic clinical definition, it has been suggested that quantitative phenotypes, that is, endophenotypes, could be useful for dissecting the genetic basis of ADHD. As hypothesized intermediates between genes and disease outcomes, endophenotypes are thought to be directly influenced by fewer genes than disease phenotypes.<sup>4</sup>

A previous study conducted on 288 individuals affected and unaffected with ADHD from the Paisa community, a population exhibiting features of genetic isolation from Colombia, South America, found a number of neuropsychological tests that met the criteria of endophenotypes.<sup>5</sup> The tests were aimed at ascertaining neuropsychological impairments frequently observed in patients with ADHD such as visual-motor functioning, executive function and intelligence. For executive function and intelligence, the following neuropsychological tests were performed: the Wechsler Intelligence Scale for Children (WISC) Block Design, performance intelligence quotient (PIQ) and full scale intelligence quotient (FSIQ). Correct responses and omissions on the 'A'-cancellation and vigilance test (ACVT) were used to assess sustained attention, that is, vigilance. Finally, the Rey-Osterrieth complex figure test (ROCF), standardized for Colombian children, was used to test visual-motor skills and immediate visual-motor memory recall.<sup>5</sup>

Starting with the above-mentioned neuropsychological endophenotypes, we hypothesized that applying genetic linkage and association analyses would implicate new genetic regions. To this end, we performed such analyses between our putative ADHD endophenotypes and genomic polymorphisms, that is, microsatellites and single nucleotide polymorphisms (SNPs). Our results provide novel findings that extend previous approaches and may

<sup>1</sup>Genomics and Predictive Medicine, Genome Biology Department, John Curtin School of Medical Research, ANU College of Medicine, Biology and Environment, The Australian National University, Canberra, ACT, Australia; <sup>2</sup>Group of Neurosciences, University of Antioquia, Medellin, Colombia; <sup>3</sup>Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA; <sup>4</sup>National Center for Indigenous Genomics, Genome Biology Department, John Curtin School of Medical Research, ANU College of Medicine, Biology and Environment, The Australian National University, Canberra, ACT, Australia and <sup>5</sup>The NYU Child Study Center at NYU Langone Medical Center, New York, NY, US and Nathan Kline Institute for Psychiatric Research, Orangeburg, NY, US. Correspondence: Dr M Muenke, Medical Genetics Branch, National Human Genome Research Institute, National Institutes of Health, Bethesda, MD, USA or Dr M Arcos-Burgos, Genomics and Predictive Medicine, Genome Biology Department, John Curtin School of Medical Research, ANU College of Medicine, Biology and Environment, The Australian National University, Building 131 Garran Road, Canberra, ACT 2600, Australia. E-mail: mamuenke@mail.nih.gov or Mauricio.Arcos-Burgos@anu.edu.au

<sup>6</sup>These authors contributed equally to this work.

Received 13 February 2015; revised 2 July 2015; accepted 5 August 2015; published online 24 November 2015

offer new insights on the etiological components of this common behavioral condition, if replicated.

## MATERIALS AND METHODS

### Sample population

We studied multigenerational and extended and nuclear pedigrees from the Paisa genetic isolate from the Medellin metropolitan area in the state of Antioquia, Colombia. Family ascertainment and sample size has been described in our previous studies.<sup>5,6</sup> Briefly, Paisa descent was considered as having all four grandparents originating from the Paisa region of Colombia. The original sample consisted of 1077 family members, 725 (67.3%) adults (17 years and older) and 352 (32.7%) children and adolescents (6–16 years old), from 141 nuclear and multigenerational families (126 nuclear and 15 extended and multigenerational families) from the Paisa genetic isolate<sup>5–8</sup> with an average family size of 38.5 (range 5–85 individuals) and an average of 3.62 generations (range 2–5). Upon obtaining written informed consent from participating subjects and/or their parents/legal guardians, pedigrees were built through a fixed sampling scheme from a parent or grandparent of an index proband as approved by the University of Antioquia Ethics Committee (Protocol: 11-13-342). This mentioned Committee also approved a subsequent collaboration with researchers from the US National Institutes of Health (Protocol: 00-HG-0058). Full details of the clinical, demographic and genetic ascertainment features, as well as the methodology of endophenotype characterization were published elsewhere.<sup>5–8</sup>

### Whole-genome scan non-parametric linkage

Genotyping of microsatellite markers was previously described.<sup>7</sup> A total of 372 markers across the whole genome, with an average distance of 8.68 cM, were genotyped in the aforementioned study (Table 1). In the present effort, given the large and multigenerational unique nature of these pedigrees, we applied non-parametric linkage analyses, as implemented in MERLIN, to the genotyped data.<sup>9</sup> Association analyses, using a variance component model to estimate an additive effect for each microsatellite, was carried out, in addition to non-parametric linkage analysis. Before association evaluation, missing genotypes were estimated

to increase statistical power. In addition to the non-parametric linkage analysis, we applied family-based association tests to the whole-genome scan data, under the null hypothesis of presence of no linkage and no association. In the case of previously linked regions, we applied family-based association tests under the null hypothesis of linkage and no association for single marker evaluation. In the case of haplotype analyses (shown in Supplementary Materials), the null hypothesis of no linkage and no association was tested. Five equidistant steps were arbitrarily defined between markers to estimate multipoint linkage statistics. Despite attempts to improve MERLIN's performance and resource use, it was computationally intensive and unable to run optimally because of the large pedigree sizes. To resolve this, we pruned some branches to decrease pedigree sizes. The pruning criteria were based on the availability of genotype and endophenotype information as well as family structure. The linkage analysis was carried out on six different endophenotypes (WISC Block Design, WISC PIQ, WISC FSIQ, ACVT Correct Responses, ACVT Omissions and ROCFT Copy scores) using age, sex, school grade and ADHD status as covariates to control for potential confounding factors.

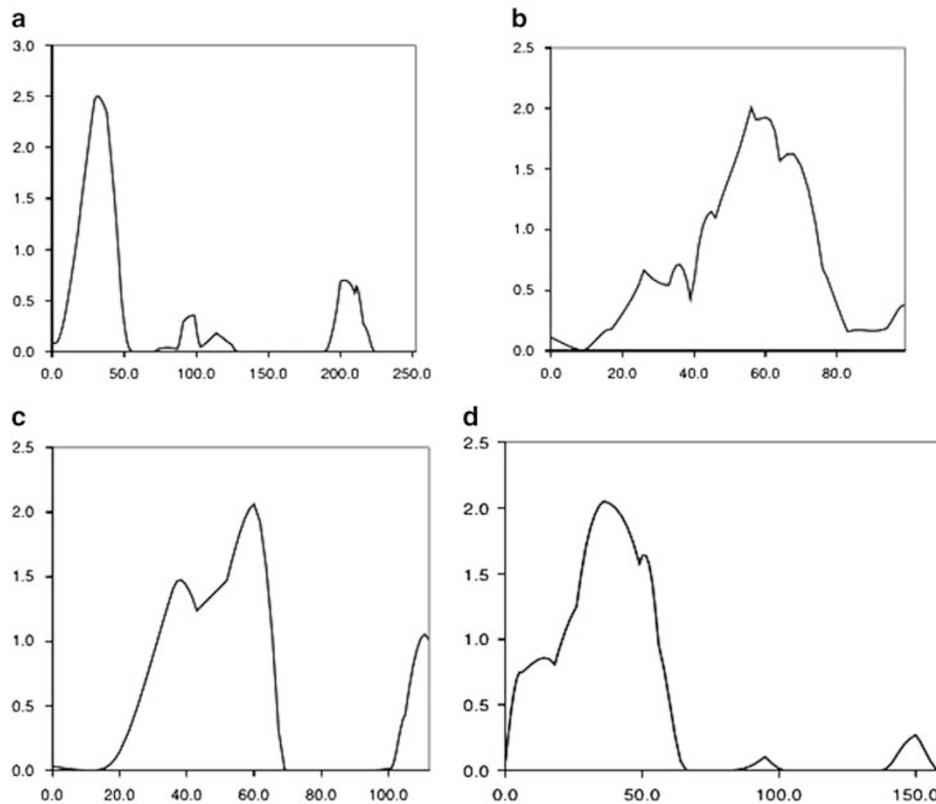
### Association analyses to loci linked to ADHD

Fine-scale targeted genetic association with a resolution of ~68 Kilo-base pairs (kb) was conducted to SNP markers spanning regions linked to ADHD, that is, 4q, 5q, 11p, 17p, 20q from the Paisa genetic sample<sup>3</sup> by using an approach similar to that previously described.<sup>10</sup> The 11p region refers to the linkage and association of variants of *DRD4* to ADHD.<sup>11</sup> *DRD4* is harbored in 11p. Unfortunately, we did not have enough data of the 11q-linked region in the set of paisa families. In the present studies, the data obtained were imported to SNP and Variation Suite (SVS) 7.6.7 (Golden Helix, Bozeman, MT, USA; <http://www.goldenhelix.com>) for association analyses.<sup>12–14</sup> The Golden Helix SVS 7.6.7 is an integrated collection of analytic tools for managing, analyzing and visualizing multifaceted genomic and phenotypic data. Parameters for excluding markers from analyses included: (i) deviations from Hardy–Weinberg equilibrium, (ii) a minimum genotype call rate of 70%, (iii) the presence of more than two alleles and (iv) monoallelic markers. Genotype and allelic frequencies were estimated by maximum likelihood. Family-based association tests as implemented in SVS 7.6.7 were applied to the whole set of markers that passed quality control. Genetic analysis, using the

**Table 1.** Summary for linkage and association analysis of peak LOD scores, *P*-values, position of peak and position relative to the closest marker

Endophenotype	Chromosome	Peak LOD	<i>P</i> -value	Peak LOD position (cM)	Closest marker	Distance from marker (cM)
WISC Block Design	2	2.51	0.00034	31.333	D2S1360	3.333
	3	1.25	0.0082	216	D3S1311	0
	5	1.11	0.0118	171	D5S1471	1
	9	1.33	0.0067	104	D9S938	0
	10	1.73	0.0024	61.334	D10S1221	1.666
	11	1.41	0.0054	109.667	D11S4464	3.333
	12	1.86	0.0017	56	D12S398	0
	13	1.19	0.0097	36	D13S325	3
	14	1.75	0.0023	109.5	D14S1434	3.5
	15	1.42	0.0053	58.667	D15S131	1.333
	16	1.02	0.015	27.5	D16S3103	4.5
	18	1.05	0.0141	28	D18S542	0
	19	1.41	0.0054	10	D19S1034	0
	WISC PIQ	2	1.68	0.0027	38	D2S405
3		1.34	0.0064	210.167	D3S2418	1.167
4		1.28	0.0076	33	D4S391	0
5		1.61	0.0032	172	D5S1471	0
9		1.21	0.0091	104	D9S938	0
12		1.78	0.0021	51.333	C12S916	2.333
13		2.01	0.00118	56	D13S317	0
15		2.06	0.00103	60	D15S131	0
18		1.07	0.0132	67.667	D18S851	3.667
19		1.33	0.0067	3.333	D19S591	3.333
WISC FSIQ	2	1	0.016	84.833	D2S1394	2.167
	3	1.06	0.0137	182	D3S2427	0
	12	2.05	0.00106	36	D12S1042	0
	19	1.12	0.0116	0	D19S591	0

Abbreviations: FSIQ, full scale intelligence quotient; LOD, logarithm of odds; PIQ, performance intelligence quotient; WISC, Wechsler Intelligence Scale for Children.



**Figure 1.** ADHD endophenotypes multipoint linkage chromosomal plots of non-parametric LOD scores  $>2.0$ . (a) WISC block design, chromosome 2p; (b) WISC PIQ, chromosome 13q; (c) the WISC PIQ, chromosome 15q; (d) WISC FSIQ chromosome 12p. LOD, logarithm of odds.

dominant model, and allelic tests of association were applied as implemented in Golden Helix's SVS 7.6.7. Each endophenotype (WISC Block Design, WISC PIQ, WISC FSIQ, ACVT Correct Responses, ACVT Omissions and ROCFT Copy scores) was independently analyzed while age, sex and school grade were considered as covariates of interest. ADHD status was considered as an interacting variable. Multiple test correction to determine significance was performed using the false discovery rate (FDR) approach. Haplotype analyses were also applied to contrast with marker-wise results (described in detail in Supplementary Materials).

## RESULTS

### Sample population—inclusion/exclusion criteria

From the 352 children and adolescents, 16 were excluded; 10 had a diagnosis of probably affected with ADHD and 6 were excluded because of incomplete clinical information. This left 336 young subjects, including 228 affected and 108 unaffected with ADHD in whom FSIQ was assessed. Only children and adolescents with  $FSIQ \geq 81$  and with regular school grades corresponding to their age were included in subsequent analyses to exclude participants potentially affected with generalized learning disorders. After applying this exclusion criterion, a final sample of 288 children and adolescents remained, including 194 (67.4%) affected with ADHD and 94 (32.6%) unaffected. The proportion of excluded children and adolescents with  $FSIQ \leq 80$  and academic problems did not differ statistically between affected (34/228; 14.9%) and unaffected children (14/108; 13.0%), (odds ratio=1.17, 95% confidence interval: 0.6–2.3, chi-square=0.2274,  $P=0.63$ ). We observed expected significant differences between ADHD affected and unaffected individuals on demographic covariates: sex ( $P < 0.00001$ ), age ( $P < 0.0001$ ) and school grade ( $P < 0.0001$ ).

### Whole-genome scan non-parametric linkage analyses

We found LOD scores  $>2.0$  for WISC Block Design on chromosome 2, marker D2S1360 (LOD=2.51,  $P=0.00034$ ); WISC PIQ on chromosome 15, marker D15S131 (LOD=2.06,  $P=0.00103$ ); and at marker D13S317 (LOD=2.01,  $P=0.00118$ ); and for WISC FSIQ on chromosome 12, marker D12S1042 (LOD=2.05,  $P=0.00106$ ) (Figure 1 and Table 1). Nominal LOD scores  $>1.0$  are presented in Table 1. Additional linkage results are presented in the Supplementary Materials.

### Association analysis to loci linked to ADHD

The targeted association analysis was carried out to SNP markers spanning regions previously described to be linked with ADHD, that is, 4q, 5q, 11p, 17p and 20q.<sup>3</sup> Table 2 shows only the significant associations after FDR correction. The ROCFT endophenotype was associated to markers rs6551660 (G allele,  $P_{raw}=0.0009$ ,  $P_{FDR}=0.0260$ ) and rs2013374 (G allele,  $P_{raw}=0.0014$ ,  $P_{FDR}=0.0260$ ), both located within the *Latrophilin 3* gene (*LPHN3*) (Figure 2). We also found that rs2282794, harbored in the *Fibroblast growth factor 1* (*FGF1*) gene, was significantly associated with ROCFT. Two markers were found to be significantly associated with WISC Block Design, rs2228130 (A allele,  $P_{raw}=0.0018$ ,  $P_{FDR}=0.0260$ ) and rs333117 (C allele,  $P_{raw}=0.0051$ ,  $P_{FDR}=0.0350$ ) located within the *Polymerase (RNA) II (DNA directed) polypeptide A* (*POLR2A*) and between the *Protein spinster homolog 3* (*SPNS3*) gene and the *Protein spinster homolog 2* (*SPNS2*) gene, respectively. Two markers were found to be significantly associated with WISC FSIQ, rs2236196 and rs3746372 located within the *Cholinergic receptor, nicotinic, alpha 4 (neuronal)* (*CHRNA4*) gene and between the uncharacterized *LOC100130152* and the *Potassium voltage-gated channel subfamily KQT member 2* (*KCNQ2*) genes, respectively. Three markers were found to be

**Table 2.** Top 20 regions of association for endophenotype and SNP markers, chromosome position, allele and frequency, closest gene, *P*-values with and without FDR correction

Endophenotype	Marker	Chromosome	Position	Allele (Frequency)	Closest gene(s)	P-value	
						Raw	FDR-corrected
ROCFT	rs6551660	4	62708149	G (0.43)	<i>LPHN3</i>	0.0009	0.0260
	rs2013374	4	62697759	G (0.37)	<i>LPHN3</i>	0.0014	0.0260
	rs2122642	4	62698263	C (0.37)	<i>LPHN3</i>	0.0016	0.0260
	rs2345041	4	62698356	C (0.37)	<i>LPHN3</i>	0.0016	0.0260
WISC block design	rs2228130	17	7404990	A (0.03)	<i>POLR2A</i>	0.0018	0.0260
ROCFT	rs4484334	4	62499840	T (0.49)	<i>LPHN3</i>	0.0024	0.0260
	rs1510921	4	62895591	T (0.19)	<i>LPHN3</i>	0.0026	0.0260
	rs7695134	4	62704851	T (0.42)	<i>LPHN3</i>	0.0027	0.0260
	rs2282794	5	141981708	A (0.11)	<i>FGF1</i>	0.0037	0.0317
WISC FSIQ	rs2236196	20	61977555	G (0.26)	<i>CHRNA4</i>	0.0043	0.0331
WISC block design	rs333117	17	4395169	C (0.45)	<i>SPNS3/SPNS2</i>	0.0051	0.0350
WISC PIQ	rs2236196	20	61977555	G (0.26)	<i>CHRNA4</i>	0.0061	0.0331
ROCFT	rs10001410	4	62474228	C (0.48)	<i>LPHN3</i>	0.0062	0.0350
	rs1948616	4	62487687	T (0.48)	<i>LPHN3</i>	0.0067	0.0350
WISC PIQ	rs1565902	4	62408619	C (0.37)	<i>LPHN3</i>	0.0069	0.0350
	rs6551678	4	63023050	G (0.31)	<i>LOC391656/LOC100131441</i>	0.0082	0.0350
WISC FSIQ	rs3746372	20	62032034	G (0.27)	<i>LOC100130152/KCNQ2</i>	0.0083	0.0350
ROCFT	rs990640	4	62698936	T (0.37)	<i>LPHN3</i>	0.0083	0.0350
ACVT-O	rs1982177	17	4119993	C (0.45)	<i>ANKFY1</i>	0.0091	0.0350
ACVTCR	rs1982177	17	4119993	C (0.45)	<i>ANKFY1</i>	0.0091	0.0350

Abbreviations: ACVT, 'A'-cancellation and vigilance test; FDR, false discovery rate; FSIQ, full scale intelligence quotient; PIQ, performance intelligence quotient; ROCFT, Rey-Osterrieth complex figure test; SNP, single nucleotide polymorphism; WISC, Wechsler Intelligence Scale for Children.

significantly associated with WISC PIQ, the two markers with the highest association being rs2236196 and rs1565902, which are located within the *CHRNA4* and *LPHN3* genes, respectively. Marker rs1982177, located within the *Ankyrin repeat and FYVE domain-containing protein 1* gene (*ANKFY1*) was found to be associated to ACVT Omissions and ACVT Correct Responses. Haplotype results are presented in the Supplementary Materials section.

## DISCUSSION

One of the long-term goals of finding associations between endophenotypes and specific loci and/or genes is the prediction of disease risk before clinical symptoms manifest fully. As a preliminary approach, this exploratory study sought possible associations between neuropsychological impairments frequently observed in patients with ADHD and novel genes. By using neuropsychological and genetic data from multigenerational families from the Paisa genetic isolate, we performed genetic linkage and association studies for several neuropsychological endophenotypes of ADHD. Linkage analysis was used to identify potential chromosomal regions. Then, association analysis was applied to specific loci identified as linked to ADHD in these pedigrees with the aim of identifying potential new candidate genes or to evaluate loci previously defined within the *LPHN3* gene.

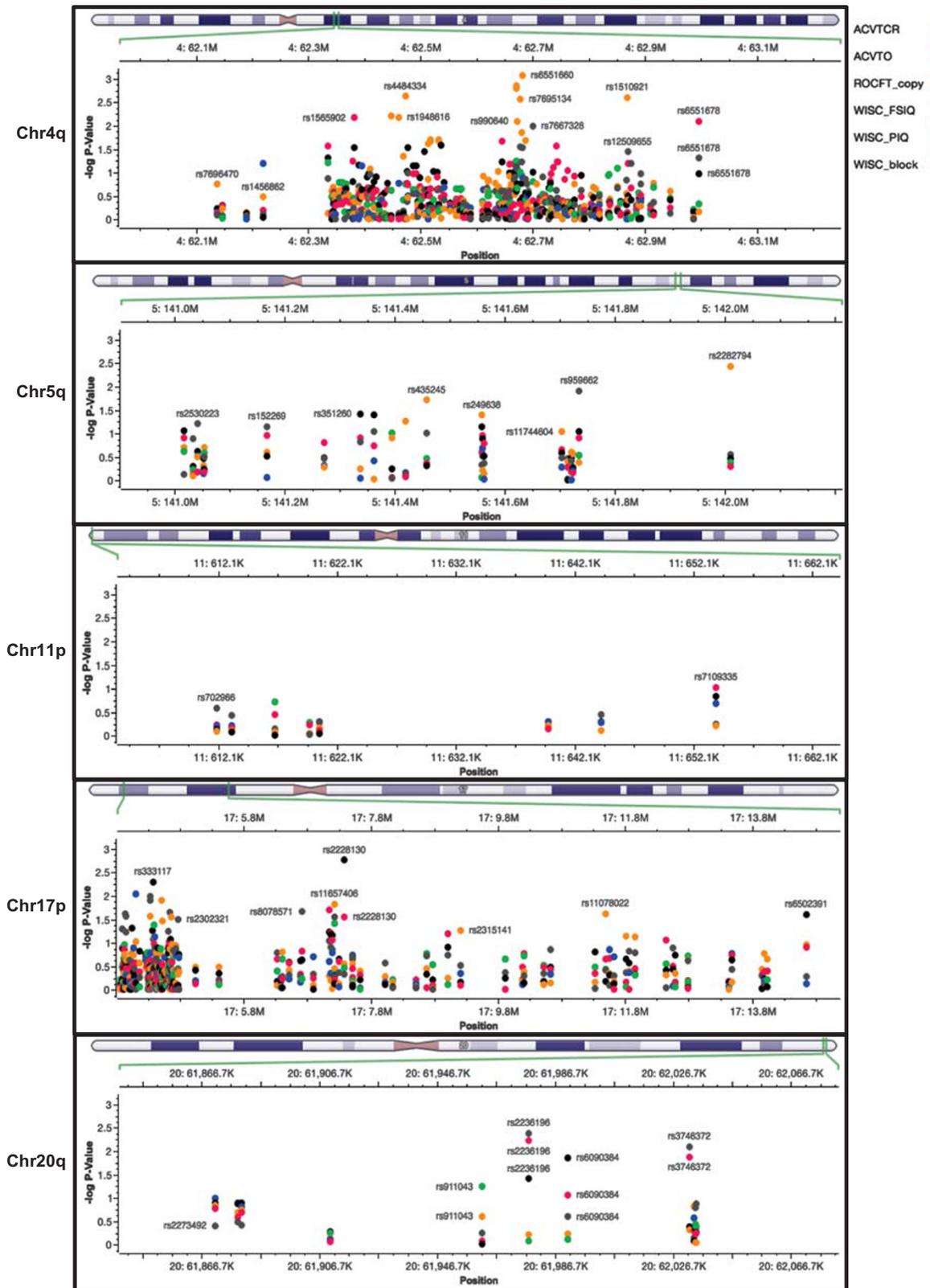
Our linkage analyses yielded nominal linkage at multiple chromosomal regions for WISC Block Design, WISC PIQ, WISC FSIQ, ACVT Correct Responses and ACVT Omissions. The six endophenotypes tested for a diverse range of performance, from fluid intelligence to sustained attention and visual-motor skills. We found genomic regions previously implicated in ADHD linked to our endophenotypes in chromosomes 4q13.2, 5q33.3, 11p15.5, 12p11.23 and 17p11. The overlapping of our linked regions to endophenotypes, that is, 2p24.2, 13q31.1, 15q23 and 12p11.23 to those reported by other studies is very difficult to demonstrate without applying a formal meta-analysis. However, our linkage results suggest that there is convergence with other

endophenotype linkage studies in chromosomes 2, 3, 4, 12, 13 and 14 (ref. 15, 16) (Figure 3).

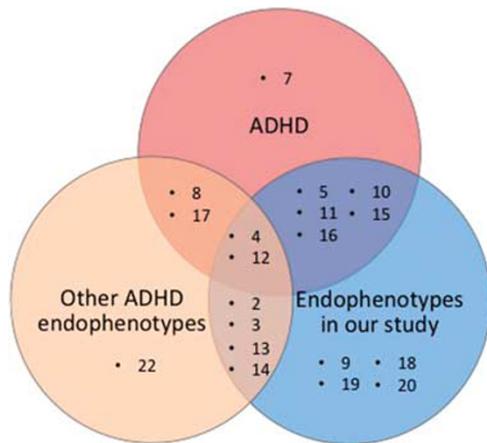
The implicated chromosomes described here and in other endophenotype studies could lead to the identification of novel candidate genes for ADHD, for example, promising linkage results from chromosome 13 led to the identification of *TUBA3* as a potential ADHD candidate gene.<sup>15</sup> These results support the concept that endophenotype measures could be a good indicator of ADHD status, but it is important to emphasize that by definition an endophenotype is not identical to a diagnosis, for example, an endophenotype is presumed to be more directly related to genetic factors, but many individuals will exhibit the endophenotype without the diagnosis, and *vice versa*.<sup>4</sup>

Association analyses carried out on selected chromosomal regions (4q, 5q, 11p, 17p and 20q) revealed significant associations for all tested endophenotypes, particularly close to the genes *LPHN3*, *FGF1*, *POLR2A*, *SPNS3*, *SPNS2*, *KCNQ2*, *CHRNA4* and *ANKFY1*. Interestingly, ROCFT was the endophenotype with the greatest significance in association studies even though it did not yield any nominal linkage signals. From the ROCFT association studies, two genes of interest were identified, *LPHN3* and *FGF1*. *LPHN3* encodes a member of the latrophilin subfamily of G protein-coupled receptors and has already been implicated in ADHD.<sup>3</sup> Latrophilins are thought to function in cell adhesion and signal transduction. *LPHN3* is highly expressed in the brain, particularly in the amygdala, caudate nucleus, pontine nucleus and cerebellum.<sup>10</sup> A loss of *LPHN3* function caused a reduction in the number and misplacement of dopamine-positive neurons in the ventral diencephalon of zebrafish which displayed a hyperactive/impulsive motor phenotype.<sup>17</sup> Hyperactivity/impulsivity or a lack of attention and motor control, prominent symptoms of ADHD, should impair the ability to perform the ROCFT copy test. Thus, the significant association between *LPHN3* and the ROCFT suggests that this endophenotype may be useful in dissecting the complex pathophysiology of ADHD.

Our ROCFT analysis also showed significant associations with *FGF1*. *FGF1* encodes for a protein in the fibroblast growth factor



**Figure 2.** Manhattan plot illustrating the independently analyzed endophenotypes with age and sex as covariates for chromosomal regions 4q, 5q, 11p, 17p and 20q. The vertical axis represents  $-\log_{10}(P\text{-value})$ ;  $-\lg(P) > 1.30$  was defined as a significant association. ROCFT copy was highly associated with LPHN3 variants harbored in the same region associated to ADHD. ACVTCR, << A >> Cancellation and Vigilance Test copy response; ACVTO, << A >> Cancellation and Vigilance Test Omissions; ROCFT\_copy, Rey-Osterrieth Complex Figure Test Copy subtest; WISC\_FSIQ, Wechsler Intelligence Scale for Children Full Scale Intelligence Quotient; WISC\_PIQ, Wechsler Intelligence Scale for Children Performance Intelligence Quotient; WISC\_block, Wechsler Intelligence Scale for Children Block Design.



**Figure 3.** Venn diagram comparing chromosomes implicated in ADHD linkage analyses, our endophenotype linkage analyses and ADHD endophenotype analyses by other investigators.

(FGF) family. The FGF family is involved in a broad range of mitogenic and cell survival activities ranging from embryonic development, cell growth, morphogenesis, tissue regeneration, tumor growth and invasion.<sup>18</sup> The FGF1 protein has a role in neuronal survival in Alzheimer's disease and is thought to be involved in its pathophysiology.<sup>19</sup> This gene could represent a novel candidate gene for ADHD by virtue of its involvement in neuronal survival, which may underlie the consistently decreased brain volume observed in ADHD.<sup>20</sup> *FGF1* was found to be highly expressed in brain regions related to major depression that might also be relevant for ADHD such as the dorsolateral prefrontal cortex and the anterior cingulate cortex.<sup>21</sup>

The association analyses also showed that *CHRNA4* was significantly associated with WISC FSIQ and WISC PIQ endophenotypes. This gene encodes a subunit of the neuronal nicotinic acetylcholine receptor, which is widely distributed in the brain and is involved in attention, memory and perception.<sup>22</sup> This gene has previously been associated with ADHD and is strongly implicated in tobacco addiction.<sup>22–24</sup> On the basis of the comorbidity of substance use disorders (particularly nicotine addiction) with ADHD,<sup>3,8,25</sup> and the distribution of receptors that would be affected by *CHRNA4* mutations, this gene and the nicotinic pathway should be examined more closely to better understand the pathophysiology of ADHD.

Association analyses also found significant associations for WISC Block Design and markers close to *SPNS3/SPNS2* and *POLR2A*. *SPNS3* is a gene that belongs to the solute carrier family 22 (SLC22) expressed in rat frontal cortex.<sup>26</sup> However, the possible functions of *SPNS3* within the brain remain unknown. With regard to *SPNS2*, there is no evidence of a specific role in brain, but it appears to be essential in cell trafficking from the bone marrow to blood.<sup>27</sup> The *POLR2A* gene encodes the primary subunit of the RNA polymerase II, which synthesizes messenger RNA in eukaryotes.<sup>28</sup> As there is scarce information about this gene, its possible involvement in ADHD remains to be studied.

A gene marker close to *KCNQ2*, a potassium channel encoding gene, was associated with WISC FSIQ. *KCNQ2* mutations are involved in forms of benign familial neonatal epilepsy, a condition that can co-occur with ADHD.<sup>29,30</sup>

Finally, the biological relevance of the association between the endophenotypes ACVT Omissions and ACVT Correct Responses with *ANKFY1* remains unknown. *ANKFY1* encodes an endosomal protein that participates in cell trafficking and is expressed in several tissues including the brain, but this gene has not been previously related to any neuropathological conditions.

Given that these pedigrees were ascertained because of ADHD clustering, our analyses controlled for ADHD as a predictor (covariate of interest) to determine genetic effects after adjusting for ADHD. By adding ADHD status to the conditional mean model and also using such status for the offset computation, we also increased the power of the family-based association test statistic substantially.<sup>31,32</sup>

This study has several limitations. Chromosome 2 had the highest linkage score for WISC Block Design (LOD=2.51) in our analyses and was shown to be a locus with significant linkage when Motor Timing was studied as a potential ADHD endophenotype.<sup>15</sup> However, we only performed association analyses on our six endophenotypes from chromosome 4 onwards. This was because of the limited availability of SNP genotyped data for chromosome 2 in our sample. As a result, we were unable to observe potential associations to candidate genes on chromosome 2.

Even though the number of individuals in this study was of sufficient size to capture endophenotype associations, the sample derived from a genetic isolate, the Paisas. This genetic isolate has been useful in identifying a number of genes associated to endophenotypes and potentially to ADHD,<sup>5</sup> but our association findings need to be replicated in independent populations and meta-analyzed to determine whether our results will apply to diverse populations. We pursued such a strategy for *LPHN3*, in which the initial finding was made in the Paisa genetic isolate and replicated in German, Spanish, American and Norwegian samples.<sup>10</sup> Thus, future replications in non-Paisa populations are planned. Further studies should also be carried out on the genes of interest that were significantly associated to the endophenotypes in this project, particularly *FGF1* and *CHRNA4*, to determine whether their variants confer susceptibility or protection for ADHD.

In conclusion, we found that neuropsychological endophenotypes were useful in discovering potential candidate genes related to ADHD that can afford greater insight into the pathophysiology of the disorder. Our analyses support the concept that the six endophenotypes are potentially linked and/or associated to ADHD. The ROCFT and WISC endophenotypes are specially promising, relative to the use of clinical categorical criteria alone, as they are able to identify individuals along a continuum. These are also widely available well-standardized measures. Furthermore, using different endophenotypes may also allow narrowing the specific neurobiological issue faced by individual subjects, because different pathways were implicated. For example, lower scores on the WISC could implicate the nicotinic pathway (because *CHRNA4* was associated with WISC endophenotypes) while lower scores on the ROCFT might implicate frontal-parietal circuits given *FGF1* association with the endophenotype. Thus, from linkage and association analyses, endophenotypes provide a powerful, objective and independent framework to current syndromic psychiatric classification in assessing the potential genetic causes underpinning ADHD susceptibility.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGMENTS

Claudio Mastronardi, Eva Pillai and Mauricio Arcos-Burgos are supported by John Curtin School of Medical Research, ANU College of Medicine, Biology and Environment, The Australian National University, Canberra, ACT, Australia, Project R42100-12. Maximilian Muenke, Ariel F Martinez, Maria T Acosta and Mauricio Arcos-Burgos are supported by the Division of Intramural Research, National Human Genome Research Institutes, National Institutes of Health, Bethesda, MD, USA. Part of this research was funded by the NHGRI-NIH and by COLCIENCIAS which funded the Project: 'Genética del Trastorno de Atención-Hiperactividad: los fenotipos complejos, los endofenotipos y la asociación con genes mayores y de susceptibilidad' Code: 1115-04-18083, Contract: 459-2005, to David Pineda, University of Antioquia and University of San Buenaventura.

REFERENCES

- 1 Polanczyk G, De Lima MS, Horta BL, Biederman J, Rohde LA. The worldwide prevalence of ADHD: A systematic review and meta-regression analysis. *Am J Psychiatry* 2007; **164**: 942–948.
- 2 Faraone SV, Mick E. Molecular genetics of attention deficit hyperactivity disorder. *Psychiatr Clin North Am* 2010; **33**: 159–180.
- 3 Arcos-Burgos M, Vélez J, Solomon B, Muenke M. A common genetic network underlies substance use disorders and disruptive or externalizing disorders. *Hum Genet* 2012; **131**: 917–929.
- 4 Gottesman II, Gould TD. The endophenotype concept in psychiatry: Etymology and strategic intentions. *Am J Psychiatry* 2003; **160**: 636–645.
- 5 Pineda DA, Lopera F, Puerta IC, Trujillo-Orrego N, Aguirre-Acevedo DC, Hincapié-Henao L *et al*. Potential cognitive endophenotypes in multigenerational families: segregating ADHD from a genetic isolate. *Atten Defic Hyperact Disord* 2011; **3**: 291–299.
- 6 Arcos-Burgos M, Castellanos FX, Lopera F, Pineda D, Palacio JD, García M *et al*. Attention-deficit/hyperactivity disorder (ADHD): feasibility of linkage analysis in a genetic isolate using extended and multigenerational pedigrees. *Clin Genet* 2002; **61**: 335–343.
- 7 Arcos-Burgos M, Castellanos FX, Pineda D, Lopera F, Palacio JD, Palacio LG *et al*. Attention-deficit/hyperactivity disorder in a population isolate: linkage to loci at 4q13.2, 5q33.3, 11q22, and 17p11. *Am J Hum Genet* 2004; **75**: 998–1014.
- 8 Palacio JD, Castellanos FX, Pineda DA, Lopera F, Arcos-Burgos M, Quiroz YT *et al*. Attention-deficit/hyperactivity disorder and comorbidities in 18 Paisa Colombian multigenerational families. *J Am Acad Child Adolesc Psychiatry* 2004; **43**: 1506–1515.
- 9 Abecasis GR, Cherny SS, Cookson WO, Cardon LR. Merlin—rapid analysis of dense genetic maps using sparse gene flow trees. *Nat Genet* 2002; **30**: 97–101.
- 10 Arcos-Burgos M, Jain M, Acosta MT, Shively S, Stanescu H, Wallis D *et al*. A common variant of the latrophilin 3 gene, LPHN3, confers susceptibility to ADHD and predicts effectiveness of stimulant medication. *Mol Psychiatry* 2010; **15**: 1053–1066.
- 11 Arcos-Burgos M, Castellanos FX, Konecki D, Lopera F, Pineda D, Palacio JD *et al*. Pedigree disequilibrium test (PDT) replicates association and linkage between DRD4 and ADHD in multigenerational and extended pedigrees from a genetic isolate. *Mol Psychiatry* 2004; **9**: 252–259.
- 12 Wong ML, Dong C, Flores DL, Ehrhart-Bornstein M, Bornstein S, Arcos-Burgos *et al*. Clinical outcomes and genome-wide association for a brain methylation site in an antidepressant pharmacogenetics study in Mexican Americans. *Am J Psychiatry* 2014; **171**: 1297–1309.
- 13 Paz-Filho G, Boguszewski MC, Mastronardi CA, Patel HR, Johar AS, Chuah A *et al*. Whole exome sequencing of extreme morbid obesity patients: translational implications for obesity and related disorders. *Genes (Basel)* 2014; **5**: 709–725.
- 14 Johar AS, Mastronardi C, Rojas-Villarraga A, Patel HR, Chuah A, Peng K *et al*. Novel and rare functional genomic variants in multiple autoimmune syndrome and Sjögren's syndrome. *J Transl Med* 2015; **13**: 1–11.
- 15 Rommelse NN, Arias-Vasquez A, Altiink ME, Buschgens CJ, Fliers E, Asherson P *et al*. Neuropsychological endophenotype approach to genome-wide linkage analysis identifies susceptibility loci for ADHD on 2q21.1 and 13q12.11. *Am J Hum Genet* 2008; **83**: 99–105.
- 16 Doyle AE, Ferreira MA, Sklar PB, Lasky-Su J, Petty C, Fusillo SJ *et al*. Multivariate genomewide linkage scan of neurocognitive traits and ADHD symptoms: suggestive linkage to 3q13. *Am J Med Genet B Neuropsychiatr Genet* 2008; **147B**: 1399–1411.
- 17 Lange M, Norton W, Coolen M, Chaminade M, Merker S, Proft F *et al*. The ADHD-susceptibility gene Iphn3.1 modulates dopaminergic neuron formation and locomotor activity during zebrafish development. *Mol Psychiatry* 2012; **17**: 946–954.
- 18 Yun Y-R, Won JE, Jeon E, Lee S, Kang W, Jo H *et al*. Fibroblast growth factors: biology, function, and application for tissue regeneration. *J Tissue Eng* 2010; **1**: 1.
- 19 Mashayekhi F, Hadavi M, Vaziri HR, Naji M. Increased acidic fibroblast growth factor concentrations in the serum and cerebrospinal fluid of patients with Alzheimer's disease. *J Clin Neurosci* 2010; **17**: 357–359.
- 20 Krain AL, Castellanos FX. Brain development and ADHD. *Clin Psychol Rev* 2006; **26**: 433–444.
- 21 Evans SJ, Choudary PV, Neal CR, Li JZ, Vawter MP, Tomita H *et al*. Dysregulation of the fibroblast growth factor system in major depression. *Proc Natl Acad Sci USA* 2004; **101**: 15506–15511.
- 22 Itier V, Bertrand D. Neuronal nicotinic receptors: from protein structure to function. *FEBS Lett* 2001; **504**: 118–125.
- 23 Wallis D, Arcos-Burgos M, Jain M, Castellanos FX, Palacio JD, Pineda D *et al*. Polymorphisms in the neural nicotinic acetylcholine receptor alpha4 subunit (CHRNA4) are associated with ADHD in a genetic isolate. *Atten Defic Hyperact Disord* 2009; **1**: 19–24.
- 24 Banaschewski T, Becker K, Scherag S, Franke B, Coghill D. Molecular genetics of attention-deficit/hyperactivity disorder: an overview. *Eur Child Adolesc Psychiatry* 2010; **19**: 237–257.
- 25 Acosta MT, Arcos-Burgos M, Muenke M. Attention deficit/hyperactivity disorder (ADHD): complex phenotype, simple genotype? *Genet Med* 2004; **6**: 1–15.
- 26 Jacobsson JA, Haitina T, Lindblom J, Fredriksson R. Identification of six putative human transporters with structural similarity to the drug transporter SLC22 family. *Genomics* 2007; **90**: 595–609.
- 27 Fukuhara S, Simmons S, Kawamura S, Inoue A, Orba Y, Tokudome T *et al*. The sphingosine-1-phosphate transporter Spns2 expressed on endothelial cells regulates lymphocyte trafficking in mice. *J Clin Invest* 2012; **122**: 1416–1426.
- 28 Ten Asbroek AL, Fluiter K, Van Groenigen M, Nooij M, Baas F. Polymorphisms in the large subunit of human RNA polymerase II as target for allele-specific inhibition. *Nucleic Acids Res* 2000; **28**: 1133–1138.
- 29 Jones JE, Watson R, Sheth R, Caplan R, Koehn M, Seidenberg M *et al*. Psychiatric comorbidity in children with new onset epilepsy. *Dev Med Child Neurol* 2007; **49**: 493–497.
- 30 Kattimani S, Mahadevan S. Treating children with attention-deficit/hyperactivity disorder and comorbid epilepsy. *Ann Indian Acad Neurol* 2011; **14**: 9–11.
- 31 Lange C, Laird NM. On a general class of conditional tests for family-based association studies in genetics: the asymptotic distribution, the conditional power, and optimality considerations. *Genet Epidemiol* 2002; **23**: 165–180.
- 32 Lange C, Laird NM. Power calculations for a general class of family-based association tests: dichotomous traits. *Am J Hum Genet* 2002; **71**: 575–584.



This work is licensed under a Creative Commons Attribution-NonCommercial-NoDerivs 4.0 International License. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in the credit line; if the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material. To view a copy of this license, visit <http://creativecommons.org/licenses/by-nc-nd/4.0/>

Supplementary Information accompanies the paper on the Molecular Psychiatry website (<http://www.nature.com/mp>)