

## Report

# Joint Analysis of the *DRD5* Marker Concludes Association with Attention-Deficit/Hyperactivity Disorder Confined to the Predominantly Inattentive and Combined Subtypes

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Attention-deficit/hyperactivity disorder (ADHD) is a highly heritable, heterogeneous disorder of early onset, consisting of a triad of symptoms: inattention, hyperactivity, and impulsivity. The disorder has a significant genetic component, and theories of etiology include abnormalities in the dopaminergic system, with *DRD4*, *DAT1*, *SNAP25*, and *DRD5* being implicated as major susceptibility genes. An initial report of association between ADHD and the common 148-bp allele of a microsatellite marker located 18.5 kb from the *DRD5* gene has been followed by several studies showing nonsignificant trends toward association with the same allele. To establish the postulated association of the (CA)<sub>n</sub> repeat with ADHD, we collected genotypic information from 14 independent samples of probands and their parents, analyzed them individually and, in the absence of heterogeneity, analyzed them as a joint sample. The joint analysis showed association with the *DRD5* locus ( $P = .00005$ ; odds ratio 1.24; 95% confidence interval 1.12–1.38). This association appears to be confined to the predominantly inattentive and combined clinical subtypes.

Attention-deficit/hyperactivity disorder (ADHD [MIM 143465]) is a common disorder of childhood onset that manifests itself as a combination of inattentive, hyperactive, and impulsive symptoms. It is known to affect

3%–9% of school-aged children worldwide (Faraone et al., in press) and may often persist into adulthood (Faraone et al. 2000). The heritability of ADHD is estimated to be 70%–90% (Levy et al. 1997; Thapar et al. 1999). Children with this disorder often develop severe problems with personal relationships and academic development. Compared with control subjects, ADHD probands exhibit lower grades, fail more courses, have worse performance on standardized tests, have fewer friends, and are rated less adequate in psychosocial adjustment (Man-

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**Table 1****Information about Individual Groups**

Group Number	Primary Investigator	No. of Cases	Publication Status <sup>a</sup>	Origin of Sample	Instrument of Diagnosis <sup>b</sup>
1	C. J. Kratochvil	41	U	United States	KSADS
2	F. Levy	66	U	Australia	ATBRS
3	L. Kent	69	U	United Kingdom	CAPA
4	S. V. Faraone	82	U	United States	K-SADS-E
5	L. A. Rohde	85	U	Brazil	K-SADS-E
6	E. Tahir	100	P <sup>c</sup>	Turkey	KSADS
7	P. Asherson	103	U	United Kingdom	CAPA
8	A. Thapar	106	P <sup>d</sup>	United Kingdom	CAPA
9	R. D. Todd	161	U	United States	MAGIC
10	M. Gill	168	P <sup>e</sup> + U	Ireland	CAPA
11	R. P. Ebstein	176	U	Israel	Clinical interview
12	C. L. Barr	178	P <sup>f</sup> + U	Canada	PICS-IV
13	R. J. Sinke	207	U	Holland	DISC
14	S. L. Smalley	438	P <sup>g</sup>	United States	K-SADS-PiL

<sup>a</sup> U = unpublished data; P = previously published data; P + U = previously published data with the addition of new unpublished data.

<sup>b</sup> KSADS = Kidi schedule for affective disorders and schizophrenia (Kaufman et al. 1997). ATBRS = Australian Twin Behavior Rating Scale (Hay et al. 2001). CAPA = Child and Adolescent Psychiatric Assessment (Angold et al. 1995). K-SADS-E = Schedule for Affective Disorders and Schizophrenia for School-Age Children—Epidemiologic Version (Orvaschel et al. 1987). MAGIC = Missouri Assessment of Genetics Interview for Children (Reich 2000). PICS-IV = Parent Interview for Child Symptoms (A. Schachar, A. Ichowicz, unpublished data). DISC = Diagnostic Interview Schedule for Children (Shaffer et al. 2000). K-SADS-PiL = Schedule for Affective Disorders and Schizophrenia for School-Age-Children Present and Lifetime version (Kaufman et al. 1997).

<sup>c</sup> Tahir et al. (2000).

<sup>d</sup> Payton et al. (2001).

<sup>e</sup> Daly et al. (1999).

<sup>f</sup> Barr et al. (2000).

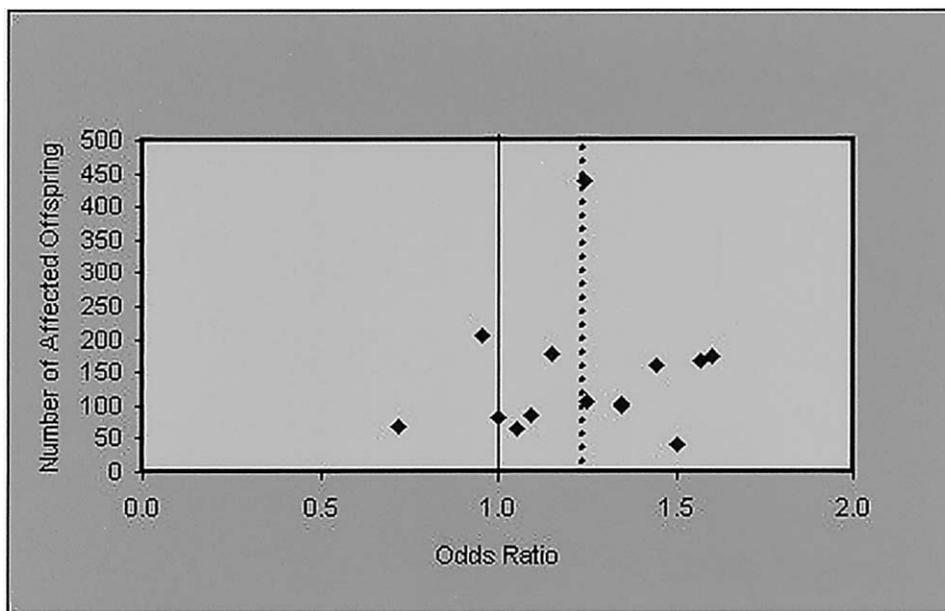
<sup>g</sup> Kustanovich et al. (in press).

nuzza and Klein 2000). In addition, individuals with this disorder are at greater risk for substance abuse (Flory et al. 2003). Furthermore, individuals with ADHD are reported to have increased driving problems; as part of a case-control study, individuals with ADHD self-reported more traffic citations—including, speeding, vehicular crashes, and license suspensions—than their counterparts in the control group (Barkley et al. 2002).

The exact etiology of ADHD is unknown, but it is widely recognized to have a significant genetic component, as demonstrated by family (Biederman et al. 1992), twin (Silberg et al. 1996), and adoption (Cadoret and Stewart 1991) studies. Although both the serotonergic and noradrenergic systems have been implicated in ADHD, the dopaminergic system is by far the most extensively explored to date. Neuropharmacological studies have demonstrated that methylphenidate and dexamphetamine—stimulant medications that are effective in ~70% of patients with ADHD (Spencer et al. 1996)—block the reuptake of dopamine by the dopamine transporter DAT1 (Amara and Kuhar 1993; Krause et al. 2000). Neuroimaging studies have shown abnormalities in the frontal lobe and subcortical structures, regions that are known to be

rich in dopaminergic neurotransmission and important in the control of attention and response to organization (Lou et al. 1990; Zametkin et al. 1990; Rubia et al. 1997). Multiple animal models have been produced, including the DAT1 knockout mouse, which expresses spontaneous hyperactivity and difficulty with learning tasks (Davids et al. 2003). In addition, molecular genetic studies have implicated susceptibility genes, including those for the dopamine D4 receptor (*DRD4*), the dopamine transporter (*DAT1*), dopamine  $\beta$  hydroxylase (*DBH*), synaptosomal associated protein 25 (*SNAP25*), and the dopamine D5 receptor (*DRD5*).

To date, the most extensively examined genes of the dopaminergic system are *DRD4* and *DAT1* (Hawi et al. 2003a). The primary focus of attention at *DAT1* has been the 480-bp allele of a 40-bp repeat located in the 3' UTR of the gene. In the case of *DRD4*, the majority of studies have reported on a 48-bp microsatellite located in exon 3 of the gene and translated to the third intracellular loop of the protein. Recently, meta-analyses have been performed on both of these markers, in an attempt to definitively confirm or refute association with ADHD (Faraone et al. 2001; Maher et al. 2002).



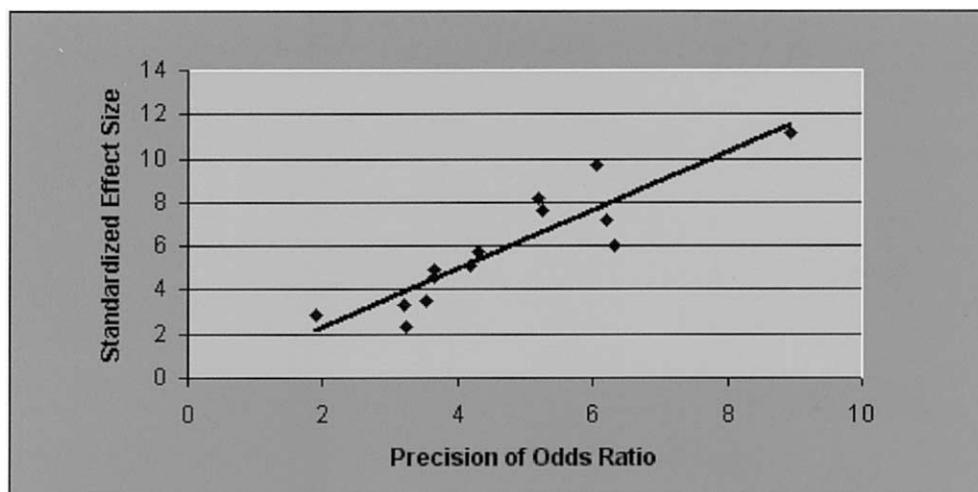
**Figure 1** Funnel plot showing the number of affected offspring versus OR. The inverted funnel shape of this plot depicts results consistent with the absence of publication bias. The solid line represents OR = 1, and the dotted line represents OR = 1.24.

In 1999, Daly et al. reported a significant association between ADHD and the 148-bp allele of a microsatellite marker located 18.5 kb 5' to the *DRD5* gene on chromosome 4. Subsequent studies of this (CA)<sub>n</sub> repeat marker have shown nonsignificant trends toward association with the same allele (Barr et al. 2000; Tahir et al. 2000; Payton et al. 2001). To avoid the premature conclusion of the presence or absence of an association with ADHD (and guided by the meta-analyses of *DRD4* and *DAT1*), a joint analysis of *DRD5* was proposed. Although there is no evidence to suggest that the D5 microsatellite is itself functional, the association reported by Daly et al. (1999) is too strong to be ignored. Therefore, we hypothesized that if the association with ADHD were true, the microsatellite may be in linkage disequilibrium (LD) with one or more functional variants. To this end, we invited all known groups with samples based on parent-proband trios to genotype their sample for the marker and present us with their data for analysis (table 1).

In the event of an overall significant association between the variant and ADHD, we proposed that, owing to the unique opportunity presented by a data set of this magnitude, we would further examine the association in relation to a number of different subdivisions. Three times more males than females receive a diagnosis of ADHD (Anderson et al. 1987), and it has been reported by teachers that affected boys are more inattentive and more hyperactive/impulsive than affected girls (Hartung et al. 2002). Furthermore, Clarke et al. (2003) have reported results indicating that girls with ADHD also ex-

hibit abnormalities in their electroencephalograms; however, there is far less variance in their profiles than appears in boys. We therefore proposed analysis by the sex of the affected child. In addition, we planned to examine the sex of the transmitting parent, because the literature has suggested the possibility of imprinting at the *DRD2* (Kirley et al. 2002) and *SNAP-25* loci (Brophy et al. 2002) in conjunction with multiple known imprinted genes in various other areas of psychiatric genetics (Davies et al. 2001). Finally, recent attention has focused on more homogeneous diagnostic symptoms and subtypes. Waldman et al. (1998) reported that, in between-family association analyses, levels of predominantly hyperactive-impulsive symptoms were related to the number of *DAT1* high-risk alleles but that levels of the inattentive symptoms were not, whereas within-family analysis showed association between *DAT1* and the combined subtype. We therefore also proposed to analyze the joint sample in relation to diagnostic subtype.

All children in the present study were given a diagnosis of one of the three clinical subtypes (predominantly inattentive, predominantly hyperactive/impulsive, or combined) of ADHD, according to criteria of the *Diagnostic and Statistical Manual*, fourth edition (DSM-IV) (American Psychiatric Association 1994). To receive a diagnosis of predominantly inattentive or predominantly hyperactive/impulsive ADHD, children must display a minimum of six of the nine symptoms from the inattentive or hyperactive/impulsive sections of the DSM-IV in a minimum of two settings (e.g., home and school). If a child has



**Figure 2** Regression of the standardized effect size versus the precision of the OR. The intercept of the line will occur close to 0, implying no bias in the samples.

six or more symptoms in both sections, then he or she receives a diagnosis of combined-type ADHD. Inclusion criteria for the present study were the presence of a DSM-IV diagnosis of childhood-onset ADHD and the genotyping of one or both parents. Exclusion criteria were the presence of pervasive developmental disorders, fragile X syndrome, major neurological disorders, fetal alcohol syndrome, Tourette syndrome, psychosis, and a full-scale IQ score <70. Information on the sex of the children was provided along with the clinical subtype. Genotypic data for the microsatellite marker were collected from each group, along with at least three DNA samples, which were genotyped at the Neuropsychiatry Genetics Laboratory, Dublin, to ensure consistent allele calling between centers. The data received from each group consisted of genotypes from affected probands and either or both of their parents.

To obtain as complete a sample as possible—and to avoid potential bias—we contacted all members of the ADHD collaborative network (Faraone et al. 2003) and invited their participation. We also searched PubMed using the keywords “ADHD” and “*DRD5*.” This revealed an earlier meta-analysis (Maher et al. 2002) that contained data from four published studies, which we have included, and from an additional unpublished sample, which we excluded because it did not meet our inclusion criteria. We are aware of five additional samples that have not yet been genotyped for this marker. This approach to sample collection should provide adequate power to confirm a gene of small effect and to minimize publication bias. A funnel plot (Egger et al. 1997) was produced to examine for evidence of sample bias in the study. This was achieved by plotting the number of affected children for each sample against its equivalent odds

ratio (OR), in conjunction with the OR for the combined sample (fig. 1). The larger samples should result in ORs that are closer to the true OR than the smaller samples. In addition, we regressed the normal SD of the OR against the precision of the OR, according to the methods used by Egger et al. (1997) through use of STATA 6.0 (StataCorp 1999). This analysis works on the same basis as the funnel plot, in that the precision of the OR increases with sample size. Egger et al. (1997) showed that, in the absence of bias, the regression would run through the origin (fig. 2). This method produces an  $R^2$  (measure of the fit of the regression to the data) and a value for the intercept of the line, along with corresponding statistics.

Initially, association analyses were performed on each individual group’s sample, using the family-based extended transmission/disequilibrium test (ETDT) (Sham and Curtis 1995). This test was designed to avoid population stratification by using parental genotypes as internal controls and to disregard duos whose inclusion could potentially lead to bias (Curtis and Sham 1995). It is adapted from the transmission/disequilibrium test (TDT) and is used to examine for linkage in the presence of association between multiallelic markers and disease phenotypes. The analysis involves the comparison of transmissions and nontransmissions of the risk allele from heterozygous parents to affected offspring.

To test for heterogeneity among the samples, we used the transmission and nontransmission information of the 148-bp allele from the TDT analysis. Two different logistic regression models were compared. The first model assumed homogeneity (i.e., that the regression coefficients were the same across studies, so only one regression coefficient was estimated); the second model assumed het-

erogeneity (i.e., that all the regression coefficients were different, so that as many regression coefficients were estimated as there were studies). Both models produced a  $-2 \log$  likelihood value, a measure that attempts to assess the suitability of the statistical method used for a given set of data. Using the formula  $(-2 \log \text{likelihood}_{\text{individual}}) - (-2 \log \text{likelihood}_{\text{combined}}) = \chi^2$ , we tested for evidence of a significant difference between the two models, which would be indicative of heterogeneity among the samples.

For the sensitivity analysis, each group's data were removed in turn from the combined total, and the remaining data were reanalyzed. This was performed to ensure that no individual group was biasing the combined result.

The attributable fraction (Levin 1953) for the marker was calculated according to the formula  $f(RR - 1)/[f(RR - 1) + 1]$ , where  $f$  is the frequency of the 148-bp allele in the sample of nontransmitted alleles and  $RR$  is the relative risk. The value was calculated as  $RR = [a/(a + c)]/[b/(b + d)]$ , where  $a$  is the number of transmissions of the risk allele,  $b$  is the number of nontransmissions of the risk allele,  $c$  is the number of transmissions of the nonrisk alleles, and  $d$  is the number of nontransmissions of the nonrisk alleles.

To test the combined sample for significant differences between the diagnostic subtype, the sex of the child, and the sex of the transmitting parent, we first performed TDT analyses on the subsets of each group (e.g., a male subset and a female subset for the group defined by sex), using the ETDT. The ORs calculated for each were then weighted according to the number of cases in each subset and were compared with each other by use of logistic regression.

TDT analyses on the individual samples tested showed a significant association between the 148-bp allele and two of the ADHD samples. The majority of the rest displayed an excess of transmission of the 148-bp allele but did not attain statistical significance. Two of the samples showed a level of transmission of the 148-bp allele that was less than the expected transmission (table 2; fig. 3).

Despite these variations, the heterogeneity test failed to detect the presence of heterogeneity between the 14 samples ( $\chi^2 = 11.98$  with 13 df;  $P = .53$ ). They were therefore combined, and the joint analysis on this data set (1,980 probands and 3,072 parents) indicated a strongly significant association of small effect ( $\chi^2 = 16.45$  with 1 df;  $P = .00005$ ; OR = 1.24; 95% CI 1.12–1.38) between ADHD and the 148-bp allele of the microsatellite marker. Sensitivity analysis (table 3) showed strong significance, regardless of the data set removed, with the  $P$  value never  $>.001$  and the OR never fluctuating  $>0.04$  in either direction from the combined result. The attributable fraction was calculated using the frequency of the 148-bp allele among nontransmitted parental alleles as a

**Table 2**

**Individual Group Results**

Group Number	No. of Affected Cases	No. of Parents	Allele Range <sup>a</sup>	Frequency of Allele 10 (%)	OR	<i>P</i>
1	41	63	6–17	49.2	1.50	.44
2	66	82	7–17	35.1	1.05	.88
3	69	121	4–18	50.8	.72	.29
4	82	126	5–18	46.5	1.00	1.00
5	85	138	7–19	39.7	1.09	.77
6	100	193	4–17	39.8	1.34	.21
7	103	179	6–18	44.9	1.35	.28
8	106	233	6–18	43.2	1.25	.42
9	161	271	6–19	44.5	1.45	.05
10	168	325	6–17	43.6	1.57	.02
11	176	333	1–16	36.3	1.60	.004
12	178	268	5–17	46.4	1.15	.38
13	207	295	6–19	44.6	.95	.75
14	438	445	5–20	38.5	1.24	.05
Total	1,980	3,072	1–20	43.1	1.24	.00005

<sup>a</sup> Allele range: allele 1 = 166 bp, allele 20 = 128 bp, and allele 10 = 148 bp.

conservative estimate of population frequency and was found to be 0.07.

In the funnel plot (fig. 1), the larger samples displayed ORs closer to that of the combined OR than did the smaller samples, resulting in a visual representation of an inverted funnel that is consistent with the absence of sample bias. The method described by Egger et al. (1997) produced a scatter plot of the data with an  $R^2$  value of 0.8036 (fig. 2), implying a good fit of the data to the line. The intercept was calculated to be 0.179 (SE = 1.100245;  $t = 0.16$ ;  $P = .874$ ; 95% CI  $-2.218645$  to  $2.575809$ ). According to Egger et al. (1997), in the absence of publication bias, the intercept of this line should be 0. If bias exists, then the intercept should be significantly  $>0$ . Our intercept of 0.179 is not significant and is therefore consistent with the funnel plot in its implication that publication bias is absent from the sample.

We examined the association in relation to the sex of the child, which was negative, and the sex of the transmitting parent, which showed a trend toward a maternal effect ( $\chi^2 = 2.6$ ; with 1 df;  $P = .11$ ) and the diagnostic subtype. The three clinical subtypes—predominantly inattentive ( $n = 445$ ), combined ( $n = 1,106$ ), and predominantly hyperactive/impulsive ( $n = 122$ )—were examined separately and found to have ORs of 1.5, 1.3, and 0.9, respectively, with corresponding  $P$  values of .0001, .0001, and .6. Comparison between the individual ORs (table 4) indicated a significant difference ( $P = .035$ ) between the inattentive and hyperactive/impulsive subtypes.

Loci conferring risk with OR  $<1.5$  require large samples for detection and replication (Dahlman et al. 2002). In the field of psychiatric genetics, this is difficult to attain;

consequently, there are multiple small studies of markers without conclusive results. This has led to the introduction of the combined, or meta-analysis, approach to this area of genetics. The principal concerns in combining multiple data sets are the possibility of heterogeneity between samples and the possibility of publication bias. In the present study, we found no evidence of heterogeneity among the samples and no publication bias, and we therefore combined the data from all 14 groups.

Using the large combined sample, we have determined that the OR for the DRD5 148-bp variant is 1.24; however, because the allele is common in the population, it has an attributable fraction of 0.07. For complex disorders involving the interaction of multiple genes and environmental factors, it is difficult to attribute an individual case to any single etiological factor. Nevertheless, the attributable fraction is still a useful index of potential public health importance, because it is defined to be the fractional reduction in the frequency of disease if the risk factor were eliminated from the population. Sensitivity analysis showed that the omission of any of the groups—including that with the smallest *P* value (group 11), that with the largest *P* value (group 13), or that containing the original findings (group 10)—did little to alter the overall results, implying that no one group's data are responsible for the combined result.

The existence of a single published result showing significant association between the 148-bp allele and ADHD

**Table 3**

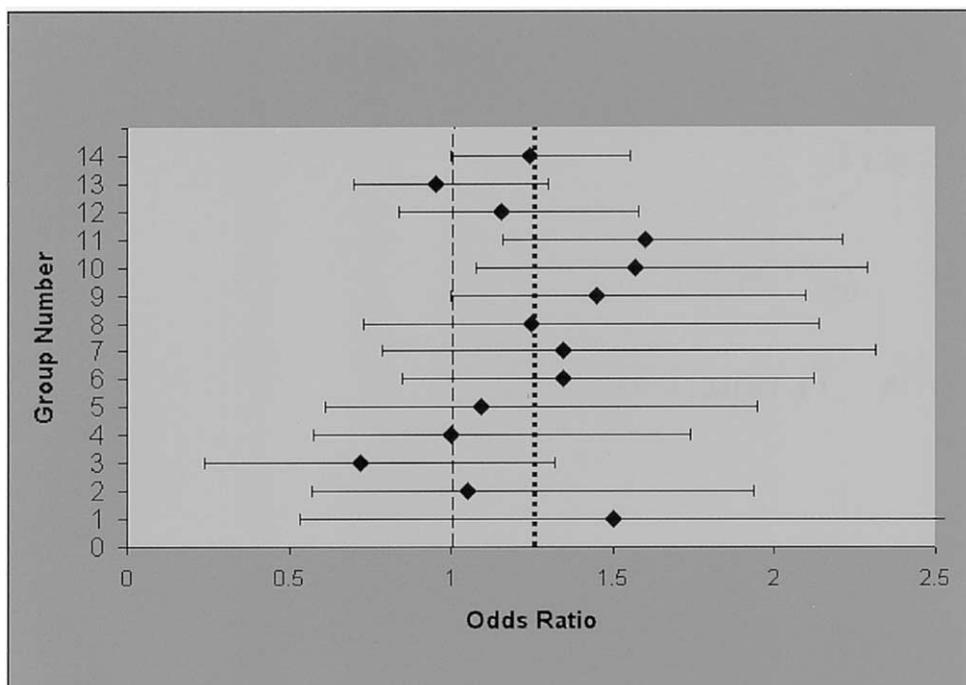
**Sensitivity Analysis**

Subtracted Group	OR	95% CI	<i>P</i>
1	1.24	1.11 – 1.38	.00007
2	1.25	1.12 – 1.39	.00004
3	1.26	1.14 – 1.40	.00002
4	1.25	1.12 – 1.39	.00004
5	1.24	1.12 – 1.38	.00007
6	1.24	1.11 – 1.38	.00011
7	1.24	1.11 – 1.38	.00009
8	1.24	1.12 – 1.38	.00007
9	1.22	1.10 – 1.37	.00027
10	1.22	1.09 – 1.36	.00041
11	1.20	1.08 – 1.34	.00103
12	1.25	1.12 – 1.40	.00007
13	1.28	1.15 – 1.43	.00001
14	1.24	1.10 – 1.40	.00039
Total <sup>a</sup>	1.24	1.12 – 1.38	.00005

NOTE.—Results are shown for the joint data set, with the subtraction of each group in turn.

<sup>a</sup> Totals indicate results when no group is subtracted.

(Daly et al. 1999), coupled with a number of nonsignificant trends in the same direction (Barr et al. 2000; Tahir et al. 2000; Payton et al. 2001), follows a pattern familiar to researchers and others engaged in association studies with small samples. It could have been



**Figure 3** ORs and respective 95% CIs for each sample, displayed in order of size, with the largest sample at the top. The solid line represents an OR of 1, and the dotted line represents an OR of 1.24

**Table 4****Logistic Regression Analysis Comparing ORs of the Diagnostic Subgroups**

Comparison	$\chi^2$	<i>P</i>	95% CI
Predominantly inattentive vs. combined	1.287	.257	.902–1.471
Predominantly hyperactive/impulsive vs. combined	2.654	.103	.659–1.039
Predominantly inattentive vs. predominantly hyperactive/impulsive	4.468	.035	.367–.963

concluded, incorrectly, that the original result was a false-positive finding. The present joint analysis, however, suggests that the original is a true finding of association between ADHD and the polymorphism and that individual sample sizes lacked sufficient power for confirmation.

Subtype analyses indicate that the association of the 148-bp allele is selective for predominantly inattentive and combined subtypes. It should be noted that although the sample size for the predominantly hyperactive/impulsive group is relatively small, the power of this sample to detect association with the disorder was >75% ( $\alpha = 0.05$ ) and 85% ( $\alpha = 0.1$ ). Whether the DRD5 association is restricted to inattentive symptoms awaits further study using QTL (Curran et al. 2001) or alternative subtyping approaches (Todd et al. 2001; Rasmussen et al. 2002).

The microsatellite marker is located 18.5 kb from the transcription start site of the DRD5 gene, and no functional role has been reported to date. This makes it an unlikely, although not impossible, functional variant. It is more probable that the microsatellite is in LD with the true functional variant (or variants) located in (or closer to) the DRD5 gene. In support of this theory, a recent publication reported a significant haplotype composed of the 148-bp allele of this marker and another microsatellite located at the 3' UTR of the gene ( $\chi^2 = 14.208$ ;  $P = .00017$ ) with a significant LD between the markers ( $D' = 0.519$ ) (Hawi et al. 2003b).

In conclusion, the present study has shown that the 148-bp allele of this DRD5 microsatellite marker is associated with ADHD in a large combined homogeneous international sample. It has also been indicated that the association may be confined to particular subtypes or symptom traits, although we acknowledge that this finding requires additional confirmation. We believe that the results of this joint analysis will encourage efforts to identify the functional variant or variants of this gene and will promote further collaboration in the field of psychiatric genetics.

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## Electronic-Database Information

The URL for data presented herein is as follows:

Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim/> (for ADHD)

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