

ARTICLE

Genome-wide linkage analysis in a Dutch multigenerational family with attention deficit hyperactivity disorder

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Attention deficit hyperactivity disorder (ADHD) is a common neuropsychiatric disorder. Genetics has an important role in the aetiology of this disease. In this study, we describe the clinical findings in a Dutch family with eight patients suffering from ADHD, in whom five had at least one other psychiatric disorder. We performed a genome-wide (parametric and nonparametric) affected-only linkage analysis. Two genomic regions on chromosomes 7 and 14 showed an excess of allele sharing among the definitely affected members of the family with suggestive LOD scores (2.1 and 2.08). Nonparametric linkage analyses (NPL) yielded a maxNPL of 2.92 ($P=0.001$) for marker D7S502 and a maxNPL score of 2.56 ($P=0.003$) for marker D14S275. We confirmed that all patients share the same haplotype in each region of 7p15.1–q31.33 and 14q11.2–q22.3. Interestingly, both loci have been reported before in Dutch (affected sib pairs) and German (extended families) ADHD linkage studies. Hopefully, the genome-wide association studies in ADHD will help to highlight specific polymorphisms and genes within the broad areas detected by our, as well as other, linkage studies.

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INTRODUCTION

Attention deficit hyperactivity disorder (ADHD) is a pervasive neuropsychiatric disorder affecting 4–5% of children and 0.5–2% of adults.¹ Genetics has an important role in the aetiology of the disorder. Data from clinical studies support the familial nature of ADHD.² Twin studies of categorically defined ADHD estimated heritability to be 60–90%.³ Smalley⁴ and Faraone *et al*⁵ reported sibling relative-risk ratios (λ s) of 4.0–8.0. Adoption studies report an increased frequency of ADHD in biological relatives of probands.^{6–8} Smalley⁴ proposed a genetic model for ADHD with an involvement of multiple genes with minor-to-moderate effect sizes interacting in an additive manner. Genome-wide exclusion mapping⁹ and molecular studies of candidate genes¹⁰ support this theory. However, the exact aetiology of ADHD is still unknown.

The search for susceptibility genes involved in the development of ADHD has focused mostly on the dopaminergic system, largely because of the therapeutic effects of methylphenidate hydrochloride, which increases extracellular dopamine levels by inhibiting re-uptake from the synaptic cleft. Many association studies on genes such as the dopamine D4 receptor gene (*DRD4*), the dopamine D5 receptor gene (*DRD5*), dopamine beta hydroxylase (*DBH*), dopamine transporter (*DAT1* or *SLC6A3*) and the synaptosomal-associated protein of 25 kDa (*SNAP25*) provide further support for the involvement of the dopaminergic pathway.^{11–13}

Several studies have also implicated the serotonergic and noradrenergic neurotransmitter systems as being involved in the pathophysiology of the disorder.¹⁴ The observation that methylphenidate decreases hyperactivity in *DAT1* knockout mice by increasing 5-HT transmission could mean the involvement of the serotonin system in the aetiology of ADHD.¹⁵ In addition, low platelet and whole-blood 5-HT levels have been reported in ADHD probands.¹⁶ There is evidence of the involvement of serotonin transporter (*5-HTT*) and receptor (*HTR2A* and *HTR1B*) genes.¹⁷ The involvement of the noradrenergic system, the α 1C, 2A and 2C adrenergic receptor (*ADRA1C*, 2A, 2C) genes, is equivocal and further study is needed.¹⁷

Most replicated polymorphism studies showed negative as well as positive findings, and conclusive evidence of the involvement of any specific gene has not yet been presented.^{13,17}

Since recently, genome-wide association studies (GWA) are becoming available for ADHD.^{18–20} Eventually, they will lead to the identification of novel genes or pathways, as genome-wide studies do not rely on current biological knowledge. To date, eight genome-wide linkage analyses (GWLA) have been performed in ADHD. These studies have been conducted mainly in affected sib pairs (5),^{9,21–25} in isolated populations (2)^{26,27} and in extended pedigrees (1).²⁸

We describe the clinical findings of a Dutch family with several members suffering from ADHD and the results from the second GWLA that applies the extended pedigree strategy in ADHD.

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MATERIALS AND METHODS

Subjects

The family (as shown in Figures 1 and 2) consisted of 37 persons in three generations, of which 24 participated in the study. In total, six individuals died before the start of this research. A total of five persons refused to participate and two did not fulfil our age inclusion criterion (> 12 years).

A total of 10 female (41.7%) and 14 male (age range: 13–83 years) were included in the study. Four children were between 13 and 16 years of age. One older family member (brother of I-2, not displayed in figures) participated in the research by donating a blood sample, but he refused to be interviewed or examined.

Signed informed consent, as approved by the Medical Ethics Committee of the Erasmus Medical Centre and the Dutch Central Committee on Human-related Research (CCMO), was obtained during the first visit. In the case of children between 12 and 18 years of age, both parents co-signed the consent form.

Diagnostic assessment

Clinical evaluation. During home visits, all participating family members underwent a semi-structured interview (MV) to assess the presence of ADHD symptoms on the basis of DSM-IV criteria (American Psychiatric Association²⁹), the history or presence of other psychiatric or somatic disorders and the use of medication. If possible, parents were interviewed regarding ADHD symptoms during the school age period of their children. Each subject also filled in a self-report questionnaire regarding the presence of ADHD symptoms according to the DSM-IV criteria in childhood and adulthood.

Six or more of the inattentive and/or hyperactive/impulsive criteria in childhood had to be present in order to be diagnosed as ADHD. A second psychiatrist (MvdL), experienced in diagnosing ADHD in adults, performed a

clinical assessment of the family members. The final diagnosis of ADHD was based on a consensus between the two psychiatrists. In addition, school reports, medical and psychological reports, if present, were studied for clues for ADHD.

Questionnaires. All subjects were administered the Structured Clinical Interview for DSM-IV Axis I disorders³⁰ (validated Dutch version: Groenestijn et al^{B1}) and Axis II Personality Disorders³² (validated Dutch version: Weertman et al^{B3}), from which we only evaluated the presence of borderline and antisocial personality disorder.

Phenotyping

Our behavioural phenotypes consisted of ‘ADHD definitely affected’, in which subjects met the full childhood DSM-IV ADHD criteria with onset of symptoms before the age of 7 years and with persistence of clearly impairing symptoms in more than one setting, and ‘ADHD possibly affected’ if they failed to meet the DSM-IV ADHD criteria, particularly with respect to impairment or by meeting only five instead of the six criteria of the inattentive and/or hyperactive subtype in childhood. Individuals who did not meet a minimum of five out of six criteria of the inattentive and/or hyperactive subtype in childhood (DSM-IV ADHD criteria) were classified as ‘likely unaffected’.

Laboratory analysis

Blood samples were collected from 24 available family members. Genomic DNA was extracted from peripheral blood using the Puregene DNA purification kit from Gentra Systems (Minneapolis, MN, USA).

A genome-wide screen was carried out with 382 fluorescently labelled markers from the ABI PRISM MD-10 Linkage v. 2.5 Mapping Set (Applied Biosystems, Foster City, CA, USA), covering all autosomes with an average spacing of 10 cM. Additional markers from the Marshfield integrated genetic map (available online at <http://research.marshfieldclinic.org/genetics/GeneticResearch/compMaps.asp>)

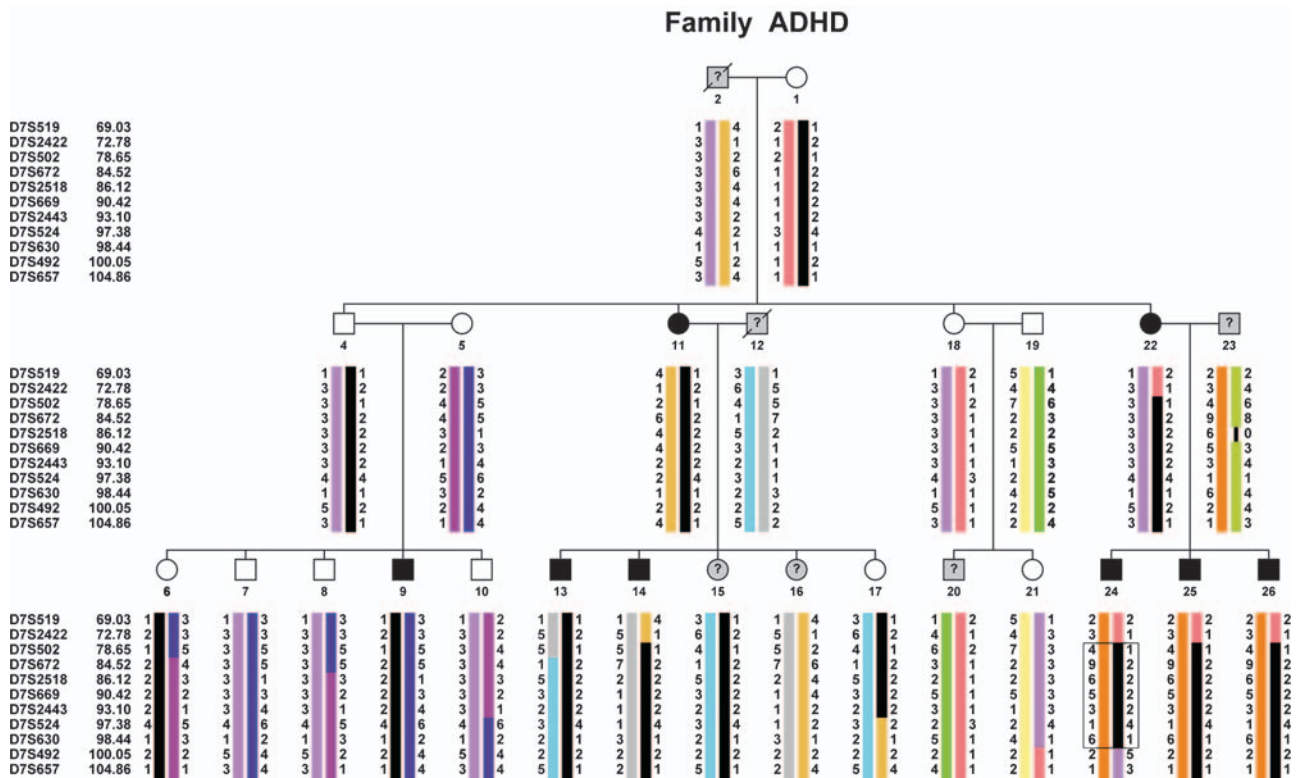


Figure 1 : Family tree showing microsatellite haplotypes corresponding to the 7p15.1–q31.33 region. Marker order is according to the sex-averaged Marshfield map. A black bar represents the haplotype segregating with the disease. The minimum candidate region determined by recombinations is boxed (person III-24). Clinical phenotypes are as follows: blackened symbol ‘ADHD definitely affected’, (?) ‘ADHD possibly affected’ or not examined (deceased) and white symbol ‘likely not affected’. For linkage analysis, individuals classified as ‘possibly affected’ and ‘likely not affected’ were coded as ‘diagnosis unknown’. Haplotypes from persons I-2 and II-12 (both deceased) are inferred.

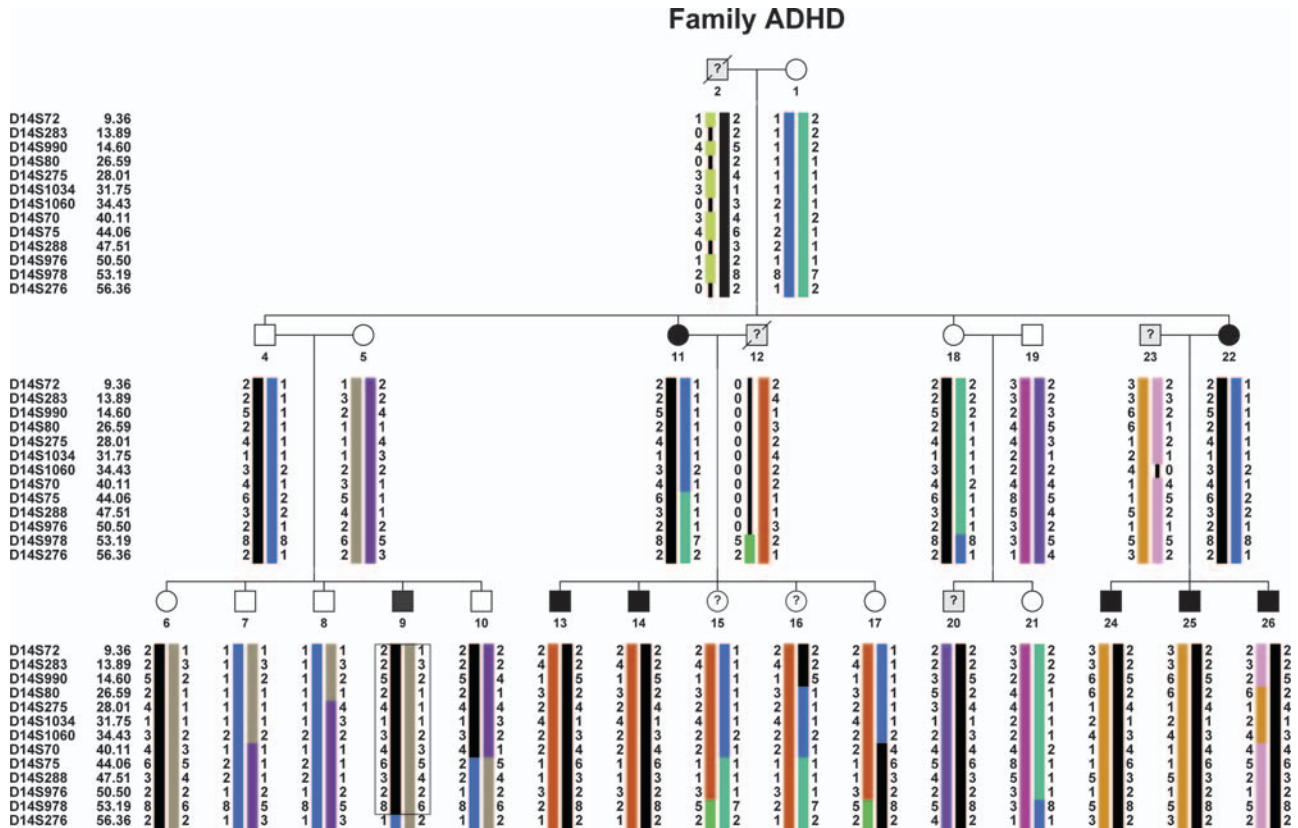


Figure 2 Family tree showing microsatellite haplotypes corresponding to the 14q11.2–q22.3 region. See legend of Figure 1. The minimum candidate region for chromosome 14 is boxed (person III-9).

were genotyped for fine mapping of ‘positive’ regions. PCR products were pooled and loaded onto an ABI 3100 automatic DNA sequencer (Foster City, CA, USA); data were analysed using GeneMapper v.2 (Applied Biosystems). Mega2³⁴ was used to process the genetic data into the appropriate format.

Linkage analysis

Owing to the uncertainties related to the correct genetic model in ADHD, parametric and nonparametric linkage analyses (NPL) were performed using Simwalk2 software (version 2.9, Eric Sobel, University of California, Los Angeles (UCLA), CA, USA).³⁵ For parametric analysis, we specified an autosomal dominant mode of inheritance, a mutant allele frequency of 1 in 1000 and equal marker allele frequencies. Marker order and genetic distances were based on the Marshfield integrated linkage map. For some markers with an unresolved order, the Icelandic (deCODE) genetic map was used. A phenocopy rate of 1 in 1000 and a reduced penetrance of 80% were applied. A conservative affected-only design was used, thus all individuals in whom ADHD was not clinically proven (ADHD possibly affected and likely unaffected) were classified as ‘diagnosis unknown’.

A total of 1000 simulations (SLINK and MSIM programs, Jurg Ott, Rockefeller University New York, NY, USA)³⁶ were performed using the genetic model specified above. To evaluate the use of a more conservative (and less powerful) ‘affected only’ design, two simulation analyses were performed. The first considered an ‘affected only’ disease status definition and the second design included all clinical information available.

Pedigree location scores (directly comparable with multipoint LOD scores) were calculated.

For nonparametric analysis, the maximum-tree statistic (max-tree, the largest number of affected members inheriting an allele from one founder allele)³⁷ is reported. This statistic was designed for traits best modelled by dominant inheritance. The NPL-All statistic, a measure of whether a few founder alleles are overly present in affected members, is reported as well (designed for traits best modelled by additive inheritance).³⁷ Empirical *P*-values

(10 000 simulations) were also obtained. This *P*-value is the probability of obtaining a value for that statistic that is equal to or greater than the observed value, if the traits were not linked to markers.

For positive regions, haplotypes were constructed using Simwalk2 and were subsequently checked manually on the basis of the minimal number of recombinations. To display haplotypes, the programme HaploPainter³⁸ (Holger Thiele, Gene Mapping Center, Max Delbrueck Center for Molecular Medicine, and Cologne Center for Genomics, University of Cologne, Germany) was used.

RESULTS

Psychiatric diagnosis

A total of 8 out of 24 family members (33%) were diagnosed as ADHD ‘definitely affected’ (three had ADHD of the combined type, four had the inattentive type and one had the hyperactive/impulsive type). ADHD ‘possibly affected’ was diagnosed in four members (17%) (see Figure 1).

Comorbidity. Except for tic disorders (more frequent in the unaffected or possibly affected group) and nicotine use disorder (equally present in both groups), we found more comorbidity in the ADHD-diagnosed group than in the unaffected or possibly affected group (ie, obsessive compulsive disorder was found in 38% of the ADHD group compared with 19% in the unaffected or probably affected group, Table 1). The average number of comorbidities per patient was higher in the ADHD-diagnosed group as compared with that in the unaffected or possibly affected group (3.4 *versus* 2.3, respectively). The percentage of patients having one or more comorbid disorders was 63% in the ADHD group *versus* 69% in the unaffected and possibly

affected group (Table 1). We did not diagnose the presence of a borderline or antisocial personality disorder in any member of this family. The majority (75%) of ADHD patients were male (six out of eight). In the group diagnosed as unaffected or possibly affected, the male–female ratio was 1:1.

Genetic studies. At least one individual of the family is an ‘obligate carrier’ (Figure 1 and 2, individual II-4); this person transmits the disease gene but the phenotype description and anamnesis were normal. As male-to-male transmission was observed, a causal gene located on the X chromosome was excluded.

Simulation analysis to estimate the probability of detecting genetic linkage, given the pedigree structure (statistical power), was performed. An average LOD score of 1.7 with a maxLOD of 3.3 was

obtained when all clinical information was included. A conservative ‘affected only’ approach yielded an average LOD score of 1.3 and a maxLOD of 2.1.

Despite the loss in statistical power, we decided to use the ‘affected only’ design owing to reduced gene penetrance (a clinically unaffected person could be carrying the disease gene, which could lead to false linkage results).

We performed parametric and NPL for all autosomes. Four regions on chromosomes 3, 7, 14 and 21 showed an excess of allele sharing among the patients of the family. Two of these regions, on chromosomes 7 and 14, showed suggestive LOD scores³⁹ 2.1 and 2.08, respectively (Table 2), very close to the maxLOD predicted by the simulation studies (2.1).

By haplotype analysis, we could exclude the regions on chromosomes 3 and 21, as one patient (III-14) was not sharing the ‘affected’ haplotype (data not shown). We then refined the regions on chromosomes 7 and 14 by testing 17 extra markers. Parametric linkage analysis again yielded suggestive LOD scores: 2.1 for markers D7S502, D7S672, D7S2518 and D7S669 (at 0 recombination fraction). The maximum NPL (2.92) in the region was observed for marker D7S502 ($P=0.001$), followed by markers D7S672 and D7S669 (NPL=2.8, $P=0.002$).

By haplotype analysis on 7p15.1–q31.33 (Figure 1), we observed that all eight patients share the same haplotype extending from D7S2422 (72.8 cM, 51.1 Mb) to D7S492 (100.1 cM, 89.2 Mb). Recombination events occurring in patients II-22, III-14 and III-24 determined the upper (p-arm) and lower (q-arm) border. The minimum region extended 20 cM (D7S502–D7S630, 78.7–98.4 cM, 66.7–88.3 Mb), containing 161 genes (NCBI build 36.3).

For chromosome 14, the maxLOD score (2.08) was obtained for markers D14S80–D14S984 (Table 2), with a maxNPL score of 2.56 for marker D14S275 ($P=0.003$). Haplotype analysis on 14q11.2–q22.3 showed a common haplotype shared by all patients extending from the centromere to 14q (D14S72–D14S276), comprising at least 47 cM.

Table 1 Lifetime psychiatric comorbidity in a Dutch family with ADHD (N (%))

DSM-IV axis I	ADHD N=8	ADHD LU/PA, N=16
Alcohol-use disorder ^a	2 (25)	2 (13)
Cannabis-use disorder ^b	1 (13)	1 (6)
Nicotine-use disorder ^c	4 (50)	8 (50)
Depressive disorder ^d	3 (38)	4 (25)
Anxiety disorder	3 (38)	2 (13)
Tic disorder	1 (13)	5 (31)
Obsessive compulsive disorder	3 (38)	3 (19)
Comorbid disorder(s) ^e	5 (63)	11 (69)

Abbreviations: ADHD: attention deficit/hyperactivity disorder; LU/PA: likely unaffected and possibly affected; NOS, not otherwise specified.

^aDependence or abuse.

^bDependence or abuse.

^cDependence or abuse.

^dMajor depressive disorder, single or recurrent episode, Dysthymic disorder, depressive disorder NOS.

^eOne or more comorbid disorder.

Table 2 Summary of the linkage analysis results

Chromosome	Physical map (Mb) ^a	Genetic map (cM) ^b	Marker name ^c	NPL-ALL	P-value	LOD score
7	66	78.6	D7S502	2.92	0.001	2.1
7	71.5	84.5	D7S672	2.8	0.002	2.1
7	75.2	86.1	D7S2518	2.74	0.002	2.1
7	77.7	90.4	D7S669	2.8	0.002	2.1
7	79.7	93.1	D7S2443	2.7	0.002	2.08
7	84.5	97.4	D7S524	1.72	0.02	1.64
7	88.3	98.4	D7S630	1.65	0.03	1.73
14	20.4	9.4	D14S72	1.48	0.03	2.04
14	21.7	13.9	D14S283	1.88	0.01	2.07
14	22.6	14.6	D14S990	1.92	0.01	2.07
14	25.8	28	D14S275	2.56	0.003	2.08
14	26.8	26.6	D14S80	2.5	0.003	2.08
14	32.5	34.4	D14S1060	2.54	0.003	2.08
14	33.5	40.1	D14S70	2.45	0.003	2.08
14	36.5	44.1	D14S75	2.17	0.007	2.08
14	43.2	47.5	D14S288	2.17	0.007	2.08
14	47.6	50.5	D14S976	2.22	0.006	2.08
14	49.2	52.5	D14S984	2.2	0.006	2.08
14	51	53.2	D14S978	1.74	0.018	1.95
14	52.9	55.3	D14S989	1.2	0.06	-0.42

^aAccording to NCBI build 36.3.

^bAccording to the sex-averaged Marshfield map.

^cMarkers displayed in italic were tested during the fine mapping phase.

A recombination event observed in patient III-9 delimited the lower border of this large region (Figure 2).

We also observed the presence of the 'disease haplotype' in individuals free of symptoms: II-4 (carrier) and III-6 for chromosome 7, and II-4, II-18 and III-6 for the chromosome 14 region.

DISCUSSION

We clinically characterized a family with more than 30% of the participating members diagnosed with ADHD. The overall frequency of DSM-IV Axis I comorbidity in the definitely affected group (63%) is lower than the frequencies found in most studies among adults with ADHD (75%).^{2,40} The frequency of tic disorder was high, particularly in the 'possibly affected' and 'likely unaffected' group (31%). A total of 25% (6 out of 24) of the investigated relatives had a tic disorder.

Owing to the scarceness of families with a sufficient number of patients allowing a powerful linkage analysis, most GWLA in ADHD were limited to sib pair cohorts^{9,21–25} and to isolated populations in the region of Antioquia, Colombia²⁶ and the southwest of The Netherlands.²⁷ Recently, Romanos *et al*²⁸ published the only GWLA performed in unrelated extended pedigrees. Factors complicating linkage analysis, such as reduced penetrance and bilinear inheritance, were present in several families.

We performed a GWLA in a Dutch family using parametric and nonparametric affected-only analysis designs. With both analyses, two regions on chromosomes 7 (72.8–100.1 cM) and 14 (9–56.36 cM) yielded suggestive LOD scores on the basis of Lander and Kruglyak's established criteria.³⁹

From the nonparametric analysis, two statistics were examined: the max-tree (dominant model) and the NPL-All (additive model). For both regions, the NPL-All statistic performed better (ie, for D7S502 max-tree NPL 2.2, $P=0.007$ and for NPL-All 2.9, $P=0.001$), suggesting that an additive disease model better fits the genetic data than a dominant model.

Interestingly, both loci have been reported before. In view of the complexity of a psychiatric disorder such as ADHD, it is likely that multiple genes with a limited effect size are involved in disease aetiology even in single multigeneration families. The alternative hypothesis is that only one region represents a true linkage finding.

A Dutch sib-pairs study (117 ASPs with ADHD, strict diagnosis criteria, DSM-IV) identified suggestive linkage in a region on chromosome 7p13 (MLS 3.04 for markers D7S1818, 69.9 cM and for D7S2422, 72.8 cM).²¹ In addition, in a 155 German ADHD sib-pairs study, both regions (D7S2490, 88 cM and D14S306, 46 cM)²² showed (nominal) linkage signals. Furthermore, in eight extended families with ADHD of German origin, GWLA revealed linkage signals at 7q21.11 (LOD 3.14, 84–89 cM) and 14q12 (LOD 4.17, 16 cM), when analysing families together under a narrow diagnostic criteria.²⁸ However, when the families were analysed independently, other chromosomal regions were highlighted. Recently, a meta-analysis of seven GWLA in ADHD (five affected sib pairs and two multigenerational pedigrees studies) identified a significant linkage signal on chromosome 16 and several regions with nominal linkage signals on chromosomes 5, 6, 7, 8, 9, 15 and 17.⁴¹ The region on chromosome 7 (60–91 cM) overlaps the region detected in our family (72.8–100.1 cM).

A recent study converged evidence from their GWA (500K SNP arrays) and from extended pedigrees GWLA (50K), and found several common candidate loci that overlapped across the studies.⁴² They reported the locus on 14q11.2–12, which overlaps with the region found in our family. With the GWA analysis, two genes from the area were highlighted: *SLC7A8* and *NOVA1*.⁴²

It is remarkable that in a clinically and genetically heterogeneous disorder such as ADHD, overlapping genomic regions are reported by studies conducted in different populations, using different assessment, genotyping and analysis strategies. Hopefully, the ongoing GWA studies^{18–20} will help to highlight specific SNPs and genes within the broad areas detected by our, as well as other, linkage studies. In conclusion, we performed a GWLA in a relatively large Dutch family with eight ADHD patients and found two (replicated) loci showing suggestive evidence of linkage on chromosomes 7p and 14q.

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