Copy number variants in attention-deficit hyperactive disorder: identification of the 15q13 deletion and its functional role

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Objectives **Evidence has supported a role for rare copy number variants in the etiology of attention-deficit hyperactivity disorder (ADHD), in particular, the region 15q13, which is also a hot spot for several neuropsychiatric disorders. This region spans several genes, but their role and the biological implications remain unclear.**

Methods **We carried out, for the first time, an analysis of the 15q13 region in an Italian cohort of 117 ADHD patients and 77 controls using the MLPA method, confirmed by a genome single-nucleotide polymorphism array. In addition, we probed for downstream effects of the 15q13 deletions on gene expression by carrying out a transcriptomic analysis in blood.**

Results **We found 15q13 deletions in two ADHD patients and identified 129 genes as significantly dysregulated in the blood of the two ADHD patients carrying 15q13 deletions compared with ADHD patients without 15q13 deletions. As expected, genes in the deleted region (*KLF13*, *MTMR10*) were downregulated in the two patients with deletions. Moreover, a pathway analysis identified apoptosis, oxidation reduction, and immune response as the mechanisms that were altered most significantly in the ADHD patients with 15q13 deletions. Interestingly, we showed that deletions in *KLF13* and *CHRNA7* influenced the expression of genes belonging to the same immune/inflammatory and oxidative stress signaling pathways.**

Conclusion **Our findings are consistent with the presence of 15q13 deletions in Italian ADHD patients. More interestingly, we show that pathways related to immune/inflammatory response and oxidative stress signaling are affected by the deletion of *KFL13* and *CHRNA7*. Because the phenotypic effects of 15q13 are pleiotropic, our findings suggest that there are shared biologic pathways among multiple neuropsychiatric conditions.** ***Psychiatr Genet*** **25:59–70**

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Introduction

Attention-deficit hyperactivity disorder (ADHD) is a common psychiatric condition. Meta-analysis shows that 5.3% of youth have this disorder and that the prevalence does not differ markedly worldwide (Polanczyk *et al.*, 2007). Particularly in Italy, a national registry under the control of the Italian National Health Service has estimated the prevalence of this disability to be within the range of 0.43–3.6% (Al-Yagon *et al.*, 2013). In a sample of Italian students, the prevalence was estimated of 3%, in line with other reports in European countries (Bianchini *et al.*, 2013).

It is characterized by behavioral and cognitive alterations leading to inattention, impulsivity, and hyperactivity. The etiology is complex, with contributions from both genetic and environmental factors. The heritability has been estimated to be 76% (Faraone and Mick, 2010). Studies of common variants of candidate genes have not identified any genes definitively conferring a risk for ADHD (Gizer *et al.*, 2009). In addition, genome-wide association studies have been too underpowered to detect genome-wide significant associations with common single-nucleotide polymorphisms (SNPs) (Neale *et al.*, 2010), fitting with the polygenic and multifactorial model for ADHD, where many common variants of small effects contribute toward the pathological phenotype. Although no genome-wide significant SNPs for ADHD have been discovered, meta-analysis has confirmed the existence of a statistically significant polygenic background (Lee *et al.*, 2013; Yang *et al.*, 2013).

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Moreover, cross-disorder genome-wide association studies show that ADHD shares common risk variants with other psychiatric disorders (Cross-Disorder Group of the Psychiatric Genomics Consortium *et al.*, 2013).

In addition to the common variants, rare deletions or duplications in the genome known as copy number variants (CNVs) also contribute toward the high heritability of the disorder. Thus, it seems likely that the high heritability of ADHD is because of both common and rare variations. Several studies have found an increased burden of large, rare CNVs in ADHD, some of which overlap with findings in autism (Elia *et al.*, 2010; Williams *et al.*, 2010, 2012; Lionel *et al.*, 2011). A CNV region of particular interest is 15q13, a hot spot for several neuropsychiatric disorders such as schizophrenia (Stefansson *et al.*, 2008; Stone *et al.*, 2008; Van Bon *et al.*, 2009; Stephens *et al.*, 2012), epilepsy (Dibbens *et al.*, 2009; Helbig *et al.*, 2009), autism (Pagnamenta *et al.*, 2009), developmental delay (DD), intellectual disability (ID), and dysmorphic features (Sharp *et al.*, 2008; Ben-Shachar *et al.*, 2009; Miller *et al.*, 2009), as well as ADHD (Lionel *et al.*, 2011; Williams *et al.*, 2012). The frequency of 15q11q13 CNVs was estimated by Williams *et al.* (2010) to be 1.91% in European cohorts.

In this region, several significant deletions or duplications were found in which the beginning and end points vary across individuals. The region implicated by 15q13 CNVs spans several genes, including *CHRNA7* (cholinergic receptor, nicotinic, alpha 7), *KLF13* (Kruppel-like factor 13), *TRPM1* (transient receptor potential cation channel, subfamily M, member 1), *MTMR10* (myotubularin-related protein 10), and *OTUD7A* (OTU domain containing 7A) (Sharp *et al.*, 2008; Ben-Shachar *et al.*, 2009; Miller *et al.*, 2009; Van Bon *et al.*, 2009). Among these, only *CHRNA7* has been associated nominally with ADHD in common variant studies (Stergiakouli *et al.*, 2012; Williams *et al.*, 2012). This gene has been identified as the major candidate gene responsible for the predominant manifestations of 15q13.3 microdeletion syndrome (Hoppman-Chaney *et al.*, 2013; Le Pichon *et al.*, 2013).

To date, the mechanisms by which genes within the deleted region exert their effect are unclear. A recent paper, using immortalized lymphoblastoid cell lines, reported genome-wide differential expression of genes implicated in neurodevelopment and muscular function from a patient with 15q13.3 homozygous microdeletion syndrome (Le Pichon *et al.*, 2013). The 15q13.3 microdeletion syndrome is characterized by a wide range of phenotypic features, including ID, seizures, autism, and psychiatric conditions. This deletion is inherited in ~75% of cases and has been found in mildly affected and normal parents, consistent with variable expressivity and incomplete penetrance.

We followed up on these previous findings by carrying out, for the first time, an analysis of the 15q13 region in an Italian cohort of 117 ADHD patients and 77 healthy individuals. We also sought to investigate the molecular mechanisms associated with 15q13 region deletions by carrying out a microarray gene expression study in the blood of two drug-naive ADHD patients carrying 15q13 deletions and nine drug-naive ADHD patients without 15q13 deletions.

Methods

Participants

ADHD patients were enrolled by a network of Clinical Centres: Adolescent Neuropsychiatry Unit of Fatebenefratelli and Oftalmico, Milan; Department of Childhood and Adolescent Neuropsychiatry, Spedali Civili Brescia; Childhood and Adolescent Neuropsychiatry (UONPIA), Spedali Riuniti, Bergamo; Azienda Ospedaliera, Cremona, Rho, and Mantova. Patients were diagnosed with ADHD according to the *Diagnostic and Statistical Manual of Mental Disorders*, 4th ed. (DSM-IV) criteria (American Psychiatric Association, 2000) and the guidelines of the Italian Institute of Health (2005). Moreover, revised Touwen neurological tests were performed. Exclusion criteria included childhood schizophrenia, autism, intelligence quotient (IQ) up to 70 [Wechsler Intelligence Scale for Children (WISC)], epilepsy, encephalitis, Tourette syndrome, and conduct disorder. They had moderate to severe ADHD.

The age at data collection was 11.37 ± 2.70 years and the proportion of males was 89.4%. Stratification according to diagnostic subtypes evidenced 70.8% for the ADHD combined type, 27.8% for the predominantly inattentive type, and 1.4% for the predominantly hyperactive–impulsive type.

The control group included unrelated volunteers not affected by ID, chronic and medical diseases, inflammatory diseases, and allergies, undergoing blood tests for a presurgical screening. They were also selected to exclude ADHD or conduct disorder. The age at data collection was 10.25 ± 2.15 years and the proportion of males was 77.9%.

All the participants enrolled in this study were Caucasoid and living in Northern Italy.

The study protocol was approved by the Local Ethics Committee and as the participants were all under-age youth, their parents were requested to provide written informed consent for the study as indicated on the approval note by the Local Ethics Committee. The parents of 11 ADHD patients provided written informed consent to be recalled for a blood Paxgene sample for whole expression studies. This study has therefore been carried out in accordance with the ethical standards established in the 1964 Declaration of Helsinki and its later amendments.

Genetic analysis

The DNA of all participants was extracted from blood samples or saliva using commercial standard kits. The MLPA assay was performed using the MLPA Kit P343-C1 produced by MRC-Holland (Amsterdam, the Netherlands) according to the manufacturer's protocol. The kit includes 49 probes: 26 probes contain complementary sequences of exons for genes in the 15q11-q13 region and 11 probes for genes in the 16p11.2 region.

MLPA statistical analysis

Analyses of results were carried out on the basis of the peak areas of each probe obtained using GeneMapper software v4.0 (Applied Biosystems, Foster City, California, USA). Coffalyser software (MRC-Holland, Amsterdam, the Netherlands) v9.4 was used to analyze the MLPA data for CNVs. Bin sizes were adjusted accordingly for the peak sizes observed. Data were normalized by dividing the peak area of each probe by the average peak area of the seven control probes in the probe mix obtained from the sample set. The normalized data were then divided by the median peak area of all samples to obtain an indication of copy number variation for each probe. A value of 0.7 or below and 1.3 and above were set as thresholds for loss and gain, respectively.

We tested the MLPA assay by analysis of three positive controls, obtained from the Tor Vergata General Hospital (Rome) and IRCCS Fatebenefratelli (Brescia), with known deletions and duplications in regions targeted by the probes. For all controls, the correct CNV was detected by MLPA analysis (data not shown).

SNP array analysis and generation of CNVs calls

Both samples from ADHD patients with the 15q13 deletions were genotyped by Affymetrix Human Mapping GeneChip 6.0 arrays with a total of two millions of probes, half of which were polymorphic. DNA was processed according to the instructions provided in the Affymetrix Genome-Wide Human SNP Nsp/Sty 6.0 Assay Manual. Initial analysis of the array to calculate the intensity data was carried out using Affymetrix GeneChip Command Console Software (AGCC, Santa Clara, California, USA). The AGCC probe cell intensity data were then analyzed using Genotype Console 3.01 (GTC3.01) to obtain genotype data. The copy number state calls were generated from the BRLMM-P-Plus algorithm implemented in GTC 3.0.1. This algorithm compared the intensity signal of each marker in each sample against a reference pool formed from a group of 270 samples derived from the HapMap database. After this comparison, the software generates a median intensity value for each marker. This value was then used by the Affymetrix segmentation algorithm to identify CNVs. To reduce the presence of false-positive CNVs, the segmentation algorithm parameters were set to consider as a CNV only those regions larger than 100 kb, comprised of at least 25 contiguous markers without a diploid state and with an average probe density lower than 10 kb.

RNA isolation and microarray gene expression analyses

Blood samples from the two drug-naive ADHD patients with the 15q13 deletions and drug-naive nine ADHD patients without 15q13 deletions were obtained by venipuncture in the morning using PaxGene Tubes (Qiagen, Hilden, Germany). The two ADHD patients with the 15q13 deletions and nine ADHD patients without 15q13 deletions were age and sex matched (mean age of participants with 15q13 deletions 11.50 ± 4.95; mean age of participants without 15q13 deletions 12.22 ± 3.27; $t = 0.26$, $P = 0.80$; 100% males).

RNA isolation was performed using the PaxGene Blood RNA Kit (Qiagen) according to the manufacturer's protocols and the quality and integrity of RNA were assessed using Nanodrop 2000 (ThermoScientific, Waltham, Massachusetts, USA).

Gene expression microarray assays were performed using Human Gene 1.1 ST array strips (Affymetrix Inc., Santa Clara, California, USA) on the Affymetrix Gene Atlas platform following the manufacturer's instructions (*http://www.affymetrix.com/support/technical/manuals.affx*).

Data analysis and pathway analysis

Gene expression microarray data were imported from the Gene Atlas instrument into Partek Genomics Suite 6.0 (Partek, St Louis, Missouri, USA) as CEL files using default parameters. Quality controls were performed using Affymetrix expression console software, whereas the analysis of variance to obtain a list of genes modulated differentially in the two groups was carried out using the Partek Genomic Suite. Pathway analysis was carried out using Pathway Studio Software 5.0 (Ariadne, Lausanne, Switzerland) with the standard Gene Set enrichment analysis, originally developed by the Broad Institute (*http://www.broad.mit.edu/gsea/*) (Mootha *et al.*, 2003). This algorithm uses a correlation-weighted Kolmogorov–Smirnov statistic on all gene expression changes and computes pathway enrichment scores by considering gene set membership information, gene list ranking, and gene–gene dependencies that reflect real biology.

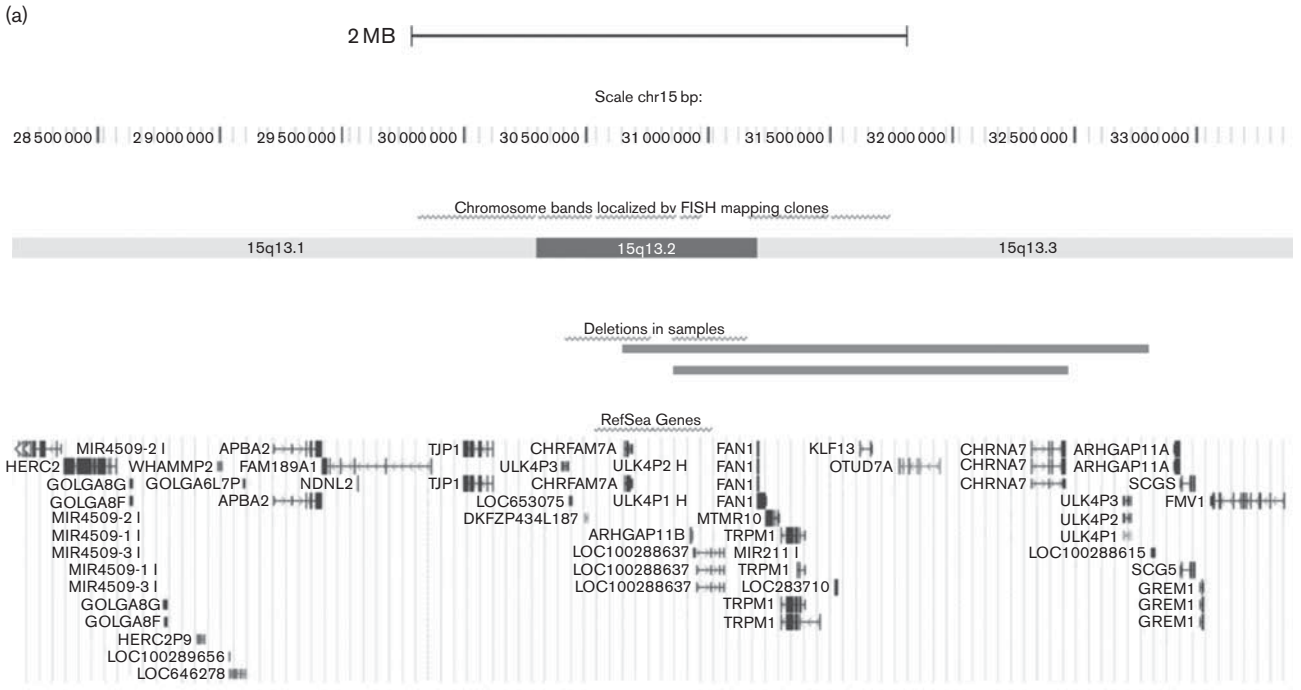
Finally, we carried out a target prediction analysis (Ariadne) to determine the pathways influenced by the genes belonging to the 15q13 deletion (*KLF13, MTMR10, CHRNA7*).

Results

Out of 117 ADHD patients and 77 healthy individuals, we excluded one patient and six participants from the control group because of low-quality DNA. Among the patients, we identified two with a significant reduction in the peak areas for the probes of the *TRPM1*, *KLF13*, and

Fig. 1

(a)



(b)

Patient number	Imbalance	Size (Mbp)	Chromosome band	Chromosomal region
1	Heterozygous deletion	2.39	15q13.2-q13.3	Chr15:30,450,356-32,843,110
2	Heterozygous deletion	1.79	15q13.2-q13.3	Chr15:30,743,132-32,539,525

(a) Schematic representation of the deletions found at the 15q13.2-q13.3 region in two ADHD patients. (b) Size and chromosomal positions of the two deletions (assembly: GRCh37/hg19). ADHD, attention-deficit hyperactivity disorder.

CHRNA7 genes in the 15q13 region. From the statistical MLPA data analysis, we obtained probe signal values of 0.48, 0.43, and 0.45 for *KLF13*, *CHRNA7*, and *TRPM1* for patient 1 and values of 0.5, 0.44, and 0.46 for patient 2, defining two heterozygous deletions in 15q13.3 (Fig. 1a). The clinical features of these patients are reported in Table 1.

No genomic rearrangements in the 15q11-q13 region were found in the control samples. The 1.7% rate of 15q13 CNVs in cases did not differ significantly from the 0% rate in controls (Fisher's exact test, $P=0.53$). Moreover, no genomic rearrangements in the 16p11.2 region were found in the patient and the control samples by MLPA analysis.

The two CNVs observed in ADHD patients were confirmed and fine mapped using Affymetrix Human Genome-Wide SNP Arrays, defining a deletion of 2.39 Mbp in patient 1 and 1.79 Mbp in patient 2 (Fig. 1b). These two CNVs have an overlapping region, which spans the genes *ARHGAP11B* (Rho GTPase activating protein 11B), *FAN1* (FANCD2/FANCI-associated nuclease 1), *MTMR10*, *TRPM1*, *KLF13*, *OTUD7A*, and *CHRNA7* (Fig. 1a). No other CNVs greater than 100 kbp were found in nonpolymorphic regions for both patients. For patient 1, the breakpoints fell into two regions with low probe coverage. In particular, the most centromeric breakpoint fell in a region of 123 kbp, which was covered by only four probes. Because of the low coverage, however, we cannot formally exclude that the CNV breakpoint maps 123 kbp downstream and that the CNV is closer in size. As for the telomere breakpoint, the coverage is higher and the actual breakpoint falls in a range of 14 kbp.

As for patient 2, we can reasonably exclude that the CNV is smaller than what we reported as the internal boundaries of the deletion are well covered. We can reasonably exclude that the CNV is smaller than what we

Table 1 Clinical features of the two ADHD patients carrying 15q13 deletions

Features	Patient 1	Patient 2
ADHD rating scale	Predominantly inattentive type	Combined type
Demographic features		
Age, sex	8, Male	15, Male
Height (cm)	156	164
Weight (kg)	49	66.5
Cognitive and neuropsychological assessment		
Memory performance (TEMA, Digit Span of WISC)	No	Yes
Total intelligent quotient (WISC)	92	81
Verbal intelligent quotient	98	85
Performance intelligent quotient	87	81
Campanelle test (accuracy, sustained attention, row data/*z*)	127/−5.5	111/−3.2
Campanelle test (rapidity, selective attention, row data/*z*)	41/−3.38	47/−1
Continuous performance test (omission errors, mean/*z*)	2/2.4	–
Psychopathological features		
Conners for parents	79	87
Conners for teachers	49	90
Anxiety, depressive symptoms (CDI, K-SADS-PL)	No	Yes
Learning problems	No	No
Aggressiveness	No	Yes
Comorbidity features		
Autism	No	No

Note: Campanelle test (Biancardi and Stoppa, 1997), continuous performance test (paper format) values are presented in row data/*z*-scores (cut off = −2). IQs are obtained from the WISC.
ADHD, attention-deficit hyperactivity disorder; CDI, Children's Depression Inventory; IQ, intelligent quotient; K-SADS-PL, Kiddie-Schedule for Affective Disorders and Schizophrenia-Present and Lifetine version; TEMA, Test of Memory and Learning; WISC, Wechsler Intelligence Scale for Children.

reported as the internal boundaries of the deletion are well covered. However, we cannot exclude that the CNV extends upstream of the more centromeric deleted probe for 74 kbp.

To assess the impact on the biological processes regulated by genes within the deleted region, we carried out gene expression microarray analyses in the blood obtained from the two ADHD patients carrying 15q13 deletions and nine ADHD patients without 15q13 deletions.

The analysis of variance analyses identified 129 significantly dysregulated transcripts [$P < 0.05$ and fold change (FC) < -1.5 or > 1.5] (Table 2). We then visualized the most significantly changed transcripts using a more stringent *P* value ($P < 0.01$ and FC < -1.5 or > 1.5) in the heatmap generated by hierarchical clustering analysis (Fig. 2).

As expected, some genes located in the 15q13 deleted region were downregulated in the two ADHD patients carrying 15q13 deletions: *KLF13*, *P* value: 3.26×10^{-5}, FC: −1.9; *MTMR10*, *P* value: 0.0032, FC: −1.9. *CHRNA7* did not show significant *P* values ($P = 0.9$, FC: −1.01), possibly because of its very low expression levels in the blood.

We then used the 129 transcripts that were modulated significantly in ADHD patients carrying the 15q13 deletion to carry out a pathways analysis and found several pathways to be significantly dysregulated in the ADHD deletion carriers ($P < 0.005$ after multiple test correction): apoptosis ($P = 2.44 \times 10^{-76}$), oxidative stress ($P = 1.75 \times 10^{-39}$), as well as immune response ($P = 2.93 \times 10^{-35}$) signaling (Table 3).

Interestingly, when we carried out a target prediction analysis for the genes belonging to the 15q13 deletion region (*KLF13*, *MTMR10*, *CHRNA7*) and that were significantly downregulated in ADHD patients carrying the 15q13 deletion (*KLF13*, *MTMR10*), we observed (Fig. 3) that many of these gene targets belonged to the same immune/inflammatory and oxidative stress signaling pathways. In Fig. 3, we show the main gene networks activated by the *KLF13* and *CHRNA7* genes. Interestingly, the same pathways that we found to be significantly modulated in the data set of the 129 dysregulated transcripts are also the main network activated by genes affected by the presence of 15q13 deletions.

Discussion

Our results showed the presence of 15q13 deletions in two ADHD patients, whereas no genomic rearrangements of this region were found in the control samples. The low frequency of 15q13 CNVs observed in our patients (1.72%) is similar to that observed for 15q11q13 CNVs by Williams *et al.* (2010) (1.91%), who carried out a genome-wide analysis of 410 children with ADHD, all of white UK origin.

To our knowledge, this is the first report of this recurrent CNV in Italy for ADHD patients. These findings are in line with other ADHD studies (Lionel *et al.*, 2011; Williams *et al.*, 2012), and also with studies that identified 15q13 CNVs in other psychiatric disorders, such as schizophrenia (Stefansson *et al.*, 2008; Stone *et al.*, 2008; Van Bon *et al.*, 2009; Stephens *et al.*, 2012), autism (Pagnamenta *et al.*, 2009), and epilepsy (Dibbens *et al.*, 2009; Helbig *et al.*, 2009). The overlap in CNV loci among disorders suggests pleiotropy of genes predisposing to these diseases (Moskvina *et al.*, 2009).

Moreover, both ADHD patients with 15q13 deletions had total IQs that are in the lower quartiles of normal (92 and 81). This confirms that the 15q13 deletion is associated with lower overall IQ in ADHD patients, as also reported in the study by Williams *et al.* (2010).

We also found a significant difference in gene expression profiles between ADHD patients carrying deletions in 15q13 and ADHD patients without deletions in that region. As expected, two genes from the 15q13 deleted region (*KLF13, MTMR10*) were significantly downregulated in the two patients carrying 15q13 deletions.

Table 2 One hundred and twenty-nine genes significantly dysregulated in the blood of the two ADHD patients with deletions in 15q13 compared with nine ADHD patients without 15q13 deletions ($P < 0.05$ and fold change < -1.5 or > 1.5)

	Gene symbol	Gene assignment	*P* value (deletion)	Fold change
1	SIDT1	SID1 transmembrane family member 1	1.56E−05	−1.6
2	KLF13	Kruppel-like factor 13	3.26E−05	−1.9
3	CD38	CD38 molecule	3.72E−05	−1.7
4	STARD9	StAR-related lipid transfer (START) domain containing 9	6.87E−05	−1.6
5	CTU1	Cytosolic thiouridylase subunit 1 homolog (*Schizosaccharomyces pombe*)	7.19E−05	−1.5
6	CD81	CD81 molecule	7.34E−05	−1.6
7	IL27RA	Interleukin 27 receptor, alpha	8.16E−05	−1.7
8	HIST1H2BM	Histone cluster 1, H2bm	0.000170717	−1.9
9	FAM78A	Family with sequence similarity 78, member A	0.000221351	−1.5
10	ZAP70	Zeta-chain (TCR) associated protein kinase 70 kDa	0.000263754	−1.7
11	HIST1H3I	Histone cluster 1, H3i	0.000422049	−1.9
12	LLGL2	Lethal giant larvae homolog 2 (Drosophila)	0.000693742	−1.6
13	DUSP18	Dual specificity phosphatase 18	0.000852471	−1.5
14	YPEL1	Yippee-like 1 (Drosophila)	0.000882967	−1.5
15	SLA2	Src-like-adaptor 2	0.00109583	−1.5
16	USP20	Ubiquitin-specific peptidase 20	0.00120489	−1.5
17	TTC38	Tetratricopeptide repeat domain 38	0.0013294	−1.5
18	HIST2H3C	Histone cluster 2, H3c	0.00133776	−1.6
19	PI3	Peptidase inhibitor 3, skin-derived	0.00210276	3.1
20	SYT11	Synaptotagmin XI	0.00230888	−1.6
21	ZNF597	Zinc finger protein 597	0.0023228	−1.6
22	CEP78	Centrosomal protein 78 kDa	0.00247458	−1.9
23	TULP3	Tubby-like protein 3	0.00253636	−1.6
24	MRPL49	Mitochondrial ribosomal protein L49	0.0026203	−1.5
25	SCARNA9L	Small Cajal body-specific RNA 9-like	0.00309212	−2.3
26	FUT3	Fucosyltransferase 3 [galactoside 3(4)-L-fucosyltransferase]	0.00316799	1.7
27	MTMR10	Myotubularin-related protein 10	0.00324594	−1.9
28	VNN1	Vanin 1	0.00430811	1.7
29	TLE1	Transducin-like enhancer of split 1 [E(sp1) homolog. Drosophila]	0.0044082	−1.8
30	PRF1	Perforin 1 (pore-forming protein)	0.00460118	−1.6
31	JAZF1	JAZF zinc finger 1	0.00462456	−1.8
32	OPTC	Opticin	0.00503031	1.6
33	LOC100133315	Transient receptor potential cation channel, subfamily C	0.00514394	−1.8
34	EFTUD1	Elongation factor Tu GTP-binding domain containing 1	0.00563133	−1.6
35	TOX	Thymocyte selection-associated high-mobility group box	0.00657298	−1.7
36	KRTAP4-3	Keratin-associated protein 4-3	0.00659143	1.7
37	ARHGAP23	Rho GTPase activating protein 23	0.0066427	−1.9
38	PRDX6	Peroxiredoxin 6	0.00668549	−1.8
39	SH2D2A	SH2 domain containing 2A	0.00674759	−1.5
40	OR7G3	Olfactory receptor, family 7. Subfamily G, member 3	0.007437	1.7
41	HIST1H2BH	Histone cluster 1, H2bh	0.00752556	−1.7
42	OR10H2	Olfactory receptor, family 10. Subfamily H, member 2	0.00860143	1.6
43	UBE2F	Ubiquitin-conjugating enzyme E2F (putative)	0.0102957	−1.7
44	ELOF1	Elongation factor 1 homolog (*Saccharomyces cerevisiae*)	0.0108196	−1.6
45	EMC3	ER membrane protein complex subunit 3	0.0113859	−1.6
46	GPR56	G protein-coupled receptor 56	0.0115175	−1.9
47	SNUPN	Snurportin 1	0.0115562	−1.5
48	RPIA	Ribose 5-phosphate isomerase A	0.0116402	−1.7
49	TMEM116	Transmembrane protein	0.0126389	−1.6
50	DEXI	Dexi homolog (mouse)	0.0128963	−1.9
51	SHISA4	Shisa homolog 4 (*Xenopus laevis*)	0.01313	1.6
52	ARHGAP23	Rho GTPase activating protein	0.013844	1.9
53	YOD1	OTU deubiquinating enzyme 1 homolog (*S. cerevisiae)*	0.0142472	−1.6
54	DIP2A	DIP2 disco-interacting protein 2 homolog A (Drosophila)	0.015922	−1.6
55	FAM99A	Family with sequence similarity 99, member A (nonprotein coding)	0.0162297	1.7
56	LRRC18	Leucine-rich repeat containing 18	0.0163249	1.8
57	ZFP90	Zinc finger protein 90 homolog (mouse)	0.0167504	−1.6
58	LINC00299	Long intergenic nonprotein coding RNA	0.0176272	−1.6
59	HIST1H2BC	Histone cluster 1, H2bc	0.0178361	−1.6
60	RPA2	Replication protein A2, 32 kDa	0.0182596	−1.6
61	ACADM	Acyl-CoA dehydrogenase. C-4 to C-12 straight chain	0.0184106	−1.5
62	ASTE1	Asteroid homolog 1 (Drosophila)	0.0184753	−1.6
63	SERPINB3	Serpin peptidase inhibitor, clade B (ovalbumin), member 3	0.0196835	1.6
64	ATP6V1D	ATPase, H+ transporting, lysosomal 34 kDa, V1 subunit D	0.0201942	−1.6
65	SRXN1	Sulfiredoxin 1	0.020326	1.7
66	SCGB1A1	Secretoglobin, family 1A, member 1 (uteroglobin)	0.0204639	1.8
67	RBMX	RNA-binding motif protein, X-linked	0.0206876	−0.2
68	TTI2	TELO2 interacting protein 2	0.0211204	−1.8
69	HARS	Histidyl-tRNA synthetase	0.0215574	−1.6
70	CGB5	Chorionic gonadotropin, beta polypeptide 5	0.0216971	1.5
71	RPSA	Ribosomal protein SA	0.0218604	−1.7
72	HIST2H2AA3	Histone cluster 2, H2aa3	0.0221445	−1.6
73	SLCO4C1	Solute carrier organic anion transporter family, member 4C1	0.02266	−1.6
74	CCR3	Chemokine (C–C motif) receptor 3	0.0227878	−1.8

Table 2 (*continued*)

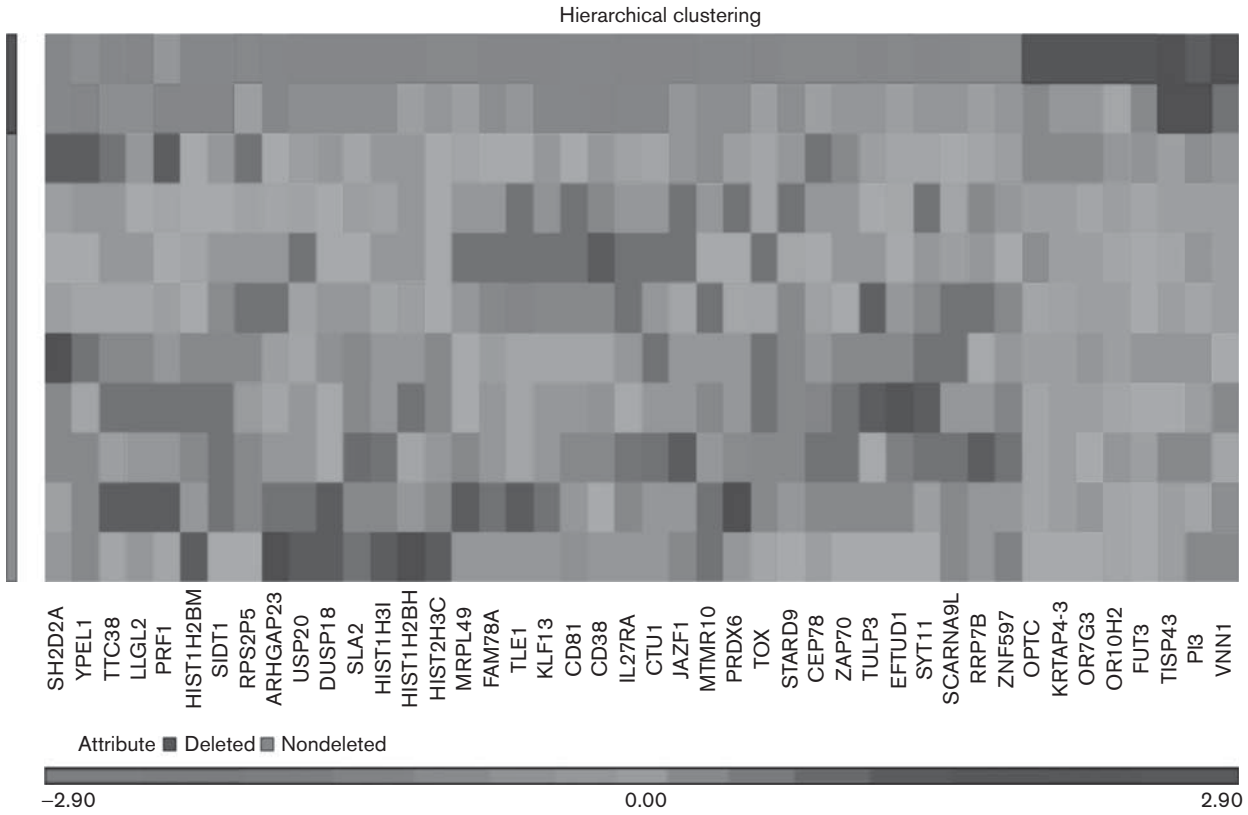
	Gene symbol	Gene assignment	*P* value (deletion)	Fold change
75	JAKMIP2	Janus kinase and microtubule interacting protein 2	0.0232163	−1.6
76	OR51B4	Olfactory receptor, family 51. Subfamily B, member 4	0.0236548	1.6
77	TBC1D7	TBC1 domain family, member 7	0.0236723	−1.6
78	CGB7	Chorionic gonadotropin, beta polypeptide 7	0.0243057	1.5
79	FAM48B1	Family with sequence similarity 48, member B1	0.024683	1.8
80	ANKRD11	Ankyrin repeat domain 11	0.0251498	−1.9
81	CSDA	Cold shock domain protein A	0.0256404	−1.6
82	CDRT1	CMT1A duplicated region transcript 1	0.0262512	1.6
83	LGALS14	Lectin, galactoside-binding, soluble 14	0.0262856	1.6
84	MUC3B	Mucin 3B, cell surface-associated	0.0265501	1.7
85	MCM8	Minichromosome maintenance complex component 8	0.0268405	−1.6
86	IGKV1D-42	Immunoglobulin kappa variable 1D-42 (nonfunctional)	0.0283412	−1.7
87	SH2D1B	SH2 domain containing 1B	0.0287854	−2.1
88	KRT18	Keratin 18	0.0290164	−1.6
89	OR51E2	Olfactory receptor, family 51. Subfamily E.	0.0294379	1.6
90	OR14I1	Olfactory receptor, family 14. Subfamily I, member 1	0.0306603	1.6
91	RFPL4A	Ret finger protein-like 4A	0.031631	1.5
92	RPS12	Ribosomal protein S12	0.0322135	−1.5
93	CHORDC1	Cysteine and histidine-rich domain (CHORD) containing 1	0.0329611	−1.5
94	SNORD38B	Small nucleolar RNA, C/D box 38B	0.0333204	−1.5
95	SLC48A1	Solute carrier family 48 (heme transporter), member 1	0.0343111	−1.5
96	HSPA2	Heat shock 70 kDa protein 2	0.0345332	1.6
97	OR3A1	Olfactory receptor, family 3. Subfamily A, member 1	0.0348752	1.7
98	DHFR	Dihydrofolate reductase	0.0351019	−1.6
99	CYP4Z1	Cytochrome P450, family 4. Subfamily Z, polypeptide 1	0.0353701	1.6
100	PPDPF	Pancreatic progenitor cell differentiation and proliferation factor	0.0355663	−1.6
101	MBNL3	Muscleblind-like splicing regulator 3	0.0361796	−1.9
102	PTPN22	Protein tyrosine phosphatase, nonreceptor type 22 (lymphoid)	0.0362042	−1.6
103	ENC1	Ectodermal-neural cortex 1 (with BTB-like domain)	0.0363468	−1.5
104	MUC12	Mucin 12, cell surface-associated	0.0365138	3.2
105	FCRL6	Fc receptor-like 6	0.0366632	−1.8
106	TPRX1	Tetra-peptide repeat homeobox 1	0.036786	1.8
107	TRAV19	T-cell receptor alpha variable 19	0.0377103	−1.7
108	LCN8	Lipocalin 8	0.0387392	1.9
109	CTSE	Cathepsin E	0.0392981	−1.7
110	TBC1D28	TBC1 domain family, member 28	0.0399368	1.6
111	KRTAP10-5	Keratin-associated protein 10-5	0.0400279	1.6
112	IGSF21	Immunoglobin superfamily, member 21	0.0401894	1.5
113	GRAP	GRB2-related adaptor protein	0.040266	−1.6
114	SCARNA9L	Small Cajal body-specific RNA 9-like	0.0405693	−1.5
115	CMKLR1	Chemokine-like receptor 1	0.0406512	−1.5
116	HIST1H2AJ	Histone cluster 1	0.0409278	−1.7
117	C14orf45	Chromosome 14 open reading frame 45	0.041883	−1.7
118	MICA	MHC class I polypeptide-related sequence A	0.0419817	−1.6
119	CKMT1A	Creatine kinase, mitochondrial 1A	0.0432747	1.7
120	CKMT1A	Creatine kinase, mitochondrial 1A	0.0432747	1.7
121	SRRD	SRR1 domain containing	0.0439037	−1.6
122	GPR128	G protein-coupled receptor 128	0.0440475	2.8
123	MUT	Methylmalonyl CoA mutase	0.0446626	−1.5
124	EFCAB4B	EF-hand calcium-binding domain 4B	0.0461407	−1.6
125	ANKRD50	Ankyrin repeat domain 50	0.0467662	−1.6
126	SRD5A3	Steroid 5 alpha-reductase 3	0.0475235	−1.7
127	ABCE1	ATP-binding cassette. Subfamily E (OABP), member 1	0.0476485	−1.5
128	G6PC2	Glucose-6-phosphatase, catalytic 2	0.0491228	1.6
129	ABCD1	ATP-binding cassette. Subfamily D (ALD), member 1	0.0498771	−1.6

ADHD, attention-deficit hyperactivity disorder.

Our pathways analyses indicated that apoptosis, oxidative stress, and immune response signaling were the most significantly differentially modulated pathways linked to 15q13 deletions. Interestingly, we found that deletions in *KLF13* and *CHRNA7* genes affected the expression levels of genes implicated in the same immune response signaling, inflammatory as well as stress oxidative pathways. Furthermore, three genes from our list of genes that were significantly altered in ADHD patients with 15q13 deletions [*IL27RA*, interleukin 27 receptor, alpha; *ZAP70*, zeta-chain (TCR)-associated protein kinase 70 kDa; *FUT3*, fucosyltransferase 3 (galactoside 3(4)-L-fucosyltransferase, Lewis blood group)] were part of immune/inflammatory response signaling. Similarly, the *PRDX6* (peroxiredoxin 6) gene encoding a thiol-specific antioxidant protein, in our gene list, found to be down-regulated in ADHD patients with 15q13 deletions, belonged to the oxidative stress pathway (Fig. 3).

The *KLF13* gene encodes a transcription factor; it plays an important role in activating *CCL5* (RANTES) gene expression in T lymphocytes (Song *et al.*, 1999). CCL5 is

Fig. 2



Heatmap showing the results of hierarchical clustering of our dataset. It identifies two main groups (left side). Black: patients carrying deletions in 15q13; gray: patients without deletions in 15q13. Thirty-five genes are downregulated and 8 genes upregulated in ADHD patients carrying deletions in 15q13 versus ADHD patients without deletions in 15q13. ADHD, attention-deficit hyperactivity disorder.

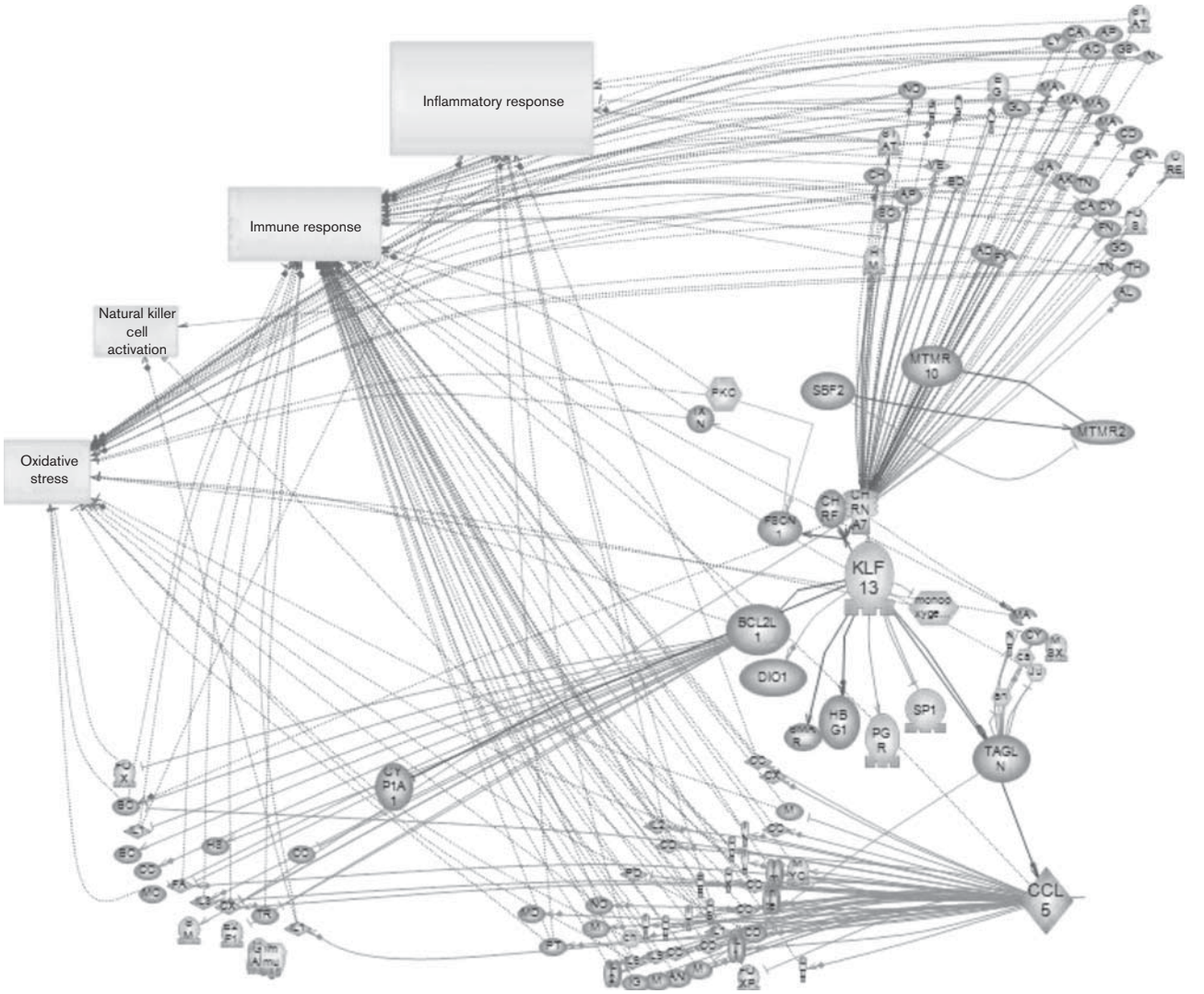
Table 3 Pathways regulated by 15q13 deletion genes

Name	*P* value
Apoptosis	2.44E−76
Oxidation reduction	1.75E−39
Immune response	2.94E−35
Oxidoreductase activity	2.44E−20
Oxidoreductase activity. Acting on single donors with incorporation of molecular oxygen, incorporation of two atoms of oxygen	0.000332
Cell cycle regulation	1.37E−10
Hedgehog pathway	4.12E−06
Nicotinate and nicotinamide metabolism	1.51E−05
Axon guidance	1.76E−05
Guanylate cyclase pathway	0.000134
Apoptosis regulation	0.000191
B-cell activation	0.000305
Gap junction regulation	0.000543
Insulin action	0.000923
Respiratory chain and oxidative phosphorylation	0.001402
Metabolism of triacylglycerols	0.004377
NK-cell activation	0.005798

a member of the chemokines family and is involved in immune/inflammatory events. *KLF13* may regulate multiple stages of both B-cell and T-cell development, in accordance with evidence emerging from murine models (Outram *et al.*, 2008). As expected, we observed downregulation of this gene, which is further confirmed by another study that reported a decrease in the mRNA levels of this gene in a proband with a homozygous 15q13.3 microdeletion compared with controls (Le Pichon *et al.*, 2013). Thus, because it has been suggested that immune response (Ceylan *et al.*, 2012) as well as inflammation (Donev and Thome, 2010) play a key role in the etiology of ADHD, as well as in autism and schizophrenia (Gibney and Drexhage, 2013), decreased *KLF13* expression could be indirectly involved in these pathologies.

CHRNA7 is located within the deleted region, but shows low expression levels in our blood samples. Our target prediction analysis, however, suggests that it also influences immune response and inflammatory signaling, both of which may be involved in the pathogenesis of ADHD (Donev and Thome, 2010; Ceylan *et al.*, 2012) or other psychiatric disorders (Gibney and Drexhage, 2013). We cannot differentiate the effects of *CHRNA7* from other deleted genes from the same region, but we can speculate that *CHRNA7* is likely to contribute significantly toward immune response and inflammatory signaling pathways. It has been reported that stimulation of *CHRNA7* on human polymorphonuclear neutrophils and

Fig. 3



A target prediction analysis showed the pathways influenced by the genes belonging to the 15q13 deletion (*KLF13, MTMR10, CHRNA7*). *KLF13* and *CHRNA7* activated inflammatory response, immune response, and oxidative stress networks.

blood mononuclear phagocytes *in vitro* attenuates the expression of leukocyte markers involved in cell recruitment and adhesion, and release of tumor necrosis factor-α and other proinflammatory cytokines (Vukelic *et al.*, 2013).

KLF13 and *CHRNA7* influence the oxidative stress pathway. Some evidence showed that oxidative stress might suppress the expression of the *CHRNA7* at protein and mRNA levels during the early stages of damage in PC12 cells (Guan *et al.*, 2001) as well as in polymorphonuclear neutrophils and blood mononuclear phagocytes *in vitro* (Vukelic *et al.*, 2013). Moreover, several studies have shown the implication of reactive oxygen species in the regulation of RANTES (Lin *et al.*, 2000; Barlic and Murphy, 2007; Tripathy *et al.*, 2007, 2010).

There are findings that support that oxidative metabolism may play a role in the etiopathogenesis of ADHD, with a meta-analysis of extant studies showing increased markers of oxidative stress among unmedicated ADHD patients compared with controls [Joseph *et al.* (in press)]. Alterations in the oxidative stress pathway are also observed for other psychiatric disorders (Ghanizadeh *et al.*, 2013; Wu *et al.*, 2013).

Thus, our results indicate that immune/inflammatory and oxidative stress pathways dysregulated in ADHD patients carrying the 15q13 deletion appear to play a role not only in ADHD but also in other psychiatric disorders such as schizophrenia and autism. This strengthens the issue on the pleiotropic effects of 15q13 deletions and thus on the existence of shared biologic signaling among

multiple neuropsychiatric disorders. Evidence for shared genetic causes among disorders has also been shown for common variants (Cross-Disorder Group of the Psychiatric Genomics Consortium *et al.*, 2013). Furthermore, for inflammatory response, it has also been reported that ADHD patients are at a higher risk for asthma (Fasmer *et al.*, 2011; Mogensen *et al.*, 2011; Kwon *et al.*, 2014). Investigation of the mechanisms associated with deletion could explain the common origin of different psychiatric pathologies.

We acknowledge that this study has some limitations. Because 15q13 CNVs are rare, it is possible that any pathophysiologic insights from CNV carriers may not be generalizable to other ADHD patients. However, it is possible that ADHD CNVs impact the same biological pathways as common variation. If so, our results would be relevant to a larger subset of patients. It is also important to underline that the frequency of 15q13 CNVs observed in our patients is similar to that observed in other European courts. Another limitation is that our gene expression study used ADHD patients without deletions as controls. Although this allows us to differentiate the effects of the deletions from the effects of other sources of ADHD's etiology, it is possible that our power was reduced to detect pathways that have heterogenous etiologies.

Our gene expression study was carried out in leukocytes, a peripheral tissue. Although not all genes expressed in the brain are also expressed in blood, several considerations suggest that peripheral gene expression studies can be useful (Sullivan *et al.*, 2006; Rollins *et al.*, 2010). Moreover, although the brain is clearly the locus for much of psychiatric pathophysiology, numerous studies implicate processes such as inflammation and abnormal immune responses, which are expressed in peripheral tissues (Gladkevich *et al.*, 2004), and would be expected to impact gene expression in blood cells. It is also likely that some gene expression profiles may be epiphenomena of brain activity. A systematic review of the literature shows that peripheral measures of neurotransmitters and their metabolites are associated significantly with brain levels (Marc *et al.*, 2011). Consistent with this, in a recent meta-analysis, we showed that four peripheral measures of monoamine metabolism significantly discriminated ADHD and non-ADHD samples (Scassellati *et al.*, 2012). Although these brain-related changes in neurotransmitters and metabolites in the periphery are not caused by blood cell gene expression, they likely have effects on gene expression that are useful for differentiating ADHD cases from controls.

Finally, a further limitation is linked to *CHRNA7* expression. Despite the importance of this gene in a variety of neuropsychiatric phenotypes (Miller *et al.*, 2009; Shinawi *et al.*, 2009), including ADHD (Stergiakouli *et al.*, 2012; Williams *et al.*, 2012), it is not sufficiently expressed in blood to have been informative for our analyses. However, contrasting results for the expression of this gene in blood have been reported (Sato *et al.*, 1999; Benfante *et al.*, 2011; Van der Zanden *et al.*, 2012; Le Pichon *et al.*, 2013). Higher expression of *CHRNA7* in the brain highlights the potential importance of this gene in the central nervous system (Le Pichon *et al.*, 2013).

Conclusion

Our findings are consistent with the presence of 15q13 deletions in Italian ADHD patients. More interestingly, we show that pathways related to immune/inflammatory response and oxidative stress signaling are affected by the deletion of *KFL13* and *CHRNA7*. Because the phenotypic effects of 15q13 are pleiotropic, our findings imply the existence of shared biologic pathways among multiple neuropsychiatric conditions.

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Conflicts of interest

In the past year, Dr Faraone received consulting income and/or research support from Shire, Akili interactive Labs, VAYA Pharma, SynapDx, and Alcobra and research support from the National Institutes of Health (NIH). His institution is seeking a patent for the use of sodium–hydrogen exchange inhibitors in the treatment of ADHD. In previous years, he received consulting fees or was on Advisory Boards or participated in continuing medical education programs sponsored by Shire, Alcobra, Otsuka, McNeil, Janssen, Novartis, Pfizer, and Eli Lilly. Dr Faraone receives royalties from books published by Guilford Press: Straight Talk about Your Child's Mental Health. For the remaining authors there are no conflicts of interest.

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