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A Genome Wide Copy Number Variations Analysis in Autism Spectrum Disorder (ASD) and Intellectual Disability (ID) in Italian Families

Mucciolo Mafalda[1], Chiara Di Marco[1,3], Roberto Canitano[2], Sabrina Buoni[2], Elisa Frullanti[1], Maria Antonietta Mencarelli1[3], Bizzarri Veronica[1], Sonia Amabile[1], Lucia Radice[2], Margherita Baldassarri[1,3], Caterina Lo Rizzo1[3], Ilaria Meloni[1], Joussef Hayek[2], Alessandra Renieri[1,3] and Francesca Mari[1,3]

[1]*Medical Genetics-Department of Biotechnology, University of Siena-Policlinico S. Maria alle Scotte, 53100 Siena, Italy*
[2]*Department of Child Neuropsychiatry, University Hospital of Siena, 53100 Siena, Italy*
[3]*Department of Medical Genetics, Azienda Ospedaliera Universitaria Senese, Siena, Italy*

Abstract

Background: Autism Spectrum Disorders (ASD) and Intellectual Disability (ID) represent lifelong conditions with severe impact on behavior and lifestyle of patients and their families. Array comparative genomic hybridization (array-CGH) has clarified the underlying genetic causes of ASD and ID by CNVs identification in several chromosomal regions with susceptibility to different levels of severity of ASD or ID in up to 1% of patients.

Methods: Using oligo array-CGH we analyzed 476 unrelated subjects with ASD or ID, thoroughly investigated by both child neuropsychiatrists and clinical geneticists. The inheritance of the CNV was tested in the majority of cases (82% of positive cases).

Results: A total of 198 rearrangements were identified in 154 cases. CNVs were classified in three groups: i- CNVs previously known to be associated with ASD or ID (28/198, 14%), including 16p11.2, 15q13.3, 17p12 and 17q12; ii- CNVs including genes known to be associated with either ASD or ID (9/198, 4.5%); iii- CNVs of unknown significance (161/198, 81.3%).

Conclusion: Our study confirmed that array-CGH analysis is able to detect the underlying genetic cause in about 18% of ASD or ID patients, highlighting it as an essential diagnostic tool for patient's assessment. Overall, a prevalence of duplications with respect to deletions was observed (62% and 38%, respectively) but among the deleted cases an enrichment of microdeletions in ASD cases (p=0.03) is present. Furthermore, we shown a prevalence of multiple CNVs in ASD cases compared to ID (p=0.05), pointing out the complex nature of ASD.

Keywords: Array-CGH; CNVs; Autism spectrum disorders; Intellectual disability; Genomic disorders

Abbreviations: ASD: Autism Spectrum Disorders; ID: Intellectual Disability; DSM-IV: Diagnostic and Statistical Manual 4th Edition; ADI-R: Autism Diagnostic Interview-Revised; ADOS-G: Autism Diagnostic Observation Schedule Generic; Array-CGH: Array Comparative Genomic Hybridization; CNV: Copy Number Variation

Introduction

Autism Spectrum Disorders (ASDs) and intellectual disability (ID) are the most common development disorders in humans representing an important health burden in the population [1]. ASDs are a spectrum of psychological conditions characterized by impairments in communication, dysfunctional reciprocal social interaction and the presence of restricted, repetitive and stereotyped patterns of behavior. In addition to Autism, ASDs include Asperger syndrome and Pervasive Developmental Disorder Not Otherwise Specified (PDD-NOS) [2]. ASDs present complex and heterogeneous etiology with a strong evidence of genetic involvement placing it among the most heritable neurodevelopmental disorders. ID is a condition characterized by below average intellectual functioning (IQ<70) together with significant limitation in both intellectual and adaptive functioning [3]. ASD is associated in 70% of individuals with intellectual disability. To date, no single gene has been shown to account for a majority of ASD or ID susceptibility [4]. The complex heterogeneity of both conditions, possibly resulting from the interaction of several genes and environmental factors, makes the identification of contributory genes extremely difficult.

Comparative genomic hybridization (CGH) technology has been widely used in research studies and in clinical practice of ASDs in order to detect copy number variants (CNVs) throughout the genome. CNVs represent a significant source of genetic variability and are responsible of disease susceptibility for several neurobehavioral phenotypes. Several studies revealed clinical relevant CNVs detection rate variable from a 10–20% [5,6] depending on the resolution of the applied array platform and by clinical selection of patients on the basis of family history and associated anomalies. Overall, array-CGH analysis increased the diagnostic yield in ASDs and ID, allowing the identification of new genetic causes. In addition to the well-known recurrent pathogenic rearrangements, several new microdeletions and microduplications have been identified in ASDs and ID patients as potentially pathogenic [5-7].

Here, we report the results of array-CGH analysis performed on a panel of 476 ASD or ID patients for which accurate clinical assessment and genetic counseling were performed.

***Corresponding author:** Alessandra Renieri, MD, PhD Department of Medical Biotechnology Medical Genetics Unit University of Siena Viale Bracci, 253100 – Siena, Italy, Tel: +39 0577 233303; Fax: +39 0577 233325; E-mail: alessandra.renieri@unisi.it

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Methods

A cohort of 476 unrelated patients referring to the Medical Genetics Unit of Siena (Italy) and classified as Autism Spectrum Disorder (ASD) (267/476; 56%) or intellectual disability without ASD (ID) (206/476; 44%), was selected for this study. Among these, 333 were males and 143 were females (M:F=2.3:1). Considering the 2 classes, the M:F ratio is 3.8:1 for the ASD group, in accordance with literature data, and 1.3:1 for the ID group. A cognitive evaluation for all subjects was carried out by the team of the Neuropsychiatric Unit of Siena based on standardized Diagnostic and Statistical Manual 4th Edition (DSM-IV) criteria using Autism Diagnostic Interview-Revised (ADI-R) and/or Autism Diagnostic Observation Schedule Generic (ADOS-G) standards. The patients were therefore classified based on the severity of intellectual disability: mild, moderate, severe. The patients were classified based on the severity of intellectual disability. Among the ASD group, 141 out of 267 cases also presented ID and were classified as follows: 34 patients were severe cases, 71 were moderate and 36 were mild. In ID group, the majority of patients were moderate or mild (60 and 50 respectively) and 24 were severe (Table 1).

All patients also underwent a comprehensive evaluation by a clinical geneticist (FM or MAM) who excluded a diagnosis of a recognizable syndrome. All families had genetic counseling and biological samples of parents were available in the majority of cases (82%). All patients, except those with microcephaly, have been tested for FMR1 gene expansion and resulted negative.

Biological samples of these patients and their parents, whenever available, were collected after obtainment of informed consent by patients' guardians/parents and stored in the "Cell Lines and DNA Bank of Rett syndrome, X linked mental retardation and other genetic diseases", member of the Telethon Network of Genetic Biobanks.

Array-CGH analysis

Genomic DNA of the patients was isolated from an EDTA peripheral blood sample using the QIAamp DNA Blood Kit according to the manufacturer's protocol (Qiagen, Valencia, CA, USA). Genomic DNA of normal male and female controls was obtained from Agilent (Agilent Technologies, Santa Clara, CA, USA). One microgram of genomic DNA from the patient (test sample) and the control (reference sample) were labeled with Cy5 and Cy3 fluorocrome, respectively.

Array-CGH analysis was performed using commercially available oligonucleotide microarrays containing about 60.000 60-mer probes (Human Genome CGH Microarray 60 K Kit, Agilent Technologies, Santa Clara, CA, USA) according to manufacturer's standard protocol. The median functional resolution was 45 Kb. Copy number variations (CNVs) were considered significant if they were defined by three or more oligonucleotides, and were not present in the Database of Genomic Variants (http://dgv.tcag.ca/dgv/app/home). Confirmation of results was performed by a second independent experiment. Segregation analysis of the identified rearrangements was performed with the same technique. Map positions are based on hg19 GRCh build 37 (Feb 2009) and listed based on ISCN 2013.

Results

CNVs discovery

Our cohort included 476 patients with ASD (56%) or ID without ASD (44%) (Table 1).

Among the 476 patients, 154 exhibited at least one rearrangement. In these 154 patients, a total of 198 rearrangements were identified: 76 deletions and 122 duplications. CNVs were classified in three groups: i) CNVs (28/198, 14%) previously known to be associated with ASD or ID, including 16p11.2, 15q13.3, 17p12 and 17q12 (Table 2); ii) CNVs (9/198, 4.5%) including genes known to be associated with either ASD or ID (Table 3); iii) CNVs (161/198, 81.3%) of unknown significance (Table 4 and Figure 1). The size of rearrangements ranged from 1 Kb to 13 Mb with a mean size of 868 Kb. Observing the size of the CNVs in the three classification groups we noted a prevalence of large CNVs in group i) and ii) for both ASD and ID cases. In the group of variant of unknown significance we observed instead a prevalence of small CNVs (Figure 2).

Out of 154 patients, 122 (79.2%) patients had only one rearrangement and 32 (20.8%) had two or more rearrangements (four in one case) (Table 2). Among the 32 patients with more than one rearrangement, 21 (65.6%) were classified as ASD, while the remaining 11 (34.4%) were in the ID group (Figure 3). The data show a prevalence of multiple CNVs in ASD cases compared with ID cases (p=0.05). In addition, we noted that the deletions are more represented in ASD than in ID patients (49 and 27, respectively), while the duplications are similarly represented in ASD and ID (65 and 58 respectively) (Table 2 and Figure 4). These results suggest a stronger association between the presence of a microdeletion and the ASD phenotype (p=0.03).

	ASD n=267 (56)			**ID n=209 (44)**		
	Positive n=83 (31)	**Negative n=184 (69)**	**Tot n=267**	**Positive n=71 (34)**	**Negative n=138 (66)**	**Tot n=209**
Sex						
Male	66 (79.5)	146 (79.4)	212 (79.4)	40 (56.3)	81 (58.7)	121 (57.9)
Female	17 (20.5)	38 (20.6)	55 (20.6)	31 (43.7)	57 (41.3)	88 (42.1)
Cognitive impairment						
Severe	11 (13.2)	23 (12.5)	34 (12.7)	8 (11.3)	16 (11.6)	24 (11.5)
Moderate	17 (20.5)	54 (29.3)	71 (26.6)	17 (23.9)	43 (31.1)	60 (28.7)
Mild	10 (12.0)	26 (14.1)	36 (13.5)	19 (27.8)	31 (22.5)	50 (23.9)
n.a.	45 (54.2)	81 (44)	126 (47.2)	27 (38)	48 (34.8)	75 (35.9)
EEG and/or MRI abnormality						
present	17 (20.5)	39 (21.2)	56 (21.0)	28 (39.4)	59 (42.7)	87 (41.6)
absent	12 (14.5)	32 (17.4)	44 (16.5)	13 (18.3)	19 (13.8)	32 (15.3)
n.a.	54 (65)	113 (61.4)	167 (62.5)	30 (42.2)	60 (43.5)	90 (43.1)

Brackets data are presented as %

Table 1: Characteristics of samples.

ID #	Sex	Phenotype	Chr	Cytoband	CNV start	CNV stop	CNV	Size	Inheritance	References
2476	M	ASD	1	1q21.1	145,632,334	145,747,214	Loss	115 Kb	Mat	Pinto et al. [5]
326*	M	ASD	1	1q21.1	145,632,334	145,747,214	Loss	115 Kb	Mat	Pinto et al. [5]
2117	M	ID	1	1q21.1	145,632,334	145,747,214	Loss	115 Kb	Mat	Pinto et al. [5]
180	M	ASD	2	2q31.1q33.2	180,306,799	193,335,172	Loss	13 Mb	De novo	Cocchella et al. [33]
168	M	ID	3	3p14.1p14.3	54,452,525	65,609,348	Loss	11 Mb	De novo	Okumura et al. [36], De la Hoz et al. [37]
387	F	ID	4	4p16.3	51,413	2,079,430	Loss	2,03 Mb	De novo	Battaglia et al. [40]
762	M	ASD	7	7q11.23	72,420,745	74,139,390	Gain	1,72 Mb	De novo	Velleman and Mervis [41]
803	F	ID	9	9q31.1q32	107,970,048	114,341,159	Loss	6.37 Mb	De novo	Mucciolo et al. [42]
952	F	ID	14	14q32.31	101,697,865	107,258,824	Loss	5,6 Mb	De novo	Engels et al. [43]
2720	M	ASD	15	15q11.2q13	20,849,110	28,525,401	Gain	7,7 Mb	De novo	Thomas et al. [26]
174	M	ID	15	15q11.2q13	23,739,358	28,525,460	Loss	4,8 Mb	Unknown	Thomas et al. [26]
221	M	ASD	15	15q13.3	32,021,733	32,510,804	Loss	489 kb	Unknown	Bacchelli et al. [27]
846	M	ID	15	15q13.3	32,021,733	32,438,943	Gain	417 kb	Mat	Szafranski et al. [28]
278	F	ASD	15	15q26.3	98,612,748	101,320,461	Gain	2,7 Mb	Pat	Tatton-Brown et al. [44]
1537	M	ASD	16	16p13.11	14,944,560	15,960,084	Gain	1 Mb	Mat	Ullmann et al. [45]
1383	F	ID	16	16p13.11	14,944,560	16,305,677	Gain	1,36 Mb	Pat	Ullmann et al. [45]
7*	M	ASD	16	16p11.2	29,673,954	30,198,553	Loss	524 kb	De novo	Jacquemont et al. [25]
518	M	ASD	16	16p11.2	29,673,954	30,197,341	Loss	523 kb	De novo	Jacquemont et al. [25]
41	M	ID	16	16p11.2	29,673,954	30,119,712	Loss	446 kb	Unknown	Jacquemont et al. [25]
1573	M	ASD	16	16p11.2	29,673,954	30,198,553	Loss	525 kb	Pat	Jacquemont et al. [25]
778	M	ID	16	16p11.2	29,673,954	30,198,600	Gain	525 kb	Mat	Jacquemont et al. [25]
936	F	ASD	17	17p11.2	16,603,130	20,434,018	Gain	3,8 Mb	De novo	Potocki et al. [46]
27	F	ID	17	17p11.2	16,892,401	20,193,196	Gain	3,3 Mb	De novo	Potocki et al. [46]
1	M	ASD	17	17q12	34,851,537	36,473,234	Gain	1,62Mb	Pat	Nagamani et al. [47]
1129	M	ASD	22	22q11.2	18,896,972	21,379,958	Gain	2,48 Mb	Mat	Wentzel et al. [48]
66*	M	ASD	X	Xp22.31	7,555,292	8,266,181	Gain	710 kb	Mat	Esplin et al. [49]
1046	F	ID	X	Xp22.31	6,457,403	8,266,240	Gain	1,80 Mb	Pat	Esplin et al. [49]
656	F	ID	X	Xq28	152,764,591	154,841,455	Gain	2,08 Mb	De novo	Bijlsma et al. [50]

*Patients presenting a second CNV of unknown significance (Table 5)

Table 2: CNVs affecting known deleterious regions.

ID #	Sex	Phenotype	Chr	Cytoband	CNV start	CNV stop	CNV	Size	Inheritance	Genes	References
1070 *	M	ID	2	2q13	110,841,715	110,980,342	Gain	138 Kb	Pat	NPHP1	Yasuda et al. [8]
1740	M	ASD	7	7q32.3q33	131,948,767	133,002,068	Gain	1,05 Mb	De novo	PLXNA4	Suda et al. [9]
1302 *	M	ASD	9	9p24.3	611,628	762,947	Gain	151 kb	Pat	KANK1	Vanzo et al. [10]
302	M	ID	9	9q22.32	97,843,040	98,659,815	Gain	817 kb	Mat	PTCH1	Izumi et al. [11]
1986 *	F	ID	12	12p12.1p12.2	20,038,565	25,826,850	Loss	5,8 Mb	De novo	SOX5	Lee et al. [12]
2256	F	ASD	16	16q24.2	87,340,135	87,420,919	Gain	80 kb	Pat	FBXO31	Handrigan et al. [13]
681	M	ASD	20	20p12.1	14,824,372	15,268,002	Loss	443 kb	Mat	MACROD2	Jones et al. [14]
36	M	ID	X	Xp22.11	22,836,324	23,411,163	Loss	575 kb	Mat	PTCHD1	Chaudhry et al. [17]
1420	M	ID	X	Xp11.22 Xp22.12	52,892,965 19,904,414	53,325,084 20,553,212	Gain Gain	432 kb; 649 kb	Mat Mat	IQSEC2, KDM5C - RPS6KA3	Fieremans et al. [15], Matsumoto et al. [16]

*Patients presenting a second CNV of unknown significance (Table 5)

Table 3: CNVs affecting known deleterious regions.

CNVs affecting known deleterious regions

To identify specific CNVs which may contribute to ASD or ID phenotype, we first looked for CNVs in well-known ASD/ID associated region (Table 3). Among the validated CNVs, we identified a common hotspot at 16p11.2 (four deletions and one duplication), whose pathogenicity in ASD has been longtime established. In order of frequency in our cohort, we found three maternally inherited deletion in 1q21, two 15q13.3 duplication, two duplications in 17p11.2, two Xp22.31 duplication, a deletion and a duplication in 16p13.11 and a deletion and a duplication in 15q11.2q13. Additional CNVs occurring in single cases are listed in Table 2.

The 46.4% of these diseases associated CNVs are inherited (28.6% maternal and 17.8% paternal) while the 42.8% occurring *de novo*.

CNVs affecting ASD/ID associated genes

We evaluated our data looking also for CNVs involving genes already known to be involved in ASD or ID (Table 4). We identified the following rearrangements: a duplication of NPHP1 gene in 2q13 [8]; a *de novo* 7q32.3 gain involving PLXNA4 [9]; a 9p24.3 duplication involving KANK1 gene [10]; a duplication of PTCH1 in 9q22 [11]; a 5.8 Mb deletion on 12p12.2p12.1 including SOX5 [12]; a duplication of the 16q24.2 region [13]; an inherited 20p12.1 deletion interrupting the MACROD2 gene [14]; a Xp11.22 duplication involving KDM5C and IQSEC2 [15]; a duplication in Xp22.12 including the RPS6KA3 gene [16]; and the deletion of PTCHD1 in Xp22.11 [17].

The 72.7% of these CNVs are inherited (45.5% maternal and 27.2% paternal) while the 18.2% occurring *de novo*.

ID #	Sex	Phenotype	Chr	Cytoband	CNV start	CNV stop	CNV	Size	Inheritance
1151	M	ASD	1	1p31.1	78,470,596	79,383,315	Gain	912 kb	Pat
853	M	ASD	1	1q43	236,631,519	236,748,164	Gain	117 kb	Pat
2327	M	ID	1	1q44	247,136,811	247,348,787	Gain	212 kb	Unknown
445	M	ASD	2	2q36.3	229930692	230824760	Gain	894 kb	Unknown
460	M	ASD	2 7	2p16.3 7q34	48,012,867 142,759,860	48,085,059 142,881,336	Gain Loss	72 kb 121 kb	Pat Pat
2449	F	ID	2	2q14.2	120,126,884	120,567,392	Gain	440 kb	Unknown
122	F	ID	2	2q14.2	120126884	120567451	Gain	440 kb	Pat
364	M	ASD	3 18	3p21.32p21.31 18q12.3	44,517,678 38,972,042	46,719,256 39,600,614	Loss Gain	2,2 Mb 629 kb	Mat Pat
314	M	ASD	3 10 12	3q13.11 10q11.22 12p12.1p12.2	105,294,119 47,148,490 21,011,274	105,294,179 51,594,486 21,349,852	Gain Gain Loss	60 kb 4,4 Mb 339 kb	Mat Pat Mat
2329	F	ASD	3	3q25.33q26.1	160,576,359	160,734,451	Loss	158 kb	Pat
1519	M	ASD	3	3p25.3	11,296,962	11,301,588	Gain	4,6 kb	Pat
1757	F	ID	3	3q11.2	94,798,716	95,275,664	Gain	477 kb	Mat
2868	M	ID	3 4 4	3q25.1 4q21.23 4q22.1	152,851,538 84,709,012 89,867,186	153,025,201 84,803,435 90,170,143	Loss Gain Gain	173 kb 94 kb 303 kb	Mat Pat Pat
318	M	ASD	4	4p16.3	72,447	113,524	Gain	41 kb	Pat
159	F	ASD	4	4q12	53,483,283	54,424,231	Loss	941 kb	Mat
100	M	ASD	4 1	4q35.2 1p22.3	189,609,241 87,406,704	190,896,674 87,501,238	Loss Loss	1,29 Mb 94 kb	Pat Mat
886	M	ASD	4 9	4p16.3 9q34.3	2,932,298 138,236,224	3,144,568 138,309,199	Gain Gain	212 kb 73 kb	Mat Pat
224	M	ASD	4	4p16.1	10,077,404	10,141,989	Gain	65 kb	Pat
1842	M	ID	4	4p16.3	72,447	113,524	Gain	41 kb	Pat
197	M	ASD	5	5q21.1	100,191,817	100,373,993	Gain	182 kb	Pat
352	F	ASD	5	5q11.2	56,471,611	56,538,065	Loss	66 kb	De novo
1621	M	ASD	5	5q12.3	64,085,869	64,217,093	Loss	131 kb	Mat
1734	M	ASD	5	5q15	93,728,464	93,918,134	Loss	190 kb	Mat
1877	M	ID	5	5p15.33	435,961	548,072	Gain	112 kb	De novo
2798	M	ID	5	5q23.1	115,551,734	115,628,469	Gain	76 kb	Unknown
1302**	M	ASD	6	6q14.1	82,840,207	83,335,748	Gain	495 kb	Pat
283		ASD	6	6q27	168,343,841	168,776,873	Gain	433 kb	Pat
420	F	ASD	6	6q27	170,228,674	170,890,108	Loss	661 kb	De novo
1494	F	ID	6	6p11.2	57,467,120	58,014,473	Gain	547 kb	Pat
7*	M	ASD	6	6q22.31	123,539,625	124,166,602	Gain	Mat	627 kb
2658	M	ASD	7	7q21.13	89,621,308	89,626,894	Loss	5,6 kb	Mat
2553	M	ASD	7	7q21.13	88,956,630	89,445,171	Gain	488 kb	Unknown
274	F	ASD	7	7q34	142,429,334	142,487,095	Gain	58 kb	De novo
41	M	ASD	7 16 20	7q36.3 16q24.3 20p11.23	156,518,109 90,059,273 18,020,282	156,626,420 90,096,088 18,167,715	Loss Gain Gain	108 kb 36 kb 147 kb	Mat Unknown Mat
57	M	ID	7 12	7q22.1 12q24.12	100,998,732 112,184,121	101,092,135 112,308,872	Gain Gain	93 kb 125 kb	Mat Mat
303	M	ASD	8	8p23.1	9,895,739	9,992,928	Loss	97 kb	Pat
857	F	ID	8	8q12.1	56,428,093	56,922,601	Gain	494 kb	Unknown
183	M	ASD	9	9p13.2p13.1	37,857,269	38,050,719	Loss	193 kb	Mat
1022	M	ASD	9	9p24.1	6,328,511	6,331,140	Loss	2 kb	Mat
1704	M	ASD	9	9p22.1	18,882,921	19,047,891	Gain	165 kb	Pat
1082	F	ID	9	9q22.33	99,795,072	99,799,469	Gain	4,4 kb	Mat
1986**	F	ID	10 16	10q24.1 16q23.2	97,443,497 81,228,359	97,489,429 81,314,441	Gain Gain	46 kb 86 kb	Pat Mat
235	M	ASD	10	10q21.1	60,274,699	61,008,293	Gain	733 kb	Mat
193	F	ASD	10	10q24.2	99,379,380	99,508,437	Gain	129 kb	Pat
2759	M	ID	10	10q22.2	75,004,669	75,010,521	Gain	6 kb	Pat
1560	M	ASD	11 X	11p15.5 Xq23	202,958 114,142,995	251,529 114,821,025	Gain Gain	48 kb 678 kb	Mat Mat
1071	M	ID	11	11p11.2	44,151,640	44,228,368	Gain	77 kb	Pat
452	F	ID	11	11p15.2	12,848,840	12,923,522	Loss	75 kb	Mat
1157	M	ID	11	11q14.2	86,317,846	86,374,738	Gain	569 kb	Unknown

815	M	ASD	12 4	12p11.22p11.23 4q32.2	27,378,911 162,186,946	27,768,451 162,680,616	Gain Gain	390 kb 494 kb	Mat Pat
405	M	ASD	12	12q23.2	102,541,996	102,591,550	Loss	49 kb	Pat
1650	M	ASD	12	12q24.33	132,421,843	132,564,931	Gain	143 kb	Pat
2173		ASD	12 19	12q21.31 19q13.42	81,447,520 55,070,419	82,577,288 55,146,304	Loss Loss	1,13 Mb 76 kb	Mat De novo
2818	M	ASD	12	12q24.33	133,589,041	133,767,927	Loss	179 kb	Pat
2292	F	ID	12	12q24.21	114,336,264	114,397,847	Gain	62 kb	Mat
1061	F	ID	13	13q12.11	20,797,139	21,059,910	Gain	260 kb	Unknown
598	M	ID	13	13q13.2	35,619,499	36,124,728	Gain	505 kb	Unknown
1	F	ID	13	13q21.33	73,255,663	73,330,259	Gain	75 kb	Unknown
1061	F	ID	13	13q12.11	20,797,139	21,059,910	Gain	260 kb	Unknown
1871	M	ASD	14	14q21.1	41,067,302	41,374,628	Gain	307kb	Pat
384	M	ASD	14	14q21.1	41,018,728	41,310,931	Loss	292 kb	Pat
798	M	ASD	15	15q15.3	43,888,927	44,043,043	Loss	154 kb	Pat
1070**	M	ID	16	16p13.11	15,131,723	15,154,746	Loss	23 kb	Mat
1536	M	ASD	16 4	16p13.12 4p32.1	14,464,480 156,274,983	14,564,129 156,294,358	Loss Loss	99,7 kb 19 kb	De novo Pat
2260	M	ASD	16	16p13.3	2,636,803	3,187,007	Gain	550 kb	Pat
613	F	ID	16	16p11.2	28335078	28601225	Gain	266 kb	Unknown
865	F	ID	16	16p12.2	21,599,687	21,837,551	Loss	238 kb	Pat
1594	M	ID	16	16p13.2	8,846,009	8,958,889	Gain	113 kb	Pat
1041	M	ID	16	16p13.2p13.3	6,427,764	6,755,045	Loss	327 kb	Unknown
1900	F	ID	16	16q23.2	80,518,009	80,913,149	Gain	395 kb	Unknown
345	F	ID	16 14	16q23.2 14q23.1q23.2	80,518,009 62,050,287	80,803,969 62,199,228	Gain Gain	286 kb 149 kb	Unknown Unknown
807	M	ASD	17 21 21 4	17q12.12 21q22.12 21q21.1 4q13.3	33,687,356 37,408,252 17,205,639 75,239,841	33,738,467 39,655,123 18,791,789 75,971,503	Loss Gain Gain Gain	51 kb 2,25 Mb 1,59 Mb 731 kb	Unknown Unknown Unknown Unknown
1501	M	ASD	17 21	17q12 21q22.11	33,687,356 33,650,665	33,738,408 33,947,130	Loss Gain	51 kb 296 kb	Mat Mat
2840	M	ASD	17	17q23.3	61,947,138	61,998,256	Loss	51 kb	De novo
2073	M	ID	17	17p11.2	21,320,065	21,501,883	Gain	182 kb	De novo
1315	F	ID	17	17q24.3q25.1	70,879,778	71,425,811	Loss	546 kb	Mat
1511	M	ID	17 18	17p13.3 18p11.31p11.23	526,813 6,942,021	876,813 8,015,631	Gain Gain	350 kb 1,07 Mb	Pat Pat
66 *	M	ASD	17	17p13.3	1,009,242	1,184,475	Gain	175 kb	Mat
326 *	M	ASD	19 22	19q13.2q13.31 22q11.23	43,024,253 25,664,674	43,096,807 25,892,194	Loss Loss	72,5 kb 227 kb	Unknown Unknown
1035	F	ASD	19	19q13.42	54,754,462	54,845,360	Gain	91 kb	Pat
8	F	ID	19	19p13.3	2,890,901	2,918,258	Loss	27 kb	Mat
1614	M	ID	19	19q13.42	53,882,840	53,954,264	Loss	71 kb	Mat
277	M	ASD	21	21q21.3	29,912,404	30,026,553	Loss	114 kb	Pat
1778	F	ASD	21	21q21.2	24,493,619	24,622,114	Loss	128 kb	Pat
1839	M	ID	21	21q22.3	45,673,257	45,835,771	Gain	162 kb	Pat
1090	M	ID	21	21q22.3	45,877,333	46,001,538	Loss	124 kb	Pat
1178	M	ASD	22 6	22q13.2 6q27	42,663,298 170,627,209	43,415,658 170,865,950	Loss Gain	840 kb 80 kb	De novo Mat
2567	M	ID	22	22q11.22	22,323,105	22,569,822	Loss	247 kb	Unknown
2452	M	ASD	X	Xp22.2	10,845,239	11,135,472	Gain	290 kb	Mat
142	M	ASD	X	Xq12	65,815,490	65,895,015	Loss	79 kb	De novo
940	M	ASD	X	Xq22.3	106,351,712	106,629,060	Gain	277 kb	Mat
1681	M	ASD	X	Xq26.2	130,571,153	130,960,558	Gain	389 kb	Mat
36	M	ID	X	Xp22.11	22,836,324	23,411,163	Loss	575 kb	Mat
2159	M	ID	X	Xp22.31	8,498,107	8,759,709	Gain	262 kb	Mat
375	F	ID	X	Xp22.31	8,498,107	9,082,705	Gain	585 kb	Pat
1240	F	ID	X	Xq23	115,591,058	115,864,916	Gain	274 kb	Mat
135	M	ID	Y Y	Yp11.2 Yp11.2	3,389,860 4,879,636	3,560,315 4,937,890	Gain Gain	170 kb 58 kb	Pat Pat
94	M	ASD	6	6p25.3	1,580,622	1,663,440	Gain	80 kb	Unknown
1478	M	ASD	9	9q34.3	140,707,451	141,008,863	Loss	300 kb	De novo
296	F	ID	6	6q16.1	95,958,454	96,238,765	Loss	280 kb	Pat

1346	M	ASD	2	2q11.2	100,625,292	100,722,540	Gain	100 kb	Mat
177	M	ASD	19	19q13.41	53,424,222	53,569,529	Gain	150 kb	De novo
331	F	ASD	16	16p12.2	21,475,060	21,806,299	Gain	330 kb	Mat
313	M	ID	3	3p22.2	38,502,327	38,830,481	Gain	330 kb	Mat
	M	ID	6	6q25.1	152,201,766	152,367,137	Gain	170 kb	Unknown
1258	M	ASD	3	3q12.2	100,354,612	100,451,345	Amp	97 kb	Pat
2695	M	ID	10	10q11.21	45,872,003	46,017,634	Gain	150 kb	Mat
1366	F	ID	11	11p11.2	48,081,727	48,466,844	Gain	390 kb	Unknown
172	M	ASD	1	1q21.1	145,413,388	145,609,172	Loss	200 kb	De novo
535	M	ASD	2	2q32.2	189,865,631	189,925,490	Loss	60 kb	Mat
2740	M	ID	5	5p13.2	37,142,520	37,516,603	Loss	370 kb	Unknown
315	F	ID	7	7q35q36.3	148,255,537	153,360,454	Loss	5.1 Mb	De novo
746	M	ASD	15	15q11.2	22,784,523	23,085,096	Loss	300 kb	Mat
1275	M	ID	6	6q22.31	123,539,625	124,166,602	Gain	630 kb	Unknown
107	F	ASD	5	5p13.2	37,516,603	37,697,730	Gain	180 kb	Mat
2578	M	ASD	5	5q35.3	177,756,155	178,507,278	Gain	750 kb	Mat
116	M	ID	X	Xq25	124,439,330	127,799,936	Loss	3.36 Mb	Mat
850	F	ASD	17	17q11.2q12	31,917,720	32,858,733	Gain	940 kb	Unknown
556	F	ID	8	8q22.2q22.3	101,947,980	103,870,397	Loss	1.92 Mb	De novo
271	M	ASD	2	2q32.2	189,307,596	189,682,921	Gain	380 kb	Mat
956	F	ASD	16	16q24.3	89,849,284	89,909,419	Gain	60 kb	Unknown
1852	M	ASD	2 6	2p21 6q24.1	45,616,537 140,669,555	45,909,120 141,354,777	Gain Gain	290 kb 690 kb	Mat De novo
1475	M	ASD	3 11	3q29 11p12	197,574,293 40,282,913	197,803,764 41,385,462	Gain Loss	230 kb 1.1 Mb	Mat Mat
1505	F	ASD	1 2	1p21.2 2q13	101,474,231 110,841,715	101,503,523 110,980,342	Loss Loss	30 kb 140 kb	Pat Pat
605	F	ASD	21 17	21q21.3q22.2 17p11.2	33,374,279 19,683,783	37,721,662 19,850,774	Loss Gain	4.3 Mb 170 kb	De novo De novo
268	M	ID	3 4 4	3q25.1 4q21.23 4q22.1	151,368,848 84,489,988 89,648,163	151,542,511 84,584,411 89,951,120	Loss Gain Gain	170 kb 90 kb 300 kb	Mat Pat Pat
400	M	ID	10 4 Y	10q21.3 4q34.1 Yp11.2	69,991,540 174,675,826 6,414,449	70,406,148 175,673,767 9,442,908	Gain Gain Loss	410 kb 1 Mb 3.03 Mb	Pat Mat Pat

*Patients presenting a CNV affecting known deleterious regions (Table 3)
**Patients presenting a CNV involving ASD or ID genes (Table 4)

Table 4: CNVs of unknown significance.

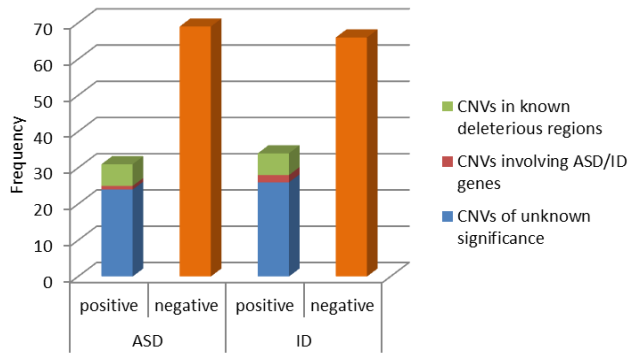


Figure 1: Graphical representation of positive and negative cases in ASD and ID group according to the three groups: CNVs previously known to be associated with ASD or ID (green); CNVs including ASD or ID associated genes (red); CNVs of unknown significance (blue).

CNVs with potential clinical impact

In addition to the already reported ASD/ID associated CNVs or genes, we identified several CNVs never reported in the literature and thus classified with unknown significance (Table 5). In this group of variants, we found 6 potentially pathogenic CNVs involving genes playing a role in the nervous system. Among these we identified two Xp22.31 duplications encompasses the KAL1 (Kallmann syndrome 1 sequence) gene, a gene involved in embryonic development of the kidney and human central nervous system, including the spinal cord, olfactory bulbs, olfactory nerves and retina [18]. Loss-of-function mutations of the KAL1 gene are a known cause of Kallmann syndrome, but neither complete nor partial duplications of KAL1 have been associated to specific clinical symptoms [19]. Sowińska-Seidler et al. [19] reported a patient manifesting hyperosmia and ectrodactyly accompanied by mild intellectual disability, unilateral hearing loss, genital anomalies and facial dysmorphism. Hemizygous tandem duplication on Xp22.31, encompassing the promoter region and the first two exons of KAL1, has been identified in that patient. We also identified two duplications in 2q14.2 including DBI (diazepam binding inhibitor) gene. This gene encodes an acyl-CoA binding protein that is an allosteric binder of GABA receptors involved in lipid metabolism and in signal transduction at type A gamma-aminobutyric acid receptors located in brain synapses [20]. Due to the prior evidence of the importance of GABAergic genes in autism, it is possible that duplication of DBI could affect signaling and lead to deficits in neuronal function [21]. We found a 2q36.3 duplication involving Delta/Notch-like EGF-related receptor (DNER) gene. DNER is a trans-membrane ligand for Notch that is specifically expressed in the somatodendritic domain in CNS neurons and is essential for precise cerebellar development [22,23]. Finally, we

	Positive ASD n=83			Positive ID n=71		
	CNVs in known deleterious regions	CNVs involving ASD/ID genes	CNVs on unknown significance	CNVs in known deleterious regions	CNVs involving ASD/ID genes	CNVs on unknown significance
CNV orientation						
Deletions	6	1	42	6	2	19
Duplications	7	4	54	7	4	47
No. of CNV						
Single CNV	11	3	53	12	4	44
2 CNVs	3	1	14	-	2	6
3 CNVs	-	-	2	-	-	3
4 CNVs	-	-	1	-	-	-

Table 5: Characteristics of CNVs in ASD and ID group.

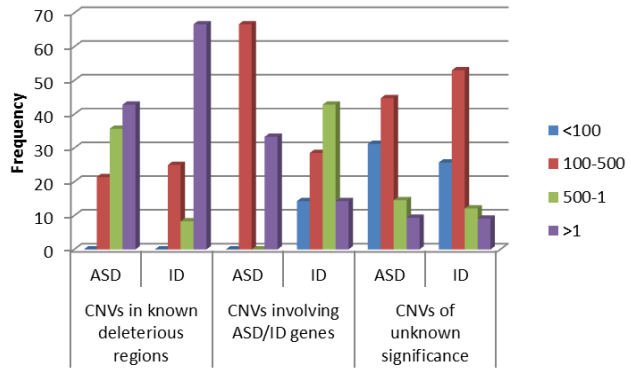
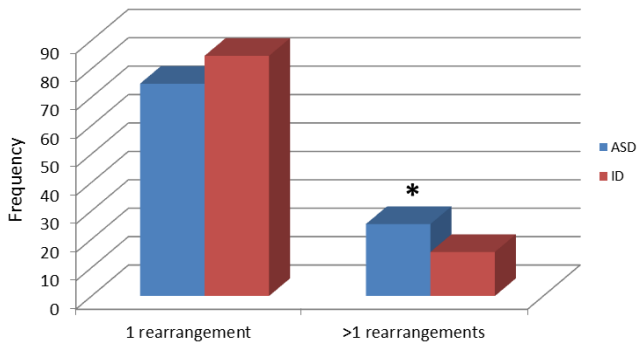


Figure 2: Distribution of CNVs in ASD and ID group based on rearrangement size. Large CNVs (>500 kb) enriched in first and second group.

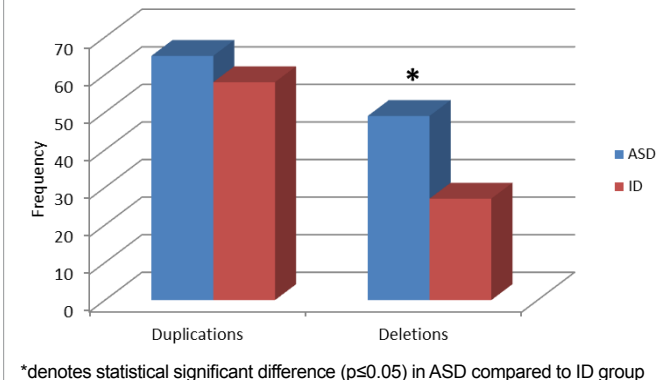


*denotes statistical significant difference ($p \leq 0.05$) in ASD compared to ID group

Figure 3: Frequency of single (left panel) and multiple (right panel) CNVs in ASD (blue) and ID (red) group. A prevalence of multiple CNVs is observed in ASD cases compared with ID cases.

found a deletion in 4q12 including the USP46 gene. Huo et al. [24] recently reported USP46 as the deubiquitinating enzymes specific for Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor (AMPARs) both *in vitro* and *in vivo*. AMPARs have been shown to regulate neuronal development and mediate the excitatory synaptic transmission in the brain. Knockdown of USP46, due to siRNAs or shRNAs, leads to an amount of AMPAR ubiquitination and consequently to a reduction in AMPAR protein amount in neurons [24].

The 68.9% of the unknown CNVs are inherited (50% maternal and 50% paternal) while the 11.2% occurring *de novo*.



*denotes statistical significant difference ($p \leq 0.05$) in ASD compared to ID group

Figure 4: Duplication and deletion distribution in ASD (blue) and ID (red) group. A prevalence of deletion is observed in ASD cases compared with ID cases.

Discussion

In the present study, we report the investigation of CNVs in a cohort of 476 patients classified as ASD and ID without ASD. We identified clinical relevant CNVs in 28 cases, which correspond to about 18.2% of the positive cohort, falling within the range of detection rate reported in literature [6].

Among the rearrangements in regions already associated to ASD, alterations in 16p11.2 are the most frequent occurring in our population (5/154, 3.2%). Deletions of this region have been associated with ASD while the reciprocal duplication with schizophrenia [25]. The 15q11.2q13 duplication is considered one of the common genomic causes for autism, occurring in 1-3% of cases [26]. However in our cohort it was less frequent than the CNV at 16p11.2 (we found a duplication and a deletion in two patient classified as ASD and ID, respectively). In our cohort we detected also a 15q13.3 duplication encompassing CHRNA7 in two cases. Chromosome 15q13.3 recurrent microdeletions are causally associated with a wide range of phenotypes, including ASD, ID, seizures, and other psychiatric conditions [27]. The pathogenicity of the reciprocal microduplication is less certain. Recently, Szafranski et al. suggested that the CHRNA7 duplication may confer a predisposition to neurodevelopmental and neuropsychiatric phenotypes, including ASD, possibly in association with other genetic modifiers [28]. Three maternally inherited losses including *PDZK1* (1q21) were identified in two ASD patients and in one patient with ID. Casey et al identified ASD-specific risk haplotypes at 1q21.1 in three different population cluster and PDZK1 was the only gene including in the genetic region shared by all three haplotypes [29]. Furthermore, Bernier et al. recently reported an increased prevalence of macrocephaly and increased ASD symptom severity in patients currying the 1q21 duplication [30].

We also found 2q31.1q33.2 deletion of 13 Mb and "*de novo*" origin in a patient with intellectual disability, macrocephaly, behavioural disturbance, speech defect and facial dysmorphisms, in accordance with previously reported patients [31-33]. Current literature provides more than 30 patients with interstitial deletions in chromosome 2q31q33. The critical region points to a few genes, namely NEUROD1, ZNF804A, PDE1A and ITGA4, which are good candidates to explain the cognitive and behavioural phenotype, as well as the severe speech impairment associated with the this deletion and are included in our CNV [33]. Finally we found a large deletion of 11 Mb, in region 3p14.1p14.3, of "*de novo*" origin. Interstitial 3p deletions have been very rarely reported and the phenotype-genotype correlation is not well understood. Previous reports have documented a chromosome 3p14 deletion in several patients with global developmental delay, intellectual disability, language impairment and autistic features, but without any other major malformations and only mild facial dysmorphism [34-37]. The region deleted in our patients includes 78 genes of those FEZF2, CADPS, SYNPR, ATXN7, PRICKLE, and MAGI1, are known or presumed to have a role in neurodevelopment.

We also reported here a number of CNVs involving genes previously implicated in ASD.

We identified a paternally inherited duplication in 9p24.3 involving KANK1 gene. Deletions of KANK1 have been associated with neurodevelopmental disease including congenital cerebral palsy, hypotonia, quadriplegia and ID. Since a random monoallelic expression has been suggested for this gene [10], a tight regulation of the expression of this gene is hypothesized and thus we cannot exclude a contributing role of KANK1 duplication in the phenotype of our patient. A 1.05 Mb *de novo* gain involving PLXNA4 was identified in an ASD case. Decreased expression of axon-guidance proteins, as PLXNA4, was found in the brains of people with ASD, suggesting that dysfunctional axon-guidance protein expression may play an important role in the pathophysiology of autism [9]. Handrigan data highlight 16q24.2 as a region of interest for ASD, ID and congenital renal malformations. These conditions are associated, albeit without complete penetrance, with deletions affecting C16orf95, ZCCHC14, MAP1LC3B and FBXO31. The function of each gene in development and disease warrants further investigation [13].

In one patient an inherited 20p12.1 deletion of nearly 450 Kb interrupting the MACROD2 gene (previously known as C20orf133) has been identified. This gene is a strong positional candidate risk factor for autistic-like traits in the general population [14]. A maternally inherited duplication of band 9q22 was found in an ID case. The rearrangement is associated with growth retardation, mild ID and mild facial dysmorphisms. Based on the described functions of duplicated genes, PTCH1 represents a candidate gene that may be responsible for the phenotypic findings [11]. Deletions or loss-of-function mutations of PTCH1 gene result in basal cell nevus syndrome (Gorlin syndrome). We also found de novo 5.8 Mb deletions on 12p12.2p12.1 involving SOX5. The SOX5 gene encodes a transcription factor involved in the regulation of nervous system development and chondrogenesis. Deletion involving this gene is reported to be associated with global developmental delay, intellectual disability, expressive language delay, mild motor impairment, distinct features and multiorgan involvement [12]. KDM5C and IQSEC2 are located adjacent to each other at the Xp11.22 locus. Deletion and mutations in either of these genes are associated with severe ID in males while female carriers are mostly unaffected [15]. Here, we identified a maternally inherited duplication in a male patient who also presented duplication in X22.12 including the RPS6KA3 gene. This gene is responsible for Coffin-Lowry syndrome (CLS), which is characterized by ID and facial and bony abnormalities but also affects non-syndromic X-linked ID [16]. In another male we identified a maternally inherited Xp22.11 deletion. Hemizygous PTCHD1 loss of function is known to cause an X-linked neurodevelopmental disorder with variable degrees of ID and prominent behavioral issues [17]. Thus, also in this case, the use of array-CGH technique has enabled the detection of a clinically relevant rearrangement.

Within the unknown significance group we speculate about the potential pathogenicity of six CNVs, mainly basing on gene content: two duplications in Xp22.31, encompassing the KAL1 gene, two duplications in 2q14.2 involving the DBI gene, duplication in region 2q36.3 including the DNER gene and a deletion in 4q12 involving the USP46 gene. All these three genes were particularly interesting because of their expression and function in the nervous system.

The KAL1 gene encodes a protein, anosmin-1, that is known to directly stimulate tyrosine kinase activity of the fibroblast growth factor receptor 1 (FGFR1), an important signaling molecule involved in a wide range of developmental processes. Tole et al. demonstrated that FGF signaling is required for generating telencephalic midline structures, in particular septal and glial cell types and all three cerebral commissures [38]. The DBI gene encodes a protein that is involved in lipid metabolism and the displacement of beta-carbolines and benzodiazepines, which modulate signal transduction at GABAa receptors located in brain synapses. Experiments *in vivo*, demonstrated that DBI can promote neurogenesis in the subventricular zone, counteracting the inhibitory effect of GABA, while the DBI gene product acted as positive allosteric modulators of GABAa receptors in prolonging the duration of IPSCs in reticular nucleus, so it could be endogenously effective by modulating seizure susceptibility [39]. DNER gene is strongly expressed in Purkinje cells in the cerebellum; it contributes to the morphological and functional maturation of Bergmann glia via the Notch signaling pathway, and is essential for cerebellar development. However, with the exception of KAL1, complete or partial duplications of the other two genes have not been reported in the literature. Thus, clinical symptoms associated with duplications and/or increased gene expression remains unknown. These VOUS might still deserve further investigations for any possible association with neurodevelopmental disorders. We also identified a deletion in 4q12 which involves the USP46 gene. USP46 encodes for a deubiquitinating enzyme that plays a role in behavior, possibly by regulating GABA action. Huo et al. identified USP46 as the deubiquitinating enzyme for AMPARs (Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptor) that are the primary mediators for inter-neuronal communication and play a crucial role in higher brain functions including learning and memory [24].

However, our findings also underline that the majority of identified CNVs are still of unknown significance highlighting the challenging role of the clinical geneticists in interpreting correctly the role of these CNVs and estimating the exact recurrence risk. The pathogenicity of these unknown variants can be determined based on different characteristic also highlighted in this study: the orientation (deletion or duplication) of the CNV, the size and gene content.

In conclusion, our study confirmed that array-CGH analysis is able to detect the underlying genetic susceptibility factors in a consistent number of ASD and ID patients, strongly indicating that it has became an essential diagnostic tool for assessing these patients. Moreover our data shown a general amount of duplications in the cohort of positive patients, but no differences are detectable among the ASD and the ID

group. Otherwise when the deletion and duplication are considered as single classes, we note a strong association among the presence of a microdeletion and the ASD phenotype. This association confirms once again observation that deletions have a stronger effect than their reciprocal duplications. The variability in genetic susceptibility to ASD from one subject to another one is today well established. In some cases a single *de novo* rearrangement is sufficient to cause ASD while in other cases a combination of multiple CNVs is reported. In our study we found that the majority of cases carrying more than one rearrangement are classified as ASD (65% of cases) with a prevalence of small CNVs. These data confirm that a synergic effect of multiple CNVs occur in ASD phenotype and further highlight the multifactorial nature of ASD already reported in literature. We also noted that the majority of the CNVs in the entire three groups are inherited. These data should not lead on to a wrong interpretation of the CNVs. The phenotypic differences among proband and a carrier parent may in fact be associated to subtle phenotypic signs, different chromosome rupture point or other independent factors as epigenetic and environment. It is therefore important to carefully consider all these aspects in the interpretation of CNVs, particularly for those of unknown significance.

Further studies in sufficiently large cohort of ASD and/or ID cases are however required not only to refine our understanding of previously isolated genes and regions in ASD and ID, but also to identify novel molecular pathways involved in the etiology of autism and other neurodevelopmental disorders.

Competing Interests

The authors declare that they have no competing interests.

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