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Array-comparative genomic hybridization analysis in a cohort of 130 children with Autism Spectrum Disorders: A single Center Italian Study

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Abstract

Introduction Autism spectrum disorder (ASD) is a heterogeneous clinical condition, and its genetic basis is widely confirmed. The chromosomal microarray analysis (CMA) is a first-line diagnostic test that identifies copy number variants (CNVs). Some of these genomic rearrangements are associated with ASD, but the meaning of most of them is still unknown.

*Materials and methods***.** We performed a comparative genome hybridization (array-CGH) analysis in 130 children with confirmed ASD. Genetic results were analyzed and compared to clinical phenotype.

Results and discussion. 61/130 children carry CNVs, 44 presenting variants of unknown significance (u-CNVs), and 17 with susceptibility-CNVs (c-CNVs). Clinical evaluation showed no differences in cognitive abilities, language and EEG abnormalities, ASD symptoms among CNVs group and other patients. Finally, we highlight the role of *GPHN*, *IMMP2L* and *ZMYND11*, as ASD susceptibility genes.

Conclusions. Our findings underscore the importance of array-CGH in ASD children since new CNVs and emerging genes appear to be associated with different clinical pictures. ***Clin Ter 2023; 174 (6):509-517 doi: 10.7417/CT.2023.5018***

Key words: *Array-CGH, Autism, Copy Number Variants (CNVs)*

Introduction

Autism spectrum disorder (ASD) is a heterogeneous, early-onset clinical condition characterized by impaired social communication and restricted, repetitive behaviors (1) with high prevalence in the general population (2).

ASD is more common in males than females and is usually associated with other concurrent neuropsychiatric conditions, including ADHD, intellectual disability, and epilepsy(3, 4).

Although multiple studies have been performed, the pathogenesis of ASD remains still largely unclear and complex gene-environment interactions are highlighted (5, 6).

Much effort has been made in recent years to understand the role of genetics on ASD, with increasing evidence of a strong impact on neurodevelopmental disorders, as shown in studies on twins (7).

However, it is estimated that a genetic cause could be identified in 10-20% of cases of ASD patients (8). Among all, "syndromic ASD" is usually characterized by dysmorphisms, epilepsy, neurological symptoms and multiple congenital abnormalities and is more often associated with known genetic causes, often consisting in monogenic alterations (i.e., X Fragile Syndrome, TSC) (9).

Over the years, array-comparative genomic hybridization (array-CGH) analyses assumed a well-established role in characterizing ASD patients, though now whole-exome sequencing is considered the first-line diagnostic test in ASD.

Numerous copy number variations (CNVs), consisting of DNA structural alterations characterized by duplication or deletion of DNA segments (10), are associated with ASD and commonly considered a significant contributing factor in ASD susceptibility (11).

The 16p11.2 and 15q11-13 are known regions with high recurrence of CNVs associated with ASD (12). As it is known, many ASD-risk genes are often involved in synaptic transmission pathways as well as in transcription, splicing, and chromatin remodeling suggesting complex interactions(13, 14).

However, clinical interpretation of such genomic rearrangements is often challenging and inconsistent genotype-phenotype correlation is found (15). So, despite the improvement in genetic diagnosis, ASD remains an extremely heterogeneous condition with indefinite genetic configuration(16).

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We aim to identify and characterize genome abnormalities in 130 patients with ASD by analyzing SNP-array results concerning CNV frequency, inheritance, and pathogenetic role. We also define the diagnostic sensitivity of the SNP array in our cohort in comparison with literature data.

Finally, we correlate patient's phenotype (cognitive skills, symptoms severity, language and EEG abnormalities) to CNV presence and subtype.

Materials and Methods

Participants

130 children with ASD were recruited for the study between February 2016 and August 2021 at the Child Neuropsychiatry Unit of Umberto I Hospital, Sapienza University.

All ASD subjects had to meet the following criteria:

1. Clinical diagnosis of ASD, according to commonly accepted criteria of the Diagnostic and Statistical Manual of Mental Disorders, Fifth Edition (persistent deficits in social communication and social interaction plus at least two of four types of restricted, repetitive behaviors) supported by ADI and ADOS administration to assure all met the cut off for ASD;
2. Physical and neurological examination performed by physicians experienced in this field;
3. Evaluation of cognitive abilities through the Griffiths Mental Development Scales Extended Revised for ages 2–8 (GMDS-ER).
4. Clinical and severity assessment of ASD symptoms using standardized tests (ADI and ADOS);
5. Genetic investigation: molecular analyses by array-CGH were performed in each participant, using the same platform.

Exclusion criteria included:

1. Genetic investigation and ASD diagnosis without clinical assessment of cognitive abilities and ASD symptoms.
2. "Complex" physical phenotypes with multiple minor anomalies and/or systemic congenital malformations.

The study was conducted with the approval of the local ethics committee at our Institution. Written informed consent was obtained from all ASD patients' parents or legal guardians for the present study.

Procedures

Clinical evaluation

The patient's history and anamnesis were collected through a parent interview.

We focused on neurodevelopmental and medical history, looking for the presence of language delay, epilepsy, or other neuropsychiatric disorders. Family history was also investigated in all patients.

Finally, all participants underwent physical and neurological examinations. In a subgroup of patients EEG and/or brain MRI were performed.

To evaluate ASD symptoms, two standardized and diagnostic instruments were utilized: later Autism Diagnostic Observation Schedule-Generic, Second Edition (ADOS-2) (17) and Autism Diagnostic Interview-Revised (ADI-R)(18). Both were administered and scored by licensed clinicians who have reached clinical reliability on the instrument.

The ADOS-2 is a semi-structured, standardized assessment of Communication, Social interaction, Play, Restricted and repetitive behaviors for children with suspected ASD. It provides a calibrated quantification of autism symptoms.

The ADI-R is a standardized semi-structured interview addressed to children and adults with a mental age above 2.0 years. It follows the DSM-5 diagnostic criteria for autism. Scores provide results for three subdomains of autistic symptoms: Language/Communication, Reciprocal Social Interactions, and Repetitive Behaviors/Interests.

The clinicians assessed the presence or absence of language production through direct observation. The cognitive or developmental level was assessed by the brief intelligence quotient (IQ) from Leiter-Ror (19) by the general quotient (GQ) from Griffiths Mental Development Scales Extended Revised for ages 2–8, GMDS-ER 2–8 (20). Intellectual disability or developmental delay was considered when children had an IQ or GQ score lower than 70.

In a subgroup of patients (n 76) we also evaluated epileptic seizures and anomalies by performing an electroencephalogram (EEG).

A comparative genomic hybridization (CGH) analysis was conducted for each child using specific probes for single nucleotide polymorphisms (SNP-array). DNA extracted from patient blood samples was analyzed using the SNP-array platform (Affymetrix Genome-Wide Human 6.0; Affymetrix, Palo Alto, CA) according to the protocol provided by the manufacturer. The platform consists of approximately 1.8 million markers (906,600 SNPs and 946,000 oligonucleotide probes), with a 680 bp probe spacing with an effective average resolution of 75 Kb. Data from 270 healthy controls (International HapMap Project) were used as a reference. The data analysis was carried out using the Chromosome Analysis Suite software version 4.0 (Affymetrix). Parental testing was performed using Real-Time PCR to determine CNV origin, except for a single case for which parental samples were unavailable. All the CNVs were compared with those present in other databases such as Database of Genomic Variant (DGV), DECIPHER, Clinvar. The analysis of the genomic regions and the gene content of the CNVs was based on using UCSC Genome Browser, OMIM, SFARI. Molecular analysis of FMR1 gene was also carried out for each child, and only children who tested negative were enrolled in the study.

CNVs associated with Autism Spectrum disorders or neurodevelopmental disorders were selected. For patients meeting inclusion criteria, we collected information about their CNVs (location, coordinates, size, genes encompassed, and inheritance). CNVs interpretation was made based on the American College of Medical Genetics (ACMG) guidelines (21), with some modifications, considering the different types of evidence (functional, genetic, population, in silico etc.) in order to give consistence to the final classification based on five CNV classes: pathogenic or likely pathogenic, VUS, likely benign or benign)(21).

In our study CNVs were classified u-CNV (unknown-CNV), also known as variants of unknown significance (VUS), and c-CNVs (causative or potentially causative-

CNVs). In detail, we used the term "VUS" or u-CNV when insufficient evidence for definitive clinical significance was available; in contrast, we used the term c-CNVs when a CNV was described in the literature as associated with ASD or neurodevelopmental disorders, also when represent a risk factor with low penetrance The same classification has been given to CNVs involving genes already associated with ASD and/or neurodevelopmental disorders even though the CNV itself has not yet been described (likely pathogenic-CNVs or c-CNV). When the SNP-array analysis was negative and showed no rearrangement, we classified the patients as w-CNVs (without-CNVs).

Statistical Analysis

Statistical analysis was performed using SPSS. The ANOVA test has been performed to compare u-CNVs, c-CNVs and w-CNVs groups in order to study significant correlation between the phenotype (cognitive skills, symptoms severity, language and EEG abnormalities) and the subtype of CNV. A p<0,05 was considered statistically significant.

Results

Demographic characteristics and sample composition

Our sample consists of 130 individuals, including 2 pairs of twins and 2 pairs of siblings. Concerning sex, 109 were males (84%) and 21 were females (16%). The M:F ratio was 5:1 following literature data reporting values of 4-5:1 (22). The mean age at the time of evaluation was 3 years and 4 months (range 2 to 6 years). In our cohort, high familiarity was observed, with a positive family history for neurodevelopmental and other neuropsychiatric disorders in 50% of patients.

Diagnostic yield of CMA and characterization of CNVs

Among 130 children with a confirmed diagnosis of ASD, 61 of our 130 patients (34%) tested positive for imbalances, while 53% of cases (69 patients) showed negative SNP-array analysis (w-CNVs). A total of 72 CVNs detected; in one case, the SNP array identified a region of homozygosity (ROHs). Within the CNV group, 17 children (13%) demonstrated pathogenic or likely pathogenic CNV.

The diagnostic yield of the SNP array in our clinical sample was 13% when considering c-CNVs. Regarding sensitivity,

SNP analysis did not show any statistically significant variability within the different subgroups of patients.

When considering CNVs distribution, 48 (79%) children showed a single CNV and among total CNVs, 47 were duplications (gain), 25 were deletions (loss).

All except one imbalance were studied for the parental origin: 36 were maternally inherited, 31 paternally inherited and 4 de novo.

As for CNVs size, 51 (70%) of CNVs were between 100 and 999 kb in size, 17 (24%) were minimal (<100 kb), and only 4 (%) CNVs were >1 Mb (for a summary of CNVs characteristics see Table 1).

Table 1. Summary of demographic characteristics

N	Males (%)	Females (%)	Mean Age (range)	Positive family history for neuropsychiatric disorders (%)
130	109 (84)	21 (16)	3,4 (2 to 6)	65 (50)

Interestingly, the de novo CNVs were all deletions and larger than the average size of the total sample, but this difference was not statistically significant in the ANOVA test.

Among the total of 72 CNVs, 17 were considered as c-CNVs 24%) and 55 (76%) u-CNVs. Moreover, 69 (53%) patients carried no CNVs (w-CNVs).

The chromosomes most often involved in a CNV were the X chromosome (11 cases), and chromosomes 2 and 3, with 7 cases each. When considering c-CNVs, chromosomes 15 and 7 were more often implicated.

Clinical and neuropsychiatric features in patients with ASD according to CNVs

When considering several features (i.e., positive family history, dysmorphisms, cognitive quotients, language, Eeg abnormalities), we did not find any significant difference among groups (c-CNV, w-CNV and u-CNV) on ANOVA analyses.

Regarding clinical features, 8 of 130 participants (6%) had mild somatic dysmorphisms. However, no statistically significant differences were observed among the CNV and w-CNV group concerning dysmorphisms (p=0,07).

The cognitive or developmental level was assessed by the brief intelligence quotient (IQ) from Leiter-R, available for 30 children and/or by the general quotient (GQ) from GMDS-ER, available for 97 cases.

Globally speaking, 38 children showed a GQ/IQ>85 and could be considered "high-functioning autism". Hence, 89 children showed a GQ/IQ<85; among them, 66 cases presented an IQ value <50, 14 between 50-70, and 3 an IQ>75.

However, no significant differences were observed among the c-CNV, w-CNV and u-CNV groups, regarding cognitive or developmental level (p=0,055).

Similarly, with 61 non-verbal patients and 69 verbal children, oral language skills showed no difference among CNVs groups (p=0,068).

Autism severity was assessed by ADOS test and available for 127 cases, resulting in mild in 8 subjects (6%), moderate in 76 subjects (60%) and severe in 43 patients. In the c-CNVs group only a child had a mild severity score, while 10 children (59%) showed a moderate score and 6 children (35%) had a severe score.

EEG was available for 76 cases. In 9 patients, EEG showed epileptic abnormalities, while in 28 patients non-specific abnormalities emerged.

Globally no significant differences were found among CNV and no-CNV groups for epilepsy or isolated EEG anomalies (p=0,083).

See Table 2 for detailed clinical characteristics of the study population organized by SNP-array results (CNVs group).

Table 2. Summary of CNVs characteristics.

SEGREGATION	Median size	N	Standard Deviation	Minimum size	Maximum size
de novo	639,75	4	516,930	263	1400
maternal	309,08	36	274,397	77	1200
paternal	254,00	31	266,156	36	1200
not evaluable	405,00	1			
Total	305,07	72	293,152	36	1400

Dimension CNVs	Frequency	u-CNVs	c-CNVs
< 100 kb	17	17	0
100-999 kb	51	37	14
≥1000 kb	4	1	3
Total	72	55	17

Potential pathogenic role of CNVs in autism

We identified some CNVs with potential clinical relevance, already associated with autism and/or other neuropsychiatric conditions. GPHN, IMMP2L and ZMYND11 are interesting ASD susceptibility genes and will be discussed later. Clinical and molecular features of present patients with pathogenic CNVs are reported in Table 3.

Table 4 reported the coordinates of u-CNVs and c-CNVs .

Table 3. Clinical characteristics of patients organized by SNP-array results.

	Total	SEX		DYSMORPHISMS (p=0,065)		EEG (p=0,083)		
		male	female	not detected	detected	negative	Non-specific anomalies	Epileptic anomalies
w-CNV	69	55	14	65	1	12	16	5
u-CNV	44	40	4	34	6	16	8	4
c-CNV	17	14	3	15	1	11	4	0
total (N)	**130**	**109**	**21**	**114**	**8**	**39**	**28**	**9**
w-CNV	53%	50%	67%	57%	13%	31%	57%	56%
u-CNV	34%	37%	19%	30%	75%	41%	29%	44%
c-CNV	13%	13%	14%	13%	13%	28%	14%	0,0%
total (%)	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**

	Total	Neuropsychiatric Familiarity (p=0,068)		ADOS score (p=0,053)		
		absent	present	mild	moderate	severe
w-CNV	69	35	34	3	39	26
u-CNV	44	21	23	4	27	11
c-CNV	17	10	7	1	10	6
total (N)	**130**	**66**	**64**	**8**	**76**	**43**
w-CNV	53%	53%	53%	37%	51,3%	61%
u-CNV	34%	32%	36%	50%	35,5%	25%
c-CNV	13%	15%	11%	13%	13,2%	14%
total (%)	**100%**	**100%**	**100%**	**100%**	**100%**	**100%**

	Total	VERBAL	NOT VERBAL (p=0,068)	IQ (p=0,055)	
				> 85	< 85
w-CNV	69	36	34	21	46
u-CNV	44	24	19	14	29
c-CNV	17	9	8	3	14
total (N)	130	69	61	38	89
w-CNV	53%	52%	56%	55%	52%
u-CNV	34%	35%	31%	37%	33%
c-CNV	13%	13%	13%	8%	16%
total (%)	100%	100%	100%	100%	100%

Table 4..Clinical and molecular features of patients with pathogenic CNVs

C-CNVs	Sex	Dimension	Segregation	Genes	Language	QI/ Qs	ADOS score	Familiarity	EEG AGE OF ONSET
Microduplication 16p13.11 ID 1 (15481920-16516109)	M	1 Mb	pat	NDE1, ABCC6, MYH11	Verbal	140	Severe	Absent	Negative 5
Microdeletion 14q23.3 ID 2 (67031866-67415874)	M	348 kb	de novo	GPHN	Averbal	< 85	Moderate	Speech delay in maternal line	Non-specific 3 Anomalies.
Microdeletion 14q23.3 ID 3 (67111394-67341052)	M	230Kb	pat	GPHN	Averbal	50-70	Severe	Absent	Non-specific. 3 anomalies
Microdeletion 7q31.1 ID 4 (109389060-110338543)	M	150 Kb	pat	IMMP2L	Averbal	50-70	Severe	Absent	Negative 3.9
Microdeletion 7q31.1 ID 5 (109389060-16021388)	M	949 Kb	mat	IMMP2L	Verbal	117	Moderate	Absent	Not available 3.9
Microduplication 10p15.3 ID 6 and ID 7 (100026-277232)	F	177 kb	mat	ZMYND11	Averbal	<50	Moderate	Speech delay in paternal line	Rare focal paroxysmal 3 anomalies
Microduplication 10p15.3 ID 7 (100026-277232)	F	177Kb	mat	ZMYND11	Averbal	<50	Moderate	Speech delay in paternal line	Negative 3.9
Microduplication Xp22.33 ID 8 (539247-1226834)	M	688 kb	mat	SHOX	Averbal	<50	Moderate	An Autistic brother	Negative 2,4
Microduplication 9q33.1 ID 9 (119005705-119431557)	M	466 kb	mat	ASTN2	Verbal	50-70	Moderate	Two sisters with speech delay	Negative 2
Microduplication 18p11.31 ID 10 (4054162-4182981)	M	129 kb	pat	DLGAP1	Averbal	<50	Severe	Negative	Negative 3
Microdeletion 15q11.2 ID 11 (22770421-23282799)	F	513 kb	pat	NIPA1	Verbal	<50	Moderate	Negative	Negative 2.8
Microdeletion 15q11.2 ID 12 (22770421-23283811)	M	512 Kb	de novo	NIPA2	Verbal	<50	Severe	Negative	Non-specific. 3.3 - anomalies
Microdeletion 15q11.2 ID 13 (22770421-23283811)	M	512 Kb	pat	CYF1P1	Verbal	<50	Moderate	Negative	Negative 3.3
Microduplication 15q11.2 ID 14 (22770421-23283811)	M	425 kb	pat	NIPA1, NIPA2, CYF1P1	Verbal	109	Mild	Speech delay in paternal line	Negative 3.4
Microdeletion 6q26 ID 15 (16188913-163121898)	M	1,2 Mb	pat	PARK2	Averbal	<50	Moderate	Autism in paternal line	Negative 4
Microduplication Xq26.2 ID 16 (130672818-130969981)	M	297 kb	mat	FIRRE	Verbal	<50	Moderate	Absent	Not available 3.4
Microdeletion 12p13.33 ID 17 (2275662-3735847)	M	1,4 Mb	de novo	CACNA1C	Verbal	<50	Severe	Absent	Negative 3,8

*ID 6 and ID 7 are monozygotic twins

Table 5 (Coordinates of u-CNVs and c-CNVs)

CNV	CHR		Start	End
u-CNV	4	4q12	57265105	57528065
u-CNV	15	15q13.3	31676869	31754147
u-CNV	3	3p21.33	43703137	43858845
u-CNV	2	2q34	213098545	213257267
c-CNV (ABCC6, NDEI, MYH11)	16	16p13.11	15481920	16516109
u-CNV	5	5q33.1	151280288	151370667
c- CNV (GPHN)	14	14q23.3	67031866	67415874
u-CNV	22	22q13.31	44582698	44682810
u-CNV	6	6p12.3	47366251	47453556
u-CNV	9	9p22.1	18960472	19224325
u-CNV	5	5q33.1	150236368	150346397
u-CNV	X	Xq27.2	140405022	141061444
c-CNV (CACNA1C)	12	12p13.33p13.32	2275662	3735847
u-CNV	X	Xp22.33	1374716	1460944
u-CNV	3	3q27.3	186386545	186422122
u-CNV	7	7p12.3	47723548	47802084
u-CNV	16	16p13.3	8635486	8732487
u-CNV	2	2q36.2	225207853	225313045
u-CNV	15	15q26.1	90369994	90579851
u-CNV	8	8p23.1	10384665	10510511
u-CNV	5	5q35.3	179194643	179848955
u-CNV	10	10p11.21	34783096	35120647
u-CNV	12	12p21.31	80545610	80634327
u-CNV	22	22q13.2	42466023	42541947
u-CNV	16	16q22.2	72249593	72449154
c-CNV (IMMP2L)	7	7q31.1	111106611	111256664
u-CNV	4	4q22.1q22.2	93680732	93789776
c-CNV (ZMYND11)	10	10p15.3	100026	277232
c-CNV (ZMYND11)	10	10p15.3	100026	277232
u-CNV	17	17q11.2	29088272	29218205
u-CNV	1	1p36.11	27340564	27423929
u-CNV	2	2p25.3	3455691	3750246
c-CNV (NIPA1)	15	15q11.2	22770421	23195725
c-CNV (FIRRE)	X	Xq26.2	130672818	130969981
c-CNV (NIPA1, NIPA2, CYF1P1)	15	15q11.2	22770421	23283811
u-CNV	X	Xq24	120499811	120791674
u-CNV	8	8p21.2	26808968	26909933
u-CNV	X	Xq21.1	78567049	79432015
u-CNV	X	Xq21.1	79525952	79659941
c-CNV (SHOX)	X	Xp22.33	539247	1226834
c- CNV (NIPA1, NIPA2, CYF1P1)	15	15q11.2	22770421_	23282799
u-CNV	3	3p14.1	68399827	69633735
u-CNV	10	10p11.21	34754578	35231183
u-CNV	X	Xq23	109496686	109750335
u-CNV	X	Xq23	109496686	109750335

u-CNV	14	14q23.1	58495516	58572821
u-CNV	2	2p24.2	18657654	19081245
u-CNV	9	9q33.3	127484621	127583436
c-CNV (ASTN2)	9	9q33.1	119005705	119431557
u-CNV	6	6q24.2	143659033	143750414
u-CNV	3	3p26.3	285806	42591
c-CNV (DLGAP1)	18	18p11.31	4054162	4182981
u-CNV	X	Xq21.33	96763621	96844569
u-CNV	2	2q31.1	170506833	170638324
u-CNV	6	6q23.123.2	131060365	131524134
u-CNV	15	15q11.2	22770421	23282905
u-CNV	2	2q24.3	165437721	165536656
u-CNV	X	Xp22.33 or Yp11.32	287730_ or 237730_	372028 or 322028
u-CNV	2	2q11.1	95764508	96082096
u-CNV	14	14q11.2	21424364	21705869
u-CNV	3	3p26.1	5259269	5408983
u-CNV	3	3p26.1	7344172	7379955
u-CNV	11	11q14.2	86101766	86300594
u-CNV	11	11q13.5	76229880	76588285
u-CNV	11	11q21	92893824	93261774
c-CNV (GPHN)	14	14q23.3	67111394	67341052
u-CNV	3	3p11.1	88159133	88565521
c-CNV (PARK2)	6	6q26	16188913	163121898
u-CNV	9	9p23	9232351	9488649
c-CNV (IMMP2L)	7	7q31.1	109389060	110338543
u-CNV	5	5q35.1	170361477	170537807
u-CNV	8	8p22	15753954	16021388

Discussion

Our study aimed to identify and characterize genome abnormalities in 130 patients with ASD through the analysis of SNP-array results, evaluating possible associations between CNVs and different clinical characteristics (cognitive skills, symptoms severity, language and EEG abnormalities). No relevant differences were found among CNVs groups concerning clinical correlates, except for ASD symptoms that were more represented in the c-CNVs group than in other patients (without statistically significant differences). Our data align with the literature on CMA as a diagnostic tool in ASD patients. In our cohort, the CMA diagnostic yield was 13%, in line with previously reported values of 10-20% (23). Notwithstanding the well-established role of CMA as a diagnostic tool in ASD, relatively new techniques such as Whole-genome sequencing (WGS) and whole-exome sequencing (WES) showed greater diagnostic utility and some authors suggest performing WES before or simultaneously with the CMA in the case of NDD (9);(24). When comparing CNVs results to different clinical correlates (positive family history, language, IQ/GQ and EEG abnormalities), no significant differences appeared between c-CNV, u-CNv and w-CNV groups. As for IQ/CG, a number of studies have found increased percentages of c-CNVs in ASD individuals with low cognitive functioning or intellectual disability, while others, similarly to us, did not support these findings(23);(25). These differences could be explained by using different evaluation tools and scales, and by different sample selection criteria due to the inhomogeneous characteristics of the population under examination. On the other hand, a recent study(26) confirmed that children who carry c-CNVs have higher ADOS scores than the w-CNV group, suggesting that genomic alterations may contribute to significant ASD symptoms. Here, some relevant clinical and molecular features of patients with c-CNV will be discussed. First, we found duplication in the 16p13.11 region (15481920-16516109), including Morbid genes ABCC6, NDE1, MYH11, in a male patient with the severe phenotype (ID1), reinforcing the potential pathogenic role of the NDE1 gene in ASD genesis(27). Moreover, ID2 and ID3, were both male with moderate and severe ADOS symptoms, respectively. Both were nonverbal with low cognitive functioning (SQ <85 and SQ 50-70, respectively) and EEG showed nonspecific anomalies. They carry a microdeletion of the long arm of chromosome 14 in the region 123.3 (14q23.3), including the GPHN gene (OMIM Morbid 603930). GPHN is a haploinsufficient gene encoding a key scaffolding protein in

the neuronal postsynaptic membrane, with well-established functional links with synaptic proteins implicated in ASD, such as neuroli- gins and neurexins

The involvement of this gene in our patients supports recent data in the literature, reinforcing the possible role of GPHN in susceptibility to autism, intellectual disability, and epilepsy(28);(29) .On the other hand, the role of IMMP2L, is more controversial. IMMP2L encodes an inner mitochondrial membrane protease-like protein, which is required for processing of cytochromes inside mitochondria (30).

Two of our subjects showed CNVs overlapping IMMP2L. One of them presented high cognitive functioning and he was verbal, the other one, instead, was averbal with low cognitive functioning. Recent evidence suggested an association between IMMP2L and developmental delay, speech delay, epilepsy, behavioral disorders, and autistic traits (30);(31) even if previous meta-analyses did not identify any association between IMMP2L gene deletions and ASD (32). Our data supported a possible contribution of IMMP2L gene deletions/duplications to the development of ASD, with incomplete penetrance and variable expressivity. Our study also underlined the role of ZMYND11, supported by recent findings which underline the association of this gene with autistic-like phenotypes(33) ZMYND11, in particular is speculated to regulate RNA polymerase II during the elongation step of transcrip- tion. It has been proposed that ZMYND11 is important for the "fine-tuning" of gene expres- sion rather than acting as an essential regulator (33).

Other potential causative genes are SHOX and ASTNT as shown in Table 3.

Finally, 4 subjects of our clinical sample showed CNVs (loss/gain) within the critical region of the 15q11.2 microdeletion syndrome, already considered a predisposing factor for neurodevelopmental disorders (34-38).

5. Conclusions

CMA results in our cohort overlap with those in the literature, showing no significant differences in clinical characteristics between c-CNVs, u-CNV, and w-CNV groups. These results suggest a much more detailed analysis of larger samples is needed. Moreover, our study supports the possible role of some ASD susceptibility genes, including NDE1, GPHN and ZMYND11.

Some important limitations must be noted. The sample size could be expanded, moreover some children did not perform EEG and MRI, which could be interesting data to correlate with CNVs. Hence, future perspectives include the acquisition of new data to increase the research in this field.

Author Contributions: A short paragraph specifying their individual contributions must be provided for research articles with several authors. The following statements should be used "Conceptualization, A.P.; L.B.; C.S. methodology, A.P.; L.B.; C.S..; software, C.S.; validation, A.P.., C.S.. and L.B..; formal analysis, F.D.P.; investigation, V.S., M.M.; M.N.; F.F. resources, data curation, . V.S..; M.M.; M.N.; F.F.; F.D.P.; C.S.;N.G.;F.C. A.P; L.B.;.; writing—original draft preparation, . V.S..; M.M.; M.N.; F.F.; F.D.P.; C.S.; A.P; L.B.; writing—review and editing, V.S..; M.M.; M.N.; F.F.; F.D.P; C.S.; A.P; L.B..;N.G.;F.A.; visualization, V.S..; M.M.; M.N.; F.F.; C.S.; A.P; L.B.;N.G.; F.A.; supervision, V.S..; M.M.; M.N.; F.F.; C.S.; A.P; L.B.;F.A; N.G; project administration, V.S..; M.M.; M.N.; F.F.; C.S.; A.P; L.B.; funding acquisition, V.S..; M.M.; M.N.; F.F.; C.S.; A.P; L.B.;. All authors have read and agreed to the published version of the manuscript." Please turn to the CRediT taxonomy for the term explanation. Authorship must be limited to those who have contributed substantially to the work reported.

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