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Heterozygosity for Neuronal Ceroid Lipofuscinosis predisposes to Bipolar Disorder

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Abstract

Objective. Bipolar Disorder (BD) is an heritable chronic mental disorder causing psychosocial impairment, affecting patients with depressive/manic episodes. The familial transmission of BD does not follow any of the simple Mendelian patterns of inheritance. The aim of this study is to describe a new large family with twelve affected BD members: WES was performed in eight of them, three of which were diagnosed for BD, and one was reported as a "borderline" individual.

Materials and Methods. WES data allowed us to select variants in common between the affected subjects, once including and once excluding a "borderline" subject with moderate anxiety and traits of obsessive-compulsive disorder.

Results. Results were in favor of new predisposing BD genes, electing a heterozygous missense variant in *CLN6* resulting in a "borderline" phenotype that if combined with a heterozygous missense variant in *ZNF92* is responsible for the more severe BD phenotype. Both rare missense changes are predicted to disrupt the protein function.

Conclusions. Loss of both alleles in *CLN6* causes Neuronal Ceroid Lipofuscinosis, a severe progressive neurological disorder of childhood. Our results indicate that heterozygous *CLN6* carriers, previously reported as healthy, may be susceptible to bipolar disorder late in life if associated with additional variants in *ZNF92*.

Keywords: Bipolar Disorder; WES; *ZNF92*; *CLN6*.

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1. Introduction

Bipolar Disorder (BD) is a common and chronic mental disorder causing psychosocial impairment, affecting patients with depressive/manic episodes [1, 2]. Bipolar phenotypes are defined only according to clinical features, and, to date, specific diagnostic tests do not yet exist [2]. Different subtypes of BDs are recognised, including BD type I and BD type II, well described by the Diagnostic and Statistical Manual of Mental Disorders, 5th edition (DSM-5) [3].

Bipolar I is defined by the presence of at least one manic episode, which might have been preceded or followed by a hypomanic episode or major depressive disorder [3]. During a manic episode, which generally lasts about a week, mood may shift rapidly from euphoric to anger or depression, presenting psychotic features, necessitating hospitalization to prevent harm to self or others. The lifetime risk of suicide in individuals with bipolar disorder is estimated to be at least 15 times that of the general population [3].

Bipolar II is instead characterized by the presence of at least one current or past hypomanic episode of about four days, and a major depressive episode; manic episodes never occur [2-3]. During hypomanic episodes, disturbance mood is not severe enough to cause social impairment or necessitate hospitalization; recurrent thought of death and suicidal ideation without a specific plan are common [3]. Bipolar-like phenomena which do not satisfy the criteria for BD I or II are classified as "Unspecified bipolar and related disorders" [2-3]. Several studies demonstrated the BD early age of onset, wherein more than 70% of individuals manifest the typical characteristics of the illness before the age of 25 years [4-6]. Multiple evidence have also strongly demonstrated that BD is a highly heritable phenotype [4-10]. In patients with established disease, a family history of mood or psychotic illness is common, with an estimated heritability of approximately 85% [2, 4-6]. The familial transmission of BD does not follow any of the simple Mendelian patterns of inheritance, and several analyses show that BD cannot be accounted for as one highly penetrant susceptibility gene [2, 11]. Nevertheless, according to the current knowledge, the fundamental biological basis of BD still remains unknown [1]. Initial research for BD risk loci were chiefly focused on linkage analysis [12]; however, linkage methods do not work well in the face of complex patterns of inheritance, so they failed to produce definitive, replicable findings [12]. In the past decade, large-scale Genome-Wide Association Studies (GWAS) of Single-Nucleotide Polymorphisms (SNPs) and analyses of Whole-Exome Sequencing (WES) performed on families revealed several tens of genetic loci related to BD, even if these loci explain only 18% of BD susceptibility [13-15]. *ANK3* (chromosome 10q21.2), *CACNA1C* (chromosome 12p13), *TRANK1* and *DCLK3* (chromosome 3p22, both) were some of the earliest genes to be implicated in BD by GWAS [12]; WES studies on multiple families identified promising variants in *RGS12* (chromosome 4p16.3), which has been reported in previous next-generation sequencing studies of schizophrenia [14]. Not less important, at least two locus are associated to recurrent Copy Number Variations (CNVs) in large, BD case–control samples: duplications on cytoband 16p11.2, and deletions on 3q29 [12]. Finally, prenatal and postnatal environmental risk factors have been implicated in a number of mental illnesses, including BD [16]. Prenatal infections, childhood maltreatment and psychological stressors significantly affect the course of BD. Risk factors for bipolar are numerous, both genetic and environmental, but low attributable risk, inconsistency of results, inability to identify the temporality of the

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relationship, lack of a clear biological mechanism and the nonspecific nature of many risk factors means that causation is difficult to assign in an individual patient [16].

The aim of this study is to describe a new large family with twelve affected BD members: WES was performed in eight of them, three of which were diagnosed for BD, and one was reported as a "borderline" individual. The analysis allowed us to highlight new genes not previously reported in literature in association with BD, suggesting new perspectives on the genetics of BD and expanding the current knowledge on it.

2. Materials and Method

2.1 Evaluated Individuals

In the present study, we describe a family with twelve affected BD members through five generations, with an apparently Mendelian inheritance pattern. The study was performed on eight subjects: three were affected by BD I or BD II, four without any signs of mental disease, one reported with moderate anxiety and traits of obsessive-compulsive personality. Patients came to the Medical Genetics department after being evaluated by the Unit of Psychiatry, Department of Mental Health (University of Siena, Policlinico "Santa Maria alle Scotte"). They were examined according to the DSM-5 criteria, with the purpose to investigate their psychopathological condition. Each of them was initially evaluated using the *MOOD Spectrum – Self Report, Lifetime version (MOODS-SR)* [17] and three psychometric scales: *Clinical Global Impression (CGI)* [18], *Montgomery Asberg Depression Rating Scale (MADRS)* [19] *and Young Mania Rating Scales (YMRS)* [20]. The same psychometric approach was then used to evaluate the improvements derived from the beginning of the therapy. For each patient genetic counseling was then performed, in order to evaluate each individual phenotype. All the subjects involved gave their written informed consent to the study that was carried out according to the Helsinki declaration.

Genomic DNA was extracted from EDTA peripheral blood samples using MagCore HF16 (Diatech Lab Line, Jesi, Ancona, Italy) according to the manufacturer's instructions. DNA quantity and integrity were estimated using the NanoDropTM 2000/2000c Spectrophotometer (ThermoFisher Scientific).

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2.2 Whole Exome Sequencing

Sample preparation was performed following the *Illumina DNA Prep with Enrichment* and *oligo Illumina Exome Panel* manufacturer protocol. The workflow uses a bead-based transposome complex to mediate a uniform tagmentation reaction of genomic DNA, which fragments and then tags DNA with adapter sequences in one step. A subsequent target enrichment workflow is then applied. Following pooling, the double stranded DNA libraries are denatured and biotinylated. *Illumina Exome Panel (CEX)* probes are hybridized to the denatured library fragments. After hybridization, Streptavidin Magnetic Beads (SMB) then capture the targeted library fragments within the regions of interest. The captured and indexed libraries are eluted from beads and further amplified before sequencing. The whole exome sequencing analysis was performed on the Illumina *NovaSeq 6000* (Illumina San Diego, CA, USA) according to the NovaSeq 6000 systemguide. Reads were mapped against the hg19 reference genome by using the Burrow-Wheeler aligner BWA [21]. Variant calling was obtained using an in-house pipeline which takes advantage of the GATK Best Practices workflow [22]. We obtained mean coverage of 105x for targeted sequenced regions (range, 95- 145x). WES data were analyzed using the enGenome-eVai (CE-IVD) software. In order to identify any potential pathogenic variants segregating among the family, we firstly approached the concept of "Reverse Phenotyping" on all the affected subjects. In this step, no filters in the prioritization of variants were applied. The aim was to check for the presence of any alterations involved in the manifestation of BD, in genes not previously described to be associated with mental disorders. Once excluded this first step, variants' prioritization was carried out and obtained by: selecting rare variants (Minor Allele Frequency, MAF <0,01) found only in BD subjects; evaluating genes previously associated with BD in literature, from GWAS and WES studies (Supplementary, Table 1) [12; 14-15; 23]; using Human Phenotype Ontology (HPO) terms: bipolar affective disorder (HP:0007302), depression (HP:0000716), obsessive-compulsive behaviour (HP:0000722), mania (HP:0100754), psychosis (HP:0000709), anxiety (HP:0000739), suicide ideation (HP:0031589). HPO terms were specifically selected according to the symptomatology presented by each evaluated individual. Missense variants were predicted to be damaging by CADD-phred prediction tools [24] and splice site variants by four *in silico* tools: SpliceSiteFinder-like, MaxEntScan, NNSPLICE, GeneSplicer.

The UCSC genome browser (https://genome.ucsc.edu ; December 2021), ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/ ; January 2022), Intervar (Clinical Interpretation of genetic variants by ACMG/AMP 2015 guideline, https://wintervar.wglab.org/; December 2021), VarSome (https://varsome.com/ ; January 2022), CADD (Combined Annotation-Dependent Depletion, https://cadd.gs.washington.edu/ ; January 2022) databases, and the Alamut Visual software application v.2.11 (Jan 2018 Interactive Biosoftware, Rouen, France) were

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used in order to evaluate the genomic coordinates, the probable deleterious effect on the protein, the allele frequency, the possible splicing effects and the American College of Medical Genetics (ACMG) pathological classification of each variants. OMIM (Online Mendelian Inheritance in Man- https://www.MIM.org/ ; December 2021), DECIPHER (DatabasE of Chromosomal Imbalances and Phenotypes using Ensembl Resources - https://www.deciphergenomics.org/ , February 2022) and BipEx Consortium (Bipolar Exosome, https://bipex.broadinstitute.org/ , February 2022), databases were used in order to evaluate the involvement of the genes in BD, and if they had been previously identified in affected subjects.

3. Results

3.1. Clinical Description

Clinical findings of evaluated patients are described as follows. The family pedigree is shown in Figure 1. Psychometric evaluations were carried out for each patient by the Unit of Psychiatry, Department of Mental Health.

Patient 1 (Figure 1; V;5)

Patient 1 is a 27 years old woman. She was born at term from spontaneous delivery. She experienced sleep disturbances until the age of six, for which she started therapy with Noprom. She manifested her first psychopathological symptoms when she was eighteen years old, when she began to attend university. She developed symptoms of demoralization, obsessive-compulsive behavior, anxiety, mood swings and irritability. The family doctor prescribed her an unspecified antidepressant. After three days of intaking, her mood expanded, she developed sub-total insomnia, logorrhea, tangential thought process, ease in relationship with strong behavioral disinhibition, episodes of dysphoria and impulsiveness, mystical and megalomaniac delusions regarding conviction of possessing paranormal powers. Hospitalization followed this event, during which a diagnosis for BD type I was established. Over the years her psychiatric history has been characterized by the alternation of satisfying conditions and symptomatic re-exacerbations. At 24 years old, the patient experienced an additional severe maniac exacerbation, marked by reduced energy, hypersomnia, lethargy, side effects to the therapy, such that a second hospitalization was necessary. Currently, the patient is in good psychopathological condition. She currently takes Valproic Acid 600 mg per day, Lorazepam 1mg, Cariprazine 1,5mg two per day, Lamotrigine 50 one per day.

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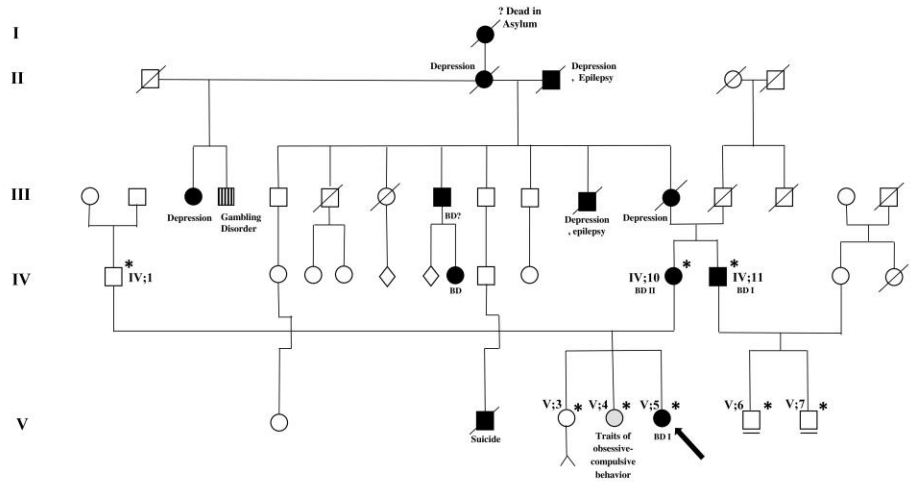

Figure 1. Family pedigree. WES analysis was performed on patients highlighted with *****. The black arrow indicates the index subject, Patient 1(V;5). A square represents a male individual; a circle, a female individual; a rhombus: undetermined sex. Black symbols: subjects affected by BD symptomatology. Striped symbols: subjects affected by various disorders. In gray: subject with traits of obsessive-compulsive behavior. Genetic counseling was carried out on the family with internal code #17949.

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Patient 2 (Figure 1; IV;10)

Patient 2 is the 55 years old proband's mother affected by Bipolar Disorder II. Her first signs of depression arose following the death of the mother; the age of her first symptoms is not available. After being treated with a diuretic due to the diagnosis of Meniere Syndrome, she started to experience depression, anxiety, sleep disturbances, labile mood, irritability, inner tense and polarization about her daughter's health problems. Later on, she developed sadness, apathy, and abulia. Over the years her psychiatric history has been characterized by the alternation of satisfying conditions and symptomatic re-exacerbations. Currently she displays significant clinical improvement. She is in therapy with Valproic acid CHR 300 mg, Citalopram 10 mg.

Patient 3 (Figure 1; IV;11)

Patient 3 is the maternal uncle of the index subject. He is a 63 years old male affected by BD type I. He manifested subclinical depression since he was young. At 57 years old, he was hospitalized for episodes of depression,

lack of volition, insomnia, delusions of failure, partial insight, and further manic episodes and behaviors in line with the DSM-5 criteria for BD I. Over the years, his psychiatric history has been characterized by the alternation of satisfying conditions and symptomatic re-exacerbations. He was released in good psychopathological condition after being treated with: Olanzapine 5 mg, Lamotrigine 150 mg, Sertraline 50 mg, lithium carbonate 900 mg.

Patient 4 (Figure 1; V;4)

Patient 4 is the middle sister of Patient 1. She is 30 years old. Psychiatrists reported moderate anxiety and traits of obsessive-compulsive personality. No other relevant clinical data were described. The girl was referred to be in good health.

3.2. Molecular Characterization

Results obtained by WES analysis are summarized in Table 1. All variants refer to the Human Genome Assembly GRCh37/hg19. We searched for common variants among the affected subjects, and we found heterozygous missense and synonymous variants in 10 genes: *PDE1C*, *HERC2*, *CHD7*, *MRC2*, *SPON1*, *ZNF92*, *TTC19*, *RBM12*, *CLN6*, *WDR62* (Table 1). However, variants in *PDE1C*, *HERC2*, *CHD7*, *MRC2* and *TTC19* were noticed not only in BD subjects, but also in healthy ones (Supplementary, Figure 1), and were thus excluded from the analysis. Because of their low CADD phred scores (<15) [24], variants in *SPON1*, *RBM12* and *WDR62* were excluded too (Table 1).

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Table 1. Variants in common among BD subjects from WES analysis.

Gene symbol (#OMIM)	Transcript	HGVS genomic	HGVS_coding	HGVS_protein	Variant Effect	Classification (ClinVar/ InterVar)	dbSNP	gnomAD	Splicing effect	CADD phred
PDE1C (#602987)	Chr 7, NM_001191057.4	g.31904581 A>G	c.725A>G	p.Tyr242Cys	Missense	Unreported/ Uncertain	n.a.	n.a.	n.a.	26.5
HERC2 (#605837)	Chr15, NM_004667.6	g.28386774 G>A	c.11819G>A	p.Arg3940Gln	Missense	Unreported/ Uncertain	rs370282963	0.0012%	n.a.	27.6
CHD7 (#608892)	Chr 8, NM_017780.4	g.61735198G >C	c.3094G>C	p.Glu1032Gln	Missense	Unreported/ Uncertain	rs1355615827	0.00040%	n.a.	23
MRC2 (#612264)	Chr 17, NM_006039.5	g.60769687 C>T	c.4315C>T	p.Arg1439Cys	Missense	Unreported/ Uncertain	rs776632141	0.0013%	n.a.	29.2
SPON1 (#604989)	Chr 11, NM_006108.4	g.13963049 C>A	c.145C>A	p.Arg49Ser	Missense	n.a.	n.a.	n.a.	n.a.	1.98
ZNF92 (#603974)	Chr 7, NM_152626.4	g.64864204 G>A	c.1177G>A	p.Gly393Arg	Missense	Unreported/ Uncertain	rs764034193	0.0024%	n.a.	15.95 /
		g.64864203G >T	c.1176G>T	p.Thr392Thr	Synonymous	Unreported/ Likely Benign	rs566012007	0.00037%	n.a.	
TTC19 (#613814)	Chr 17, NM_017775.4	g.15928441G >T	c.787G>T	p.Ala263Ser	Missense	Uncertain	rs141892030	0.029%	It creates an alternative splicing site at 3' in exon 8 for two predictive in silico tools (SpliceSiteFinder-like; MaxEntScan)	27

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RBM12 (#607179)	Chr 20, NM_006047.6	g.34242641 A>G	c.604A>G	p.Met202Val	Missense	Unreported/ Likely Benign	rs146171962	0.0040%	It creates an alternative splicing site at 3' in exon 3 for two predictive in silico tools (SpliceSiteFinder-like; MaxEntScan)	13.23
CLN6 (#606725)	Chr 15, NM_017882.3	g.68504183 C>T	c.316C>T	p.Arg106Cys	Missense	Uncertain	rs202226970	0.0067%	n.a.	24.3
WDR62 (#606725)	Chr 19, NM_001083961.2	g.36595687 G>A	c.4329G>A	p.Gln1443Gln	Missense	Uncertain/ Likely Benign	rs771131709	0.0028%	n.a.	0.10

In "Classification", a unique output correlates to both ClinVar and InterVar interpretations. CADDphred was calculated for missense variants only [24]. HGVS: Human Genome Variation Society (https://www.hgvs.org). Chr: chromosome; n.a.= not available.

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On a first analysis, we therefore considered mutated genes only in BD subjects, and we obtained that the variant in common was found in *ZNF92* (Figure 2). As a second step, we considered the mutated genes in common also with the "borderline" subject, and we obtained the missense variant in *CLN6* (Figure 2). According to this data, we could hypothesize that the variant in *CLN6* alone predisposes to the obsessive-compulsive phenotype, but when combined with the variant in *ZNF92* is causative for the BD phenotype, according to an oligogenic inheritance.

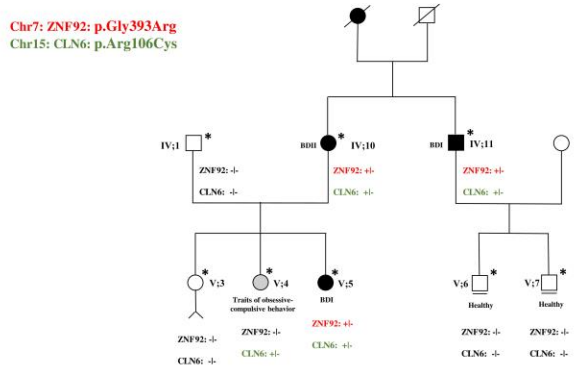

Figure 2. Genes in common between affected and borderline family members. A square represents a male individual; a circle, a female individual. + and – refer to the presence or the absence, respectively, of the heterozygous variant in the indicated gene. The missense variant in *ZNF92* is exclusively shared by BD affected subjects; the missense variant in CLN6, also by the «borderline» subject.

Finally, assuming that the effect of some of the identified variants could be aggravated by the concomitant presence of any Structural Variants (SVs), we analyze WES raw data from affected subjects using the read count–based tool EXCAVATOR2 [doi:10.1093/nar/gkw695]. The tool uses BAM files of WES experiments to extract the depth of sequencing coverage, correct it for technical biases and identify regions with altered copy number [25]. Each affected/ "borderline" subject was compared with a same global control sample that results from pooling all unaffected samples by running the tool in "pooling" mode. Then, Copy Number Variations (CNV) common to affected subjects (w/wo "borderline") were merged into copy-number variable regions (CNVRs) and annotated through AnnotSVv2.2 (https://lbgi.fr/AnnotSV ; Supplementary, Bipolar Disorder Family_SVs). The tool uses BED or SV files to produce a tab-separated values file, scoring and ranking the SVs into five classes from pathogenic to benign [26]. Results are summarized in Table 2. The analysis showed the presence of 12 CNVRs among two out of three BD individuals, plus the borderline one. Probably due to insufficient coverage, WES data from Patient V;5 were excluded from the

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comparison of the shared SVs in order to prevent this sample leading to biased results. The sample has indeed passed the quality control required by the EXCAVATOR2 pipeline, but it showed a distortion in the results of all the chromosomes that could lead to wrong interpretations (Supplementary, Figure 2). None of the 12 observed SVs has been confirmed among healthy subjects. Some deletions have been scored as Likely Pathogenic (score 4); none of them involves genes previously associated with BD.

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Table 2. Analysis of CNVs from Excavator 2 and AnnotSV.

AnnotSV ID/ SV type	SV Chr	SV Start	SV End	SV size (Kb)	Gene Name	Transcript	GnomAD_ID	AnnotSV ranking	Association to Bipolar Phenotypes
1_2181795_2185513_DEL	1	2181795	2185513	3.71 Kb	***SKI***	NM_003036	gnomAD_v2_DEL_1_331	4	No
2_168811559_168855462_DEL	2	168811559	168855462	43.9 Kb	***STK39***	NM_013233	n.a.		No
3_6786589_6863041_DEL	3	6786589	6863041	76.4 Kb	***GRM7-AS3***	NR_110123	gnomAD_v2_DEL_3_34441	2	No
4_56436502_56446416_DEL	4	56436502	56446416	9.9 Kb	***PDCL2***	NM_152401	n.a.	2	No
7_5121870_5182726_DEL	7	5121870	5182726	60.8 kb	***ZNF890P***	NR_034163	gnomAD_v2_DEL_7_90969;gnomAD_v2_DEL_7_90970;gnomAD_v2_DEL_7_90981	2	No
8_144111099_144116293_DEL	8	144111099	144116293	5.19 Kb	**No gene**	-	gnomAD_v2_DEL_8_114898	2	No
10_133456235_133465931_DEL	10	133456235	133465931	9.96 Kb	**No gene**	-	n.a.	2	No
11_117833717_117838299_DEL	11	117833717	117838299	4.58 Kb	**No gene**	-	gnomAD_v2_DEL_11_144738	2	No
12_113206065_113246063_DEL	12	113206065	113246063	39.9 Kb	***RPH3A***	NM_001347952	n.a.	2	No
17_81444368_81463817_DEL	17	81444368	81463817	19 Kb	**No gene**	-	-	2	No
19_4719845_4770779_DEL	19	4719845	4770779	50.9 Kb	***DPP9***	NM_139159	n.a.	2	No
19_4719845_4770779_DEL	19	4719845	4770779	50.9 Kb	***MIR7-3***	NR_029607	n.a.	2	No
19_4719845_4770779_DEL	19	4719845	4770779	50.9 Kb	***MIR7-3HG***		n.a.	2	No
19_15238361_15244668_DEL	19	15238361	15244668	6.3 Kb	**No gene**	-	gnomAD_v2_DEL_19_200694	2	No

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AnnotSv ranking scores span from 1-5 according to the AMCG technical standards for the interpretation of constitutional copy number. Pathogenic CNVs: class 5; Likely Pathogenic: class 4; Variant of Uncertain Significance: class 3; Likely Benign: class 2; Benign: class 1. The analysis was carried out among Patients IV;10, IV;11, V;4 (Figure 1). Probably due to the low coverage regarding WES data from Patient V,5 analysis could not be ruled out on it. N.a: not available.

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4. Discussion

BD is a very heterogeneous condition, due to tens of genetic causes still not explained at all [1]. Over the years, GWAS analyses allowed to identify up to fifty genome-wide significant loci [7;12;13;15], and WES analyses performed on families allowed to characterize up to hundred candidate genes for BD, including *de novo* or segregating variants through generations [8;10;14]. In the present study, we describe WES results identified in a new BD affected family, performed on three affected members, four healthy individuals and one member with some traits of psychiatric disorder (obsessive-compulsive disorder). The analysis allowed us to highlight missense heterozygous variants in ten relevant genes: *PDE1C, HERC2, CHD7, MRC2, SPON1, ZNF92, TTC19, RBM12, CLN6, WDR62*. In order to identify the variants involved in the phenotypic manifestation, we focused firstly on those shared by the affected subjects, and secondly on those in common also with the symptomatic individual with moderate anxiety and traits of obsessive-compulsive personality. Based on this, *CLN6* was selected as the predisposing gene for obsessive-compulsive disorder, manifesting the BD phenotype when worsened by other concomitant variants in other genes such as that identified in *ZNF92*.

Ceroid Lipofuscinosis Neuronal, 6 (*CLN6*, #606725) is a gene involved in a group of lysosomal storage disorders with a predominantly autosomal recessive inheritance [27]. Neuronal Ceroid Lipofuscinosis (NCL) caused by biallelic variants in the *CLN6* gene usually presents in early to late childhood to juvenile, between 1.5 and 8 years of age, with slow motor degeneration, ataxia, loss of vision, epilepsy, and mental disabilities [28]. Early death by 12–15 years of age is reported [28]. Variants in *CLN6* may also lead to a rare adult-onset form of NCL in which the symptoms present in adulthood, typically between 24 and 38 years old [29,30] The symptomatology includes ataxia, tremor and cognitive impairment around the age of 41 [30]. Homozygous variants in *CLN6* have been reported also in association with depression and anxiety, and some evidence supports the link with psychiatric problems and obsessive-compulsive behavior [29,30]. Although, to date, heterozygous *CLN6* carriers do not seem to show any distinct clinical phenotype, nor most of times psychiatric conditions [30,31], we describe a subject with different phenotypic features and propose to pay particular attention to the possible implications that these variants may have in the development of "borderline" phenotypes, in the context of a multifactorial disease worsened by other concomitant variants in other genes such as that identified in *ZNF92*.

Zinc Finger protein 92 *(ZNF92*, #603974*)* belongs to the ZnF transcription factors family, which contains the Kruppel-associated box, or KRAB domain [32]. The ZNF family presents motifs in DNA- and RNA-binding proteins

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and appears to be involved in many cellular functions, such as DNA recognition, RNA packaging, transcriptional activation, apoptosis regulation, protein folding and integration [33]. Increasing evidence suggests that genetic variants on *ZNFs* influence the susceptibility to psychiatric disease, especially in BD [12; 33]. Here, we propose *ZNF92* as a new gene involved in the pathogenesis of bipolar phenotype.

Despite the small number of the evaluated subjects (8), this study provides new molecular information regarding the genetics of BD. We cannot completely define the role that the observed variants may have in the onset of the psychiatric condition, since they're not already reported in the ClinVar database, nor in other specific BD databases, such as BipEx Consortium. This last browser in particular shows no evidence of association with BD for these two identified variants, in a dataset of 14210 affected cases and 14422 negative controls (https://bipex.broadinstitute.org/). In a subset of 2726 samples analyzed through the enGenome- eVai (CE-IVD) software, only one sample, already diagnosed for Alport syndrome and reported negative for it, presented the same *CLN6* variants; the *ZNF92* variant was reported only in the BD affected subjects presented in this study. None of these subjects reports the combination of these two variants at the same time. Given its rarity in the general population, and given its recurrence throughout the family in analysis, it is highly probable that this combination is private. The bipolar phenotype could be due to the impairment of the protein structure encoded by *CLN6*, previously reported as a gene involved in mental disorders; anyway, a multifactorial pathway should be considered, in which additional genes or the family environment could contribute to the final phenotype. Actually, there is no strong basis for the assumption that any of the shared variants would be fully penetrant, and those found in common with the healthy subjects may play a role in the family's illness. For instance, it is well known that mutations in *HERC2* produce clinical syndromes in which key neurodevelopmental events are altered, resulting in intellectual disability and other neurological disorders [34]. Genetic variation in *PDE1C* associates with multiple measures of human cognitive function [35]; *CHD7* is a critical player in the epigenetic regulation of neuronal differentiation, disruptions of which are believed to be associated with schizophrenia and autism [36]; *TTC19* mutations have been identified in a family with severe psychiatric manifestation [37]; finally, truncating mutations in *RBM12* are associated with psychosis [38]. Based on specific prediction scores reported in DECIPHER database (Table 3)[39,40], some of these genes, such as *CHD7* and *HERC2*, seem to be intolerant to loss-of-function variations. It is thus possible that variants in these genes may contribute to the full manifestation of the psychiatric disease, even if they are present also in unaffected individuals. We cannot even be sure that the subjects so far identified as "healthy" cannot manifest psychiatric disorders during their lifetime. It could be interesting to calculate a polygenic risk score for each genotyped individual; unfortunately, our group of individuals is too small to define significant data that should be compared with an extended set of controls. There is no reported polygenic risk score for BD in the

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Polygenic Score Catalog (https://www.pgscatalog.org/), but even if it was present, it was not exportable to a different population.

Table 3. Predictive Scores for each gene identified from WES analysis according to the DECIPHER database.

Genes	pLI	% HI
PDE1C	0.00	22.08
HERC2	1.00	50.09
CHD7	1.00	2.39
MRC2	0.67	43.17
SPON1	-	-
ZNF92	0.00	92.19
TTC19	0.00	51.13
RBM12	0.00	14.22
CLN6	0.00	41.58
WDR62	0.00	51.07

pLI: Probability of Loss-of-function Intolerance. The pLI score is the probability that a given gene falls into the Haploinsufficient category, and therefore is extremely intolerant of loss-of-function variation. Genes with high pLI scores (pLI ≥ 0.9) are extremely LoF intolerant, whereby genes with low pLI scores (pLI ≤ 0.1) are LoF tolerant **[39].**

% HI: Probability of being a Haploinsufficient gene. A haploinsufficient gene is one which requires two functional copies to produce the standard phenotype. The probability that a gene is haploinsufficient based on a set of functional, evolutionary, and network properties of the gene. High ranks (e.g. 0-10%) indicate a gene is more likely to exhibit haploinsufficiency, low ranks (e.g. 90-100%) indicate a gene is more likely to NOT exhibit haploinsufficiency [40].

Based on these scores, among these genes *HERC2* and *CHD7* seem to be intolerant to loss-of-function variations; the rest of the genes, including *CLN6* and *ZNF92*, seem to be more tolerant to variations. It is possible that *HERC2* and *CHD7*, although present in healthy individuals, could contribute to a lesser extent to the observed phenotype.

Finally, none of the twelve identified CNVs showed clinical correlation with BD; some of these have been classified as "Likely Pathogenic", but the absence of clear associations with psychiatric disorders does not help in their

interpretation. This further study reinforces the role of the two genes, *ZNF92* and *CLN6*, found by WES in the manifestation of the disease.

In conclusion, this study suggests new perspectives regarding the genetics of BD. Compared to the other WES studies, it is performed in a smaller group of subjects, and it lacks reproducibility in other families; on the other hand, however, all the investigations ruled out, including the analysis of SVs from WES raw data through EXCAVATOR2 (not reported in BD studies so far), lead to a unique result, and so the involvement of *CLN6* and *ZNF92* in BD and obsessive-compulsive behavior. Further analysis on larger cohorts should be performed to clarify the roles of the genes and the impact of the selected variants in the manifestation of the bipolar phenotype. If additional variants in *ZNF92* and *CLN6* are detected in psychiatric disorders, this work could support their correlation to them, help in identifying subjects at risk for their development and improve their clinical management, recommending appropriate therapies and monitoring their health over time.

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Author Contribution: F.P. analyzed data and drafted the manuscript; F.V, G.D, C.F, F.A. and S.F. helped in the analysis of data and in drafting the manuscript; A.M.T, A.G and A.F provided psychological information about patients; G.B and R. D.A analyzed CNVs from WES data; F.M and A.R. performed genetic counseling on the family. All authors have read and agreed to the published version of the manuscript.

Ethical Statement: The patients gave their informed consent for diagnostics testing and research studies. Studies were performed and samples were obtained in accordance with the Helsinki Declaration of 1964, as revised in October 2013 in Fortaleza, Brazil.

Data Availability Statement: Data sharing is not applicable to this article as no datasets were generated or analyzed during the current study.

Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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