Seizure: European Journal of Epilepsy 80 (2020) 145–152

Contents lists available at ScienceDirect

Seizure: European Journal of Epilepsy

journal homepage: www.elsevier.com/locate/seizure

Targeted re-sequencing in malformations of cortical development: genotype-phenotype correlations

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ARTICLE INFO

Keywords:
Malformations of cortical development
Next-generation sequencing
Gene panel
Brain MRI

ABSTRACT

Purpose: Malformations of cortical development (MCD) are a phenotypically and genetically heterogeneous group of disorders, for which the diagnostic rate of genetic testing in a clinical setting remains to be clarified. In this study we aimed to assess the diagnostic rate of germline and pathogenic variants using a custom panel in a heterogeneous group of subjects with MCD and explore genotype-phenotype correlations.

Methods: A total of 84 subjects with different MCD were enrolled. Genomic DNA was isolated from peripheral blood. Fifty-nine tartget genes were assessed using a custom next-generation sequencing (NGS) panel.

Results: Genetic causes were identified in one-fourth of our cohort (21.4 %). Overall, we identified 19 pathogenic or likely pathogenic single-nucleotide variants in 11 genes among 18 subjects, including *PAFAH1B1 (LIS1)* (n = 3), *TUBA1A* (n = 3), *DYNC1H1* (n = 3), *ACTG1* (n = 2), *TUBB2B* (n = 1), *TUBB3* (n = 1), *DCX* (n = 1), *FLNA* (n = 1), *LAMA2* (n = 1), *POMGNT2* (n = 1) and *VLDLR* (n = 1). The diagnostic yield was higher in patients with lissencephaly/pachygyria (60 %) (p = 0.001), cobblestone malformation (50 %), and subcortical

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https://doi.org/10.1016/j.seizure.2020.05.023
Received 17 April 2020; Received in revised form 15 May 2020; Accepted 29 May 2020

band heterotopia (SBH) (40 %). Furthermore, five out of six subjects with suspect tubulinopathies on imaging harboured pathogenic variants in tubulin genes. Overall, germline pathogenic variants were more likely to be identified if MCD were diffuse ($p = 0.002$) and associated with other central nervous system malformations ($p = 0.029$). Moderate to severe intellectual disability was also more commonly associated with pathogenic variants ($p = 0.044$).

Conclusion: Customized gene panels may support the diagnostic work-up for some specific MCD, especially when these are diffuse, bilateral and associated with other brain malformations.

1. Introduction

Malformations of cortical development (MCD) encompass a heterogeneous group of disorders related to the disruption of tightly regulated processes of cortex formation, due to various genetic, infectious, or vascular etiologies [1]. Clinical presentation and outcome in individuals with MCD are highly variable, including intellectual disability and refractory epilepsy at the most severe end of the spectrum [2,3]. The identification of underlying mechanisms is thus crucial for a more accurate prognosis, familial recurrence risk counselling, and better health management. Moreover, in a few specific conditions, elucidation of the specific cause of MCD opens the possibility for tailored treatments, such as mTOR inhibitors. However, our current understanding of MCD and genetic diagnosis in the clinical setting remain limited [4].

MCD are typically classified into three major groups that recapitulate the primary developmental steps of cell proliferation, neuronal migration, and cortical organization [5]. Any event, either genetic or acquired, that occurs at one of these crucial stages can dramatically impair the process of cortex formation, resulting in various MCD.

The widespread availability of new MRI techniques with improved spatial resolution in the last decades has enhanced our ability to characterize even subtle cortical malformations, significantly expanding the spectrum of MCD with consequent constant revision and update of their classification. In parallel, the advent of next-generation sequencing (NGS) has uncovered a large number of novel genes associated with MCD, tremendously advancing our understanding of pathways and molecular processes of both normal and abnormal cortical development. To date, more than 100 genes related to MCD have been identified, challenging the genetic diagnosis in a clinical setting [1,6]. The diagnostic rate in some conditions, e.g. double cortex syndrome, pachygyria and periventricular nodular heterotopia, is limited by the fact that, in up to 30 % of cases, mutations could be found in the affected tissue only at a mosaic level (i.e. postzygotic) and are thus undetectable in blood [7]. Moreover, the recent discovery of genetic variants almost exclusively at a mosaic level in the PI3K-AKT-mTOR pathway, causing a spectrum of conditions ranging from focal cortical dysplasia to complex brain overgrowth disorders, has added a further line of complexity in the genetic diagnosis of MCD [8].

In recent years, gene panel sequencing has been proved to be a powerful and cost-effective diagnostic tool for the detection of pathogenic variants in many neurological disorders such as intellectual disability, autism spectrum disorder [9,10] and epilepsy [11–13], while little is known concerning its use in MCD, mostly limited to small cohorts and a limited number of genes related to specific neuroradiological patterns [14,15]. Recently, Lee et al. [16] provided the first evidence of diagnostic yield of 23 %, using a custom panel of 96 genes in a cohort of 81 subjects with MCD.

The main aim of this study was to assess the diagnostic rate of germline pathogenic variants using a custom panel in a heterogeneous group of subjects with MCD. Secondary aims were to identify imaging and clinical features that were associated with pathogenic or likely pathogenic variants detected at NGS panel analysis and to explore genotype-phenotype correlations.

2. Methods

This multicenter, retrospective/prospective cohort study, coordinated by the Gaslini Children's Hospital (Genoa, Italy) was approved by our institutional research ethics board. Written informed consent was obtained from each participant or legal representative.

2.1. Subject selection

We selected eighty-four unrelated non-consecutive patients with MCD who underwent next-generation analysis from January 2016 to June 2019 at our Institute. Sixty patients were recruited at Gaslini Children's Hospital, while the remaining from thirteen different Italian institutes. We included patients with i) MCD identified at postnatal brain MRI, ii) available data on extra-CNS malformations and dysmorphism detected at clinical evaluation, iii) available data on neurological assessment.

We excluded i) subjects with hemimegalencephaly and focal cortical dysplasia, given the high rate of somatic mutations in genes belonging to the PI3K-AKT-mTOR pathway [8] that we currently investigate in affected tissues (e.g. brain specimen) through another specific in-house gene panel, ii) subjects with Tuberous sclerosis complex since *TSC1* and *TSC2* are included in the gene panel of the PI3K-AKT-mTOR signalling [8] along with other negative regulatory genes of the GATOR pathway (*DEPDC5*, *NPRL2*, *NPRL3*) [17], iii) schizencephaly since *COL4A1*, the main gene mutated in a minority of subjects with schizencephaly, is included in another vascular gene panel available at our Institute, iv) history of teratogen exposure during pregnancy, v) established diagnosis of congenital infections (i.e. TORCH complex), vi) poor MR imaging quality, and vii) previously identified pathogenic copy number variants (CNVs) .

2.2. Neuroimaging

Brain MRI studies were acquired in different centres with different protocols, but all included diffusion-weighted images, T2-weighted, and FLAIR images on the 3 planes and 3D T1-weighted sequences. A pediatric neuroradiologist with 10 years of experience (MS) and a paediatrician with expertise in neurogenetics (AA) reviewed the images in consensus, based on the 2012 Barkovich classification [5,18], including six main neuroradiological patterns: i) polymicrogyria, ii) cobblestone malformations, iii) lissencephaly spectrum, iv) periventricular nodular heterotopia (PNH), v) subcortical band heterotopia (SBH), and v) dysgyria and/or simplified gyral pattern. MCD were stratified based on their location and extension in "unilateral" or "bilateral" (i.e. involving one or both cerebral hemispheres, respectively), and "focal" (i.e. involving one or less than one lobe in a cerebral hemisphere, even if multiple) or "diffuse" (i.e. involving multiple lobes in a cerebral hemisphere). Additional brain malformations, including basal ganglia abnormalities, cerebellar malformations, brainstem abnormalities, enlarged dysmorphic ventricles/ventriculomegaly, corpus callosum abnormalities and white matter signal alterations were also noted.

2.3. Clinical evaluation

Demographic information and clinical features were obtained by

reviewing medical records and included data on neurological and motor impairment, developmental state, the severity of intellectual disability according to the latest available neuropsychological assessment, occipital circumference, epilepsy diagnosis, type of seizures, seizure onset age, response to epilepsy treatment, and presence of other congenital malformations and dysmorphisms (considering relevant at least the presence of three craniofacial dysmorphic features).

Developmental milestones were evaluated by the Griffiths Mental Developmental Scales-Extended and Revised (GMDS-ER) below the age of 5 years, while cognitive impairment was assessed by the Wechsler scales in older children or with the Leiter scales in non-verbal or severely intellectually disabled patients [19]. Global developmental delay (GDD) was defined according to the Diagnostic and Statistical Manual of Mental Disorders- 5th edition (DSM-5) as a persistent (at least for 6 months) delay in two or more developmental domains, including motor, speech, cognition, social functioning, and activities of daily living, in children below the age of 5 years. The term intellectual disability (ID) was applied to older children in whom intelligence quotient (IQ) testing could be performed, estimating the degree of intellectual disability: mild (IQ < 70), moderate (IQ = 40–54), severe (IQ = 25–39), profound (IQ < 25). Other neuropsychiatric features were recorded such as the presence of autism spectrum disorder and other behaviour issues. Results of basal metabolic screening or other genetic tests such as karyotype, CGH-array, and target genetic testing were recorded, when available.

2.4. Targeted gene panel sequencing

We designed a custom panel including 59 OMIM genes associated with MCD. These genes were selected when they were supported by at least two independent studies based on an extensive literature review. The list of genes included and their corresponding phenotypes are illustrated in the Supplemental material Table 1. Libraries were prepared according to the manufacture's protocol from patients' DNA, extracted from 1 mL of blood. Library preparation and sequencing was made simultaneously for 13 individuals for each run utilizing barcode adapters. Targeted sequencing was performed using Ion Ampliseq™ Custom Panel kit for analysis with Ion 316™ Chip Kit v2 and Ion-PGM platform (Life Technology – Thermo Fisher Scientific, Waltham, MA, USA). We used this technology for constructing a fragment library by multiplex PCR of 294,000 amplicons with 12 primer pools representing all coding exons, untranslated regions (UTRs) and splicing regions of the 59 selected genes.

The reads were mapped to the reference human genome sequence (GRch37/ hg19) by using CLC Bio Genomics Workbench 7.5.1 software (CLC Bio, Aarhus, Denmark), after removal of duplicates. We set at 95 % the minimum fraction of targeted regions that should be covered at least 20 × .

Single-nucleotide polymorphisms (SNPs) and short indels were called with CLC Bio Workbench by filtering outcalls with a read coverage < 20x and an average quality of < 20.

Variants were annotated by CLC Bio software using dbSNP147 and ExAC databases. For the interpretation of sequence variants, we used the American College of Medical Genetics and Genomics guidance (ACMG) classification system. Variants were prioritized as follows: (1) pathogenic or likely pathogenic variants previously reported in ClinVar, (2) novel non-synonymous, splice-site coding and synonymous variants with a minor allele frequency ≤0.01 in Exac database. Selected variants were validated by Sanger sequencing and segregation analysis has been performed when parental DNA was available. CNVs were analyzed

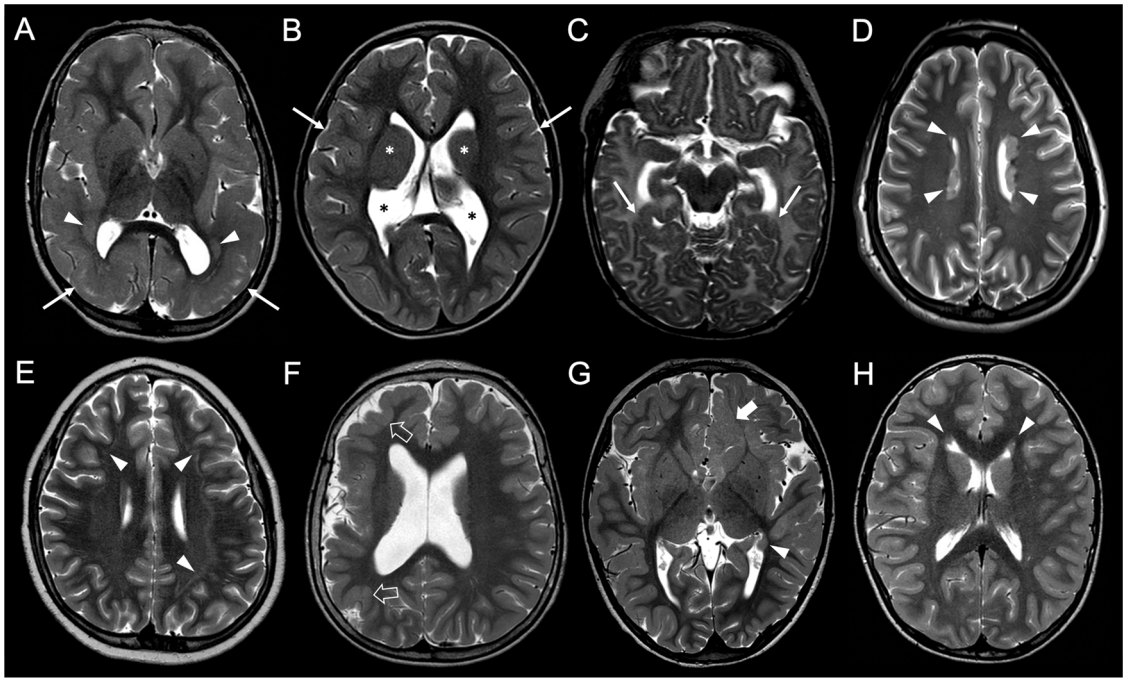

Fig. 1. Brain MRI axial T2-weighted images of patients with positive (A–D) and negative (E–H) results at NGS panel. A) In this subject (S7) harbouring a *DYNC1H1* variant, there is bilateral diffuse lissencephaly with a posterior-anterior gradient (arrows) and nodules of heterotopic neurons in the temporal lobes white matter (arrowheads). B) Subject 5 harbouring a *TUBB3* variant present bilateral diffuse dysgyria, especially at the level of the insular lobes (arrows), associated with basal ganglia anomalies (white asterisks) and enlarged/dysmorphic lateral ventricles (black asterisks). C) In this subject (S17) carrying a *LAMA2* variant, bilateral focal cobblestone malformation is noted at the level of mesial temporal lobes (arrows). D) In this girl (S18) harbouring an *FLNA* mutation, there are bilateral multiple nodules of heterotopic neurons along the lateral ventricles (arrowheads). E) This patient with negative results at NGS panel (S70) presents with bilateral incomplete and subtle subcortical band heterotopia (arrowheads). F) Similarly, in this subject with unilateral diffuse polymicrogyria (empty arrows) and reduced hemispheric volume (S49), no pathogenetic variants are found. G) This subject with multiple but focal MCD (S56), i.e. left frontal polymicrogyria (arrow) and a single periventricular nodular heterotopia (arrowhead), the NGS panel yields negative results. H) In this subject with negative NGS panel (S78), there are few bilateral periventricular nodular heterotopias located only at the level of frontal horns (arrowheads).

Table 1
Genetic findings and neuroradiological features in subjects with malformation of cortical development.

Subjects	Gene	Inheritance/ Segregation	Transcript	Nucleotide change	Amino acid change	Zygosity	ACMG classification	Previous literature	Malformation of cortical development
S1	*TUBA1A*	AD, dn	NM_006009.4	c.535A > G	(p.Thr179Ala)	Heterozygous	Likely pathogenic	*novel*	Bilateral diffuse dysgyria (tubulinopathy pattern)
S2	*TUBA1A*	AD, dn	NM_006009.4	c.641 G > A	(p.Arg214His)	Heterozygous	Pathogenic	ClinVar	Bilateral diffuse dysgyria (tubulinopathy pattern)
S3	*TUBA1A*	AD, na	NM_006009.4	c.545 T > C	(p.Val182Ala)	Heterozygous	Likely pathogenic	*novel*	Bilateral diffuse dysgyria (tubulinopathy pattern
S4	*TUBB2B*	AD, dn	NM_178012.5	c.758 T > C	(p.Leu253Pro)	Heterozygous	Likely pathogenic	*novel*	Bilateral perisylvian PMG (tubulinopathy pattern)
S5	*TUBB3*	AD, dn	NM_006086.4	c.862 G > A	(p.Glu288Lys)	Heterozygous	Likely pathogenic	ClinVar	Bilateral diffuse dysgyria (tubulinopathy pattern)
S6	*DYNC1H1*	AD, na	NM_001376.5	c.10282_10284delCAG	(p.Gln3428del)	Heterozygous	Likely pathogenic	*novel*	Bilateral diffuse PMG
S7	*DYNC1H1*	AD, dn	NM_001376.5	c.926 G > A	(p.Arg309His)	Heterozygous	Pathogenic	ClinVar	Bilateral diffuse Lissencephaly (P-A gradient)
S8	*DYNC1H1*	AD, dn	NM_001376.5	c.1798 G > A	(p.Ala600Thr)	Heterozygous	Likely pathogenic	*novel*	Bilateral diffuse Lissencephaly (P-A gradient)
S9	*PAFAH1B1*	AD, na	NM_000430.4	c.193-1G > A		Heterozygous	Pathogenic	*novel*	Bilateral diffuse Lissencephaly/ pachygyria (P-A gradient)
S10	*PAFAH1B1*	AD, na	NM_000430.4	c.482_487delGCGGCAins CCTGGTCGAAC	(p.Ser161Thrfs*14)	Heterozygous	Pathogenic	*novel*	Bilateral diffuse Lissencephaly
S11	*PAFAH1B1*	AD, dn	NM_000430.4	c.766C > T	(p.Gln256*)	Heterozygous	Pathogenic	*novel*	Bilateral pachygyria, PNH
S12	*ACTG1*	AD, dn	NM_001614.5	c.767 G > A	(p.Arg256Gln)	Heterozygous	Likely pathogenic	*novel*	Bilateral pachygyria (P-A gradient)
S13	*ACTG1*	AD, na	NM_001614.5	c.88 G > T	(p.Val30Leu)	Heterozygous	Likely pathogenic	*novel*	Bilateral pachygyria (P-A gradient)
S14	*DCX*	AD, dn	NM_000555.3	c.910 G > A	(p.Gly304Arg)	Heterozygous	Pathogenic	*novel*	Bilateral diffuse lissencephaly (A–P gradient), SBH
S15	*VLDLR*	AR	NM_003383.5	c.323_324delGCinsAA	(p.Cys108*)	Compound heterozygous[a]	Pathogenic	*novel*	Bilateral diffuse Lissencephaly
S16	*POMGNT2*	AR	NM_032806.6	c.511 G > A	(p.Asp171Asn)	Homozygous	Likely pathogenic	ClinVar	Bilateral frontal dysgyria
S17	*LAMA2*	AR	NM_000426.3	c.5374 G > T c.8787_8793del CCATGTT	(p.Glu1792*) (p.Phe2929Leufs*34)	Compound heterozygous	Pathogenic Pathogenic	ClinVar *novel*	Bilateral temporal-mesial cobblestone
S18	*FLNA*	X-linked, dn	NM_001110556.2	c.5237C > T	(p.Pro1746Leu)	Heterozygous	Likely pathogenic	*novel*	Bilateral diffuse PNH

[a]Subject 15 resulted compound heterozygous for a 0.203 Mb deletion in 9p24.2 (2.602.123-2.805.636) (hg19), encompassing *VLDLR*.
Legend: ACMG American College of Medical Genetics and Genomics, A anterior, AD autosomal dominant, AR autosomal recessive, dn *de* novo, na not available, PMG polymicrogyria, PNH periventricular nodular heterotopia, S subject, SBH Subcortical band heterotopia, P posterior.

by Copy number variant detection tool (CLC Bio) to identify CNVs in target sequencing data. The identified CNVs were confirmed by CGH-Array.

2.5. Statistics

Continuous variables were summarized as mean, and categorical variables were summarized as frequencies and percentages. The associations between imaging and clinical features and presence of pathogenic or likely pathogenic variants at NGS panel analysis were evaluated by the Chi-squared and Fisher exact test. Statistical significance was set at $p = 0.05$. Statistical analyses were performed using SPSS Statistics software, v21 (IBM, Armonk, NY, USA).

3. Results

3.1. Demographics and clinical-neuroradiological features

Forty-six of the eighty-four subjects were males (mean age 8.2 years, range 0.08–53). The most common MCD type was polymicrogyria (n = 35, 41.7 %), followed by PNH and dysgyria/simplified gyral pattern (both n = 19, 22.6 %), lissencephaly/pachygyria (n = 15, 17.8 %), and SBH (n = 10, 11.9 %) (Fig. 1). Only two patients had cobblestone malformation. MCD were bilateral and diffuse in most cases (73/84, 86.9 %, and 54/84, 64.3 %, respectively). Fourteen patients (16.7 %) had two or more different types of MCD. Specifically, six subjects had polymicrogyria with PNH, six individuals had lissencephaly/pachygyria with SBH (n = 4), PNH (n = 1) or nodules of heterotopic neurons (n = 2), while dysgyria and/or simplified gyral pattern were associated with polymicrogyria (n = 3), SBH (n = 1) or PNH (n = 1). Among the PNH group, 42 % (8/19) were isolated. Of note, 17 % of patients with polymicrogyria had associated unilateral cerebral hemispheric hypoplasia (6/35) that was found only in this MCD type. Unilateral MCD were mostly polymicrogyria (8/11), followed by PNH (2/11) and SBH (1/11).

In addition to MCD, 50 patients (59.5 %) had other CNS abnormalities, including cerebellar malformations (n = 20), brainstem abnormalities (n = 24), enlarged/dysmorphic ventricles (n = 16), corpus callosum abnormalities (n = 34) and white matter abnormalities (n = 10). A tubulinopathy pattern, defined by the association of MCD with ventricular enlargement/dysmorphism, pons hypoplasia/asymmetry, cerebellar hypo-dysplasia, callosal abnormalities and dysmorphic basal ganglia, was identified in 6/84 subjects (7 %).

Overall, 80.9 % (68/84) of subjects with MCD had global developmental delay (GDD). Intellectual disability was diagnosed in 79.3 % of cases (46/58), being mild in 23.9 % (11/46) and either moderate or severe in 76.1 % (35/46). Autistic spectrum disorders (ASD) were observed in 6.1 % of children (4/65) while other behavioural problems were found in 17.9 % (14/78). Epilepsy occurred in 62.6 % (52/83) of cases. Median age of seizure onset was 1.75 year (lower quartile = 0.48, upper quartile = 7, interquartile range ¼ 6.52). Four patients had neonatal seizures (4/52, 7.7 %), while 23 subjects (23/52, 44.2 %) had infantile-onset epilepsy. Microcephaly was detected in 27.3 % of our cohort (18/66). Demographics, clinical features and neuroimaging data of all patients are summarized in Supplemental material Table 2.

3.2. Targeted gene sequencing

Overall, the diagnostic yield in our cohort was 21.4 % (18/84). In particular, we identified 19 pathogenic or likely pathogenic single-nucleotide variants in eleven genes, including *PAFAH1B1 (LIS1)* (n = 3), *TUBA1A* (n = 3), *DYNC1H1* (n = 3), *ACTG1* (n = 2), *TUBB2B* (n = 1), *TUBB3* (n = 1), *DCX* (n = 1), *FLNA* (n = 1), *LAMA2* (n = 1), *POMGNT2* (n = 1) and *VLDLR* (n = 1). Variants in *LAMA2* were in the compound heterozygous state, thus accounting for a total number of 19 mutations in 18 subjects. Fourteen of these variants were *novel*, i.e. absent in public databases and previously unreported. Of note, the patient harbouring a single pathogenic variant in *VLDLR* also carried a deletion encompassing *VLDLR* on the other allele. We also identified a variant affecting the canonical splice-site of the *ERMARD* gene in a female with PNH. This variant, maternally inherited, was classified as a variant of unknown significance (VUS). The genetic findings of our patients are summarized in Table 1.

3.3. Genotype-phenotype associations

The diagnostic yield widely varied among different MCD types. Pathogenic variants were relatively frequent in patients with lissencephaly/pachygyria (60 %, 9/15) ($p = 0.001$), cobblestone malformation (50 %, 1/2), SBH (40 %, 4/10) and dysgyria/simplified gyral pattern (27.8 %, 5/18). The genetic cause of polymicrogyria and PNH was elucidated only in 5.7 % (2/35) and 5.3 % (1/19), respectively. None of the individuals with unilateral MCD harboured pathogenic variants, including those with polymicrogyria and unilateral cerebral hypoplasia.

Pathogenic variants in *PAFAH1B1* were the most represented among individuals with lissencephaly (n = 3), followed by *DYNC1H1* (n = 2), *DCX* (n = 1) and *VLDLR* (n = 1). Two pathogenic variants in *ACTG1* were identified in two patients with pachygyria.

Mutations in tubulin genes, i.e. *TUBA1A* (n = 3), *TUBB2B* (n = 1), and *TUBB3* (n = 1), were detected only in patients with imaging features consistent with tubulinopathies. Conversely, one patient with a typical tubulin-related imaging pattern resulted negative at NGS panel. Two compound heterozygous variants in *LAMA2* and a homozygous pathogenic variant in *POMGNT2* were identified in two subjects with muscular dystrophy-dystroglycanopathies. Of note, only one of them displayed the typical cobblestone malformation at MRI. Finally, only one subject who had isolated bilateral PNH harboured a pathogenic variant in *FLNA*.

The associations between clinical-neuroradiological features and positive genetic results are summarized in Table 2. Individuals with diffuse MCD were more likely to harbour pathogenic variants in genes included in the panel ($p = 0.002$), whereas there was only a tendency in patients who had two or more different MCD types ($p = 0.079$). Subjects with MCD and other associated CNS malformations, such as basal ganglia abnormalities, cerebellar and brainstem hypoplasia or ventriculomegaly, were more likely to have positive genetic test results (30 %, 15/50, $p = 0.029$). Among these, brainstem abnormalities were more frequently associated with positive germline variations (13/23, 54.2 %, $p < 0.001$), together with ventricular enlargement/dysmorphism (7/16, 43.8 %, $p = 0.036$). When clinical characteristics were compared, we found that moderate to severe intellectual disability was more commonly found in patients with positive genetic testing ($p = 0.044$). Conversely, no significant associations have been found between positive genetic findings and neuropsychiatric problems, neurological abnormalities, epilepsy, dysmorphisms, and extra-CNS malformations.

4. Discussion

The present target gene panel enabled to uncover the molecular diagnosis in one-fourth of our patients (21.4 %), in line with a recent study by Lee et al. [16], despite some differences in the selecting criteria of the two studies. Of note, germline pathogenic variants were more likely to be identified if MCD were diffuse, reaching a diagnostic yield of 33.3 %, while the diagnostic rate rose to 51.4 % when MCD were also bilateral and associated with other CNS abnormalities. These results are similar to several diagnostic rates of NGS used in the clinical setting for other neurodevelopmental disorders [9–13], suggesting that gene panel sequencing might be a powerful diagnostic tool also in subjects with MCD.

Our findings confirm a high genetic heterogeneity in patients with MCD. In particular, we observed a variable diagnostic rate among

Table 2
Clinical and neuroimaging features associated with positive results at MCD NGS panel.

Clinical and neuroimaging features	Pathogenic/likely pathogenic variants	P value
Clinical features:		
Positive Family history (18/83)	2/18 (11.1 %)	0.355
Consanguinity (5/82)	1/5 (20 %)	1
Global developmental delay (68/81)	17/68 (25 %)	0.281
Intellectual disability (46/58)	12/46 (26 %)	0.261
Moderate and severe ID (35/46)	12/35 (34.3 %)	**0.044**
ASD (4/65)	1/4 (25 %)	1
Other psychiatric disorders (14/78)	2/14 (14.3 %)	0.722
Focal neurological deficits (42/65)	12/42 (28.6 %)	0.38
Epilepsy (52/83)	10/52 (19.2 %)	0.584
Microcephaly (18/66)	5/18 (27.8 %)	0.531
Extra CNS malformations (17/84)	2/17 (11.8 %)	0.343
Significant Dysmorphisms (30/76)	5/30 (16.7 %)	0.569
Neuroimaging features:		
Bilateral MCD (73/84)	18/73 (24.7 %)	0.11
Diffuse MCD (54/84)	17/54 (31.5 %)	**0.002**
Multiple MCD (15/84)	6/15 (40 %)	0.079
PMG (35/84)	2/35 (5.7 %)	**0.003**
Cobblestone malformation (2/84)	1/2 (50 %)	0.385
Lissencephaly/Pachygyria (15/84)	9/15 (60 %)	**0.0001**
PNH (19/84)	1/19 (5%)	0.217
SBH (10/84)	4/10 (40 %)	0.210
Dysgyria/simplified gyral pattern (19/84)	6/19 (31.6 %)	0.223
Unilateral hemispheric hypoplasia (6/84)	0/6 (0%)	0.333
Other CNS malformations (50/84)	15/50 (30 %)	**0.029**
Basal ganglia malformations (12/84)	5/12 (41.7 %)	0.120
Cerebellar malformation (20/84)	7/20 (35 %)	0.119
Brainstem anomalies (24/84)	13/24 (54.2 %)	**< 0.0001**
Enlarged/Dysmorphic ventricles (16/84)	7/16 (43.8 %)	**0.036**
Callosal anomalies (34/84)	11/34 (32.4 %)	0.059
White matter anomalies (10/84)	3/10 (30 %)	0.440

Legend: ASD autistic spectrum disorders, CNS central nervous system, ID intellectual disability, MCD malformation of cortical development, PMG polymicrogyria, PNH periventricular nodular heterotopia, SBH subcortical band heterotopia. Significant statistical results are indicated in bold fonts.

different MCD types. Indeed, the diagnostic yield was higher in patients with lissencephaly/pachygyria, cobblestone malformations, SBH and dysgyria, compared to PNH and polymicrogyria. Similarly, Lee et al. [16] found a high diagnostic rate in individuals with lissencephaly and SBH, suggesting that the genetic background of these malformations has been better explored.

In details, pathogenic variants in *PAFAH1B1* were the most represented among subjects with lissencephaly, similar to previous studies [20]. These were followed by *DYNC1H1* mutations, previously reported only in a minority of lissencephaly cases (i.e. 3% of subjects), showing predominantly a posterior-anterior gradient [20]. Although DCX mutations account for about 20 % of lissencephaly (mostly males) and 25–85 % of sporadic SBH cases [21,22], we were able to identify only one DCX mutation in a subject with lissencephaly, SBH and bilateral nodules of heterotopic neurons in temporal lobe white matter. Of note, the diagnostic rate significantly varied between SBH associated with other cortical malformations and isolated SBH. Indeed, we found germline pathogenic variants in the *PAFAH1B1*, *TUBB3, DYNC1H1* and *ACTG1* genes when SBH was associated with either lissencephaly/pachygyria or neuronal heterotopias, whereas we did not identify any pathogenic variant in isolated SBH cases. As previously observed by Hehr et al. [23], these data suggest that, at least in part, SBH may be due to somatic DCX mutations, undetectable in DNA extracted from peripheral blood cells.

Both pathogenic *ACTG1* variants were found in two individuals with pachygyria, with opposite gradients of severity (i.e. anteroposterior and posteroanterior), and partial agenesis of corpus callosum. One subject also showed vermis and pons hypoplasia. Of note, none of them had congenital abnormalities or dysmorphisms suggestive of Baraitser-Winter syndrome (MIM 614583), confirming the high clinical heterogeneity of this neurodevelopmental disorder [24].

One patient with cobblestone malformation harboured compound pathogenic variants in *LAMA2*. Cobblestone malformation is a recognizable neuronal migration disorder characterized by protrusions of neurons beyond the first cortical layer at the pial surface of the brain [25]. It is usually seen in association with a wide range of muscular dystroglycanopathies, including Fukuyama muscular dystrophy, muscle-eye-brain disease and Walker-Warburg syndrome, and more than 20 causative genes have been identified so far [26,27]. The genetic heterogeneity and the overlapping clinical phenotype of the related conditions make cobblestone malformation suitable for gene panel investigation. Interestingly, we also identified a homozygous pathogenic variant in *POMGNT2,* a further dystroglycanopathy gene (MIM 614830), in a subject showing typical clinical and posterior cranial fossa features of this neuromuscular disorder [28] but in the absence of cobblestone malformation. Remarkably, the absence of this neuroradiological hallmark has been reported only in another patient with POMGNT2-related disorder [29].

Concerning the PNH group, we were able to elucidate the genetic cause only in one subject with isolated PNH, who harboured a pathogenic variant in *FLNA*. Besides, in a female presenting with PNH and CC hypodysgenesis, we identified a splice variant (c.1317 + 1G > A) in the *ERMARD* gene. PNH along with polymicrogyria, hydrocephalus, cerebellar malformations and callosal abnormalities, is part of the phenotypic spectrum of 6q27 deletion syndrome, encompassing the *ERMARD* gene [30] that has been reported mutated only in a minority of PNH cases [31]. However, constraint metrics in gnomAD suggested a tolerance to loss-of-function variations (pLI = 0). Therefore, we classified this variant as a VUS according to the ACMG guidelines. Overall, our findings reflect the wide clinical and genetic heterogeneity observed in various PNH cohorts [32,33]. Indeed, isolated PNH is mostly due to FLNA mutations in subjects with bilateral PNH and epilepsy [34]. In contrast, PNH associated with other neuronal migration defects (e.g. polymicrogyria and lissencephaly), is more likely linked to mutations in other neuronal migration-related genes [23,35].

The lowest diagnostic rate was found in subjects with polymicrogyria (5.7 %). Indeed, the genetic cause was elucidated only in two of the 35 subjects with polymicrogyria, who harboured a TUBB2B and a DYNC1H1 mutation, respectively. A low diagnostic rate of 13 % was also found in the polymicrogyria cohort of Lee et al. [16]. Polymicrogyria is characterized by abnormal cortical lamination and folding pattern resulting in an excessive number of small gyri. It accounts for approximately 20 % of all MCD [36] and more than 50 causative genes have been identified so far [37]. Polymicrogyria and other neuronal migration defects like PNH, could also be due to acquired etiologies, such as intrauterine infection [38,39], vascular insults [40] and exposure to teratogens during pregnancy [41], although the exact rate of these nongenetic causes remain elusive. Remarkably, all individuals with unilateral MCD, mostly including polymicrogyria, yielded negative genetic testing, pointing to possible underlying acquired etiologies or somatic mutations.

In our series, the coexistence of other CNS abnormalities was associated with positive findings at gene panel testing. In particular, brainstem abnormalities and ventricular enlargement/dysmorphism were more frequent in positive cases, followed by corpus callosum abnormalities, cerebellar malformations, basal ganglia abnormalities and white matter abnormalities. Notably, ventriculomegaly was also more frequent among positive cases in the case series described by Lee et al. [16]. Moreover, a third of subjects with ventricular enlargement/dysmorphism from our cohort harboured pathogenic variants in tubulin genes, confirming that the dysmorphic appearance of the frontal horns is very important to suspect these conditions, especially when

associated with other features, such as enlarged tectum, pons hypoplasia/asymmetry, cerebellar hypo-dysplasia, callosal abnormalities and dysmorphic basal ganglia [42,43]. To date, genes encoding different isotypes of tubulin, namely alpha-tubulin (*TUBA1A*), beta-tubulin (*TUBB2A*, *TUBB2B*, *TUBB3*, *TUBB4A*, *TUBB*) and gamma-tubulin (*TUBG1*) have been linked to a wide range of malformations of cortical development, including lissencephaly/microlissencephaly, polymicrogyria, dysgyria and simplified gyral pattern [43]. Of note, in the present series, all but one individuals with a neuroimaging pattern consistent with a tubulinopathy harboured pathogenic variants in one of the tubulin genes, namely *TUBA1A, TUBB2B* and *TUBB3*, thus suggesting that tubulinopathies are an additional MCD pattern for which gene panel sequencing might be employed as a first diagnostic work-up. The negative result in one subject with a typical neuroradiological pattern of tubulinopathy may be related to a possible no optimal coverage for a specific genomic region. Alternatively, a possible pathogenic variant may lie in a deep intronic region not investigated by our target gene panel. Furthermore, we cannot rule out that this neuroradiological presentation could be due to mutations in a novel tubulin-like gene.

Regarding clinical features, in our cohort, moderate/severe ID was associated with positive genetic findings, suggesting that individuals with a more severe phenotype in terms of brain abnormalities and ID severity would benefit of MCD gene panel as a first line of investigation. Conversely, we did not find any association between genetic findings and extra-CNS malformations, epilepsy, neuropsychiatric problems and dysmorphisms.

4.1. Limitations

Our study has several limitations. First, a target gene panel allows analyzing exclusively genes included at the time of the study design, making impossible to explore newly discovered genes or deep intronic pathogenic variants, as instead ensured by whole-exome and genome sequencing (WES, WGS). Although gene panels will be replaced in the next future by WES-WGS in a clinical setting, we showed that at least some MCD types would benefit from a gene target analysis as a first diagnostic work-up. This could be pursued through a bioinformatics customized panel, periodically updated according to the most recent literature review to seek for pathogenic variants among the selected genes in the WES-WGS dataset. A further limitation is related to the fact that parental samples were not available for all subjects included in the study and we may have missed some *de novo* variants, underestimating the diagnostic yield of our panel. Moreover, we did not explore possible somatic mutations in other tissues. The low diagnostic rate in patients with isolated SBH might be explained by underlying somatic mutations, thus suggesting that buccal swab should be performed in all cases yielding negative germline results. In addition, the lack of detailed EEG studies in several patients may have limited the phenotypic description and genetic correlations. Lastly, this study might have been hampered by the use of different MRI protocols . However, 3D T1-weighted images were always available, poor MRI imaging was an exclusion criteria, and images were reviewed by an experienced pediatric neuroradiologist, thus improving MCD detection and characterization.

4.2. Conclusions

Overall, our findings suggest that customized gene panels may support the diagnostic work-up of MCD, especially when these abnormalities are diffuse, bilateral and associated with other CNS malformations. In particular, cobblestone malformations, lissencephaly/pachygyria, tubulinopathies and SBH may benefit more than other MCD types of a target gene panel as a first diagnostic tier. Larger series are needed to further assess the best approach for the genetic diagnosis of MCD, also considering alternative or complementary genetic studies such as WES and WGS. The integration of phenotypic information with molecular data in larger studies might also help to elucidate novel pathomechanisms, especially for those MCD that still have a low diagnostic rate.

Ethical standards

This was a multicenter, retrospective/prospective cohort study, coordinated by the Gaslini Children's Hospital (Genoa, Italy). This study has been performed following the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

Acknowledgements

This work was developed within the framework of the DINOGMI Department of Excellence of MIUR 2018-2022 (law 232 of 2016). We acknowledge the support from Enea-'Eroi in ricerca onlus' (www.eneaonlus.org).

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.seizure.2020.05.023.

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