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ORIGINAL RESEARCH

Neuroradiologic, clinic and genetic characterization of cerebellar heterotopia: a pediatric multicentric study

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ABSTRACT

Background and purpose: Cerebellar heterotopia (CH) is a neuroradiological abnormality poorly reported and investigated in the literature. It can be observed as an isolated finding, but it has been mainly reported in the context of cerebellar dysgenesis and in syndromic conditions. The aim of this study is to provide a comprehensive neuroradiological, clinical, and genetic characterization of a cohort of pediatric patients with cerebellar heterotopia.

Materials and methods: Patients with a diagnosis of CH were systematically selected from the neuroimaging databases of the four Italian Centers participating in this retrospective study. For each patient, information regarding demographic, clinical, genetic and neuroradiological data were collected.

Results: Thirty-two pediatric patients were recruited and subdivided into two groups: patients with isolated CH and/or cerebellar malformations (n= 18) and patients with CH associated with cerebral malformations (n=14). Isolated CH consistently showed a peripheral subcortical localization in the inferior portion of cerebellar hemispheres, with either unilateral or bilateral distribution. Ten patients belonging to the second group had a diagnosis of CHARGE syndrome, and their nodules of CH were mainly but not exclusively bilateral, symmetric, located in the peripheral subcortical zone and in the inferior portion of the cerebellar hemispheres; the remaining 4 patients of the second group, showed either bilateral or unilateral CH, located in both peripheral cortex and deep white matter and in the superior and inferior portions of cerebellum. Patients with isolated CH showed high prevalence of language development delay; neurodevelopmental disorders were the most represented clinical diagnoses. Recurring features were behavioral problems and motor difficulties. A conclusive genetic diagnosis was found in 18/32 patients.

Conclusions: We found distinctive neuroradiological patterns of CH. Genetic results raise the possibility of a correlation between cerebellar morphological and functional developmental disruption, underscoring the importance of CH detection and reporting to orient the diagnostic path.

Abbreviations

CH Cerebellar heterotopia**; MRI** Magnetic resonance imaging **; CC** Corpus callosum**; ASD** autism spectrum disorder**; IVH** inferior vermian hypoplasia.

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Disclosure of potential conflicts of interest

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SUMMARY SECTION

PREVIOUS LITERATURE:

Cerebellar heterotopia (CH) has been previously classified as a subtype of focal cerebellar dysplasia but a standardized description of CH and associated clinical and genetic findings has never been performed. Genetic causes of CH are not known but there is evidence that CH may result from disruptions of cerebellar development. The prevalence of the finding of macroscopic cerebellar heterotopia is unknown, but such finding has been reported in the context of cerebellar dysgenesis and in syndromic conditions, such as trisomy 13, trisomy 18, and CHARGE syndrome, mainly in association with other brain malformations.

KEY FINDINGS: Distinctive neuroradiological patterns of CH have been outlined. Isolated CH consistently showed a peripheral subcortical localization in the inferior portion of cerebellar hemispheres, with either unilateral or bilateral distribution. Patients with isolated CH showed high prevalence of language development delay; neurodevelopmental disorders were the most represented clinical diagnosis.

KNOWLEDGE ADVANCEMENT: Cerebellar heterotopias are likely to be associated with at least some degree of developmental delay. The not inconsiderable genetic results raise the possibility of a correlation between cerebellar morphological and functional developmental disruption, highlighting the importance of CH detection to orient the diagnostic workout.

INTRODUCTION

Cerebellar heterotopia (CH) is a neuroradiological finding characterized by the presence of abnormal areas resembling cerebellar cortex within the cerebellar white matter. In 2002, for the first time, Patel and colleagues classified CH as a subtype of focal cerebellar dysplasia[1]. Cerebellum development is a complex process which can be summarized into four steps, namely organization of the cerebellar territory, establishment of cerebellar progenitors, migration of granule cells, and formation of cerebellar nuclei and circuitry[2]. The pathogenesis of CH is still debated and complex, but it may be described as the result of under migration of Purkinje cells, over-migration of granule cells, impairment of programmed cell death[1], or a combination of these developmental mechanisms. While genetic causes have not been clearly elucidated so far, CH may result from disruptions in normal cerebellar development. In essence, CH may arise from alterations in protein function and genetic pathways that affect cerebellar migration or cell death/survival programming. For example, dysfunction of intercellular matrix proteins or vascular endothelial growth factors

implicated in granule cell migration might play a role in CH determination [3]. Additionally, mice with pathogenic mutations in genes encoding cellular guidance proteins may exhibit phenotypes with CH [4,5]. To date, no single gene has been conclusively associated with CH.

Microscopic cerebellar cell rests have commonly been observed in fetuses and newborns, autopsies of infants with isolated visceral and skeletal malformations, infants with trisomy defects and infants with no obvious malformation, as reported by Rorke and colleagues[6]. Four histologic subtypes of cerebellar cell rests have been described and appear to be related to cerebellar localization in infants, with the majority found in children without somatic or cerebral malformations. These four types of cell rests include compact groups of mature neurons, focal and perivascular immature granule cell collections, well-organized mixed cell rests composed of all components of a cerebellar folium arranged in normal relationships (heterotopias), and poorly-organized mixed cell rests [7]. Rorke and colleagues [6] found that heterotopias were the least common malformation in all groups, with a higher percentage of trisomic infants. On the other hand, the discovery of macroscopic cerebellar heterotopia, detectable by magnetic resonance imaging (MRI), has been rarely reported to date, thus its prevalence is unknown. Although it can be observed as an isolated finding, cerebellar heterotopia has been mainly reported in the context of cerebellar dysgenesis and in syndromic conditions, such as trisomy 13, trisomy 18, and CHARGE syndrome[8,9].

It is worth noting that the presence of ectopic neurons within cerebellar white matter does not appear to cause cerebellar-specific symptoms [10]. Therefore, a clear clinical correlation of CH is not expected.

Given the absence of a standardized description of CH, here we aim at providing a comprehensive neuroradiological, clinical, and genetic characterization of a cohort of pediatric patients with CH detectable on MRI.

MATERIALS AND METHODS

Recruitment and inclusion criteria

This is a retrospective multicenter study involving four Italian Centers, namely Mondino Foundation (Pavia), Medea (Bosisio Parini Lecco), Gaslini Children's Hospital (Genova) and Ospedale dei Bambini Vittore Buzzi (Milano). Written informed consent was obtained from the parents or legal representatives of all involved patients. The study complied with institutional regulations for anonymized retrospective studies and was approved by the local ethics committee of National Neurological Institute C. Mondino (N° 0099934/21).

Patients with a diagnosis of CH were selected from the neuroimaging databases of the four Centers from 2013 to 2023. CH was defined as the presence of one or more nodules with signal intensity identical to the cerebellar cortex in all sequences within cerebellar white matter. MRI exams of all patients were reviewed by three neuroradiologists (PA , MS and FA) with 15 to 25 years of experience in pediatric neurororadiology, who confirmed the presence of CH and assessed other relevant imaging findings.

Patients were subdivided into two groups according to MRI findings:

-Group A: patients with isolated CH or cerebellar malformations

-Group B: patients with CH associated with other major malformations (i.e. cerebral malformations of cortical development, midline malformations, and brainstem malformations). Patients with cerebellar hypoplasia and mild cerebral dysmorphism not meeting criteria for malformations were classified under Group A.

Information regarding demographic, clinical, neuroradiological, and genetic data were collected for each patient.

Clinical examination

A standardized evaluation of clinical presentation was performed using clinical records.

The clinical data collection included: developmental history, neurological and general examination, dysmorphological evaluation, screening for extra-neurological involvement, cognitive/developmental assessment, electroencephalogram.

Genetic testing

Genetic testing i.e. karyotype, CGH array, whole exome sequencing (WES) have been variably performed in the patients, as part of their diagnostic workout. Only (likely) pathogenic variants according to American College of Medical Genetics and Genomics (ACMG) Classification guidelines were considered.

Imaging studies

Brain MRI studies were performed using both 1.5T (7 patients) and 3T (25 patients) scanners with at least T1 and T2-weighted sequences with a slice thickness of 3 mm or less. All subjects had T2-weighted sections in at least 2 planes (axial and coronal) with a slice thickness of 1.5/3 mm. T2 FLAIR sequences were acquired in 31/32 patients, with 3D sequences in 11 cases. DWI/DTI sequences with 3 plane reconstructions were available in 31/32 patients. SWI or T2*-FFE sequences were available in 21 subjects. All available sequences and planes were used for CH evaluation.

The number of nodules, their symmetry or asymmetry, and location (peripheral subcortical

or in the deep white matter; in the superior or inferior cerebellum with respect to a plane passing through the horizontal fissure) were recorded in all patients.

Associated cerebral dysmorphisms or malformations were defined, also according to biometric reference measures[11,12]. Corpus callosum anomalies classified in accordance with Garel et al. [11]; a corpus callosum was considered thin or thick if its measurements were beyond 2 standard deviations from the normal values for age provided in the paper. A band-like corpus callosum was defined as having a uniform thickness along its entire course and lacking an isthmic indentation.

Statistics

Quantitative data were presented as mean and standard deviation or median and interquartile range, and categorical data, as frequencies and percentages.

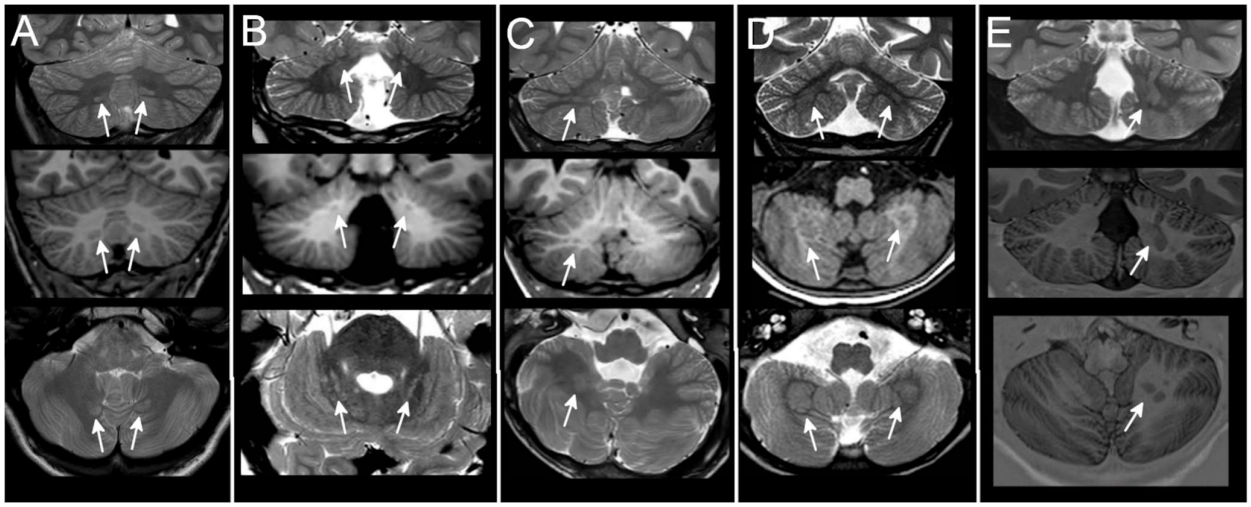

FIG 1. **Pattern of cerebellar heterotopias**

In the top row, coronal T2 sections are shown; in the middle row, coronal and axial T1 sections are presented; while in the bottom row, axial T2 sections are illustrated, except for the patient in E, where an axial IR section is shown.

Inferior bilateral (A), and superior bilateral (B) small nodules in the peripheral subcortical white matter are shown. Bigger nodules could be unilateral (C) or bilateral (D) in the deep white matter of the inferior cerebellum or they could be located in both the inferior and superior part of the hemisphere (E). In all sequences, the cerebellar heterotopia nodules exhibit signal intensity isointense to the cortex and show no signs of edema.

RESULTS

The recruited cohort included 32 patients, of whom 9 were females (28%) and 23 were males (72%). The mean age was 9.2 years (range 1 to 18 years). Demographic data and summarized clinical, genetic and imaging features are shown in Online Supplemental Data, while detailed clinical findings for each patient are reported in supplementary online data, Online Supplemental Data.

Considering the entire cohort, heterotopic nodules were mostly located in the peripheral subcortical white matter (n=28/32; 87,5%) and typically in the inferior part of the cerebellar hemispheres (n=27/32; 84%) *Figure 1*. In one case, nodules were detected in the deep white matter and in three patients in both deep and peripheral subcortical regions. Superior location was found in four patients and superior plus inferior distribution in only one. Nodules were more frequently bilateral (n=20/32; 62,5%) and usually had a lentiform or ovoid shape, with overall length of a few millimeters. In very few cases they were lobulated with a diameter up to 10 mm. The lobulated appearance of larger lesions may be due to the presence of closely situated small nodules. In other cases, the nodules were generally limited to a maximum of 1 or 2 per patient.

According to the associated imaging phenotype, eighteen patients were assigned to group A and fourteen to group B.

Group A (n=18)

Clinical findings:

In this group, 14/18 (78%) patients had a history of developmental delay of different extent. In particular, 12/18 (67%) reported language delay. Seven (39%) patients received a diagnosis of

neurodevelopmental disorder other than global developmental delay and language disorder, including specific learning disorder, autism spectrum disorder (ASD), intellectual disability, developmental coordination disorder. Abnormal behavior, including social problems and emotional difficulties were present in the majority of the patients (12/18; 67%). Five (27%) patients presented clumsiness/ coordination problems. Specific cerebellar signs, namely oculomotor apraxia and dysmetria, and ataxic gait were observed only in two patients.

Electroencephalogram recordings showed nonspecific, non-epileptiform generalized abnormalities (n=3) and epileptiform abnormalities, associated with either focal or generalized epilepsy (n=2).

Extra-neurological signs and symptoms were rarely detected (n=5) and included nonspecific facial dysmorphisms, growth deficiency, cardiovascular and appendicular malformations.

Genetic results:

Fourteen (78%) out of 18 patients underwent genetic testing (either karyotype, Fragile X expansion evaluation, array comparative genomic hybridization and/or whole exome sequencing) and overall a genetic diagnosis was reached in five patients. Of these, four carried pathogenic *de novo* heterozygous variants in autosomal dominant genes: *ANKRD11* (c.2404_2407del; p.Leu802LysfsTer60), *ANKRD11* (c.2398_2401del; p.(Glu800Asnfs*62), *KDM6B* (c.2705del; p.Leu902HisfsTer13); and *PAK1* (c.A427G: p.Met143Val); and the remaining one had a 1,5Mb *de novo* deletion of chromosome 1p35-1p34.3.

The patients carrying a pathogenic variant of *PAK1* gene, *KDM6B* and *ANKRD11* have been previously published[13].

Imaging findings:

Nodules of cerebellar heterotopia were either monolateral (n=8; 44%) or bilateral with symmetric distribution (n=10; 55%). Localization was peripheral subcortical and in the inferior portion of cerebellar hemispheres in all patients. *See* Online Supplemental Data

Eight patients presented with single or multiple brain dysmorphisms / minor malformative findings associated with CH. Minor dysmorphisms of corpus callosum were observed in seven (39%) patients, including thin (n=2), thick (n=3), dysmorphic (n=1) or band-like (n=1) shape. Five (28%) patients had mild vermian hypoplasia, mainly involving the inferior vermis in three *Figure 1, Supplementary material*. Additional observed findings were a small posterior fossa (n=1), a small area of periventricular white matter damage with cavitation (n= 1), and platybasia (n=1). *No signs of cerebellar hemorrhage were identified.*

Group B (n=14)

Clinical findings:

Ten patients had a clinical diagnosis of CHARGE syndrome. The remaining four patients had the following clinical diagnosis: syndromic intellectual disability (n=2); epileptic encephalopathy (n=1) and Down syndrome (n=1). All patients had a history of developmental delay. Specific cerebellar signs were present in only one patient. An extra-neurological involvement was found in all patients.

Genetic results:

In the 10 patients with CHARGE syndrome, genetic testing confirmed the presence of pathogenic variants in the *CHD7* gene. Of the remaining four cases, three received a genetic diagnosis, including

a pathogenic heterozygous variant in the *DYNC1H1* gene (c.10247_10279dup;p.Leu3416_Asn3426dup), a complex chromosomal rearrangement (4q34-qter monosomy and 13q231-qter trisomy), and trisomy 21.

Imaging findings:

In ten patients with CHARGE syndrome, nodules of CH were mainly but not exclusively bilateral, symmetrical, and mainly located in the peripheral subcortical white matter and in the inferior cerebellar hemispheres.

In four patients, CH was associated with complex brain malformations: one showed dysgyric cortex, vermian hypoplasia, ectopic neuro-hypophisis, small adenohypophysis, thin optic nerves, thick lamina quadrigemina, and abnormal inner ear structures; the second one had cerebellar dysplasia, periventricular nodular heterotopia and dysmorphic temporal horns; a third presented polymicrogyria, heterotopic subependymal nodules, malrotated hippocampi and dysmorphic basal ganglia; finally, the fourth patient had pons hypoplasia. *Figure 2 and Figure 3 Supplementary material*.

In these cases, CH was found to be either bilateral or unilateral, located in both peripheral subcortical and deep white matter and in the superior and inferior portions of cerebellum. *Figure 1 supplementary material*. No signs of cerebellar hemorrhage were identifie*d*.

DISCUSSION

CH has received poor attention in previous hindbrain malformation classifications [8,16] . Lack of awareness of this easily overlooked imaging finding may be the primary driver behind lack of identification.

This retrospective multicenter study provides a description of CH neuroradiological patterns in a cohort of pediatric patients, clinically and genetically characterized. Such comprehensive evaluation of CH imaging features and associated clinical characteristics and genotype represents a first attempt to highlight the importance of this finding in the diagnostic pathway. In fact, neurodevelopmental and functional outcome of several cerebellar malformations is far from being defined and the phenotypic spectrum is often broad, ranging from normal or near-normal functioning to profound disability for a given malformation [17]. Like in other brain regions, cerebellar neuronal migration relies on appropriate spatiotemporal patterns [18]. The migration process takes place both prenatally and postnatally and is controlled by several molecules, leading to the establishment of elaborate compartments and circuitry.

Wright and colleagues in 2019 [9] described CH as a recurrent finding in a cohort of 35 patients with CHARGE syndrome, with a prevalence of 77%. Moreover, CH has been previously reported in patients with trisomy 21 or trisomy 18 and rarely associated with other genetic conditions such as Turner syndrome [19,20], ornithine carbamoyltransferase deficiency [21] , MKS3-related Meckel syndrome [22], occipital horn syndrome[23], OPHN1-related syndrome [24] and Fryns syndrome [25] in single patients. Importantly, cerebellar hemorrhages, should be excluded since these could be mistaken for HC, especially in premature infants; in the presented cohort no history of cerebellar hemorrhage, no imaging signs of blood products, and only one patient was born preterm.

In our cohort, clinical findings of patients with CHARGE syndrome are in line with the literature, showing a pattern of CH characterized by recurrent appearance and location. More precisely, the distribution pattern mainly resulted in a bilateral, symmetrical and peripheral subcortical disposition, typically located in the inferior portion of cerebellar hemispheres [9]. CHARGE syndrome is part of the CHD7- related disorder spectrum, caused by point mutations or deletions of the *CHD7* gene (*MIM *608892*) [26], which is known to be involved in embryonic development. Indeed, Reddy

and colleagues demonstrated a critical role for CHD7 in the formation, differentiation and migration of neural crests [27]. In 2013 Yu et al [28]. showed that reduced FGF8 expression, which is a critical signal for early cerebellar development, results from CHD7 haploinsufficiency and is responsible for cerebellar vermis hypoplasia, a common finding in CHARGE. Moreover, during earlier stages of cerebellar development, CHD7 regulates the accessibility, histone acetylation and RNA Polymerase II binding at gene enhancers implicated in cerebellar morphogenesis [27]. Collectively, these data suggest that CHD7 governs multiple phases of cerebellar development through the accurate regulation of gene transcription; folding and migration anomalies may arise from these deregulated cellular processes that consequently reorganize themselves. Thus, unsurprisingly, CH is well represented in this genetic condition, together with vermian hypoplasia and cerebellar dysgenesis which are other common findings.

In the present study, when CH was associated with other complex brain malformations outside the CHARGE spectrum, it was coarse, localized in the deep white matter, and

distributed both in the superior and inferior portions of cerebellum. Associated malformations involved both infra- and supra-tentorial brain.

A relevant contribution of the present study is the description of patients with isolated CH or CH combined with minor malformative/dysmorphic findings. In these cases, CH consistently showed peripheral subcortical localization in the inferior portion of cerebellar hemispheres, with either unilateral or bilateral distribution. Up to 35% of these patients had corpus callosum dysmorphisms, and a similar percentage showed vermian hypoplasia, mainly involving the inferior vermis. Corpus callosum dysgenesis or dysmorphisms can often be found in association with other minor malformations or brain dysmorphisms such as periventricular heterotopia [29] in patients with neurodevelopmental disorders and variable clinical presentation.

Inferior vermian hypoplasia (IVH) is characterized by a volumetric reduction of the inferior portion of the cerebellar vermis and patients with isolated IVH have been reported to show delayed development, gross and fine motor disabilities, as well as social-communication deficits, and behavioral problems [17].

In our cohort, patients with isolated CH showed high prevalence of developmental delay, with an even greater occurrence of language development delay. Unlike isolated cerebral heterotopias, which can be completely devoid of clinical correlates, cerebellar heterotopias are thus likely to be associated with at least some degree of developmental delay.

Neurodevelopmental disorders were the most represented clinical diagnoses, including intellectual disability, autism spectrum disorder, and specific learning disorder. Recurring features in the cohort were also behavioral problems including social skills impairment, and motor difficulties. Of note, specific cerebellar signs were observed only in two patients and only one patient showed extra neurological malformations. Indeed, specific cerebellar signs were not expected to be determined by the presence of CH; conversely, the imaging finding of CH can provide further insight to comprehensively assess neurodevelopmental disorders, considering the impairment of specific cerebro-cerebellar circuits that may be relevant, for instance, to the development of ASD, language and behavioral issues. And, according to the presented results, cerebellar heterotopias are most often associated with at least some degree of developmental delay

A direct comparison of clinical features of group A and group B was not carried out for a two-fold reason. Firstly, considering the complexity, the localization, and extent variability of associated brain malformations of group B as opposed to the recurrent finding of almost isolated CH in group A, we assume that in the former major events of disruption are involved in the developmental process, thus unsurprisingly leading to composite and heterogeneous syndromic phenotypes. Moreover, group B was mainly represented by the group of CHARGE patients, who do have a well-known spectrum of

clinical features and the remaining 4 patients were then few and with such a heterogeneous neuroimaging finding that a mere comparison with group A was deemed as trivial.

The genetic etiology of many brain malformations remains poorly understood and the yield of genetic testing has been widely reported to be low in patients with minor cerebellar malformative findings [30]. Nevertheless, access to standardized extensive genetic testing is not always available and this could negatively bias this outcome. The same is applicable to our cohort, in which 76% patients with isolated CH underwent some genetic testing, but a detailed assessment inclusive of whole exome sequencing was performed only in 35% representing one considerable limitation of this study, being it retrospective. Overall, a genetic diagnosis was reached in 23% of patients.

A novel *de novo* frameshift variant in the *KDM6B* gene was found in a patient with peripheral subcortical monolateral inferior CH and neurodevelopmental disorder. *KDM6B (MIM *611577)* pathogenic variants have been recently described as associated with a rare "neurodevelopmental disorder with coarse facies and mild distal skeletal abnormalities' ' syndrome [29]. Neuroradiological features in only three single patients have been reported, showing mild cerebellar cortical and subcortical atrophy and/or paracerebellar ventricular enlargement [31]. *KDM6B* is highly expressed in cerebellar neurons, where it plays an important role in neuronal migration during development, as well as in non-neuronal cells such as Bergmann glia, which constitute the scaffold for neuronal migration [32,33]; moreover, the clinical features associated to this syndrome (e.g. hypotonia, ASD and ADHD traits) represent a link to possible cerebellar circuits dysfunction. A *de novo* pathogenic variant in the *ANKRD11* gene was found in two patients showing peripheral subcortical, bilateral symmetric, inferior CH, associated with inferior vermian hypoplasia and thick CC in one case and dysmorphic CC in the other. Pathogenic variants in *ANKRD11* are responsible for KBG syndrome (*MIM *611192*), typically characterized by developmental delay, short stature, and characteristic dysmorphic findings. To date, only unspecific neuroradiological defects have been reported in KBG patients, such as white matter abnormalities, corpus callosum defects, cerebellar vermis hypoplasia [34], cortical abnormalities including periventricular nodular heterotopia and, only recently, CH [15]. *ANKRD11* encodes for a protein mainly expressed in neurons and glial cells of the developing brain, playing a crucial role in proliferative processes of cortical neural precursor cells [35,36]. Moreover, *ANKRD11* contributes to the global regulation of transcription, possibly modulating the expression of other genes playing a role in regulation of cortical development [35]. Among these genes, *NCOR2 (MIM *600848)* was reported to be co-expressed in Purkinje cells with *ANKRD11* and *CHD7*. As for *CHD7, ANKRD11* might also be implicated in potential molecular and cellular mechanisms by which chromatin remodelers contribute to brain morphogenesis during development and disease [34].

A third patient with peripheral subcortical, bilateral, inferior CH, who featured intellectual disability and epilepsy, was found to carry a *de novo* pathogenic variant in the *PAK1* gene (*MIM *618158*) gene, which encodes a member of serine/threonine p21-activating kinase family that regulates cell motility and morphology and is expressed in the cerebellum as well [13]. Previous reports on associated brain MRI findings included single descriptions of periventricular and subcortical white matter abnormalities. All these observations, along with the emerging literature linking cerebellar functions to neurodevelopmental disorders such as ASD [13, 37], prompt further studies to better understand the role of the above-mentioned genes in cerebellar development.

Three of the four non-CHARGE patients belonging to group B received a genetic diagnosis. One patient carried a d*e novo* variant in the *DYNC1H1* gene (*MIM *614563*), which encodes for a cytoplasmic dynein ubiquitously expressed in the brain and with functions in intracellular motility. Neuroradiological findings in patients with *DYNC1H1* mutations include cerebellar hypoplasia and dysplasia [38]. A second patient had a complex clinical phenotype associated with trisomy 21. Cerebellar abnormalities are frequently observed in association with Down syndrome; trisomy 21 is

linked with a delay in ciliogenesis, recognized as a cause of dysregulation of neuron outgrowth and cell migration [39]. The third patient had a complex chromosomal rearrangement involving chromosomes 4 and 13, leading to both neurological and extra-neurological multiple malformations.

A clear male predominance (78%) has been observed, nevertheless up to date no X-linked mutations have been found. Even though speculative, a possible explanation could be the presence of unknown regulatory genes on X chromosomes linked to cerebellar development.

In the overall cohort CH showed a higher prevalence of inferior cerebellum localization, with a clear predominance in group A. Superior CH has been described in four patients, three of whom belong to group B. The paucity of patients may reflect the relative inferior prevalence of such condition although a precise mechanism for such an occurrence remains unknown. Patients with superior CH show a more severe neurodevelopmental phenotype although genotype-phenotype correlations cannot be drawn given the reduced number of patients and the frequent association with other structural brain anomalies. One possible explanation of this finding is that in light of the different origin of superior and inferior cerebellum, distinct cell types arising from diverse subregions of a primordium can be affected by a developmental disruption, thus leading to a different contribution to the assembly of a complex three-dimensional structure, according to long fate genetic mapping of cell movements [40] . Alternatively, the clinical phenotype may be predominantly dependent on the genetic cause of the condition. Further studies investigating functional consequences of CH on brain function are needed. The genetic diagnostic yield was predictably higher in patients presenting CH and other major brain malformations compared to isolated CH with or without associated brain dysmorphisms. Nevertheless, overall, considering the limited access to complete genetic testing for patients with isolated CH, who underwent WES in 35% of cases, the diagnostic yield registered in the latter group of patients appears to be relevant. Given the presented results, the presence of CH in association with developmental delay and or syndromic features, might thus represent a negative prognostic sign. Moreover, the presence of a superiorly located CH seems to correlate with a more severe clinical picture: this element might be relevant for the counseling. Regarding the imaging protocol for detecting CH, we believe that T1-weighted sequences in the coronal plane often raise the initial suspicion of heterotopia, which should then be confirmed in at least one other plane. Therefore, 3D T1-weighted sequences with a maximum voxel size of 1 mm are highly beneficial for diagnosis. Additionally, assessing signal intensity on other sequences (particularly T2-weighted and, if available, FLAIR) is essential to establish isointensity of the anomalies relative to the cerebellar cortex. Diffusion-weighted imaging (DWI) sequences are especially helpful in differentiating CH from small gliotic lesions that may affect the cerebellum. While the morphology and signal characteristics of heterotopias on standard morphological sequences are often highly indicative of CH, DWI can provide additional specificity, for instance, in cases of gliosis where T1 and T2 signal characteristics might overlap with those expected for gray matter. It is important to emphasize that due to the often millimetric size of cerebellar heterotopias, evaluation in multiple planes is essential to avoid missing these anomalies.

One of the main limitations of this study is indeed the incomplete availability of genetic testing in the overall cohort. Furthermore, the small sample size and retrospective study design precludes detailed correlation of CH with neurological deficits, and therefore, this association does not necessarily imply causation. Another potential limitation is the lack of advanced MRI techniques to help explore the impact of CH on the overall architecture of the brai. The acquisition of high-resolution DWI data and the use of advanced modeling methods (like Constrained Spherical deconvolution) have provided interesting information on the reorganization of white matter bundles in many supratentorial malformations [40] and could potentially be applied to this cohort to unveil structural modifications of the white matter

which were not detected by standard anatomical sequences.

CONCLUSIONS

In conclusion, we present the most extensive sample of CH to date. Not only we do confirm CH as a recurrent feature of CHARGE syndrome, highlighting its specific distribution pattern, but we also show that CH can occur both in an isolated form, with a mainly peripheral subcortical inferiorly located pattern, or associated to minor malformative findings (mainly corpus callosum dysmorphisms and vermis hypoplasia) in patients with different phenotypes of neurodevelopmental disorders. These results confirm a possible correlation between cerebellar morphological and functional developmental disruption, underlining the relevance of taking into account the presence of CH both in the diagnostic process and in the genetic counseling.

Future studies on larger cohorts, and, possibly, a more extensive and homogeneous genetic assessment will likely provide further elements to better classify and comprehend the pathogenesis and clinical correlations to this intriguing malformation.

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SUPPLEMENTAL FILES

Online Supplemental Data. Clinical, neuroradiological and genetic features of the cohort

	Group A (18 patients)	**Group B- Non CHARGE (4 patients)**	**Group B- CHARGE (10 patients)**
Clinical features			
Developmental delay	14/18 (78%)	4 /4 (*100%*)	10/10 (*100%*)
Language delay	12/18 (*67%*)	4/4 (*100%*)	10/10 (*100%*)
Intellectual disability	7/18 (*39%*)	3 / 4 (*75%*)	10/10 (*100%*)
<u>Neurological findings</u>			
Hypotonia	5/18 (*28%*)	2/4 (*50%*)	4/10 (*40%*)

Clumsiness/ coordination deficit	5/18 (*28%*)	2/4 (*50%*)	2/10 (*20%*)
Specific cerebellar signs	*2/18 (11%)* *Oculomotor apraxia and dysmetria, ataxic gait*	1 /4 (*25%*)	-
<u>Behavioral and psychiatric comorbidities</u>			
Social problems	4/18 (*22%*)	-	-
Externalizing problems	6/18 (*33%*)	-	1/10 (*10%*)
Internalizing problems	2/18 (*11%*)	-	-
Neurodevelopmental disorder	10/18 (*55,5%*) *Specific learning disorder (n=1); Autism spectrum disorder (ASD) (n=2); language disorder and developmental coordination disorder (n=1); language disorder (n=2); intellectual disability (n=3); global developmental delay (n=1)*	-	-
<u>Extra neurological findings</u>			
Facial dysmorphisms	2/18 (*11%*)	1 / 4 (*25%*)	-
Growth deficit/ retardation	3/18 (*17%*)	-	2/10 (*20%*)
Skeletal	2/18 (*11%*)	2/4 (*50%*)	4/10 (*40%*)

malformations			
Cardiovascular malformations	2/18 (*11%)*	3 /4 (*75%*)	5/10 (*50%*)
Neuroradiological features			
<u>CH pattern</u>	Monolateral peripheral subcortical inferior (n=8) (*44%*) Bilateral peripheral subcortical inferior (n= 10) (*55%*)	Peripheral subcortical and deep WM unilateral/bilateral superior / inferior (n=3) (*75%*) Bilateral peripheral subcortical Superior (n=1) (*25%*)	Deep WM bilateral superior (n=1) (*10%*) Bilateral peripheral subcortical inferior (n=7) (*70%*)
<u>Associated cerebral findings</u>			Monolateral Peripheral Subcortical inferior (n=2) (*20%*)
Vermian hypoplasia	5/18 (*28%*)	3 /4 (*75%*)	
Corpus Callosum dysmorphisms	7/18 (*39%*)	4/4 (*100%*)	10/10 (*100%*)
Other	-	-Dysgyric cortex, vermian hypoplasia, ectopic neuro-hypophisis, small adenohypophysis, thin	4/10 (*40%*) Inner ear malformation; olfactory

		optic nerves, thick lamina quadrigemina, and abnormal inner ear structures; -cerebellar dysplasia, periventricular nodular heterotopia and dysmorphic temporal horns; -polymicrogyria, heterotopic subependymal nodules, malrotated hippocampi and dysmorphic basal ganglia; -pons hypoplasia	nerve hypoplasia; coloboma; brainstem hypoplasia; Cerebellar dysplasia
Genetic features				
Pathogenic variants	5/18 (*28%*)	3 /4 (*75%*)	10/10 (*100%*)	

ADHD= Attention Defict Hyperactivity disorder ; ASD= Autism spectrum disorder; CH= Cerebellar Heterotopia; WM= White matter

Online Supplemental Data. Neuroradiological findings of the cohort

Patient	Group	CH Pattern	Corpus Callosum	Vermis	Other findings
1	A	Peripheral subcortical monolateral, inferior	-	Inferior vermian hypoplasia	-
2	A	Peripheral subcortical bilateral	-	-	-

		symmetric, inferior			
3	A	Peripheral subcortical bilateral symmetric, inferior	Thick	Vermian hypoplasia	Platybasia
4	A	Peripheral subcortical bilateral symmetric, inferior	Thin	Inferior vermian hypoplasia	-
5	A	Peripheral subcortical , monolateral , inferior	-	-	-
6	A	Peripheral subcortical l, monolateral , inferior	band-like shape	-	-
7	A	Peripheral subcortical bilateral superior	-	-	Small periventricular cavitation
8	A	Peripheral subcortical bilateral inferior	-	-	-
9	A	Peripheral	-	-	-

		subcortical monolateral (right) inferior				
10	A	Peripheral subcortical monolateral (right) inferior	-	-	-	
11	A	Peripheral subcortical monolateral (left) inferior	-	-	-	
12	A	Peripheral subcortical monolateral (left) inferior	Thin	Mild vermian hypoplasia	Small fossa	posterior
13	A	Peripheral subcortical bilateral inferior	-	-	-	
14	A	peripheral subcortical monolateral (left) inferior	-	-	-	
15	A	peripheral subcortical bilateral inferior	-	-	-	
16	A	luxtacortical bilateral inferior	Thick	-	-	
17	A	peripheral subcortical	Thick	Inferior vermis	-	

		bilateral inferior		hypoplasia	
18	A	peripheral subcortical bilateral inferior	Dysmorphic	-	-
19	B - CHARGE	Deep WM bilateral superior	Dysmorphic	Vermis hypoplasia	Inner ear malformation Brainstem hypoplasia
20	B - CHARGE	Peripheral subcortical bilateral inferior	Dysmorphic	Vermis hypoplasia	Inner ear malformation Olfactory nerve hypoplasia Brainstem hypoplasia
21	B-CHARGE	Peripheral subcortical monolateral (right) inferior	-	Vermis hypoplasia	Inner ear malformation Brainstem hypoplasia
22	B - CHARGE	Peripheral subcortical bilateral inferior	-	Vermis hypoplasia	Inner ear malformation Olfactory nerve hypoplasia Dysmorphic hippocampi Brainstem hypoplasia
23	B-	Peripheral	-	Vermis	Inner ear

	CHARGE	subcortical bilateral inferior		hypoplasia	malformation Olfactory nerve hypoplasia Ventricular dilatation
24	B - CHARGE	Peripheral subcortical monolateral (right) superior	-	Mild vermis hypoplasia	Inner ear malformation Ventricular dilatation
25	B - CHARGE	Peripheral subcortical bilateral inferior	-	Vermis hypoplasia	Inner ear malformation Olfactory nerve hypoplasia
26	B-CHARGE	Peripheral subcortical bilateral inferior	Thin CC	Vermis hypoplasia	Pontine hyoplasia Cerebellar dysplasia Hypoplastic clivus Inner ear malformations Ventricular dilatatation Persistent trigeminal artery Dysmorphism of hippocampi Olfactory nerve hypoplasia

27	B - CHARGE	Peripheral subcortical bilateral inferior	Mild thinning CC	Vermian hypoplasia	Pons hypoplasia Right carotid severe hypoplasia Inner ear malformation Olfactory nerves agenesis
28	B - CHARGE	Peripheral subcortical bilateral inferior		Vermian hypoplasia	Pons hypoplasia Skull base malformation Malformed hypothalamus Inner ear malformation Olfactory nerves agenesis
29	B	Peripheral subcortical and deep unilateral inferior	Thin	Inferior vermian hypoplasia	Dysgyric cortex ectopic neurohypophisis and small adenohypophysis thin optic nerves Thick lamina quadrigemina abnormal inner ear structures
30	B	Peripheral subcortical and deep white matter /	Thin	-	Cerebellar dysplasia Periventricular nodular

		unilateral, superior and inferior			heterotopia Dysmorphic temporal horns
31	B	Peripheral subcortical bilateral superior	dysplastic CC and small anterior commissure	mild vermian hypoplasia	Bilateral polymicrogyira; heterotopic subependymal nodules, malrotated hippocampi, dysmorphic basal ganglia
32	B	Peripheral subcortical and deep WM bilateral inferior	Thin	Vermian hypoplasia	Pons hypoplasia cerebral atrophy

Group A : isolated CH; Group B : CH associated with other brain malformations

CC: corpus callosum; CH: cerebellar hypoplasia; WM= white matter

Supplementary TABLE 1. Clinical and genetic characteristics of each patient.

Patient	***Diagnosis***	***Developmental delay***	***Language delay***	***ID (if more than 5 yo)***	***OFC***	***Facial dysmorphisms***
Patient 1	Specific learning disorder and internalizing problems	Mild	Yes	Borderline	Normal	Absent
Patient 2	AuSD	Mild	Yes	NA	Normal	Absent

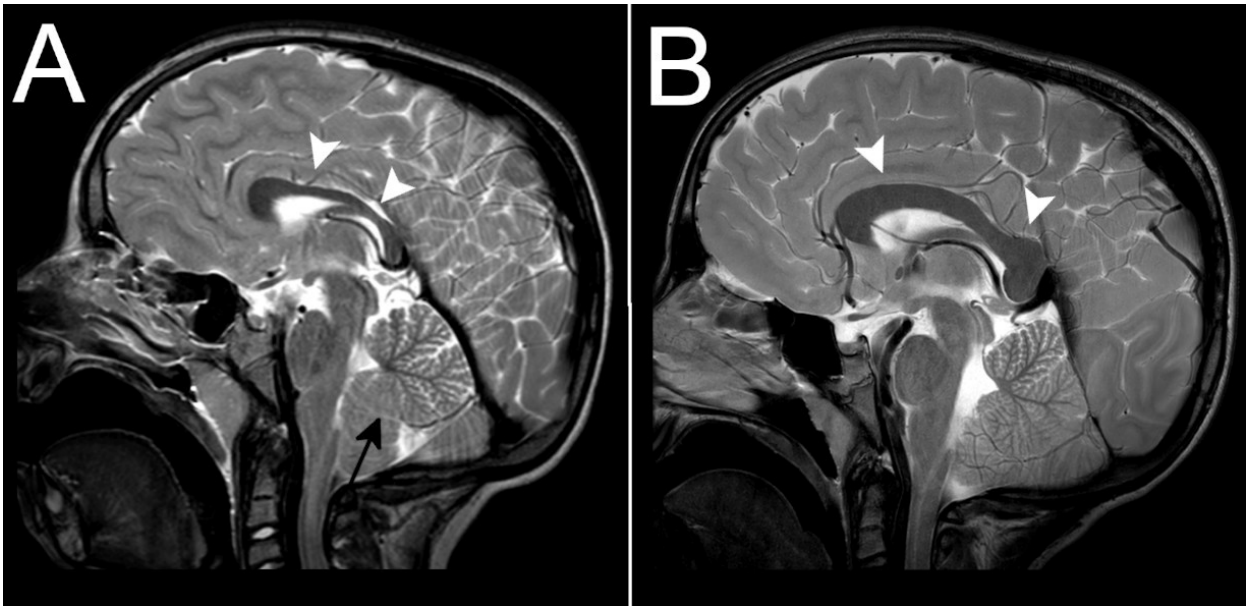
Patient 3	KBG syndrome	Mild	Yes	Poor adaptive functioning	Normal	Bushy eyebrows and micrognathia
Patient 4	Global developmental delay, oculomotor anomalies, developmental coordination disorder and language disorder in a patient with CH and 1p35-1p34.3 (1,5Mb) microdeletion	Mild	Yes	NA	Normal	Absent
Patient 5	KDM6B-related neurodevelopmental disorder	Mild	No	Absent	Normal	Absent
Patient 6	AuSD grade 3, visual impairment, nodular CH	Moderate	Yes	Severe	Normal	Absent

Patient 7	Congenital cardiac malformation	Absent	NA	NA	normal	Absent
Patient 8	Myopathy of unknown origin	Mild	yes	Mild	Normal	Absent
Patient 9	Language disorder and behavioural problems	Absent	Yes	Mild	Normal	Absent
Patient 10	Language disorder and delayed growth	Absent	Yes	NA	Relative macrocephaly	Absent
Patient 11	Intellectual disability	Mild	Yes	Moderate	Normal	Absent
Patient 12	Intellectual disability	Moderate	Yes	Moderate	Normal	Absent
Patient 13	School and social phobia	Absent	No	Absent	Normal	Absent
Patient 14	Benign epilepsy (SeLECTs)	Absent	No	No	Normal	Absent
Patient 15	Complex uropathy	Absent	No	No	Normal	Absent
Patient 16	PAK1-related disorder	Moderate	Yes	Moderate	>97th p	Absent
Patient 17	Intellectual disability	Moderate	Yes	Moderate	Normal	Absent
Patient 18	KBG syndrome	mild	Yes	NA	Normal	broad forehead, wide anterior fontanelle, low-set ears with increased posterior angulation, sparse medial eyebrows, wide nasal bridge, anteverted nares, long and smooth philtrum, (+ right hand type B polydactyly)

Patient 19	CHARGE syndrome	Moderate	Yes	Moderate	<<3rd p	Absent
Patient 20	CHARGE syndrome	Moderate	Moderate	Moderate	<3rd p	Absent
Patient 21	CHARGE syndrome	Moderate	Moderate	Moderate	Normal	Absent
Patient 22	CHARGE syndrome	Moderate	Moderate	Moderate	<3rd p	Absent
Patient 23	CHARGE syndrome	Mild	Mild	Mild	Normal	Absent
Patient 24	CHARGE syndrome	Mild	Mild	Mild	Normal	Absent
Patient 25	CHARGE syndrome	Moderate	Moderate	Moderate	Normal	Absent
Patient 26	CHARGE syndrome	Moderate	Absent	Moderate	NA	NA
Patient 27)	CHARGE syndrome	Severe	Absent	Severe	3rd p	Absent

Patient 28	CHARGE syndrome	Severe	Absent	Severe	<3rd p	NA
Patient 29	Severe delay	Severe delay	Severe	normal	Normal	saddle nose; pick feet
Patient 30	Complex cereberal malformation	Absent	Mild	Absent	Normal	Absent
Patient 31	DYNC1H1-related Epileptic encephalopathy	Severe	Severe	Severe	<<3rd p	Absent
Patient 32	Congenital cardiac malformation in Down syndrome	Moderate	Moderate	Moderate	<<3rd p	Absent

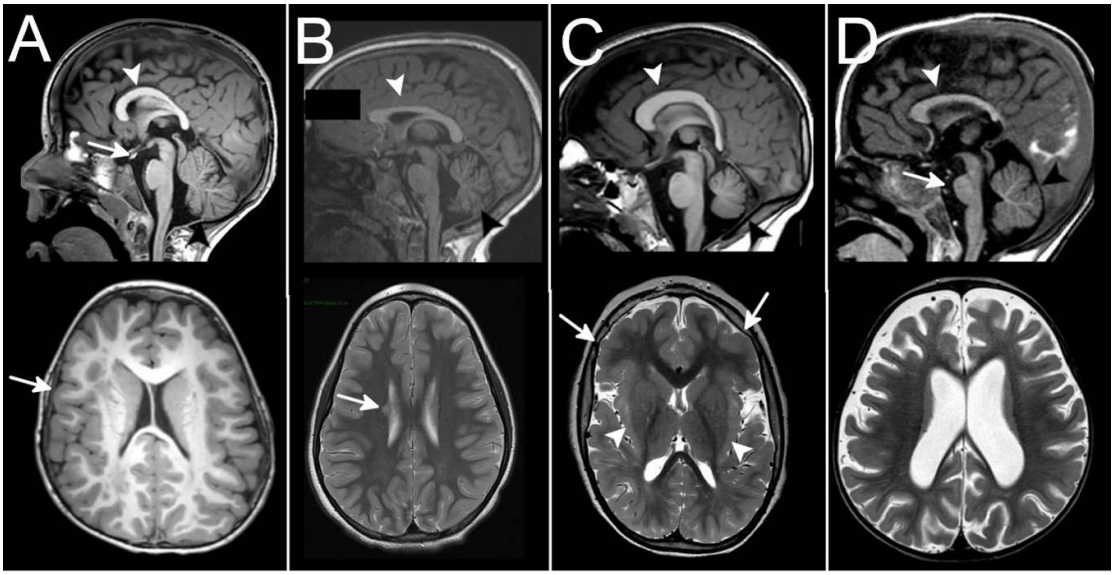
AuSD: Autism spectrum disorder; ADEM: Acute disseminated encephalomyelitis; SeLECTs: self limited epilepsy with centrotemporal spikes; OFC: occipitofrontal head circumference; PFO: patent foramen ovale; PDA: patent ductus arteriosus; ID : iintellectual disability.



Supplementary FIGURE 1. Associated brain findings in group A

T2-weighted sagittal sections

Patients of group A could show minor dysmorphisms of the brain like thinning (A) or thickening (B) of the corpus callosum (arrowheads) and/or mild vermian hypoplasia

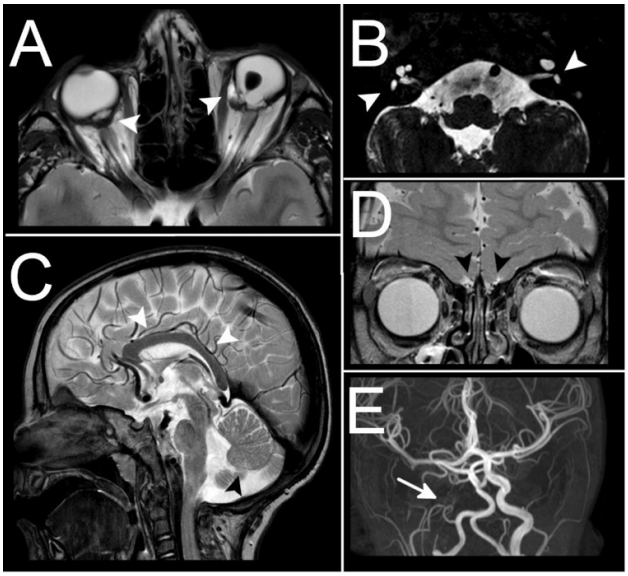


Supplementary FIGURE 2. Associated brain findings in group B, other than CHARGE

T1-weighted sagittal and T1 and T2-weighted axial sections are shown

The 4 patients of group B not affected by CHARGE showed in all cases complex dysmorphisms of the corpus callosum (white arrowheads in A-D) and hypoplasia or dysplasia of the vermis and cerebellum (black arrowheads in A-C).

Patient A also had an ectopy of the neurohypophysis (white arrow in the upper panel) and a pattern of dysgyric cortex (white arrow in the lower panel). Patient B had an heterotopic periventricular nodule (white arrow) while patient C showed polymicrogyria (white arrows) and abnormal basal ganglia (white arrowheads in the lower panel). Patient D had severe pons hypoplasia and diffuse cerebral atrophy (related to a cardiovascular event)



Supplementary FIGURE 3. Common findings in CHARGE patients

As part of the spectrum, CHARGE patients could show colobomas (white arrowheads in A), malformation of the inner ear and semicircular canals (white arrowheads in B), vermian hypoplasia (black arrowhead in C), corpus callosum dysmorphisms and thinning (white arrowhead in C) and thinning/agenesia of the olfactory tracts (black arrowheads in D). One patient also had an agenesis of the right carotid artery (white arrow in E)