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TITLE

CLINICAL AND MOLECULAR CHARACTERIZATION OF CORPUS CALLOSAL ABNORMALITIES: TOWARDS A BETTER UNDERSTANDING OF THEIR GENETIC BASIS

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1. The Corpus Callosum

1.1 Introduction: Interhemispheric commissural formations

The human encephalon needs interhemispheric connections aimed at coordinating the activities of contralateral cortical areas.

These connections are made by axons crossing the telencephalic midline, grouped into five main cerebral commissures: (*commissura*) anterior, posterior, hippocampal, abenular and corpus callosum. [1]

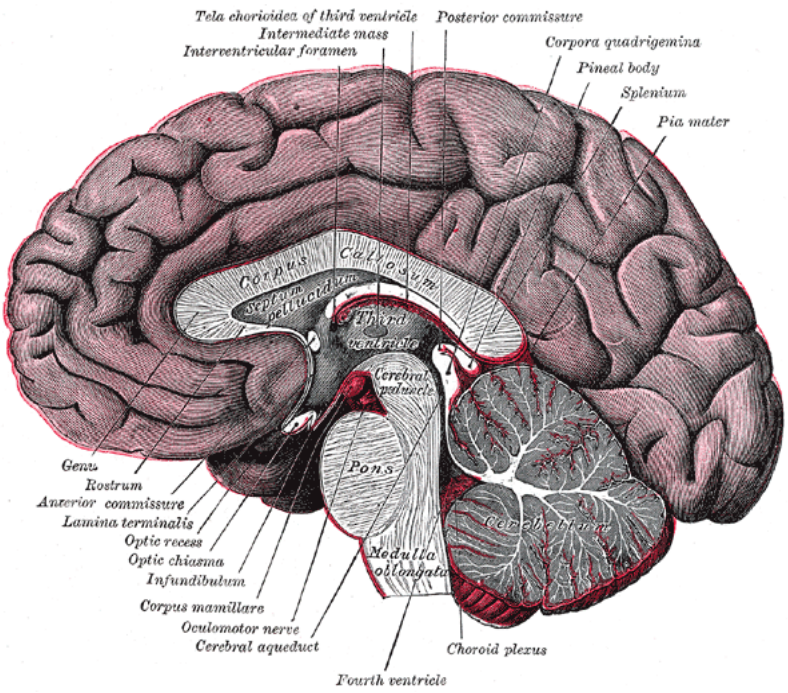


Figure 1. Henry Vandyke Carter - Henry Gray (1918) Gray's Anatomy, Plate 720

The distribution of the commissural fibers in the cortex is very unequal, since some areas are richly provided, while others lack any commissural connection.

It is based on two fundamental principles, not without exceptions:

- The commissural fibers interconnect symmetrical cortical areas of the two hemispheres (principle of homotopy);

- A cortical area has commissural connections not only with the corresponding symmetrical area but also with the contralateral areas that are symmetrical with respect to all the regions of the ipsilateral hemisphere with which it is connected (principle of the heterogeneity of Mettler). The main exceptions to the latter reside in the fact that interhemispheric connections are much less widespread than intra-hemispherical ones.[2]

The anterior commissure is a structure of the paleocortex and olfactory bulbs but it also contains fibers of neocortical origin, so much so that the scarce neocortical interhemispheric connections in the mammalian acallosal species are all achieved through this commission.

The posterior commissure constitutes the lower lip of the epiphyseal peduncle and is composed of fibers that largely combine mesencephalic formations, such as the upper quadrigeminal tubercles, the pretectal nuclei, the interstitial nucleus of Cajal and the somatomotor nuclei and viscera effector of the 3rd pair of cranial nerves; other fibers of white matter originate from the nucleus of the posterior commissure (of Darkschewitsch) of both sides. Fibers descending from the posterior commissure also enter the constitution of the medial longitudinal fasciculus.

The hippocampal commissure mutually connects the hippocampal formations of the two sides and can be considered a proper structure of the archibrain .

The abenular commissure forms the upper lip of the epiphyseal peduncle. It consists of a thin middle stripe crossed by fibers, mainly of olfactory origin, which connect the two nuclei of the abenula.

The corpus callosum, on the other hand, is a structure that has developed in parallel with the evolution of the neocortex: non-mammalian vertebrates and lower mammals, such as the Monotremes and some of the Marsupials, are characterized by little or no development of the neocortex and, according to this, their brain lacks the corpus callosum.[3]

It represents the largest tract of white matter present in the human brain, containing about 200 million axons that form homotopic or heterotopic connections between regions of the cerebral cortex of the right and left hemispheres.[4]

This structure was first described in 1836 by Owen, who identified it as the unique distinctive feature of placental mammal.[4]

1.2 Embryogenesis

The formation of cerebral commissures in the human brain is a very organized and regulated process, in which both the expression of specific transcription factors by the cells that give rise to the commissural fibers and mechanisms external to these cells play a key role, including the formation of glial median structures and their expression of specific guide molecules. In addition to known axonal driving factors (Slit, Neutrins and their receptors, among others), new molecular mechanisms have proved to be crucial in the formation of cerebral commissures: they involve *Notch, FGFR1, NFIB, EphA4*, members of the Wnt family and components of the extracellular matrix, such as heparan solfa proteoglycans[5]

Furthermore, a very recent study by *Richards et al group 2016* has shown that the midline crossing of the callosal axons depends on the primitive remodeling and degradation of the interhemispheric fissure[6].

This remodeling event would start from the astroglia and both sides of the interhemispheric fissure, which interleaves into one another and degrades the intervening leptomeninges. The callosal axons would then preferentially extend over these specialized astroglial cells to cross the midline. A key moment in interhemispheric remodeling is the differentiation of the astroglia from the radial glia, which is initiated by the Fgf8-MAPK-NFI signal.[6]

It is known that the fibers of the corpus callosum originate from the large pyramidal cells of the telencephalic cortex, often as collateral of projection fibers; therefore, these are commissural fibers from neocortical areas, with a precise topographical organization.[7]

The morphogenesis of the corpus callosum in humans begins as early as the eighth week of gestation. At this time, the portion of the terminal lamina immediately adjacent to the corioid canvas on the midline thins (lamina reuniens) and forms the primordio of the septal area.[1] The pioneering axons, originating from the cingulate cortex, cross the lamina reuniens to ninth week. The progression of these fibers is achieved thanks to the guidance of the commissural glial cells and the indusium griseum which, through attractive and repulsive signals, support the pioneer axons in crossing the midline, which occurs at 12-13 weeks of fetal life. The anterior section begins to form around the 14-15th week of gestation, while the posterior section begins between the 18th and 19th week. While the anterior commissure, the hippocampal commissure and the splenium develop through the lamina reuniens, the anterior

portion of the corpus callosum (which circumscribes the pellucid septum) has a different development process, which is achieved by crossing the meninges of the interhemispheric fissure[1]

The corpus callosum is completely formed at 18-20 weeks of gestation, and in the following weeks it continues to thicken and grow caudally through axonal growth mechanisms, showing a progressive maturation that lasts throughout childhood and part of adolescence through mechanisms of pruning ("pruning") axonal.[8]

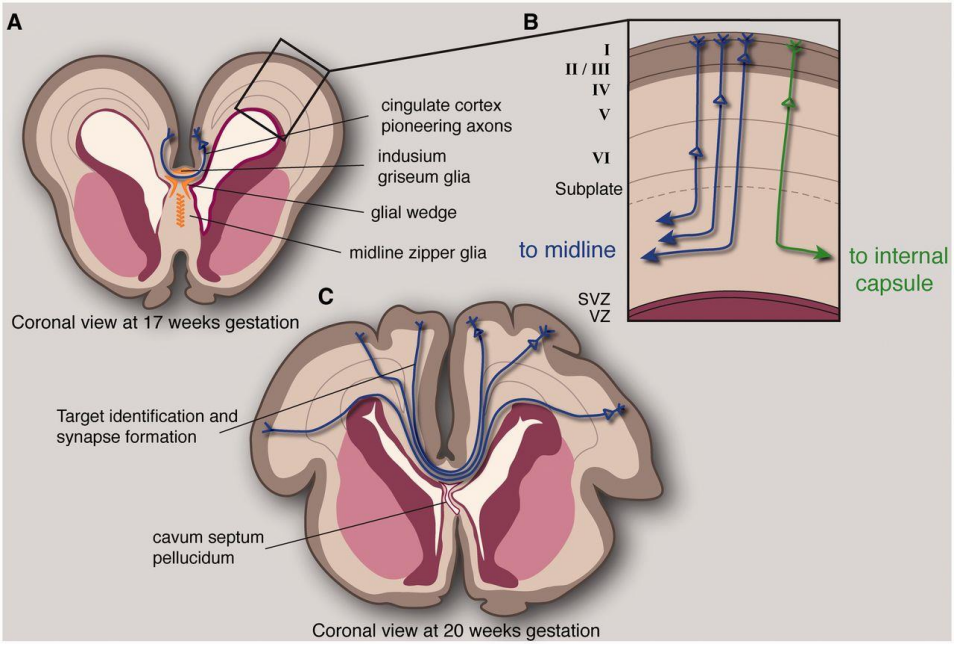


Figure 2. (**A**). Callosal neurons originate from the cortical layers I, II / III, V and VI. The layer in which a neuron resides is not sufficient to define its specialization as a projection callosal neuron; what instead seems to identify the callosal neurons is the expression of the transcription factor SATB2. These neurons project an axon radially across the midline (B). Once the axons reach the contralateral hemisphere, they must recognize their target area and create synapses with the target neurons, presumably by means of molecular recognition mechanisms. (C). An exuberant axonal growth continues after birth and is accompanied by axonal "pruning" mechanisms, which continue during childhood and adolescence. [9]

At birth, in a full-term newborn, the corpus callosum has taken its final form but is still thin: the thickness (vertical dimension) of the corpus callosum increases during childhood and adolescence, with a more pronounced growth of

the front sections in the first 10 years of life compared to that of the posterior segments, which predominates during adolescence.

However, there are significant inter-individual variations in the shape and size of the corpus callosum.

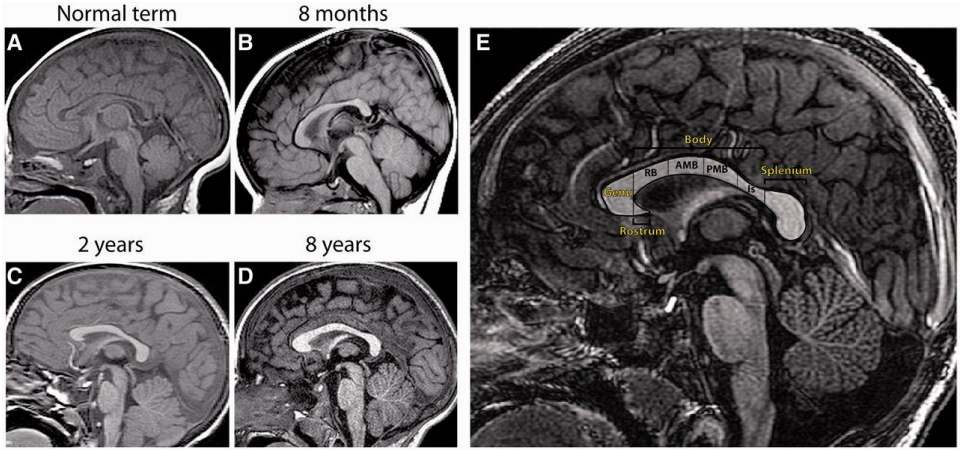


Figure 3. Brain T1-weighted magnetic resonance images in sagittal projection, showing the physiological conformation of the corpus callosum in the newborn at term (A), at 8 months (B), at 2 years (C), at 8 years (D) and in the adult (E). [9]

Given the complexity of the development of the corpus callosum, the causes of the anomalies can be multiple and are often associated with other abnormalities of brain development.

1.3 Anatomy

The corpus callosum has traditionally been divided into four distinct segments, in anteroposterior direction: the rostrum (rostrum), hooked around the anterior commissure; the knee (genum), which curves following the lower limit of the frontal lobe; the trunk or body, which in turn is distinguished in isthmus and front, intermediate and posterior segments; the splenium (splenium), a thick

bulge lying on the quadrigeminal lamina, which marks the posterior limit of the corpus callosum.[1]

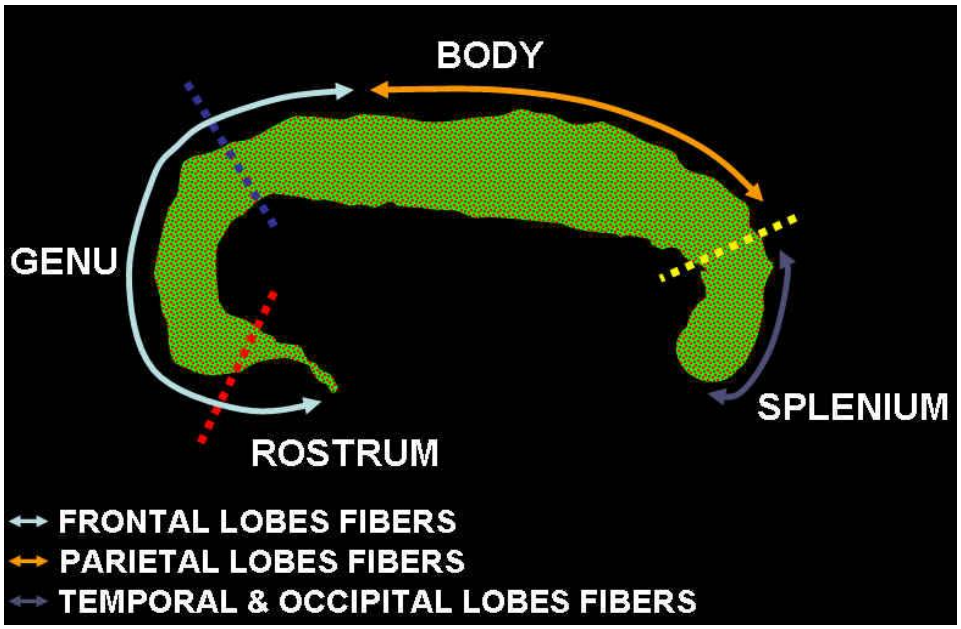


Figure 4. Conformation of the corpus callosum in the adult subject and origin of the fibers that pass through it. The dotted lines indicate the limits of the different regions. Curved arrows indicate the origin of the fibers.

The corpus callosum has a topographical organization: the anterior sections interconnect mostly ventral regions of the cortex, while the posterior sections interconnect the more dorsal associative areas of the temporal and parietal lobes, and the occipital lobes.[10, 11]

This organization is associated with regional differences in the composition of callosal fibers, parallel to the type of information transmitted, which differs according to the different interconnected cortical areas. In fact, the analysis of the composition of the corpus callosum of primates shows a wide variability:

- the callosal regions that interconnect the prefrontal and temporoparietal associative areas are mostly composed of poorly myelinated, small-caliber, slow-conduction fibers.
- - in the callosal regions that connect primary and secondary sensory-motor areas, there is a concentration of highly myelinated fibers, with a diameter greater than 3 μm, at rapid conduction speed. [10, 12]

The callous connections can be both inhibitory and excitatory: the former become responsible for the reciprocal inhibition of the two hemispheres and, therefore, of their independent functioning, while the latter allow the integration of the information between them. Most fibers are excitatory.[4]

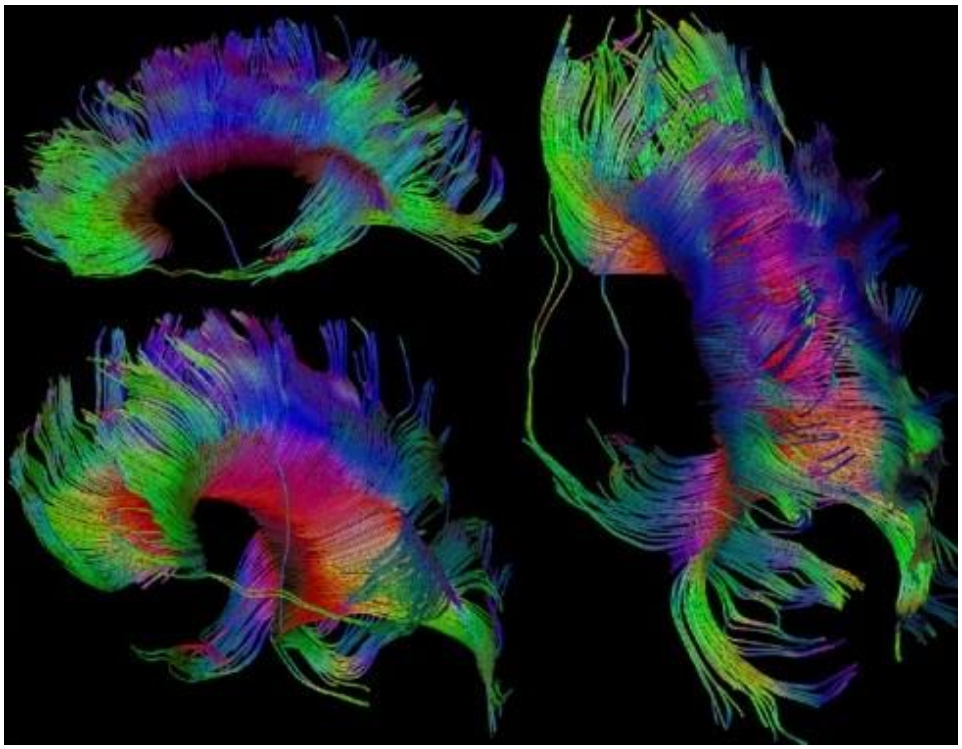


Figure 5. Spatial tensor diffusor imaging (DTI) showing the corpus callosum. Colors are assigned based on the dominant direction of the fibers.

1.4 Physiology

The main functions of the corpus callosum are the coordination and transfer of information between the two cerebral hemispheres: in this way this structure promotes the functional integration of sensitive, motor and visuo-motor information, as well as higher cognitive functions such as language and abstract reasoning. The corpus callosum plays a key role in bimanual coordination.[13, 14]

On the other hand, it participates in the hemispheric specialization of lateralized functions, such as the prevalent use of a hand and the processing of language at the level of the left hemisphere. To this end, the callosal inhibitory fibers would create a channel through which a hemisphere can inhibit the contralateral to dominate a given function.[15]

Of great interest is the finding that neurons of cortical areas interconnected by callosal fibers show synchronous oscillations of their action potentials for variable periods of time: this mechanism of synchronous activation of multiple contralateral target cortical areas is important for some aspects of perception such as binding (a process in which information on color, movement and form is integrated into a unified perception) as well as for more general phenomena such as consciousness. [16, 17]

2 Corpus Callosal Abnormalities

The corpus callosum abnormalities (CCA) have a prevalence estimated between 0.3% and 0.7% in patients who perform an magnetic resonance imaging of the brain.[9] They represent the most frequent cerebral malformations observed in humans.

CCA can be isolated, but more often they are associated with a broad spectrum of brain development disorders and organ malformations, so they are part of a complex syndrome. The agenesis of the corpus callosum (*OMIM # 217990*), for example since the genetic etiology was understood, more than 200 different congenital syndromes have been described as associated with this type of malformation.

The most frequent anomalies associated with CCA include those of the posterior cranial fossa, interhemispheric cysts and neuronal migration disorders, found in approximately 45.8% of cases.[9]

To date, therefore, no classification system seems to be able to predict the functional outcome of patients with CCA. [20]

What is certain is that the presence of extracallosal cerebral anomalies are predictive factors of a worse neurodevelopment, while the isolated agenesis of the corpus callosum is considered the condition with better prognosis. However, the finding of severe intellectual disability in patients with isolated agenesis leaves the debate open. In the absence of epidemiological studies on an unselected population, the description of large series of patients with CCA would be very useful.'[20, 21]

They constitute a broad spectrum of commissural defects which include complete agenesis of the corpus callosum (cACC, about 1/4000 live births), partial agenesis (pACC), Thin corpus callosum (ThinCC) and Thick corpus callosum (ThickCC).

2.1 Complete Agenesis of Corpus Callosum (cACC)

Complete agenesis of the corpus callosum is, in almost all cases, accompanied by the absence of the hippocampal commissure and, often, also by the absence or hypoplasia of the anterior commissure, thus defining itself more precisely as "classical commissural agenesis". Their absence is immediately identifiable on the sagittal plane in MRI.

However, it should be noted that this is not an actual agenesis of the callosal fibers, but rather a form of heterotopia; in fact these have formed in themselves, but, given the lack of intersection along the median line, they take on a right angle direction in both hemispheres and go inside the medullary veil forming a parasagittal bundle that invades the area above the lumen of the lateral ventricles.

This bilateral bundle is called the Probst bundle, named after Moriz Probst, a neuroanatomist and psychiatrist who first described it in 1901.[19]

It is therefore a thick bundle of white matter that mediates intrahemispheric connections, rather than interhemispherical ones, and which, after myelination, in T1-weighted sequences appears more hyperintense than the remaining white matter and vice versa in T2-weighted ones it appears more hypointense due to its intrinsic compact axonal composition (Figure 6).

The Tensor Diffusion imaging (DTI) allows not only the direct visualization of the probst bundles but also to visualize any aberrant intrahemispheric connections affecting the remaining white matter dossiers.

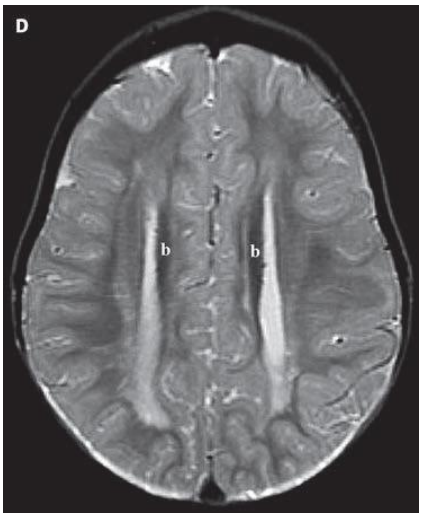


Figure 6 : T2-weighted image on the axial plane showing the Probst beams, more hypointense than the remaining white matter. Barkovich Pediatric neuroimaging.

On the coronal plane, a typical sign of complete agenesis is even more appreciable, highlighting the simultaneous presence of the Probst bundles and their expansion in the area mesially located at the lateral ventricles, from which the altered conformation and arrangement of the ventricles themselves. This sign is called "moose head sign" or "viking helment sign", that is, literally, "sign of the moose head" (or even more commonly with "bull horns") or "sign of the viking helmet" (Figure 9). Also the underlying gyrus does not form and similarly absent the pericallosal sulcus, from which the typical and anomalous deep radial convergence of the furrows of the medial surfaces of the cerebral hemispheres will result, which is typically definable in MRI on the inter-hemispheric sagittal plane.

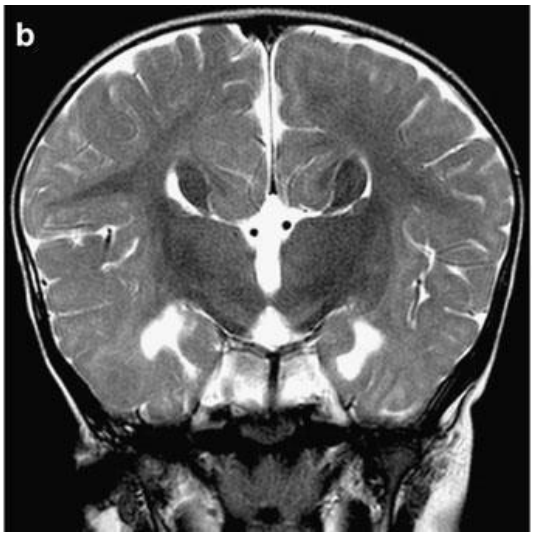


Figure 7 : scansione coronale T2-pesata in cui appare evidente il “moose head sign”. Raybaud C. (2010) The corpus callosum, the other great forebrain commissures, and the septum pellucidum: anatomy, development, and malformation. Neuroradiology vol. 52 (6): 447–477

This radiological finding highlights, as mentioned above, the influence that the alteration of the development of the corpus callosum has on the entire aspect of the median and paramedian brain area, which appears completely disrupted.[20]

In fact, the interhemispherical fissure appears much wider and the ventricular structure is significantly modified: the lateral ventricles appear in fact separate, far from the median line and parallel to each other, with an aspect on the half-moon coronal plane of the front horns and bodies, which configures the so-called "teardrop" conformation. The third ventricle is instead enlarged and located at the top, reaching the area occupied by the corpus callosum and the foramina of Monro are enlarged.

The ascent and expansion of the chorioid tissue, i.e. the roof of the third ventricle, can be associated with the development of communicating interhemispheric cysts, which, together with the non-communicating ones, which derive instead from a multilocular cystic dysplasia affecting the meninges,

constitute the two main types of interhemispheric cysts.[21]

The definition of communicating or non-communicating precisely defines the presence or absence of communication with the ventricles. One of the most important examples of callosal agenesis associated with multilocular (therefore non-communicating) cysts is the Aicardi syndrome

On the axial plane, this type of conformation of the ventricular system gives rise to another finding defined as "racing car sign", given its similarity with its shape (Figure 8).

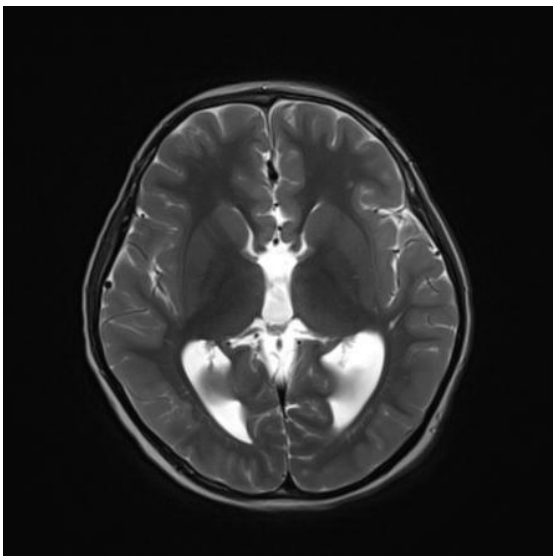


Figure 8: T2-weighted axial plane image showing the racing car sign

On the axial plane, therefore, it can be seen how the atria and occipital horns are instead widely enlarged, an aspect which is called "colpocephaly"; this finding is commonly explained on the basis of the absence of the forceps major [21] (Figure 9).

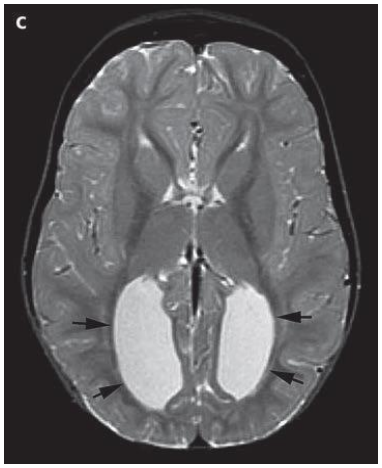


Figure 9: T2-weighted image on the axial plane showing the enlargement of the occipital horns of the lateral ventricles (colpocephaly). Barkovich A.J., Raybaud C. (2012) Pediatric neuroimaging. 5. Ed

Some of these pictures as already specified, a range of possible findings found in case of commissural agenesis, therefore it is not necessary to expect to find them in their entirety; in particular the Probst bundles are not always present, as well as the persistence of the anterior commissure which in some cases may even be hypertrophic, due to probable vicariance phenomena.

2.2 Partial agenesis (pACC)

As regards instead the partial commissural agenesis, also called commissural hypogenesis or dysgenesis or dysplasia, it should be remembered that at the 14th week of gestation the corpus callosum already has its own complete form from the point of view of separation into segments, albeit obviously in very small scale.

In fact, in the most typical form of partial commissural agenesis, the commissural plate is much shorter and squat, but grossly complete (Figure 10): in fact, a relatively large knee appears, a stocky body with a small septum pellucidum, a visible splenium and with a retained adhesion to the arches through the commissura hippocampus.

The rostrum is usually less identifiable. Since the entire structure is shorter, on the coronal plane there is an approximately normal appearance in the anterior sections, but in the posterior sections there are the typical finds of agenesis, including the Probst bundles. Thanks to current knowledge, it is clear that this is the result of a lack of expansion and accumulation of fibers that, normally, would have dorsally "pushed" the hippocampal commissure and the fibers of the splenium, and that it does not therefore result from a lack of formation of the posterior part commissures, as previously thought.

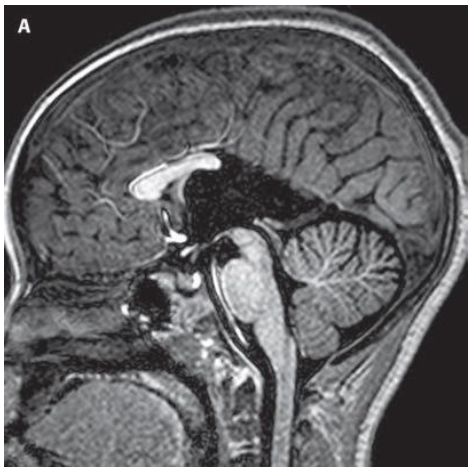


Figure 10: T1-weighted image on the midline on the sagittal plane showing a "classical" partial commissural agenesis.Barkovich Pediatric neuroimaging. 5. Ed

In addition to the classical type, there are numerous other types of partial commissural agenesis: for example, there are more extreme cases in which there is only a small and rudimentary sketch located more frequently in the anterior site, which is thought to be a small callosal knee, or a hippocampal commissure that has never moved posteriorly (in this case the pellucid septum is more likely to be absent), or vice versa it is possible to find a sketchy corpus callosum in the absence of the other two commissures.

Furthermore, there may be a "segmental" callosal agenesis, in which the dorsal part of the anterior portion of the corpus callosum does not merge with the splenium, thus giving rise to two segments that appear separate on the sagittal plane and of which the front borders on the pellucid septum, while the posterior one is in contact with the fornix; the precise etiology of this type of anomaly has not yet been clarified.

Interesting is the possible finding, in case of partial commissural agenesis, of so-called "sigmoid bundles" (ie sigmoid bundles), or axonal fiber bundles that run from one hemisphere to another not in a perfectly homotopic way (as in the case of the normal callosal fibers), but above all heterotopic, connecting different brain areas of the two hemispheres to each other (Figure 11). Studies in DTI have been important for the recognition of these bundles.[22]

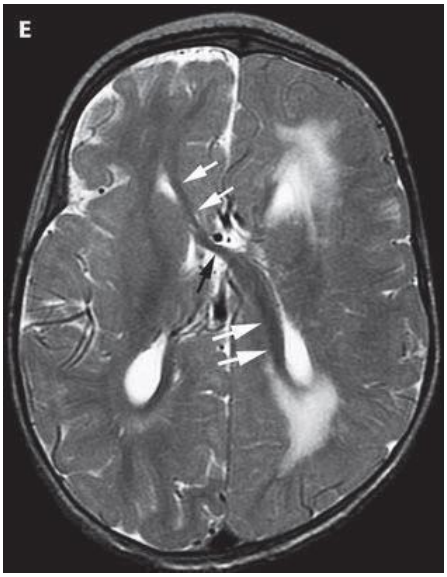


Figure 11: T2-weighted image on the axial plane in which a singmoidal beam is clearly evident. Barkovich Pediatric neuroimaging. 5. Ed

In fact, there is an extremely large number of different callosal and commissural hypo-dysplasias that may not necessarily represent different malformations but be part of a large malformative continuum.

4

2.3 Thin Corpus Callosum (ThinCC)

Thin corpus callosum is meant a proportionate and diffuse malformation thinning of the corpus callosum in its entirety, with an unaltered relationship between the length and morphology of the various segments.[23] (Figure 12).

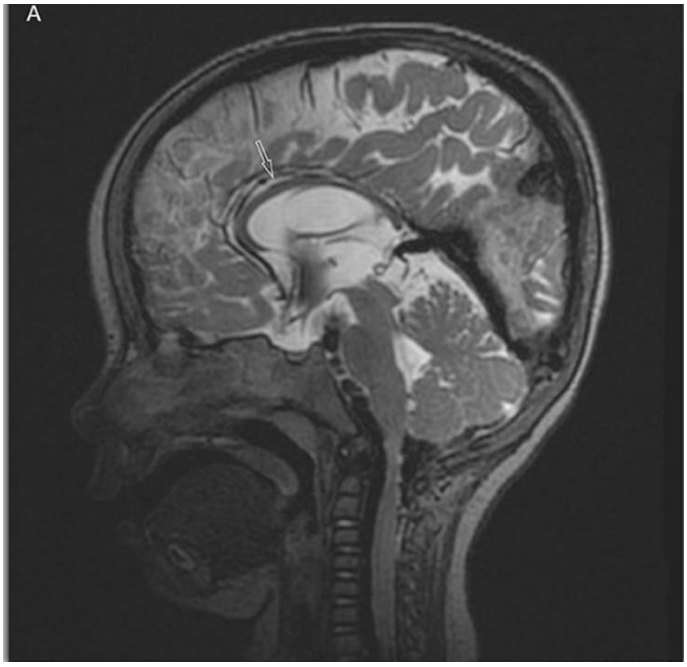


Figure 12: T2- sagittal weighted image showing a hypoplastic corpus callosum Barkovich Pediatric neuroimaging. 5. Ed

It is a rare and difficult to diagnose malformation anomaly before the age of 3 years, as we know that the myelination processes of all the cerebral white matter, which are the basis of the physiological thickening process of the corpus callosum, end almost definitively between 2 and 3 years of age.

Disorders such as leukoencephalopathies, congenital metabolic disorders (e.g. some mucolipidosis), or outcomes of hypoxic-ischemic encephalopathy or other types of perinatal or postnatal damage such as fetal alcohol syndrome, HIV or severe neonatal hypoglycaemia can be accompanied by the presence of a thin corpus callosum in whole or in part, without however being part of a malformative spectrum but simply

representing the result of the loss of white matter fibers and therefore of commissural fibers.

For this reason, of course, all patients with associated metabolic or clastic pathologies, acquired or congenital, especially if of the white matter and similarly the fetal-alcoholic syndromes, TORCH and HIV infections and all other viral connatal infections have been excluded from the series. , since they are thin corpus calluses but not hypoplasia.[22]

2.4 Thick corpus callosum (ThickCC)

Thick corpus callosum is meant a malformative thickening of the corpus callosum (Figure 13) associated or not with its dysmorphisms.

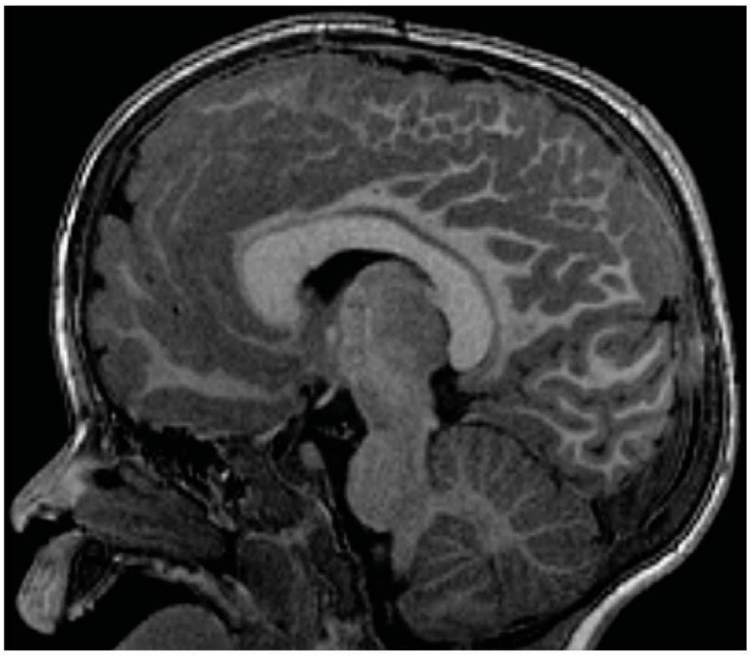


Figure 13: T1-weighted image on the sagittal plane that highlights a hyperplastic corpus callosum Gaetano Terrone, Norine Voisin, Ali Abdullah Alfaiz, Gerarda Cappuccio, Giuseppina Vitiello, Alessandra D'Amico, A James Barkovich et al. (2016) De novo PIK3R2 variant causes polymicrogyria, corpus callosum,hyperplasia and focal cortical dysplasia. European Journal of Human Genetics (2016) 24, 1359–1362

Biometry of corpus callosum in children was known by large cohort described by Garel et al 2011.[24] Recent work reviewed small series of children with ThickCC and their genetic etiology that remains still unknown.[25]

2.5 Etiology

There are numerous possible causes of CCA: genetic abnormalities, gestational disorders including metabolic disorders, infections and teratogens. [21]

To date, the cause of agenesis of the corpus callosum (ACC) is detectable only in 30-45% of cases: in about 10% of patients chromosomal abnormalities are found, while in the remaining 20-35% a known genetic syndrome is diagnosed. [23] In 55-70% of patients with cACC, on the other hand, the etiology is unknown and the agenesis of the corpus callosum is often an isolated finding.[21]

Despite environmental factors, among which alcohol during pregnancy and maternal phenylchetonuria, have been described as risk factors for an abnormal development of the corpus callosum.[26] On the contrary, the inheritance of the CCA both isolating and syndromic provides strong support for the hypothesis of a genetic etiology. Chromosomal anomalies, Copy Number Variants (CNVs), point mutations can be equally causative and, probably, oligogenic alterations are foreseen by this type of conditions. [5, 26, 27] The known genes so far associated with CCA encode proteins involved in very different pathways. The seemingly sporadic nature increases the difficulty of genetic studies.[9]

The CCA have been associated with several major chromosomal rearrangements in more than 200 syndromes, both autosomal and X-linked.[9, 5] This extreme genetic heterogeneity of CCA is probably attributable to the considerable complexity of the embryological processes that lead to the development of the corpus callosum. .

Recent studies on cohorts of a few hundred affected subjects have allowed to identify many new loci associated with CCA, they have highlighted how the CGH array (Comparative Genomic Hybridization) is a valid diagnostic approach[29]. It is hypothesized that many cytogenetic defects have yet to be identified and therefore new

genes, included in these rearrangements, will be identified as possible candidates for this type of condition. [26,27]here are various pathological conditions associated with this finding: these ThickCC are mostly rare and recently found syndromes, including the MCAP (megalencephaly-capillary malformation syndrome) and MPPH (megalencephaly, polymicrogyria, polidactyly, hydrocephalus) syndrome generally associated with callosal hyperplasia (despite cases of hypoplasia reported), megaloencephaly, polymicrogyria,polydactyly and hydrocephalus, where the mTOR pathway that is involved in the control of the main cellular functions is variously altered, including protein synthesis, metabolism, cell cycle, survival, cell growth and proliferation.

kinase B (AKT) -mammalian target of rapamycin (mTOR) pathway and loss of function in these and other genes involved in the pathway

Note the association between callosal hyperplasia and type 1 neurofibromatosis (NF1) as well as the association with autism spectrum disorders.[25]

Furthermore the introduction of next-generation sequencing technologies (Next-generation sequencig NGS) that allow the molecular analysis of several genes simultaneously (panels targeted or extended), of the exome or the set of DNA coding sequences (Whole Exome Sequencing, WES) or of the entire genome (Whole Genome Sequencing WGS), are increasing the diagnostic rate in rare diseases to date of unknown etiology and could make an important contribution in the identification of new neurodevelopmental genes related to CCA.

2.6 Diagnosis

Antenatal imaging performed before 18-20 weeks of gestation (time at which ultrasound screening is generally performed) is not able to detect a developmental anomaly of the corpus callosum. [28]

Some indirect ultrasound signs may suggest the presence of an ACC: absence of the cavum septi pellucidi, colpocephaly (dilation of the occipital horns of the lateral ventricles), anomalous course of the pericallosal artery, widening of the interhemispherical fissure, radial arrangement of the furrows in the internal projections of the hemispheres brain. [29,30]

Once an anomaly of the corpus callosum is suspected, it is difficult to distinguish an isolated form from a syndromic. For this purpose, a fetal morphological ultrasound in a specialist center and an invasive diagnosis by amniocentesis are strongly recommended, with analysis of the fetal karyotype and cytogenetic-molecular deepening with array-CGH.[2]

The karyotype is abnormal in 17.8% of cases and often these cases present associated malformations on neuroimaging.[30,31]

Screening for congenital infections on maternal blood and amniotic fluid (if available) is also recommended, although the positivity rate is generally low. [20]

Ultrasound often fails to detect more subtle cases of partial agenesis or callosal hypoplasia. Therefore, fetal magnetic resonance imaging (MRI) represents the best imaging technique for direct visualization of the corpus callosum in cases where an CCA is suspected, as well as associated anomalies that are not visible on ultrasound.

The growing use and increasing resolution of pre- and post-natal neuroimaging techniques (ultrasound and MRI) have led to the increasingly frequent recognition of different types of CCA. When CCA are recognized in prenatal setting and karyotype/CGH array are negative, they remain a really prenatal dilemma. Agenesis of corpus callosum is one of the first causes of brain anomalies associated to therapeutic abortion .

In particular, in the postnatal period, progress in the field of diffusion tensor tractography (DTI) has significantly improved our knowledge of the connections that

the corpus callosum makes between the different cortical areas in healthy individuals and how these connections are disturbed and diverted in patients with CCA. [29]

2.7 Clinical features

A broad spectrum of clinical phenotypes have been described in patients with CCA. [33]

Developmental delay, speech impairment, intellectual disability, motor deficit and epilepsy may occur in such individuals in variable combinations.[33]

Severe neuromotor deficit is observed in about 80% of patients with syndromic CCA, and in a significantly smaller percentage of cases of isolated ACC. Among the latter, subjects with isolated non-syndromic agenesis show the best skills, with neurological physical examination normal in more than half of the cases. [27] Cognitive and linguistic skills have the same profile as neuromotor skills, with a more evident severe delay in patients with syndromic ACC compared to isolated forms. However, cognitive disorders of varying degrees are found in more than 60% of patients with isolated ACC, and severe language impairment in more than 50% of cases.[27]

Individuals with complete agenesis do not show the interhemispheric disconnect that is observed in patients with split brain surgical resection, but seem to have a slight deficit in the interhemispheric transfer. [19, 9] Visual evoked potentials show that in patients with total callosal agenesis visual stimuli do not propagate through the midline; however, these individuals are capable of comparing simple visual stimuli (letters and colors) between visual fields and normally completing elementary bimanual tasks.[31] This suggests that simple conceptual and motor information can be transferred between the two hemispheres despite the absence of the corpus callosum, probably through other commissural systems, and demonstrates the extraordinary plasticity of human brain

development. The role of callosal agenesis in tasks requiring complex cognitive functions is more evident.[35, 36]

Patients with IQ (Intellectual Quotient) values within the normal range (generally they are individuals with isolated agenesis), have more subtle alterations in higher cognitive abilities, including the understanding of non-literal linguistic expressions or verbal humor (word games, jokes, irony, etc.). [37, 38] In particular, recent studies have confirmed that the understanding and interpretation of proverbs are significantly compromised in patients with corpus callosum agenesis.[36, 38] So, although the agenesis of the corpus callosum does not seem to have a direct and dramatic impact on cognitive skills or basic language skills, there are often slight anomalies in the understanding and use of non-literal verbal forms, and disabilities in problem solving, in the speed of processing and in abstract reasoning.[37, 38]

Often this aspect leads to relationship difficulties similar to those observed in so-called high-functioning autistic patients, including reduced self-awareness in a social context[37], the difficulty in imagining the social perspective of others[39], poor dialectic and conversational skills, as well as narrowness of verbal expression and emotional experience.[36] Memory deficits, equally shared between verbal and visuospatial memory, are frequent, as well as learning disabilities, also equally distributed between writing, reading and calculation.[9]

A third of adult patients with CCA showed behavioral traits compatible with the diagnosis of autism spectrum disorder (ASD), while often in childhood these patients did not reach the cut-off for diagnosis in childhood, a sign that these characteristics are more evident with the time.[18]

Epilepsy is found in about 40% of patients with syndromic ACC and in about 15% of isolated forms. Degree of severity and responsiveness to antiepileptic drugs seem worse in syndromic forms (especially in the presence of associated cortical

developmental malformations). There are no significant differences in incidence between focal and generalized crises. In most cases of ACC with epilepsy, the onset of seizures is early, within the first year of life. Electroencephalographic studies do not allow to define a specific pattern for the different CCA subtypes. However, rapid rhythms, asymmetric and irregular background activity, focal epileptiform abnormalities and sporadic slow waves without clinical correlation are observed in about a quarter of patients with non-epileptic CCA. [19, 9]

3. Patients and methods

3.1 Italian Corpus Callosum Abnormalities (CCA) Study Group

Starting from the sharing of data and experiences on CCA by a multidisciplinary team composed of pediatric neurologists, clinical geneticists, neuroradiologists and neuropsychologists, and from the collaborative effort between centers and specialists from different hospitals, an Italian research group on CCA has been formed, the Italian Corpus Callosum Abnormalities (CCA) Study Group.

Given the above premises, some significant aims have been identified.

First of all, to create a detailed neurological, neuropsychological and neuroradiological characterization of a cohort of patients with syndromic CCA and non syndromic ones: this was the initial aim pursued by our group in our *retrospective study*.

As regards the *perspective study,* , the team of expert neuroradiologists related to the project has prepared a common study protocol for in-depth analysis by tractography and resting connectivity, which will be performed in neuroradiology centers equipped with adequate equipment for this project and distributed on the national territory.

The study includes a clinical and neuropsychological protocol, a neuroradiological protocol and a genetic diagnostic and research protocol in karyotype and CGH-array negative patients using Whole Exome Sequencing (WES).

In particular, our perspectives study provide use of this genetic postnatal protocol for specific groups of CCA:

- Non syndromic isolated ACC with normal cognition
- Family CCA
- ThickCC project
- Syndromic CCA

The project has the aim of improving knowledge on the prognosis of anomalies of the corpus callosum, in order to provide help for clinicians (geneticists, pediatricians, neurologists, maternal-fetal medicine specialists and others) who follow people affected by this condition and their families after the diagnosis of CCA in the postnatal setting, but especially in the prenatal setting.

In addition, the known and newly identified genes can be used for the construction of a NGS diagnostic panel for CCA, useful for the prognostic definition in the pre / postnatal diagnosis phase.

This clinical-molecular research project is a multicenter study involving numerous Italian and foreign centers with the aim of identifying the genetic basis of ACC, through a multidisciplinary experimental diagnostic approach.

The Coordinating Center of the study was the Department of Translational Medical Sciences - Section of Pediatrics, Specialist Unit of Pediatric Neurology and Developmental Pediatrics, University Hospital "Federico II" of Naples, with principal investigator Prof. Ennio Del Giudice.

Numerous Italian and International centers, highly qualified in the field of pediatric neurology and / or genetic diseases, participated in the project.

	Centres	*Responsable*
1	Azienda Ospedaliera Universitaria Federico II, Napoli	Prof. Ennio Del Giudice Prof. Generoso Andria Prof. Lucio Nitsch Prof. Achille Iolascon Prof. Nicola Brunetti-Pierri
2	Università di Pavia	Prof.ssa Orsetta Zuffardi
3	Policlinico Universitario Tor Vergata, Roma Università degli studi dell'Aquila	Prof. Francesco Brancati,
4	IRCSS Fondazione Stella Maris, Pisa	Prof. Giovanni Cioni
5	IRCCS E Medea di Bosisio Parini, Lecco	Prof. Renato Borgatti
6	Istituto Carlo Besta, Milano	Prof.ssa Daria Riva
7	Istituto di Ricovero e Cura a Carattere Scientifico, Arcispedale S.Maria Nuova, Reggio Emilia	Prof.ssa Livia Garavelli
8	Università degli Studi di Roma la Sapienza, Roma	Prof. Antonio Pizzuti Prof. Vincenzo Leuzzi Prof Alberto Spalice Prof. Luigi Tarani Prof. Pasquale Parisi
9	Ospedale Pediatrico Bambino Gesù, Roma	Prof. Federico Vigevano Prof. Enrico Bertini
10	Università degli Studi di Salerno, Salerno	Prof. Giangennaro Coppola
11	Azienda Ospedaliero-Universitaria Consorziale Policlinico di Bari	Prof.ssa Lucia Margari
12	Policlinico S. Orsola Malpighi, Bologna	Prof. Duccio Maria Cordelli
13	IRCCS, Istituto delle Scienze Neurologiche di Bologna, Ospedale Bellaria, Bologna	Prof. Gobbi
14	Ospedale Salesi di Ancona	Dott.ssa Anna Ficcadenti
15	Azienda Ospedaliero Universitaria "Policlinico Vittorio Emanuele", Catania	Prof.ssa Agata Fiumara
16	Università degli Studi di Brescia, Spedali Civili, Brescia	Prof.ssa Elisa Fazzi
17	RCCS Istituto G. Gaslini, Genova	Prof. Pasquale Striano Dott.ssa Mariasavina Severino Dott.ssa Maria Margherita Mancardi
18	Azienda Ospedaliera di Padova, Padova	Prof.ssa Agnese Suppiej
19	Fondazione Monza e Brianza per il Bambino e la sua Mamma	Prof. Angelo Selicorni
20	UNIL (Universitè de Lausanne) Centre for Integrative Genomics, Losanna, Svizzera	Prof. Alexandre Reymond
21	SIEOG (Società Italiana di Ecografia Ostetrica e Ginecologica)	Prof. Pasquale Martinelli, Prof. Dario Paladini, Prof. Federico Prefumo, Prof Giuseppe Rizzo

Table 1. Centres involved in Italian CCA Study Group.

The project was submitted and approved to the Ethics Committee of the Federico II University. An informed consent has been prepared for the collection of sensitive data, for extraction and conservation of DNA and for cytogenetic and molecular investigations.

3.2 Retrospective study : methodology

A cohort of 642 patients were recruited by the Italian CCA Study Group in the period from December 2014 to May 2019.

Inclusion criteria were the presence of anomalies of the corpus callosum, both syndromic and non-syndromic. Acquired CCA patients (e.g. vascular / hypoxic insults, prenatal exposure to alcohol, and congenital, perinatal and neonatal infections, such as CMV, Rubella, toxoplasmosis and influenza) and cases with scanty data, were excluded from the study.

A standardized and anonymized Clinical questionnaire was created for the purpose of collecting and reviewing the clinical, neuropsychological and neuroimaging and genetics data of all patients recruited by the Italian CCA Study Group.

The data relating to sex, age, the presence of affected family members, the availability of a blood / DNA sample, the type of CCA from which it is affected were collected for each patient recruited.

In this regard, our series was reviewed and classified agreeing on the following definitions:

- Isolated CCA in the absence of other central nervous system (CNS) malformations;

- Plus CCA when callosal anomalies are associated to other CNS

malformation;

- Non Syndromic (NS-CCA) when brain anomalies are not associated

to facial dysmorphisms and other extra CNS malformations;

- Syndromic CCA (S-CCA) when anomaly of the corpus callosum associated with other malformations of the CNS or other organs / systems and facial dysmorphisms.

The morphological classification of the CCA adopted by the Group and to which the Clinical Card refers to the distinction into four subgroups:

1. Complete or total agenesis

2. Partial commissural agenesis or Dysplasia

3. Thin corpus callosum or Harmonic hypoplasia

4. Thick corpus callosum or Hyperplasia with or without dysplasia,

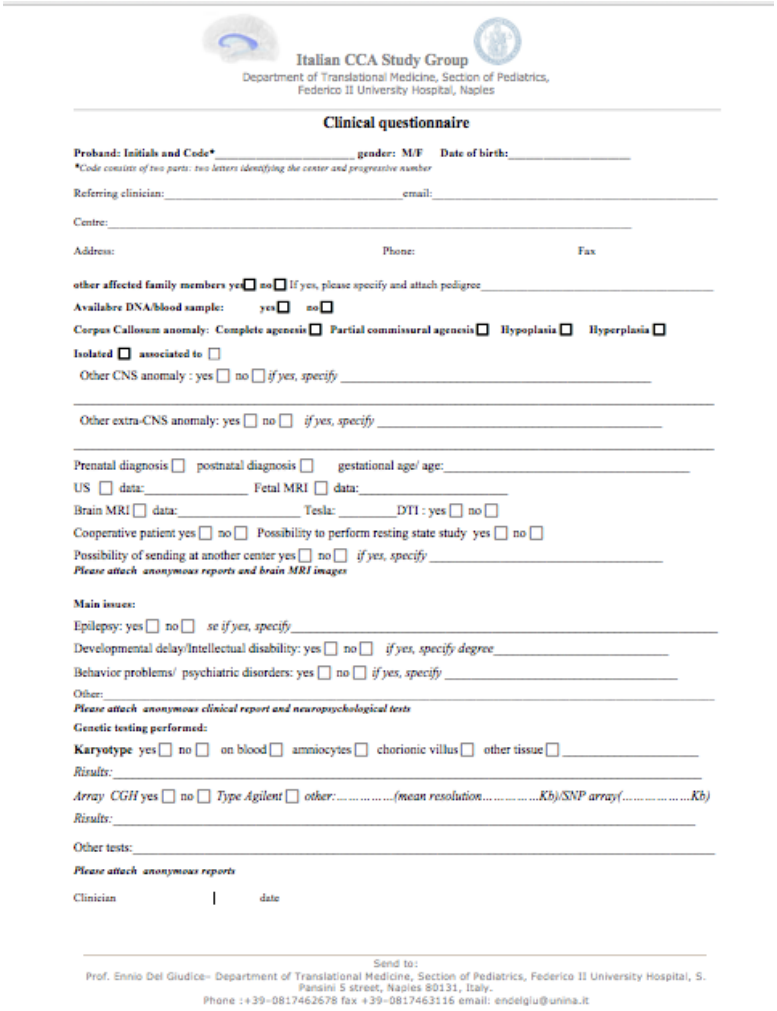
according to *Garel et al.*[24]

For patients with harmonic hypoplasia the minimum age for neuroradiological classification is fixed at 3 years.

The Clinical Questionnaire also investigates the time of diagnosis (prenatal or postnatal), the imaging technique used (ultrasound, fetal MRI, postnatal brain MRI) and the possibility of further investigations such as the examination of tractography and the study of connectivity at rest. All patient are followed for almost 3 years after birth.

The main clinical problems of the patient are then requested, including cognitive functioning and behavioral problems and psychometric assessments with standardized quantitative scores.

Finally, cytogenetic and molecular data, for which it is required to specify the tissue and the methods of execution, the type of platform used and the average resolution were required. Furthermore, it was requested to attach reports of cytogenetic and molecular analyzes.



Italian CCA Study Group
Department of Translational Medicine, Section of Pediatrics,
Federico II University Hospital, Naples

Clinical questionnaire

Proband: Initials and Code* _____ **gender: M/F** **Date of birth:** _____
**Code consists of two parts: two letters identifying the center and progressive number*

Referring clinician: _____ email: _____

Centre: _____

Address: Phone: Fax

other affected family members yes ☐ no ☐ If yes, please specify and attach pedigree _____

Availabre DNA/blood sample: yes ☐ no ☐

Corpus Callosum anomaly: Complete agenesis ☐ Partial commissural agenesis ☐ Hypoplasia ☐ Hyperplasia ☐

Isolated ☐ associated to ☐

Other CNS anomaly : yes ☐ no ☐ *if yes, specify* _____

Other extra-CNS anomaly: yes ☐ no ☐ *if yes, specify* _____

Prenatal diagnosis ☐ postnatal diagnosis ☐ gestational age/ age: _____

US ☐ data: _____ Fetal MRI ☐ data: _____

Brain MRI ☐ data: _____ Tesla: _____ DTI : yes ☐ no ☐

Cooperative patient yes ☐ no ☐ Possibility to perform resting state study yes ☐ no ☐

Possibility of sending at another center yes ☐ no ☐ *if yes, specify* _____

Please attach anonymous reports and brain MRI images

Main issues:

Epilepsy: yes ☐ no ☐ *se if yes, specify* _____

Developmental delay/Intellectual disability: yes ☐ no ☐ *if yes, specify degree* _____

Behavior problems/ psychiatric disorders: yes ☐ no ☐ *if yes, specify* _____

Other: _____

Please attach anonymous clinical report and neuropsychological tests

Genetic testing performed:

Karyotype yes ☐ no ☐ on blood ☐ amniocytes ☐ chorionic villus ☐ other tissue ☐ _____

Results: _____

Array CGH yes ☐ no ☐ *Type Agilent* ☐ *other:……(mean resolution……Kb)/SNP array(……Kb)*

Results: _____

Other tests: _____

Please attach anonymous reports

Clinician | date

Send to:
Prof. Ennio Del Giudice– Department of Translational Medicine, Section of Pediatrics, Federico II University Hospital, S. Pansini 5 street, Naples 80131, Italy.
Phone :+39-0817462678 fax +39-0817463116 email: endelgiu@unina.it

Figure 14. Clinical Questionnaire.

Furthermore, brain MRI imeges were attached to the questionnaire, selected by the participating neuroradiologists and uploaded on a single server, in order to be able to collect and re-discuss them for common reclassification in the relevant meetings.

The clinical, neuropsychological and cytogenetic and molecular analyzes data collected were entered in a protected web-based database. All clinical and molecular data were anonymized and all information relating to each patient was identified by a unique numerical code.

All data entered have been coded according to *Human Phenotype Ontology HPO* (http://human-phenotype-ontology.github.io/about.html). All the CNVs reported have been re-evaluated through international tools of the variants (UCSC, DECIPHER, DGV, ClinVar) and the most updated scientific literature.

4. Results:

4.1 Retrospective study: our series

Although more than 600 patients were initially recruited, as previously specified, some were progressively excluded from the case history due to criteria that were not strictly responsive (anomalies secondary to metabolic defects, fetal-alcoholic syndrome, type 1 neurofibromatosis, etc.). Therefore, the study was ultimately conducted on 556 patients, of which 57% male and 43% female. 99.3% of cases were sporadic cases, while only 4 CCA families were included (0.7%).

Based on the classification into syndromic and non-syndromic anomalies as previously described, 332 cases out of 556, or 60%, are among syndromic CCA (S), while 224 out of 556, about 40%, are non-syndromic ones (NS). (Table 2)

	N.	cACC	pACC	ThinCC	ThickCC
S-CCA	332 (60%)	93 (28%)	136 (41%)	49 (15%)	54 (16%)
NS-CCA	224 (40%)	109 (49%)	67 (30%)	23 (10%)	25 (11%)
Total	556	202	203	72	79

Table 2

Reviewing on the basis of the classification into “isolated” and “plus” anomalies, it has been observed that the isolated anomalies result to be in a slight majority, that is 296/556 (53%) and the plus 260/556 (47 %).

Going to evaluate the single types of anomalies in both these classes see Table 3.

	N.	cACC	pACC	ThinCC	ThickCC
Isolated	296 (53%)	121 (40%)	98 (33%)	40 (14%)	37 (13%)
Plus	260 (47%)	81 (32%)	105 (40%)	32 (12%)	42 (16%)
Total	556	202 (36%)	203 (37%)	72 (13%)	79 (14%)

Table 3

As regards the diagnosis and data available, 132 cases (30% of available data) were detected in prenatal setting and following during first 3 year of life at least, 56% were non syndromic CCA, while 44% was syndromic complex conditions.

However, it is necessary to consider the fact that a limitation of this retrospective data arises from the existence of a bias linked to the origin of the data from mainly pediatric structures and does not contain data relating to possible therapeutic abortions.

Timing Of Diagnosis	Tot	NS-CCA	S-CCA
Prenatal	132/443 (30%)	74 (56%)	58 (44%)
Postnatal	311/443 (70%)	118	193
NA	113	32	81
Total	556	224	332

Table 4

4.1.1 CNS Anomalies

Anomalies concerning the CNS found in association with the CCA of our cases are very heterogeneous.

In the order (Table 5):

Interhemispheric cysts were found in 4% of CCA, all ACC. This responds to the fact that, as previously explained in chapter II, the formation of such cysts can be just a morphologically consequent of the total or partial lack of formation of the corpus callosum.

For similar reasons, a hydrocephalus was found in 14 ACC cases.

In line with embryological and developmental reasons, anomalies of the white matter were found in a rather significant percentage of 7%.

Cortical brain anomalies were present in 18%of CCA. Nodular grey matter heterotopias were reported in 10% of CCA

These occur mainly in the form of nodular heterotopias, i.e. the formation of gray matter nodules in sites normally used to accept white matter, mainly periventricular.

20% of anomalies of the brainstem and cerebellum were found in our series.

A cerebellar hypoplasia and vermis hypoplasia was found in 10-14% of CCA.

Other numerically minor anomalies are pericallosal lipomas, agiria and pachygiria, dysgiria, anomalies of the cranial, hypothalamic nerves, basal ganglia , as well as encephaloceli and arachnoid cysts. (Table 5).

	cACC	**pACC**	**ThinCC**	**ThickCC**	**Tot**	**P value**
	N=202	N=203	N=72	N=79	N=556	
Interhemispheric	16	7	0	0	23 (4%)	0,003
Lipomata	0	6	1	1	8 (1%)	0,096
White matter anomalies	8	15	9	6	38 (7%)	0,095
Cranial nerve anomalies	7	5	1	3	16 (3%)	0,754
Hypotalamic alterations	1	7	0	0	8 (1%)	0,026
Brain cortex malformations	40	36	9	14	99 (18%)	0,587
Polymicrogyria	25	21	1	4	51 (10%)	0,021
Nodular heterotopia	26	23	1	5	55 (10%)	0,023
Lis spectrum	3	4	2	1	10 (2%)	0,88
Dysgiria	4	1	5	6	16 (3%)	0,002
Basal ganglia anomalies	6	4	1	4	15 (3%)	0,46
Brainstem and cerebellar anomalies	32	47	19	18	116 (21%)	0,144
Cerebellar hypoplasia	16	26	8	4	54 (10%)	0,157
Cerebellar vermis hypoplasia	23	32	13	12	80 (14%)	0,444
Pons anomalies	14	32	12	11	69 (13%)	0,027
Encephalocele	1	2	0	0	3 (0.5%)	0,66
Hydrocephalus	7	7	0	0	14 (2.5%)	0,146
Aracnoid cysts	0	2	1	2	5 (1%)	0,223

Table 5

The presence of macrocephaly, microcephaly or asymmetries of the skull is also reported (Table 6).

	cACC	pACC	ThinCC	ThickCC	Total
	N=202	N=203	N=72	N=79	N=556
Macrocephaly	28 (32%)	37 (43%)	8 (9%)	14 (16 %)	87/556 (16%)
Microcephaly	8 (24 %)	11 (33%)	2 (6 %)	12 (36%)	33/556 (6%)
Asymmetric skull	7	5	0	1	13 (2%)

Table 6

4.1.2 Extra CNS Anomalies

The anomalies of other organs and systems turned out to be (Table 7):

- Dysmorphisms in 257 cases out of 556 (i.e. 46.%), in particular 20 cases out of 257 (8%) in non-syndromic patients and 237 cases out of 257 (92%) in syndromic patients.

- Visual disturbances in 101 cases out of 556 (18 %), 9 (9%) among non-syndromic and 92 (91%) among syndromic ones.

- Hearing disorders in 46 cases out of 556 (8%), 2 (4%) among non-syndromic patients and 44 (96%) among syndromic patients.

- Cardiovascular pathologies were found in 81 cases out of 556 (15%), 5 (6%) in non-syndromic patients and 76 (94%) among syndromic patients.

- Respiratory pathologies were found only in syndromic patients, 22 cases (ie 4% of the total of 556 patients).

- Gastrointestinal pathologies were observed in 26 patients out of 556 (5%), 2 (8%) among the non-syndromic and 24 (92%) among the syndromic CCA.

- Skeletal anomalies in 99 patients out of 556 (18%), 10 (10%) among non-syndromic and 89 (90%) among syndromic forms.

- Urogenital abnormalities in 75 patients out of 556 (16%), 8 (11%) among non-

syndromic and 67 (89 %) among syndromic ones.

	NS-CCA	S-CCA	TOT.
	N=224	N=332	N=556
Dysmorphisms	20 (8%)	237 (92 %)	257 (46%)
Vision impairment	9 (9%)	92 (91%)	101 (18%)
Hearing loss	2 (4%)	44 (96%)	46 (8%)
Congenital heart defects	5 (6%)	76 (94%)	81 (15%)
Respiratory problems	0	22 (100%)	22 (4%)
Gastrointestinal problems	2 (8%)	24 (92%)	26 (5%)
Skeletal anomalies	10 (10%)	89 (90%)	99 (18%)
Urogenital anomalies	8 (11%)	67 (89%)	75 (14%)
Table 7			

4.1.3. Neurodevelopmental features and CCA

Reviewing clinical questionnaire of this heterogeneous series, we try to evaluate results and some considerations. (Table 8, 9, 10, 11). Epilepsy was present in 13% of NS-CCA versus 39% of syndromic ones.In particular, seizures was reported in 9%of Isolated NS-CC and 3% S-CCA without other brain anomalies, detectable by brain MRI and DTI study.

Developmental delay and intellectual disability were reported in 93 % od S-CCA and 55% of NS patients, with prevalence of mild impairment (61%).

These percentage reduced in isolated CCA (51% NS vs 87%). While no significant difference of cognitive involvement was evident in S-CCA, as you can see in Table 9, in NS-CCA isolated normal cognitive was reported in 65% of complete ACC and mild cognitive impairment in 80% of patient with intellectual disability (35%).High percentage of patients with isolated NS corpus callosum hypoplasia (74%) and thickness (72%). As expected these value improve in NS-CCA plus with other brain anomalies, but cACC confirmed a better prognosis than others CCA.

Behavior problems were referred in similar percentage (23-20%) in NS and S-CCA. Autism was present in 8% of NS-CCA and 9% in isolated NS-CCA. Very low was

the percentage of autism in NS-cACC isolated and plus (2%), confirming its best prognosis.

	NS-CCA	Isolated	S-CCA	Isolated	Tot
	N=224	N=164	N=332	N=128	N=556
Epilepsy	31 (13%)	15/164 (9%)	132 (39 %)	40/128 (3%)	163 (29%)
DD/ID	121(55%)	83/164 (51%)	294 (93%)	111/128 (87%)	415 (75%)
Mild	74 (61%)	53/83 (63%)	105 (36%)	44/111 (40%)	179 (32%)
Moderate	31 (26%)	20/83 (24%)	75 (26%)	35/111 (32%)	106 (19%)
Severe	16 (13%)	10 (13%)	114 (38%)	32/111 (28%)	130 (23%)
Autism	17 (8%)	14 (9%)	9 (3%)	6 (5%)	26 (5%)
Behaviour problems	51 (23%)	37 (23%)	57 (17%)	25 (20%)	108 (20%)

Table 8

	Syndromic CCA n=332 **Available NPI data 311** **Not available data 13/332 (4%)**					
	Mean age	cACC	pACC	ThinCC	ThickCC	Tot n=319
		n=89	n=133	n=45	n=52	
Normal IQ	8	4 (4%)	16 (12%) (9 isolated)	2 (4%) (2 isolated)	3 (6%)	25/319 (8%)
DD/ID	12,7	85 (96%)	117 (88%)	43 (96%)	49 (94%)	294/319 (93%)
Mild	10	26/85 (31%)	38/117 (32%)	16/43 (37%)	25/49 (51%)	105/319 (33%)
Moderate	14	18/85 (21%)	35/117 (30%)	9/43 (21%)	13/49 (27%)	75/319 (24%)
Severe	13	41/85 (48%)	44/117 (38%)	18/43 (42%)	11/49 (22%)	114/ 319 (36%)
Behaviour problems		12/89 (13%)	18/133 (14%)	13/45 (29%)	14/52 (27%)	57/319 (18%)
Autism		1/89 (1%)	3/133 (2%)	3/45 (7%)	2/52 (4%)	9/319 (3%)

Table 9

	Non-Syndromic isolated CCA n=168 Not available data 4/168 (2,4%)					
	Median age	cACC n=86	pACC n=41	ThinCC n=19	ThickCC n=18	Tot n=164
NORMAL IQ	12.8	56 (65%)	15 (37%)	5(26%)	5 (28%)	81/164 (49%)
ID/DD	11	30 (35%)	26 (63%)	14 (74%)	13 (72%)	83/164 (51%)
Mild	9	24/30 (80%)	15/26 (58%)	6/14 (43%)	8/13 (62%)	53/83 (63%)
Moderate	14	3/30 (10%)	6/26 (23%)	8/14 (57%)	3/13 (23%)	20/83 (24%)
Severe	11	3/30 (10%)	5/26 (19%)	0	2/13 (15%)	10 (13%)
Behavior problems	13,4	8/86 (9%)	11/41 (27%)	8/19 (42%)	10/18 (56%)	37/164 (23%)
Autism	8,9	2/86 (2%)	4/41(10%)	1/19 (5%)	7/18 (39%)	14/164 (9%)
Table 10						

	Non-Syndromic CCA plus n=56 Not available data 1/56 (2%)					
	Median age	cACC n=19	pACC n=25	ThinCC N=4	ThickCC N=7	Tot n=55
Norma IQ	12.8	8 (42%)	9 (36%)	0	0	17/55 (31%)
ID/DD	11	11 (48%)	16 (64%)	4 (100%)	7 (100%)	38/55 (69%)
Mild	9	7/11 (64%)	9/16 (56%)	1/4 (25%)	4/7 (57%)	21/38(55%)
Moderate	14	3/11 (27%)	6 (38%)	1/4 (25%)	1/7 (14%)	11/38 (29%)
Severe	11	1/11 (9%)	1 (6%)	2/4 (50%)	2/7 (29%)	6/38 (16%)
Behaviour problems	13,4	1/19 (5%)	8/25 (32%)	2/4 (50%)	3/7 (43%)	14/55 (25%)
Autism	8	0	2/25 (8%)	0	1/7 (14%)	3/55(5%)
Table 11						

4.1.4 Clinical and genetic testing

Our series counts S-CCA (37% of all S-CCA) with known clinical and molecular etiology.

Within this group, we found a wide variety of clinical phenotypes demonstrating

genetic heterogeneity. Fifty-one percent of them had a clinical diagnosis of a monogenic known syndrome, in some cases confirmed by molecular testing (Table. 12). The most frequent condition (13 patients) was Aicardi syndrome (OMIM 304050) in which agenesis of corpus callosum is one of the mandatory diagnostic features. CCA was a frequently/occasionally-associated sign among the other syndromes.

Patient N.	Diagnosis	OMIM	Type of CCA	Recognized CCA syndrome
2*	L1 Syndrome	308840	pACC	+++
13	Aicardi Syndrome	304050	cACC	+++
1	Polimicrogyria, CC hyperplasia		ThickCC	-
1	Rett syndrome	312750	pACC	++
2	ARX Syndrome	300382	cACC	+
1	Angelman Syndrome	105830	ThinCC	+
1	Smith-Lemli-Opitz Syndrome	270400	ThinCC	+
1	Mucopolysaccharidosis type ii	309900	ThinCC	+
3*	Sotos Syndrome§	117550	cACC, pACC	+++
2*	Tuberous Sclerosis Syndrome	191100	cACC, pACC	+
1*	Kabuki Syndrome	147920	pACC	+
4*	Mowat-Wilson§	235730	cACC, pACC	+++
1*	Malpuech Syndrome	248340	pACC	+
1*	FG Syndrome	305450	pACC	+
1*	Hypomelanosis of Ito	300337	cACC	+
2*	Goldenhar Syndrome	164210	cACC, pACC	+
1*	MMR Syndrome (megalocornea mental retardation)	249310	pACC	+
1	Sekel syndrome	210600	pACC	+
2	Phelan Mc Dermid Syndrome	606232	pACC, ThickCC	+
2	Wolf-Hishorn syndrome#	194190	pACC	+
1	Sindrome ATR-X	301040	cACC	+
1	Prader Willi syndrome°	176270	cACC	+
1	Smith-Magenis syndrome§	182290	ThickCC	
1	Acrocallosal syndrome	200990	cACC	+++
1	Syndromic craniostenosis	123100	pACC	+
1	Willams-Beuren syndrome	194050	ThickCC	-
1	Greig syndrome§	175700	ThinCC	+
1	TUBB2 realated syndrome	615763	pACC	+++
1*	Neurofibromatosis type 1 syndrome§	613113	pACC	+/-
1	Cri du chat syndrome§	123450	cACC	+
1’	TBCK mutation	616899	ThinCC	+
1	DDX3X mutation	300160	pACC	+

Table 12. Monogenic Disorders in Syndromic CCA.

*àsome cases recently published by Romaniello et al. 2016

‘ recently published by Chong JX et al. 2017

§ point mutation or chromosomal microdeletion at CMA

chromosomal deletion at karyotype

° Uniparental disomy at methylation test

Among patients with a known diagnosis, 18% (5% of all S-CCA) presented karyotype alterations. Among these, common trisomies, such as two cases of trisomy 21

(Down's Syndrome), one case of Klinefelter syndrome and one of Edwards syndrome, as well as five patients with chromosome 8 rearrangements, were referred. For the first time we described X monosomy with ThickCC (Table 13).

Pz N.	Chromosomal Rearrangement	Type of CCA	Clinical features
1	45,X0	ThickCC	
1	46,XY/47,XY,+8	cACC	Mild DI, PDA, renal hypoplasia
1*	47,XY+18	pACC	
1*	47,XXY	pACC	Mild DD, epilepsy
2*	47,XY+21	pACC	DD
1*	46XYder(21)	cACC	Severe ID, extracallosal brain anomalies, microcephaly, dysmorphisms, PDA
1*	46,XX, der(21)t(8;21)(p23;22)	pACC	DD, dysmophisms
1	dup10p11.2-ter del 10q26-qter	cACC	Severe ID, epiplepsy, prominent metopic suture, microcephaly, dysmorphisms
1*	46,XY,del(10)(q26.1)	pACC	Moderate DD, microcephaly hearing loss, VSD, dysmorphism S
1*	46,XY,der(13)t(13q34;18q23)	pACC	Moderate DD, microcephaly, PDA, hearing loss, left double renal district, dysmorphisms
1*	46,XY,del(1)(q43)	cACC	Severe DI, severe microcephaly,hearing loss, dysmorphisms
1*	46,XY,invdup(8)(p23)	pACC	Severe DI, macrocephaly, dysmorphisms, tetraparesis
1*	46,XX,invdup(8)(p23)	cACC	Severe mental retardation, microcornea, hypotonia, facial dismorphisms, cleft palate, atrial septal defect
1	46, XX, Invdup del8	cACC	DI, brainstem atrophy
1	45, XY, -18[3]/46, XY, r(18)(p11.2 2p22)[47]	ThinCC	Severe ID, dysmorphisms, left ventricular hypertrophy, hypospadia
1	46,XX,del(13)(q32q32)	pACC	Severe ID, retinal vessels tortuosity, hearing loss, ASD, dysmorphisms
1	46,XYi(18)(qter-> q10::q10->qter)	ThinCC	Severe DD, dysmorphisms, epilepsy
1	46,XX,der(13)t(6;13)(q27;q3)	cACC	Severe ID, epilepsy microcephaly, ASD, dysmorphisms
1	46,XY,der(10)t(4;10)(p15.2;p15)	pACC	Severe ID, epilepsy, dysmorphisms
1	8p- syndrome	cACC	Severe ID, microcephaly, ASD, dysmorphisms
1	mos 45,XY,-18[3]/46, XY, r(18)(p.11.2p22)[47]	ThinCC	Severe ID, dysmorphisms, cleft palate, left ventricle hypertrophia, urogenital anomalies
1	46,XY,der(20)t(9;20)(p24.1;p13)mat.ish der(20)(D20S1157-, 305J7-T7+).	pACC	Mild ID, hyposmia, pectus excavatum, feet anomalies

Table 13. Chromosomal rearrangement on high resolution Karyotype

* some cases recently published by Romaniello et al. 2016

Microdeletion/microduplication syndromes were found in the remaining 31% of

cases with a known genetic diagnosis.

Overall CMA was reported in 55% of cases, 32% NS-CCA (n=99) and 68% S-CCA (n=206). See Table 14-15 with CNVs divided in larger/smaller than 1 Mb.

In S-CCA detection rate of CMA was 23%, while negative in 55%. In 22% variant of unknown significance (VUS) were found.

ThickCC was found in microdeletions, never reported in literature, Williams-Beuren, Smith-Magenis and Phelan McDermid syndromes and del3p14.1 dup11p14.3, del13q14, del1p36.3 condition. This type of callosal dysplasia was also present in other large rearrangements such as: del1p36 (OMIM#607872), del3p14.1, del13q14, del15q24, dup11p14.3, dup12p13 and dup14q11.2, dup1q34 A case of pAgCC due to del17q11.2 was found in a patient with NF1 syndrome.

In NS-CCA detection rate was 2% CNVs likely pathogenic, while negative cases were 66% and VUS 32%

ThickCC of corpus callosum was also described for the first time in an NS-CCA patient with a microduplication 1q21 (1.2 Mb, OMIM#612475), paternally inherited, with autism spectrum disorders and familial positive anamnesis for psychiatric illness (bipolar disorder, depression). In a patient with NS cACC a microdeletion 16p13.3 (2.3 Mb), inherited from the mother, was reported (Table 14).

In 8 cases (6 NS-CCA and 2 S-CCA) the CNVs reported were probably pathogenic. In particular, two del16p13.3 (OMIM#610543), in patients with cACC and Hypo CC, both maternally inherited, encompassing *RBFOX1* gene, were associated to epilepsy and neuropsychiatric disorders.[40] Two male patients (NS-CCA and S-CCA) presented Xq28 duplication (OMIM#300815), encompassing *GDI1 e MECP2* genes, previously associated to CCA.[28] A case was also reported with pACC, developmental delay, behavioral problems and with a deletion of 58 kb in Xq28, encompassing the gene *RPL10* (OMIM # 312173), recently described in X-linked ID condition[41] and in a syndromic disease with cerebellar involvement and Spondylo-Epiphyseal Dysplasia[42]. A maternally

inherited 15q13.3 duplication (460 Kb), encompassing *CHRNA7* gene (OMIM# 118511), was reported in one case (Table 15).

Pat N. Gender	Cr Banda	Size	Origin	OMIM	CCA	NS/S	Clinical features	CNVS associated to CCa
1 M	dup 1q21	1,2 Mb	Pat	612475	ThickCC	NS	Autism	-
1 M	del 1q44	1,3 Mb	de novo		pACC	S	ID moderate, microcephaly, lipomas, wthite matter anomalies, epilepsy, ASD, dismorphisms	+
1* M	del 1q44	5,1 Mb	-		cACC	S	ID severe, ASD, dismorphisms	+
1*	del 1p36.3	1.5 Mb	de novo	607872	pACC,	S	ID	+
M								-
1					ThickCC		ID, macrocephaly, extra callosal CNS anomalies	
M								
2					ThinCC		ID severe, congenital multiple malformations	
M/F								
1 F					pACC		DD severe, epilepsy, anomalia di Ebstein anomalie, tetraparesis, dismorphisms	+
1 M	del 3q29	2,3 Mb	de novo	609425	ThinCC	S	DD, developmental problems, ADHD	+
1 M	del 3q13.31	2.2 Mb	de novo	615433	pACC	S	DD severe, prominent metopic ridge	+
1 F	del 3q13.11	14,9 Mb	de novo		pACC	S	ID, dismorphisms	+
1 M	del 3p14.1	12,3 Mb	de novo		ThickCC	S	DD severe, cerebral gyral anomalies, foramen magnus stenosis	+/-
1	del 4q34.3	1 Mb	de novo	615656	pACC	S	ID severe, extra callosal CNS anomalies	-
M	del 15q11.2						dismorphisms	
1 M	dup 11p14.3	1,7 Mb	-		ThickCC	S	ID, macrocephaly, megalencephaly	+/-
1	dup 12p13	15,78 Mb	de novo		ThickCC	S	DD, ocular anomalies, dismorphisms	+/-
M	dup 14q11.2	7,99 Mb						
1 M	del 13q21.1	1,3 Mb	NA		ThinCC	S	DD moderate, microcefaly, dismorphisms	+
1 M	del 13q14	9,2 Mb	de novo	613884	ThickCC	S	ID, dismorphisms, hypospadia	+/-
1* M	del 14q12	3 Mb	de novo	613457	pACC	S	ID moderate, epilepsy, dismorphisms, extra callosal CNS anomalies	+
1* M	del 14q12	1 Mb	de novo	613457	cACC	S	ID moderate, microcephaly, epilepsy, dismorphisms	+
1 M	del 14q12	1.3 Mb	de novo	613457	pACC	S	DD severe, microcephaly, dismorphisms	+
1 F	del 15q24	1.3 Mb	de novo	613406	ThickCC	S	ID severe, microcephaly, dismorphisms, multiple malformations	+
1 F	dup 16p13.3	2 Mb	-	613458	cACC	S	ID mild moderate, dismorphisms	+
1 F	del 16p13.3	2,3 Mb	de novo	610543	cACC	S	ID mild, dismorphisms, ASD	+
1 M	del 17p13.3	5.6 Mb	-		ThinCC	S	DD, cerebellar hypoplasia, empty sella, dismorphisms, truncal obesity, short stature, behaviour problems	+
1 F	del 17q21.31	2 Mb	de novo		ThinCC	S	DD, dismorphisms	+

Table14: Large CNVs >1 Mb/ Microdeletion-duplication syndromes reported

Gender	del/ dup	Chr band	Size	Hg	Coordinates	*origin*	*Genes*	CCA	NS/S	Phenotype	Diagnosis
F*	Dup	1p36	296	19	Chr1:50922171-51218439	*de novo*	*Exon 5-18 FAFI*	pACC	S	Cerebellar vermis hypoplasia, microcephaly, esophoria, dysmorphisms,	VUS
F	Del	1q23.1	73	19	chr1:155,882-155,963	*pat*		Hypo CC	S	Severe DI, pachygyria, cerebellar hypoplasia, dysmorphisms	VUS, likely benign
	Del	6q26	163		chr6:163,025-163,299	*mat*					VUS, likely benign
M	Dup	3p24	294	19	chr3:28,025,781-28,320,086	*pat*	*CMC1*	cACC	NS	normal IQ, speech problems	VUS
	Dup	5p13.3	47		chr5:32,110,734-32,158,429	*de novo*	*PDZD2, GOLPH3*				VUS, likely benign
	Del	11q21.1	40		chr11:59,824,041-59,864,046	*mat*	*MS4A3, partial* *MS4A2*				VUS
F	Del	3p14.2	228	19	chr3:59026972-59799	*pat*	*C3orf67, FHIT*	pACC	S	ID, behaviour problems epilepsy, dysmorphirms,VSD, cardiac hypetrophy, myopia	VUS
M*	Dup	4q25	508	19	Chr4:113121551-1136	*pat*	*APIAR,TIFA, ALPK1, NEUROG2, c4orf21, ZGRF1, LARP7*	cACC	S	Mild ID, mild back hypertrichosis, esotropy, broad nose with high nasal bridge, posteriorly rotated ears, wide deep philtrum, small mandible, high palate, supplementary nipples	VUS
M	Dup	4p16.3	61	19	chr4: 55715-116860	*na*	*ZNF718 - ZNF595 - ZNF718*	cACC	NS	Normal IQ	likely benign
F	Del	5q14.2	158	19	chr5:81833016-81991	*pat*	-	cACC	S	Severe ID, hypoplasia right brain hemisphere, polymicrogyrya, focal epilepsy	VUS, likely benign
	Del	Xq24Xq25	182		chrX:120783916-1209	*mat*	-				VUS, likely benign
F*	Del	6q22.31	57	19	chr6: 118,823,223-118	*pat*	*first 2 exons PNL, exons 5,6 CEP85L*	pACC	S	Moderate DD, epilepsy, ASD, VSD, inguinal hernia	VUS *PLN is associated to Hypertrofic cardiomyopathy (OMIM 613874) e dilatativ (OMIM 609909*
F	Del	6q23.2	75	18	chr6:131,666,377-132,	*de novo*	*ARG1, MED23,ENPP1*	Hypo CC	S	Moderate ID, dysmorphisms	VUS
F*	Dup	8p11.21	395	19	chr8:42.938.288 - 43.3	*mat*	*FNTA, SGK196, HGSNAT, POTEA*	pACC	S	ID moderate, microcephaly, white matter anomalies, laryngomalacia	VUS
F*	Dup	8q21.3	126	19	chr8:87175213-87300	*mat*	*SLC7A13*	cACC	NS	Severe ID, epilepsy	Likely benign
M	Dup	9p24.3	135	19	chr9:875558-1010349	*mat*	first 2 exon DMRT1, DMRT3	cACC	NS	Learning disability	Likely benign
M	Dup	10p11.21	216	19	chr10:35,220,579-35,437,319		*CUL2, CREM*	Hyper CC	NS	Moderate ID, autism	VUS
M	Dup	11q23.3	76	19	chr11:117,955,758-118	*pat*	*TMPRSS4,OMIM Morbid SCN4B*	Hypo CC	S	Epilepsy, spastic cerebral palsy	VUS
Na	Del	12p12	344	18	chr12:21032465-21377	*mat*	*SLC01B3,SLC01B7, SLC01B1*	Hypo CC	S	ID, white matter anomalies	VUS
	Dup	18p11	188		chr18:12691414-12797	*pat*	*PSMG2, CEP76, PTPN2*				
F	dup	12q24.31	242	19	chr12:120,968,373..121,210,427		*RNF10 - POP5 - CABP1 - MLEC - UNC119B - ACADS - SPPL3*	Hypo CC	S	Mild DD, dysmorphysms	VUS
M	Del	13q33.1	76	19	chr13:101,212,939-101	*na*	*NALCN*	Hypo CC	S	DI, dysmorphysms, congenital contractures	Pathogenetic CLIFAHDD, (OMIM#616266)

Table15: Part I

Gender	del/ dup	Chr band	Size	Hg	Coordinates	origin	Genes	CCA	NS/S	Phenotype	Diagnosis
M	Del	14q32.33	66	19	chr14:105194478-105	*de novo*	*AKT1*	Hyper CC	NS	Moderate ID, behaviour problems	VUS
F	Del	14q32.33	225	19	chr14:106538421-106763947	*na*	*LINC00226*	cACC	NS	Moderate DD, Hypeactyvity, macrocephaly, dysmorphisms	likely benign
	Del	20p12.1	19		chr20:14416991-14436873		*MACROD2*				
F	Dup	15q13.2	660	19	chr15:30,943,903-31,609,679	*mat*	*LOC100288637, HERC2P10, MTMR10, FAN1,TRPM1,MIR211, LOC283710*	pACC	NS	DD	VUS
M	Dup	15q13.3	460	19	chr15:31,981,638-32,444,196	*mat*	*CHRNA7*	pACC	NS	DD	Pathogenetic *CHRNA7* (OMIM# 118511)
F	Del	15q21.1	161	19	chr15:46,125,094-46,286,679	*pat*		pACC	S	Mild DI, periventricular heterotopia, obesity	Likely benign
F	Del	16p13.3	219	19	chr16:6,920,942-7,139,773	*mat*	*RBFOX1*	cACC	S	ID, hypotonia	Probably Pathogenetic *RBFOX1 (OMIM# 605104)*
M	Del	16p13.3	79	19	chr16:7180401-7260025	*mat*	*RBFOX1*	Hypo CC	S	Moderate ID, dysmorphisms	Probably Pathpgenetic *RBFOX1 (OMIM# 605104)*
F	Del	16q22.1	133	19	chr16:70,152,776-70,286,564	*na*	*PDPR, CLEC18C, SMG1P7, EXOSC6 (PARZIALE), AARS(PARZIALE)*	Hypo CC	NS	Mild ID	VUS
F	Dup	17q11.2	96	19	Chr17:29108874-29204874	*mat*	*CRLF3, first 15 exons ATADs*	pACC	S	Severe ID, cerebellar vermis hypoplasia, spastic tatraparesis, microcephaly,	VUS
M	Dup	19p13.12	367	19	chr19:15,124,588-15,491,798	*de novo*	*ILVBL - NOTCH3 - EPHX3 - BRD4 - AKAP8 - AKAP8L - WIZ - RASAL3 - PGLYRP2*	pACC	NS	ID	VUS
M	Del	19p13.3	656	19	chr19:64,447-721,353	*de novo*	*WASH5P, FAM138F,FAM138A, OR4F17, LINC01002, MIER2, PPAP2C, THEG, C2CD4C, SHC2, ODF3L2, MADCAM1, TPGS1, HCN2, POLRMT, FGF22, BSG, RNF126, PRSS57, PAL,FSTL3*	pACC	NS	ID, cerebellar hypoplasia	VUS
F*	Del	21q22.3	25	19	Chr21:47561572-47577572	*pat*	*First 10 exons of FTCD*	cACC	S	severe ID, microcephaly, hypertelorism, epilepsy	VUS
F	Dup	22q11.23	244	19	chr22:22,642,519-22,887,125	*pat*	*BMS1P20 - ZNF280B - ZNF280A -*	pACC	NS	ID	VUS, likely benign
M	Dup	Xq22.2	67	19	chrX:103,220,412-103,288,063	*na*	*Mir1256, TMSB15B, H2BFXP,H2BFWT*	Hypo CC	NS	ID, macrocephaly	VUS
M	Del	Xq28	58	19	chrX:154,396,791-154,425,825	*na*	*RPL10, SNORA70, DNASE1L1, TAZ*	pACC	NS	DD, behavour problems	Probably pathogenetic *RPL10* (OMIM #312173)
M	Dup	Xp22.33	276		chrX:2,066,581-2,343,577	*mat*	*DHRSX*	cACC	NS	Normal IQ	VUS
	Del	12p13.31	67		chr12:7,985,489-8,053,460	*mat*	*SLC2A14*				VUS
	Dup	16p12.2	164		chr16:21,574,908-21,739,885	*mat*	*METTL9, IGSF6, OTOA*				VUS
M	Dup	Xq12	80	19	chrX:65815490-65895015		*EDA2R*	Hypo CC	S	Severe ID, epilepsy, cerebellar hypoplasia	VUS
	Del	6p24.3	112		chr6:9866302-9977793		*OFCC1*				VUS
	Dup	7q36.2	386		chr7:154623222-155008840		*DPP6,PAXIP1,HTR5A*				VUS
	Del	22q12.3	220		Chr22:33961343-34181601		*LARGE*				VUS
M	Dup	Xq28	773	19	chrX: 153059079-153832724	*mat*	*Diveris geni tra cui GDI1 e MECP2*	pACC	NS	ID moderato, white matter anomalies	Pathogenetic *(OMIM#300815) GDI1 e MECP2*
M	Dup	Xq28	250	19	chrX:153577888-153832694	*mat*	*GDI1, FLNA, RPL10,EMD, TAZ, G6PD,*	pACC	S	ID moderate, cerebellar atrophy, epilepsy	Pathogenetic *(OMIM#300815) GDI1*

Table 15: Part II

4.2 Results, perspective study: preliminary data

4.2.1 Isolated NS-ACC and normal cognition

Our series includes 56 patients with isolated non syndromic ACC without intellectual disability, after psychometric evaluation age related by our neuropsychological protocol.

Array CGH was performed in 14 patients (25%) and was negative in 7 patients (50%) and VUS were identified in 7 patients.

WES was performed in one female patient with cACC an IQ 120 but no pathogenic mutations were identified.

4.2.2 Family CCA

Our cohort included four CCA family with two or more members affected by CCA with different clinical findings in the same family. Array CGH was performed in all family and was negative.

WES was performed in all family, of which in two S-ACC and in two NS-ACC ones.

The proband of first syndromic case (Figure 15), a 5-year-old boy, had facial dysmorphisms such as noting plagiocephaly, prominent metopic suture, prominent ears, short neck, thumb adducted to the hands bilaterally moderate developmental delay, absence of expressive language. He also had a peculiar gait with bent knees and extra-rotated feet. The parents had had a second pregnancy hesitated in therapeutic interruption for the confirmation in the male fetus of agenesis of the corpus callosum at the ultrasound evaluation of the II trimester of pregnancy. The family history showed on the maternal side the presence of three uncles suffering from severe intellectual disabilities and behavioral problems. In one of these, agenesis of the corpus callosum was reported.

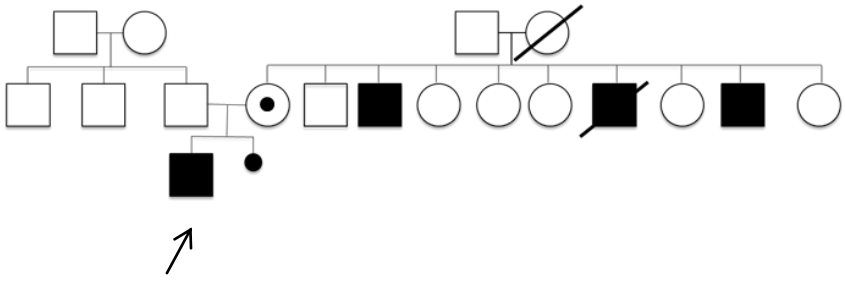
Array CGH reported only VUS parental inherited.

WES variations analysis in quartet (trio+uncle) highlighted a maternally inherited hemizygous mutation c.1108G> A (p.Gly370Arg) in exon 9 of the *L1CAM gene*.

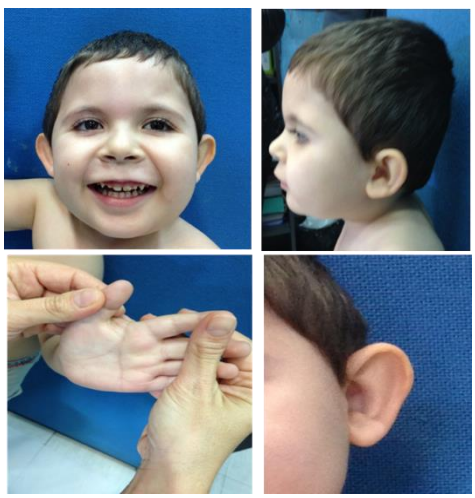
This mutation was described by Ruiz et al., 1995 (HGMD: # CM950736) in a family with MASA syndrome, characterized by the presence of agenesis of the corpus callosum.

L1CAM mutations (OMIM # 308840) were associated with several clinical phenotypes including Agenesis of the corpus callosum (OMIM # 304100¬), hydrocephalus from Silvio aqueduct stenosis (OMIM # 307000), CRASH syndrome (Corpus callosum hypoplasia, Retardation , Adducted thumbs, Spastic paraplegia, and Hydrocephalus, OMIM # 303350) and MASA syndrome (Mental retardation, Adducted thumbs, Shuffling gait, and Aphasia OMIM # 303350).[43]

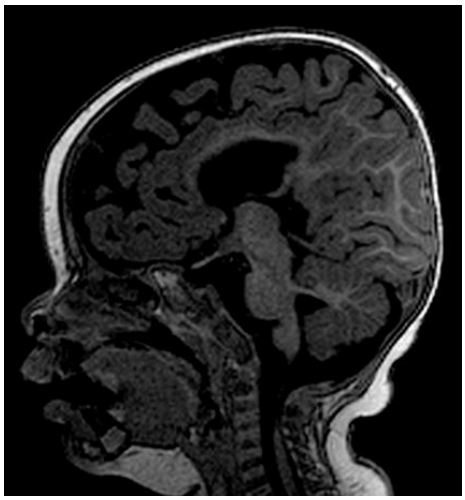
Even the members of our family, despite having the same variant, presented very heterogeneous phenotypic pictures, demonstrating the variable expressiveness of mutations of the *L1CAM* gene.



A



B.



C.

Figura 15. A. genealogical tree, B. proband, note plagiocephaly, prominent metopic suture, prominent ears, short neck, adducted thumb. C. MRI brain, sagittal plane at T1-weighted sequences showing marked hypoplasia of the corpus callosum and megacisterna magna.

Our second ACC family proband has mild developmental delay and complete ACC diagnosed by prenatal setting and confirmed in postnatal brain MRI. At family history his father had myoclonic epilepsy and pACC by brain MRI. The grandfather also had incidental diagnosis of cACC, during brain TC examination for a ictus cerebri.

WES performed in trio and all paternal uncles samples, identified paternally inherited heterozygous variation in exon 16 of *DCC* gene NM_005215.3:c.2426A>G (p.Tyr809Cys).This variation was also identified in one paternal uncle with negative brain MRI. Monoallelic *DCC* mutations were associated to congenital mirror movements, isolated ACC, or both.[44] Biallelic *DCC* mutations were reported in patients with a developmental splitbrain syndrome (DSBS).[45]

The variation c.2426A>G (p.Tyr809Cys) detected in our family was not present in *EXAC and Gnomad or Clinva*r and predicted as pathogenic by principals protein prediction tools (*mutation taster, polyphen2,m-cap*). According to recommendation of American College of Medical Genetics and Genomics and the Association for Molecular Pathology[46,47] and some of its tool (*Varsome*), this variation was a VUS. Agree with its pathogenic rule, we confirmed in three family members with different clinical phenotype, assuming a possible incomplete penetrance, previously reported in other works. [46,47]

We are revaluating clinical phenotype of this family to exclude or confirm congenital mirror movement and to determine cognitive profile by our neuropsychological protocol.

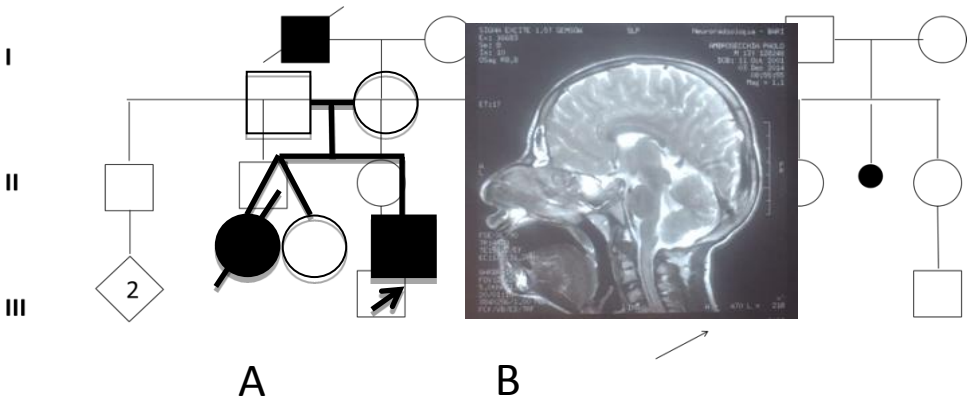


Figure 16: genealogical tree of our *DCC* family.

Third family proband had complex phenotype including pACC microcephaly, facial dysmorphisms, growth retardation, dystonic tetraparesis, stereotypic movement, generalized epilepsy, severe intellectual disability and absence of expressive language. Family history reported a sister died at 2 years of age with developmental delay, facial dysmorphisms, club feet, and at brain MRI pACC, cerebellum (vermis) hypoplasia and retrocerebellar aracnoid cyst, cortical dysplasia and white matter hypomyelination (Figure 17).

CMA was negative and WES analysis in trio samples was negative. DNA sample of affected sister was not available.

Figure 17: genealogical tree of S-pACC family and sagittal MRI scan of proband (A,B).

Our fourth family includes three generation of pACC and pontocerebellar hypoplasia and intellectual disability and behavior problems in three sisters (two monozygotic twins) , and their mother and grandmother. One of the sisters also present generalized epilepsy only partially drug responder, while her mother reported generalized epilepsy (absences) until adult age. CMA was negative. WES analysis in all family members are in progress.

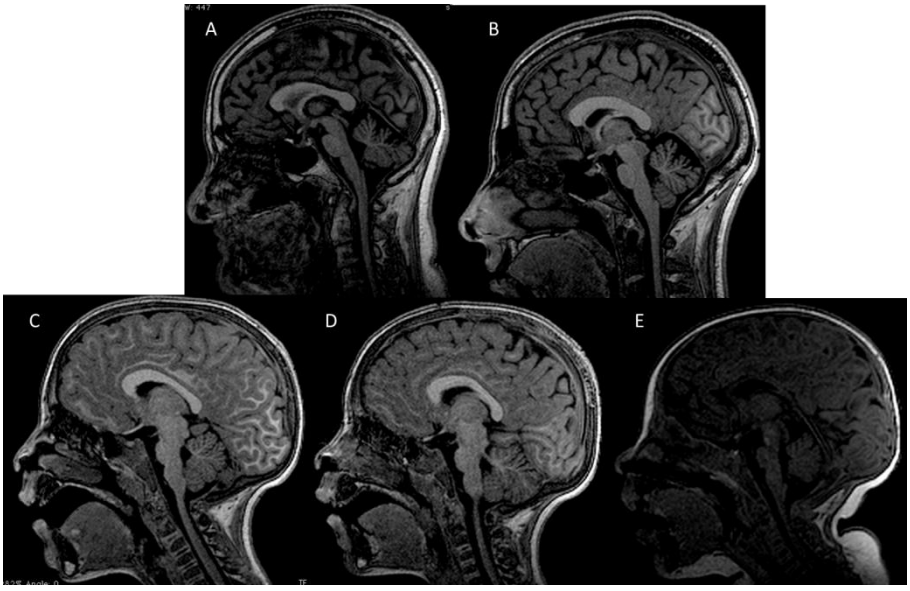


Figure 18.MRI brain scans T1weight of grandmother (A), mother (B), daughters (C, D, E)

4.2.3 ThickCC project

Our series includes 79 Thick CC patients (69% male and 31% female) all sporadic case, not including NF1 patients. S-ThickCC was 69% in our series. In 42/79 we found other brain anomalies (Plus 53%) and in remaining cases were isolated CCA.

Developmental delay and or intellectual disability was reported in 80% of NS-ThickCC and 93% of syndromic ones. Epilepsy was reported in 28% of non-syndromic patients and in 19% in syndromic ones. Autism was present in high percentage (32% and 39 in isolated) on NS patient.

Regarding genetic results 33% of S and only 8%of NS had known etiology, by

karyotype and CMA in genetic condition previously mentioned. WES analysis was performed in six cases. In one isolated NS-Thick CC no pathogenic mutations were found. Three WES in syndromic form are in progress and positive in two.

Of these WES analyses found a *PIK3R2 (*OMIM# 603157de novo mutation was detected in a patient 8-year old with polymicrogyria and focal cortical dysplasia (Figure 18).[48]

The proband also presented spastic hemiplegia on the left and dysmorphic notes, which included, sinofria, flat nasal bridge, anteverse nostrils, thumbs and wide toes. The brain MRI examination performed at the age of 3 years showed bilateral frontoparietal polymicrogyria and incomplete perisylvian opercolarization of the right hemisphere, showing thickening and blurring of the junction between cortex and white matter at the level of the right temporal cortex with reduced myelination of the white matter and hypoplasia of the right cortico-spinal bundle, cystic lesion in the right frontal operculum and hyperplasia of the corpus callosum.

Activating somatic mutations in the PI3K – AKT – mTOR pathway have recently been identified as causing hyper-growth syndromes. De novo post-zygotic variants in the PIK3CA gene are responsible for the spectrum of mutation-related overgrowth conditions (PROS) related to mutations in this gene, including megalencephaly and capillary malformation syndrome (MCAP) and hemimegalencephaly syndrome (MCAP) (hemimegalencephaly, HMEG). De novo germinal mutations in the *PIK3R2* and *AKT3* genes, which are components upstream of the mTOR pathway, have been found in patients with megalencephaly-polymicrogyria-polydactyly-hydrocephalus MPPH syndrome and in patients with perisylvian polymicrogyria.

The new mutation found in our patient c.1669G>C (p.D557H) in the *PIK3R2* gene affects a highly conserved residue at the interface with the α catalytic subunit of PIK3. (Figure 19)

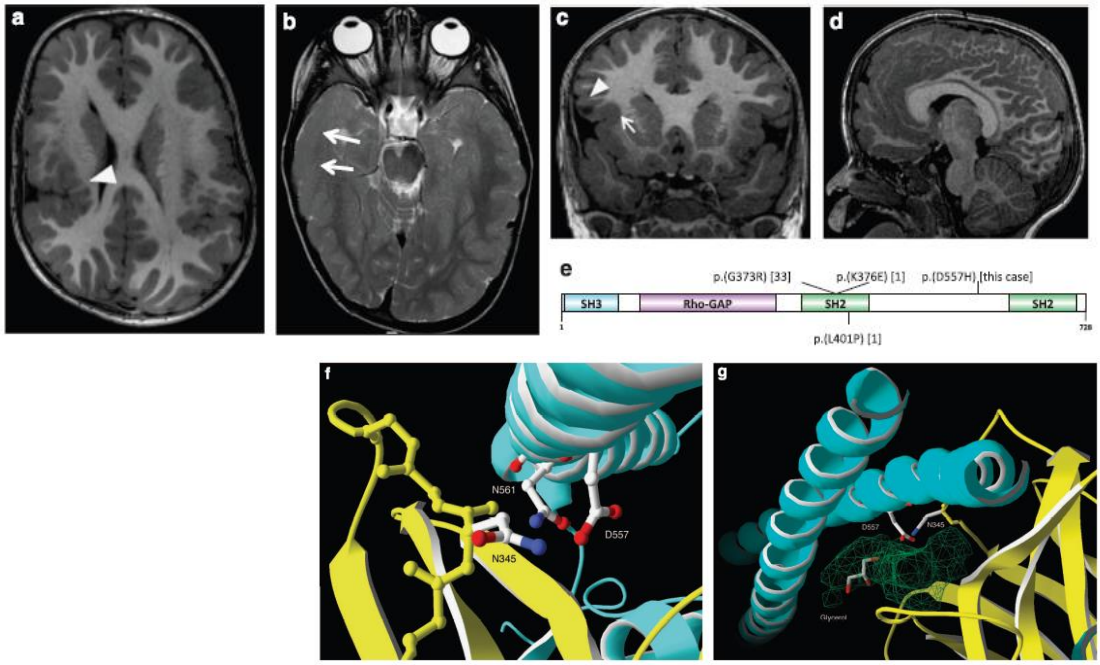


Figure 19: brain MRI, localization and modeling of the mutated residue of *PIK3R2*. (a) on the axial plane scan at T1-weighted sequences shows bilateral frontoparietal polymicrogyria and incomplete perisylvian opercolarization of the right hemisphere (white arrow); (b) axial scan to T2-weighted sequences show thickening and blurring of the junction between cortex and white matter at the level of the right temporal cortex with reduced myelination of the white matter and hypoplasia of the right cortico-spinal bundle. (c) the scan on the coronal plane at T1-weighted sequences shows cortical thickened infolding with blurring of the subcortical white matter in the anterior right portion of the insula (white arrow) and cystic lesion in the right frontal operculum (white arrow head). (d) thickened corpus callosum. (e) Schematic representation of the protein domains and mutations in *PIK3R2* reported in the literature and of the patient described by us c.1669G>C (p.D557H). (f, g) interaction between the residue of *PIK3R2* D557 and PIK3CA N34 by reconstruction with UNIPROT. (Terrone et al 2016)[48]

About the second case solved by WES analysis, it is a sporadic case of a 9 year old boy with intellectual grade disability moderate, short stature and a complex brain malformation pattern characterized by corpus callosum hyperplasia, gyration anomalies, neuronal heterotopias, variant Dandy-Walker, and hypoplasia of the frontal lobes. In addition the patient had had a severe delay in the acquisition of the stages of development due to important axial hypotonia.

WES analysis performed on the family resulted in the identification of different variants after bioinformatics analysis , among which we have elected p.Glu195del in the *MAST1* gene (Microtubule-Associated Serine / Threonine Kinase 1; OMIM # 612256). This variant is not present in ExAC, nor is it present in 1000 genomes, had a very low allelic frequency, but above all prediction tools was reported as pathogenic on protein

function. A patient with point mutation in the gene was described in the literature *MAST1* and infantile cerebral palsy.[49] In addition, *MAST1* is involved in microdeletion of the 19p13.13 region, associated with macrocephaly and intellectual disability.[49] Furthermore *MAST1* interacts with the *PTEN* gene, le whose dominant germline mutations cause macrocephaly and autism (OMIM # 605309).

This gene codes for a phosphatase with dual tumor suppressor activity. specification that antagonizes the PIK3 signal, through its phosphatase activity on the lipids and negatively regulates the MAPK pathway with its phosphatase activity on protein.

From the collaboration with other international groups 6 were found patients with different mutations in *MAST1* gene. All patients had varying degrees of intellectual disability, speech disorder, history of important hypotonia and problems of coarse and marching motor skills. All patients had Thick CC, cortical anomalies (pachygyria, polymicrogyria, subependymal heterotopias), cerebellar hypoplasia and brainstem thinning.

On animals harboring Mast1 microdeletions, we find that the PI3K/AKT3/mTOR pathway is unperturbed, whereas Mast2 and Mast3 levels are diminished, indicative of a dominant-negative mode of action. Finally, we report that de novo MAST1 substitutions are present in patients with autism and microcephaly, raising the prospect that mutations in this gene give rise to a spectrum of neurodevelopmental diseases.[50]

Table 1. Clinical Summary of Patients with MCC-CH-CM

Patient	P1	P2	P3	P4	P5	P6
Mutation (chr19)	19:12958677delGAG	chr19:12962798delGAA	chr19:12962804delGTT	19:12975903G>A	19:12975903G>A	19:12975903G>A
Mutation (NM_014975.2)	Glu194del c.580_582del	Lys276del c.825_827del	Leu278del c.831_833del	Gly517Ser c.1549G>A	Gly517Ser, c.1549G>A	Gly517Ser, c.1549G>A
CADD v1.3	16,71	17,35	21,4	27,9	27,9	27,9
Inheritance	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*	*de novo*
Ethnicity	Caucasian	Caucasian	Caucasian	Moroccan	Caucasian	Caucasian
Geographic ancestry	Italian	Hungarian	French	Moroccan	Unknown	French
Sex	Male	Male	Female	Female	Female	Female
Age at last evaluation	9 years	11.5 years	6 years	6 years	10 years	1.5 year
OFC[a] at birth	Unknown	36.5 cm (50^{th}–75^{th} percentile)	Unknown	Unknown	Unknown	35 cm (50^{th} percentile)
OFC[a]	52 cm	53 cm (25^{th}–50^{th} percentile)	51 cm	52 cm (+0.6 SD)	49.2 cm at 2 years, 9 months (50^{th} percentile)	46.5 cm at 18 months
Cortical dysgenesis	Dysplastic longitudinal gyri; subependymal heterotopias	Extensive undersulcated gyral pattern over frontal, temporal, and perisylvian regions; dysplastic longitudinal gyri	Tubulinopathy-like dysgyria with mildly thick cortex and diffuse very shallow sulci	Periventricular lesions	Diffuse subtle dysgyria; radial/shallow sulci with some deep infolds; dysplastic longitudinal gyri	Subtle frontal dysgyria; dysplastic longitudinal gyri
Basal ganglia	Normal	Poorly developed	Normal	Normal	Normal	Normal
Cerebellum	Vermis hypoplasia (++); counterclockwise rotation of cerebellar vermis	Mild diffuse cerebellar hypoplasia (+) with prominent sulci	Hypoplasia (++)	Hypoplasia (++)	Vermis hypoplasia (++) and mild hemispheric hypoplasia	Vermis hypoplasia (++) and mild hemispheric hypoplasia
Brainstem	Hypoplasia (++)	Small pons (+)	Hypoplasia (++)	Pontine hypoplasia (++)	Severe brainstem hypoplasia with a very small pons (++), thin midline cleft, long and mildly enlarged medulla	Severe brainstem hypoplasia (++), small pons with relative sparing of its buldging
Corpus callosum	Hyperplasia, mostly over genu and anterior body (++)	Very thick and dysplastic corpus callosum (mega corpus callosum) (++)	Hyperplasic (++)	Hyperplasia, mostly over genu and anterior body (+)	Thick corpus callosum (+)	Thick corpus callosum (+)
Ventricular dilation	Enlarged 4^{th} ventricle (+)	Enlarged 3^{rd} ventricle (++)	Enlarged 3^{rd} ventricle (++)	Enlarged 4^{th} ventricle (++)	Enlarged 3^{rd} and 4^{th} ventricles (++)	(+)
Cognitive abilities	Moderate cognitive impairment	Intellectual disability	Severe encephalopathy	Severe intellectual disability	Global developmental delay	Global developmental delay
Verbal abilities	Poor	Non-verbal	Non-verbal	Non-verbal	Non-verbal	Only some sounds (vowels)

(*Continued on next page*)

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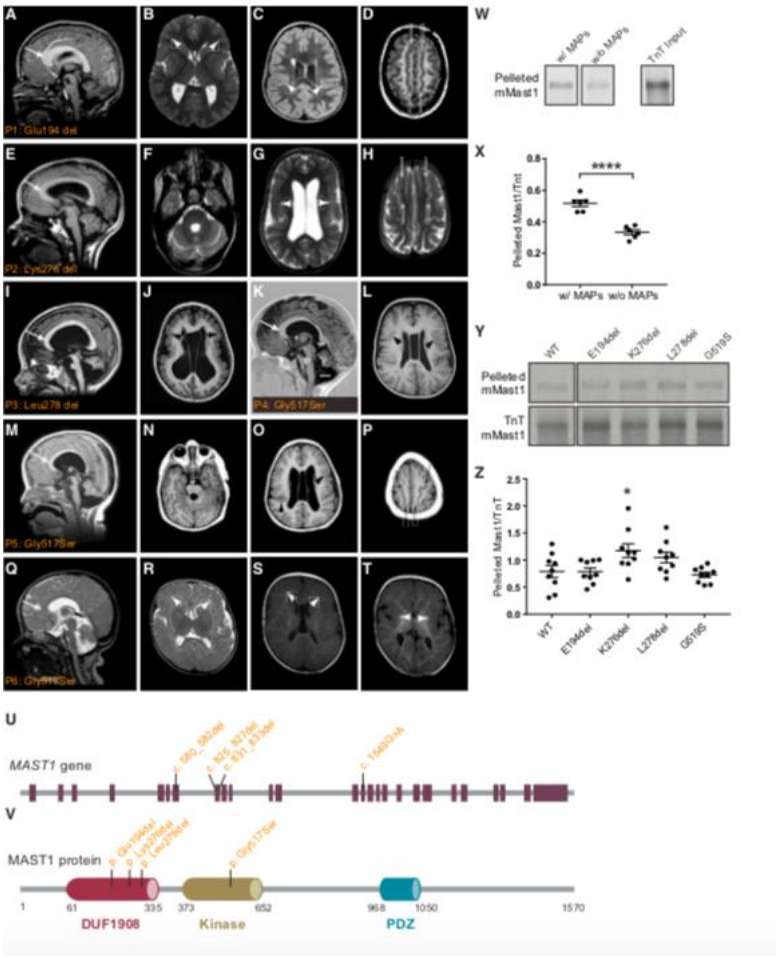

Figure 20 Bran MRI patients and schematic representation of the *MAST1* genomic locus shows the position of the mutations identified in patients P1–P6. Our case A,B,C,D scans.[50]

4.2.4 Syndromic CCA with unknown etiology, first results

In our series more than 64% of S-CCA remains without a molecular diagnosis.

WES analysis are in progress in some cases. In one we found de novo mutation c.641_643delTCA, p.(Ile214del) in DDX3X gene in a female patient with facial dysmorphisms, postnatal microcephaly, severe intellectual disability with absent speech and seizures with infantile onset. We described this case in a collaborative work of three cases thanks to Telethon Undiagnosed Disease Programm funding.51

Brain MRI studies revealed a similar malformative pattern in all patients (Figure 21), characterized by bilateral frontal and perysilvian polymicrogyria (Patients 1 and 3) and fronto-insular dysgyria (Patient 2), variable degrees of callosal hypo-dysgenesis,

dysmorphic basal ganglia with indistinct anterior limbs of internal capsules, small olfactory bulbs, pontine and inferior vermis hypoplasia. White matter was globally reduced, especially at the level of ventral cingulum, with enlargement of lateral ventricles and peculiar temporal horn dilatation. DTI studies showed marked hypoplasia of the corpus callosum with prevalent posterior involvement, and reduced volume of the anterior limbs of the internal capsule and ventral cingulum.

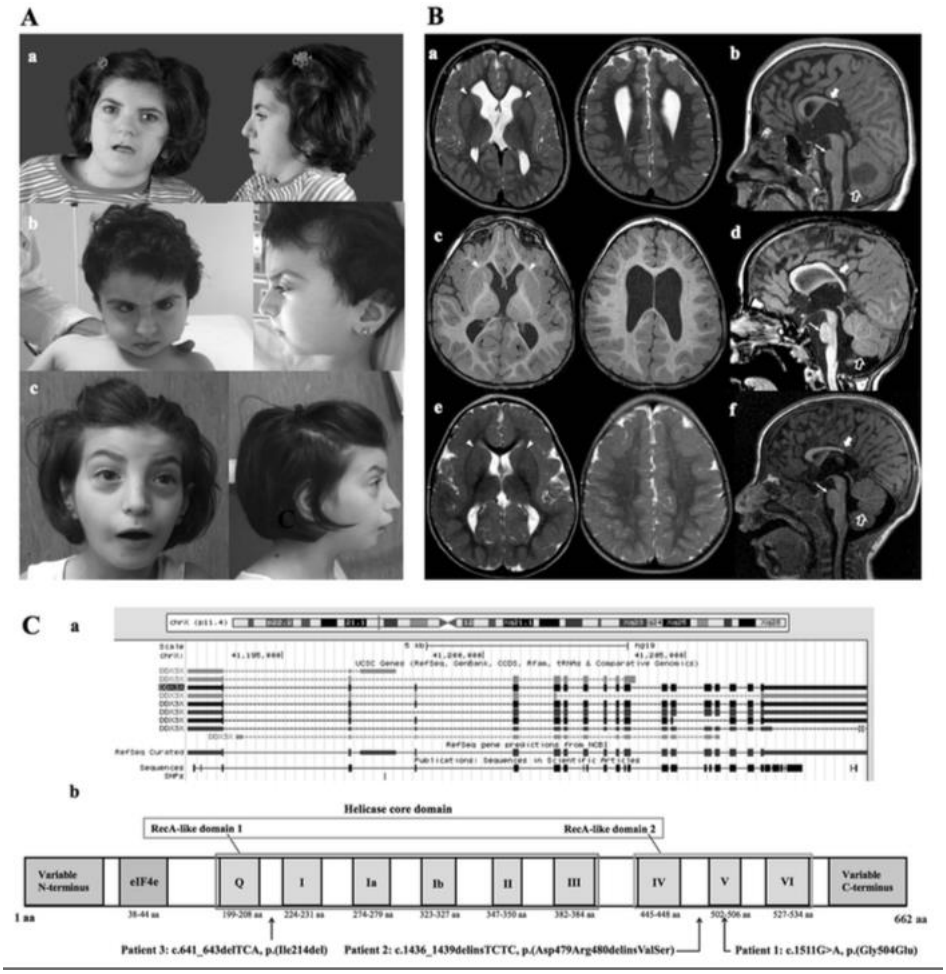

Figure 21. Facial features of patients in paper (our is C patient) ; Brain MRI and different mutations discovered in *DDX3X* gene[51]

5. Discussion

Developmental anomalies of the corpus callosum may occur as isolated brain malformations or as part of a more complex syndrome with a wide range of associated clinical phenotypes. Our study focuses on a series of 556 Italian patients with syndromic or non-syndromic CCA, diagnosed in the prenatal or postnatal period. To the best of our knowledge, this is the largest series collected and described in the literature. It emerges, as expected, that CCA constitutes a very heterogeneous group of developmental CNS anomalies with a high percentage of sporadic cases, 99% in our series.

Nevertheless, cACC is more frequent in NS-CCA (49%), whereas pACC is more frequent in the syndromic forms (41%). These observations suggest that the underlying basic defects entail the involvement of genes correlated with the overall development of the brain rather than specific genes only pertaining to the proliferation and guidance of callosal fibers. These data were in line with previous works.[20]

In our series 30% of the recruited patients received a prenatal diagnosis, while 70% were diagnosed in the postnatal period. This finding suggests the need for greater attention in the search for these malformations in the course of fetal ultrasound screening. Moreover, out of the 131 prenatal diagnoses, 56% turned out to be NS-CCA forms and 44% S-CCA. A limitation of our retrospective analysis comes from the bias that data are mainly sourced from pediatric hospitals inasmuch as information related to abortions and molecular data in these pregnancies is lacking.

In our sample, cortical brain anomalies was reported in 18% of CCA patients. Although the CC is part of the midline telencephalic pattern, the predominance of cortical malformations suggests that the mechanisms involved in neuronal migration, organization, and axonal guidance play a prominent pathogenic role as reported in several studies.[6] However, a recent study highlights that developmental defects in

interhemispheric remodeling are likely to be a primary etiology underlying human callosum agenesis.[52.]

Cerebellar and brainstem anomalies were reported in 20%of CCA. These possible association was in agreement with literature.[53]

Although epilepsy is frequently associated with CCA (13% and 39% of NS-CCA and S-CCA respectively), we did not observe any typical pattern, with comparable percentages of focal and generalized forms. But our data were heterogeneous and in some cases incomplete. Seizures were referred in 9%of isolated NS-CCA.

The role of corpus callosum in epilepsy is enigmatic and the mechanisms by which CCA contribute to epileptogenesis remain unknown. However, CCA per se cannot act as a start point of epilepsies., it is possible to explane with the straight association with cortical anomalies, not always macroscopic brain alterations.[54]

Our clinical data support the hypothesis that individuals with isolated NS forms have the best prognosis (in terms of neurodevelopment) and the milder phenotype. The absence of associated malformations, more than the degree or type of abnormality of the CC, seems to play the most important role in determining a more favorable outcome in these patients.

The percentages of DD/ID in patients with NS-CCA 55%(49% isolated e 56% plus vs. 93% S-CCA) are in line with those reported in other studies.[20]

More than sixty percent of NS-CCA have mild DD/ID and isolated NS cACC has better prognosis, because intelligence is within the normal range for nearly 3/4 of the children. However, they frequently have mild learning difficulties, in line with a recent meta-analysis study.[20,21.] Moreover, 13% of isolated NS-CCA patients presented severe intellectual disability, leaving the question open.

Overall, the high percentage of developmental delay/intellectual disability problems in CCA suggests disruption of the normal neuronal connections involved in cognitive functions during brain development.[20,21,22]

Our study strengthens the association between CCA and autism in 8% of NS CCA. These percentage increased in NS-Thick CC (39%).

Overall, the necessity for open data sharing to provide a more solid ground for the discovery of neuroimaging biomarkers within the context of the wide human neuroanatomical diversity is evident .[55] However, individuals with CCA should be screened for autism spectrum and CCA should be considered in autism diagnostic workup.[56]

Regarding etiology, this study further supports the notion that CCA represents a nonspecific brain malformation associated with a wide range of complex, genetically heterogeneous phenotypes.

Syndromic forms represent 60% of all cases, in agreement with the previous literature, and among them 63% are of unknown etiology and only 37% of known etiology. Among the latter 18% had a karyotype alteration equal to 6% of all S-CCA, 51% had a clinical diagnosis of a known monogenic syndrome, and 31% had a condition of microdeletion/microduplication, that accounted for only 23% of the S-CCA in which CMA examination was performed. VUS were found in 22% of S-CCA.

Regarding isolated NS-CCA, likely causative CNVs were found in 2% CGH-arrays performed In most cases, however, CMA was either negative or identified benign variants (66%) and VUS (32%). Therefore, the diagnostic rate of CMA in our cohort of patients ranges from 2% to 23% (NS-CCA to S-CCA), not much different from previous studies.[57]

Moreover, ThickCC was found, in our cohort, in microdeletion/microduplication syndromes, such as Williams-Beuren and Smith-Magenis syndromes, Phelan McDermid and in other large rearrangement including del 1p36 (OMIM#607872), del3p14.1, de13q14, del15q24, dup11p14.3, dup12p13 and dup14q11.2. Hyperplasia of corpus callosum in Williams-Beuren and Smith-Magenis and Phelan McDermid syndromes was not previously reported in the literature.

WES analysis appears a quite promising, though expensive and time-consuming, method to detect new gene mutations that cause CCA. Data from the literature, and the few cases we reported, indicate that where CMA analysis is negative WES can give an answer to the diagnostic burden, possibly also in cases of NS-CCA. As a matter of fact, a germline mutations was recently found by WES in the DCC gene as a cause of NS-CCA.

Prenatal diagnosis of ACC should prompt a specific anamnesis regarding any neurological disorder, as well as intentional physical examination of both parents aimed to detect mirror movements. In suspicious cases, detection of DCC pathogenic variants might markedly improve the predicted prognosis, alleviate the parental anxiety, and possibly prevent pregnancy termination.[44,45]

For the first time we describe ThickCC in patient with de novo mutations in *PIK3R2* and *MAST1* genes, identifying new genes involved in brain development and neuronal apoptosis.[48,50]

Our ThickCC series is the largest in literature and represent a important focus for further studies according to recent interest in pediatric and adult neurodevelopmental and neurodegenerative conditions.[56,58]

In conclusion, despite the fact that developmental anomalies of the corpus callosum are the most frequent brain malformations, their etiology is still uncertain, as well as their outcome. The diagnostic rate of the methods of classical and molecular cytogenetics in

the syndromic forms is around 23%, while it is very low in the isolated forms of CCA. NGS -CMA combined strategies analysis allow to identify CCA molecular basis, to improve the diagnostic rate in prenatal and postnatal setting. The increase on molecular knowledge about human brain connectome will allow to better understanding cognitive functions and behavior alterations.

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