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DI PAVIA

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Aicardi Syndrome:
clinical, genetics and therapeutic aspects

PhD Tutor: Prof. Pierangelo Veggiotti

PhD dissertation of

Dr. Silvia Masnada
Matr. N. 450263

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INDEX

1. HISTORICAL OVERVIEW	1
1.1 <i>Introduction</i>	1
1.2 <i>History of Diagnostic Criteria</i>	2
1.3 <i>Neurological epileptological features</i>	5
1.4 <i>Ophthalmological findings</i>	6
1.5 <i>Neuroradiological findings</i>	7
1.6 <i>Extraneurological findings</i>	9
1.7 <i>Differential diagnosis</i>	10
1.8 <i>Genetics aspects</i>	13
2. AICARDI SYNDROME MULTICENTRE STUDY: CLINICAL AND NEURORADIOLOGICAL PHENOTYPE CORRELATIONS IN 67 CASES	15
2.1 <i>Introduction</i>	15
2.2 <i>Material and Methods</i>	15
2.3 <i>Results</i>	18
2.4 <i>Discussion</i>	28
3. LONG TERM EEG AND CLINICAL FOLLOW-UP OF AICARDI SYNDROME: EEG AT ONSET PREDICT DIFFERENT EVOLUTIONS	35
3.1 <i>Introduction</i>	35
3.2 <i>Material and Methods</i>	35
3.3 <i>Results</i>	37
3.4 <i>Discussion</i>	52

2 4. AICARDI SYNDROME: KEY FETAL MRI FEATURES AND PRENATAL DIFFERENTIAL DIAGNOSIS	55
4.1 <i>Introduction</i>	55
4.2 <i>Material and Methods</i>	56
4.3 <i>Results</i>	58
4.4 <i>Discussion</i>	68
5. A 3D CRANIOFACIAL MORPHOMETRIC ANALYSIS IN AICARDI PATIENTS	72
5.1 <i>Introduction</i>	72
5.2 <i>Material and Methods</i>	73
5.3 <i>Results</i>	75
5.4 <i>Discussion</i>	79
6. HYPO-AGENESIS OF CORPUS CALLOSUM AND AICARDI SYNDROME: FOUR CASES WITH DEBATED DIAGNOSIS	82
6.1 <i>Introduction and cases</i>	82
6.2 <i>Discussion</i>	87
7. THERAPEUTIC ASPECTS	90
7.1 <i>Literature revision and data from multicenter study on 67 cases</i>	90
7.2 <i>Cannabidiol Expanded Access Program for Patients with Dravet Syndrome and Lennox-Gastaut Syndrome</i>	91
8. GENETIC AND REVISED DIAGNOSTIC CRITERIA INTERNATIONAL COLLABORATION	96
8.1 <i>International collaboration</i>	96
8.2 <i>Summary of the Consensus Conference on Aicardi syndrome: from defining the</i>	

<i>phenotype to unravelling the genotype</i>	97
<i>8.2.1 Genetic Studies</i>	97
<i>8.2.2 Clinical Features and Diagnostic Criteria</i>	103
9. CONCLUSIONS	107
10. REFERENCES	109

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Key statement of the work

- With my work I have performed the most extensive neuro-radiological revision on Aicardi patients, which allowed to better characterize the neuroradiological phenotype of the syndrome and describe new previously unreported findings
- the multicenter collection of Aicardi patients permitted to define possible precocious predictors (MRI and EEG) of long term clinical outcome, with significant implications in clinical practice
- The prenatal and postnatal MRI comparison, allowed to define key fetal MRI features of the syndrome, with important implications in pregnancy and early neonatal management
- Through a 3D morphometric analysis, we have recognized similar facial measurements in Aicardi cases; these findings will help clinicians in classifying atypical or doubt cases and so in performing more definite Aicardi diagnosis
- We have created an International collaboration with the aim of define new Aicardi diagnostic criteria and shed light on the etiology of the syndrome

1. HISTORICAL OVERVIEW

1.1 Introduction

Aicardi Syndrome (AS) is a rare congenital condition originally described by the classical triad congenital chorioretinal lacunae, corpus callosum dysgenesis and epileptic spasms. The rate of incidence was reported about 1:105.000 and 1:167.000 live births in the United States and between 1:93.000 and 1:99.000 in some European Countries (respectively Netherlands and Sweden). Despite the real prevalence of AS is unknown, an estimated worldwide prevalence was over 4000 (Kroner, Preiss et al. 2008). The mortality is high, although from analysis of more than 200 subjects in 2007, life expectancy was higher than previously thought, with a median survival age estimated as 18.5 (± 4) years; the oldest surviving individuals reported was a 32 and 49 years old women (Glasmacher, Sutton et al. 2007). The syndrome unusually affects primarily females, although few males with XXY karyotype (one with lissencephaly and oloprosencephaly which was atypical for AS and the other three with incomplete imaging information) (Hopkins, Humphrey et al. 1979; Chen, Chao et al. 2009; Zubairi, Carter et al. 2009; Shetty, Fraser et al. 2014), or more recently three male XY cases were reported (Aggarwal, Aggarwal et al. 2000; Chappelow, Reid et al. 2008; Anderson, Menten et al. 2009), although atypical features, such as severe mychrocephaly, give doubt on the diagnosis.

Because of the absence of a specific genetic hallmarks, the diagnosis is still based on clinical features, therefore since the first definition, multiple revisions of the diagnostic criteria were advanced.

1.2 *History of Diagnostic Criteria*

Jean Aicardi firstly observed this nosological framework and recognized the importance of this symptomatic triad in 1965, describing eight patients with atrophic pseudotoxoplasmic choroiditis in a cohort of patients with infantile spasms in flexion and corpus callosum agenesis (Aicardi et al. 1965). These patients had negative TORCH serology screening. This clinical association was defined as a distinct syndrome only few years later, in a report of 15 cases showed similar clinical features, published in the French literature in 1969 (Aicardi, Chevrie et al. 1969). Afterwards, the main features of the syndrome were wide delineated in a study of 184 cases conducted by Aicardi and Chevrie (Chevrie and Aicardi, 1994).

Taking into account the improvement of brain and eye imaging techniques, in 1994 Aicardi reconsidered the diagnostic criteria underlined the importance of other features, such as periventricular heterotopias, choroid plexus and intracranial cysts. He therefore suggested the possibility to make the diagnosis even in the absence of one component of the classic triad if two or more of the “*major features*” (periventricular and subcortical heterotopias, cysts around the 3d ventricle and/or choroid plexuses, optic disc coloboma) were present; he also identified some “*supporting features*” which could support the diagnosis such as vertebral and costal abnormalities, microphthalmia and/or other eye abnormalities, “split brain” EEG (dissociated suppression-burst tracing), gross hemispheric asymmetry and abnormalities of gyration (Aicardi et al., 1994). The classification was further changed by Aicardi in 1999 which added to the previous “*major features*” polymicrogiria and choroid plexus papillomas, and mostly revised in 2005 when Aicardi merged the previous classical triad and the major features of 1999 in

new major features. Subsequently, in 2005, Sutton and colleagues revised the criteria with the current definition of *the major and supporting features*. In details, the presence of three classical features is diagnostic for Aicardi Syndrome and the existence of two classical features and at least two other major or supporting features is strongly suggestive of the diagnosis (Aicardi 1999) (Sutton, Hopkins et al. 2005) (Figure 1). More recently, cases with a favourable outcome and normal neurological examination highlighted the wide variability in severity (Lee, Kim et al. 2004) (Guerriero, Sciruicchio et al. 2010; Paula Grigorian and Scott Lowery 2012) (Prats Vinas, Martinez Gonzalez et al. 2005; Aziz, Sisk et al. 2010). Furthermore the identification of “atypical cases” without infantile spasms or with normal corpus callosum, absence of polymicrogyria even if associated with major and/or supporting feature have expanding the phenotype, giving however significant problems in the diagnostic classification (Grosso, Lasorella et al. 2007). Therefore, new diagnostic criteria are needed to better classified these atypical patients and define the phenotypic spectrum of Aicardi Syndrome.

Classical Triad (1969)		
Agenesis of corpus callosum (total or partial)		
Infantile spasms		
Chorioretinal lacunae		
Proposed new diagnostic criteria for the diagnosis of Aicardi Syndrome (1994)		
Classical Triad	New major features	Supporting Features
Infantile Spasms Chorioretinal lacunae Agenesis of the corpus callosum (total or partial)	Periventricular and subcortical heterotopia Cysts (ependymal?) around the 3 ^d ventricle and/or choroid plexuses Coloboma of optic disc	Vertebral and costal abnormalities Microphthalmia and/ or other eye abnormalities Split brain' EEG (dissociated suppression-burst tracing) Gross hemispheric asymmetry Abnormalities of gyration
New Criteria for the Diagnosis of Aicardi Syndrome (1999)		
Classical Triad	New major features	Supporting Features
Infantile Spasms Chorioretinal lacunae Agenesis of the corpus callosum (may be partial)	Cortical malformations (mostly microgyria) Periventricular and subcortical heterotopia Cysts around the 3 ^d ventricle and/or choroid plexuses Papillomas of choroid plexus Optic disc/nerve coloboma	Vertebral and costal abnormalities Microphthalmia and/or other eye abnormalities Split brain' EEG (dissociated suppression-burst tracing) Gross hemispheric asymmetry
Proposed diagnostic criteria for the diagnosis of Aicardi syndrome, Aicardi (2005)		
Major features		Supporting Features
Infantile spasms ^a , Chorioretinal lacunae ^b , Coloboma of the optic disc (and nerve) often unilateral Agenesis of the corpus callosum (total or partial), Cortical malformations (mostly microgyria) ^b Periventricular (and subcortical) heterotopias ^b , Intracranial cysts (probably ependymal) interhemispheric or around third ventricle, Papillomas of choroid plexuses		Vertebral and costal abnormalities Microphthalmia and/or other eye abnormalities 'Split brain' EEG (dissociated suppression-burst tracing) Gross hemispheric asymmetry
^a may be replaced by other types of seizures (usually focal)		
^b present (or probably present) in all cases		
Sutton Modified Diagnostic Criteria (Sutton et al. 2005)		
Classical Triad	Major Features	Supporting Features
Agenesis of the corpus callosum Chorioretinal lacunae Infantile Spasms	Cortical malformation (mostly polymicrogyria) Periventricular and subcortical heterotopias Cysts around the 3 rd cerebral ventricle and/or choroid plexus Optic disc/nerve coloboma	Prominent premaxilla, with upturned nasal tip and sparse lateral eyebrows Vascular malformation or vascular malignancy Vertebral and costal abnormalities Microphthalmia "Split-brain" EEG Gross cerebral hemispheric asymmetry

Figure 1.
Revisions of diagnostic criteria

1.3 Neurological epileptological features

Neurological examination commonly reveals a wide clinical spectrum ranging from axial hypotonia, hemiparesis to severe spastic tetraplegia with extrapyramidal signs (Aicardi 2005). Usually head circumference is normal but mild to severe acquired microcephaly can be observed (Aicardi 1999). Aicardi patients have frequently poor outcome with early severe epileptic encephalopathy and moderate to severe global development delay (Rosser, Acosta et al. 2002). Severe global developmental delay is often present (Menezes, MacGregor et al. 1994). In a cohort of 70 patients, 91% percent attained milestones no higher than 12 months, sixteen girls older than 1 year of age (21%) were able to walk, nine girls (12%) with minimal or no assistance; three girls over 2 years of age (4%) were able to speak in short sentences (Rosser, Acosta et al. 2002). Moreover, AS patients with a favourable outcome have increasingly been reported, which further expands the phenotype of the disorder (Lee, Kim et al. 2004; Guerriero, Scirucchio et al. 2010). Infantile Spasms (IS) are the most characteristic seizures observed, frequently asymmetric or also unilateral, mostly with onset in the firsts months of life. In epilepsy the evolution, focal seizures are frequently observed, isolated or in association with IS, also at the epilepsy onset. In AS, the classical finding of hypsarrythmia related to spasms was not constantly observed. Typical AS Electroencephalographic (EEG) pattern, named « split brain », is characterized by bilateral independent bursts of multifocal epileptiform abnormalities occurring on a burst-suppression pattern showing complete asynchrony and asymmetry between the two hemispheres. Over the evolution of epilepsy, EEG abnormalities tend to remain stable during time, and almost never evolve to a definite Lennox Gastaut Syndrome

(Fariello, Chun et al. 1977; Ohtsuka, Oka et al. 1993; Aicardi 2005). During time, usually AS patients developed a drug resistant epilepsy, with polymorphic seizures including myoclonic, generalized tonic-clonic, atonic, tonic, atypical absence, focal/complex partial seizures, also audiogenic reflex seizures were reported (Grosso, Farnetani et al. 2007) (Glasmacher, Sutton et al. 2007).

1.4 Ophthalmological findings

Chorioretinal lacunae were historically considered pathognomonic of the condition (Aicardi et al., 1994). These are present since birth and the size don't change during years. At funduscopy examination they look like yellowish or whitish, flat, depigmented, usually round or ovoid defects in the choroid, sharply demarcated, and can be as large as the optic disc; they are multiple although of variable extent and generally bilateral. The largest lacunae tend to cluster around the disc, whereas small pinkish lesions tend to be more peripheral. The size normally varies from 0.1 to more than 3 disc diameters and does not change with age. Pigment deposits are frequently present at their periphery or even in the central part. They are on the same plane as the retina so that blood vessels do not bend on crossing their borders (Aicardi 2005). Microscopical examinations revealed a marked disturbance of retinal architecture (proliferative changes, detachment, pigment migration, disorganization, the replacement of normal layers by thin glial network, photoreceptor folds), choroidal vessel decreased in numbers and caliber, scattered rosettes were seen too (Del Pero, Mets et al. 1986; Font, Marines et al. 1991). Menezes and colleagues, in their report of 14

patients, found a correlation between macular involvement and the size of chorioretinal lacunae with the visual function and the clinical outcome. Particularly, a better clinical outcome in term of motor and language development and visual function was observed in patients with small lacunae and normal fovea (Menezes, Lewis et al. 1996). These data were confirmed in a subsequent study on three cases, in which unilateral chorioretinal lacunae, small chorioretinal lacunae which respect the central retina and the macula were found related to a better outcome in term of psychomotor development, survival and as good visual development (Galdos, Martinez et al. 2008). A significant asymmetry of both ocular and brain lesions of Aicardi syndrome was reported (Cabrera, Winn et al. 2011). Moreover, the syndrome was also associated with numerous other less specific ocular malformations: microphthalm, cataracts, retinal detachment, hypoplastic papilla and optic disc, iris or choroid coloboma (Aicardi et al., 1994).

1.5 Neuroradiological findings

Since the first description of corpus callosum agenesis, the development of CT scan and MRI, have allowed to better delineate and describe the complex of brain abnormalities in AS. Partial or complete agenesis of corpus callosum is never isolated and, currently, is not enough to make a definite diagnosis. For this reason, in the revised criteria cortical polymicrogyria, cerebral heterotopias and cysts were included as major findings (Aicardi 1999) (Sutton and Van den Veyver 1993). Corpus callosum agenesis is complete in most of the cases, and when partial most frequently the posterior part lacks. Through an imaging revision of 23 AS patients, Hopkins and colleagues found

polymicrogyria in 100% of the patients, mainly with anterior involvement (91%), an asymmetric distribution and often associated with underdevelopment of the operculum; the areas of cortical dysplasia in anatomopathological studies correspond to polymicrogyric cortex, indeed the broad appearance of the gyri being due to fusion of the molecular layers of facing convolutions. In the opinion of Aicardi, polymicrogyria may play the major role in the determination of mental retardation, seizures and neurological signs (Aicardi 2005; Hopkins, Sutton et al. 2008). In the same study, Hopkins found heterotopias in 100%, mainly periventricular, with a bilateral and asymmetric distribution, but also subcortical, thalamic, cerebellar and in IV ventricle has been described (Hopkins, Sutton et al. 2008). The intracranial cysts, reported in 95% of the cases, are preferentially observed in the interhemispheric fissure, in the third ventricle's region, in the pineal gland zone. Choroid plexus cysts are the most frequent, presents in more than 50% of cases. By the few available pathological examinations, these cysts have probably a neuroepithelial glial-ependymal origin or they can be arachnoid cysts. Imaging studies confirmed a signal higher than that of CSF in T2-weighted sequences, probably because of a high protein content typical of neuroepithelial cysts. The nature of posterior fossa cysts remains unclear. Most of the cysts can have large dimensions without producing significant compression, however in some cases can be the responsible of hydrocephalus, which necessitate of surgical drainage (Aicardi 2005) (Barkovich, Simon et al. 2001). The discovery of callosal agenesis in association with intracranial or choroid plexus cysts strongly suggests the prenatal suspicion of AS (Columbano, Luedemann et al. 2009) (Gacio and Lescano 2017). Choroid plexus papilloma, holoprosencephaly, embryonic tumours, and posterior fossa abnormalities (cerebellar dysplasia, vermis hypoplasia, enlarged cisterna magna)

were also described (Tagawa, Mimaki et al. 1989; Hopkins, Sutton et al. 2008; Burch-Smith, Ordonez et al. 2012). These “typical” MRI features, in term of the multiple malformations described, contributes to distinguishing AS to the other types of callosal agenesis (Aicardi 2005).

1.6 Extraneurological findings

Among the extraneurological features, orthopaedic problems are the most common: costo-vertebral defects are reported in almost 39% of patients (Donnenfeld, Packer et al. 1989; Glasmacher, Sutton et al. 2007), particularly hemivertebrae (23% of patients), block or fused vertebrae, also named butterfly vertebrae, and uni or bilaterally absent or bifurcated ribs (10%), all of them can be responsible for severe deformity; indeed, scoliosis is found in 50-55% of cases (Rosser, Acosta et al. 2002; Aicardi 2005; Glasmacher, Sutton et al. 2007) and frequently required surgical attention (Grigoriou, DeSabato et al. 2015). Respiratory problems are frequent: in a cohort of 67 patients, pneumonia was reported in 45% of patients, chest congestion in 67% and aspiration in almost half of the patients (49%) (Glasmacher, Sutton et al. 2007). Endocrinological problems were reported, particularly a decline in growth rate, both weight and height, after 7-10 years of age and precocious puberty (42%). An involvement of gastrointestinal system is described as constipation, abdominal pain; reflux and dysphagia are frequent problems, which lead to a feeding tube or percutaneous endoscopic gastrostomy alimentation (Glasmacher, Sutton et al. 2007). A distinctive facial phenotype, including a prominent premaxilla, upturned nasal tip, decreased angle

of the nasal bridge, and sparse lateral eyebrows, were described (Sutton, Hopkins et al. 2005). Small hands, an increased incidence of hand malformations have been also reported (Sutton, Hopkins et al. 2005) and cleft lip and palate occasionally occurred (Robinow, Johnson et al. 1984; Sato, Matsuishi et al. 1987; McPherson and Jones 1990; Umansky, Neidich et al. 1994; Glasmacher, Sutton et al. 2007). Tumors are frequently reported in patients with AS; the most commons are: choroid plexus papillomas, which should be monitored because of their slow and insidious growth, but also lipomas, angiosarcomas, hepatoblastomas, medulloblastoma, retinoblastoma, embryonal carcinoma, gastric polyposis, and embryonal carcinomas were observed. A single case of large-cell medulloblastoma has been also reported (Tanaka, Takakura et al. 1985; Tagawa, Mimaki et al. 1989; Tsao, Sommer et al. 1993; Trifiletti, Incorpora et al. 1995; Palmer, Nordborg et al. 2004; Frye, Polling et al. 2007; Kamien and Gabbett 2009; Burch-Smith, Ordonez et al. 2012; Akinfenwa, Chevez-Barrios et al. 2016). An increased incidence of vascular malformations, such as palatal hemangioma and pigmentary lesions has been observed (Kiristioglu, Kilic et al. 1999; Sutton, Hopkins et al. 2005).

1.7 Differential Diagnoses

1. *Corpus callosum agenesis*. This anomaly can be isolated or associated with other brain malformations or can be a part of a syndrome. The association of corpus callosum agenesis with cyst that do not communicate with the ventricles and the presence of subependymal heterotopia and polymicrogyria are relatively

- specific for AS (Barkovich, Simon et al. 2001)
2. *Chorioretinopathy in congenital intrauterine infections.* Congenital toxoplasmosis, CMV or rubella fetopathy, were historically the first differential diagnosis suggested. Nevertheless, the lacunae configuration and topography, associated to pigmentary changes, can help in differentiate AS lacunae from the congenital infections chorioretinopathy (Willis and Rosman 1980)
 3. *Oculocerebrocutaneous syndrome (OCCS),* also called Delleman syndrome, is an association of ocular malformations (orbital cyst, anophthalmia or microphthalmia), focal skin defects and brain malformations including agenesis of the corpus callosum, polymicrogyria, periventricular nodular heterotopias and enlarged lateral ventricles. The pathognomonic feature of this syndrome is tectal dysplasia with cerebellar hypoplasia and vermis agenesis. This syndrome is more commonly diagnosed in males than females (Moog, Jones et al. 2005)
 4. *Microcephaly with or without chorioretinopathy, lymphedema or mental retardation (MCLMR) (OMIM #152950) and Autosomal recessive microcephaly associated to chorioretinopathy (MCCRP1 OMIM #251270, MCCRP2 OMIM #616171 and MCCRP3 OMIM #616335).* Contrarily to AS patients, these patients present mild to severe microcephaly without neuronal migration defects (e.g. polymicrogyria or heterotopia), no optic nerve coloboma, while chorioretinal abnormalities had a typical peripheral localization different from AS. Moreover, MCLMR has an autosomal dominant transmission with variable expression and the majority of diagnosed patients showed a *KIF11* mutation, never found in AS patients (Mirzaa, Enyedi et al. 2014)
 5. *Amniotic band syndrome.* Chorioretinal lacunae are also reported in this

- syndrome in association with hand and feet malformation, facial cleft, corpus callosum agenesis and ventriculomegaly (Hashemi, Traboulsi et al. 1991)
6. *Orofaciodigital syndrome type VIII*. The ocular abnormalities reported in this syndrome are similar to those seen in AS. Differentially from AS, the chorioretinal atrophy of colobomatous origin is classically associated with other typical oro-facial-digital features (Gurrieri, Sammito et al. 1992)
 7. *Goltz syndrome-focal dermal dysplasia (FDS)*. This is an X-dominant disorder sharing common features with AS including corpus callosum agenesis, microphthalmia, coloboma, seizures, skeletal anomalies and facial asymmetry. FDS typically presents linear skin defects, adipose tissue herniation and papillomas of the skin or mucous membrane, not commonly observed in AS patients. This condition is lethal in males (Van den Veyver 2002)
 8. *Microphthalmia with linear skin defects (MLS; OMIM #309801)*. This disease is characterized by uni or bilateral microphthalmia and/or anophthalmia associated with congenital facial skin defects. Some other ocular abnormalities (corneal malformations, orbital cysts, cataracts), brain malformations, epilepsy and developmental delay can be present. The diagnosis is based on the identification of mutation in three genes localized in the Xp22.31 region (*COX7B*, *HCCS*, *NDUFB11*). In AS patients, Xp22 region was longer studied, but no candidate gene were detected (Van den Veyver 2002; Yilmaz, Fontaine et al. 2007)

1.8 Genetic aspects

Congenital infections on the basis of AIC were excluded and several genetic way were explored by different research group, without solve this challenge.

Considering the female prevalence, a dominant X-linked inheritance of an X-linked gene, lethal early in development for male, was the first hypothesis advanced (Ropers, Zuffardi et al. 1982). Taking into account the absence of familial recurrence, the mutation was supposed arise de novo, and the presence of discordant monozygotic twins suggested a postzygotic event (Costa, Greer et al. 1997). A 46Xt(X;3) (p22;q12) translocation was found in a patient sharing some of the aspects found in AIC (callosum aganesis, retinal lacunae, michrophtalmia, vertebral and ribs defects) and a de novo complex deletion in 1p36 chromosome region was detected in another case with infantile spasms, coloboma, callosal agenesis and cardiac defect, although these translocation/deletion was not confirmed in all the other cases tested (Ropers, Zuffardi et al. 1982; Donnenfeld, Packer et al. 1989; Bursztejn, Bronner et al. 2009). A skewed X-inactivation was demonstrated (Neidich, Nussbaum et al. 1990; Eble, Sutton et al. 2009), but not throughout confirmed (Costa, Greer et al. 1997; Hoag, Taylor et al. 1997). Filamin inclusion were found in astrocytes of AS patients, but genetic analysis on FLNA gene fail to confirm the histological data (Van den Veyver, Panichkul et al. 2004; Anderson, Menten et al. 2009). No copy number variations (CNVs) were detected with full coverage X chromosomal BAC arrays on 18 AIC patients (Yilmaz, Fontaine et al. 2007) and through CGHaray analysis validated with qPCR in a group of 38 AIC cases (Wang, Sutton et al. 2009). Moreover also the advanced technique of Next Generation Exome and Genome Sequencing fail to detect a genetic etiology; indeed, the

de novo nonsense variant in TEAD1 and OCEL1 found each respectively in two different cases, were do not confirmed in a subsequent studies on a larger cohort of 38 cases (Schrauwen, Szelinger et al. 2015; Lund, Striano et al. 2016; Wong, Sutton et al. 2017). No disease genetic variant was detected on CDKL5 gene in a choort of 10 patients with AIC (Nemos, Lambert et al. 2009). Taking into account the possibility of epigenetic DNA modifications causing AIC, DNA metylation pattern was studied and confirmed the presence of different myelination pattern in proband with AIC in several neurodevelopmental and neuroimmnological network (Piras, Mills et al. 2017); however, despite the enormous effort, the genetic cause of AIC remains a mystery.

2. AICARDI SYNDROME MULTICENTRE STUDY: CLINICAL AND NEURORADIOLOGICAL PHENOTYPE CORRELATIONS IN 67 CASES

2.1 Introduction

Aicardi Syndrome (AS) is a rare congenital condition defined by the presence of corpus callosum agenesis, chorioretinal lacunae and epileptic spasms or by the modified diagnostic Criteria (Aicardi 2005; Sutton, Hopkins et al. 2005). From the first descriptions, imaging studies better delineated the neuroradiological phenotype which manifests also with the presence of polymicrogyria, nodular heterotopias and intracranial cysts (Hopkins, Sutton et al. 2008). Usually patients are severely neurologically disabled (Aicardi, Chevrie et al. 1969; Donnenfeld, Packer et al. 1989; Rosser, Acosta et al. 2002); the clinical outcome is frequently complicated by the development of different life threatening comorbidities such as pneumological, orthopedic and gastrointestinal problems (Trifiletti, Incorpora et al. 1995; Glasmacher, Sutton et al. 2007; Grigoriou, DeSabato et al. 2015); however rare cases with favorable outcomes and normal neurologic examination are reported (Iturralde, Meyerle et al. 2006; Grosso, Lasorella et al. 2007). Here it is reported a multicenter retrospective revision of the imaging studies and the clinical-neurological outcome of 67 Aicardi patients, in order to find associations among neuroradiological features, electroencephalographic trace, and clinical-neurological outcomes.

2.2 Material and Methods

This was a multicenter retrospective study, which involved different centers from Italy, France, and four centers from Switzerland, Denmark and Germany. This study adheres

to the principle of Helsinki Declaration and was carried out through routine diagnostic activity. Exclusively patients who satisfied classical diagnostic criteria or Sutton modified criteria were included in the study. A retrospective collection of clinical, epileptological and neurological data were performed. Patients' clinical motor outcome was retrospectively classified on the basis of the Gross Motor Function Classification System - Expanded and Revised (GMFCS) and the Manual Ability Classification (MACS) (Paulson and Vargus-Adams 2017). Neurological examination was also classified with a scoring system based on clinical evaluation: patients with normal neurological examination scored 0, presence of slight pyramidal signs 1, hemiplegia or dyplegia 2, severe diffuse hypotonia 3 and spastic tetraplegia 4. Taking into account the poor cognitive and language outcome of patients with AS, a classification system measuring the ability to say sentences (class 0), single words (class 1), babbling (class 2), vocalization (class 3), absent (class 4) was used. Eating and drinking Ability Classification System (EDACS) was used to assess eating and drinking safety and efficiency (Paulson and Vargus-Adams 2017). For statistical analysis seizures were classified in spasms, focal seizures, and status epilepticus isolated or in variable associations. Seizure frequency has been considered in daily, weekly, and monthly episodes.

A retrospective systematic revision of brain Magnetic Resonance Imaging (MRI) or Computerized Tomography (CT) imaging was performed with a standardized revision protocol (Details in Table 1). Imaging reports of 75 MRI and 8 brain CT of 67 patients diagnosed with Aicardi Syndrome were reviewed. Imaging studies, 64 MRI and 7 CT, of 55/67 patients were available for a systematic revision. Imaging revision was performed by two experienced neuroradiologist, and a third neuroradiologist was

involved in case of discrepancy. MRI studies were performed using different 1.5 T scanners according to standard protocols: T1 spin echo (SE) sagittal sequences (representative parameters: slice thickness 3 mm; repetition time (TR) 500-550 ms; echo time (TE) 8-15 ms), T2 Turbo Spin Echo (TSE) axial and coronal images (representative parameters: slice thickness 3 mm; TR 5.300-6.000 ms; TE 120-200 ms), fluid attenuated inversion recovery (FLAIR) axial and coronal images (slice thickness 3 mm; TR 8.000-10.000 ms; TE 120-125 ms; inversion time (IT) 2.800 ms) and inversion recovery (IR) coronal sequences (slice thickness 3 mm; TR 2.800 ms; TE 10-15 ms; IT 400 ms) were obtained. When available, Diffusion Weighted Imaging (DWI) data were also reviewed. For statistical analysis patients were classified on the basis of MRI features. For statistical analysis MRI features analyzed were classified as follow: partial/complete agenesis or hypoplasia of corpus callosum. About the gray matter (GM) involvement and distribution of cortical dysplasia, monofocal (defined single areas of cortical dysplasia), multifocal (defined multiple but not adjoining areas of cortical dysplasia), or diffuse cortical malformations were distinguished. Nodular heterotopias were characterized according to the number (<4 or >4), Cysts were classified according to localization (supratentorial, subtentorial or both, and cysts and/or papilloma of choroid plexus). About posterior fossa abnormalities were considered mild abnormalities (as the cases of slight inferior vermis hypoplasia) and severe more complex malformations (e.g. cerebellar cortical dysplasia, romboencephalosynapsis). A detailed list of all the imaging parameters analyzed and their classification is provided in table 1.

The sample was described with the usual descriptive statistics: mean and standard deviation (SD) or median and interquartile range (IQR) for continuous variables and

proportions for categorical ones. The Pearson's chi square test (or Fisher's exact test) were used to evaluate differences between categorical variables, whereas one-way analysis of variance or the Student's t-test were used to disclose differences in continuous variables. If the assumption of normality (tested with the Shapiro Wilk's test) was not met, an analogous non-parametric test (Mann-Whitney's or Kruskal-Wallis' tests) was used. This analysis was followed by the Student's t-test or Kruskal-Wallis test and post-hoc testing, adjusting for multiple comparisons. Statistical significance was taken at the ≤ 0.05 level, unless adjusting for multiple comparisons (applying the Bonferroni correction) was needed. All analyses were performed using STATA/SE for Windows, version 14.2.

2.3 Results

Clinical phenotype

This multicenter study allowed us to collect a cohort of 92 patients with suspected AS; only 67 out of these cases were selected and included in the study according to the Classical or Modified Diagnostic Criteria (Sutton, Hopkins et al. 2005). All the cases are female. Five patients were deceased at the time of the study (range of age 5-28 y). 15/54 cases (27,78%), in which the pregnancy history was available, presented with complications during pregnancy (threats of abortion, hypertension, polyhydramnios, oligohydramnios, placenta abruption, reduced fetal movements, HCV infection). 16/44 (36,36%) mothers had previous history of abortion. Mean maternal age was 31,93 (range 17-42), mean paternal age 35,35 (range 19-51). A head circumference follow-up was available for 35 patients: at birth 31 (91,17%) patients had normal head circumference, 3 patients macrocephaly, one microcephaly; at the last evaluation, 40% (14/35) developed microcephaly (13 from a condition of normal head circumference,

one case from a previous macrocephaly). In 58/67 (86,56%) cases ophtalmological examination revealed the classical chorioretinal lacunae; for five patients ophtalmological examination was not available or inconclusive. 33/61 (54,09%) of the patients have coloboma, five of them (8,20%) associated with microphthalmos. 5/61 (8,20%) patients have only microphthalmos. Seizures onset was reported with a mean age of 75.45 days (2,5 months) (range 1-540 days). At onset 50,75% (34/67) of the patients displayed epileptic spasms, 10,45% (7/67) cases focal seizures, 38,81% (26/67) patients different types of seizures in associations: spasms in association with focal seizures (22/26), spasms and generalized seizures (2), focal seizures and status epilepticus (1), focal and myoclonic seizures (1), with a frequency of multiple daily seizures in 100% of the patients. Electroencephalographic reports were evaluable in 54/67 patients at onset; in 48,15% (26/54) of the patients EEG was characterized by a poor background activity with multifocal epileptiform discharges (EDs); 29,63% (16/54) had a definite hypsarrythmic pattern, while a 22,22% (12/54) of the cases had a suppression burst pattern. The severity of EEG at onset was statistically directly associated with the severity of the motor and language outcome, particularly, patients with a suppression burst pattern had a level of V in GMFCS and MACS and a level of 3-4 at the language clinical scale: GMFCS (p-value: 0.0337), MACS (p-value: 0.0258) and language scale (p-value=0.0281). Moreover, statistical analysis revealed a worse manual outcome in patients which present at onset focal seizures alone (p value 0.0112). During a mean follow-up of 123,05 months (range 9-324 months), 4/64 patients (6,25%) had only epileptic spasms in their epilepsy history, 7 patients (10,94%) only focal seizures, while most of the cases, 53/64 (82,81%), displayed multiple type of seizures: spasms and focal seizures (24), spasms, focal and generalized seizures (10),

spasms, focal seizures and status epilepticus (7); also myoclonic seizures (7) were reported in associations with other type of seizures. Status epilepticus was reported in 13 patients (20,31%). At the last evaluation only one patient was seizures free (at the age of 5 years old), the rest of the cases 61/62 (98,38%) developed a drug-resistant epilepsy, with a failure of three antiepileptic drugs (AED) appropriately chosen and used; only 14,51% (9/62) achieved a partial seizures control. In details, for whom an accurate seizures frequency evaluation were available, 57,41% (31/54) displayed multiple daily seizures, 31,48% (17/54) had weekly seizures (1-7 seizures/week), 9,26% (5/54) had ≤ 4 seizures/month. During their epilepsy history patients had tried more than three AED during time till 17 AED. Parents and clinicians reported a reduction in seizures frequency with Vigabatrin, ACTH, Valproic Acid, Lamotrigine. Five patients tried ketogenic diet without clear results on efficacy. Statistical analysis revealed a direct correlation between the poorer seizures control and neurological outcome, in neurological scale (p.value 0.0076) and in manual abilities, particularly more than 50% of the patients with multiple daily seizures had a level of V at MACS Scale (p-value=0.0285).

Developmental milestones were delayed in 66/67 patients, two patient had only language delay. Concerning neurological outcome, at the last evaluation, 46,77% (29/62) of the patients had a severe spastic tetraplegia, eight patients (12,90%) a severe diffuse hypotonia, 22/62 (35,48%) displayed an hemiplegia or dyplegia, two patients (3,22%) had slight pyramidal signs; in 21 patients extrapyramidal signs, particularly dystonia and dyskinesia were reported; only one patient had a normal neurological examination at the last evaluation (5 years old).

Considering motor functions, most of the patients, 66,66%, had severe limitations in the ability to maintain antigravity control and need a wheelchair (level of V and IV at the GMFCS), however 26,19% of the cases use wheeled mobility for long distances but had the possibility to walk with aid (level III and II), only four patients are independent walkers, with minimum balance or coordination problems (level I). 67,24% of the cases had limited or any possibility to handle objects, while 31,03% could had a residual capacity with some difficulties, and one patient had only few limitation in manual abilities with a some independence in daily activities. Language was completely absent or with the possibility of say only simple vocalizations, or babbling in 80,95%; 11,1% of the patients could pronounce a few of single words and only five patients (7,9%) could speak with simple sentences. Feeding problems were frequently reported (74%) with different degree of severity. Details of clinical outcome scales are reported in Table 2. Other comorbidities could be established on the basis of the medical history in a subset of the cohort: 28/40 (70%) suffered from scoliosis, and 11/40 (27,5%) had vertebral dysmorphisms (fused or cleft vertebræ, hemi or butterfly vertebræ); 20/35 (57,14%) had recurrent respiratory infections till pneumonias in 13/35. Sleep problems, particularly awakenings and/or sleep apnea were reported in 19/35 patients (54,28%). 24/32 (75%) patients suffered from constipation, one patient had a diagnosis of colitis ulcerosa. In twelve patients was available the information about height during follow-up, 7 out 12 patients presented a height growth in average with the general population for sex and age till the age of seven, when they presented a progressive reduction; five patients presented a normal growth till the age of 3 or 6 years and after respectively they presented a progressive reduction in growth compared the population; only one patients

showed a lower height since her birth. 22/25 (88%) presented behavioural problems, particularly hands stereotypic movements and/or aggressivity.

CORPUS CALLOSUM DYSGENESIS 100% (67/67)			VENTRICULAR ABNORMALITIES 96,36% (53/55)		CORTICAL DYSPLASIA 98,43% (63/64)				POSTERIOR FOSSA MALFORMATION 63,63% (42/66)	
complete agenesis	partial agenesis	hypoplasia	ventriculo megaly	dysmorphisms	diffuse	multi focal	focal	not detected	mild	severe
50,75% (34/67)	40,3% (27/67)	8,96% (6/67)	61,51% (34/55)	89,09% (49/55)	51,56% (33/64)	35,94% (23/64)	10,94% (7/64)	1,5% (1/64)	42,42% (28/66)	21,21% (14/66)

CYSTS 96,36% (53/55)											BASAL GANGLIA DYSMORPHISMS 76,36% (42/55)	
LOCALIZATION				NUMBER			PATTERN		TYPE*		mild	severe
supra tentorial	infra tentorial	supra and infra	choroid plexus	absent	one	≥2 til 10	uniloculated	multiloculated	2b	2d		
61,19% (41/67)	8,96% (6/67)	17,91% (12/67)	33,33% (22/66)	3,63% (2/55)	49,09% (27/55)	47,27% (26/55)	81,13% (43/53)	9,4% (5/53)	49,05% (26/53)	43,39% (23/53)	52,38% (22/42)	47,61% (20/42)

NODULAR HETEROTOPIAS 98,18% (54/55)														
LOCALIZATION					DISTRIBUTION		NUMBER			PATTERN				
sub cortical	periventricular				bilateral	mono lateral	absent	<4	>4	single	confluent	mixed		
	body	trygon	frontal horn	tempora l horn	occipital horn	IV ventricle								
5,55% (3/54)	62,96% (34/54)	59,25% (32/54)	59,25% (32/54)	31,48% (17/54)	42,59% (23/54)	1,85% (1/54)	77,77% (42/54)	22,22% (12/54)	1,8% (1/55)	20,63% (13/63)	73,02% (46/63)	29,62% (16/54)	22,22% (12/54)	40,74% (22/54)

Table 1. Sistematic revision of MRI studies, according the the classification proposed by Hopkins and colleague (Hopkins, Sutton et al. 2008). *classification of cysts type according the Barkovich classification (Barkovich, Simon et al. 2001)

	I	II	III	IV	V	Total n. patients
GMFCS	4 (6,30%)	8 (12,69%)	9 (14,28%)	9 (14,28%)	33 (52,38%)	63
MACS	1 (1,72%)	7 (12,06%)	7 (18,96%)	10 (17,24%)	29 (50%)	58
EDACS	13 (26%)	5 (10%)	7 (14%)	8 (16%)	17 (34%)	50
language	5 (7,90%)	7 (11,1%)	9 (14,28%)	13 (20,63%)	29 (46,03%)	63

Table 2. Clinical scales. Gross Motor Function Classification System - Expanded and Revised (GMFCS) and the Manual Ability Classification (MACS), Eating and drinking Ability Classification System (EDACS) (Paulson and Vargus-Adams 2017). Language classification system measuring the ability to say sentences (class 0), single words (class 1), babbling (class 2), vocalization (class 3), absent (class 4).

Neuroradiological results

Imaging were performed at a median age of 45 months (range first day of life-235 months). The results of imaging revision are described in details in Table 1. 91,04% of the patients had corpus callosum malformations, and considering patients with partial agenesis, lack of the posterior part of the body and splenium and rostrum was predominant (71,42%) (Figure 1). Callosal malformation was statistically directly correlated with the neurological clinical scale (p-value: 0.0378; statistical differences from 3 versus 1, p. 0.0158), and with GMFCS (p-value: 0.0283) and MACS (p-value: 0.0267).

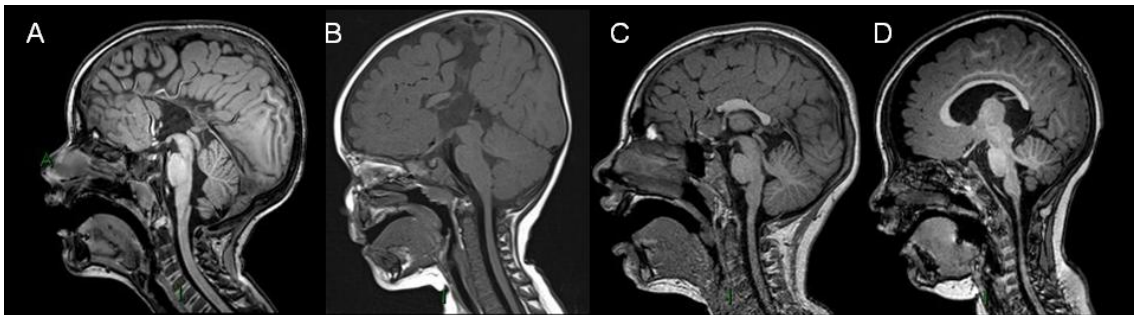


Figure 1. Corpus callosum (cc) malformations: T1 - weighted sagittal MRI images show complete (a) or partial (b,c) agenesis of cc, lacking of the posterior part of the body, the splenium and the rostrum (b) or only the genu and the rostrum (c); complete but diffusely hypoplastic cc (d) were also described.

Considering the 55 images reviewed, cysts were present in 96,36% of the cases. Taking into account their localization, most of the patients (61,19%) had supratentorial cysts, 33,33% had choroid plexus cysts or papilloma and most of them (49,05%) showed ≥ 2 cysts. A uniloculated cyst was the most frequent pattern detected (81,13%). According to Barkovich classification, patients displayed a similar rate (49,05% vs 43,39%) between respectively a type 2b cysts (hyperintense to CSF on T1-w images) and a type 2d (CSF-like signal as arachnoid cysts) (Figure 2).

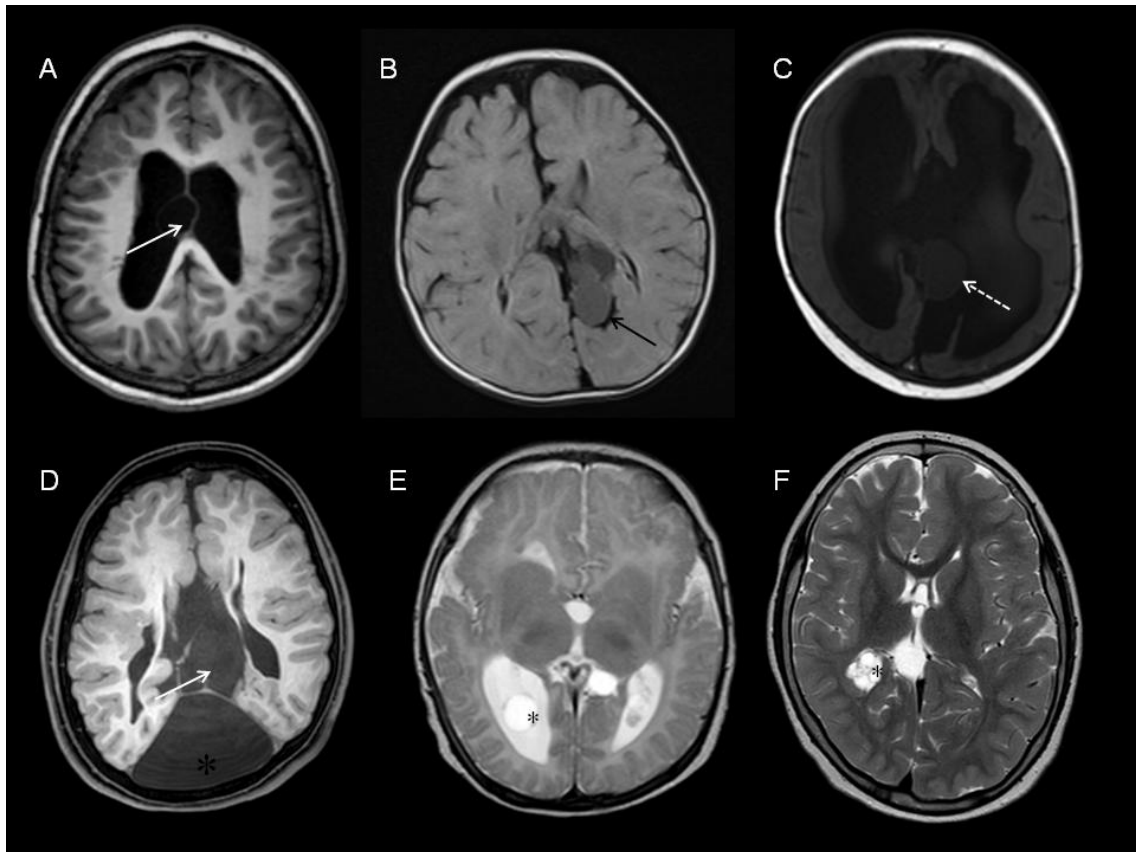


Figure 2. Cysts: T1 weighted axial images (a,d) show single uniloculated interhemispheric supratentorial arachnoid cysts (white arrow), with CSF-like signal (type 2d according to Barcovich classification); in image d) associated with an infratentorial arachnoid cyst (*). T1 weighted axial images (b,c) show multiloculated (black arrow,b) and uniloculated (dashed white arrow, c) supratentorial interhemispheric cysts with hyperintense-to-CSF signal, supposed to be glioependymal (type 2b according to Barcovich classification) cysts. T2 weighted axial scans (e,f) show cyst (asterisc, e) and papilloma (asterisc, f) of choroid plexuses.

For 64/67 patients a systematic description of abnormal cortical involvement was possible: 87,5% of the patients displayed diffuse bilateral or multifocal cortical dysplasia, while only 10,94% had a focal dysplasia. Taking into account the imaging reviewed, in 72,22% of the patients dysplasia resembled a polymicrogyric pattern. In three cases polymicrogyria was associated with schizencephaly. In 72,22% of the patients an anterior-posterior gradient of the cortical malformation was identified, particularly involving the frontal, opercular and sylvian cortex (Figure 3). In 40,74% of

the cases an asymmetric distribution of the dysplastic cortex was evident. 54,54% of the cases had a gross asymmetry in term of cerebral hemispheres volume. Hippocampal dysmorphisms were detected in 52/55 patients, in term of a stubby and vertical aspects. Analysis revealed a statistically significant association between cortical malformations and seizures at onset (p-value= 0.032), and a higher levels in all the clinical outcome scales, particularly $\geq 50\%$ of the patients with diffuse bilateral abnormal cortical pattern had a level of V in GMFCS, MACS and EDACS and a level of 4 (the most severe) at the neurological and language clinical scale: GMFCS (p-value: 0.0007), MACS (p-value: 0.0048), EDACS (p-value: 0.0027), language (p-value: 0.0146), neurological clinical scale (p-value: 0.0222). Moreover, a statistical direct correlation between abnormal cortical pattern and EEG at onset was found (p-value 0.005).

Considering the 55 brain scans reviewed, 98,18% presented heterotopias. According to the anatomical distribution, periventricular heterotopias were observed mainly around lateral ventricles and involving mainly anterior portions, less frequently occipital and temporal horns. In three cases subcortical nodules were associated. Most of the cases had more than 4 nodules and displayed a bilateral and asymmetric distribution. Most frequently both patterns were observed (Figure 3). The number of nodular heterotopias was statistically related with the GMFCS, particularly $\geq 50\%$ patients with more than 4 nodules had a Scale level of V (p-value=0.0306).

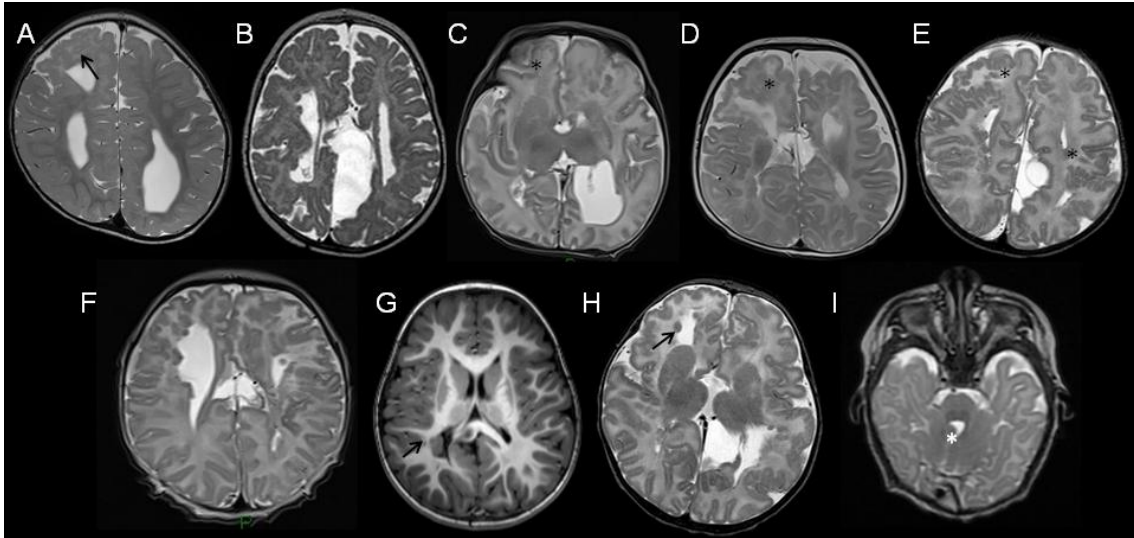


Figure 2. Cortical dysplasia: T2 weighted axial images show different patterns of cortical dysplasia with focal (arrow, a), multifocal (c, d e) or diffuse (b) distribution, in most cases resembling a polymicrogyric pattern (asterisk, c, d, e), mostly with anterior-to-posterior gradient (c, d).

Nodular heterotopias: T2 weighted axial images (f, g, h) and IR T1 axial scan (g) show the presence of periventricular heterotopias. Nodules can be numerous and spread asymmetrically around lateral ventricles (f) with singular (arrow, h), confluent (arrow, g) or both pattern (f). A single case of subependymal heterotopia of the IV ventricle was detected (asterisk, i).

In 63,63% of the patients posterior fossa malformations were detected; among these, 2/3 of the cases had mild abnormalities (in particular vermis hypoplasia and/or vermian rotation) associated in 42,85% patients with enlarged cistern magna, while 1/3 showed complex posterior fossa malformations, from severe cerebellar hypoplasia (12/14) predominantly associated with cerebellar cortical dysplasia (8/10), less frequently to romboencephalosinapsis (2/14), Dandy-Walker continuum (1/14), brainstem hypoplasia (1/14), (Figure 4).

In 42/55 (76,36%) patients MRI also revealed basal ganglia dysmorphisms, both mild and severe forms. The milder forms were characterized by a stubby and globular aspect or slight hypoplasia of the striatum, often associated with an irregular and straight profile of the lateral profile of putamen. The more severe ones (20/42) were associated

to thalamic adhesion (11,90%) or agenesis of anterior limb of internal capsulae (35,71%). Among this last group, 9/15 had also microcysts long the irregular and straight profile of the putamen, likely due to small dilated perivascular spaces, more evident on one side (Figure 4). In the nine cases the radiological evidence of agenesis of anterior limb of internal capsulae prompted a genetic screening on tubuline genes, which resulted negative.

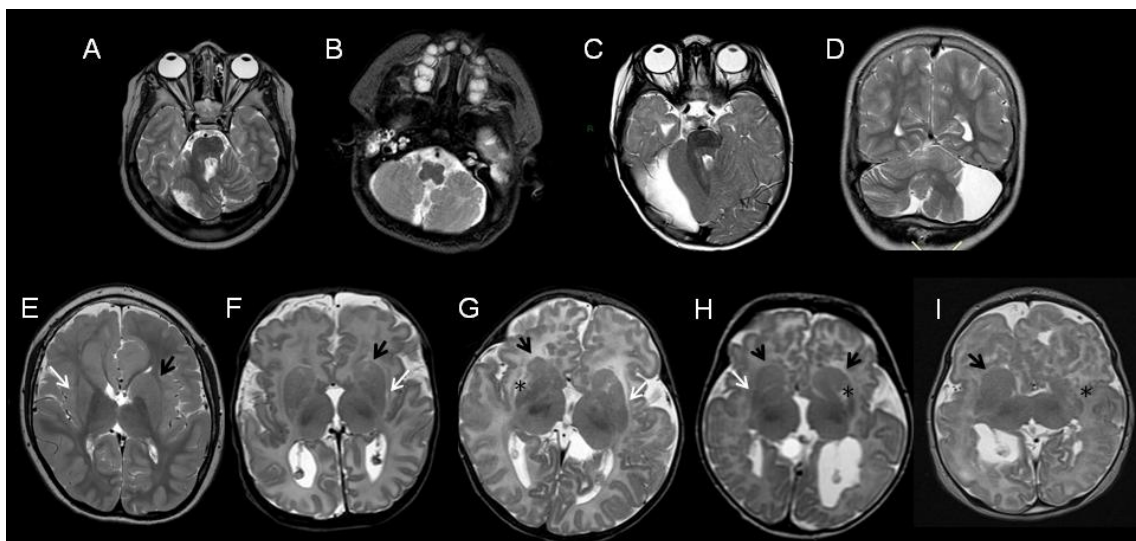


Fig.4 Posterior fossa dysmorphisms: T2-weighted axial (a, b, c) and coronal (d) MRI images show complex posterior fossa malformation with romboencephalosynapsis and emispheric schizencephalia cortical dysplasia on left cerebellar emisphere and inferior vermis hypoplasia (c) cortical dysplasia on right cerebellar emisphere and fusion with the vermis, in likely incomplete romboencephalosynapsis (d) cortical dysplasia on left and superior aspect of right cerebellar emisphere and vermis mild abnormalities (in particular vermis hypoplasia and/or vermian rotation) enlarged cistern magna complex posterior fossa malformations, from severe cerebellar hypoplasia predominantly associated with cerebellar cortical dysplasia, less frequently to romboencephalosynapsis, Dandy-Walker continuum, brainstem hypoplasia Basal Ganglia dysmorphisms: T2-weighted axial images show a stubby and globular aspect or slight hypoplasia of the striatum with an irregular and straight profile of the lateral profile of putamen (white arrow e, f, g, h), variably associated with microcysts long the irregular profile of the putamen (asterisk (*) g, h, i), likely referable to small dilated perivascular spaces.

Severe dysmorphisms with talamic adhesion (i) and/or agenesis of anterior limb of internal capsulae (black arrow) are described.

Regarding the ventricular system, 61,51% of the patients had ventriculomegaly, asymmetric in six cases. In 89,09% cases ventricular dysmorphisms were detected, from the classical colpocephalic aspect, classically associated to corpus callosum agenesis, to more complex cases with focal enlargements of ventricular system and/or indentation deformities due to nodular heterotopias and/or due to choroid plexus cysts.

Excluding hyperintense or blurred signal of the subcortical white matter (WM) that could be associated with malformed cortex, no other WM anomalies were observed.

Lastly, coloboma could be detected on MRI/CT in 32/55 patients, bilaterally in 12 ; in 22/48 (45,83%) patients in whom was assessable, optic nerves and chiasm were thin, asymmetric in a half of the cases.

Pituitary gland was well recognizable both in adeno- and neurohypophyseal portions in all the cases, showing a regular morphology.

2.4 Discussion

Aicardi Syndrome is a rare sporadic congenital condition, classically defined by the triad chorioretinal lacunae, epileptic spasms and corpus callosum agenesis (Aicardi, Chevrie et al. 1969). Out of the three classical features, chorioretinal lacunae, were considered pathognomonic by Aicardi (Aicardi 2005); in our sample, these were found in most of the patients (86,56%), confirming they are a typical sign, which should alert clinicians to consider the diagnosis. In line with the literature, all of our patients displayed an early onset epilepsy mostly with spasms, isolated or in association, with an high seizures frequency. In 51,85% at onset, EEG trace showed a severe destructuretion

of background activity, from an hypsarrythmic (29,63%) till a suppression burst pattern (22,22%). According to the literature (Aicardi 2005), which reports a severe seizures outcome, also in our sample, in a mean follow-up of 10,25 years, till for one case 27 years, 98,38% of the cases developed a drug-resistant epilepsy, with different type of seizures; an high rate of status epilepticus was observed, which could be explicated by the absence of corpus callosum inhibitory interemispheric circuits (Safouris, Popa et al. 2014). At the last evaluation only one patient was seizures free on AEDs, with also a normal neurological examination at the age of 5; in this patient the diagnosis is undoubted because of the concomitant presence of the classical triad plus polymicrogyria, nodular heterotopias, interemispheric and choroid plexus cysts. Usually AS patients are severely neurologically disabled (Uccella, Accogli et al. 2019) (Menezes, MacGregor et al. 1994; Rosser, Acosta et al. 2002), nevertheless patients with a favorable outcome till normal neurologic examination were reported (Abe, Mitsudome et al. 1990; Lee, Kim et al. 2004). Also in our sample, despite the severe epilepsy outcome, regarding motor functions, a more objective methods of evaluation allowed us to detect residual capacities: 26,19% of the cases use wheeled mobility for long distances but had also the possibility to walk with aid, and four patients are independent walkers. We found a 31,03% which could had the possibility of handle object, and one patient had some independence in daily activities. Language was more impaired than the motor functions. Concerning comorbidities, feeding problems were frequently reported, particularly most of the cases had severe limitation to safety and/or necessity of tube feeding or percutaneous endoscopic gastrostomy. In line with literature (Grigoriou, DeSabato et al. 2015), also in our sample scoliosis, vertebral dysmorphisms and respiratory complications were frequently observed. Growth pattern reflects the

literatures results (Glasmacher, Sutton et al. 2007). Previously unreported, behavioral problems, hands stereotypic movements and/or aggressivity, were frequent (88% of the cases).

In parallel with the wide clinical phenotype observed, we made a detailed imaging revision. Specifically, callosal dysgenesis was constantly found, mostly as agenesis. We confirm cysts as a typical finding in AS, found in 96,36% of our sample, mostly supratentorial; we have found a slight prevalence of 2b type versus the arachnoid ones, in line with the hypothesis advanced by Aicardi (Aicardi 2005) and subsequently corroborated subsequent studies (Hopkins, Sutton et al. 2008) (Uccella, Accogli et al. 2019). Usually cortex is severely involved, with the exception of few patients with more focal dysplasia and a patient without an apparent cortical malformation, despite an history of spasms which suggest a probably microscopic cortical involvement not detectable with our current imaging techniques. Over the known asymmetric distribution of the dysplasic cortex, as previously suggested by Hopkins and colleagues (Hopkins, Sutton et al. 2008), an anterior-posterior gradient of severity could be recognized in most of the patients. Nodular heterotopias were almost universally present, frequently multiple, asymmetric, with a periventricular involvement, and with an anterior predominance location. Considering both our and Hopkins sample, we can speculate that this anterior-posterior gradient (both involving cortex and heterotopias) associated with the cerebral asymmetry, can be considered as a specific cortical pattern for AS, which can suggest or reinforce the diagnosis. As previously reported (Hopkins, Sutton et al. 2008), we found posterior fossa abnormalities, from mild dysmorphisms to severer cerebellar cortical dysplasia, and also previously unreported romboencephalosinapsis. Basal ganglia evaluations revealed an unexpectedly high rate

of dysmorphisms (76,36% of patients) that ranged from mild to more severe forms, associated with thalamic adhesion or agenesis of anterior limb of internal capsulae. Milder and sever forms had a similar stubby and globular aspect of the basal nuclei, associated with an irregular and straight profile of the putamen; moreover most of the patients with agenesis of anterior limb of internal capsulae had microcysts associated along the basal profile of the putamen, ascribed to small dilated perivascular spaces. Taking into account the high prevalence of this findings in our sample, we can speculate that these dysmorphisms, could be considered a specific feature of basal ganglia in Aicardi Syndrome when evident; futures studies on other cohorts will allow to confirm our findings. Interestingly, in most of the patients (9/15) with the most severe BG dysmorphisms, tubuline genes were negative and five of them underwent also exome sequencing without significant results. Therefore, these findings could open the way for a new genetic research pathway on the syndrome. Karyotype (Donnenfeld, Packer et al. 1989), research of candidates genes (FLNA, TEAD1, OCEL1) (Van den Veyver, Panichkul et al. 2004; Schrauwen, Szelinger et al. 2015; Wong, Sutton et al. 2017), methylation array (Piras, Mills et al. 2017), and more recently the advanced genetic analysis of Whole Exome Sequencing (Lund, Striano et al. 2016) carried out from different research groups, did not solve the genetic challenge of AS. Our wide cohort allowed us to observed that the severe and diffuse involvement of multiple brain structures in our patients resemble the wide spectrum observed in thubulinopathies. These last conditions commonly involve commissure, corpus callosum can be hypoplastic, dysplastic or agenetic, they are characterized by different degree of severity of cortical sulcation and gyration anomalies, and periventricular heterotopias, hippocampal anomalies, and vermian hypoplasia or rotation, cerebellar dysplasia, and

the mostly characteristic involvement of deep gray nuclei as globular/hypertrophy appearance, poorly defined anterior limb of internal capsule. Indeed oculomotor nerve and optic nerve involvement are reported (Goncalves, Freddi et al. 2018). Our study demonstrate an increasing neuroradiological complexity in AS, characterized by a global subversion of multiple brain structure, so we can speculate that possible genes coding for microtubules, or for the numerous family of microtubules-associated protein or other microstructures of cytoskeleton, which integrity and functions is essential in drive cells proliferation, migration, synaptogenesis, may be a way forward to solve the mystery of AS. Supporting the hypothesis of a defect in the cytoskeleton which may underling the pathogenesis, filamin inclusion were found in astrocytes of AS patients, but genetic analysis on FLNA gene did not confirm the histological data (Van den Veyver, Panichkul et al. 2004). Further genetic studies may corroborate our hypothesis. The first aim of our study, so find associations among neuroradiological features, electroencephalographic trace, and clinical-neurological outcomes, was widely supported by statistical analysis which reveal significant associations between the severity of MRI features, EEG trace at onset and clinical-neurological outcome. As previously only hypothesized by Hamano and colleagues (Hamano, Yagishita et al. 1989), which suggested a milder outcome in patients with partial agenesis, a statistically directly correlation between the severity of corpus callosum agenesis and the neurological clinical evaluation and the motor scales, both gross motor functions and manual abilities was found. However, the severity of callosal agenesis cannot be the only responsible for the clinical outcome. Romaniello et al in a cohort of 162 patients with corpus callosum agenesis observed more severe neuromotor deficit and cognitive impairment in syndromic patients and non-syndromic with associated cerebral

malformations, compared with non-syndromic patients with isolated agenesis (Romaniello, Marelli et al. 2017). Our statistical analysis excluded a statistical influence of polymicrogyria (p value 0.100) and heterotopias (p value 0.526) on corpus callosum; however, 90,90% patients with complete agenesis have usually severe diffuse polymicrogyria and an high number of heterotopias, which can partially explain the correlation between severe callosal agenesis and worse clinical outcome. A more severe abnormal cortical pattern was statistically associated with higher scores in all the clinical outcome scales, GMFCS, MACS, neurological and language scale. Moreover the number of nodular heterotopias was directly related with the severity of GMFCS. Interestingly, cortical malformations are, even, related with long term feeding problems (EDACS), EEG and seizures at onset. Our results corroborate the first hypothesis advanced by Aicardi, which considered cortical dysplasia the most determinant of mental retardation, seizures and neurologic signs (Aicardi 2005). No correlation was found between posterior fossa dysmorphisms and clinical outcome, despite the possible influence of cerebellum and brainstem on language profile (Romaniello, Marelli et al. 2017); we can speculate that this results could be influenced by the severity of cognitive outcome which cannot allow to discriminate cognitive and language functions. A further interesting prognostic factor found was EEG at onset, previously only hypotesized or observed but not statistically defined (Ohtsuka, Oka et al. 1993; Prats Vinas, Martinez Gonzalez et al. 2005; Grosso, Lasorella et al. 2007), which in our sample was found correlated with worse gross motor and manual functions and with language scales. Neidich and coll. (Neidich, Nussbaum et al. 1990), in their small cohort, 7 cases, observed a relation between drug resistance and the severity of developmental delay; in the same way, in our study, seizure outcome was directly correlated with neurological

and motor outcome at MACS scale; these observations are in line with the well-known effects of encephalopathic changes on motor functions and cognition which depend on seizures control (Pindrik, Hoang et al. 2018).

Our study, the most extensive neuro-radiological sample reviewed to date, allowed us to delineated and describe in details the complex AS neuroradiological phenotype, which manifests as a mutiple brain malformations, not only limited to corpus callosum dysgenesis, but almost constantly associated with polymicrogyria, nodular heterotopias, and intracranial cysts. Moreover, our study underlines the potential frequent association with posterior fossa abnormalities and, previously unreported, basal ganglia dysmorphisms. The correct global detection of all these neuroradiological features, with their specific features, variable degree of severity and localization, should be considered in the diagnostic work-up of AS. Moreover, our data underline the importance of the MRI and EEG data, not only for a correct diagnosis, but as the most significant and precocious prognostic factors in predicting the long term clinical-neurological outcome. Future studies and, possibly, the identification of the genetic basis of this syndrome will allow to better characterize the wide phenotypic spectrum in AS and to detect the potential genetic prognostic factors of this complex syndrome.

3 LONG TERM EEG AND CLINICAL FOLLOW-UP OF AICARDI SYNDROME: EEG AT ONSET PREDICT DIFFERENT EVOLUTIONS

3.1 Introduction

Aicardi syndrome (AIC) is a rare developmental condition, classically described as corpus callosum agenesis, chorioretinal lacunae and epileptic spasms, by Jan Aicardi in 1965 (Aicardi 1965). Infantile spasms are the most characteristic type of seizures observed, presented both during epilepsy onset and also during the epilepsy evolution, which is characterized by a drug resistant epilepsy; other types of seizures, focal, tonic, generalized tonic-clonic, myoclonic, atonic seizures and status epilepticus, are frequently reported (Glasmacher, Sutton et al. 2007). Typically at onset, Electroencephalographic (EEG) pattern was described with bilateral independent bursts of multifocal epileptiform abnormalities occurring on a burst-suppression pattern showing complete asynchrony and asymmetry between the two hemispheres, named «split brain EEG» (Fariello, Chun et al. 1977; Ohtsuka, Oka et al. 1993). In their small cohort of six cases, Ohtsuka et al. reported that in some cases, the condition evolves to Lennox Gastaut Syndrome (Ohtsuka, Oka et al. 1993), although Aicardi suggested that almost never the EEG tend to evolve to a definite slow spike-waves pattern (Aicardi 2005). With the exception of two previous studies both on six cases, literatures lacks of consistent EEG follow up studies on larger cohorts. Aims of this study were describe the long term EEG evolution in a cohort of 11 cases with AIC and find possible early predictors of the clinical and EEG outcomes.

3.2 Material and Methods

This was a multicenter retrospective study, which involved different Italian centers.

This study adheres to the principle of Helsinki Declaration and was carried out through routine diagnostic activity. Exclusively patients who satisfied classical diagnostic criteria or Sutton modified criteria (Sutton, Hopkins et al. 2005) and only cases with a complete electroencephalographic, clinical and neuroradiological follow up were included in the study. A retrospective systematic revision of Electroencephalographic data were performed by two experienced epileptologists with a protocol which include: classification on EEG Organization with a scoring system: *score of 0*-abnormal background activity consisting of loss of dominant posterior rhythms for wake EEG and loss of normal sleep-graphoelements and/or the larger amounts of EDs or severely abnormal background consisting of continuous or invariable delta activity with no activity, suppression– burst pattern, or hypsarrhythmia; *score of 1*-mildly abnormal background consisting of slowing of dominant posterior rhythms for age and/or dominant posterior rhythms detectable only on one hemisphere, with superimposed frequent EDs, for sleep EEG rarely detectable normal sleep-graphoelements and frequent EDs; *score of 2*-of normal or near-normal background consisting of the presence of dominant posterior rhythms within normal limits for age and rare EDs, during sleep well detectable normal sleep-graphoelements and rare EDs. Evaluation of Interictal EEG Epileptiform Discharges (EDs), Photic Stimulation Response, Hyperventilation, Electrocardiogram, Pneumogram, Electromyography (when available), Ictal Recording (clinical and electroencephalographic pattern) were also performed. This revision protocol was applied both at wakeful and sleep EEG registrations. A retrospective systematic revision of brain Magnetic Resonance Imaging (MRI) was performed by two experienced neuroradiologists; MRI features analyzed were classified as follow: partial/complete agenesis or hypoplasia of corpus callosum. About the gray matter (GM) involvement

and distribution of cortical dysplasia, monofocal (defined single areas of cortical dysplasia), multifocal (defined multiple but not adjoining areas of cortical dysplasia), or diffuse cortical malformations were distinguished. Nodular heterotopias were characterized according to the number (<4 or >4).

A retrospective collection of clinical, epileptological and neurological data were performed. Patients' clinical motor outcome was retrospectively classified on the basis of the Gross Motor Function Classification System - Expanded and Revised (GMFCS) and the Manual Ability Classification (MACS) (Paulson and Vargus-Adams 2017). Neurological examination was also classified with a scoring system based on clinical evaluation: patients with normal neurological examination scored 0, presence of slight pyramidal signs 1, hemiplegia or dyplegia 2, severe diffuse hypotonia 3 and spastic tetraplegia 4. Taking into account the poor cognitive and language outcome of patients with AS, a classification system measuring the ability to say sentences (class 0), single words (class 1), babbling (class 2), vocalization (class 3), absent (class 4) was used. Eating and drinking Ability Classification System (EDACS) was used to assess eating and drinking safety and efficiency (Paulson and Vargus-Adams 2017).

3.3 Results

Eleven patients were included in the study, median age at follow-up was 11 years (range 2,5 and 23 years); two patients were deceased at the time of the study. EEG evaluation allowed us to define two different groups of patients, according to the EEG at onset and evolution.

At the epilepsy onset, in the first group including six patients: three patients presented a definite asynchronous suppression burst pattern (SB); in one case SB persisted in the

first year of life, in one patient SB evolved into an hypsarrhythmia at six months, another case developed an hemihypsarrhythmia associated with a SB pattern (Figure 1). Another single case patient presented at the onset a definite hypsarrhythmic pattern. Two cases showed a poor organized background activity with multifocal asynchronous EDs which evolved in four months in an hypsarrhythmic pattern (Figure 2). In all these cases an absent background activity persisted during all the follow-up (score of 0), both at sleep and wake EEG registrations, with a clear asymmetry on the two hemispheres. EDs were multifocal, bilateral, asynchronous but tend to be also synchronous and diffuse during sleep stages; only in one case a clear prevalence in side of EDs was detectable, stable during evolution. Typically these patients showed frequent EDs, with a significant increase in frequency during sleep since the first two years of life and which persists for several years into adolescence. They had a mean age at epilepsy onset of 1.34 months (range 1 day-3 months), the only case with epilepsy onset at 3 months of age had a premature birth, so she had a corrected age of 1 month. All the cases presented epileptic spasms at onset, multiple clusters per day, three cases associated with focal seizures, two with generalized hypertonic seizures. In their epilepsy evolution patients frequently present spasms (5/6), focal seizures (4/6), generalized (3/6), atonic seizures (1/6) and in two cases also status epilepticus were reported. All the cases developed a drug resistant epilepsy, with multiple/daily clusters. Two patient were died at the time of the study, at 5 and 23 years of age. Concerning imaging evaluation, four patients presented complete corpus callosum agenesis, two partial; four cases had a severe diffuse GM involvement, one case a multifocal (for a case only MRI report were available so a definite classification of GM involvement was not possible). All the patients presented more than 4 nodules of heterotopias. All the cases had a spastic

tetraparesis, and received at GMFCS a score of 5. Five cases did not have the possibility to handle objects (score of 5 at MACS) and one have a very limited possibility to manage objects (score of 4 at MACS). Language was absent in four the cases, one patients can only vocalize. Four cases had severe dysphagia (score of 5 at EDACS), one some limitation to efficiency and safety (score of 3 at EDACS); for one case, two years old, feeding difficulties was not reported till now (Details in Table 1a and 1b).

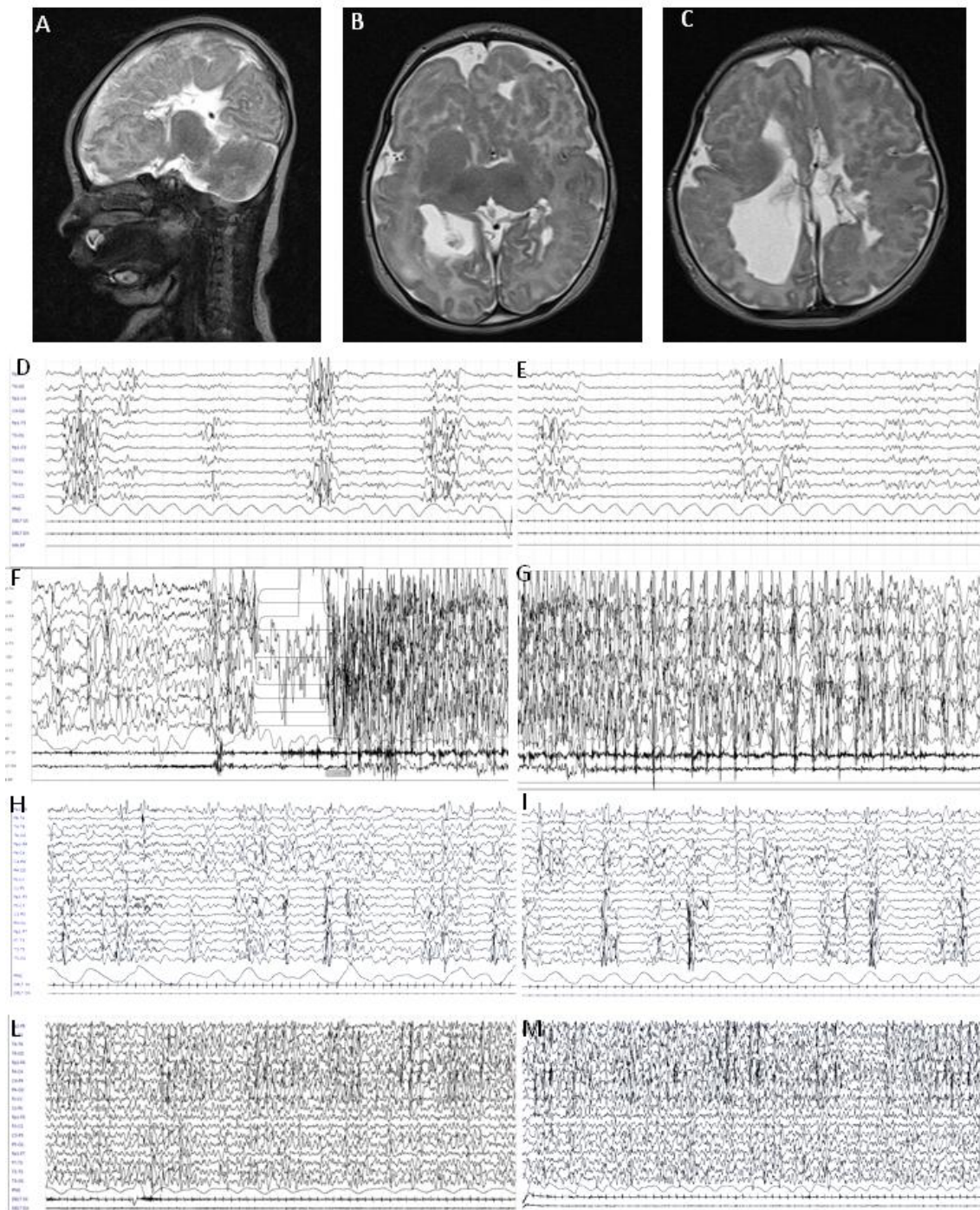


Figure1.

Case 1. MR performed at sagittal (A) and axial (B, C) T2-weighted TSE images showing complete corpus callosus agenesis (A), diffuse dysplastic cortex resembling polymicrogyria (PMG) with antero-posterior gradient and heterotopic nodules (B, C) . At 3 weeks of life (D, E) interictal EEG shows independent burst of high voltage (100-200uV) spikes, spikes-waves and

delta waves complexes asynchronous on two hemispheres alternated with interburst with low voltage activity, lasting 4-8 seconds, configuring a suppression burst pattern present both during sleep and wake recordings. Ictal EEG (F, G) shows high voltage (200-250 uV) spike-waves complexes (1,5 Hz) centro temporal right, which rapidly spread on the left hemisphere and became more frequent (1 Hz) and followed by high voltage (200-250 uV) spike-slow waves complexes, clinically patient presented a generalized hypertonia followed by bilateral clonic movements more pronounced on the right arms. Seizures lasted 1 minute.

At 2 months of life (H, I) interictal EEG shows absence of physiological background activity, hemihypsarhythmic pattern on the right hemisphere and a suppression bursts pattern on the left hemisphere, present both during sleep and awake recording.

At 23 months of age (L, M) interictal EEG shows asynchronous multifocal epileptiform discharges (EDs), high voltage (150-250 uV) spikes, spike-waves, spikes and slow waves complexes localized on fronto-temporal regions and on left hemisphere, more pronounced on the right hemisphere and during sleep (I)

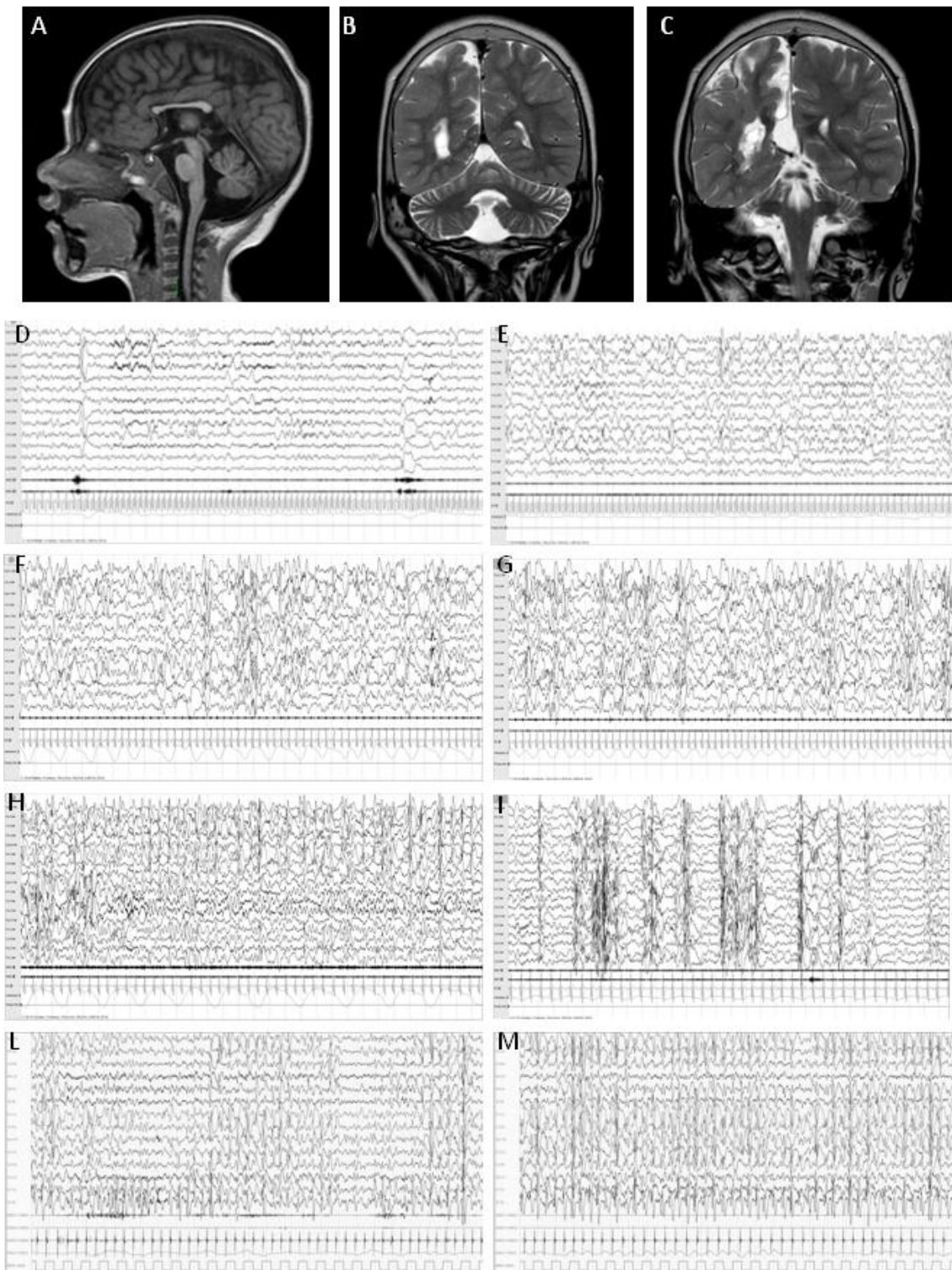


Figure 2. Case 3. MR performed at sagittal (A) T1-weighted image and coronal (B, C) T2-weighted TSE images showing partial corpus callosum agenesis (A), multifocal polymicrogyria, frontal, parietal, occipital, more on right hemisphere, and heterotopic nodules on temporal occipital right horn, confluent (B, C) .

At 3 months of age (corrected age 1 month) wake EEG (D) shows a poorly organized background activity, with diffuse theta delta rhythms, spikes, sharp-waves and low amplitude sequences of fast rhythm on central-temporal regions, bilateral; recorded two episodes of high voltage (100-250 uV) delta waves more amplitude on right hemisphere, associated with bilateral asymmetric (first left contraction) upper limbs flexion and adduction. Sleep EEG (E) shows spindles on left frontal regions and bilateral high voltage (100-200 uV) spikes-waves, spikes-slow waves complexes, on frontal central regions, more frequent on right hemisphere.

At 7 months of age (correct age of 5 months) wake (F) and sleep (G) EEG shows bilateral high voltage (200-450 uV) delta waves, spikes, spikes-waves, spikes-slow waves complexes, more pronounced on right hemisphere, which configure an hypsarrhythmic pattern.

At 3 years and 6 months wake EEG (H) shows bilateral asynchronous high voltage (200-250 uV) spikes, spikes-waves, spikes-slow waves complexes more frequent and with more amplitude on right hemisphere. During sleep (I) EEG show generalized high amplitude (250-300 uV) spike- and polyspikes-waves complexes and less frequent asynchronous multifocal EDs

At 7 years of age, wake EEG (L) shows frequent bilateral synchronous and asynchronous high voltage (250-300 uV) spike and spike-waves, spike-slow waves complexes; during sleep (M) EDs are subcontinuous and more synchronous

In the second group of five cases, at epilepsy onset, EEG wake registration showed poorly organized background activity (score of 1), during both awake and sleep registration, and physiological sleep-graphoelements (sleep spindles, vertex waves, Kcomplexes), spindles synchronous and asynchronous. During EEG follow-up, they maintained a good/poor background activity (score of 1/2): in most of the EEG recordings a posterior dominant rhythmic, physiological for age, bilateral, in three with a slight asymmetry, and spindles, synchronous and asynchronous, were recognized. In all these patients, EDs were asynchronous, with a more focal distribution, which usually has remained constant, in term of localization and side during all their EEG follow-up was detectable. In all the cases an increase in frequency of EDs during sleep were

detected, typically at 4-6 years of age, which persisted till 9-10 years of age (Figure 3-4). Imaging revealed partial corpus callosum agenesis in three patients, complete in one, hypoplasia in one case; GM involvement was absent in one case, one patient presented a focal dysplasia, three cases a multifocal involvement. Three patients showed few nodules of heterotopias, only one case more than four. Concerning clinical and epilepsy evolution, they had a mean age at epilepsy onset of 4 months (range 3-8 months), with clusters of spasms; in their epilepsy history all the cases displayed also focal seizures. All the patients had a partial response to antiepileptic drugs (AEDs), particularly with monthly/weekly seizures and only one case presented one seizure/day at last evaluation. Four of these cases presented hemiparesis, one patient slight pyramidal signs; three cases had the possibility to walk without limitation (score of 1 at GMFCS) and two without aid but with some difficulties (score of 2 at GMFCS); two patients handled object with only somewhat reduced quality and/or speed of achievement (score of 2 at MACS), and one case with some difficulties (score of 3 at MACS). Three patients were able to speak with sentences, one with single words, and one only with bubbling. All the cases eat and drink safely, only one case with some limitation in efficiency were reported (EDACS score of 1-2) (Details in Table 1a and 1b).

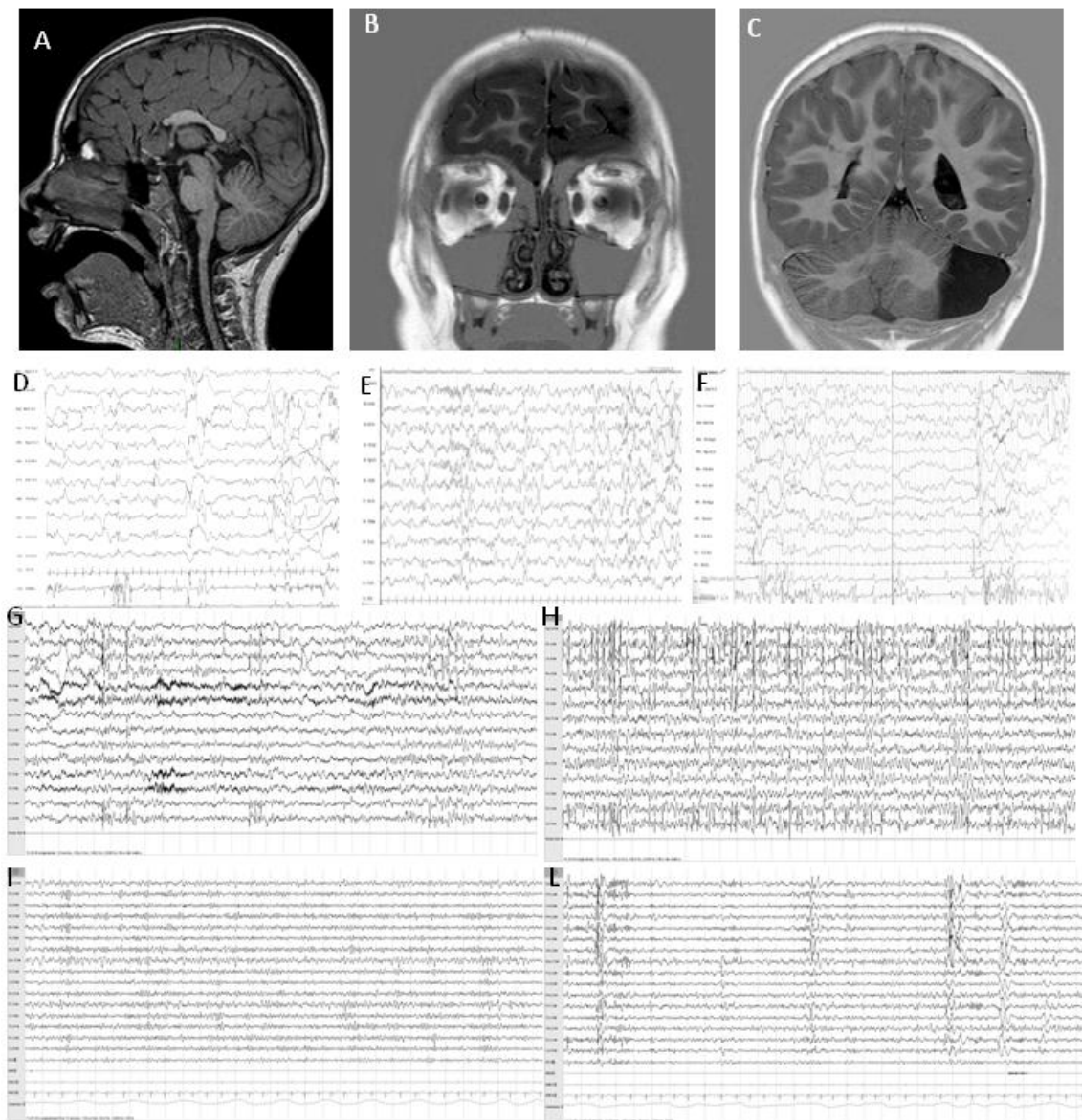


Figure 3. Case 8. MR performed at sagittal (A) and axial (B, C) T2-weighted TSE images showing partial corpus callosum agenesis (A), dysplastic cortex resembling polymicrogyria on frontal right lobe and multiple heterotopic nodules (B, C).

At 3 months of age, interictal EEG shows absence of physiological background activity, high voltage (100-200 uV) spikes, spike-waves complexes on frontal-central-temporal left and right regions, asynchronous (D). During sleep (E) shows spindles on right frontal regions, multifocal asynchronous Eds: spikes, spike-waves complexes on frontal-central-temporal left and right regions. Ictal EEG (F) shows high voltage (>300 uV) delta waves more pronounced on right hemisphere followed by fast activity associated with asymmetric spasms.

At 4.5 years of life interictal EEG shows bilateral symmetric posterior dominant rhythm, alfa, 7-

8 Hz, and high amplitude (100-200 uV) spikes, spike-waves complexes on frontal-central-parietal-occipital right regions and less frequent on central temporal parietal left regions (G) . Sleep EEG (H) shows rare spindles on left hemisphere, subcontinuous high voltage (200-300 uV) spikes and spikes-waves complexes on right frontal-central-temporal-parietal regions and less frequent high voltage (100-150uV) spikes, spike-waves on fronto central left regions

At 15 years of age interictal EEG shows bilateral symmetric posterior dominant rhythm, alfa, 8-9 Hz; slow activity on left frontal region and rare spikes, spike-waves on frontal-central-temporal right regions, amplitude 80-90 uV, and more rare spikes, spike-waves on frontal-temporal left regions, amplitude 80 uV (I). During sleep (L) shows bilateral synchronous and asynchronous splindles, spikes, high voltage 100-170 uV spike-waves complexes on frontal-central-temporal right regions and less frequent on frontal- temporal left regions

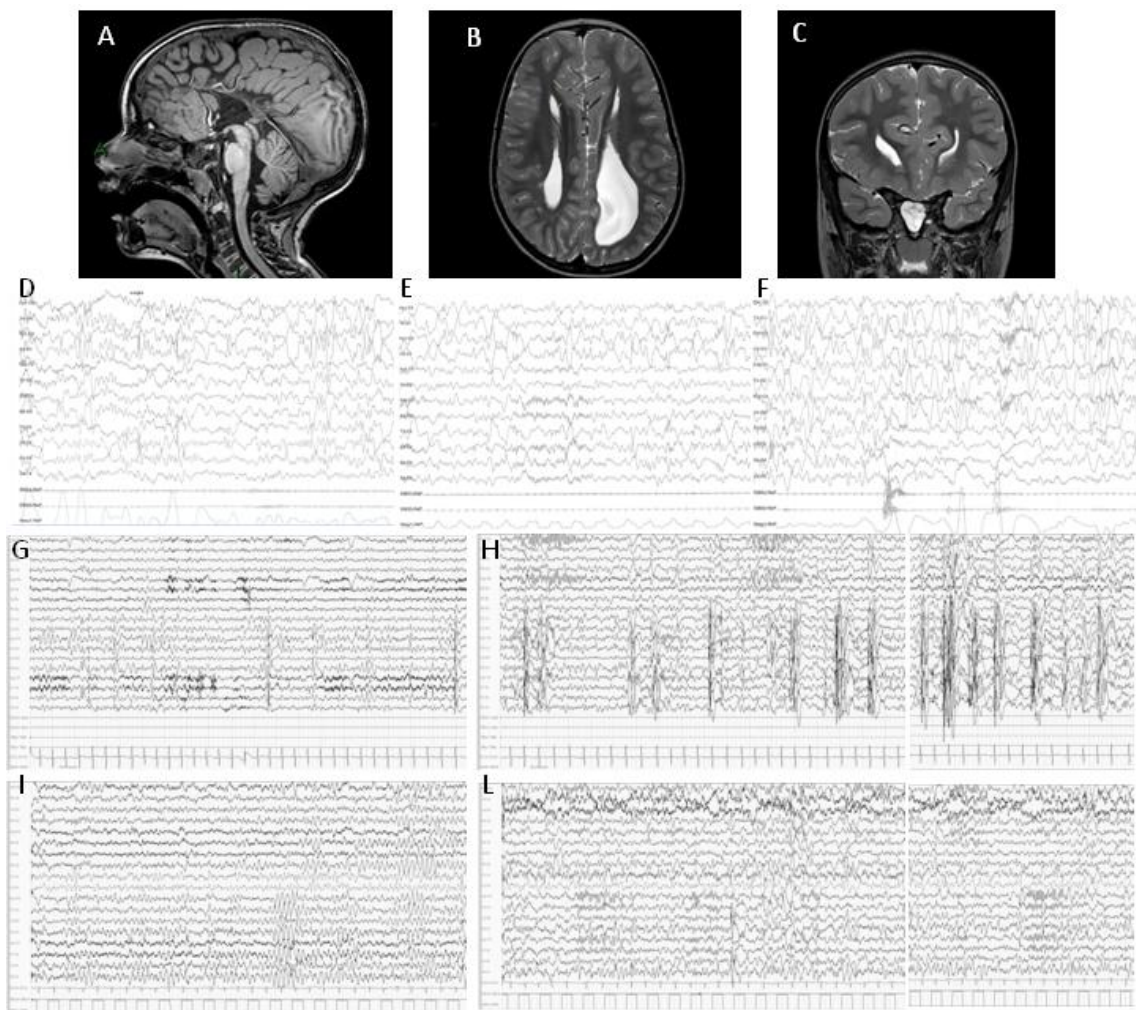


Figure 4. Case 9. MR performed at sagittal (A) and axial (B, C) T2-weighted TSE images showing complete corpus callosum agenesis (A), multifocal polymicrogyria on frontal bilateral regions and two heterotopic nodules (B, C).

At 3.5 months of age interictal EEG shows interhemispheric asymmetry with slow activity on left hemisphere and high voltage (100-200 uV) spikes, spike-slow waves complexes on central left regions (D). During sleep (E) shows spindles only on right frontal regions, and high voltage (100-200 uV) spikes, spike-slow waves complexes on central left regions. Ictal EEG (F) shows low voltage fast activity on left hemisphere associated with asymmetric spasms

At 8 years of life interictal EEG shows slow activity on left hemisphere and high amplitude (100-150 uV) spike-waves, spikes-slow waves and sharp waves on central-parietal left regions, rare spike-waves on right temporal-parietal regions (G). Sleep EEG (H) shows spindles on right hemisphere, and an activation of EDs during sleep: frequent high voltage (200-300 uV) spikes and spikes-slow waves complexes on left central-temporal-parietal-occipital regions sometimes with diffusion on right hemisphere and less frequent high voltage (100uV) spike-slow waves and slow waves on central-parietal right regions

At 11 years and 7 months of age interictal EEG (I) shows slow activity on left hemisphere and short part of posterior dominant rhythm, alpha, 7-8 Hz on right hemisphere. Presence of high amplitude (100-150 uV) waves on central-parietal left regions, rare sharp waves on right temporal-parietal regions. Sleep EEG (L) shows bilateral asynchronous spindles, spike-spike-waves on central-parietal regions bilateral, more on left hemisphere

Considering the EEG revision of all the 11 cases, in 9/11 the persistence of epileptic spasms was observed during the entire follow-up; spasms could be observed both during awakening and sleep registrations, could be isolate and/or in short or long lasting clusters.

In the early EEG registrations, in a context of a poor background activity with some physiological elements detectable, three cases presented subtle episodes of fast activity, in some case preceded by high voltage slow waves, associated with sudden head and limbs flexion and adduction (Figure 5). Although both at EEG registration and clinically these episodes can be subtle, should be considered epileptic spasms because they might

precede the appearance of the classical EEG and clinical epileptic spasms.

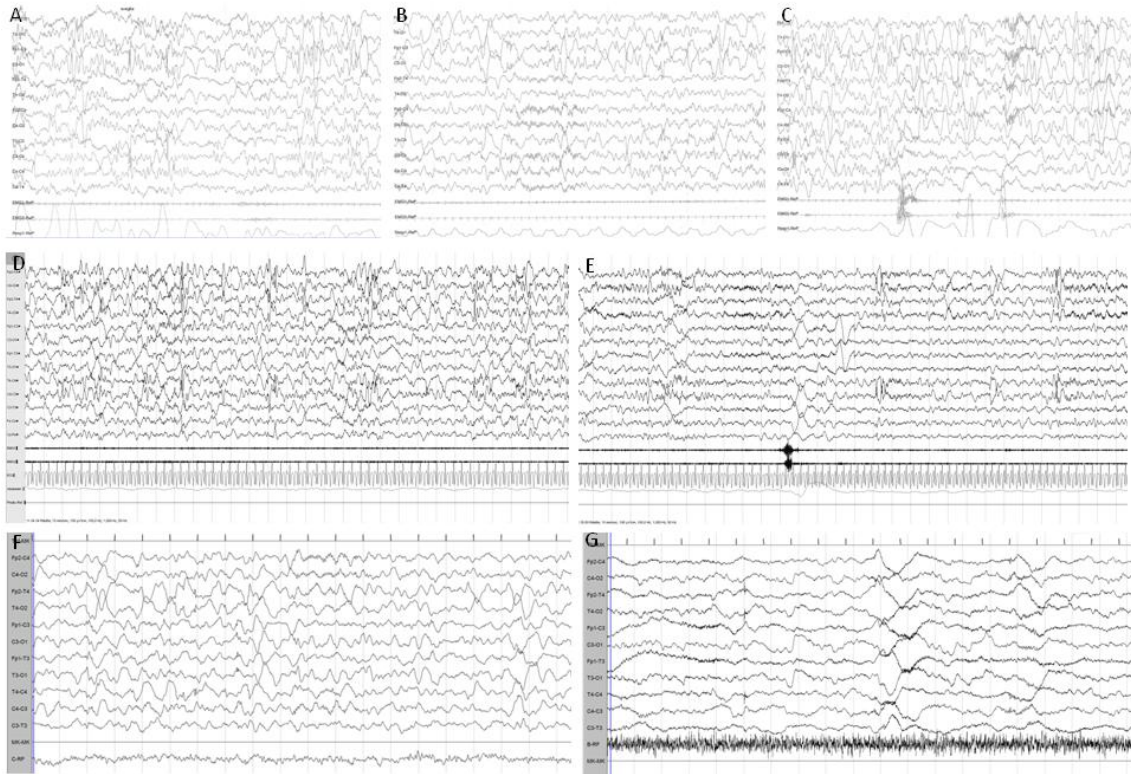


Figure 5. Case 9. At 3.5 months of age interictal EEG shows interhemispheric asymmetry with slow activity on left hemisphere and high voltage (100-200 μ V) spikes, spike-slow waves complexes on central left regions (A). During sleep (B) shows spindles only on right frontal regions, and high voltage (100-200 μ V) spikes, spike-slow waves complexes on central left regions. Ictal EEG (C) shows low voltage fast activity on left hemisphere associated with asymmetric spasms

Case 3. At 3 months of age (corrected age 1 month) wake EEG (D) shows a poorly organized background activity, with diffuse theta delta rhythms, spikes, sharp-waves and low amplitude sequences of fast rhythm on central-temporal regions, bilateral; one episode of fast activity on right hemisphere associated with bilateral asymmetric (first left contraction) upper limbs flexion and adduction on polygraphic registration(E).

Case 7. At 3 months of age sleep EEG (F) shows spindles on the right frontal regions and multifocal bilateral high voltage (200 μ V) spikes and spikes-waves complexes. Ictal EEG (G) shows diffuse high voltage (250 μ V) slow waves followed by fast activity, associated with head and limbs flexion and adduction

Most of the patients presented an asymmetric background activity both during wakefulness and sleep EEG; this asymmetry were more clear during awakenings, when a diffuse slow activity typical of the sleep stages persisted on one hemisphere and a wake EEG activity can be recorded on the other hemisphere (Figure 6).

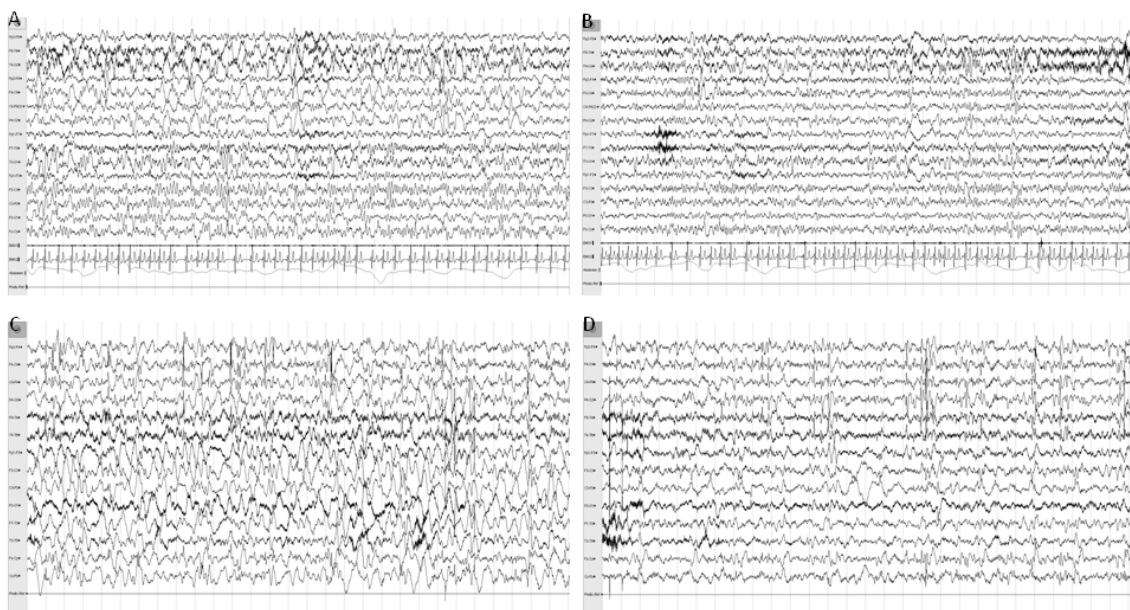


Figure 6. Case 7. At 8 years of life, interictal EEG shows during awakening interhemispheric asymmetry, diffuse high voltage (200-250 uV) delta waves on the right hemisphere and theta rhythm on the left hemisphere with rare high voltage delta waves on occipital regions, after 1 minute EEG shows more symmetric theta activity on the two hemispheres and rare sharp waves (voltage 100-150 uV) on centro temporal right hemisphere.

Case 8. At 5 years and 5 months of age, interictal EEG shows during awakening interhemispheric asymmetry, diffuse high voltage (200-250 uV) delta waves on the left hemisphere and theta waves mixed with rare high voltage (200 uV) delta waves on left hemisphere. Spike-waves complexes are detected on frontal-central-parietal-occipital right regions and less frequent on central temporal parietal left regions

ONSET - FIRST YEAR OF LIFE

1-6 YEARS OF LIFE

	age	background activity		EDs		classification	Age at sz onset	background activity		EDs		seizures
		awake EEG	sleep EEG	awake EEG	sleep EEG			awake EEG	sleep EEG	awake EEG	sleep EEG	
Pt 1	2 y	0 asymmetric slow R	0 asymmetric slow R	multifocal, R>L	multifocal, bilat, increase in frequency	SB, after 1mo hypsarrhythmia R, SB L	1 day, GTCS, spasms	0 asymmetric	0 asymmetric	multifocal, R>L	multifocal, R>L, subcontinuous since 14 mo	focal sz, spasms
Pt 2	died at 5 y	0 asymmetric	0	multifocal asynch	multifocal asynch	1 mo SB, 6 mo hypsarrhythmia	1 mo, focal bilat, GTS, spasms	0 asymmetric	0 asymmetric	multifocal asynch, diffuse bouffées	multifocal asynch, diffuse bouffées, increase in frequency since 1 y	focal sz, spasms, SE
Pt 3	8 y	0 1	0 1	multifocal, R>L	multifocal, R>L	hypsarrhythmia at 5 mo CA	1 mo CA, spasms	0 1 asymmetric slow R	0 1 rare spindles asynch/synch, asymmetric slow R	multifocal, R>L	multifocal, R>L, diffuse bouffée, increase in frequency since 2 y to date	spasms, atonic sz, focal sz, GTS, eyes myoclonia
Pt 4	23 y	0	0	multifocal asynch	multifocal asynch	SB	1,5 mo, focal, spasms	0 asymmetric slow L	0 1 rare spindles asynch	multifocal bilateral asynch	multifocal asynch, diffuse bouffée, activation since 2 y to date	spasms, GS, atypical absences, focal sz, SE
Pt 5	died at 23 y	0 asymmetric	/	multifocal asynch	/	hypsarrhythmia asynch	1,5 mo, spasms	0 asymmetric	0 asymmetric	multifocal R>L, diffuse bouffées	multifocal R>L increase in frequency since 2,5 y, diffuse bouffées to 17 y	GS
Pt 6	6 y	1 asymmetric	/	multifocal L>R	/	hypsarrhythmia at 5 mo	1 mo, focal, spasms	0 asymmetric	0 rare spindles asynch	multifocal, diffuse bouffées	multifocal bilat, diffuse bouffées since 1 y	focal, spasms
Pt 7	7 y	0 1	1 spindle s synch/asynch	multifocal	multifocal	/	3 mo, spasms	1 2 7-8 Hz, slight asymmetric slow R	1 2 spindles synch/asynch, asymmetric slow R	F C T bilat	F C T bilat, increase in frequency at 5 years	focal sz
Pt 8	17 y	1 asymmetric slow R	1 2 spindle s	F C T R>T O L	F C T R>T O L	/	3 mo, spasms	1 2, 7-8 Hz asymmetric slow activity L	1 2 asymmetric slow activity L, spindles synch/asynch	F C T R>C T O L	F C T R>C T O L, diffuse bouffées, subcontinuous from 4,5 y to 10 y of age	focal, spasms
Pt 9	14 y	1 2 asymmetric slow L	1 spindle s synch/asynch R>L asymmetric slow L	C T bilat L>R	C T bilat L>R	/	3 mo, spasms	1, 7-8 Hz asymmetric slow activity L	1 spindle s synch/asynch R>L, asymmetric slow activity L	C T bilat L>R	C T bilat L>R, diffuse bouffées, increase in frequency from 6 y to 9 y	focal, spasms
Pt 10	9 y	1	/	F T bilat R>L	/	/	3 mo, spasms	1	0 1	F T bilat	F T bilat increase in frequency from 5 y	focal, spasms, focal SE
Pt 11	4 y	1 slight asymmetric slow R	1 spindle s bilat synch, slow R	C T bilat R>L	C T bilat R>L, rare diffuse bouffées	/	8 mo, spasms	2, 7-8 Hz, bilat	1 spindle s bilat synch, slow activity R	no EDs	C T bilat R>L, diffuse bouffées, increase in frequency from 4 y	focal, spasms

Table 1a.

LONG TERM F-UP >6 YEARS

	background activity		EDs		seizures	neur ol	EDA CS	MA CS	lang uage	GM FCS	CC	Poly	heter otopi a
	awake EEG	sleep EEG	awake EEG	sleep EEG									
Pt 1						4	1	5	4	5	3	3	2
Pt 2						4	5	5	4	5	2	3	2
Pt 3	0	0	multifo cal subcon tinuous synchr/ asynch	multifocal subcontinuo us synchr/asyn ch, diffuse bouffées	spasms, atonic sz, focal sz, GTS, eyes myoclonia	4	3	5	3	5	2	2	2
Pt 4	0 asym metric	0	multifo cal bilateral asynchr on	multifocal bilateral asynchron, diffuse bouffée	spasms,G S, atypical absences, focal sz	4	5	5	4	5	3	3	2
Pt 5	0 asym metric	0 asymmetric	multifo cal R>L	multifocal R>L	GS	4	5	5	4	5	3		
Pt 6						4	5	4	4	5	3	3	2
Pt 7	2 8-9 Hz	1	multifo cal	multifocal	focal sz	2	1	2	0	1	2	1	1
Pt 8	1 2, 8-9 Hz asym metric slow activit y L	1 2 asymmetric slow activity L, spindles synch/asynch	F C T R> C T O L	F C T R> C T O L, diffuse bouffées, after 7 y EDs reduction	spasms	2	1	2	0	1	2	2	2
Pt 9	1, 10 Hz, asym metric slow activit y L	1 spindles synch/asynch R>L, asymmetric slow activity L	rare C T bilat L>R	rare C T bilat L>R, rare diffuse bouffées	focal, spasms	2	1	3	2	2	3	3	1
Pt 10	1	/	F C T bilat R>L	/	focal, spasms	2	2	2	1	2	2	2	1
Pt 11						1	1	2	0	1	1	0	1

Table 1b. asynch asynchronous; C central; CA corrected age; EDs epileptiform discharges; F frontal; GS

generalized seizures; GTCS generalized tonic clonic seizures; GTS generalized tonic seizures; L left; O occipital; R right; synch synchronous; SB suppression burst; SE status epilepticus; T temporal; y years

Discussion

Our study described the long term EEG and clinical evolution of 11 cases with AIC Syndrome and allowed to identify possible early predictors of the clinical and EEG outcomes. The results permitted to delineated two distinct EEG “phenotype”, a “*Classical Severe Phenotype*”, which corresponds to the cases previously describes in literature (Fariello, Chun et al. 1977; Ohtsuka, Oka et al. 1993; Aicardi 2005) and a “*Mild Phenotype*”, with different Electroencephalographic features at the epilepsy onset and during epilepsy evolution, which corresponds to different clinical outcomes.

Patients with the *Classical Severe Phenotype* presented a very early epilepsy onset, before 3 months of life, with spasms frequently associated with other type of seizures, and a severe destructurement of EEG traces, which persist over all the EEG follow up in the first one; patient with the *Mild Phenotype* had a later epilepsy onset compared the first group, after 3 months of age, with only spasms, associated with a poorly organized background activity, without configuring an hypsarrythmic nor a suppression burst pattern and the persistence of physiological sleep-graphoelements during sleep registrations. Considering this last EEG features, our study warns about the possible detection of subtle episodes of fast activity, or high voltage slow waves, associated with subtle head and limbs flexion and adduction in a context of a not definite hypsarrythmic pattern, which should be recognized and promptly treated as epileptic spasms. Over time, patients with Mild Phenotype maintained a mildly abnormal background activity both at wakefulness and sleep registrations, with a more focal EDs while in the *Classical Severe Phenotype* EDs tend to be more frequent and synchronous, particularly

during sleep which correspond to the more frequent generalized seizures. Both the two groups presented a sleep activation of EDs, limited in time in the Mild Phenotype compared the Classical Severe Phenotype who presented a prolonged sleep activation, from the first years of life which persisted into adulthood. In line with the previous description of Aicardi (Aicardi 2005), although the sleep activation and the synchronization of EDs associated with the possible recurrence of atonic and generalized seizures, we did not observe an evolution into a definite pattern typical of Lennox Gastau Syndrome. All these cases displayed a drug-resistant epilepsy, although a partial response in the Mild group was observed. The group can be distinguished also from clinical and neurological point of view: the *Classical Severe Phenotype* presented a severe clinical-neurological and functional outcome (severe tetraparesis); the second displayed a less severe neurological and clinical phenotype, with hemiparesis or slight pyramidal signs, possibility to walk with/without help, handle objects and eat. Imaging evaluation revealed in most of the *Classical Severe Phenotype* cases a severe cerebral malformation, with complete corpus callosum agenesis, diffuse cortical anomalies and multi nodules of heterotopias; while the second group presented mostly partial corpus callosum agenesis, a more focal cortical anomalies and few nodules.

Nevertheless the sample is small for statistical analysis, our study demonstrated that the severity of EEG at onset appears to be related to the severity of cortical anomalies and callosal agenesis. Moreover, the severity of EEG tend to be stable over time and associated with the severity of clinical neurological outcome. This data are in line with the previous multicenter study on 67 AIC cases in which statistical analysis revealed a statistical differences between abnormal cortical pattern and EEG at onset and significant associations between the severity of EEG at onset and severity at

GMFCS, MACS and language scales (in these study EEG were evaluated mostly from reports not from EEG traces evaluations). Moreover in the same study, complete agenesis of corpus callosum was directly correlated to higher scores on neurological clinical scale, GMFCS and MACS. Our recent results sustain the hypothesis that MRI images and EEG in the first year of life could be considering possible prognostic factor in predict EEG and clinical long term evolution.

In conclusion data from our long term EEG and clinical study delineated two different phenotype of AIC Syndrome, with different severity on MRI studies, EEG at onset which remain constant over time, which can predict the clinical outcome. Future studies on larger choort will sustain our first results.

4 AICARDI SYNDROME: KEY FETAL MRI FEATURES AND PRENATAL DIFFERENTIAL DIAGNOSIS

4.1 Introduction

Aicardi Syndrome (AIC) is a rare congenital syndrome classically defined by the presence of total or partial corpus callosum (CC) agenesis, chorioretinal lacunae and epileptic spasms (Aicardi 2005; Sutton, Hopkins et al. 2005). Over time imaging studies have better defined the neuroradiological phenotype of the syndrome which manifests with the concomitant presence of additional multiple brain malformations, including polymicrogyria (100%), nodular heterotopias (100%) and intracranial cysts (95%) (Hopkins, Sutton et al. 2008); so that, Aicardi suggested a list of revised criteria for AIC, including these neuroradiological findings among the major features of the syndrome. However, the pre-natal diagnosis of AIC remains still difficult lacking of the clinical data. Intrauterine magnetic resonance imaging (iuMRI) has become a powerful diagnostic tool to detect fetal malformations, even at early gestational age (Righini, Zirpoli et al. 2004; Girard, Chaumoitre et al. 2006), though the pre-natal suspect of AIC has been reported at least in few anecdotal cases (Columbano, Luedemann et al. 2009) (Hergan, Atar et al. 2013; Vinurel, Van Nieuwenhuysse et al. 2014; Gacio and Lescano 2017).

Considering the lack of consistent and extensive data about the pre-natal imaging presentation of the syndrome, we aimed to describe in a relatively large cohort of clinically confirmed AIC patients, the brain iuMRI presentation of the syndrome, also comparing the prenatal findings with the post-natal ones. Moreover, a retrospective revision of brain iuMRIs of a large group of fetuses with CC dygenesis-agenesis and cortical malformations (AIC mimickers) was performed and compared in consensus

with AIC iuMRI cases, in order to identify among them the neuroradiological findings potentially predicting AIC and so differentiating the syndrome from similar fetal conditions.

4.2 Material and Methods

First part of the study: iuMRI versus postnatal MRI in confirmed AIC patients

In the first part of the study, clinically confirmed AIC patients, selected in a multicentre setting involving six Italian centers and one French center, were retrospectively collected. Patients who exclusively satisfied classical diagnostic criteria or Sutton modified criteria were included (Sutton, Hopkins et al. 2005). Only cases with both iuMRI and postnatal MRI data were included. Data on gestational age (GA) at iuMRI and sex were recorded, together with post-natal MRI data and clinical follow-up information. Then, a systematic blind revision of AIC iuMRIs and postnatal MRIs was performed separately by two different équipes of pediatric neuroradiologists by using a revised neuroradiological protocol described by Hopkins (Hopkins, Sutton et al. 2008) (Protocol details in Table 1 and Table 2). A dataset including cerebral and ocular biometric and morphological findings was created. IuMRI examinations were performed at 1.5 Tesla (with a cardiac or abdominal phased-array coil) and protocol included at least -multiplanar single-shot fast spin-echo T2-weighted sequences (in plane res. about 1mm^2 ; section thickness, 3–4 mm), and T1-weighted gradient-echo or fast-spin-echo (FSE) 4-5 mm'thick-sections sequences. Study complied with Institutional regulations for retrospective studies on fetal MR imaging. Postnatal MRI studies were performed using 1.5 Tesla scanners according to standard protocols including at least T1-weighted spin-echo (SE) sagittal sequence T2-weighted FSE axial

and coronal images, fluid attenuated inversion recovery (FLAIR) axial and coronal images and inversion recovery (IR) coronal sequences. Diffusion Weighted Imaging (DWI) was also included .

In this first part, we only tested the diagnostic accuracy of the iuMRI in detecting ocular coloboma compared to postnatal MRI as the reference standard, because the diagnostic performance of the former has not been tested so far (Righini, Avagliano et al. 2008). Sensitivity, specificity, positive and negative predictive values were calculated.

Second part of the study: AIC fetal iuMRIs versus similar fetal conditions (AIC mimickers)

In the second part of the study, from a database involving more than 4000 iuMRI studies performed at Children's Hospital V. Buzzi from 2004 to 2019, all fetal cases carrying CC dysgenesis-agenesis and cortical gyration anomalies (AIC mimickers), with or without interhemispheric cysts were selected. Exclusion criteria were: iuMRI protocol not including the minimal image sequence type and number, neuroradiological findings clearly referable to conditions unequivocally different from AIC (such as lissencephaly, severe microcephaly, anomalies of ganglionic eminence region, severe cranio-facial dysmorphisms); moreover all the fetal cases without clinical and neuroradiological follow-up information were excluded. Taking into account the few male cases with AIC syndrome described in literature, with XY or XXY karyotype (Hopkins, Humphrey et al. 1979; Aggarwal, Aggarwal et al. 2000; Anderson, Menten et al. 2009; Shetty, Fraser et al. 2014), we decided to include in this control group even male fetuses. Then we statistically compared the iuMRI findings of these AIC mimickers with those of the AIC cases previously described, to identify possible differences among them and thus iuMRI

findings predictors of AIC. Statistical analysis was performed considering one dependent variable, the presence or absence of AIC diagnosis, and the following independent categorical variables: ventricular abnormalities, gross cerebral asymmetry, intracranial cysts, choroid plexus cysts and/or papilloma, nodular heterotopias, basal ganglia dysmorphisms, cerebellar abnormalities, coloboma. Gyration anomalies were divided into focal, focal next to the cysts and diffuse; CC abnormalities were classified as complete, partial and dysgenesis. Sex was also included among the independent variables. Chi-square test was performed on the independent variables comparing expected and observed proportions of AIC for each variable. Binary logistic regression was employed to identify the most predictive independent variables. Finally, receiver operating characteristics (ROC) curves were generated to quantify the area under the curve (AUC) of the single variables and of the combination of the strongest predictors in identifying AIC. Statistical analysis was performed using IBM SPSS software Ver.20.

4.3 Results

iuMRI versus postnatal MRI in confirmed AIC patients

Ten iuMR imaging exams and thirteen post-natal MR imaging exams from a total of nine female patients were evaluated. IuMRI exams were performed at a median GA of 28.3 weeks. Postnatal MRI exams were performed at a mean age of 28.2 months (from 6 days to 7 years and 9 months range) (details in table 1).

iuMRI were performed because of ultrasound signs of ventriculomegaly, colpocephaly, CC agenesis, intracerebral and/or intraventricular cysts, isolated or in combination.

	<i>Age at MRI</i>	<i>CC</i>	<i>Ventricles</i>	<i>ventric dysmorphisms</i>	<i>gross asymmetry</i>	<i>Cysts (localiz, type, number, pattern)</i>	<i>Heterotopia (localiz, number, pattern)</i>	<i>Gyration anomalies and gradient</i>	<i>posterior fossa abnormalities</i>	<i>BG dysmorphisms</i>	<i>Ocular abnormalities</i>
Pt1 pre	28wks 3 d	C	R	bilat	no	IT, 2d, 1, uniloc	Diffuse bilat, >4, mixed	diffuse dysgyria, no gradient	DMJ	N	bilat coloboma, microphthalmos
post	7 d	C	R	bilat	no	IT, 2b, 1, uniloc	frontal, temporal, occipital, body, trygon bilat, >4, mixed	severe diffuse polymicro, A-P	DMJ	globular aspect, not defined anterior limb of internal capsulae	bilat coloboma, microphthalmos, optic nerve chiasm atrophy
Pt2 pre	29 wks 6 d	C	bilat	bilat	no	IT, 2d, multiple, multiloc, choroid	frontal, occipital, <4, single	diffuse dysgyria, no gradient	no	no	bilat coloboma
post	6 d; 3 mo 11d	C	R	bilat	no	IT, 2b, multiple, multiloc, choroid	frontal horn, body bilat, 3, single	frontal bilat polymicro, A-P	small fossa	not defined anterior limb of internal capsulae	L coloboma, optic nerve chiasm thin
Pt3 pre	30 wks 1 d	P	R	yes		IT, 2d, 2, uniloc, choroid	trygon R, 1, single	occipital bilat dysgyria, P-A	no	no	doubt R coloboma
post	5y 4 mo; 7y 4 mo	P	R	L	no	IT, 2d, 4, uniloc, choroid	temporal horn R, 1, single	diffuse abnormal gyration pattern, more occipital bilat, L, P-A	no	no	R coloboma, optic nerve chiasm thin
Pt4 pre	31 wks 5 d	P	no	asymmetric	one side	CPF, 1, uniloc	monolat, side cortical malf, >4, single	dysgyria, bilat, more one side, A-P	CPF, cerebellar asymmetry	no	no
post	2 mo, 2y 5 mo	P	bilat, more R	bilat more R	no	IT 2d, CPF, 2, uniloc	frontal horn, body bilat, trygon L, >4, confluent	severe diffuse bilat polymicro, A-P	CPF, L cerebellar hemisphere dysplasia, vermis hypoplasia	no	bilat coloboma, optic nerve chiasm thin
Pt5 pre	33 wks	C	no	more R	more R	IT, 2d, 1, uniloc	frontal horn, occipital bilat, temporal L, >4, mixed	dysgyria polymicro-like, bilat, A-P	no	no	bilat coloboma
post	3 mo	C	yes	yes	more R	IT, 2b 2d, uniloc	frontal horn R, trygon L, >4, mixed	frontal bilat dysplasia, R>L; A-P	enlarged cisterna, hemisph asymmetry, vermis hypoplasia	stubby	bilat coloboma
Pt6 pre	28 wks 1 d	C	yes	yes	bilat asym	IT, 2d, uniloc	diffuse, >4, mixed	diffuse dysgyria, A-P	no	not defined anterior limb of internal capsulae	bilat coloboma
post	2 y 3 mo	C	yes	yes	more R	IT, 2b, 1, uniloc	frontal, temporal horn bilat, body R, >4, mixed	Multifocal polymicro, more R, frontal lobes, perisylvian, A-P	no	not defined anterior limb of internal capsulae	bilat coloboma
Pt7 pre	21 wks 6 d	P	no	yes	R	IT, 2d, 1, uniloc, choroid	parietal, occipital horn R, >4, confluent	diffuse dysgyria, more R, A-P	cerebellar malformation	no	no
post	2 y 3 mo; 6 y 10 mo	P	no	R>L		IT, 2d, CPF, 2, multiloculated, choroid	temporal, occipital horn R, >4, confluent	frontal, parietal occipital R polymicro, no gradient	wide IV v, hemisph atrophy, vermis hypoplasia	slight more R	R coloboma, optic nerve chiasm thin

Pt8 pre	34 wks	C	Y	Y	Y	IV, 1, uniloc	bilat, >4, confluent	diffuse bilat dysgyria, A-P	cerebellar dysmorphisms	BG dysmorphisms	coloboma bilat
post	10 d, 27 d	C	bilat	bilat	R < L	IT, 2b, 1, IV, 1, uniloc, choroid	bilat, >4, confluent	diffuse bilat polymicro, A-P	hemisph asymm, vermis hypoplasia, cortic dysplasia	severe BG dysmorphisms	bilat coloboma, optic nerve chiasm thin
Pt9 pre	23 wks 2 d; 26 wks 2 d	P	monol at	anteriorly bilat	no		frontal horn bilat, more one side, >4, confluent	focal polymicro unilat, A-P	no	no	doubt coloboma
post	7y 9 mo	P	no	more L	L	CPF, 1, uniloc, choroid	body, temporal horn bilat, trygon R, >4, mixed	multifocal polymicro and frontal R dysplasia, A-P	small cysterna, vermis hypoplasia	stubby head of caudatum R	optic nerve chiasm thin

Table 1. iuMRI and postnatal MRI comparison of 9 AIC syndrome cases. A-P anterior-posterior gradient of severity; BG basal ganglia; C complete; CC corpus callosum; CPF cyst of posterior fossa; IT interhemispheric cysts; L left; N normal; mo months; P partial; R right; wks weeks

The comparison between the diagnostic performance of iuMRI and postnatal MRI revealed (details in table 1): CC agenesis was complete in 5 cases and partial in 4 cases, both assessed by iuMRI and postnatal MRI studies. Cortical gyration anomalies were detected in all the patients both in prenatal and postnatal studies, particularly 8/9 had a severe diffuse dysgyria at iuMRI, which evolved in a polymicrogyric pattern in the postnatal period, and 1 case showed a focal polymicrogyria. Concerning the severity of the cortical malformations, an anterior-posterior gradient was detected prenatally in 5 cases and postnatally in 7 cases (Figure 1). A gross asymmetry in the distribution of the dysplastic cortex and cerebral hemispheres volume was detected in 6 cases at iuMRI and in 5 postnatally. Ventriculomegaly and ventricular dysmorphism were present in 6/9 cases during prenatal evaluation and 7/9 cases postnatally. Intracranial cysts were detected in 8/9 in prenatal studies and in 9/9 patients on postnatal MRI. During fetal period all intracranial cysts had a CSF-like signal, as type 2d according to Barkovich classification (Barkovich, Simon et al. 2001), while postnatal studies allowed to differentiate 2d from 2b cysts, hyperintense to CSF on T1-weighted images (details for number, localization type and pattern in table 1) (Figure 2). One-hundred percent of the patients showed nodular heterotopias, evident in both the prenatal and postnatal MRI; the agreement was complete for number and localization and partially complete (7/9 cases) regarding pattern (details in table 1).

Posterior fossa abnormalities were displayed in 5 cases prenatally and in 7 cases postnatally; basal ganglia dysmorphism was detectable in 2 cases at iuMRI and in 7 at postnatal imaging. Five out of nine patients had ocular coloboma at iuMRI and 7/9 patients at postnatal MRI. Optic nerves, chiasm and pituitary gland were recognizable in all the iuMRI exams, but their possible abnormality (i.e. thinning) could not be assessed

due to spatial resolution limit. On the contrary, postnatal MR imaging revealed normal pituitary gland morphology in all cases, but thinning of optic nerves and chiasm in 7/9 cases.

Concerning the diagnostic performance of iuMRI vs post-natal MRI to detect optic coloboma, statistical analysis revealed a good sensitivity (77%) but lower specificity (60%), with significant effect in predicting the postnatal confirmation of coloboma (positive predictive value 83%). Negative predictive value of iuMRI for coloboma was 50%.

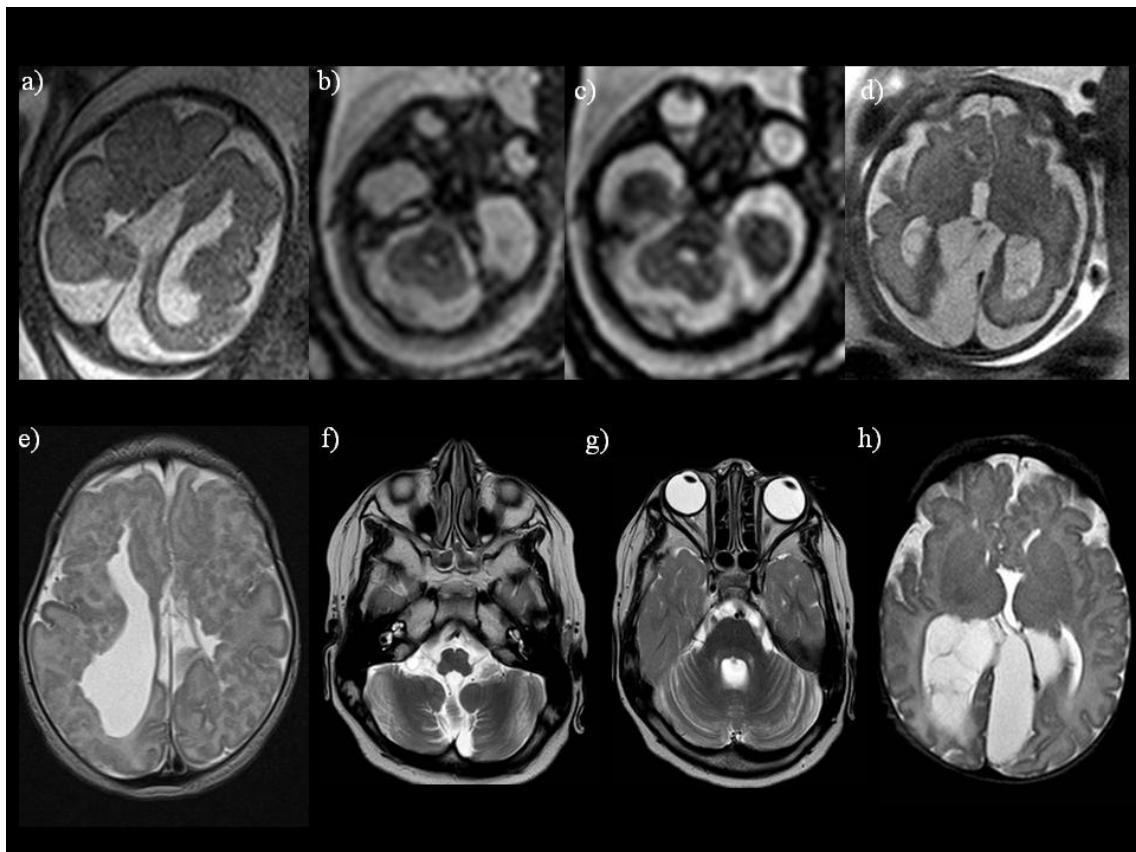


Figure 1. First column (a,e): Patient 1. in postnatal axial FSE T2weighted image(e) the presence of the interhemispheric cyst is confirmed but it shows a different signal (2b type, according to the Barkovich classification) respect to iuMRI axial ssFSE T2 weighted images (a) performed at 28 weeks of g.a.

Second column (b-f): Patient 7. postnatal axial FSE T2 weighted image(f) shows the presence of a posterior fossa cyst caudally to the right ponto-cerebellar angle, that was no present/visible on the iuMRI axial ssFSE T2 weighted image (b) performed at 21 weeks of g.a.;

Third column (c-g): Patient 7. post-natal axial FSE T2 weighted image (g) demonstrates a small posterior defect of the optic papilla on the right due to the presence of coloboma of the optic nerve head; this findings was not appreciable on the iuMRI axial ssFSE T2 weighted image (c)

Fourth column (d,h): Patient 2. postnatal axial FSE T2 weighted image (h) shows a dismorphic appearance of basal ganglia without a clear definition of the anterior limb of internal capsula, not appreciable on the iuMRI axial ssFSE T2 weighted image (d) at 29 weeks of g.a.

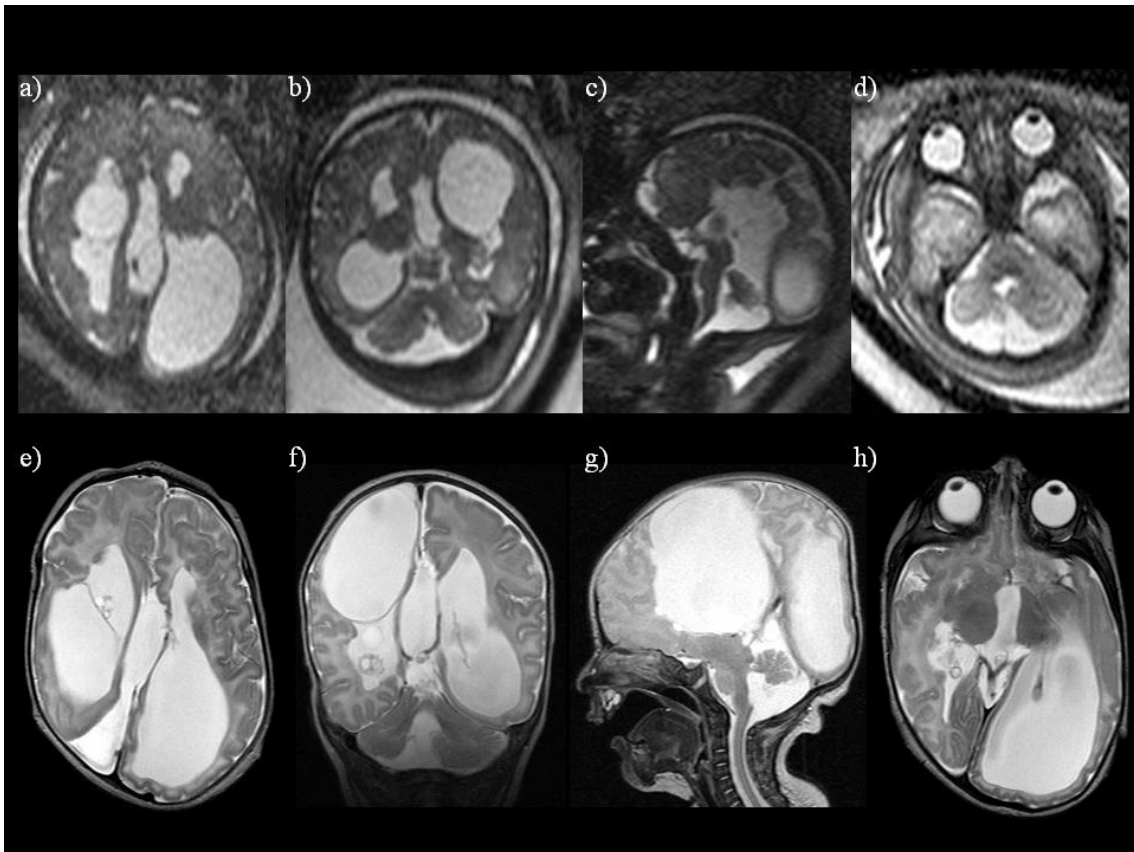


Figure 2: Case 8; 34 gw, AS. ssFSE T2 weighted iuMRI and postnatal MRI images: axial and coronal (a,b, e,f) images show dismorphism and asimmetrically enlargement of the lateral ventricle mostly due to intraventricular cysts; a diffuse dysgyria is also evident both on prenatal and postnatal studies; on sagittal image (c, g) complete corpus callosum agenesis is evident; more over the superior edge of cerebellar vermis is distorted, probably due to the presence of a cyst in the posterior fossa. In figures d and h, axial

ssFSE T2 weighted image shows a bilateral optic nerve coloboma detected in both prenatal and postnatal studies; also note the cerebellar asymmetry.

AIC fetal iuMRIs versus similar fetal conditions (AIC mimickers)

From a database of 4015 iuMR imaging reports, 122 examinations with the concomitant presence of CC dysgenesis-aggenesis and cortical gyration anomalies were selected (AIC mimickers). After imaging revision, 48/122 cases satisfied the neuroradiological inclusion/exclusion criteria; however only for 12 fetuses clinical and neuroradiological postnatal data were available and they were considered in the study as AIC mimickers group. Among them, one patient was affected by tubulinopathy (mutation in TUB1A gene) and one by a chromosomopathy (chr. 7p deletion), while no definite diagnosis was available for the other 10 patients, but medical history or neuroradiological follow up allowed to definitely exclude AIC diagnosis.

The results from the comparison between AIC and AIC mimickers iuMRIs (21 fetuses) are summarized in table 2.

	G A	sex	CC dysgenesises	Ventric anomalies	asymmetry	Cysts/papilloma of choroid plexus	heterotopias	gyration anomalies	other	MRI f-up	clinical f-up
Case 1	25	F	C	Y	N	N	bilat, >4, single	sawtooth irregularity mesial bilat, no gradient	N	confirm	no-AIC
Case 2	22	M	C	Y	N	N	bilat, 2, single	sawtooth irregularity focal, no gradient	N	confirm	no-AIC
Case 3	33	M	C	Y	Y	N	monolat, 1, single	polymicro mesial unilateral, no gradient	N	confirm	no-AIC
Case 4	26	M	C	Y	Y	N	irregularity of ependimal edge	polymicro, focal unilat, A-P	N	confim	no-AIC
Case 5	27	M	C	Y	N	N	occipital horn, atri, >4, single	polymicro, mesial unilateral, A-P	N	confim	chrom 7p del
Case 6	26	F	P	Y	Y	N	monolat, >4, confluent	polymicro focal unilateral, A-P	doubt coloboma	posterium fossa cyst	AIC
Case 7	27	F	P	Y	Y	IT 2d, R, >4 multiloc	N	anomalus sulcus focal, next to cyst	N	confirm	no-AIC
Case 8	23	M	P	Y	N	IT, 2d, 1, uniloc	N	focal sawtooth irregularity,next to cyst	N	confirm	no-AIC
Case 9	34	M	P	Y	Y	IT, 2d, 1, uniloc	N	focal polymicro, next to cysts, A-P	N	confirm	no-AIC
Case 10	23	F	P	Y	Y	IT, 2d, 1, uniloc	N	focal polymicro, next to cysts	BG dysmorphisms	confirm	no-AIC
Case 11	35	M	C	Y	Y	IT, 2d, 1, uniloc	N	focal polymicro, next to cysts	N	confirm	no-AIC
Case 12	34	F	C	Y	Y	IV uniloc	bilat, >4, confluent	dysgiria, diffuse bilat, A-P	BG and cerebellar dysmorphisms, coloboma	confirm	AIC
Case 13	31	F	C	Y	Y	IT, 2d, 1, uniloc	bilat, <4, single	dysgiria, diffuse, no gradient	N	confirm	no-AIC
Case 14	30	M	D	Y	Y	PV 1, uniloc	posterior, 1, single	dysgiria diffuse, no gradient	BG dysmorphisms	confirm	TUBA1 A mutation
Case 15	28	F	C	Y	N	IT, 2d, 1, uniloc	bilat, >4, mixed	dysgiria diffuse, no gradient	colobomamic rophtalmos, butterfly sign	confirm	AIC
Pt16	29	F	C	Y	N	IT, 2d, multiple, multiloc, choroid	frontal, occipital, <4, single	dysgiria diffuse, no gradient	coloboma bilat	confirm	AIC
Case 17	30	F	P	Y	N	IT, 2d, 2, uniloc, choroid	mono, 1, single	occipital bilat dysgiria P-A	doubt R coloboma	confirm	AIC
Case 18	31	F	P	Y	Y	CPF cyst,1, uniloc	mono, >4, single	dysgiria, bilat, A-P	cyst, cerebellar asymmetry	coloboma bilat	AIC
Case 19	33	F	C	Y	Y	IT, 2d, 1, uniloc	bilat,>4, confluent	dysgiria polymicro like, bilat, A-P	coloboma bilat	confirm	AIC
Case 20	28	F	C	Y	Y	IT 1, 2d, uniloc	bilat, >4, mixed	dysgiria diffuse, A-P	GB dysmorphisms	coloboma	AIC
Case 21	21	F	P	Y	Y	IT, 2d, 1, uniloc, choroid	mono, >4, confluent	dysgiria, diffuse, A-P	cerebellar malformation	coloboma	AIC

Tabela 2. iuMRI of AIC and non-AIC cases. A-P anterior-posterior gradient of severity; BG basal ganglia; C complete; CC corpus callosum; CPF cyst of posterior fossa; F female; IT interhemispheric cysts; L left; N normal; M male; mo months; P partial; R right; wks weeks; Y yes

The most relevant data regarded the cortical malformation category; while in the AIC group the most frequent pattern of cortical malformation was a diffuse bilateral cortical dysgyria (8/9) and only 1 case of focal polymicrogyria, in the AIC mimickers group prevailed a focal distribution (10/12) of the cortical anomaly; in particular in this latter group, 5/10 were associated and localized adjacent to interhemispheric cyst, mostly appearing as a “saw tooth” irregularity of the cortical rim or as abnormal invaginated sulcus (4/10) in fetuses at earlier GA and as focal polymicrogyria (6/10) in older ones (Figure 3). In this group only 2/12 fetuses had diffuse cortical rim irregularity, consisting in one case in tubulinopathy diagnosis, the other was under investigation but clinical data excluded AIC.

Considering the heterotopic nodules category, they were present in all the AIC fetuses and they appeared mostly multiple (>4 in 7/9) and confluent (6/9), while in the AIC mimickers fetuses they were present in 7/12 and appeared mostly sporadic (<4 in 5/7) and single (5/7). No significant differences in terms of callosal anomaly, ventricular abnormality, hemisphere asymmetry or cysts number and distribution were noted.

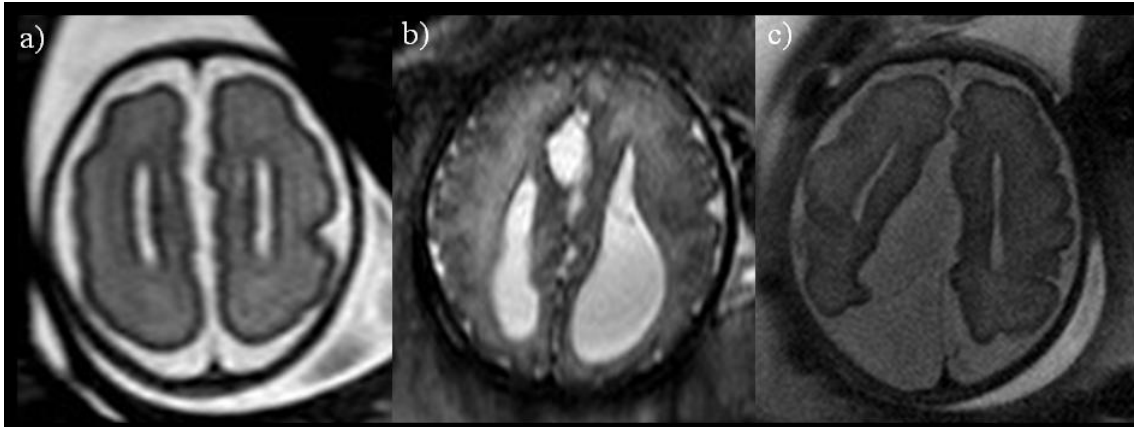


Figure 3: pattern of cortical malformation : AS vs non AS

- a) Case 5, 27 weeks of g.a, non AS; SSFSE T2w axial image shows unilateral frontal mesial polymicrogyria and irregularity of the adjacent ependymal edge, suspected for subependymal heterotopic nodules. There is no interhemispheric cyst in this case.
- b) Case 9, 34 weeks of g.a., non AS; SSFSE T2w axial image shows a focal area of polymicrogyria along the cortical rim just next to the interhemispheric cyst
- c) Case 16, 29 weeks of g.a. AS; SSFSE T2w axial image shows diffuse anomalous appearance of the cortical rim, called dysgyria, with multiple interhemispheric cysts and diffuse nodularities along the ependymal edge as for heterotopias .

Statistical Analysis Results

A statistically significant difference in the two groups of AIC and AIC mimickers fetuses were detected regarding: sex ($p = .005$), nodular heterotopias ($p = .045$), cortical gyration abnormalities ($p = .004$), posterior fossa abnormalities ($p = .021$) and optic nerve coloboma ($p = .002$).

Binary logistic regression analysis revealed five independent variables with significant effect on the predictive model: sex ($p = .002$), diffuse cortical gyration abnormalities ($p = .004$), cysts or choroid plexus papilloma ($p = .031$), heterotopias ($p = .027$), posterior fossa abnormalities ($p = .010$) and optic nerve coloboma ($p = .001$). Considering the variables with the most significant effect, namely sex, cortical gyration abnormalities

and coloboma, the model correctly predicted AIC diagnosis in 95.2% of the cases (sensitivity of 100%, specificity of 91.7%). ROC curves were generated using SPSS and the predicted probability obtained from the regression model. The areas under the curve for sex, cortical gyration abnormalities and optic nerve coloboma were respectively 0.83 (0.64-1.00), 0.84 (0.64-1.00), 0.83 (0.63-1.00); the AUC of the combination of those three variables was 0.98 (0.94-1.00).

4.4 Discussion

Our study underlines the key role of iuMRI in the pre-natal suspicion of AIC, being able to detect the hallmarks of AIC even at early gestational age, also highlighting some peculiar findings characteristic of the syndrome.

To date, in the few sporadic fetal AIC cases reported in literature, iuMRI illustrated only the main features of the syndrome, such as callosal dysgenesis-agenesis, ventriculomegaly and cortical malformations (Hergan, Atar et al. 2013); in few case reports, cysts and nodular heterotopias were also described (Columbano, Luedemann et al. 2009; Vinurel, Van Nieuwenhuyse et al. 2014; Gacio and Lescano 2017). In our 9 AIC cases, iuMRI demonstrated even at early fetal life the main anomalies described in the syndrome (callosal agenesis-dysgenesis, cysts, gyration anomalies, nodular heterotopias) and moreover highlighted other less frequently reported findings: ocular coloboma, posterior fossa abnormalities, and basal ganglia dysmorphisms. Although minor callosal anomaly or normal CC have been described in some AIC cases (Iturralde, Meyerle et al. 2006; Grosso, Lasorella et al. 2007), all our fetal cases presented with partial or complete CC agenesis. Cortical malformation was present in all 9 AIC cases, with a perfect agreement between iuMRI and post-natal MRI about the

severity and distribution; in particular iuMRI detected a peculiar pattern characterized by a diffuse and severe cortical dysgyria in 8/9, with multiple anomalous invaginated sulci then appearing as polymicrogyria at postnatal MRI; only 1 fetus had a focal polymicrogyria even at iuMRI. Moreover, as previously described by Hopkins et al. (Hopkins, Sutton et al. 2008; Cabrera, Winn et al. 2011), an anterior-posterior gradient in term of severity was predominantly observed (7/9 cases), even at iuMR imaging, and a gross asymmetry in the distribution of the dysplastic cortex and cerebral hemispheres volume was also confirmed (6/9 at iuMRI vs 5/9 postnatal MRI). Observations about cortical involvement at iuMR imaging suggest that in AIC syndrome global brain mantle derangement occurs precociously. Moreover, in all the 9 cases, cortical gyration anomalies were associated with multiple nodular heterotopias. These results confirm the important role of iuMRI in the evaluation of cortical development malformation (Glenn, Cuneo et al. 2012), even in those examinations performed at early gestational age (before 24 weeks of GA as observed in our cases). Postnatal imaging studies confirmed the prenatal data revealing some additional findings particularly regarding cysts, ocular coloboma, posterior fossa anomalies and basal ganglia dysmorphisms. In some patients with prenatal detection of arachnoid cysts, postnatal imaging revealed an increase in number; moreover, in one AIC case, postnatal imaging revealed a posterior fossa cyst not evident in the fetus, suggesting that cysts could occur and grow postnatally. Concerning cysts type, the different technique of iuMRI with respect to postnatal MRI might explain the discrepancies about the different type of cysts (2d vs 2b) respectively detected in prenatal and postnatal imaging. In the AIC group, our study confirmed the possible association with posterior fossa abnormalities, as cerebellar dysplasia or asymmetry and vermian hypoplasia, 5/7 well depicted at iuMRI. We also reported DMJ

malformation (2/9) and basal ganglia dysmorphism in AIC fetuses (7/9), the former detected by both iuMRI and post-natal imaging, the latter identified only in 2/7 cases by iuMRI, probably due to the low spatial resolution of the technique for the small dimension of these structures in fetuses. Concerning ocular coloboma, our work confirmed the iuMRI ability to detect ocular globe anomalies (Righini, Avagliano et al. 2008) , even if the optic nerves and chiasm are difficult to assess.

The second part of our study confirmed that a diffuse dysgyric cortical pattern with a frontal predominance is typical in AIC and this is mostly associated with multiple and confluent heterotopic nodules, while interhemispheric cysts are not so specific; nevertheless in literature few AIC cases with a milder neuroradiological phenotype are described (Lee, Kim et al. 2004; Grosso, Lasorella et al. 2007), expanding the spectrum. Moreover, in presence of interhemispheric cyst, none of our AIC fetuses demonstrated focal gyration anomaly localized unilateral to the cyst, as it happened in those AIC mimickers cases with callosal anomaly, interhemispheric cyst and cortical malformation; we can speculate that in these latter, the presence of a midline cyst can interfere at some level with neurons migration resulting in a focal cortical malformation, while in AIC syndrome the cysts and the diffuse cortical malformation are part of the same spectrum of brain development derangement (Wieck, Leventer et al. 2005); (Fuchs, Moutard et al. 2008). Comparing the two groups (AIC and AIC mimickers) a statistically significance difference were detected regarding sex, nodular heterotopias, cortical gyration abnormalities, posterior fossa abnormalities and optic nerve coloboma. Thus, according to our results, AIC can be suspected in fetal period when diffuse cortical gyration abnormalities and callosal malformation associated with cysts or choroid plexus papilloma, heterotopias, posterior fossa abnormalities and ocular

coloboma are present in female fetuses. In particular the association of female sex, diffuse cortical gyration abnormalities and ocular coloboma is highly predictive for AIC syndrome.

Considering the differential diagnosis, congenital infections, particularly toxoplasmosis, CMV or rubella fetopathy, were historically the first suggested (Willis and Rosman 1980), but also rare congenital syndromes were reported: *Oculocerebrocutaneous syndrome* (Moog, Jones et al. 2005), *Amniotic band syndrome* (Hashemi, Traboulsi et al. 1991), *Goltz syndrome-focal dermal dysplasia* (Van den Veyver 2002). In our sample one interesting differential diagnosis regarded tubulinopathy (TUBA1A mutation). Mutation in tubulin genes are frequently the responsible of a global subversion of multiple brain structures (commissures, cortical sulcation and gyration, posterior fossa and basal ganglia) which can resemble the complex brain malformation described in our AIC patients (Fallet-Bianco, Laquerriere et al. 2014).

Although our cohort of AIC fetuses was the larger described to date, statistical analysis has limits because of the small sample size; further studies on larger cohorts are needed to corroborate our observations.

In conclusion, our study revealed the diagnostic power of iuMRI in the prenatal diagnosis of AIC, even at early gestational age, with important implications for parental counseling and early neonatal management. Moreover we confirmed the key role of iuMRI in the diagnostic work-up of fetuses with ultrasound evidence of ventriculomegaly and CC agenesis-dysgenesis: in female fetuses with associated diffuse and severe cortical dysgyria and ocular coloboma the suspicion of AIC should promptly rise.

5. A 3D CRANIOFACIAL MORPHOMETRIC ANALYSIS IN AICARDI PATIENTS

5.1 Introduction

Aicardi syndrome (AIC) is a rare congenital condition, described for the first time in his classical triad, corpus callosum agenesis, chorioretinal lacunae and epileptic spasms, by Jan Aicardi in 1965 (Aicardi 1965). The increasing of the cohorts and case report described in literature, allowed a better definition of the phenotype: chorioretinal lacunae, considered pathognomonic, are round, depigmented areas of the retinal pigment epithelium underling choroid with variably dense pigmentation at their borders, frequently associated with other ocular abnormalities such as coloboma, microphthalmos and cataracts. Infantile spasms are the most characteristic type of seizures, but also other types of seizures, focal, tonic, generalized tonic-clonic, myoclonic, atonic seizures and status epilepticus, are reported (Glasmacher, Sutton et al. 2007). Corpus callosum agenesis is never an isolated findings, but constantly associated with a complex brain malformation consisting of polymicrogyria, interhemispheric and/or choroid plexus cysts, nodular heterotopias, and possible posterior fossa abnormalities (Hopkins, Sutton et al. 2008). Extensive genetic studies carried on so far by several international research groups, particularly skewed X-inactivation analysis (Eble, Sutton et al. 2009), candidate genes studies (Van den Veyver, Panichkul et al. 2004), methylation array (Piras, Mills et al. 2017), exome and genome sequencing, failed to solve the mystery of AIC etiology (Wang, Sutton et al. 2009; Lund, Striano et al. 2016). Considering the absence of a genetic hallmark, AIC diagnosis is still a challenge because diagnostic criteria are based only on clinical and radiological features (Aicardi 2005; Sutton, Hopkins et al. 2005). Examining 40 girls with AIC, Sutton and colleagues noticed consistent facial features, prominent premaxilla, upturned nasal tip, decreased angle of the nasal bridge, and

sparse lateral eyebrows, in over half of the cases (Sutton, Hopkins et al. 2005). Several studies have wide delineated that different syndromes are characterized by a typical facial phenotype that can drive clinicians toward the diagnosis (Pucciarelli, Bertoli et al. 2017; Pucciarelli, Bertoli et al. 2017; Dolci, Pucciarelli et al. 2018); considering these studies and taking into account the first Sutton observations, aim of the study was to perform 3D stereo photogrammetric assessment in a cohort of Italian cases with AIC in order to identify a specific AIC facial phenotype which can help the clinicians in the diagnosis.

5.2 Material and Methods

Recruitment of subjects and 3D acquisition

Exclusively patients who satisfied Aicardi Syndrome classical criteria or Sutton Modified Criteria were included in the study (Sutton, Hopkins et al. 2005). An informed consent was signed by parents or tutors of everyone, in accordance with the Declaration of Helsinki. The experimental project was approved by the local university ethical committee (26.03.14; n° 92/14).

Patients underwent 3D facial photographs through stereophotogrammetry (VECTRA-3D®: Canfield Scientific, Inc., Fairfield, NJ). The instrument comprises three pods, each with one high resolution black-and-white camera and one color camera; the cameras image the facial soft tissues from different points of view with a single shot lasting less than 2 ms, and a digital 3D model is provided. The scanning procedure is minimally disturbing, not invasive and without biological risks. A set of 20 landmarks were labeled on each face before the acquisition stage (Ferrario and Sforza 2007). Each

subject was acquired in rest position, with close or partially open mouth compatibly with her collaboration.

Each 3D facial model was elaborated by VAM® software (Canfield Scientific, Inc., Fairfield, NJ, USA). In total, 15 linear measurements and 11 angles were automatically calculated through Faces software (developed by our laboratory specifically for the extraction of metrical parameters from coordinates), after the selection of 50 facial landmarks defined according to Farkas (Farkas LG, Anthropometry of the head and face, Raven Press, New York (USA), 1994) (Tables 1, 2,3). For each patient, a group of control girls/women of the same age and ethnicity was selected from the database of the laboratory and underwent the same analysis (Table 2). Exclusion criteria were facial deformities, previous orthodontic therapy, neurological impairments and signs of recent or previous traumatic injuries affecting face.

Statistical analysis

The comparison between patients and the corresponding group of healthy subjects was performed by calculating z-scores:

$$z\text{-score} = (x - \mu) / \sigma$$

where x is the value of each measurement calculated in the patient, and μ (mean) and σ are mean and standard deviation of the same measurement computed on the healthy subjects, respectively. The smaller the z-score, the closer the patient values to the reference ones.

Possible statistically significant differences in z-score for each measurement between patients and control subjects were assessed through Mann-Whitney test ($p < 0.01$).

5.3 Results

In total, 859 subjects were analyzed: 9 females with a definite Aicardi Syndrome and 850 control subjects. The control subjects were selected to be paired for age, sex, ethnicity to the AIC patients. Each reference group comprised a minimum of 29 subjects.

Nine female Caucasoid patients aged between 7 and 32 years (mean age: 18.6 ± 9.1 years) affected by AIC were recruited for the study. Seven patients presented classical chorioretinal lacunae, in one case ophthalmological examination was normal, in one patient revealed right coloboma and lacunae only at the first examination soon after birth.

Descriptive statistics of the z-scores for each measurement are shown in Table 3. Among linear distances, four measurements showed a statistically significant difference between all the patients and healthy subjects: in detail, patients showed lower superior, middle and inferior facial depths (mean z-scores of -1.2, -2.2 and -2.4, respectively) and wider nasal breadth (mean z-score: 1.6, Fig. 1).

No statistically significant differences were found in angular measurements between patients and healthy subjects.

			Definition
Linear distances	Horizon tal	t_r, t_l	Middle facial width
		go_r, go_l	Lower facial width
		$zy_r - zy_l$	Facial width
		$al_r - al_l$	Nasal width
	Vertical	$tr - n$	Forehead length
		$n - sn$	Nasal height
		$n - prn$	Length of the nasal bridge
		$os_r - or_r$	Right orbital height
		$os_l - or_l$	Left orbital height
	Sagittal	$n - t_m$	Upper facial depth
		$sn - t_m$	Midfacial depth
		$pg - t_m$	Lower facial depth
		$pg - go_m$	Mandibular body length
		$t_m - go_m$	Mandibular ramus length
		$prn - sn$	Nasal protrusion
	Angles	Horizon tal	$t_r - n - t_l$
$t_r - prn - t_l$			Middle facial convexity
$t_r - pg - t_l$			Lower facial convexity
$go_r - pg - go_l$			Mandibular convexity
$t_r - go_r - pg$			Right gonial angle
$t_l - go_l - pg$			Left gonial angle
$al_r - prn - al_l$			Alar slope angle
Frontal		$os_r - or_r$ vs TH	Right inclination of the orbital height versus the true horizontal plane
		$os_l - or_l$ vs TH	Left inclination of the orbital height versus the true horizontal plane
Sagittal		$sn - n - prn$	Nasal convexity
		$n - prn - sn$	Nasal tip angle
		$os_r - or_r - t_r$	Right angle between orbital height and or-t distance
		$os_l - or_l - t_l$	Left angle between orbital height and or-t distance

Table 1: list of linear distances and angles analyzed in the present study

	Age	N° control subjects
Patient 1	32	38
Patient 2	21	205
Patient 3	12	48
Patient 4	9	45
Patient 5	28	205
Patient 6	16	30
Patient 7	14	45
Patient 8	7	29
Patient 9	28	205

Table 2: number of control subjects selected for each patient

			Definition	z-score	SD	p
Linear distances	Horizontal	$t_r - t_l$	Middle facial width	0.0	1.7	0.671
		$go_r - go_l$	Lower facial width	1.1	1.4	0.203
		$zy_r - zy_l$	Facial width	0.6	1.4	0.671
		$al_r - al_l$	Nasal width	1.6	0.7	<0.001
	Vertical	$tr - n$	Forehead length	-0.9	1.3	0.034
		$n - sn$	Nasal height	0.0	1.0	0.382
		$n - prn$	Length of the nasal bridge	-0.1	0.9	0.671
		$os_r - or_r$	Right orbital height	-0.6	0.9	0.202
		$os_l - or_l$	Left orbital height	0.0	0.8	0.671
	Sagittal	$n - t_m$	Upper facial depth	-1.2	0.9	0.003
		$sn - t_m$	Midfacial depth	-2.2	1.3	<0.001
		$pg - t_m$	Lower facial depth	-2.4	1.7	<0.001
		$pg - go_m$	Mandibular body length	0.4	0.9	0.203
		$t_m - go_m$	Mandibular ramus length	-1.7	1.1	<0.001
		$prn - sn$	Nasal protrusion	0.2	1.0	0.383
	Angles	Horizontal	$t_r - n - t_l$	Upper facial convexity	1.3	1.7
$t_r - prn - t_l$			Middle facial convexity	1.9	2.3	<0.001
$t_r - pg - t_l$			Lower facial convexity	2.6	2.8	0.034
$go_r - pg - go_l$			Mandibular convexity	0.1	1.0	0.203
$t_r - go_r - pg$			Right gonial angle	-1.3	2.0	0.203
$t_l - go_l - pg$			Left gonial angle	-1.0	1.6	0.034
$al_r - prn - al_l$			Alar slope angle	0.4	0.4	0.034
Sagittal		$sn - n - prn$	Nasal convexity	0.3	0.8	0.203
		$n - prn - sn$	Nasal tip angle	-0.3	1.1	0.383
		$os_r - or_r - t_r$	Right angle between orbital height and or-t distance	0.0	1.1	0.203
		$os_l - or_l - t_l$	Left angle between orbital height and or-t distance	0.4	1.3	1.000

Table 3: descriptive statistics of the z-scores and relevant p-values (Student's t-test) for each measurement

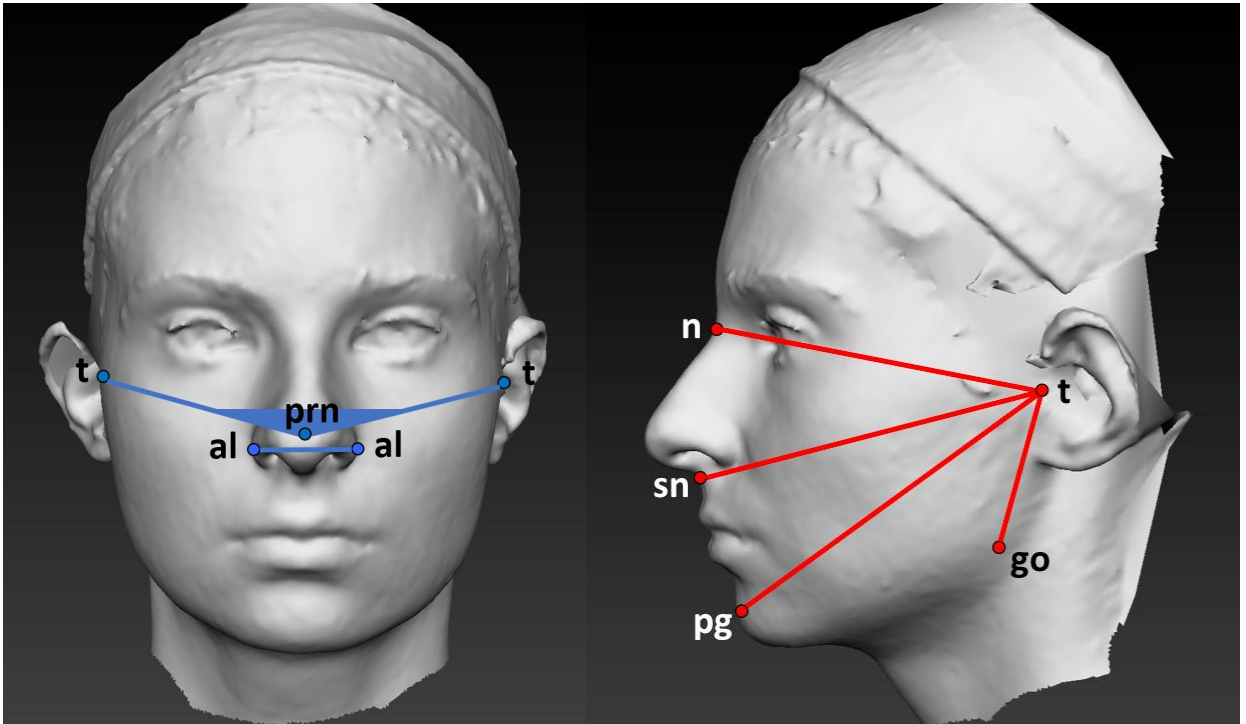


Fig. 1: measurements showing statistically significant differences between patients and healthy subjects: red lines show values that are smaller in patients than in healthy subjects, blue lines show values that are longer in patients than in healthy subjects

5.4 Discussion

Up to date Aicardi syndrome diagnosis remain clinical, based only on clinical and neuroradiological features. During years, patients without one out of the three classical criteria, without callosal agenesis (Iturralde, Meyerle et al. 2006) or spasms (Prats Vinas, Martinez Gonzalez et al. 2005) or chorioretinal lacunae, who received the diagnosis according the Modified Sutton Criteria (Sutton, Hopkins et al. 2005), and atypical cases (Lee, Kim et al. 2004) are growing up, making a challenge to perform a definite diagnosis in some cases. In absence of a genetic or biological markers, became needful find more definite parameters in order to reinforce the diagnosis. Despite the clinical and neuroradiological variability of the Syndrome, as previously demonstrated

in other studies in which a 3D morphometric analysis had proved itself powerful in definite specific facial features of definite syndromes (Pucciarelli, Bertoli et al. 2017; Pucciarelli, Bertoli et al. 2017; Dolci, Pucciarelli et al. 2018), our pilot study allowed us to identify common facial features in Aicardi Syndrome. In detail, statistical analysis revealed lower superior, middle and inferior facial depths and wider nasal breadth, presented in all the nine Aicardi cases scanned, with significant differences compared the age-, sex-, and ethnicity-matched control evaluated. The lacks of the etiology of the disease doesn't help in the understanding of these observations; can not be excluded that steroids therapies, such as Adrenocorticotrophic hormone (ACTH) usually used for epilepsy, might be influence the facial features observed.

Not differences between our patients and control were detected on prn-sn-ls (pronasal-subnasal-labral angle), and prn-n-sn (pronasal-nasion-subnasal angle-nasal convexity), so the prominent premaxilla, upturned nasal tip, decreased angle of the nasal bridge, and sparse lateral eyebrows detected in the previous study by Sutton and colleagues (Sutton, Hopkins et al. 2005) cannot be confirmed with our results.

The detection of these similar facial measurements in all the AIC cases, if it will be confirm in larger cohorts of AIC patients, it had significant applications in clinical practice. From one side, it will help clinicians in performing a definite AIC diagnosis, in classified atypical or doubt cases, moreover it will helps researchers in performing genetic analysis in more selected and homogeneous cases, opening the way to new potential clarification of the etiology of the syndrome.

Considering the encouraging results obtained from this first pilot study, 3D morphometric analysis has been scheduled on more cases with AIC, in order to amplify

the cohort in study, increase the power of the statistical analysis and so confirm our first results.

**6. HYPO-AGENESIS OF CORPUS CALLOSUM AND AICARDI SYNDROME:
FOUR CASES WITH DEBATED DIAGNOSIS**

6.1 Introduction and cases

Aicardi Syndrome (AIC) is a rare congenital syndrome characterized historically by a classic triad of total or partial agenesis of the corpus callosum, distinctive chorioretinal lacunae, and epileptic spasms. The development of refined brain imaging techniques led to expand the neuro-radiological features of the syndrome (Hopkins, Sutton et al. 2008). Therefore, Aicardi emphasized the relevance of other features, such as periventricular heterotopias, choroid plexus cysts, and coloboma, and, in 2005, Sutton and colleague proposed Modified Diagnostic Criteria: the concomitant presence of either all three of the Classical Triad or the existence of two of the Classical Triad plus at least two other Major or Supporting Features as strongly suggestive of the diagnosis of Aicardi Syndrome (Aicardi 2005; Sutton, Hopkins et al. 2005). Here we report four patients in whom the diagnosis was debated.

Case 1

This 21 months old female is the fourth child of Senegalese, 26 (mother) and 53 years old parents. During pregnancy, ultrasound imaging detected a complex brain malformation delineated on prenatal brain magnetic resonance imaging (MRI), characterized by agenesis of the corpus callosum, diffusely abnormal cortical sulcation, nodular heterotopias, and multiloculated interhemispheric cysts. She was born at term by vaginal delivery; soon after birth, several episodes of eyelid myoclonus, generalized hypertonicity and a clusters of spasms were noticed. Electroencephalogram (EEG) showed a burst-suppression pattern and clusters of high voltage slow waves prominent in

left frontal region coincident with adduction of the right arm. MRI performed at 7 days of life confirmed the severe brain malformation. Moreover, basal ganglia were asymmetrically dysmorphic, mainly on the left, and she had a diencephalic-mesencephalic junction dysplasia (DMJD) (Figure 1-a,b,c). Ophthalmological evaluation detected chorioretinal lacunae, and left microphthalmos (Figure 1-m). The clinical picture fulfilled all classic criteria for the diagnosis of AIC. Currently neurological examination shows diffuse hypotonia, severe neurodevelopmental delay with no postural acquisition, and visual impairment, and she experiences daily clusters of spasms despite multiple antiepileptic drugs. CGH-array and a gene panel with 180 genes involved in epilepsy and cortical malformations was unrevealing.

Case 2

The female was the first child of healthy of 36 (mother) and 37 years old parents of Italian origin. She was born at term after an uneventful pregnancy; TORCH investigations were negative. At 8 months, parents noticed several episodes of sudden head and upper limb flexion, in clusters. EEG revealed poor background activity, multifocal epileptiform discharges (EDs), mainly on posterior regions, and clusters of high voltage slow waves with four-limb adduction. Partial seizures control was established with vigabatrin. Brain MRI at 11 months of age revealed corpus callosal hypoplasia, a bulky posterior fossa cyst, choroid plexus cysts, and periventricular heterotopias. The cerebellum was severely dysmorphic: the right hemisphere was dysplastic and fused with vermis; no abnormalities were detected in the cortical gyri (Figure 1-d,e,f). Ophthalmological examination revealed chorioretinal lacunae (Figure 1-n). Because of the chorioretinal lacunae, epileptic spasms, nodular heterotopias, choroid plexus and arachnoid cysts, a diagnosis of AIC was confirmed. At 3 years of

age, her neurological examination is characterized by diffuse hypotonia, and intellectual disability, although she can walk with aid. She experiences spasms, and focal seizures, and multiple seizures each week.

Case 3

This girl, the third child of 25 (mother) and 37 years old parents of Italian origin, was born at term by cesarean delivery after an unremarkable pregnancy; TORCH serologies were normal. At 4 months of age, several clusters of flexor spasms were noticed. Interictal EEG revealed fronto-temporal EDs. She was treated with ACTH and vigabatrin leading to transitory control of her seizures. A month later, seizures recurred as spasms, drop attacks, and focal seizures. MRIs performed at 4 months, 6 months, and 1 year of age showing a thinned corpus callosum and dysplasia involving the frontal gyrus and parieto-occipital fissure of the right hemisphere (Figure 1-g,h,i). Ophthalmological evaluation reviewed with two experts confirmed the presence of chorioretinal lacunae (Figure 1-o). The girl had two cutaneous abdominal and back nuchal angiomas. At 2.5 years, she started walking without support but with an ataxic wide-based gait. She is intellectually disabled. Currently, neurological examination revealed diffuse hypotonia, slight pyramidal signs on left side of the body, and clumsiness in fine motor tasks. She has multiple daily spasms. Because of these spasms, chorioretinal lacunae, and brain malformation, a diagnosis of AIC was considered.

Case 4

This 4 years old girl is the third child of unrelated of 38 (mother) and 32 years old parents of Italian origin. She was born at term by cesarean section after an unremarkable pregnancy and delivery. At five months of age, multiple episodes of eye deviation, vomiting, and staring were noticed. EEG revealed the presence of high voltage, slow

waves from the left hemisphere. Seizures were partially controlled with valproic acid. MRI, performed at 1 and 3 years of age, revealed partial corpus callosal agenesis, interhemispheric cysts, diffusely simplified patterns of cortical gyration, polymicrogyria more evident on the right hemisphere, bilateral frontal cortical dysplasia, periventricular nodular heterotopias, and basal ganglia dysmorphism (Figure 1-j,k,l). The ocular fundi were normal. At 8 months, she started to sustain a sitting position, and she is able to walk without support after 23 months of age. First words were achieved at 7 months, and currently she can say and understand simple sentences. Neurological examination at 4 years revealed left hemiplegia and gait ataxia. On monotherapy, she has been free from seizures after two years of age.



Figure 1.

Patient 1. MR performed at axial (a,b) and coronal (c) T2-weighted (w) TSE images showing diffuse dysplastic cortex resembling polymicrogyria (PMG) with antero-posterior gradient (a,b); adhesio intertalamica (b, asterix); multiloculated interhemispheric cysts on the left side (black

arrow, a); basal ganglia dysmorphism, more on the left side (white arrow, b) with anterior arm of the internal capsule non recognizable. Diencephalic-mesencephalic junction dysplasia, with the “butterfly sign” (c) ; heterotopic nodules (multiple white arrows,c); complete agenesis of the corpus callosum (CC) and presence of Probst bundles (c). m) multiple chorioretinal lacunae

Patient 2. MR performed at sagittal T1-SE w (d), axial (e) and coronal (f) T2-TSE w images showing respectively corpus callosum hypoplasia (d), dysmorphic right cerebellum and vermis with bundled folia, imprinted by a bulky infratentorial cyst (e); dysmorphic temporal horns, mostly on the right (arrow, e). An heterotopic nodule is evident on left periventricular side (arrow, f). n) multiple chorioretinal lacunae

Patient 3. MR performed at sagittal (g), axial (h) and coronal (i) T2-TSE w images showing a hypoplastic CC (g), right frontal dysplasia (h, asterix) and dysmorphic right parieto-occipital sulcus (i, arrow) o) multiple chorioretinal lacunae

Patient 4. MR performed at sagittal T1-SE w (j), axial (k) and coronal (l) T2-TSE w images. Agenesis of CC, with only partial genu detectable (j). A simplified gyral pattern with right frontal dysplasia and heterotopic nodules on the right periventricular side is also evident (k). T2-w coronal image (l) shows basal ganglia dysmorphism (right side, arrow) involving the striatum and absence of the septum pellucidum

6.2 Discussion

With the growth of experience with AS, more inclusive criteria have been suggested till we applied the redefinition (Sutton, Hopkins et al. 2005). The more inclusive Modified Diagnostic Criteria allowed us to expand the phenotypic spectrum, so the number of atypical patients has increased: without spasms or without corpus callosal agenesis, widely variable in severity, from the most severe to some with favorable outcomes and normal neurologic examinations (Lee, Kim et al. 2004; Guerriero, Sciruicchio et al. 2010). Moreover, cases with only one of the classic triad but supporting features have

been reported, raising substantial issues with the diagnostic classification (Grosso, Lasorella et al. 2007).

Although in one view these criteria are more inclusive, from the other they are fixed with the presence of at least two out of the triad, plus other major or supporting features, but they lack to include the concomitant presence of all the complex brain malformations, corpus callosal agenesis, polymicrogyria, nodular heterotopias, intracranial cysts, and/or choroid plexus cysts or papillomas, central for the diagnosis, that over time has been outlined.

In specific, the Modified Diagnostic Criteria used to date allow us to diagnose for both Case 2 and Case 3 above; specifically Case 2 received the diagnosis because of the presence of two out of the three classical criteria, although her corpus callosum was only hypoplastic, and cortical malformation has not been confirmed. To our knowledge, only one other case has been reported with a normal corpus callosum, no cortical malformations, normal development, but choroid plexus papilloma; however, this patient was considered “markedly atypical” by Aicardi (Aicardi 2005). In our third patient epileptic spasms, chorioretinal lacunae, brain cortical malformation, and angiomas allowed to apply the AS diagnosis. However, her brain imaging is highly atypical: only thinning of the corpus callosum and cortical dysplasia were present, so in Case 3 a definite diagnosis of AS remains debated. Otherwise, in Case 4, the brain MRI is typical for AS, but because of the absence of two out of the three classical features (chorioretinal lacunae and epilepsy), the classic diagnostic criteria are not met. The presence of all the features which characterize the complex brain malformations deemed typical for AS sustain the diagnosis in Case 1 despite the DMJD (“butterfly sign”), underling the importance of these features in sustaining the diagnosis. However a dual

diagnosis cannot be excluded. This sign is not specifically confined to one precise condition but may be observed in other disease states including L1CAM-related disorder, Chiari II malformation, 6q terminal deletion syndrome (Severino, Righini et al. 2017), and in persons with mutations in *PCDH12* gene (Guemez-Gamboa, Caglayan et al. 2018).

Our cases underling the fragilities of the actual AS diagnostic criteria, which risk inclusion of patients who are different and distant from the patients with classic AS, such Case 3; on the other hand exclude patients who are similar, as Case 4 without spasms and lacunae but with typical brain malformation and epilepsy. The criteria, indeed, lack to enhance the importance of the complex brain malformations typical of the syndrome, which should be consider at the same level of the Triad: chorioretinal lacunae, epileptic spasms and so not include only callosal agenesis.

In conclusion, because of the absence of an unique genetic signature for the syndrome, defining the clinical diagnosis of Aicardi syndrome is still a challenge; the number of patients with atypical features is growing. The central role of all the complex brain malformation, including corpus callosal agenesis with polymicrogyria, nodular heterotopias, intracranial cysts, and choroid plexus cysts and/or papillomas, should be emphasized at the same level as the Classic Triad for inclusion in the diagnostic schema. New more strict and defined diagnostic criteria that allow better classification of atypical patients are needed, while we wait for the identification of the genetic basis of these disorders.

7. THERAPEUTIC ASPECTS

7.1 Literature revision and data from multicenter study on 67 cases

In their epilepsy evolution, Aicardi Syndrome patients displayed different type of seizures: infantile spasms, focal seizures which are the most characteristic type of seizures, but also tonic, generalized tonic-clonic, myoclonic, atonic seizures and status epilepticus are reported. Epilepsy is usually refractory to all treatments (Glasmacher, Sutton et al. 2007). In 2004 Chau et al. reported two case with AIC in which the early treatment with vigabatrin had good effect both on EEG, seizures and neurological outcome (Chau, Karvelas et al. 2004); although these results, all the cases collected with the multicenter revision I have performed had tried at their epilepsy onset vigabatrin, which does not seem to improve seizures outcome. In our first multicenter revision on 67 cases with AIC, at the last evaluation 98,38% developed a drug-resistant epilepsy, with a failure of three antiepileptic drugs (AED) appropriately chosen and used, particularly for whom an accurate seizures frequency evaluation were available, 57,41% displayed multiple daily seizures, 31,48% had weekly seizures (1-7 seizures/week), and only 9,26% had ≤ 4 seizures/month. During their epilepsy history patients had tried more than three AED during time till 17 AED. Parents and clinicians reported a reduction in seizures frequency with Vigabatrin, ACTH, Valproic Acid, Lamotrigine. Five patients tried ketogenic diet without clear results on efficacy.

Positive results on seizures and clinical-developmental outcome were described with surgery approaches (callosotomy, hemispherectomy, lobe resection, vagus nerve

stimulation); particularly a reduction in seizures frequency and developmental improvement/progression were reported in 7/9 cases (details in Figure 1).

	Age at surgery	Stype of surgery	Seizures outcome	Developmental outcome
Rosser et al. 2002	no data	hemispherectomy	seizures free on one anti epileptic drug	improved from a 2 mo to a 9 mo level
Palmer et al 2007	11 y	right parietal resection	seizures free for 4 y	functional age of 7 y at 15 y, walks and runs
Salto et al. 2002	3 y?	callosotomy, functional hemispherectomy	seizures free for 7 mo	no data
Kasasbeh et al. 2016	7 y	callosotomy	90% seizures reduction, drop attacks free for 20 months	slight improve language, walk, behaviour
Kasasbeh et al. 2016	8 y	callosotomy	no sz modification	less interactive
Kasasbeh et al. 2016	16 y	VNS	seizures improvement	no data
Kasasbeh et al. 2016	7 y	VNS	seizures worseing	no data
Podkorytova et al 2014	6 y	left functional hemispherectomy	seizures free for 6 months	improved in walk, more alert
Podkorytova et al 2014	1 y	right fronto parietal lobectomy	50% seizures reduction	head and trunk control improved slightly

Figure 1. Table shows a literature revision on surgery results on Aicardi cases; particularly age at surgery, type of surgery, seizure and developmental outcome of the cases.

7.2 Cannabidiol Expanded Access Program for Patients with Dravet Syndrome and Lennox-Gastaut Syndrome

Recently Devinsky et al. demonstrated a significant reduction in seizures frequency in an open label study with highly purified cannabidiol (CBD) in patients with Aicardi Syndrome, CDKL5, Dup15q and Doose Syndrome. AIC patients have a reduction >50% in seizures frequency during 28 days of treatment which was stable after also 12 weeks; in this study 71% of the Aicardi patients have a seizures reduction >50% (Devinsky, Verducci et al. 2018). Cannabidiol is a non-psychoactive phytocannabinoid derived from the Cannabis sativa plant which has shown antiseizure effects in

preclinical models of seizures and epilepsy. The precise mechanism by which cannabidiol exerts antiseizure activity remains unknown. Cannabidiol neither binds directly to nor activates the cannabinoid CB1 and CB2 receptors at clinically relevant concentrations, but it is known to show affinity and functional agonism or antagonism at multiple 7-transmembrane receptors, neurotransmitter transporters and ion channels (Perucca 2017). Two well controlled double-blind trials have been recently completed in patients with Lennox-Gastaut syndrome (Mazurkiewicz-Beldzinska et al, 2017; Devinsky et al, 2018). In the first one the monthly frequency of drop seizures decreased by a median of 42% for the cannabidiol 20 mg/kg group and 37% for 10 mg/kg group compared with the placebo group (17%; $p = 0.005$ and 0.002 , respectively). A significant reduction in total seizures was also demonstrated in both groups of patients treated with cannabidiol compared with placebo. Same results showed the second trials in which cannabidiol treatment was associated with a median percent reduction in monthly drop seizures of 44% vs. 22% observed in the placebo group ($p = 0.0135$).

In 2018-2019 Italy adhered to the “Cannabidiol Expanded Access Program for Patients with Dravet Syndrome and Lennox-Gastaut Syndrome” and we have the possibility to include in this study a case with AIC who satisfied the diagnostic inclusion criteria. The patient presented a mean of 17 seizures per months (range 12-25) before therapy onset, particularly spasms, focal seizures, atonic seizures and tonic seizures during sleep. A plant-derived standardized oil-based liquid oral formulation of cannabidiol was dispensed to the patient initially with a dosage of 5 mg/kg/day which was gradually increased till 15 mg/Kg by increments of no more than 5 mg/kg at intervals of no less than one week (according to the protocol). Adverse effects and liver function tests were performed after 2 weeks of treatment, and every 2 months during cannabidiol therapy.

EEG evaluation were performed before, at 1 and 6 months after therapy onset. At 1-6 months of therapy Clinical Global Impression (CGI) rating scales-Italian Version, Child Behavior Checklist (CBCL) Scales - Parent Reported Form and Bruni Scale for Sleep Disturbances were performed.

CBD was started on December 2018. Figure 2 reports in details the dosage of CBD and seizure frequency during the six months of protocol.

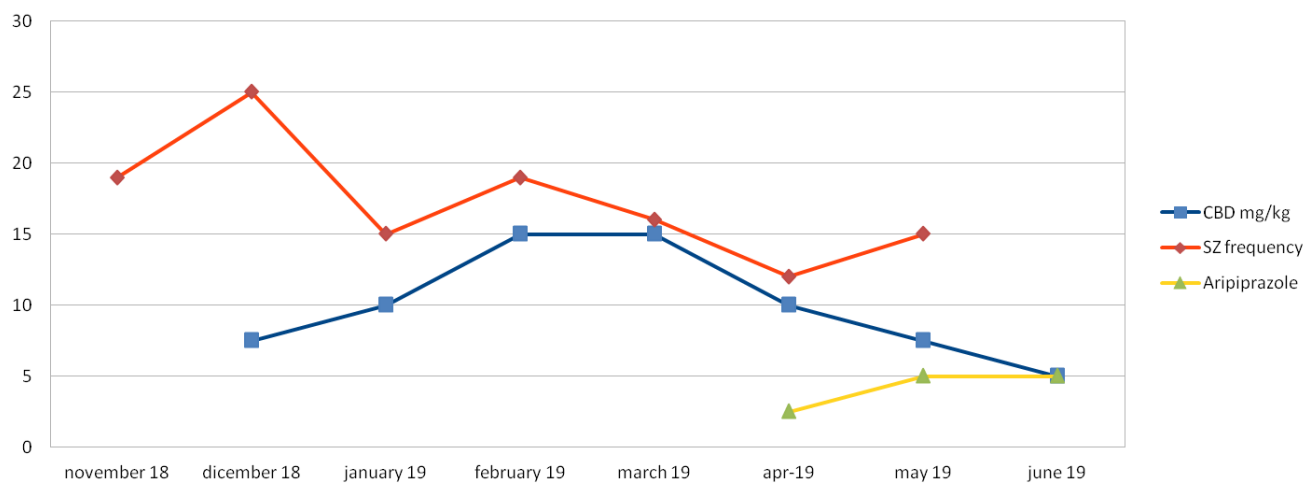


Figure 2. Cannabidiol dosage on mg/Kg/day, seizures frequency (SZ), aripiprazole dosage on mg/Kg/day during six months of therapy, from December 2018 to June 2019

At CGI rating scale the patient received a score of 6 (severe disease) both at onset and after six months of therapy, with a score of 3 and a score of 10 (slight improvement without significant adverse events due to the therapy) as index improvement at the 6 months evaluation.

CBCL Parent Reported Form revealed scores in normal range at anxious, withdrawn/depressed, thought problems scales before and at 6 months of therapy, a transition from normal range to borderline concerning rule breaking behavior (from a standard score of 57 to 67) and aggressive scales (from 58 to 66), borderline scores at

social (from a standard score 66 to 68) and attention problems scales (from a standard score 65 to 68) before and at 6 months of therapy, clinical score at somatic complaints scale before and at 6 months of therapy (from a standard score 73 to 77).

Concerning sleep disorder, Bruni Scale parent reported revealed borderline scores before the onset at all subscales and pathological scores after 6 months of therapy at the subscale “beginning of sleep and maintenance” DIMS, “arousal disorder” DA, “excessive drowsiness disorder” DES and total scale (details in Figure 3).

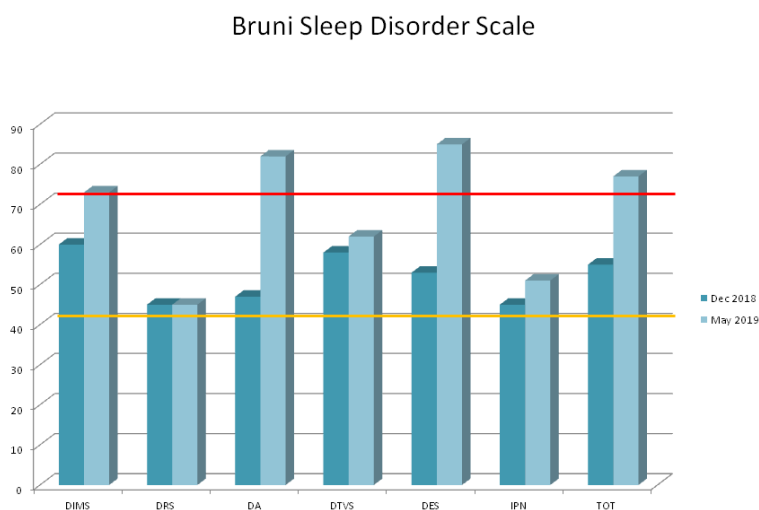


Figure 3. Bruni Sleep Disorder Scale. Total score: Tot, beginning of sleep and maintenance DIMS, sleep breathing disorders DRS, arousal disorder DA, waking-sleep transition disorder DTVS, excessive drowsiness disorder DES, nighttime hyperhidrosis IPN.

During six months of therapy seizure frequency did not drop out significantly (Figure 1), parents reported a worsening of behavioral problems (aggressive behavior only against parents) and excessive drowsiness (Figure 2), so the parents decided to withdraw the therapy on June 2019.

Studies on larger cohort of patients with AIC will allowed to better evaluate the clinical response to cannabidiol and delineate adverse event of this therapy.

To date Aicardi Syndrome remain a drug resistant condition, future clinical, genetic and pathophysiological studies may allowed to detect the cause of the syndrome and may orient targeted treatments.

8. GENETIC AND REVISED DIAGNOSTIC CRITERIA INTERNATIONAL COLLABORATION

8.1 International collaboration

We have created an international collaboration of different research groups who are working on clinical and genetics aspects of Aicardi Syndrome: the Italian (Dr. Silvia Masnada, Prof. Pierangelo Veggiotti, Dr. Anna Pichiecchio, Dr. Manuela Formica, Dr. De Giorgis Valentina, Prof. Emilio Perucca, Dr. Federico Zara), the Australian group from Adelaide (Dr. Jozef Gecz and Dr. Mark Corbett), the French group from Paris (Dr. Nadia Bahi-Buisson, Dr. Mara Cavallin and Dr. Arzimanoglou Alexis), the America groups (Dr. Igna Van Den Veyver and Dr. Elliot Sherr); on the 7th of March 2018 we have got a video meeting with the aim of discuss of genetic analysis carried out so far from the different researcher groups. Because none of the research groups have found a sure genetic hallmarks of Aicardi Syndrome, we have organized in Pavia, at National Neurologica Institute C. Mondino, an international consensus conference with the aim of redefining new more strict diagnostic criteria, discuss the genetic analysis ongoing and establish a new way of working together thus to shed light on the etiology of the syndrome. We have also organized a symposium with the aim to involve families of Aicardi patients, Italain Aicardi association and the clinicians on new knowledge about mechanisms involved in brain development and in the pathogenesis of several form of epilepsy and in recent advances in therapeutic research.

8.2 Summary of the Consensus Conference on Aicardi syndrome: from defining the phenotype to unravelling the genotype

November 16th, 2018

Attendees

Arzimanoglou Alexis (Lyon, France)

Bahi-Buisson Nadia (Paris, France)

Cavallin Mara (Paris, France)

Corbett Mark (Adelaide, Australia)

De Giorgis Valentina (Pavia, Italy)

Formica Manuela (Pavia, Italy)

Gecz Jozef (Adelaide, Australia)

Iacomino Michele (Genoa, Italy)

Masnada Silvia (Pavia, Italy)

Perucca Emilio (Pavia, Italy)

Sherr Elliott (San Francisco, U.S.A.)

van den Veyver Igna (Houston, U.S.A)

Veggiotti Pierangelo (Milan, Italy)

Zara Federico (Genoa, Italy)

8.2.1 Genetic Studies

Dr. Mark Corbett presented the genetic data for 13 patients from the Australian group, 6 with the classical Aicardi syndrome (AIC) triad (corpus callosum agenesis, chorioretinal lacunae and epileptic spasms), 4 meeting current diagnostic criteria and 3 with an AS-

like phenotype. WES was performed in all patients and 7 patients also underwent WGS. Mutations in *SLF1* – *WNT8B* – *KMT2B* – *SZT2* – *HCN1* genes were found (confidential un-published data), all of which were de novo missense mutations with high pathogenicity scores. No X-linked mutations were found. The *HCN1* gene mutation was a loss of function (LOF) mutation, but this case was subsequently reclassified as early infantile epileptic encephalopathy (OMIM: EIEE24: 615871) with partial corpus callosum agenesis with neither ocular malformations or other cortical malformations, therefore not AIC. The mutation in the *WNT8B* gene, which encodes for a protein highly expressed in brain tissue, was detected in a girl with the classical triad and consanguineous parents. Studies of the *WNT8B* and *SLF1* mutations in the morpholino zebrafish model revealed malformed fish with pigmental areas in the eyes and other brain malformations similar to those found in AIC. Studies with *KMT2B* and *SZT2* mutations revealed extra-neurological features, unimpressive defects on fish development and no corpus callosum agenesis. No somatic mutations were screened by the Australian group. Options to search for deeper somatic mutations were discussed.

Dr. Federico Zara reported that his initial 2012 studies with Exome Sequencing in 5 patients revealed no shared mutations and no new candidate genes. In 2016 a follow-up high-coverage Exome Sequencing study on chromosome X in 13 patients (including 11 trios, and for one family the proband, both parents and an hemizygotic healthy twin) revealed no variants. For 6 patients and the two monozygotic twins, saliva was also tested to look for low-frequency somatic variants. A high-density CGH-array on X chromosome was also performed in 12 trios and one proband. The genetic analysis performed by *Dr. Zara* was carried out on the group of cases clinically described in my first work “*Aicardi syndrome multicenter study: clinical and neuroradiological*

phenotype correlations in 67 cases". A review of results brought attention to potentially interesting mutations in *POLAI* (related to eye problems in the mouse model), *ACRC*, *HDAC6*, *HCFC1* (which is next to *MECP2*), though all gene mutations detected were SNPs. The ensuing discussion raised a number of suggestions for follow-up studies:

- for monozygotic twins, review not only discordant genes but also genes in common because of the possible influence of epigenetics or different penetrance of these genes;
- screen homozygous mutations, to consider the possibility of uniparental disomy;
- study more deeply selected areas of the X chromosome with high amplification;
- evaluate the possible presence on the X chromosome of predisposing genes which may interact with autosomal genes in influencing the phenotype;
- review the possible presence of overlapping CNVs in different patients;
- conduct confirmation analysis on qPCR

The possibility of a "founder X chromosome" was suggested by Igna van den Veyver because of the apparently much lower prevalence of the syndrome in Indian, Chinese or African populations however it was acknowledged that this could be ascertainment bias.

Dr. Igna van den Veyver presented her data on facial phenotyping, filamin and imaging studies and subsequently summarized her genetics studies. She performed studies of X inactivation showing skewed inactivation in AS, somatic mutation studies aimed at identifying low level mosaicisms, DNA methylation studies (no clear pattern of inactivation in common detected), balanced chromosomal rearrangements (translocation/inversion) studies, sequencing on X chromosome and WES on blood and brain tissue (two patients) which failed to detect any variants. Candidate genes that emerged included *ARX*, *CASK*, *CDKL5*, *TEAD1*, and *OCEL*. Gene expression profiling studies revealed 11 mutations, including *CSK*, *CDKL5*, *CXorf57*, *FGFR1* and *SDK1*

gene heterozygous compound mutations and homozygous mutations found in two patients, one with a very typical phenotype and one with dermatologic problems. One patient had a heterozygous compound mutation of the *KIF13A* gene, which encodes for a microtubule-dependent motor protein highly expressed in brain. She suggested to do more work more on maternal genome to look for germinal mosaicisms. She also suggested to take into consideration for the search strategy the absence of parent-to-child transmission, the occurrence of the disease only in females or Klinefelter males and to consider the possibility of epigenetics changes. A possible non-genetic cause was discussed, but considered to be unlikely.

Dr. Mara Cavallin reviewed her data with high-resolution 60 Kb array CGH on 20 patients (genetic analysis carried out on the group of cases clinically described in my first work "*Aicardi syndrome multicentred study: clinical and neuroradiological phenotype correlations in 67 cases*"). One deletion and two duplications were detected in 3 patients, without significance for the phenotype. The possibility of an abnormal X chromosome inactivation was excluded by the presence of skewed inactivation in only three patients. Studies with WGS (6 patients, all with the classical triad, neonatal onset of epilepsy and bilateral chorioretinal lacunae) and WES (16 patients) were performed. Detected candidate autosomal genes with brain expression and/or involvement in developmental included *HCN2*, *PPP1R14D*, *SDK2*, and *EFHD1*. The *EFHD1* gene had been reported in other patients with corpus callosum agenesis. RNA sequencing analysis on fibroblasts did not reveal abnormalities. No significant genes were found by cross-analysis of RNA sequencing and WGS results (confidential un-published data). In the ensuing discussion, the suggestion was made to test X inactivation not only in blood but also in other tissues.

Dr. Elliot Sherr described his cohort of patients and reported on new potentially pathognomonic MRI features consisting in washout of white matter and abnormal white matter tracts of corpus callosum on DTI studies, which do not seem to occur in patients with other corpus callosum diseases. He also reported on WES and WGS studies on 52 trios and 75 probands, additional genomic studies on 4 brain samples (particularly areas of polymicrogyria or heterotopias), and fibroblasts analysis in 7 patients. X chromosome CNVs and SNPs were also evaluated. One suggestion raised in the discussion was to map all deletions compatible with male life, and to focus the analysis on the others.

The general discussion focused on criteria to optimize selection of patients for genetic studies. There was consensus in selecting at first a core group of patients with typical phenotype, excluding patients without chorioretinal lacunae. Patients with atypical phenotypes or AS-like phenotype could be included in broader analyses as a secondary step.

There was general agreement on the following actions:

- share data in order to permit re-evaluation of genetic data in a larger pool of patients; a link to a GoogleDoc document where all members of the collaboration will be able to list their resources and which will form basis for subsequent discussion about analysis and reanalysis of the data were created
- focus on genes involved in eye development, considering the typical feature of chorioretinal lacunae
- each group could focus on different specific analysis (WES, WGS, RNA seq, CNV, different types of inheritance, X-chromosome or autosomal)
- create an Aicardi Syndrome biobank

A variety of hypotheses/searching strategies were discussed during the meeting, including:

- consider the possibility of combinations of inherited and de novo mutations
- consider the possibility of epigenetics changes, X-chromosome or autosome
- review data from monozygotic twins focusing not only on discordant genes but also on shared by the twins (but absent in the parents) because of the possible influence of epigenetics or different penetrance of these genes
- test X inactivation not only in blood but also in different tissues
- look for germinal and postzygotic somatic mosaicisms
- confirm the absence of parent-to-child transmission and the presence of the disease only in females or Klinefelter males
- screen for homozygous mutations, considering the possibility of uniparental disomy
- deeper study of very selected areas of the X chromosome with high amplification
- evaluate the possibility of predisposing genes on the X chromosome which can interact with autosomal genes in influencing the phenotype “two hit” hypothesis
- review the possible presence of overlapping CNVs in different patients; confirmation analysis by qPCR
- high quality whole genome sequencing using long-read technology (consider approaching these companies (PacBio/Illumina or Oxford Nanopore) for support)
- haplotyping X-chromosome, looking for or rejecting the hypothesis of an X-chromosome founder mutation/locus

- perform GWAS with initial, but not only, focus on the chorioretinal lacunae
- exploration and ruling out of non-genetic, eg. viral causes
- Jozef Gecz/Adelaide team suggested to consider doing GWAS on 100-200 AS cases, if we can assemble DNAs from such a cohort. Such SNP typing would also be used to look for any signatures of AS founder effect, primarily on the X-chromosome, but also beyond. The Adelaide team offered to provide funding for the SNP chips and facilitate GWAS and haplotype analyses

8.2.2 Clinical Features and Diagnostic Criteria

In the afternoon session, Dr. *Silvia Masnada* and Dr. *Mara Cavallin* presented the clinical and neuroradiological results of the Italian-French collaborative study. Because of the high prevalence of the described MRI features (corpus callosum agenesis associated with polymicrogyria, nodular heterotopias, cysts), there was consensus in considering these features together as a unique core aiming major diagnostic criteria. Other MRI features were also reported as being more frequent than previously reported in literature, including antero-posterior gradient of polymicrogyria, enlarged cisterna magna, cerebral asymmetry, cerebellar, basal ganglia and hippocampus dysmorphisms, from slight dysmorphisms to more severe abnormalities (cerebellar dysplasia and/or agenesis of anterior limb of internal capsula). The importance of excluding other genetic cause of these dysmorphisms was underlined, e.g. by screening for tubulin genes in patients with most severe basal ganglia dysmorphisms and agenesis of the anterior limb of internal capsula. The need to exclude other syndromes or disease with MRI features or ocular abnormalities similar to AS was also underlined. There was agreement that lacunae are typical for the syndrome, also when they are monolateral, and a possible

common mechanism in the formation of cysts and lacunae was suggested. The importance of an expert ophthalmologist in the diagnosis of the chorioretinal lacunae was emphasized. If possible, physicians should be asked to obtain photographic documentation of ophthalmologic features and an optic coherent tomography (OCT) in order to permit review by external expert. As for facial phenotyping, the possibility to study facial dysmorphisms with computer-based 3D methodology was suggested.

In the second part of the discussion dedicated to diagnostic criteria, *Dr. Valentina De Giorgis* reported the results of the survey conducted by *Dr. Silvia Masnada* among participants prior to the meeting. Most participants considered as major criteria corpus callosum agenesis (partial/complete) (9 participants), chorioretinal lacunae (9 participants) and epileptic spasms (8), followed by heterotopias and polymicrogyria (5 participants). Fewer participants considered as major criteria split brain EEG, intracerebral cysts, choroid plexus papilloma/cysts, gross cerebral asymmetry and female sex. Vertebral/costal abnormalities (9 participants), other cortical malformations (7), other types of seizures (6), gross cerebral asymmetry and other ocular malformations (5) were proposed as minor criteria by most participants. Optic coloboma, split brain (4), cysts (3), choroid plexus papilloma (3), heterotopias (2), partial corpus callosum agenesis (2), polymicrogyria (2), microphthalmia (1) and epileptic spasms (1) were suggested by fewer participants. Four participants considered chorioretinal lacunae to be a pathognomonic finding, unlike other features. There was no consensus on the possibility to develop major and minor criteria for a diagnose of definite AS in absence of chorioretinal lacunae. There was agreement on including in the diagnostic workup the following evaluations: EEG, MRI, chest X ray, genetic analysis (epileptic encephalopathies and cortical malformation genes, tubulin genes,

CGH array), neuropsychological, endocrinological, dermatological and oncological assessment.

The relative merits of using a new scoring system vs the “classical” system with major and minor criteria was discussed. *Elliott Sherr* described his score system and *Valentina De Giorgis* showed the score system created by the Italian group. The possibility to use machine learning or another statistical method in order to attribute scores was suggested. As for the “classical” approach, the suggestion was made to consider as major criteria chorioretinal lacunae, seizures and a specific combination of cerebral malformations (corpus callosum agenesis, polymicrogyria, heterotopias and cysts), and as minor criteria vertebral/costal malformations and other ocular abnormalities: the presence of three major criteria (or at least two, one of them being lacunae) plus minor criteria would permit a diagnosis of definite AS. The possibility to merging a new score system with the “classical” system using major and minor criteria was also discussed.

After extensive discussion, it was proposed to consider as *major criteria* chorioretinal lacunae, epileptic spasms and/or focal seizures, any degree of corpus callosum dysgenesis (complete or partial agenesis, thin corpus callosum, or corpus callosum dysmorphisms) and specific combinations of other cerebral malformations (polymicrogyria, periventricular and subcortical heterotopias, inter-hemispheric or third ventricle cysts, choroid plexus cysts and/or papilloma), and as *minor criteria* gross cerebral hemispheric asymmetry, vertebral/costal malformations, posterior fossa and cerebellar abnormalities and other ocular abnormalities. Need to elaborate on how scores are assigned and on the rationale for the choices made. I have created a database for statistical analysis with the aim of giving a score of the diagnostic criteria,

Particularly a score for “*definite AIC diagnosis*” and for “*possible AIC diagnosis*”.

In conclusion, all participants agreed on selecting a core group of definite AS patients with chorioretinal lacunae, typical clinical and MRI phenotype for the first genetic analysis. For clinical purposes, there would be value in using the new criteria for diagnosis of definite AS and possible AS.

Prof. Alexis Arzimanoglou agreed to coordinate the drafting of the consensus manuscript, with the Adelaide group coordinating the section on genetics. The manuscript should include a brief historical introduction on AS, an overview on clinical features and diagnostic criteria (including, potentially, a proposal for a revised diagnostic scheme), a section summarizing genetic studies performed to date, including the revision or not of the X-chromosome origin of AS, and a final section with suggestions for further research. A draft on consensus meeting and particularly on new diagnostic criteria is being written.

9. CONCLUSIONS

Over my three years of PhD, I had the opportunity to do a retrospective multicenter collection of AIC syndrome patients from different European Centers. I carried out different studies on the clinical-neuroradiological and electroencephalographic data collected, which allowed me to describe and better delineate the wide phenotype of the syndrome. Therefore, these studies have implications in the clinical practice and future researches.

Through neuroradiological studies on larger fetal and postnatal cohort performed, I've been able to define in details the complex and wide AIC neuroradiological phenotype, which it turns out to be a multiple brain malformation, which are not only included in the known callosal agenesis, gyration anomalies, nodular heterotopias, intracranial cysts, but implicates the frequent association with posterior fossa abnormalities and, previously unreported, basal ganglia dysmorphisms. The association between all these multiple malformation should be recognized not only in postnatal period for a correct diagnosis, but particularly in iuMRI with important implications in pregnancy and early neonatal management. iuMRI have a key role in detecting precociously all the multiple brain malformations with their peculiar presentation and associations, which are the chore of AIC. During this study, we discovered that the association of female sex, diffuse cortical gyration abnormalities and optic nerve coloboma is highly predictive of postnatal AIC diagnosis. Both postnatal and prenatal MRI studies allowed to detect a previously unreported possible differential diagnosis of the syndrome, with significant implication in future research studies.

A merger between neuroradiological, clinical and EEG data, confirmed the wide spectrum of the syndrome and underline the impact of a correct evaluation of MRI and

EEG studies at onset, which can predict long-term clinical outcome with significant implication in clinical management of the patients.

The detection of similar facial measurements in AIC cases, will help clinicians in classifying atypical or doubt cases and so in performing more definite AIC diagnosis.

The increasing of atypical cases in AIC patients brings out the necessity to redefine new AIC Syndrome diagnostic criteria. The results from the different studies carried out on clinical-neuroradiological and electroencephalographic data over my three years of PhD, and the International collaboration we have created with all the research groups from different Countries (Australia, France, USA, Italy) lay the basis of new proposed diagnostic criteria and a new collaboration on genetic research.

The new diagnostic criteria, which underline the predominant role of the complex cerebral malformation, will have significant implications: will enable a more accurate definition of the spectrum of AIC syndrome, will allow a better classification of atypical or doubt cases, moreover will help in performing genetic analysis in selected and homogeneous cases, opening the way to new potential clarification of the etiology of the syndrome.

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