NEXT GENERATION SEQUENCING FOR DIAGNOSIS IN MONOGENIC PEDIATRIC STROKE

.. from NGS panel to Whole Exome Sequencing

Marta Bertamino

University of Genova

Department of Neurosciences, Rehabilitation, Ophthalmology, Genetics, and Maternal and Children's Sciences (DINOGMI)

Tutor: Dott. Clara Malattia

Co-realtor: Dott. Maja Di Rocco

Sommario

INTRODUCTION

Definition of stroke, epidemiology and role of genetic in the different subtypes

The World Health Organization defines *stroke* as *rapidly developing signs of focal disturbance of cerebral function with symptoms lasting at least 24 hours or leading to death with no apparent cause other than of vascular origin* (WHO Project, 1998). This definition is far from ideal for children, because children with symptoms compatible with transient ischemic attack can have brain infarction shown by brain imaging despite the transient nature of their symptoms. Consequently, pediatric stroke is defined as any neurological event (including a seizure) associated with an acute infarction shown by magnetic resonance imaging. (Seidman C, 2007)

Stroke can be ischemic, hemorrhagic, or both. Ischemic stroke is more frequently caused by arterial occlusion, but it may also be caused by venous occlusion of cerebral veins or sinuses.

Stroke has been considered a rare event in neonates and children, with a reported incidence of combined ischemic and hemorrhagic pediatric stroke ranges from 1.2 to 13 cases per 100,000 children under 18 years of age (Tszee D.S., 2011; Carvalho KS, 2002; deVeber GA, 2017; Suppiej A, 2015). The overall incidence is increasing due to a combination of improved survival of those with risk factors and increased recognition. (Nikil K. Rajani, 2018)

In perinatal period, between 20 week of gestational age and 28 days of postnatal life, the incidence appears to be 17-times higher (Sebastian Grunt, May 2015; Janet Lee, February 9, 2005). However, both perinatal and pediatric stroke is likely more common than we may realize since it is frequently undiagnosed or misdiagnosed (Nidhi Agrawal, 2009; Malova M, 2017 Jan; Parodi A, 2015 Nov).

Cerebral sinovenous thrombosis (CSVT) is a rare but serious cerebrovascular disorder-affecting children from the newborn period through childhood and adolescence. The incidence is estimated at 0.6/100,000/year, with 30–50% occurring in newborns. Causes are diverse and are highly age-dependent. Acute systemic illness is the dominant risk factor among newborns. In childhood CSVT, acute infections of the head and neck such as mastoiditis are the most common, followed by chronic underlying diseases such as nephrotic syndrome, cancer (i.e. or asparaginase administration during treatment for leukemia or lymphoma), and inflammatory bowel disease (Ichord RN, 2015).

A significant proportion of cases (~13–25percentage) have no risk factors identified suggesting that undetermined genetic factors may at least partly account for this unexplained risk. Although it is a rare condition, it does not usually cluster in families and there is no evidence to suggest a Mendelian inheritance. Neither the genetic component of CSVT nor its heritability has been widely assessed mainly because of its low incidence and lack of large number of cases. (Cotlarciuc I, 2016). Inherited thrombophilias are known to cause approximately a quarter of the CSVT cases in adulthood (Ahmad A, 2006), while in pediatric and neonatal age the prevalence of hereditary thrombophilias varies significantly from study to study (from 20 to 64%) probably due to different populations studied (Se´bire G, 2005; Dlaminia N, 2010).

Hemorrhagic stroke (HS) is the result of bleeding from a ruptured cerebral artery or, in some cases, from bleeding into the site of an acute ischemic stroke. Intracerebral hemorrhage (ICH) may be either traumatic or non-traumatic (spontaneous ICH).

The reported incidence of asymptomatic and symptomatic ICH in children varies from study to study probably due to differences in the sensitivity and timing of the diagnostic imaging used (Zidan I, 2012; May Llanas M.E., 1999).

In the neonatal population, the incidence of HS was more investigated; a magnetic resonance imaging (MRI) study of full-term neonates found approximately a 25% incidence of asymptomatic intracranial hemorrhage after vaginal delivery (Looney CB, 2007). Symptomatic intracranial hemorrhage in full-term neonates is much less common, in the range of 4 per 10,000 live births. The incidence is higher, however, in instrumented births (Towner D, 1999).

Most non-traumatic hemorrhagic strokes may originate in or extend into the intraventricular, subdural, or subarachnoid space. (Pavlakis, 2008; Gabis L. V., 2002)

Studies about children with HS show that first cause is vascular malformations, which are responsible of 5%–29% of cerebral hemorrhages, whereas other causes are hematological disorders, such as thrombocytopenia or hemophilia, and malignancies. (Gabis L. V., 2002; Pavlakis, 2008)

The most common vascular malformations are arteriovenous malformations (AVMs), aneurysms, and cavernous malformations. Aneurysms and hypertension, although commonly associated with adult HS, are an infrequent cause in children. (Lynch J.K., 2005)

AVMs account for 14% to 46% of HS in children (Zidan I, 2012). Most AVMs are diagnosed in patients between 20 and 40 years, but about 18% to 20% will become symptomatic during childhood.

The incidence of intracranial aneurysms in children is about 1 per million per year, substantially less than the adult rate. About 1% to 2% of aneurysms will become symptomatic in childhood, mainly with HS. (Gabis L. V., 2002)

Hematologic abnormalities are reported to be the major risk factor in 10% to 32% of hemorrhagic strokes in most series (A. Al-Jarallah, 2000; Meyer-Heim A.D, 2003). Those includes idiopathic thrombocytopenic purpura, acute lymphoblastic anemia, sickle cell anemia, moderate and severe hemophilia A and B, and other coagulopathies (such as severe Factor XIII deficiency) (Briggs B, 2018). Brain tumors are also found to be one of the causes of ICH in children with a much lower incidence than previously mentioned causes. Anticoagulant

treatment, largely in relationship to warfarin use, has also been consistently associated with HS risk in adulthood, while in pediatric setting the role of this risk factor appears to be a minority. (Guido J. Falcone, 2017).

Mendelian forms of ICH represent only a small fraction of the total number of cases (~5percentage of hemorrhagic strokes being caused by genetic coagulopathies, some others by COL4A1-related diseases) (Guido J. Falcone, 2017; Benjamin Briggs, 2018)

Arterial ischemic stroke (AIS) accounts for about half of all strokes in children, in contrast to neonate and adults in whom 80–85% of all strokes are ischemic. This population has been the focus of our study project, and therefore we discuss below the detailed characterization

.

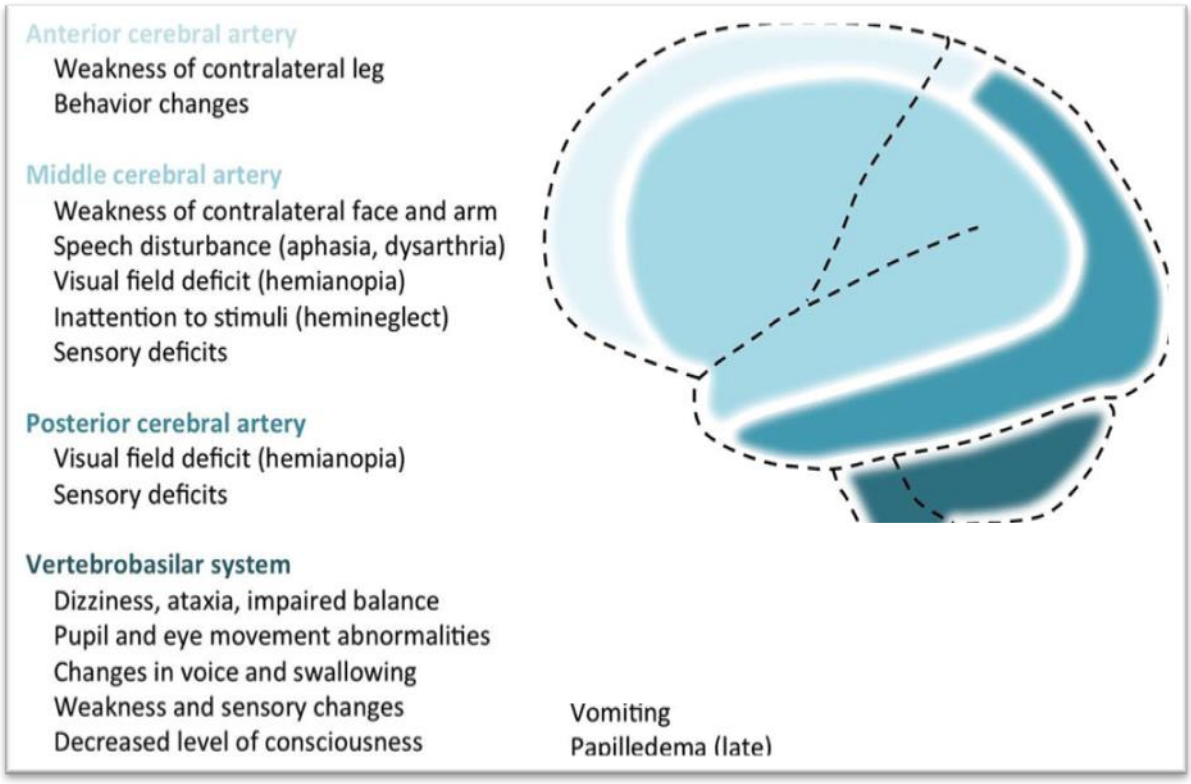
.

Epidemiology of Arterial Ischemic Stroke in Children, **Clinical Presentation** and **outcome**

Population-based studies of arterial ischemic stroke (AIS) in children (defined by age 29 days to 18 years) estimate an annual incidence of 2.4 per 100,000. (Numis A.L., 2014)

Whilst stroke classically presents with an acute focal neurological deficit such as hemiplegia, speech or gait disturbance, the presentation is highly dependent on the age of the child and may not be specific. Toddlers and infants tend to present most non-specifically, with features such as irritability, somnolence, lethargy, feeding difficulty, apnea and hypotonia. (Nikil K. Rajani, 2018). Approximately 30–50% of children with an acute neurological presentation will have non-vascular diagnoses (Mackay M, 2014). The clinical onset depends also on the involved vascular territory (Figure 1).

Figure 1. Onset symptoms in relation to involved vascular territory.



AIS can lead to significant morbidity and mortality and, contrary to commonly held views, children do not recover better than adults do after a stroke (Greenham M, 2016). Roughly,10–25% of children with a stroke will die in the first years after stroke, with a stroke-specific mortality around 5% (deVeber GA, 2017; Daniel S. Tszee, 2011).

Outcomes included neurological deficits in 60% of neonates and 70% of older children. Among neonates, deficits emerged during follow-up in around 40% (deVeber GA, 2017). Other sequelae includes subsequent seizure disorders, learning, or developmental problems (Daniel S. Tszee, 2011; Greenham M, 2016).

Recurrence risk after childhood AIS has been estimated at 12% at one year and 19% at five years (Fullerton, 2007).

AIS Risk factors and predictor of recurrency

A heterogeneous group of risk factors has been identified in association with childhood AIS. Remarkably, those risk factors are different from those found in adults. Indeed, the most frequent causes of childhood AIS are acute systemic disorders (e.e. sepsis, deyhidratation, acidosis) and non-atherosclerotic arteriopathies, such as focal cerebral arteriopathy, fibro-muscular dysplasia, and Moya-Moya syndrome, followed by congenital or acquired heart diseases, hemoglobinopathies (such as sickle cell disease - SCD), trauma, major prothrombotic states (Felling RJ, 2017; Nikil K. Rajani, 2018) (Mackay MT W. M., 2011).

The main risk factors for pediatric AIS are summarized in Table 1

Table 1. Main risk factors for pediatric AIS

Arteriopathy	53% FCA Moyamoya	 Idiopathic RNF213 gene 17q25. ACTA2 gene 10q23.3 GUCY1A3 gene 4q32.

		Down Syndrome	
		NF1	
	Dissection	Extracranial/traumatic	
		Intracranial/spontaneous	Idiopathic
			Ehlers Danlos, Marfan's
	Vasculitis	Primary	Takayasu arteritis
			Kawasaki Disease
		Secondary	Infectious
			Post Varicella Angiophaty
			immune complex deposition (i.e. SLE)
Cardiac disorders	31%		
	Uncorrected CHD with Complex right-to-left shunting		
	Cardiac surgery, ECMO		
	Cardiomyopathy with reduced LVEF		Muscular dystrophy
			Friedreich's ataxia
			Chagas' disease
Blood disorders			
	SCD		
	Prothrombotic disorders		Protein C deficiency
			APL syndrome
			Factor V Leiden
			Elevated homocysteine
			Oral Contraceptive
			Protein S deficiency
Metabolic causes			
	MELAS		
	Fabry diseases	(rare in childhood AIS)	
Other genetic cause*			

FCA = Focal Cerebral arteriopathy; CHD = Cardiac hearth diseases; ECMO = Extracorporeal Membrane Oxygenarion ; SCD = sickle cell diseases; APL antiphospholipid;
* see below.

Further, many of the risk factors listed in Table 1 may overlap or have a common mechanism. This is the case of *cerebral arteriopathy*, an "umbrella term" used to describe a broad range of cause. Cerebral arteriopathy may be the result of both underlying genetic conditions (such as NF1, PHACE syndrome or fibromuscular dysplasia), and acquired conditions such as trauma, radiation therapy or infection (i.e. VZV or other common viroses), or finally develop in a previously healthy child without any other known risk factors. (Nikil K. Rajani, 2018)

Cerebral arteriopathy is a leading mechanism for childhood stroke, accounting for 53% of pediatric AIS cases and represents the most common single cause of stroke recurrence in

children (Beslow LA, 2010; Fullerton, 2007). Recurrent AIS or transient ischemic attack (TIA) in children is predicted also by presentation without seizures, and lack of antithrombotic treatment (deVeber GA, 2017).

State of art of genetic in ischemic stroke

Stroke in childhood is more common than brain tumor, but because there is a wide spectrum in terms of etiology and most centers only see a few cases every year, there have been few large studies of genetic and environmental risk factors until recently.

Hopefully, enhanced understanding of the mechanisms underlying childhood stroke, including cerebral arteriopathies, will allow the development of mechanism-specific treatment approaches and optimize outcome and management in children. (Kirton A, 2015)

Monogenic diseases

Several rare Mendelian disorders have been described in which stroke is a prominent feature. Recognition of individuals and families carrying mutations causing Mendelian diseases with stroke as a phenotypic manifestation remains an important challenge for clinicians. Mendelian disorders can be recognized mostly when the more common risk factors are lacking or by their familial aggregation, relatively young age of onset, often more severe clinical course, and higher recurrence rates, compared with sporadic diseases. (Terni E, 2015) In these cases, the diagnosis could be aided by characteristic clinical phenotypes, often including manifestation of co-existent systemic disease; however, especially in the case of an early onset cerebrovascular disease, stroke may be the first symptom of the genetic disorder. (Bertamino M, 2018)

Monogenic diseases are responsible of about 5% of stroke cases in young adult patients; however, the percentage is likely to be underestimated because of the diagnostic complexity

and the high phenotypic variability of these conditions. The real incidence in pediatric-onset stroke is unknown yet, but certainly significantly higher.

Stroke can result from one of several pathogenic mechanisms, which might be influenced by a single-gene defect. *Cardio embolism* (i.e. cardiomyopathies, familiar atrial myxoma), *small arterial diseases* (i.e. CADASIL, CARASIL, Fabry's disease), *large artery diseases* (i.e. Homocysteinuria), *hematological disorders* (i.e. SCD, Severe prothrombotic states), *dissection* due to connective tissue disorders are the main pathogenic mechanisms reported in children and young adult. (Hassan A, 2000)

Ferro et all in 2010 described more than 50 monogenic diseases that can cause stroke in young adults. (Ferro J.M., 2010)

More recently, Munot et al have drawn attention to the association of childhood AIS with Mendelian genetic disorders, thus providing insights into disease pathogenesis, especially when non-atherosclerotic arteriopathies are involved. (Munot P, 2011)

In Mendelian disorders, the presence of a pathogenic mutation(s) of a single specific gene is usually sufficient to manifest a phenotype. However, a clear genotype-phenotype correlation is very rarely observed in monogenic strokes. At least two genetic phenomenons can influence the phenotype in both heterozygosites and homozygotes: the reduced penetrance and a variable expressivity, which refers to the type, severity and natural history of the disease. Among the factors inducing the variable expressivity, the functional effect of the mutation (complete absence of the encoded protein rather than its loss -or gain- of function), the allelic heterogeneity (different mutations in the gene), the interaction with other genes (pleiotropy) or with the environment are the most documented ones. In X-linked diseases, the clinical expression in females is possible, and the severity of the phenotype is caused by the phenomenon of X inactivation ('mosaicism'). (Terni E, 2015)

Environmental exposure (i.e., infection, hypoxemia, and vitamins) may play a crucial role in modifying genetic expression and must be carefully described in prospective studies. Moreover, the search for polymorphisms with epistatic effects, able to explain why some but not all the carriers are affected has been hampered by our poor understanding of the disease pathophysiology. (Kirkam FJ, 2003).

In deep, in cerebrovascular disorders field, advanced vascular imaging, including arteriography and Black Blood Vassal Wall (BBVW) imaging technique, will almost certainly assist with the description of stroke subtypes with different genetic predisposition in these patients.

Polygenic conditions

Several studies on young adults showed how, in most cases, it is likely that multiple genes are involved in stroke pathogenesis acting on a wide range of candidate pathways, such as the hemostatic and inflammatory system, homocysteine metabolism, and so others. (Terni E, 2015). Overall, the genetic risk factors for those polygenic forms are less well understood. In most of these cases stroke represents a complex condition, where multiple genes and the environment are both necessary to reach a threshold level, critical for the phenotype manifestation.

In this scenario, the role of a great number of candidate genes has been investigated through association studies, with controversial results. (Agerholm-Larsen B., 1997; Pfohl M., 1998; Rigat B., 1990; Cambien F. 1992; Tuncer N. 2006; Casas J.P. 2004)

The advent of GWAS technology, such as for adult stroke and other complex pathological conditions, could contribute enormously to the understanding of the genetic base of many stroke events (i.e. idiopathic perinatal stroke).

Next Generation Sequencing technique

Next generation sequencing (NGS) is a relatively new technique now being applied to genetic testing. NGS has the potential to find causal mutations, including -*de novo*, novel and familial mutations, associated with pediatric and perinatal stroke and, due the variable phenotypic presentations of the disorder, vastly improve molecular diagnosis.

First generation DNA sequencing with chain-terminating inhibitors invented by Sanger in 1977 (Sanger F., 1977), led to many genetic discoveries and has been widely used for over 30 years in research and diagnostic laboratories. Although considered a major technological breakthrough, and still useful today for variant verification, the technique has limitations, in particular when examining large regions of the genome. More recently, NGS has begun to replace Sanger sequencing due to its ability to sequence large numbers of genes, the whole exome (protein-coding regions) or entire genome at once. Thus, applications of NGS include targeted gene panels, whole exome sequencing (WES) and whole genome sequencing (WGS). Custom gene panel testing allows for screening of multiple potentially clinically relevant genes and for more flexibility in phenotype–genotype correlations than required when testing individual genes. (Poduri A., 2014)

WES focuses on the protein coding regions in the genome, comprising approximately 1–2% of the genome, which ~85% of disease related mutations are attributable to. In contrast, WGS provides information on the entire genome (both coding and non-coding regions), detecting additional mutations in regulatory regions, as well as copy number variations with higher efficiency than WES (Poduri A., 2014; Stavropoulos D. J., 2016). The ~99% of the genome not included as exome sequence also contains untranslated regions which may have a regulatory role (e.g., non-coding RNAs or transcription binding sites) along with potential protein coding sites yet to be annotated as genes. (Chrystoja C. C., 2014) The impact of variants found in non-coding regions is not currently well understood, however it is feasible that a single or a

combination of regulatory variants could have a significant impact on the pathology of conditions such as stroke. This is most evident for non-coding variants that may influence expression levels or mRNA splicing, affecting protein abundance or isoforms.

Use of NGS technologies in research and diagnostic laboratories has contributed to the rise to the rapid identification of genes associated with stroke in young adult but, to date, there are few studies on the suitability of the three above mentioned NGS techniques in pediatric and perinatal stroke, with a particular focus on their application to aiding clinicians and laboratories for a timely genetic stroke diagnosis.

NEXT GENERATION SEQUENCING BASED GENE PANEL FOR ENHANCED RAPID DIAGNOSIS IN MONOGENIC PEDIATRIC STROKE

BACKGROUND

Here, we consider insights into the pathogenesis of neonatal and childhood stroke, starting from several reports in the literature showing the association between Mendelian diseases and an increased risk of early onset stroke (Munot P, 2011).

Therefore our hypothesis is that pediatric stroke, after the exclusion of more common etiology has, in some cases, a simple Mendelian inheritance characterized by genetic heterogeneity so that several genes may be responsible of the different clinical forms of the disease.

Thus, with the present project we aim at studying through Next Generation Sequencing (NGS) genetic analysis patients, responding to strictly defined “idiopathic” stroke.

A Next Generation Sequencing strategy is being undertaken to

1) overcome poor performances of the Sanger sequencing based molecular diagnosis of heterogeneous stroke associated syndromes,
2) avoid clinical misdiagnosis
3) investigate a large number of genes and gene regions, taking into account genetic heterogeneity for patients affected with different diseases sharing cerebral stroke and whose genetics is still unknown/undisclosed.

The present project has been developed inside the multidisciplinary scientific and clinical team that have the fortunate possibility to work very closely inside the Giannina Gaslini Children's Hospital with the final aim to carefully and according to best practice manage these patients. It provides, in an increasing number of cases, the opportunity to patients and physicians to have a

clear support both for therapy (individualized rehabilitation approach, etiological therapies) and for counseling.

OBIECTIVE

The primary objective of this study was to analyze the diagnostic yield of next-generation sequencing stroke panels in selected subjects with pediatric or perinatal stroke.

Secondary objective was to perform massive sequencing (WES) of trios (proband and parents) in selected cases, negative on the NGS panel, to identify new disorder causing single genes leading to ischemic stroke and to study the variant segregation of the corresponding variants within families.

METHOD

Patients selection

We enrolled patients referred for diagnostic work-up from 2015 to 2018 to Gaslini Children Hospital (Genoa, Italy), who presented with a pediatric or neonatal onset AIS of unknown etiology. The study was conducted according to the ethical standards laid down in the 1964 Declaration of Helsinki and approved by the Internal Review Board.

Before participation, written informed consent was obtained from all parents or legal guardian.

Patients up to18 years were considered eligible when presenting one or more of the following inclusion criteria:

i) Single or multiple AIS with cerebral arteriopathy;

ii) Multiple asynchronous AIS without cerebral arteriopathy;

iii) Single or multiple AIS with multi-system arteriopathy (with or without cerebral arteriopathy)

iv) Single or multiple porencephaly with AIS and/or brain calcifications with strong family history of AIS in childhood;

v) Single or multiple asynchronous AIS in syndromic patients.

Exclusion criteria included:

i) Previous intracranial radiotherapy;

ii) Underlying conditions known to be risk factors for stroke (such as leukemia, cardiac, and other hematologic disorders);

iii) Post-infective stroke with or without focal cerebral arteriopathy (such as post-varicella angiopathy);

iv) Post-traumatic arterial dissection in patients with no sign of collagen disease.

Moreover, patients with Moyamoya disease were excluded since already included in another research project of our Institution. (Raso A, 2016)

The DNA of all the eligible patients has been stored in the a DNA repository dedicated to pediatric stroke in the Medical Genetic laboratory of the Giannina Gaslini Institute.

All patients underwent extensive hematological evaluation, to rule out major thrombophilia and sickle cell anemia, and cardiac evaluation with ECG and echocardiography (transthoracic and/or transesophageal), to exclude congenital and acquired heart diseases. Due to their high frequency in the normal population, patent foramen ovale and minor thrombophilia were not considered exclusion criteria for the genetic study.

Array-CGH analysis was performed in selected cases when a syndromic condition caused by copy number variations was suspected.

Clinical and radiological data collection

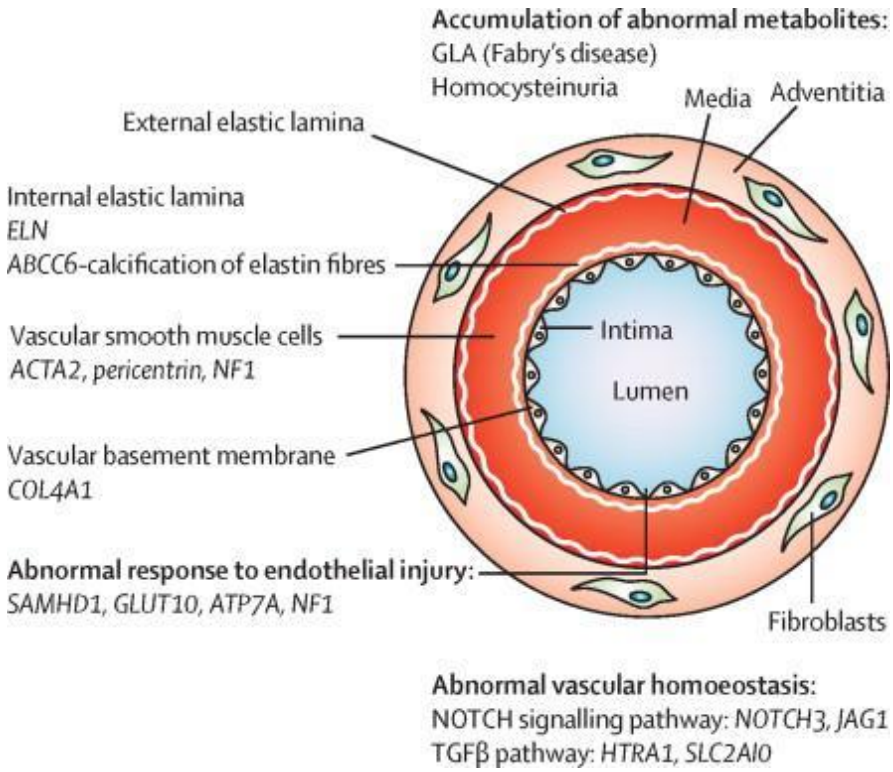
Data of eligible patients were collected from paper and/or electronic medical records. Clinical data revision focused particularly on age and signs/symptoms at onset and clinical features at the age at genetic analysis.

Brain MR studies were acquired using 3T or 1.5T MRI systems. Imaging protocols varied but always included axial T1, T2 and FLAIR images, diffusion-weighted imaging (DWI), susceptibility-weighted imaging (SWI), and intracranial MR angiography (MRA). Three-experienced pediatric neuroradiologist (MS; DT; AR) reviewed neuroradiological images. Evaluation of MRIs included the presence and localization of AIS, cerebral and/or multi-system arteriopathy, vessel and parenchymal calcifications, as well as additional brain anomalies.

Genetic Analysis

DNA was extracted from blood samples by using the kit QIAamp DNA blood (Qiagen), purified with Amicon® Ultra-0.5 Centrifugal Filter Devices and quantified with NanoDrop 2000. Patients were analyzed by the NGS approach using a customized gene panel designed through the Ion AmpliSeq designer software (http://www.ampliseq.com/browse.action) and containing 15 known genes, namely ABCC6, ACTA2, ATP7A, CBS, CECR1, CLO4A1, ELN, GLA, HTRA1, JAG1, NF1, NOTCH3, PCNT, SAMHD1, SLC2A10.

Figure 2. Main pathogenic mechanism of the 15 genes included in the NGS panel



For each of the 15 genes Table 2 reports OMIM numbers, corresponding phenotypes, and their mode of inheritance, as well as GenBank ID, number of exons, and number of base pairs in the input target and in the designed panel, whose difference corresponds to missed target base pairs and amounts to a total of 1438bp. Therefore, the experimental design represents 98.30% of the input target. Sanger sequencing covered missed regions in suspected genes. Libraries preparation and successive DNA sequencing on the Ion Torrent Personal Genome Machine PGM™ (ThermoFisher), were carried out according to ThermoFisher protocols.

Once the whole target was captured by means of 441 multiplex amplification reactions, subdivided into two PCR pools (223+218 amplicons), not all the amplicons could be amplified with the same efficiency. Those resulted with a coverage less than 10X were analyzed through Sanger sequencing. Overall, the mean coverage of the whole panel among the 38 samples was 356.61X (Table 2).

Table 2. genes responsible for simple Mendelian conditions sharing predisposition to pediatric AIS and designed gene panel

GENE	OMIM gene #	OMIM #: clinical phenotype	Inh.	GenBank ID	Coding exons	Covered (%)	Target Sequenced (bp)
ABCC6	603234	614473: Arterial calcification, generalized, of infancy, 264800: Pseudoxanthoma elasticum 177850: Pseudoxanthoma elasticum, forme fruste	AR	NM_ 001171	31	100	10975
ACTA2	102620	611788: Aortic aneurysm, familial thoracic 614042: Moyamoya disease 613834: Multisystemic smooth muscle dysfunction syndrome	AD	NM_ 001141945.2	8	100	2572
ATP7A	300011	309400: Menkes disease 304150: Occipital horn syndrome 300489: Spinal muscular atrophy, distal, X-linked	AR	NM_ 000052.6	22	100	8051
CBS	613381	236200: Homocystinuria, B6-responsive and nonresponsive types 236200 Thrombosis, hyperhomocysteinemic	AR	NM_ 001178008.2	15	100	4822
CECR1	607575	182410: Sneddon syndrome 615688: Polyarteritis nodosa, childhood-onset	AR	NM_ 001282225.1	9	100	3985
COL4A1	120130	180000: Retinal arteries, tortuosity of 611773: Angiopathy, hereditary, with nephropathy, aneurysms, and muscle cramps 607595: Brain small vessel disease with or without ocular anomalies 175780: Porencephaly 614519: Hemorrhage, intracerebral, susceptibility to	AD	NM_ 001845.5	52	100	15586
ELN	130160	123700: Cutis laxa 185500: Supravalvar aortic stenosis	AD	NM_ 001278939.1	34	100	9401
GLA	300644	301500: Fabry disease 301500: Fabry disease, cardiac variant	AR	NM_ 000169.2	7	100	2151
HTRA1	602194	600142: CARASIL syndrome 616779: Cerebral arteriopathy, autosomal dominant, with subcortical infarcts and leukoencephalopathy, type 2 610149: Macular degeneration, age-related, 610149: Macular degeneration, age-related, neovascular type	AR	NM_ 002775.4	9	94,98	3098
JAG1	601920	118450: Alagille syndrome 187500: Tetralogy of Fallot	AD	NM_ 000214.2	26	99,82	8451
NF1	613113	607785: Leukemia, juvenile myelomonocytic 162210: Neurofibromatosis, familial spinal 162200: Neurofibromatosis, type 1 601321: Neurofibromatosis-Noonan syndrome 193520: Watson syndrome	AD	NM_ 001042492.2	58	99,04	20291
NOTCH3	600276	615293: Myofibromatosis, infantile 125310: Cerebral arteriopathy with subcortical infarcts and leukoencephalopathy	AD	NM_ 000435.2	33	83,99	11461

		130720: Lateral meningocele syndrome					
PCNT	605925	210720: Microcephalic osteodysplastic primordial dwarfism, type II	AR	NM_ 006031.5	47	99,37	19084
SAMHD1	606754	614415: Chilblain lupus 612952: Aicardi-Goutieres syndrome	AD	NM_ 015474.3	16	100	4955
SLC2A10	606145	208050: Arterial tortuosity syndrome	AR	NM_030777. 3	5	98,09	2747

Bioinformatics analysis

FastQ data were analyzed by the Ion Reporter 5.0. The total variants were filtered based on the frequency in the general population reported in the databases 1000 Genomes (http://www.internationalgenome.org) and Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org/). Variants were assessed by the Ion ReporterTM Software 5.0 and a custom pipeline was additionally optimized to filter in significant variants. In particular, variants were selected based on their frequency in the general population (lower than 5% or unreported), for their impact on the encoded protein (missense, stop loss and stop gain, frameshift, and splicing variants at ±2bp from the exon ends), and for functional prediction (*in silico* analysis through online softwares, i.e. http://www.varsome.org) and/or previous pathogenicity classification (http://ww.clinvar.org). Variants thus selected were validate by standard Sanger sequencingin case they were never reported or reported as potentially damaging / damaging, their segregation was successively checked in parents, when available, and, finally, they were assessed on the basis of the clinical phenotype of probands.

To confirm the presence of the selected variants, primers were designed with Primer3Plus (https://primer3plus.com/) and aPCR protocol was set up for each variant. PCR products were purified by ExoSAP-OT (GE Healthcare) and directly sequenced by using Big Dye V.1.1 through a ABI3130 automated sequencer (Applied Biosystems, Foster City, California, USA).

RESULTS

Patients

Of the 172 patients included in the biobank (Table 3) thirty-eight subjects (23 males and 15 females) were considered eligible, based on the inclusion and exclusion criteria described above.

Table 3. Demographic features of patient in *Gaslini Stroke Biobank.*

	Total	M (%)	F (%)	Age* (SD)
Total patients	**172**	**102 (59,3)**	**68 (39,53)**	**6,95 (10,6)**
Total stroke patients	165	100 (60,61)	65 (39,39)	6,87 (10,77)
Unconfirmed diagnosis	7	4 (57,14)	3 (42,86)	
Total Perinatal	**83**	**51 (61,45)**	**32 (38,55)**	**2,54 (2,84)**
Perinatal	43	31 (72,09)	12 (27,91)	2,27 (3,17)
Presumed perinatal	27	12 (44,44)	15 (55,56)	3,58 (2,21)
Fetal	13	8 (61,54)	5 (38,46)	1,28 (2,24)
Pediatric	**75**	**47 (62,67)**	**28 (37,33)**	**7,64 (5,08)**
Adult	**7**	**2 (28,57)**	**5 (71,43)**	**50 (17,42)**

* Age of the subject at the time of blood sampling for molecular study by NGS

Eleven out of these 38 (28.9%) had a perinatal AIS onset while all the others were pediatric AIS. The mean age of at onset was 6.5 years (range: 0.2-12.5) in infantile cases while 7.1 month (range 0.2-14 month) was the mean age at onset in perinatal/presumed perinatal cases. Hemiparesis was the most common neurological symptom at onset in patients with both pediatric and perinatal stroke (55.6% and 45.4% of cases respectively), followed by seizures in either groups (33.3% and 36.3% of cases respectively). While headache, visual loss and speech disorders were quite common symptoms at onset also in our pediatric patients (14.8%, 14.8%, and 11.1%, respectively), they were never reported as symptoms at onset in our cohort of

perinatal stroke. Two patients with pediatric stroke presented an episode of inconsolable crying at the onset, in both cases associated with other focal neurologic symptoms.

Two patients (1 with perinatal stroke and one with pediatric stroke) died, 1.1 year and 4 months after the first ischemic event, respectively.

The mean age at molecular genetic analysis (NGS) was 6.5 years (range: 1 month to - 16.5 years).

Of the 27 pediatric patients, 11 subjects (40.7%) had a multisystem vascular involvement: 7 of those (25.9%) had persistent hypertension, moderate to severe, due to nephrovascular involvement; 6 patients (22.2%) had cutaneous vascular involvement (from intermittent forms of livedo reticularis to severe cutis marmorata). Surprisingly, none of them presented vascular retinal involvement, to date. However, in patients with genetic diagnosis confirmed through the NGS panel, an eye screening program has been started, aimed at identifying early eyes involvement.

To date, only one of the patients diagnosed with perinatal or presumed perinatal stroke (patient #33) has presented a multisystem vascular involvement, specifically, corneal haemorrhages and hypertension, probably of nephrovascular origin.

Three patients, with pediatric stroke, had associated suspicious characteristics for an underlying immunological disease (severe recurrent infections, generalized lymphadenopathy and / or chronic systemic inflammatory condition). None of these was genetically diagnosed with the NGS panel.

Neuroradiology

As regards the neuroradiological features of the selected case, 23/38 (60.52%) patients had multiple AISs (14/23 with negative NGS and 10/15 with positive NGS), 5/38 (13.15%) had single AIS (2/23 with negative NCG and 3/15 with positive NGS), 7/38 (18.42%) patients had porencephalic cavities (4/23 with negative NGS and 3/15 with positive NGS; one patient had also multiple AHS), and 3/38 (7.89%) patients had only an arteriopathy (all with negative NGS). Three patients out of 38 (7.89%) had also associated AHSs (2/23 with negative NGS and 1/15 with positive NGS). Calcifications were present in 6 out of 38 patients (15.78%).

Of the 24 multiple AISs, 17 (70.8%) cases were asynchronous (7/10 with positive NGS and 10/14 with negative NGS) and 3 (12.5%) cases were synchronous (2/14 with negative NGS and 1/10 with positive NGS). In 4/24 cases (16.6%) it was not possible to establish if the AIS were synchronous or asynchronous, since they were all detected in the chronic phase (2/14 with negative NGS and 2/10 with positive NGS).

Beside the presence of AIS, additional MRI abnormalities were observed in 9 out of 38 subjects (32.14%). Four out of 38 patients (10.52%) showed white matter lesions (multifocal or diffuse), while the other 4/38 (10.52%) showed a Chiari I anomaly. One patient had multisutural craniosynostoses. A moyamoya-like arteriopathy was noted in 6/38 (15.78%) cases (1/15 with positive NGS and 5/23 with negative NGS).

Cytogenetic analysis and other genetic analysis

Cytogenetic analysis has been performed in patient with associated abnormalities (dysmorphic features, malformation in another organ system) or with psychomotor delay before the first stroke or when cognitive delay was unlikely justified by the location or extent of the ischemic lesion.

Karyotype has been performed in nine patients (23.7%); only one of those, patient #28, had abnormalities (45 X0/47 XXY//46 XY), probably not related to the clinical phenotype.

Array comparative genomic hybridization (aCGH) has been performed in six patients (15.8%); two of those had structural aberrations.

In patient #22 has been identified a microduplication (12q24; 32q24.33); inherited from the healthy mother.

In patient #1 (Figure 3) have been found two duplication 12p13.31p12.3 inherited from the mother (with a history of stroke at a young age) and 11p14.2p14.1 inherited from the father (suffering from chronic pyelonephritis, secondary to urinary tract malformation). Both genetic rearrangements are also identified in the eldest sister. in the large region of the chromosome 12 is included GRIN2B, that could explain the mental retardation of the child and of the sister. Other genes (APOLD1 and ORL1), also contained in the same region, could predispose to thrombotic events; however, the role can not be defined precisely as that the older sister has never presented similar events.

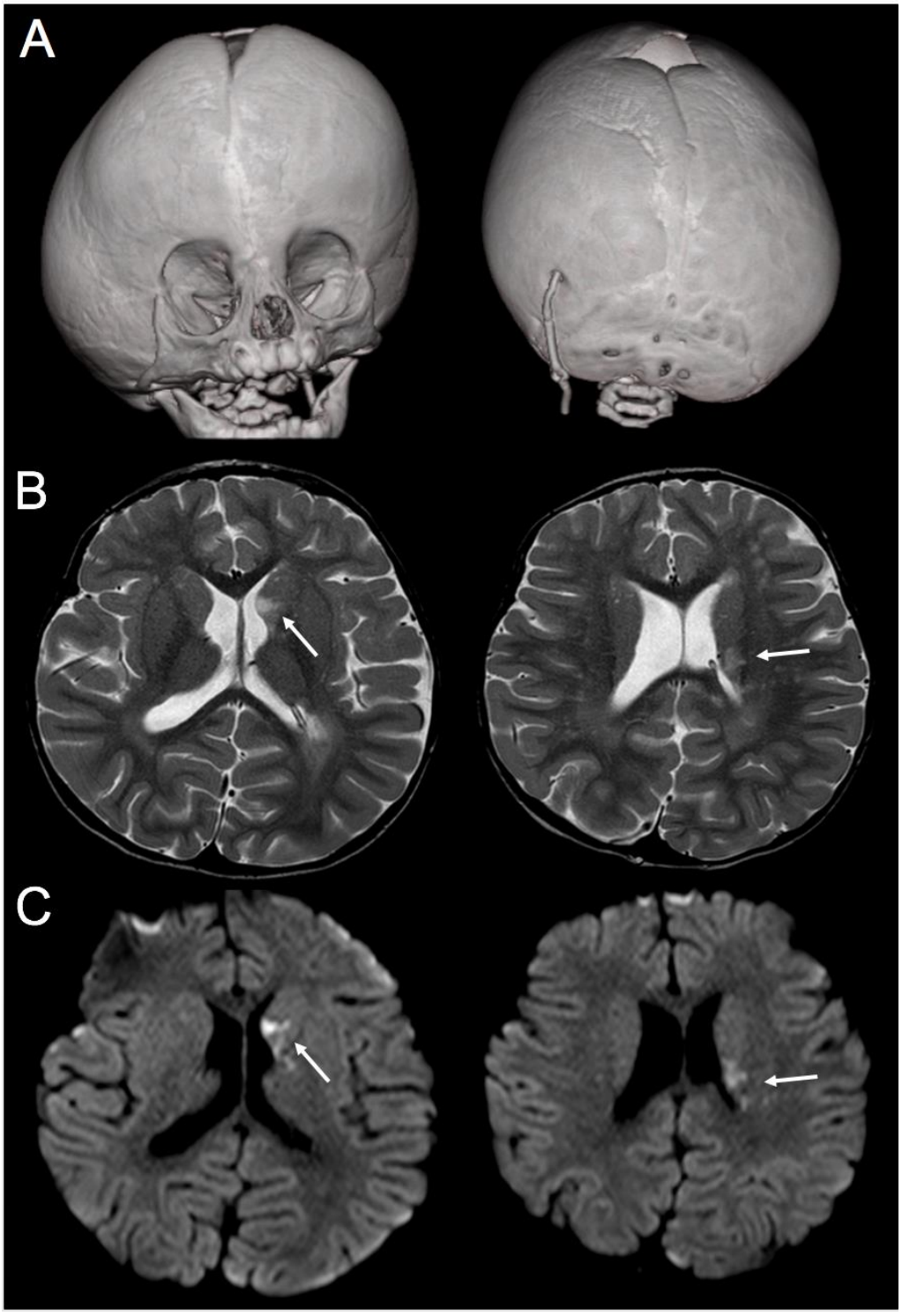


Figure 3. Neuroimaging of patient #1. A) Brain CT with 3D reconstruction. B, C) Brain MRI with multiple unilateral synchronous AISs in left MCA perforating arteries territory, without arteriopathy; multifocal bilateral WM lesions and ventricular dilatation

In two patients (#21 and #17), with suspect of Williams syndrome, has been performed FISH analysis in order to identify the submicroscopic deletion, at 7q11.23, responsible for allelic loss of elastin (ELN). Both patients results negative to the FISH analysis.

The search of the main molecular thrombophilias (Factor II G20210A and Factor V Leiden) was performed in 31 patients (81.6%). In 2 patients, both with perinatal stroke, the presence of minor thrombophilia was detected (in the patient #15 a heterozygotic mutation G20210A of factor II and in the patient #29 a mutation in heterozygosity of factor V Leiden have been identified). Both mutations could have had a role as a co-risk factor in the genesis of the ischemic event.

Patient #10, with clinical diagnosis of Sotos Syndrome, has a NSD1 *de novo* mutation in intronic splicing site (never described before). To date there are no data in the literature that report an association between Sotos syndrome and early strokes; therefore, this mutation was not considered an exclusion criterion for the NGS panel, hypothesizing a possible concausality of two distinct mutations.

Patient #27 carried out, at another center, an NGS panel aimed at the study of collagenopathies (including COL3A1, COL5A1, COL1A2, FBN1, TGFBR1, TGFBR2, MYH11, SMAD3, FBN2, MYLK, NOTCH1, TGFB2); the molecular analysis showed in exon 40 of COL1A2 gene the presence in heterozygosity of the sequence variation c.2465G> A, which results in the amino acid substitution p.Arg822His. This variant is not included in the databases available to date (i.e. ClinVar, HGMD) and is predicted of uncertain significance. Moreover, the same variant is inherited from the healthy mother.

Patient #7 has previously performed the study of the RNF213 gene, known as related to the disease of moyamoya with autosomal dominant mode. The analysis shows the presence of a single mutation, whose segregation is being validated. The presence of a RNF213 mutation, has not been considered a criterion of exclusion from genetic analysis with NGS panel, not being able to justify the clinical phenotype of the patient.

Genetic Analysis

The mean sequencing coverage depth was 356.61X, with a mean number of variants called of 144.13, and a mean of 4.49 number of variants filtered according to given criteria, namely to be investigated further. In total, 86 variants were identified, 66 of which were called only once while the other 20 were called repeatedly, from 2 to 20 times. Of those, 56 variants resulted with an allele frequency<0.05 and reasonable quality (p-value<0.01 and no distorted allelic ratio). Finally, among variants undergone Sanger sequencing validation, Table 3 reports only patients with carrying only validated (true positive) variants. Indeed, after validation and familial testing, no significant variant was left in 23 patients, that can therefore be considered as "genetically negative" for the conditions tested. Conversely, variants found in the 15 patients reported in Table 4 were taken into account as potentially accounting for their clinical phenotype and followed up with the assessment of their *de novo*/inherited nature, effect prediction through the online tools quoted in the previous Materials and Methods section, and variant allele's population frequencies.

Table 4. List of patients with validated variants.

Patient ID	GENE	cDNA	Protein	dbSNP (#rs)	Allele	AR/AD	Parental segregation	VARSOME	CLINVAR	CADD/ PHRED	MAF ExAC	Classification
3	COL4A1	c.2132G>A	p.Gly711Glu	-	Het	AD	De novo	VUS	Absent	21,4	Absent	P/LP
17	ABBC6	c.2900G>A; c.4182_4182delG	p.Trp967Ter; p.Lys1394fs	-; rs67791546	Comp Het	AR/AD?		P; P	Absent	49	Absent; 0.0000258	P/LP
	CBS	c.1111G>C	p.Val371Leu	rs372010465	Het	AR		LP	P	22,3	0.00002536	P/LP
20	ABCC6	c.2097G>T	p.Glu699Asp	rs72653784	Het	AR/AD?	NA	P	P	23,5	0.00000827	P/LP
38	ABCC6	c.1171A>G	p.Arg391Gly	rs72653762	Het	AR/AD?	Inherited by father	VUS	VUS/LP/P	23,5	0.006129	VUS
16	COL4A1	c.7C>A	p.Pro3Thr	rs751749989	Het	AD	Inherited by father	VUS	/	14,61	0.0002006	VUS
2	PCNT	c.9661C>T	p.Arg3221Trp	rs141304187	Het	AR	Inherited by father	VUS	/	22,7	0.0001239	VUS
10	ATP7A	c.2452A>G	p.Thr818Ala	rs201788154	Het	X-Link		VUS	B/LB	23,6	0.0003306	VUS
15	CECR1	c.154C>G	p.Leu52Val	-	Het	AR	NA	VUS	/	22,7	0.00002474	VUS
21	NOTCH3	p.V1952M	p.V1952M	rs115582213	Het	AD		VUS	B/LB	27,3	0.008494	VUS
25	CBS	c.65A>G	p.His22Arg	rs763151207	Het	AD		VUS	VUS	2,957	0.04419	VUS
22	ABBCC6	p.Arg1064Trp	p.Arg1064Trp	rs41278174	Het	AR/AD?	Inherited by father	VUS	B	26,4	0.02058	VUS
6	COL4A1	c.911C>T	p.Pro304Leu	rs34843786	Het	AD	NA	VUS	/	16,6	0.001112	B/LB
12	CECR1	p.Met309Ile	p.M309I	rs146597836	Het	AD		LB	LB	12,26	0.001977	B/LB
14	CECR1	c.799A>C	p.Lys267Gln	-	Het	AD	Inherited by the mother	VUS	/	10,88	/	B/LB
19	ABCC6	c.2224A>G; c.2171G>A	p.Ile742Val + p.Arg724Lys	rs59593133 ; rs58073789	Comp Het	AR/AD	De novo	LB	B	4,019 ; 5,62	0,0183 ; 0,01501	B/LB

Clinical and genetic diagnosis

Based on clinical, neuroradiological and laboratory findings, an etiological hypothesis was put forward in 17 patients, expecting mainly COL4A1 but also PCNT, ACTA2, CBS, ADA2 and ELN mutations, while the 20 others were classified as affected by an undetermined condition. NGS analysis provided results in 15 out of 38 patients (39.5%), leading to a definite etiological diagnosis in 4 of them showing pathogenic or likely pathogenic variants. Of the remaining 11 cases, 6 subjects harbored variants of unknown significance while 5 patients had benign or likely benign variants. Due to predicted low impact (either benign, likely benign, or variants of unknown significance) and inconsistent clinical features, genetic inheritance, and/or neuroradiological patterns, these mutations were not further pursued (Tables 4 and 5). Ultimately, only 2 out of 17 patients with an already suspected diagnosis and 2 out of 21 patients with an undetermined condition received a genetic diagnosis. Clinical, neuroradiological and histological data (whenever available) are detailed in Table 5 for the 15 patients with positive NGS and in Table 6 for the remaining 23 patients.

Table 5. Clinical, neuroradiological and histological data of the 15 patients with positive NGS

ID	sex	gene	Variant	genotype-phenotype correlation	AIS onset	neuroradiological features	Neurological Symptoms at onset	Neurological Symptoms at last FU	Associated clinical and histological features	gene suspected	Family hystory	array CGH or other genetic tests
3	M	COL4A1	P/LP	yes	Perinatal (2 days)	Multiple bilateral AHS, porencephaly and deep brain calcifications; no arteriopathy	Epileptic seizure, cataract, micro-ophthalmia	Multi drug-resistant epilepsy, hypertension, hyper CPK, severe psychomotor delay	Born SGA	COL4A1	Negative	-
17	M	ABCC6 CBS	P/LP P/LP	yes no	Pediatric (2.5 yrs)	AIS in the left MCA territory, non-inflammatory MCA arteriopathy and bilateral segmental ICAs agenesis with carotid rete mirabile	Right hemiparesis and multi drug-resistant hypertensive crisis.	Mild psycomotor delay, severe right hemiparesis, mild hypertension.	Multisystemic arteriopathy (infrarenal aorta, iliac arteries…) femoral artery biopsy with absent elastin in vessel wall	ELN	Negative	Normal karyotype 46XY. FISH 7q11.23 negative. Factor II e Factor V Leiden negative

20	M	ABCC6	P/LP	yes	Pediatric (5.2 yrs)	Multiple bilateral asynchronous AIS in the left PCA and right PICA arteries, with multiple asynchronous VA dissections	Neck muscle cramp, headache and visual hallucination, right hemiparesis, speech disorders	None	Mild ligamentous hyperlassity	-	Mother with recurrent flebitis,	FII and FV Leiden negative.
38	M	ABCC6	P/LP	yes	Pediatric (10.8 yrs)	Multiple unilateral asynchronous AIS in the left PICA and PCA perforators associated with multiple stenosis of the V3 and P1/P3 segments due to multiple dissections	Severe headache, visual loss and dizziness	Improvement of headache after aspirin start	Mild hypermocystein emia.	-	Negative	FII and FV Leiden negative.
16	M	COL4A1	VUS	yes	Presumed Perinatal (1.2 yrs)	Unilateral AIS in the left ACA territory and right white matter lesions	Mild right hemiparesis, neuropsycho motor develo pmental delay	Mild right hemiparesis			Negative	FII and FV Leiden negative.
2	M	PCNT	VUS	no	Presumed Perinatal (4 month)	Unilateral fronto-parietal porencephaly, hemosiderin deposits and deep calcifications.	Mild right hemiparesis	Mild right hemiparesis		COL4A1	Negative	FII and FV Leiden negative.
10	M	ATP7A	VUS	no	Pediatric (10 yrs)	Multifocal asynchronous AISs in the PCA territory	Tremors, develop-mental delay	Mild developmental delay	Sotos syndrome, Reflux nephropathy	-	Negative	NSD1 *de novo* mutation in intronic splicing site. FII and FV Leiden negative.
15	M	CECR1	VUS	no	Presumed Perinatal (2 month)	Porencephaly and deep brain calcifications.	Apparent Life treathing event	Febrile status epilepticus (1 epidose)		COL4A1	Negative	FII mutation in eterozygosis. Factor V Leiden negative.
21	M	NOTCH3	VUS	no	Perinatal (1 month)	Unilateral multiple synchronous AISs in left MCA territory and Chiari I anomaly	Hypotonia and epileptic seizures	None	Neonatal hypercalcemia, hypercalciuria and nephro-calcinosis	ELN	Negative	Karyotype normal 46XY, FISH negative for microdeletions
22	F	ABCC6	VUS	no	Pediatric (5 month)	Multiple bilateral asynchronous AISs in MCA, PCA and ACA territories in progressive (early onset) multifocal cerebral arteriopathy	Epileptic status, right hemiparesis and psychomotor regression.	Spastic tetraparesis, axial hypotonia; severe cognitive delay.	Multisistem arteriopathy (renal and cutaneous). macrocrania, dysmorphism. Long QT syndrome, intestinal malrotation. Temporal artery biopsy with absent elastin in the vessel wall	-	Negative	FII and FV Leiden negative. Kariotype 46 XX, CGH Array: microduplication (12q24; 32q24.33) of maternal origin
6	M	COL4A1	B/LB	no	Pediatric	Bilateral microischemic lesions in the hemispheric white matter and in the fronto-parietal cortex.	Headache.	Improvement of headache after aspirin start	Spherocytosis (splenectomized at the age of 3 yrs), mild hypermocystein emia.	-	Mother heterozygous for drepanocytosi s. Father has spherocytosis	FII and FV Leiden negative.

12	F	CECR1	B/LB	no	Pediatric (4 month)	Synchronous AISs in MCA territory in unilateral moyamoya-like arteriopathy; Chiari type I and lipoma of the quadrilateral lamina	Inconsolable crying, left arm hyposthenia and mildly depressed level of consciousness	Left hemiparesis, developmental delay		'	Negative	FII and FVLeiden negative.
14	F	CECR1	B/LB	no	Pediatric (10 month)	Multiple bilateral asynchronous AISs in MCA, ACA, PCA territories in early onset rapidly progressive multifocal cerebral arteriopathy	Inconsolable crying, epileptic seizures, left hemiparesis.	Dead (14 month)	Multi drug-resistant systemic hypertension in multisystemic progressive vasculopathy. Absence of actin in the medial tunica of medium and large-size vessels.	ACTA2	Paternal cousin died 5-year-old due to stroke of unknown origin Paternal aunt with repeated venous thrombosis	FII and FV Leiden negative.
19	F	ABCC6	B/LB	no	Pediatric (10.9 yrs)	Multiple bilateral asynchronous AISs in BA and PCA perforators territories (thalamic, mesencephalic) in multifocal cerebral inflammatory arteriopathy (posterior circulation)	Right hemiparesis and paresthesia	None	Severe hepato-splenomegaly and generalized lympho-adenopathy, corneal deposits, itiotic skin, joint retraction and arthritis, short stature, reduced HDL level.	'	second-cousins parents	No mutations in ABCA1, NPC1, NPC2, PSAP, SMPD1, PIM-1. FII and FV Leiden negative.
25	M	CBS	B/LB	no	Pediatric (3 yrs)	Multiple bilateral asynchronous AISs in MCA and PCA perforators territories (Basal ganglia and thalamus) without arteriopathy	Left hemiparesis and dysarthria	None	suspected immune system dysregulation (severe recurrent infections in early childhood)	'	sister with idiopathic liver fibrosis. Father, grandmother and paternal aunts with gastric cancer	FII and FVLeiden negative.

Pathogenic or likely pathogenic mutations

Among the 4 patients harboring P/LP mutations, three presented ABCC6 variants: one in compound heterozygosis (patient #17) and 2 in heterozygosis (patients #20 and #38).

In particular, patient #17 (Figure 4), presenting at 2 years of age with an AIS associated with multidrug resistance hypertension and multisystemic arteriopathy, harbored two rare heterozygous ABCC6 mutations (c.2900G>A; p.Trp967* and c.4182_4182delG; p.Lys1394Asnfs*9), each transmitted from one parent. This allowed the diagnosis of pseudoxanthoma elasticum (PXE) before the manifestation of any skin or ocular signs, as we previously reported (Bertamino M, 2018).

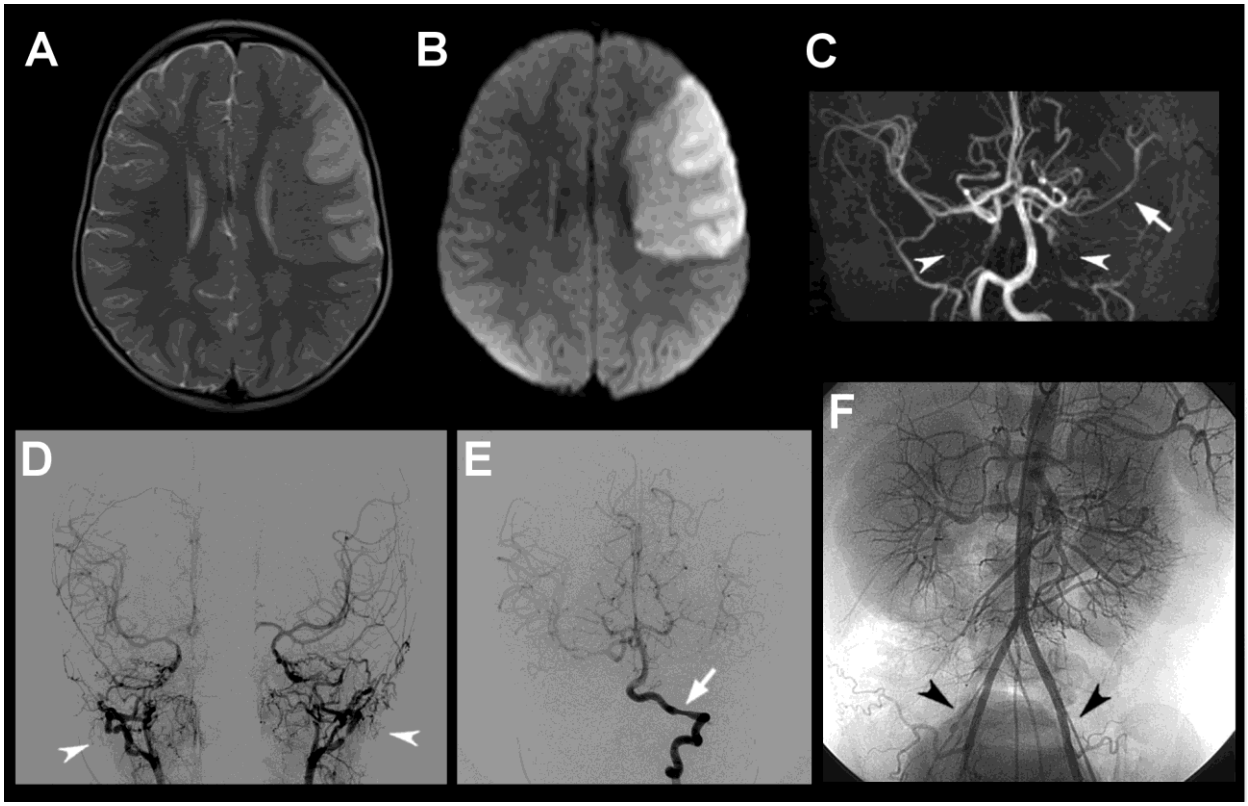


Figure 4. Neuroimaging of patient #17. Brain MRI (A, B), MRA (C) and Digital Angiography (D,E) with AIS in the left MCA territory, non-inflammatory MCA arteriopathy and bilateral segmental ICAs agenesis with carotid rete mirabile. Abdominal MRA (F) with evidence of a multisystem stenotizing arteriopathy (iliac arteries).

The other two patients #38 (Figure 6) and #20 (Figure 7), respectively harboring heterozygous p.Arg391Gly and p.Glu699Asp mutations, presented with a similar clinico-radiological phenotype characterized by multiple asynchronous AISs due to multiple dissections, in the absence typical PXE features (skin and ocular) and/or collagenopathy signs.

Finally, patient #3 (Figure 7) presented a *de novo* likely pathogenic c.2132G>A (p.Gly711Glu) mutation associated with a clinical and radiological picture suggestive for a Collagen IV-related disorder. He was born small for gestational age and experienced seizures in the second day of life. Afterwards, he presented a multidrug-resistant epilepsy associated with cataracts and intermittent marked increase plasmatic creatine kinase values (CK).

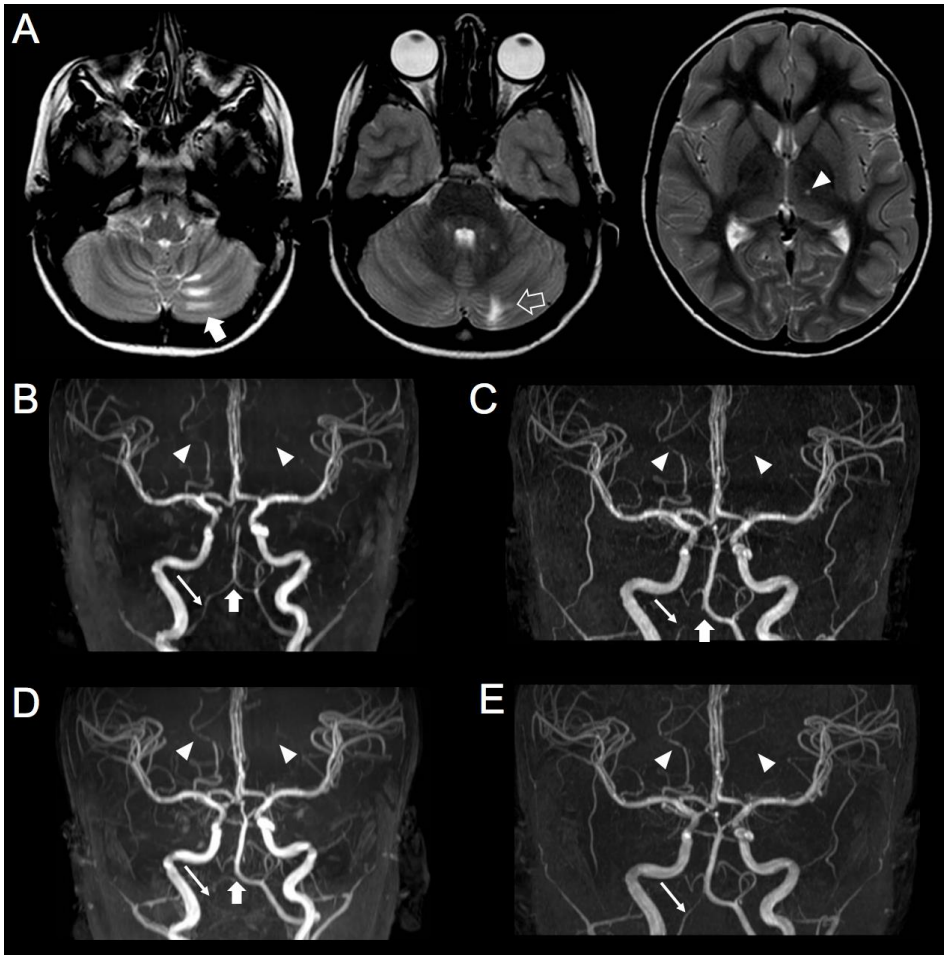


Figure 5. Neuroimaging of patient #38. Brain MRI and MRA with multiple unilateral asynchronous AIS in the left PICA and PCA perforators associated with multiple stenosis of the V3 and P1/P3 segments due to multiple dissections

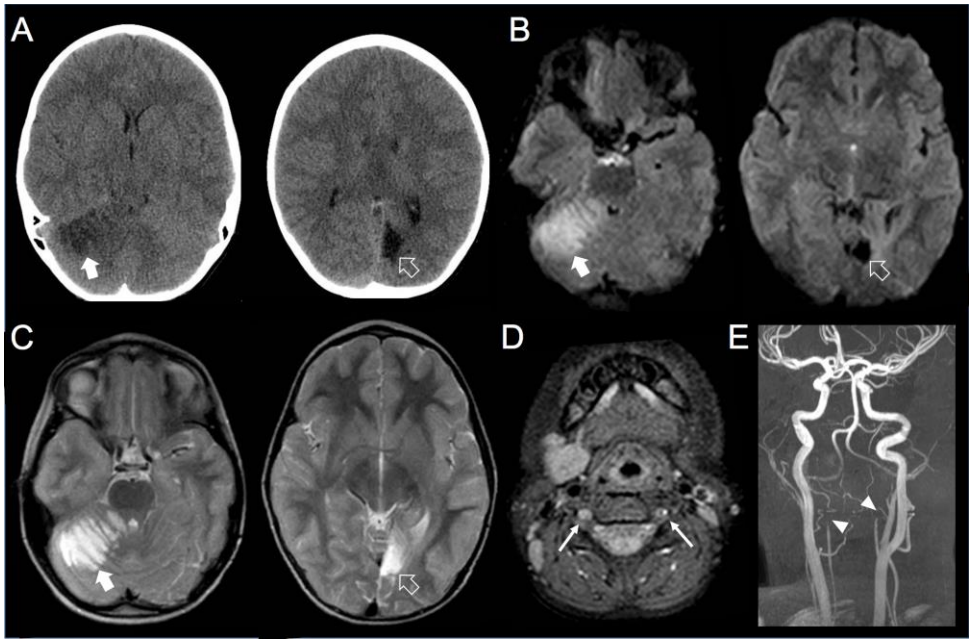


Figure 6. Neuroimaging of patient #20. Brain CT, MRI and MRA with multiple bilateral asynchronous AIS in the left PCA and right PICA arteries, due to multiple asynchronous VA dissections

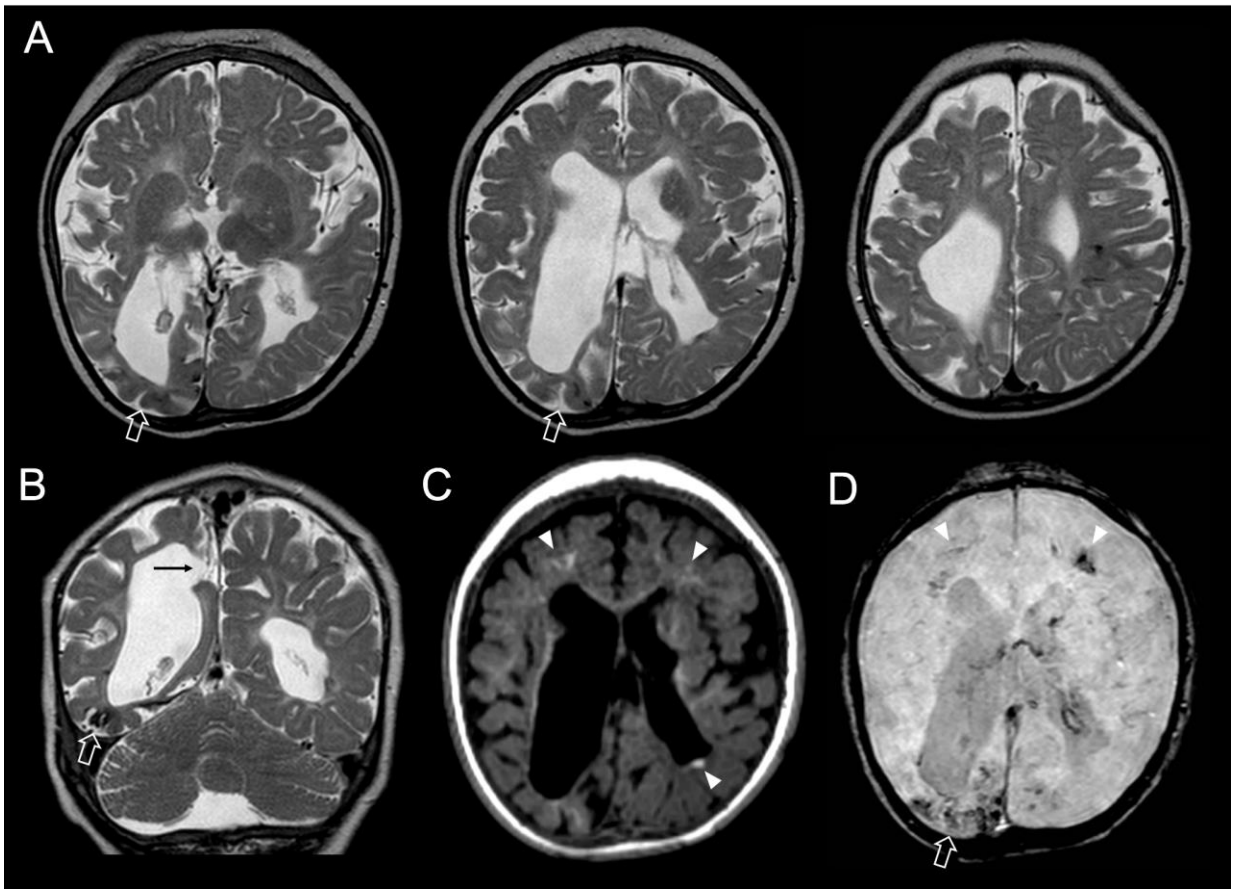


Figure 7. Neuroimaging of patient #3. Brain MRI Multiple bilateral AHS, porencephaly and deep brain calcifications.

Variants of unknown significance (VUS)

Six patients presented variants of unknown significance in different genes. Three patients (#2, #10 and #15) harbored heterozygous VUS in PCNT, ATP7A and CECR1 genes, respectively, related to autosomal recessive disorders. Moreover, their clinical and radiological phenotype was not consistent with any of these conditions.

Patient #2 and #15 had a porencephalic cavity with deep calcifications, neuroradiological findings compatible with COL-IV related disorder; patient #10 diagnosed with Sotos syndrome has been previously described.

Patients #21 presenting with neonatal AIS and hypercalcemia with hypercalciuria harbored a NOTCH3 mutation not consistent with the clinical phenotype.

Patients #22 was shown heterozygote for the ABCC6 p.Arg1064Trp but presented with a very severe phenotype characterized by intestinal malrotation, cutis marmorata and early onset and

rapidly progressive multisystemic arteriopathy causing multiple AISs. Due to the severity and complexity of the clinico-radiological picture, this patient was addressed to whole exome sequencing (WES) analysis.

Patient #16 harbored a p.Pro3Thr variant in COL4A1 inherited from the healthy father and showed a presumed perinatal AIS associated with nonspecific contralateral white matter lesions.

Benign or likely benign variants

NGS demonstrated benign or likely benign variants in CECR1, COL4A1, ABCC6, and CBS, in five patients. A single mutation in CECR1, related to an autosomal recessive disorder (ADA2 deficiency), has been found in patient #12 and #14.

Patient #12 had a non-inflammatory cerebrovascular involvement, moyamoya-like, without other signs of systemic involvement.

Patient #14 had early onset rapidly progressive multifocal cerebral and multisystemic arteriopathy, hesitated in the patient's death, a few months after the first stroke due to systemic malignant hypertension and multi organ failure. Due to the severity and complexity of the clinico-radiological picture, this patient was addressed to WES analysis.

A single mutation in CBS, related to an autosomal recessive disorder, has been found in patient #25. This male subject had multiple bilateral AISs without vasculopathy. Anamnestic history revealed a suspected regulation of the immune system. Plasma homocysteine repeatedly normal.

Patient #19 was found to carry two heterozygous ABCC6 variants, namely p.Ile742Val and p.Arg724Lys. This female patient, has a multifocal cerebral inflammatory arteriopathy with associated severe hepatosplenomegaly and generalized lymphadenopathy, corneal deposits, itiotic skin, joint retraction and arthritis, short stature, reduced HDL level. Due to the

phenotypic complexity and the known consanguinity of the parents (second cousins), the patient was referred to WES analysis.

Patient #6 presented with severe headaches with detection on brain MRI of multifocal and asynchronous cortical AISs. The patient was affected by hereditary spherocytosis and a moderate hyperhomocysteinemia. Both of these findings could be considered co-risk factors but were not sufficient to cause multiple AIS in childhood. On the other hand, neither the detected COL4A1 variant (p.Pro304Leu) was responsible of the clinico-radiological phenotype.

Genetically unclassified patients

Clinical and radiological features of those 23 patients resulted with negative NGS are reported in Table 6.

Table 6. Clinical, neuroradiological and histological data of the 23 patients with negative NGS

ID	Sex	AIS onset	Neuroradiological features	Neurological Symptoms at onset	Neurological Symptoms at last FU	Associated clinical and histological features	gene suspected	Family hystory	array CGH or other genetic tests
1	**M**	Pediatric (2.1 yrs)	Multiple unilateral synchronous AISs in left MCA perforating arteries territory, without arteriopathy; multifocal bilateral WM lesions; cranyostoses, ventricular dilatation and Chiari I anomaly	Absence seizures and psychomotor delay.	Moderate psychomotor delay. Generalized hypotonia and ligamentous hyperlassity	Facial dysmorphism		Unrelated parents. Sister with psychomotor delay and negative Brain MRI	CGH Array: duplication 12p13 inherited from mother and 11p14 inherited from father. FII and FV Leiden negative.
4	**F**	Pediatric (1.2 yrs)	Multiple bilateral asynchronous AISs in multifocal cerebral arteriopathy (anterior)	Right hemiparesis.	seazures, right hemiparesis	Systemic hypertension. Skin biopsy with deficiency of collagen type I		Consanguineous parents. Sister with asymptomatic multifocal brain vasculopathy.	
5	**M**	Pediatric (12.5 yrs)	Multiple asynchronous and rapidly progressive AIS/AHS in BA territory (brainstem) without arteriopathy; focal cerebellar hemosiderin deposit (suspect cavernoma); right cerebellar developmental venous anomaly	Ataxia and dysarthria	Normal cognitive development, residual generalized hypotonia, paraparesis, dysartria and severe dysphagia.	None		Unrelated parents. Father suffering from hypertension and hypercholesterolemia. Sister with history of mild hemorragic diatesis.	FII and FV Leiden negative.
7	**M**	Pediatric (1.2 yrs)	Multiple bilateral asynchronous AISs in the MCA territories with moyamoya-like arteriopathy (ant+post circulation) and straight appearance of MCAs; leuco-encephalopathy	Left hemiparesis	Mild left hemiparesis.	Systemic hypertension.	ACTA2	Mother with mild intellectual disability and epilepsy.	RNF213 isolated mutation.

8	**F**	Pediatric (UNK)	Multiple unilateral AISs in right ACA, MCA and PCA territories (cortical-subcortical regions) without arteriopathy; Chiari I anomaly	None	None	Congenital scoliosis due to cervico-dorsal vertebral malformations	Unrelated, healthy parents.	FII and FV Leiden negative.
9	**M**	Pediatric (UNK)	Unilateral non-inflammatoy focal arteriopathy (left terminal ICA)	Headache	Transitory ischemic attack (paresthesia, right upper limb weakness, dysarthria).	Benign neonatal convulsions (Brain MRI negative). Marfanoid habits, pectus excavatum. Chronic thyroiditis.	Unrelated, healthy parents.	FII and FV Leiden negative.
11	**F**	Pediatric (1.1 yrs)	Unilateral synchronous AISs in right MCA territory with moyamoya-like arteriopathy	Left hemiparesis and absence seizures	Unilateral Clode-Bernard-Orner Syndrome and left hemiparesis	Congenital hip dysplasia, laryngo-malacia.	Unrelated, healthy parents. Aunt with malformations of ICA and renal artery, aneurysm of the interatrial septum	Cariotype 46,XX normal; CGH Array negative. FII and FV Leiden negative.
13	**M**	Pediatric (5.9 yrs)	Multiple bilateral asynchronous AISs in bilat PCA and right PICA territories with unilateral dissective arteriopathy (intra- and extracranial VA)	Headache and right hemiparesis	None	Marfanoid hab its.	CBS	Early onset thromboembol ic events in maternal family. Mother homozygous for a MTHFR mutation	FII and FVLeiden negative.
18	**F**	Pediatric (8 month)	Multiple bilateral asynchronous AIS in ACA and MCA territories in early onset and rapidly progressive moyamoya-like arteriopathy	Seizures and right hemiparesis	tetraparesis and moderate psychomoto r delay	Connatal macrocrania, delay in the acquisition of head control. Livedo reticularis intermittent.	ACTA2	Unrelated, healthy parents.	Cariotype 46,XX normal. Array CGH negative. MELAS negative. FII and FVLeiden negative.
23	**F**	Pediatric (4 month)	Multiple bilateral synchronous AISs in left PCA and right thalamo-geniculate artery (PCA branch) with distal left PCA focal non-inflammatory arteriopathy; bilateral multifocal white matter alterations	Partial sezures and nystagmus	None			Unrelated, healthy parents.	FII and FV Leiden negative.

24	**F**	Pediatric (8 month)	Bilateral porencephalic cavities with calcifications of the globi pallidi	Syncopal episode	Lagophthalmo and mild right-eye adduction deficiency	Chilblains and livedo reticularis intermittent. Chronic renal failure. Previous cardiopathy.		Probably related, healthy parents.	
26	**M**	Presumed perinatal	Left fronto-parietal porencephaly; no arteriopathy	Mild right hemiparesis	Mild right hemiparesis	Connatal macrocrania	COL4A1	Maternal aunt with post-hemorrhagic hydrocephalus dead at 5 yrs; first-degree paternal cousin with fetal AIS/HS and poroencephaly	Cariotype 46,XY normal. FII and FV Leiden negative.
27	**M**	Pediatric (4 yrs)	Multiple bilateral asyncronous AISs in the MCA territories associated with unilateral MCA arteriopathy	Right hemiparesis and aphasia	Learning disability (dyslexia) performance anxiety.	Systemic hypertension. Marfanoid habits.	CBS	Unrelated, healthy parents.	FII and FV Leiden negative. COL2A1 mutation inherited from mother.
28	**M**	Pediatric (5 yrs)	Multiple unilateral AISs in the right MCA and PCA territories without arteriopathy; microcephaly; brainstem malformation; pituitary gland malformation	Epileptic seizure, psycomotor delay	Drug-resistant epilepsy; severe RPM.	Pituitary dwarfism, Seckel-like facies, microcrania, clinodactyly, hypospadias.	PCNT	UNK	Katiotype 45X0/47XXY//46XY, CGH array negative
29	**F**	Presumed perinatal	Right posterior frontal porencephaly	Loss of consciousness, cyanosis and generalized hypotonia episode. Mild left hemiparesis	Mild left hemiparesis		COL4A1	Unrelated, healthy parents. First-degree paternal cousin with perinatal stroke.	FII negative, heterozygosis FV Leiden.
30	**F**	Pediatric (5.8 yrs)	Multiple asynchronous AIS in the right MCA and left PCA perforators (right caudate head, left thalamus) with BA inflammatory stenosis; focal brain hemosiderin deposit	Right hemiparesis and ipsilateral visual loss	Right eye strabismus.	Livedo reticularis and steroid-dependent systemic autoinflammatory diseases (dilated myocarditis, panniculitis).	ADA2		FII and FV Leiden negative.

31	**M**	Presumed Perinatal (1.1 yrs)	Hemorrhagic porencephaly; diffuse leukoencephalopathy	Mild right hemiparesis	Epileptic seazures, right hemiparesis		COL4A1	Paternal grandfather with early ischemic cerebrovascular disease	FII and FV Leiden negative.
32	**M**	Pediatric (3 month)	Progressive leukoencephalopathy with brainstem involvement and punctate calcifications; bilateral moyamoya-like arteriopathy (ant and post)	Generalized seazures	Epilepsy	Muscle biopsy normal.			FII and FV Leiden negative.
33	**F**	Perinatal	Multiple bilateral asynchronous AHS related to AVMs associated with severe hydrocephalus	Apnea, Difficulty sucking	Death	Cataract, corneal haemorrhages and hypertension. IBDH deficiency.	COL4A1		In corso pannello con HHT
34	**M**	Perinatal (2 days)	Single unilateral AIS in the left MCA territory associated with ICA dissection	Partial sezures	None		ACTA2	grandmother with aortic dissection	FII and FV Leiden negative.
35	**M**	pediatric (2.5 yrs)	Multiple asynchronous AIS in the territories of both MCAs without arteriopathy	Left arm paresis	None				FII and FV Leiden negative.
36	**F**	Perinatal	Focal non-inflammatory stenosis of the P1 segment of the right PCA; microcephaly	Slow wave activity on EEG	slow wave activity on EEG	Dwarfism and cardiac interatrial defect	PCNT		FISH and CGH Array negative.
37	**F**	Pediatric (5 month)	Multiple asynchronous AIS and AHS in left MCA and PCA territories with bilateral moyamoya-like arteriopathy	Generalized seazures and right hemiparesis	Epilepsy and mild right hemiparesis	Sistemic hypertension and livedo reticularis.		Father with demyelinating disease	FII and FV Leiden negative.

Six of the 23 negative patients (26.1%) were perinatal or presumed perinatal stroke. Four of those were considered eligible at the NGS panel, in the suspicion of a COL4A1-related disease. Patient #26 and patient #29 were included because of a porencephalic cavity associated with a strong family history of pediatric or perinatal stroke. Patient #31 was included in the study for the detection of a porencephalic cavity, associated with a diffuse leukoencephalopathy. Patient #33 has also been studied in the suspicion of COL4A1 mutation in the light of the brain-MRI

with multiple asynchronous hemorrhagic stroke, associated with neonatal cataracts and retinal hemorrhages. Parallel to this investigation, the study of genes for familial hemorrhagic telangiectasia was started, in the light of multiple arteriovenous malformations (AVMs).
Two patients (patient #36 and patient #28) were included in the suspicion of a PCNT related disorders; both present a highly suggestive clinical and neuroradiological phenotype for a microcephalic primordial dwarfism. It is also known that this phenotype can be explained by mutations other than PCNT, namely ATR, CDC6, CDT1, CENPJ, CEP152, CEP63, ORC1, ORC4, ORC6, RBBP8, RNU4ATAC . Both of these patients were addressed to further molecular diagnostic investigations, once a chromosomal aberration was excluded through cytogenetic analysis.
Eight patients, 7 of those with pediatric stroke, had a multisystemic vasculopathy, 5 of those (patients #4, #7, #27, #33 and #37) had associated hypertension, three had cutaneous involvement (patients #18, #24 and #30), one (patient #33) has retinal haemorrhages, has already mentioned.
Three negative patients (patients #9, #13 and #27), had a non-inflammatory vasculopathy (cerebral with/without systemic involvement) and a marfanoid habitus.
The patient #32 has a non-inflammatory cerebral vasculopathy associated with alterations of the green matter, similar to a syndrome of Leight, which, moreover, has been excluded in the proband. Given the peculiarity of the neuroradiological pattern, the patient was subsequently directed to WES.

One of the remaining 7 patients had a perinatal stroke due to left internal carotid artery (ICA) dissection with family history of aortic dissection (patient #34); this patient was referred to the panel in the suspicion of a ACTA2-related disease.

Two subjects (patient #8 and #35) had multiple asynchronous pediatric AISs without arteriopathy; the first of the two, female, had associated a congenital scoliosis due to cervico-dorsal vertebral malformations.

Patient #11 had a peculiar phenotype characterized by congenital hip dysplasia and laryngomalacia, she had seizures and hemiparesis, to which a Claude Bernard Horner syndrome was add, due to unilateral synchronous AISs in right middle cerebral artery (MCA) territory with moyamoya-like arteriopathy.

Patient #23 had a bilateral non-inflammatory arteriopathy of the posterior circulation with associated bilateral multifocal white matter alterations; to date no signs of multisystem vascular involvement have occurred.

Patient #5 had a pediatric onset of multiple asynchronous and rapidly progressive ischemic and hemorrhagic stroke in posterior circulation without arteriopathy.

Lastly, the patient #1, previously genetically described in the chapter of cytogenetic investigations, has multiple unilateral synchronous AISs without arteriopathy, associated with multifocal bilateral white matter lesions, multi-sutural cranyosinostoses with ventricular dilatation and Chiari I anomaly.

In patient #17 has successfully been defined following, the detection of two heterozygous ABCC6 mutations (c.2900G>A; p.Trp967* and c.4182_4182delG; p.Lys1394Asnfs*9), each transmitted from one parent. This has, allowed to diagnose pseudoxanthoma elasticum (PXE) before the manifestation of any skin sign, as already reported (Bertamino M, 2018). Additional mutations were detected at the ABCC6 gene: patient #19 was found to carry two heterozygous ABBC6 variants, namely p.Ile742Val and p.Arg724Lys, while patients #22, #38, and #20 were shown heterozygotes for the ABCC6 p.Arg1064Trp, p.Arg391Gly, and p.Glu699Asp mutations, respectively, the two latter ones transmitted by asymptomatic fathers (Table 2).

Interestingly, patient #20 carries a pathogenic mutation that, though expected to associate with no PXE, may have contributed to his phenotype.

Mutations of the ATP7A, CECR1, NOTCH3, PCNT, and CBS genes were also detected. However due to the predicted low impact effect and to the inconsistent clinical features and neuroradiological patterns, these mutations were not further pursued (Tables 5 and 6).

Genetically unclassified patients

Clinical and radiological features of those 23 patients resulted with negative NGS are reported in Table 4. Qui va descritta secondo me la tabella 4, riportando il tipo di info che vi si possono trovare e eventuale ricorrenza tra I pazienti di sintomi rari o particolari o altra considerazione che aiuti a inquadrare questa casistica negative … I commenti nella discussione

Six of the 23 negative patients (26.1%) were perinatal or presumed perinatal stroke. Four of those were considered eligible at the NGS panel, in the suspicion of a COL4A1-related disease. Patient #26 and patient #29 were included because of a porencephalic cavity associated with a strong family history of pediatric or perinatal stroke. Patient #31 was included in the study for the detection of a porencephalic cavity, associated with a diffuse leukoencephalopathy. Patient #33 has also been studied in the suspicion of COL4A1 mutation in the light of the brain-MRI with multiple asynchronous hemorrhagic stroke, associated with neonatal cataracts and retinal hemorrhages. Parallel to this investigation, the study of genes for familial hemorrhagic telangiectasia was started, in the light of multiple arteriovenous malformations (AVMs).

Two patients (patient #36 and patient #28) were included in the suspicion of a PCNT related disorders; both present a highly suggestive clinical and neuroradiological phenotype for a microcephalic primordial dwarfism. It is also known that this phenotype can be explained by mutations other than PCNT (included in our NGS panel), namely ATR, CDC6, CDT1, CENPJ, CEP152, CEP63, ORC1, ORC4, ORC6, RBBP8, RNU4ATAC . Both of these patients were addressed to further molecular diagnostic investigations, once a chromosomal aberration was

excluded through cytogenetic analysis.

Eight patients, 7 of those with pediatric stroke, had a multisystemic vasculopathy, 5 of those (patients #4, #7, #27, #33 and #37) had associated hypertension, three had cutaneous involvement (patients #18, #24 and #30), one (patient #33) has retinal haemorrhages, has already mentioned.

Three negative patients (patients #9, #13 and #27),had a non-inflammatory vasculopathy (cerebral with/without systemic involvement) and a marfanoid habitus.

The patient #32 has a non-inflammatory cerebral vasculopathy associated with alterations of the green matter, similar to a syndrome of Leigh, which, moreover, has been excluded in the proband. Given the peculiarity of the neuroradiological pattern, the patient was subsequently directed to WES.

WES IN PEDIATRIC STROKE

Massive sequencing (WES) of selected cases, arisen negative at the above described NGS panel, has been performed to identify one or more new genes involved in the pathogenesis of pediatric stroke and to study variants segregation within families.

The whole trios (proband plus parents), or the enlarged nuclear family if more subjects are presenting a compatible disease phenotype, has been included in the WES analysis.

A pathway analysis and other both in silico and experimental approaches, useful to identify biological mechanisms affected in the disorder(s) and to confirm the gene(s) involvement, have been scheduled.

WES in patients affected with stroke, with no mutation(s) accounting for their phenotype, has been carried out in-house, by taking advantage of the Ion Proton TM (ThermoFisher) platform, available in our Institute.

To date, we have already performed WES analysis in four cases.

Patient #22

First WES has been performed in Patient #22 and her healthy non-consanguineous parents, finding compound heterozygous mutations, confirmed by Sanger sequencing, in the ABCG2 gene (Rusmini et al, unpublished).

Patient #22 had cutis marmorata at birth, macrocrania and mild facial dysmorphism, a bilateral syndactyly, intestinal malrotation and a diagnosis of long QT syndrome. First cerebral stroke was referred at the age of 5 months, with subsequent severe progression of cerebral vasculopathy. From the age of 5 years, she had a severe nephrovascular arterial hypertension in

the context of a multisystem vasculopathy, definable as fibromuscular dysplasia, as confirmed by arterial biopsy.

The CGH Array of patient #22 showed micro-duplication of the long arm of chromosome 12 (12q24; 32q24.33); this rearrangement was inherited from the healthy mother.

The mutated ABCG2 gene is a member of the ATP-binding cassette (ABC) transporter superfamily that actively export drugs and toxins from the cells, to limit adverse effects of xenotoxins 13. These variants, annotated for the transcript ENST00000237612, are p.I456V (no rs number available) and p.M514K (rs752530602). The two mutations may be pathogenic as

i) p.I456V has never been described before and

ii) p.M514K has been reported only in 1/121190 alleles in the ExAC database. In addition, both variants are predicted to have a high CADD (Combined Annotation-Dependent Depletion, http://cadd.gs.washington.edu/) score (>13), recently regarded as a reliable index of variant pathogenicity.

Despite the absence of published data regarding the involvement of this gene in arteriopathies, a pathway search of ABCG2 through the PathwayNet software (http://pathwaynet. princeton.edu/) showed a link with the HIF1 (Hypoxia-inducible factor-1) -alpha transcription factor network, consistent with the role of low oxygen levels in stimulating the production of angiogenic factors. Under conditions of normoxia, HIF-1 alpha is cytoplasmic and is rapidly degraded by the ubiquitin proteosome system. In mammalian cells, reduced oxygen levels, as it occurs in specific districts during embryogenesis, permit the accumulation of HIF-1 alpha protein in the cytoplasm. Subsequently, it translocates to the nucleus, engages HIF-1 beta, and forms the HIF-1 complex that initiates VEGF transcription and mRNA stabilization.

Functional studies are nearing completion to confirm the role of the pathogenic mutation in the genesis of the phenotype of the subject.

Patient #14

Second WES has been performed in Patient #14 and her healthy parents; parental consanguinity is suspected, having found a homozygous mutation of a gene whose product functions as a guanine nucleotide exchange factor for a small GTPase protein regulating signaling pathways downstream of integrins and growth factor receptors.

Patient #14 was born at term of a normal pregnancy; auxological parameters at birth were within normal limits. Absent dysmorphism. Well-being and normal psychomotor development up to 10 months when he presented the first stroke, with evidence of a multifocal stenosing vasculopathy (involving at first aorta and medium-sized intracranial arteries). At the age of 11 month was detected a severe hypertension, multi drug resistant, with bilateral renal artery rapidly progressive narrowing. Stenosing vascular disease it has also spread to other districts such as the supra aortic trunks, iliac and femoral artery. She experienced multiple arterial ischemic stroke despite aggressive anticoagulant and steroid treatment and death after 3 month of hospitalization due to cardiac impairment and multi organ failures.

The mutation detected in Patient #14 appears to play a role in the reorganization of the actin cytoskeleton in endothelial cells.

Further functional studies are underway to confirm the pathogenicity of the mutations found and the role in the genesis of the phenotype of the subject.

Patient #4

Third WES has been performed in Patient #4, her younger sister and their healthy related parents. Both sisters are affected by a multifocal stenosing cerebral vasculopathy; the patient #4

experienced multiple arterial ischemic stroke while the younger sister is currently asymptomatic.

Patient #4 has an early onset systemic hypertension. A skin biopsy was performed with the finding of a non-specific collagen type-I deficiency.

To date, no other signs or symptoms of systemic vascular involvement in the sister are present.

In the aforementioned family the WES study has already been done, the analysis is underway, and some candidate genes are being highlighted.

Patient #4 and her sister were sent to our attention from another center. A multidisciplinary evaluation is planned for the two patients at our center, for an accurate clinical evaluation and an advanced neuroradiological study. These data will be useful in the interpretation of the results of the WES and to better address the functional studies.

Patient #32

The last WES analysis, carried out on patient #32 and his non-consanguineous parents, is still ongoing.

The father of the proband is healthy while the mother has a malignant liver tumor. Both parents and the older brother do not show signs or symptoms of vascular or cerebrovascular disease.

Patient #32 was born at term of a normal pregnancy; auxological parameters at birth were within normal limits. Absent dysmorphism. Well-being and normal psychomotor development. At the age of 4 months he experienced epileptic seizures in apyrexia; brain MRI revealed an early onset moyamoya-like cerebrovasculopathy with a very peculiar, slowly progressive, alterations of the deep gray substance. Such alterations had led to think of a Leigh syndrome, but the metabolic profiling and muscle biopsy excluded this hypothesis.

DISCUSSION

The complexity of the different clinical phenotypes belonging to the Arterial Ischemic Stroke (AIS) spectrum is not fully accounted for by the genes and other risk factors identified so far. In particular, pediatric AIS differs from adult AIS in many respects, including the nature of the main risk factors. Indeed, while Mendelian genetic factors contribute more likely to the infantile onset, environmental and genetic predisposing risk factors may play a major role in adult stroke. Therefore, very different genetic approaches are required in the two age groups. It is generally thought that monogenic strokes have a unique phenotypic pattern and that individual mutations predispose to specific stroke subtypes [Markus HS]. However, at least in pediatric forms, none of the clinical or neuroradiological findings is specific of a given arteriopathy subtype, and the genotype-phenotype correlation apparently expand to include more genes and wider clinical spectra [Dlamini N]. This implies that single-gene testing cannot be informative enough to be used especially in a diagnostic setting, besides being too expensive and time-consuming. With reasonable sequencing costs and improved bioinformatics approaches for data analysis, NGS is increasingly used in the diagnostic workup of many pediatric diseases [Dlamini N, Abou Tayoun AN, Tonduti D]. In this study, we applied a targeted NGS technology to the parallel screening of multiple genes in children with idiopathic AIS. To avoid including a very heterogeneous set of pediatric AIS patients, we decided to focus on a precise subset based on definite inclusion criteria and with a likely genetic etiopathogenesis (i.e., familiarity, multiple lesions, multisystem vascular involvement, no post-infective or post-traumatic events). To this end, patients were selected after a careful multidisciplinary approach, including specialized clinical evaluations (physiatric, infant neuropsychiatric, psychological, cardiological, genetic), and instrumental examinations, such as electrophysiological studies and advanced neuroimaging. Despite this relatively restrictive approach, the study population remained quite heterogeneous, reflecting the complexity and diversity of the associated risk factors in children with AIS compared to the adult population. In

addition, we also decided to restricte the NGS based panel to a limited number of genes known to be responsible of simple monogenic disorders sharing stroke as one of their most common and early manifestation, including genes involved in vascular development, homoeostasis, and response to environmental factors in the pathogenesis of pediatric AIS and cerebral arteriopathies. [Munot P, McCrea N]

The present exploratory study has demonstrated the feasibility of a clinical and genetic integration by targeted NGS in the clinical workflow of pediatric and perinatal AIS of unknown etiology. In our cohort, the targeted 15-gene panel has increased the diagnostic rate of monogenic conditions associated with AIS to 10.5%, with a very relevant impact on the clinical management of patients thus diagnosed.

In particular, among positive cases we found three patients, either heterozygotes (two) or compound heterozygote (one) for *ABCC6* mutations, while one patient harbored a *COL4A1* variant. Mutations in ABCC6 are responsible for pseudoxanthoma elasticum (PXE), a rare autosomal recessive disorder characterized by the accumulation of mineralized and/or fragmented elastic fibers in the skin, eyes, vascular walls, and gastrointestinal system. Patients with PXE commonly present with skin papules and/or retinal angioid streaks, usually around the second decade of life, while vascular lesions and cerebrovascular complications are rarer but may cause severe neurologic impairment [Germain DP]. Remarkably, the patient with compound heterozygous ABCC6 mutations presented an atypical and severe phenotype characterized by early AIS associated with multi-system arteriopathy and a compensating arterial network, called carotid rete mirabile, as previously reported [Bertamino M]. In this case, NGS-based gene panel testing allowed early PXE diagnosis even in the absence of typical skin and ocular manifestations, thus leading to a timely genetic counseling, targeted therapeutic and prevention measures. Conversely, the two other patients were heterozygotes for two different missense ABCC6 mutations and presented with multiple spontaneous cervical artery dissections (sCAD) without typical skin or ocular PXE features. Of note, recent genetic studies

in young adults with sCAD have shown the presence of small deletions encompassing ABCC6 [Morcher M], while heterozygous ABCC6 variants have been described in patients with aortic dissections [Schulz V]. Moreover, histology studies in subjects with sCAD have demonstrated aberrations restricted to the elastic fiber system in the reticular dermis [Uhlig P] resembling our observation of symptomatic heterozygotes of recessive PXE, consistent with autosomal dominant transmission of forme fruste PXE (OMIM#177850), already reported with reduced penetrance [Struk B, vanSoest S].

Remarkably, in none of the cases harboring ABCC6 variants was the diagnosis suspected before genetic testing. Conversely, among the eight patients originally suspected for a collagen IV-related disease, only one received a genetic confirmation. This discrepancy may be related to the fact that the COL4A2 gene, encoding the alpha-2 chain of type IV collagen, was not included in the NGS panel, thus reducing the diagnostic yield of the study. The clinical spectrum of COL4A1 and COL4A2-related disorders is overlapping and includes small-vessel brain disease variably associated with eye defects (retinal arterial tortuosity, Axenfeld-Rieger anomaly, cataract) and systemic findings (kidney involvement, muscle cramps, cerebral aneurysms, Raynaud phenomenon, cardiac arrhythmia, and hemolytic anemia). On imaging, small-vessel brain disease causes diffuse periventricular leukoencephalopathy, lacunar infarcts, microhemorrhage, dilated perivascular spaces, and deep intracerebral hemorrhages. Moreover, porencephaly and transmantle lesions, causing hydranencephaly or schizencephaly, have also been reported.

The majority of patients in the present study remained without a genetic diagnosis, including several patients with cerebral or multisystem arteriopathies. In some instances, this might be explained by the high variability and complexity of the clinical-radiological phenotype of these patients, often including multisystemic involvement and additional syndromic or inflammatory features. These patients represent very good candidates to search for new genes through Whole

Exome Sequencing (WES) [Bamshad MJ], and a large specific gene panel for in silico analysis has already been designed and proposed [Sawyer SL].

The present study has several limitations. First, idiopathic AIS is a rare condition, accounting for about 10% of all pediatric strokes; thus, the small sample size did not allow the creation of homogeneous and reasonably large subgroups based on clinical and neuroradiological phenotypes. A tertiary pediatric centers network would allow to collect a larger amount of cases. Secondly, the number of genes included in the panel was quite limited compared to the wide phenotypic heterogeneity of the patient series. A larger and more comprehensive gene panel might improve the molecular diagnosis and therefore the clinical management of pediatric and perinatal stroke (including the hemorrhagic forms) [McCrea N].

In conclusion, the present pilot study has demonstrated a modest but satisfactory increased diagnostic rate of monogenic conditions associated with pediatric AIS, widening the phenotypic spectrum of ABCC6-related disorders and improving the genetic counseling and clinical management of positive patients. We plan to improve the present multidisciplinary and innovative approach to pediatric idiopathic AIS by updating the NGS-based gene panels and applying WES as a second-line approach to selected negative patients. This might pave the way for future larger multicentric studies, thus shedding more light on the complex pathophysiology/genetic bases of this rare condition.

CONCLUSION

Identification of a genetic basis for pediatric and perinatal stroke through NGS will strongly contribute to the full knowledge of perinatal and pediatric stroke.

This will improve the management of the patients, enabling identification of effective therapies, targeted rehabilitation and, ideally, prevention of the disease.

The use of NGS technologies represents the future of the genetic diagnosis and could become the most suitable and feasible diagnostic option also in pediatric stroke, after the exclusion of more common etiology. Presently, gene panel testing is the favored choice for genetic-stroke diagnosis due to the lower cost and higher coverage of the technology. However, as the price for WES and WGS continues to decrease, they will soon be fully integrated into the diagnostic setting, providing a wider range of diagnostic options for clinicians.

REFERENCES

A. Al-Jarallah, M. A.-R. (2000). Nontraumatic brain hemorrhage in children: etiology and presentation. *J Child Neurol*, pp. 284-289.

Adam Kirton, G. D. (2015). Paediatric stroke: pressing issues and promising directions. *The Lancet Neurology*, doi.org/10.1016/S1474-4422(14)70227-3.

Adam L. Numis, C. K. (2014). Arterial Ischemic Stroke in Children: Risk Factors and Etiologies. *Curr Neurol Neurosci Rep.*, doi: 10.1007/s11910-013-0422-8.

Agerholm-Larsen B., Tybjaerg-Hansen A., Frikke-Schmidt R., Grønholdt M.L., Jensen G., Nordestgaard B.G. (1997) ACE gene polymorphism as a risk factor for ischemic cerebrovascular disease. *Ann. Intern. Med.* 127(5):346–355.

Ahamad Hassan, H. S. (2000). Genetics and ischaemic stroke. *Brain*, Volume 123, Issue 9, Pages 1784–1812.

Ahmad A. (2006). Genetics of cerebral venous thrombosis. *J Pak Med Assoc*, 56(11):488-90.

Benjamin Briggs, K. N. (2018). Novel Factor XIII variant identified through whole-genome sequencing in a child with intracranial hemorrhage. *Cold Spring Harb Mol Case Stud.*, pii: a003525. doi: 10.1101/mcs.a003525.

Bergen AA. (2006) Pseudoxanthoma elasticum: the end of the autosomal dominant segregation myth. J Invest Dermatol. 126:704–5.

Bertamino M, M. S. (2018). ABCC6 mutations and early onset stroke: Two cases of a typical Pseudoxanthoma Elasticum. *European Journal of Pediatric Neurology*, Volume 22, Issue 4, Pages 725–728.

Beslow LA, J. L. (2010). Pediatric stroke: the importance of cerebral arteriopathy and vascular malformations. . *Childs Nerv Syst.*, 26:1263-1273.

Cambien F., Poirier O., Lecerf L., Evans A., Cambou J.P., Arveiler D., Luc G., Bard J.M., Bara L., Ricard S. (1992) Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature.* 359(6396):641–644.

Carvalho KS, G. B. (2002). Arterial strokes in children. *Neurol Clin.* , 20(4):1079-100, vii.

Casas J.P., Hingorani A.D., Bautista L.E., Sharma P. (2004) Meta-analysis of genetic studies in ischemic stroke: thirty-two genes involving approximately 18,000 cases and 58,000 controls. *Arch. Neurol.* 61(11):1652–1661

Christen-Zäch S, Huber M, Struk B, Lindpaintner K, Munier F, Panizzon RG, Hohl D. (2006) Pseudoxanthoma elasticum: evaluation of diagnostic criteria based on molecular data. Br J Dermatol;155:89–93

Chrystoja C. C., D. E. (2014). Whole genome sequencing as a diagnostic test: challenges and opportunities. . *Clin. Chem.* , 60, 724–733. 10.1373/clinchem.2013.209213.

Cotlarciuc I, M. T. (2016). Towards the genetic basis of cerebral venous thrombosis—the BEAST Consortium: a study protocol. *BMJ Open*, doi:10.1136/bmjopen-2016- 012351.

Daniel S. Tszee, J. H. (2011). Pediatric Stroke: A Review. *Emerg Med Int.* , doi: 10.1155/2011/734506 .

deVeber GA, K. A. (2017). Epidemiology and Outcomes of Arterial Ischemic Stroke in Children: The Canadian Pediatric Ischemic Stroke Registry. *Pediatr Neurol.*, pii: S0887-8994(17)30075-9.

Dlamini N. (2018). Next generation genetic considerations in paediatric stroke. *European Journal of Pediatric Neurology*, Volume 22, Issue 4, Pages 577–578.

Dlaminia N, B. L. (2010). Cerebral Venous Sinus (Sinovenous) Thrombosis in Children. *Neurosurg Clin N Am.*, 21(3-5): 511–527.

Felling RJ, S. L. (2017). Pediatric arterial ischemic stroke: Epidemiology, risk factors, and management. . *Blood Cells Mol Dis.*, 67:23-33.

Ferro J.M., M. A.-L. (2010). Aetiological diagnosis of ischaemic stroke in young adults. . *Lancet Neurol.*, 9:1085–1096.

FJ, K. (2003). Is there a genetic basis for pediatric stroke? *Curr Opin Pediatr.*, 15(6):547-58.

Fullerton, H. W. (2007). Risk of recurrent childhood arterial ischemic stroke in a population-based cohort: the importance of cerebrovascular imaging. . *Pediatrics.* , 119: 495–501.

Gabis L. V., R. Y. (2002). Time lag to diagnosis of stroke in children. *Pediatrics*, vol. 110, no. 5, pp. 924–928.

Greenham M, G. A. (2016). Outcome in Childhood Stroke. *Stroke*, ;47:1159-1164. DOI: 10.1161/STROKEAHA.115.011622.

Guido J. Falcone, D. W. (2017). Genetics of Spontaneous Intracerebral Hemorrhage. *stroke*, 48(12): 3420–3424. doi:10.1161/STROKEAHA.117.017072.

Hornstrup LS, Tybjærg-Hansen A, Haase CL, Nordestgaard BG, Sillesen H, Grande P, Frikke-Schmidt R. (2011) Heterozygosity for R1141X in ABCC6 and risk of ischemic vascular disease. *Circ Cardiovasc Genet.* 4(5):534-41.

Ichord RN, B. S.-G. (2015). Paediatric cerebral sinovenous thrombosis: findings of the International Paediatric Stroke Study. . *Arch Dis Child*, 100:174–9. doi:10.1136/archdischild-2014-306382.

Janet Lee, M. L. (2005). Maternal and Infant Characteristics Associated With Perinatal Arterial Stroke in the Infant. *JAMA*, Vol 293, No. 6 .

Lionel AC, C. G. (2018). Improved diagnostic yield compared with targeted gene sequencing panels suggests a role for wholegenome sequencing as a firstetier genetic test. *Genet Med* , 20(4):433e5.

Looney CB, J. S., LH, M., HM, W., NC, C., RM, H., & JH, G. (2007). Intracranial hemorrhage in asymptomatic neonates: prevalence on MR images and relationship to obstetric and neonatal risk factors. *Radiology*, 242(2):535-41 (ISSN: 0033-8419).

Lynch J.K., S. P. (2005). Treatment and prevention of cerebrovascular disorders in children. *Current Treatment Options in Neurology*, vol. 7, no. 6, pp. 469–480.

Mackay MT, C. Z.-C. (2014). Stroke and nonstroke brain attacks in children. *Neurology* , 82:1434-40.

Mackay MT, W. M. (2011). Arterial ischemic stroke risk factors: the International Pediatric Stroke Study. *Ann Neurol.* , 69:130-140.

Malova M, R. A. (2017 Jan;). Incidental findings on routine brain MRI scans in preterm infants. . *Arch Dis Child Fetal Neonatal* , 102(1):F73-F78. doi: 10.1136/archdischild-2015-310333.

Markus, H. S. (2011). Stroke genetics. *Human Molecular Genetics*, Volume 20, Issue R2, R124–R131.

Martin L, Maître F, Bonicel P, Daudon P, Verny C, Bonneau D, Le Saux O, Chassaing N. (2008) Heterozygosity for a single mutation in the ABCC6 gene may closely mimic PXE: consequences of this phenotype overlap for the definition of PXE. *Arch Dermatol.*144(3):301-6. doi: 10.1001/archderm.144.3.301.

May Llanas M.E., E. A. (1999). Non-traumatic cerebral hemorrhage in childhood: etiology, clinical manifestation and management. *An Esp Pediat*, 5, pp. 257-261.

Meyer-Heim A.D, E. B. (2003). Spontaneous intracranial haemorrhage in children: aetiology, presentation and outcome. *Brain and Development*, Volume 25, Issue 6, Pages 416-421.

Miksch S, Lumsden A, Guenther UP, Foernzler D, Christen-Zäch S, Daugherty C, Ramesar RK, Lebwohl M, Hohl D, Neldner KH, Lindpaintner K, Richards RI, Struk B (2005). Molecular genetics of pseudoxanthoma elasticum: type and frequency of mutations in ABCC6. *Hum Mutat*;26:235–48.

Munot P, C. Y. (2011). Paediatric stroke: genetic insights into disease mechanisms and treatment targets. *Lancet Neurol.*, 10:264-274.

Nidhi Agrawal, S. C. (2009). Imaging Data Reveal a Higher Pediatric Stroke Incidence Than Prior US Estimates. *Stroke.* 40:3415-3421.

Nikil K. Rajani, K. P. (2018). Pediatric stroke: current diagnostic and management challenges. *Quant Immaging Med Surg*, doi: 10.21037/qims.2018.11.09.

Nitschke Y, Baujat G, Botschen U, Wittkampf T, du Moulin M, Stella J, Le Merrer M, Guest G, Lambot K, Tazarourte-Pinturier MF, Chassaing N, Roche O, Feenstra I, Loechner K, Deshpande C, Garber SJ, Chikarmane R, Steinmann B, Shahinyan T, Martorell L, Davies J, Smith WE, Kahler SG, McCulloch M, Wraige E, Loidi L, Höhne W, Martin L, Hadj-Rabia S, Terkeltaub R, Rutsch F.

(2012) Generalized arterial calcification of infancy and pseudoxanthoma elasticum can be caused by mutations in either ENPP1 or ABCC6. *Am J Hum Genet.*;90:25–39

Parodi A, M. G. (2015 Nov). Lowgrade intraventricular hemorrhage: is ultrasound good enough? . *J Matern Fetal Neonatal Med.* , 28(sup1):2261-2264.

Pavlakis, S. G. (2008). Pediatric and newborn stroke. *Current Treatment Options in Neurology*, vol. 10, no. 6, pp. 431–439.

Pfohl M., Fetter M., Koch M., Barth C.M., Rudiger W., Haring H.U. (1998) Association between angiotensin I-converting enzyme genotypes, extracranial artery stenosis and stroke. *Atherosclerosis.*;140(1):161–166.

Poduri A., S. B. (2014). Genetic testing in the epilepsies-developments and dilemmas. *Nat. Rev. Neurol.*, 10, 293–299. 10.1038/nrneurol.2014.60 .

Raso A, B. R. (2016). Moyamoya vasculopathy shows a genetic mutational gradient decreasing from East to West. *J neurosurgery Sci*.

Rigat B., Hubert C., Alhenc-Gelas F., Cambien F., Corvol P., Soubrier F. (1990) An insertion/deletion polymorphism in the angiotensin I-converting enzyme gene accounting for half the variance of serum enzyme levels. *J. Clin. Invest*. 86(4):1343–1346.

Sanger F., N. S. (1977). DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci.* , 5463–5467. 10.1073/pnas.74.12.5463.

Se´bire G, B. T.-M. (2005). Cerebral venous sinus thrombosis in children: risk factors, presentation, diagnosis and outcome. *Brain*, 128, 477–489.

Sebastian Grunt, L. M.-M. (May 2015). Incidence and Outcomes of Symptomatic Neonatal Arterial Ischemic Stroke. *Pediatrics*, VOLUME 135 / ISSUE 5.

Seidman C, K. F. (2007). Pediatric stroke: current developments. *Curr Opin Pediatr*, 19:657-62.

Stavropoulos D. J., M. D. (2016). Whole-genome sequencing expands diagnostic utility and improves clinical management in paediatric medicine. . *Npj Genomic Med.* , 1:15012 10.1038/npjgenmed.2015.12 .

Suppiej A, G. C., & Italian, S. w. (2015 Jun;). Paediatric arterial ischaemic stroke and cerebral sinovenous thrombosis. First report from the Italian Registry of Pediatric Thrombosis (R. I. T. I., Registro Italiano Trombosi Infantili). . *Thromb Haemost.* , 113(6):1270-7. doi: 10.1160/TH14-05-0431.

Terni E, N. G. (2015). Genetics of ischaemic stroke in young adults. *BBA Clinical*, 96–106.

Towner D, C. M.-W. (1999). Effect of mode of delivery in nulliparous women on neonatal intracranial injury. *N Engl J Med.*, 341(23):1709-14 (ISSN: 0028-4793).

Tuncer N., Tuglular S., Kiliç G., Sazci A., Us O., Kara I. (2006) Evaluation of the angiotensin-converting enzyme insertion/deletion polymorphism and the risk of ischemic stroke. *J. Clin. Neurosci.*;13:224–227.

WHO Project, W. M. (1998). The World Health Organization MONICA Project: a major international collaboration. *J Clin Epidemiol*, 41:105-14.

Zidan I, G. A. (2012). Intracerebral hemorrhage in children. *Alexandria Journal of Medicine*, Volume 48, Issue 2, Pages 139-145.