Genetic Causes of Cerebrovascular Disorders in Childhood

Marije Meuwissen

Genetic Causes of Cerebrovascular Disorders in Childhood

ERASMUS UNIVERSITEIT ROTTERDAM

Cover photo: Robert Ludlow, UCL Institute of Neurology, Wellcome Images (used with permission)

Cover design and layout: R. Grefhorst
Printing: Druk. Tan Heck, Delft

ISBN: 978-90-6824-047-4

The work presented in this thesis was performed at the Department of Clinical Genetics at the Erasmus University Medical Center, Rotterdam

This work was financially supported by Fonds NutsOhra

Genetic Causes of Cerebrovascular Disorders in Childhood

Genetische oorzaken van cerebrovasculaire aandoeningen op de kinderleeftijd

Proefschrift

ter verkrijging van de graad van doctor aan de
Erasmus Universiteit Rotterdam
op gezag van de rector magnificus
Prof.dr. H.A.P. Pols
en volgens besluit van het College voor Promoties

De openbare verdediging zal plaatsvinden op
Woensdag 2 april om 15:30 uur

door

Marije Ellen Carolien Meuwissen

geboren te Leidschendam

ERASMUS UNIVERSITEIT ROTTERDAM

PROMOTIECOMMISSIE

Promotor

Prof. dr. R.M.W. Hofstra

Co-promotor

Dr. G.M.S. Mancini

Overige leden

Prof. dr. I.K.M. Reiss

Prof. dr. R.C.M. Hennekam

Prof. dr. K. Braun

Het verschijnen van dit proefschrift werd mede mogelijk gemaakt door de steun van de Nederlandse Hartstichting.

CONTENTS

INTRODUCTION

Chapter 1

GENERAL INTRODUCTION AND OUTLINE OF THE THESIS

GENERAL INTRODUCTION

Cerebrovascular disorders are defined as focal cerebral injury with an underlying vascular basis and can be divided in stroke resulting from arterial ischemic event, cerebral sinovenous thrombosis and hemorrhage[1]. Stroke at young age (< age 18) is not common, but is an important cause of lifelong morbidity and is among the top ten causes of death in childhood[2-4]. It has an incidence of approximately 1.3-13 per 100.000 per year in the developed countries. Causes, risk factors and pathogenic mechanisms of pediatric stroke differ in the perinatal period or in childhood.

Pediatric arterial ischemic stroke

Arterial ischemic stroke (AIS) in childhood is usually non-atherosclerotic, which is different from the adult population. In half of the children with AIS, a pre-existing condition can be identified, such as congenital heart disease or sickle cell disease; in the other part it remains uncertain and may be due to an interplay of both genetic and environmental factors. AIS can occur for example in the presence of genetic prothrombotic disorders, but also in the context of a metabolic disease (Fabry disease, congenital disorders of glycosylation) or other underlying genetic cerebral arteriopathies. In the perinatal period, most relevant risk factors are linked to maternal risk factors, such as autoimmune disease, preeclampsia, traumas, and neonatal, such as infection, systemic illness (including inborn errors of metabolism) and complex congenital heart disease. Very few genetic causes are known. In the first part of this thesis, two examples of genetic disorders leading to ischemic cerebral arteriopathy, Incontinentia pigmenti and *ACTA2* mutations, are described in more detail.

Pediatric Hemorrhagic stroke

In the main part of this thesis, we will focus on processes underlying cerebral hemorrhage occurring at young age, mostly in the perinatal and infantile period. In the childhood population, the proportion of hemorrhagic stroke is approximately 50%, versus only 15% in the adult population[1,2,4]. Hemorrhagic stroke (bleeding) is the result of a weakened vessel that ruptures and bleeds into the surrounding brain. Overall mortality of hemorrhagic stroke in children is approximately 25% and of those who survive, 42% shows significant disability[5]. Hemorrhagic stroke comprises spontaneous intracerebral (parenchymal and intraventricular) hemorrhage (ICH) and non-traumatic subarachnoid hemorrhage (SAH)[2]. A parenchymal hemorrhage is located within the brain parenchyma, whereas an

intraventricular hemorrhage is located inside the brain's ventricular system. Subarachnoidal hemorrhages are located in the subarachnoidal space, the area between the arachnoid membrane and the pia mater, that surrounds the brain and are most often due to intracranial aneurysms[6]. Common risk factors for hemorrhagic stroke at young age are listed in Table 1[7]. Most of them undely occurrence in childhood. Besides common risk factors, rare genetic causes of cerebral hemorrhage have been identified at any age, from pregnancy to adulthood and are summarized in Table 2, together with the known genetic causes of arterial ischemic stroke and our newly identified genes, which will be discussed in detail in this thesis.

Neonatal/ infantile cerebral hemorrhage is a subset of the childhood intracerebral (parenchymal or intraventricular) hemorrhage and among all types pediatric strokes is the least well characterized. It is often regarded as non-genetic, and risk factors include low birth weight and prematurity[8-11]. It usually presents as a germinal matrix hemorrhage or intraventricular hemorrhage. The germinal matrix a highly vascularized, transient layer near the ventricles that is present between 8 and 36 weeks of gestation. It has a role in the production of neurons and glial cells. The structure of the germinal matrix is selectively vulnerable to hemorrhage, which can be explained by limited astrocyte end-feet coverage of microvessels, reduced expression of fibronectin, an extracellular matrix component, and immature tight junctions[12]. In approximately 15% of infants these hemorrhages lead to venous infarction

Table 1. Risk factors for pediatric hemorrhagic stroke

Arteriovenous malformations	14-50%
Cerebral aneurysms	10-13%
Cavernous malformations	15-25%
Hematological abnormalities	10-30%
Thrombocytopenia	
Idiopathic thrombocytopenia purpura (ITP)	
Hemophilia	
Coagulopathies	
Liver failure	
Disseminated intravascular coagulation	
Iatrogenic	
Sickle cell anemia	
Brain tumors	13%

due to occlusion of the periventricular collector veins, subsequent tissue destruction and the development of porencephaly, defined as a cavity in the brain parenchyma communicating with the lateral ventricle[13]. This process is depicted in Figure 1. The hemorrhage usually occurs at late pregnancy or around birth[14]. In case of early extensive hemorrhage, the destruction can extend to the cortex and can lead to major destructive lesions such as hydranencephaly[15]. Affected neonates have a high chance of developing long term complications, such as hydrocephaly, motor impairment such as cerebral palsy, intellectual disability or epilepsy, depending on the extension of the lesions.

Already since the 1980s, familial occurrence of cerebral hemorrhage has been documented, for instance familial cases of porencephaly[16-21]. The discovery of an underlying genetic cause of childhood cerebral hemorrhage is of importance for appropriate genetic counselling. Expanding the knowledge on underlying mechanisms will hopefully result in the development of specific treatment and preventive options in the future. To achieve this, it is crucial to elucidate the pathogenic mechanisms underlying these disorders. By investigating the childhood population, we expect to find a greater influence of genetic factors compared to

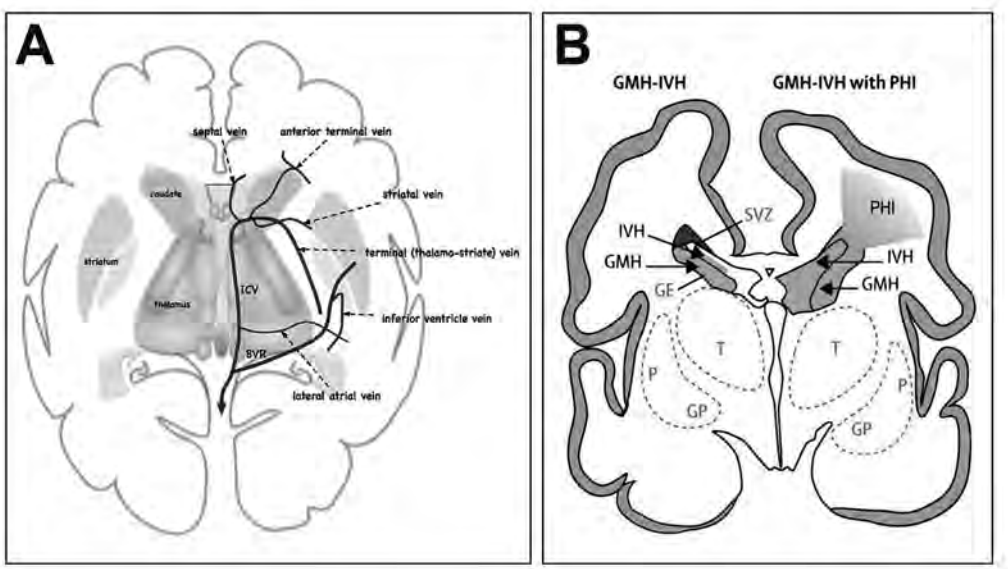


Figure 1. Schematic overview of pathogenic mechanism leading to porencephaly[119,120]. The lateral ventricles with surrounding deep veins are depicted in **A**. Porencephaly originates from a germinal matrix hemorrhage, often followed by intraventricular hemorrhage. This leads to deep venous infarctions and destruction of white matter in that area, eventually leading to a cyst communicating with the ventricle (**B**)

the adult population, since the role of additional environmental factors contributing to the hemorrhage is smaller. However, a common genetic factor may underly both neonatal and adult onset cerebral hemorrhage, due to variable expression, which is, for example, documented for *COL4A1* and *COL4A2* mutations.

Pathophysiological processes underlying the disorders described in this thesis

The identification of genetic causes of cerebrovascular disease has led to the identification of different pathophysiological processes. We will focus on two major pathophysiological processes that are mostly related to cerebral vascular integrity and are the subject of this thesis: structural changes affecting the blood vessel and blood-brain barrier integrity and dysregulation of immune response, which secondarily affects blood vessel integrity. A short overview of these processes will be provided.

Structural changes affecting blood- brain barrier integrity

The blood-brain-barrier consists of specific physical barriers, enzymes and transporters, that together form/ maintain the extracellular environment of the central nervous system. The main physical barrier consists of the endothelial cells of the blood vessel walls within the central nervous system. Of great importance for this barrier are tight junctions, which connect the endothelial cells and impair transport of large molecules across the barrier. Other components of the blood-brain barrier are astrocytes, pericytes and the extracellular matrix, especially the subendothelial basement membrane. This basement membrane is a complex formed by collagens, lamin, fibronectin, entactin, thrombospondin, heparin sulphate proteoglygans such as agrin, and chondroitin sulphate proteoglycans such as versican[22]. Collagen IV, a major component of the basement membrane, influences tight junction formation and stimulates expression of a major tight junction component, named occludin. The loss or dysfunction of one of the extracellular matrix components can lead to dysfunction of the endothelial cells of the cerebral vasculature[23].

The basement membrane: COL4A1 and COL4A2

An important component of the basement membrane of the (brain) blood vessels is collagen IV, mainly collagen IV alpha-1 and collagen IV alpha-2. These proteins form triple helices with a ration of two collagen IV alpha-1 and one collagen IV alpha-2 chain. These helices in turn form a network that gives stability to basement membranes and provides binding

sites for other extracellular matrix proteins[24,25]. These networks are not only important for vascular basement membrane stability, but they also play a role in the stability of cerebral membranes (pia mater). This latter function is important for brain developmental processes, since, during brain development, these membranes are used as anchoring points for glia cells, enabling the migration of neurons to their position in the brain cortex[26,27].

In *COL4A1* and *COL4A2* mutations, a disturbed network formation of the basement membrane is presumed, either due to an abnormal triple helix formation leading to a decrease of normal triple helices in the basement membrane, or due to a change in binding sites of the triple helix with disturbed binding of other extracellular matrix components. These processes are presumed to cause the vascular dysfunction, with disruption leading to hemorrhage, and small vessel disease[28]. Instability of the pial basement membranes can lead to brain developmental defects[26,27].

In 2005, *COL4A1* mutations were discovered as a cause of autosomal dominant hereditary porencephaly[29-31]. Over the years, the phenotype of *COL4A1* mutations has expanded from porencephaly to a multisystem disorder, comprising a broad spectrum of cerebrovascular abnormalities, including aneurysms and small vessel disease, parenchyme and cortex abnormalities, including calcifications, schizencephaly and hydranencephaly, ophthalmological abnormalities including cataract, microphthalmia and retinal vessel abnormities, renal abnormalities including cysts and hematuria, muscular findings including muscle cramps and myopathy and cardiac findings such as arrhythmias. In 2012, in several parallel studies, both familial and sporadic porencephaly were also attributed to *COL4A2* mutations[32-34]. The phenotype in both *COL4A1* and *COL4A2* mutations is extremely variable in expression, with broad intra-familial variations and evidence for reduced penetrance.

The tight junctions: Occludin and JAM3

Dysfunction of the tight junction components has been associated with cerebral hemorrhage and childhood cerebrovascular disease. Important tight junction components are occludin and junctional adhesion molecules (JAMs). Three types of JAMs are known: JAM1, which is expressed in both endothelial and epithelial cells, and JAM2 and JAM3, who are expressed in vascular endothelial cells. Occludin and JAMs play a major role in the regulation of the barrier function of the blood- brain- barrier, including the regulation of permeability and the extravasation of leucocytes. The function and position of occludin in the cell membrane is dependent on the function of microtubules and the actin cytoskeleton.

Homozygous mutations in both *OCLN* and *JAM3* cause syndromes resembling congenital infection, and are therefore named pseudo- TORCH syndromes. Mutations in *OCLN* lead to a syndrome with microcephaly, polymicrogyria and a band of gray matter calcification. Polymicrogyria is a cortical malformation which is presumed to be related to ischemic or vascular insults around five months of pregnancy. Mutations in *JAM3* cause a syndrome of hemorrhagic destruction of brain parenchyma, subependymal calcification and congenital cataracts. The precise pathophysiological mechanism in both syndromes is unknown, but dysfunction of the blood- brain- barrier is presumed to be one underlying factor[35,36].

The actin cytoskeleton

One of the major components of the cellular cytoskeleton is actin. Actin filaments are organized into polymerized fibers. These fibers are responsible for the anchoring of the cytoskeleton into the plasma membrane. Actin is essential for cell motility and the maintenance of cell shape. In the blood- brain barrier, it plays a role in the integrity of the tight junction complex by regulating the position of tight junction proteins in the cell membrane[35,37,38].

There are different subtypes of actins. One of them is *ACTA2*, which is expressed in vascular smooth muscle cells, but also in other organ systems, including the brain. The phenotype in ACTA2 mutations includes thoracic aortic aneurysms and dissections (TAAD), coronary artery disease and cerebral infarctions, cerebral aneurysms and small vessel disease. Recently, a multisystem disorder caused by specific changes in the *ACTA2* gene was identified. Cerebrovascular anomalies in this disorder comprise an abnormal course of cerebral arteries, including a moyamoya-like phenotype, diffuse periventricular leukoencephalopathy and multifocal infarcts. In addition, a broad spectrum of developmental defects can be appreciated, most due to smooth muscle cell dysfunction[39,40]. The phenotype in *ACTA2* mutations has been attributed to an abnormal function of the vascular smooth muscles. However, an abnormal function of the tight junctions may contribute to the brain phenotype, i.e. the small vessel disease.

Immune response dysregulation

Immune response in the central nervous system (CNS), comprising the brain, spinal cord, optic nerves and retina, is different from that in different organs. Due to the delicacy of the post-mitotic, non-regenerating cells within the CNS, it is of great importance that destruc-

tive, inflammatory reactions are carefully controlled. The CNS lacks lymphatic vessels, and cerebrospinal fluid functions as an equivalent of lymph[41]. Since the early 1970s, reports on the TORCH complex appeared, reporting on the concept that intrauterine infections (Toxoplasmosis gondii, Rubella, Cytomegalovirus, Herpes viruses and later also HIV) can have severe effects on the central nervous system (CNS), leading to cerebrovascular and cerebrodevelopmental disorders. Although the CNS is partly protected from toxic substances and pathogens by the blood-brain barrier, neurotropic viruses may be able to enter the CNS by crossing the barrier, by exploiting motile infected cells as Trojan horses or by using axonal transport[42].

Neuroimaging findings in the TORCH complex include intracranial calcifications, both periventricular or parenchymal, hydrocephalus and a variety of cortical anomalies, including polymicrogyria, schizencephaly, hydranencephaly, pachygyria, lissencephaly, focal cortical dysplasia, cerebellar hypoplasia and cerebral hemorrhage[43,44].

Over the years, several reports have appeared on patients with a "pseudo-TORCH" phenotype, but with absent infection parameters. These phenotypes are grouped as "pseudo-TORCH syndrome". Cardinal findings are congenital cerebral calcification, microcephaly, enlarged ventricles, cerebellar hypoplasia or atrophy and the development of icterus, hyperbilirubinemia, thrombocytopenia and hepatomegaly (in 50%) short after birth[45].

A high percentage (35%) of consanguinity in this group of patients is seen, suggesting genetic, autosomal recessive underlying conditions.

IFN type I signalling

In both viral infections, and the Aicardi-Goutières syndrome, an autosomal recessive syndrome that is one of the known genetic "pseudo-TORCH" syndromes, as well as in our newly identified "pseudo-TORCH" syndrome due to *USP18* mutations, an up-regulation of the IFN type I signalling pathway is seen.

Type I and type III interferons (IFNs) are cytokines that are critical to control early steps of viral infections[42]. Basal activity of IFNs are within the CNS appear to be low, probably owing to the toxicity of IFN to this organ[42]. IFNs act on specific cell receptors, and binding activate transduction pathways to enhance the expression of hundreds of Interferon-Stimulated Genes (ISGs), which lead the cell into an antiviral state[46]. Type I interferon comprises IFNα, produced by leucocytes, and IFNβ, produced by fibroblasts. They exhibit various biological functions, including antiviral, anti-proliferative, immunomodulatory and developmental activities. They interact with the human IFN alpha receptor (IFNAR), that is composed of two subunits (IFNAR-1 and IFNAR-2), which are present in almost any cell type[46].

The Aicardi-Goutières syndrome is caused by mutations in *TREX1*, *RNASEH2A*, *RNASEH2B*, *RNASEH2C*, *SAMHD1* or *ADAR1*. Mutations in one of these genes cause an inadequate processing of cellular nucleic acid debris, which triggers indirectly, by mechanisms not clarified to date, the innate immune response, leading to induction of IFNα expression and an up-regulation of the IFN type I signalling pathway[47].

In its classical form, the Aicardi-Goutières syndrome phenotype manifests in infancy with progressive microcephaly, spasticity and psychomotor retardation. Brain imaging includes intracranial calcification, white matter destruction and brain atrophy. Systemic manifestations are thrombocytopenia, hepatosplenomegaly, elevated hepatic transaminases and intermittent fevers. In its classis form, it has a poor prognosis, with death in early childhood in approximately 40% of cases[48]. However, several milder clinical phenotypes have been identified since the discovery of the underlying genetic defects, including retinal vasculopathy with cerebral leukodystrophy, familial chilblain lupus, systemic lupus erythematosus and non-specific inflammatory arthropathy. Only mutations in *SAMHD1* (AGS5) have been associated with a cerebral vasculopathy involving the large arteries, showing either stenosis of intracerebral large arteries with evidence of basal collaterals consistent with moyamoya syndrome, or intracranial aneurysms without occlusive disease[49-51]. Childhood cerebral hemorrhage has been reported in several patients[50,52].

NF-κB signalling

Another component that is upregulated in response to inflammation is NF-κB. NF-κB signalling is induced in response to multiple stimuli, including tumor necrosis factor α (TNFα), bacterial lipopolysaccharide (LPS) and genotoxic agents. Active NF-κB can translocate to the nucleus and activate transcription of target genes. In the CNS, NF-κB is present in neurons, astrocytes, migroglia and oligodendrocytes. It plays an important role in immune responses and protection against neuronal damage and apoptosis caused by ischemic or inflammatory brain injury and excitotoxicity caused by seizures[53]. Interestingly, NF-κB was recently identified as an important factor in the signalling pathway induced by the cytokine Interleukin (IL)-15 in cerebral vascular endothelial cells composing the blood- brain barrier, which is in turn induced by TNF α. This IL-15 signalling in endothelial cells exerts cytoprotective effects against inflammation and consequently impaired function of the blood-brain barrier[54].

Absent NF-κB activation is seen in cells harbouring a *NEMO* mutation. This is the underlying genetic cause of Incontinentia Pigmenti, a rare X-linked disorder that can present with neurological features, such as microcephaly, small-vessel disease and hemorrhagic necrosis. The cerebral abnormalities are thought to result from a combination of vascular injury/insufficiency, inflammation and a possibly disturbed apoptosis during cerebral development[55].

SCOPE OF THE THESIS

In the last decade, young patients (including neonates, infants and children) with cerebrovascular disease of unknown cause (mostly hemorrhagic stroke) were evaluated at the Departments of Clinical Genetics, Child Neurology, Pediatric Neuroradiology and Neonatology of the Erasmus University Medical Center and the Department of Neonatology of the University Medical Center Utrecht. After the discovery of *COL4A1* mutations in familial porencephaly, a close collaboration started between these departments, aiming to better understand the cause and mechanisms of perinatal hemorrhagic stroke. Our original goal was to identify the genetic factors involved in cerebral hemorrhage at young age starting from our patient´s cohort and to characterize the phenotypic spectrum of collagen IV-related cerebral disorder and other genetic cerebral vascular disorders in our young patient cohort. The results would be important to improve the diagnostics in this group of patients, to provide better counselling of families and to identify future therapeutic targets for this group of disorders.

The first part of the thesis focusses on observations on the genetic causes of ischemic cerebral arteriopathies. The second, main part of the thesis, focusses on genetic causes of perinatal, infantile and childhood cerebral hemorrhage. The third part comprises the discussion of the results.

PART I Genetic causes of ischemic stroke

Chapter 2 includes descriptions of the phenotypic variability of gene mutations normally classified among the causes of ischemic arteriopathies, but revealing themselves as broader cerebrovascular disease at young age]. In **chapter 2.1** a case report is provided on a toddler girl with a systemic disorder, including cerebral and vascular developmental defects and leukoencephalopathy, who harboured an *ACTA2* mutation. **Chapter 2.2** comprises a review of literature about neurological complications of Incontinentia Pigmenti, classically thought to result from ischemic damage and instead revealing an important hemorrhagic component.

PART II Genetic causes of hemorrhagic stroke

In **chapter 3**, an overview is provided of the current knowledge on *COL4A1* and *COL4A2* mutations is provided. In particular, a review of the clinical spectrum of *COL4A1* and *COL4A2* mutations and our experiences in a patient cohort is illustrated. Several clinical phenotypes are described in detail. A protocol for clinical management of *COL4A1* and *COL4A2* patients is provided.

In **chapter 4**, our experience with several specific *COL4A1* mutation phenotypes is described. **Chapter 4.1** describes the most severe end of the *COL4A1* mutation spectrum, comprising of severe brain destruction resembling hydranencephaly due to de novo mutations in four affected infants. **Chapter 4.2** gives a description of the severe ophthalmological phenotype leading to cataract, microcornea, microphthalmia and tunnel vision in a family with three affected patients. **Chapter 4.3** provides the description of the first patient harbouring a homozygous *COL4A1* mutation affecting a specific part of the gene, the NC1-domain.

In **chapter 5**, we describe the identification of *COL4A2* mutations as a second cause of hereditary porencephaly and small vessel disease, using a candidate gene approach. The clinical, neuroradiological and biochemical findings in two families are reported, including findings in skin fibroblasts.

In **chapter 6,** the identification of *USP18* as a novel genetic cause of pseudo-TORCH syndrome with cerebral hemorrhage is illustrated. The clinical and molecular findings in two families including five affected individuals with severe cerebrovascular disease and early demise are summarized.

Chapter 7 comprises the general discussion of the thesis, in which the most important conclusions, implications of the study for clinical practice and suggestions for future research are presented.

References

1. **Cardenas JF, Rho JM, Kirton A.** Pediatric stroke. Childs Nerv Syst 2011;27:1375-90.

2. **Beslow LA, Jordan LC.** Pediatric stroke: the importance of cerebral arteriopathy and vascular malformations. Childs Nerv Syst 2010;26:1263-73.

3. **Wang JJ, Shi KL, Li JW, et al.** Risk factors for arterial ischemic and hemorrhagic stroke in childhood. Pediatr Neurol 2009;40:277-81.

4. **Kleinman JT, Beslow LA, Engelmann K, et al.** Evaluation of intraventricular hemorrhage in pediatric intracerebral hemorrhage. J Child Neurol 2012;27:526-31.

5. **Lynch JK, Han CJ.** Pediatric stroke: what do we know and what do we need to know? Semin Neurol 2005;25:410-23.

6. **Jordan LC, Johnston SC, Wu YW, Sidney S, Fullerton HJ.** The importance of cerebral aneurysms in childhood hemorrhagic stroke: a population-based study. Stroke 2009;40:400-5.

7. **Jordan LC, Hillis AE.** Hemorrhagic stroke in children. Pediatr Neurol 2007;36:73-80.

8. **Benders MJ, Groenendaal F, Uiterwaal CS, et al.** Maternal and infant characteristics associated with perinatal arterial stroke in the preterm infant. Stroke; a journal of cerebral circulation 2007;38:1759-65.

9. **Curry CJ, Bhullar S, Holmes J, Delozier CD, Roeder ER, Hutchison HT.** Risk factors for perinatal arterial stroke: a study of 60 mother-child pairs. Pediatric neurology 2007;37:99-107.

10. **Raju TN, Nelson KB, Ferriero D, Lynch JK.** Ischemic perinatal stroke: summary of a workshop sponsored by the National Institute of Child Health and Human Development and the National Institute of Neurological Disorders and Stroke. Pediatrics 2007;120:609-16.

11. **Simchen MJ, Goldstein G, Lubetsky A, Strauss T, Schiff E, Kenet G.** Factor v Leiden and antiphospholipid antibodies in either mothers or infants increase the risk for perinatal arterial ischemic stroke. Stroke; a journal of cerebral circulation 2009;40:65-70.

12. **Raets MM, Dudink J, Govaert P.** Neonatal disorders of germinal matrix. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet 2013.

13. **Dudink J, Lequin M, Weisglas-Kuperus N, Conneman N, van Goudoever JB, Govaert P.** Venous subtypes of preterm periventricular haemorrhagic infarction. Archives of disease in childhood 2008;93:F201-6.

14. **Kirton A, Deveber G, Pontigon AM, Macgregor D, Shroff M.** Presumed perinatal ischemic stroke: vascular classification predicts outcomes. Annals of neurology 2008;63:436-43.

15. **Edmondson SR, Hallak M, Carpenter RJ, Jr.**, Cotton DB. Evolution of hydranencephaly following intracerebral hemorrhage. Obstetrics and gynecology 1992;79:870-1.

16. **Zonana J, Adornato BT, Glass ST, Webb MJ.** Familial porencephaly and congenital hemiplegia. The Journal of pediatrics 1986;109:671-4.

17. **Sensi A, Cerruti S, Calzolari E, Vesce F.** Familial porencephaly. Clinical genetics 1990;38:396-7.

18. **Berg RA, Aleck KA, Kaplan AM.** Familial porencephaly. Archives of neurology 1983;40:567-9.

19. **Shastri NJ, Bharani SA, Modi UJ, Trivedi C.** Familial porencephaly. Indian journal of pediatrics 1993;60:459-63.

20. **Mancini GM, de Coo IF, Lequin MH, Arts WF.** Hereditary porencephaly: clinical and MRI findings in two Dutch families. Eur J Paediatr Neurol 2004;8:45-54.

21. **Vilain C, Van Regemorter N, Verloes A, David P, Van Bogaert P.** Neuroimaging fails to identify asymptomatic carriers of familial porencephaly. American journal of medical genetics 2002;112:198-202.

22. **Tam SJ, Watts RJ.** Connecting vascular and nervous system development: angiogenesis and the blood-brain barrier. Annual review of neuroscience;33:379-408.

23. **Wolburg H, Lippoldt A.** Tight junctions of the blood-brain barrier: development, composition and regulation. Vascular pharmacology 2002;38:323-37.

24. **Khoshnoodi J, Pedchenko V, Hudson BG.** Mammalian collagen IV. Microsc Res Tech 2008;71:357-70.

25. **Kuhn K.** Basement membrane (type IV) collagen. Matrix Biol 1995;14:439-45.

26. **Favor J, Gloeckner CJ, Janik D, et al.** Type IV procollagen missense mutations associated with defects of the eye, vascular stability, the brain, kidney function and embryonic or postnatal viability in the mouse, Mus musculus: an extension of the Col4a1 allelic series and the identification of the first two Col4a2 mutant alleles. Genetics 2007;175:725-36.

27. **Labelle-Dumais C, Dilworth DJ, Harrington EP, et al.** COL4A1 mutations cause ocular dysgenesis, neuronal localization defects, and myopathy in mice and Walker-Warburg syndrome in humans. PLoS Genet 2011;7:e1002062.

28. **Kuo DS, Labelle-Dumais C, Gould DB.** COL4A1 and COL4A2 mutations and disease: insights into pathogenic mechanisms and potential therapeutic targets. Hum Mol Genet 2012;21:R97-110.

29. **Breedveld G, de Coo IF, Lequin MH, et al.** Novel mutations in three families confirm a major role of COL4A1 in hereditary porencephaly. J Med Genet 2006;43:490-5.

30. **Gould DB, Phalan FC, Breedveld GJ, et al.** Mutations in Col4a1 cause perinatal cerebral hemorrhage and porencephaly. Science 2005;308:1167-71.

31. **Aguglia U, Gambardella A, Breedveld GJ, et al.** Suggestive evidence for linkage to chromosome 13qter for autosomal dominant type 1 porencephaly. Neurology 2004;62:1613-5.

32. **Jeanne M, Labelle-Dumais C, Jorgensen J, et al.** COL4A2 mutations impair COL4A1 and COL4A2 secretion and cause hemorrhagic stroke. Am J Hum Genet 2012;90:91-101.

33. **Verbeek E, Meuwissen ME, Verheijen FW, et al.** COL4A2 mutation associated with familial porencephaly and small-vessel disease. Eur J Hum Genet 2012;20:844-51.

34. **Yoneda Y, Haginoya K, Arai H, et al.** De novo and inherited mutations in COL4A2, encoding the type IV collagen alpha2 chain cause porencephaly. Am J Hum Genet 2012;90:86-90.

35. **O'Driscoll MC, Daly SB, Urquhart JE, et al.** Recessive mutations in the gene encoding the tight junction protein occludin cause band-like calcification with simplified gyration and polymicrogyria. Am J Hum Genet 2010;87:354-64.

36. **Mochida GH, Ganesh VS, Felie JM, et al.** A homozygous mutation in the tight-junction protein JAM3 causes hemorrhagic destruction of the brain, subependymal calcification, and congenital cataracts. Am J Hum Genet 2010;87:882-9.

37. **Fletcher DA, Mullins RD.** Cell mechanics and the cytoskeleton. Nature 2010;463:485-92.

38. **Lai CH, Kuo KH, Leo JM.** Critical role of actin in modulating BBB permeability. Brain research 2005;50:7-13.

39. **Milewicz DM, Ostergaard JR, Ala-Kokko LM, et al.** De novo ACTA2 mutation causes a novel syndrome of multisystemic smooth muscle dysfunction. Am J Med Genet A 2010;152A:2437-43.

40. **Moosa AN, Traboulsi EI, Reid J, Prieto L, Moran R, Friedman NR.** Neonatal Stroke and Progressive Leukoencephalopathy in a Child With an ACTA2 Mutation. Journal of child neurology 2012.

41. **Ransohoff RM, Engelhardt B.** The anatomical and cellular basis of immune surveillance in the central nervous system. Nature reviews Immunology 2012;12:623-35.

42. **Sorgeloos F, Kreit M, Hermant P, Lardinois C, Michiels T.** Antiviral type I and type III interferon responses in the central nervous system. Viruses 2013;5:834-57.

43. **Bale JF, Jr.** Fetal infections and brain development. Clinics in perinatology 2009;36:639-53.

44. **Tongsong T, Sukpan K, Wanapirak C, Phadungkiatwattna P.** Fetal cytomegalovirus infection associated with cerebral hemorrhage, hydrops fetalis, and echogenic bowel: case report. Fetal diagnosis and therapy 2008;23:169-72.

45. **Vivarelli R, Grosso S, Cioni M, et al.** Pseudo-TORCH syndrome or Baraitser-Reardon syndrome: diagnostic criteria. Brain & development 2001;23:18-23.

46. **Reyes-Vazquez C, Prieto-Gomez B, Dafny N.** Interferon modulates central nervous system function. Brain research 2012;1442:76-89.

47. **Chahwan C, Chahwan R.** Aicardi-Goutieres syndrome: from patients to genes and beyond. Clin Genet 2012;81:413-20.

48. **Rice G, Patrick T, Parmar R, et al.** Clinical and molecular phenotype of Aicardi-Goutieres syndrome. Am J Hum Genet 2007;81:713-25.

49. **Thiele H, du Moulin M, Barczyk K, et al.** Cerebral arterial stenoses and stroke: novel features of Aicardi-Goutieres syndrome caused by the Arg164X mutation in SAMHD1 are associated with altered cytokine expression. Hum Mutat 2010;31:E1836-50.

50. **Ramesh V, Bernardi B, Stafa A, et al.** Intracerebral large artery disease in Aicardi-Goutieres syndrome implicates SAMHD1 in vascular homeostasis. Dev Med Child Neurol 2010;52:725-32.

51. **Xin B, Jones S, Puffenberger EG, et al.** Homozygous mutation in SAMHD1 gene causes cerebral vasculopathy and early onset stroke. Proc Natl Acad Sci U S A 2011;108:5372-7.

52. **du Moulin M, Nurnberg P, Crow YJ, Rutsch F.** Cerebral vasculopathy is a common feature in Aicardi-Goutieres syndrome associated with SAMHD1 mutations. Proc Natl Acad Sci U S A 2011;108:E232; author reply E3.

53. **Mattson MP, Camandola S.** NF-kappaB in neuronal plasticity and neurodegenerative disorders. The Journal of clinical investigation 2001;107:247-54.

54. **Stone KP, Kastin AJ, Pan W.** NFkB is an unexpected major mediator of interleukin-15 signaling in cerebral endothelia. Cellular physiology and biochemistry : international journal of experimental cellular physiology, biochemistry, and pharmacology 2011;28:115-24.

55. **Nelson DL.** NEMO, NFkappaB signaling and incontinentia pigmenti. Curr Opin Genet Dev 2006;16:282-8.

56. **Delaney HM, Rooks VJ, Wolfe SQ, Sawyer TL.** Term neonate with intracranial hemorrhage and hereditary hemorrhagic telangiectasia: a case report and review of the literature. Journal of perinatology : official journal of the California Perinatal Association 2012;32:642-4.

57. **Leblanc GG, Golanov E, Awad IA, Young WL.** Biology of vascular malformations of the brain. Stroke 2009;40:e694-702.

58. **Revencu N, Boon LM, Mendola A, et al.** RASA1 mutations and associated phenotypes in 68 families with capillary malforMation-arteriovenous malformation. Hum Mutat 2013.

59. **Orme CM, Boyden LM, Choate KA, Antaya RJ, King BA.** Capillary malformation--arteriovenous malformation syndrome: review of the literature, proposed diagnostic criteria, and recommendations for management. Pediatric dermatology 2013;30:409-15.

60. **Denier C, Labauge P, Bergametti F, et al.** Genotype-phenotype correlations in cerebral cavernous malformations patients. Annals of neurology 2006;60:550-6.

61. **Riant F, Bergametti F, Fournier HD, et al.** CCM3 Mutations Are Associated with Early-Onset Cerebral Hemorrhage and Multiple Meningiomas. Molecular syndromology 2013;4:165-72.

62. **Rodrigues VJ, Elsayed S, Loeys BL, Dietz HC, Yousem DM.** Neuroradiologic manifestations of Loeys-Dietz syndrome type 1. AJNR American journal of neuroradiology 2009;30:1614-9.

63. **Kalra VB, Gilbert JW, Malhotra A.** Loeys-Dietz syndrome: cardiovascular, neuroradiological and musculoskeletal imaging findings. Pediatric radiology 2011;41:1495-504; quiz 616.

64. **Zilocchi M, Macedo TA, Oderich GS, Vrtiska TJ, Biondetti PR, Stanson AW.** Vascular Ehlers-Danlos syndrome: imaging findings. AJR American journal of roentgenology 2007;189:712-9.

65. **Kato T, Hattori H, Yorifuji T, Tashiro Y, Nakahata T.** Intracranial aneurysms in Ehlers-Danlos syndrome type IV in early childhood. Pediatr Neurol 2001;25:336-9.

66. **Regalado ES, Guo DC, Villamizar C, et al.** Exome sequencing identifies SMAD3 mutations as a cause of familial thoracic aortic aneurysm and dissection with intracranial and other arterial aneurysms. Circulation research 2011;109:680-6.

67. **van de Laar IM, van der Linde D, Oei EH, et al.** Phenotypic spectrum of the SMAD3-related aneurysms-osteoarthritis syndrome. J Med Genet 2012;49:47-57.

68. **Guo DC, Papke CL, Tran-Fadulu V, et al.** Mutations in smooth muscle alpha-actin (ACTA2) cause coronary artery disease, stroke, and Moyamoya disease, along with thoracic aortic disease. Am J Hum Genet 2009;84:617-27.

69. **Salo AM, Cox H, Farndon P, et al.** A connective tissue disorder caused by mutations of the lysyl hydroxylase 3 gene. Am J Hum Genet 2008;83:495-503.

70. **Plaisier E, Gribouval O, Alamowitch S, et al.** COL4A1 mutations and hereditary angiopathy, nephropathy, aneurysms, and muscle cramps. N Engl J Med 2007;357:2687-95.

71. **Niemczyk M, Gradzik M, Niemczyk S, Bujko M, Golebiowski M, Paczek L.** Intracranial aneurysms in autosomal dominant polycystic kidney disease. AJNR American journal of neuroradiology 2013;34:1556-9.

72. **Chang MY, Kuok CM, Chen YC, et al.** Comparison of intracerebral hemorrhage and subarachnoid hemorrhage in patients with autosomal-dominant polycystic kidney disease. Nephron Clinical practice 2010;114:c158-64.

73. **Rosser TL, Vezina G, Packer RJ.** Cerebrovascular abnormalities in a population of children with neurofibromatosis type 1. Neurology 2005;64:553-5.

74. **Moratti C, Andersson T.** Giant extracranial aneurysm of the internal carotid artery in neurofibromatosis type 1. A case report and review of the literature. Interventional neuroradiology : journal of peritherapeutic neuroradiology, surgical procedures and related neurosciences 2012;18:341-7.

75. **Brancati F, Castori M, Mingarelli R, Dallapiccola B.** Majewski osteodysplastic primordial dwarfism type II (MOPD II) complicated by stroke: clinical report and review of cerebral vascular anomalies. Am J Med Genet A 2005;139:212-5.

76. **Hall JG, Flora C, Scott CI, Jr.**, Pauli RM, Tanaka KI. Majewski osteodysplastic primordial dwarfism type II (MOPD II): natural history and clinical findings. Am J Med Genet A 2004;130A:55-72.

77. **Kamath BM, Spinner NB, Emerick KM, et al.** Vascular anomalies in Alagille syndrome: a significant cause of morbidity and mortality. Circulation 2004;109:1354-8.

78. **Tumialan LM, Dhall SS, Tomak PR, Barrow DL.** Alagille syndrome and aneurysmal subarachnoid hemorrhage. Case report and review of the literature. Pediatric neurosurgery 2006;42:57-61.

79. **Munot P, Saunders DE, Milewicz DM, et al.** A novel distinctive cerebrovascular phenotype is associated with heterozygous Arg179 ACTA2 mutations. Brain : a journal of neurology 2012;135:2506-14.

80. **Hwang SW, Jea A.** A review of the neurological and neurosurgical implications of down syndrome in children. Clinical pediatrics 2013;52:845-56.

81. **Kassim AA, DeBaun MR.** Sickle cell disease, vasculopathy, and therapeutics. Annual review of medicine 2013;64:451-66.

82. **Wu Z, Jiang H, Zhang L, et al.** Molecular analysis of RNF213 gene for moyamoya disease in the Chinese Han population. PloS one 2012;7:e48179.

83. **Hartley LM, Khwaja OS, Verity CM.** Glutaric aciduria type 1 and nonaccidental head injury. Pediatrics 2001;107:174-5.

84. **Bishop FS, Liu JK, McCall TD, Brockmeyer DL.** Glutaric aciduria type 1 presenting as bilateral subdural hematomas mimicking nonaccidental trauma. Case report and review of the literature. J Neurosurg 2007;106:222-6.

85. **Nassogne MC, Sharrard M, Hertz-Pannier L, et al.** Massive subdural haematomas in Menkes

disease mimicking shaken baby syndrome. Childs Nerv Syst 2002;18:729-31.

86. **Arita JH, Faria EC, Peruchi MM, Lin J, Rodrigues Masruha M, Vilanova LC.** Menkes disease as a differential diagnosis of child abuse. Arq Neuropsiquiatr 2009;67:507-9.

87. **Bayram E, Topcu Y, Karakaya P, et al.** Molybdenum cofactor deficiency: review of 12 cases (MoCD and review). Eur J Paediatr Neurol 2013;17:1-6.

88. **Teksam O, Yurdakok M, Coskun T.** Molybdenum cofactor deficiency presenting with severe metabolic acidosis and intracranial hemorrhage. Journal of child neurology 2005;20:155-7.

89. **Carmi-Nawi N, Malinger G, Mandel H, Ichida K, Lerman-Sagie T, Lev D.** Prenatal brain disruption in molybdenum cofactor deficiency. Journal of child neurology 2011;26:460-4.

90. **Fazekas F, Enzinger C, Schmidt R, et al.** MRI in acute cerebral ischemia of the young: the Stroke in Young Fabry Patients (sifap1) Study. Neurology 2013;81:1914-21.

91. **Li J, Lee JJ, Chu E, Baehring JM.** Reversible leukoencephalopathy with stroke-like presentation in a patient with 5-dihydropyrimidine dehydrogenase deficiency treated with continuous 5-fluorouracil infusion. Clinical colorectal cancer 2012;11:215-7.

92. **Ishikawa N, Tajima G, Ono H, Kobayashi M.** Different neuroradiological findings during two stroke-like episodes in a patient with a congenital disorder of glycosylation type Ia. Brain & development 2009;31:240-3.

93. **Dinopoulos A, Mohamed I, Jones B, Rao S, Franz D, deGrauw T.** Radiologic and neurophysiologic aspects of stroke-like episodes in children with congenital disorder of glycosylation type Ia. Pediatrics 2007;119:e768-72.

94. **Richards M, Lavigne Lissalde G, Combescure C, et al.** Neonatal bleeding in haemophilia: a European cohort study. British journal of haematology 2012;156:374-82.

95. **Kulkarni R, Lusher JM.** Intracranial and extracranial hemorrhages in newborns with hemophilia: a review of the literature. Journal of pediatric hematology/oncology 1999;21:289-95.

96. **Lapecorella M, Mariani G.** Factor VII deficiency: defining the clinical picture and optimizing therapeutic options. Haemophilia : the official journal of the World Federation of Hemophilia 2008;14:1170-5.

97. **Kenet G, Lutkhoff LK, Albisetti M, et al.** Impact of thrombophilia on risk of arterial ischemic stroke or cerebral sinovenous thrombosis in neonates and children: a systematic review and meta-analysis of observational studies. Circulation 2010;121:1838-47.

98. **Akawi NA, Canpolat FE, White SM, et al.** Delineation of the clinical, molecular and cellular aspects of novel JAM3 mutations underlying the autosomal recessive hemorrhagic destruction of the brain, subependymal calcification, and congenital cataracts. Hum Mutat 2013;34:498-505.

99. **Crow YJ, Rehwinkel J.** Aicardi-Goutieres syndrome and related phenotypes: linking nucleic acid metabolism with autoimmunity. Hum Mol Genet 2009;18:R130-6.

100. **Meuwissen ME, Mancini GM.** Neurological findings in incontinentia pigmenti; a review. European journal of medical genetics 2012;55:323-31.

101. **Minic S, Trpinac D, Obradovic M.** Systematic review of central nervous system anomalies in incontinentia pigmenti. Orphanet journal of rare diseases 2013;8:25.

102. **Bessieres-Grattagliano B, Foliguet B, Devisme L, et al.** Refining the clinicopathological pattern of cerebral proliferative glomeruloid vasculopathy (Fowler syndrome): report of 16 fetal cases. European journal of medical genetics 2009;52:386-92.

103. **Thomas S, Encha-Razavi F, Devisme L, et al.** High-throughput sequencing of a 4.1 Mb linkage interval reveals FLVCR2 deletions and mutations in lethal cerebral vasculopathy. Hum Mutat 2010;31:1134-41.

104. **Meyer E, Ricketts C, Morgan NV, et al.** Mutations in FLVCR2 are associated with proliferative vasculopathy and hydranencephaly-hydrocephaly syndrome (Fowler syndrome). Am J Hum Genet

2010;86:471-8.

105. **Kato M, Das S, Petras K, et al.** Mutations of ARX are associated with striking pleiotropy and consistent genotype-phenotype correlation. Hum Mutat 2004;23:147-59.

106. **Chabriat H, Joutel A, Dichgans M, Tournier-Lasserve E, Bousser MG.** Cadasil. Lancet neurology 2009;8:643-53.

107. **Fukutake T.** Cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL): from discovery to gene identification. Journal of stroke and cerebrovascular diseases : the official journal of National Stroke Association 2011;20:85-93.

108. **Richards A, van den Maagdenberg AM, Jen JC, et al.** C-terminal truncations in human 3'-5' DNA exonuclease TREX1 cause autosomal dominant retinal vasculopathy with cerebral leukodystrophy. Nature genetics 2007;39:1068-70.

109. **Polvi A, Linnankivi T, Kivela T, et al.** Mutations in CTC1, encoding the CTS telomere maintenance complex component 1, cause cerebroretinal microangiopathy with calcifications and cysts. Am J Hum Genet 2012;90:540-9.

110. **Henneke M, Diekmann S, Ohlenbusch A, et al.** RNASET2-deficient cystic leukoencephalopathy resembles congenital cytomegalovirus brain infection. Nature genetics 2009;41:773-5.

111. **Joutel A, Corpechot C, Ducros A, et al.** Notch3 mutations in CADASIL, a hereditary adult-onset condition causing stroke and dementia. Nature 1996;383:707-10.

112. **Hara K, Shiga A, Fukutake T, et al.** Association of HTRA1 mutations and familial ischemic cerebral small-vessel disease. The New England journal of medicine 2009;360:1729-39.

113. **Sproule DM, Kaufmann P.** Mitochondrial encephalopathy, lactic acidosis, and strokelike episodes: basic concepts, clinical phenotype, and therapeutic management of MELAS syndrome. Annals of the New York Academy of Sciences 2008;1142:133-58.

114. **Drera B, Barlati S, Colombi M.** Ischemic stroke in an adolescent with arterial tortuosity syndrome. Neurology 2007;68:1637; author reply

115. **Kalbhenn T, Neumann LM, Lanksch WR, Haberl H.** Spontaneous intracerebral hemorrhage and multiple infarction in Williams-Beuren syndrome. Pediatric neurosurgery 2003;39:335-8.

116. **Lee WD, Hsu JJ, Huang FC, Chao MC, Chang YL, Huang MH.** Ischemic stroke in Williams-Beuren syndrome: a case report. The Kaohsiung journal of medical sciences 2009;25:212-6.

117. **Rossi G, Giaccone G, Maletta R, et al.** A family with Alzheimer disease and strokes associated with A713T mutation of the APP gene. Neurology 2004;63:910-2.

118. **Olafsson I, Thorsteinsson L, Jensson O.** The molecular pathology of hereditary cystatin C amyloid angiopathy causing brain hemorrhage. Brain Pathol 1996;6:121-6.

119. **Cornette L, DeVeber G, Govaert P.** Perinatal stroke. Seminars in fetal & neonatal medicine 2009;14:243-4.

120. **Volpe JJ.** Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. Lancet neurology 2009;8:110-24.

Table 2. Genetic causes of cerebrovascular disease

Arteriovenous malformations (AVMs)

Syndrome (inheritance)	Gene (OMIM) Chomosome	Protein	Protein function	Cerebrovascular phenotype	Additional features	Refs
Hereditary Hemorrhagic Telangiectasia (AD)	ENG (187300) 9q34.1	Endoglin	Transmembrane protein, component of TGFBR complex	Cerebral AVMs, ICH, SAH	Telangiectases; epistaxis; pulmonary, liver, gastrointestinal AMVs	56,57
	ALK1 (600376) 12q11-q14	Activin Receptor-Like Kinase 1	Cell surface receptor of TGFB-family ligands			
Hereditary Hemorrhagic Telangiectasia with juvenile polyposis coli (AD)	SMAD4 (175050) 18q21.2	Mothers Against Decapentaplegic Homolog 4	Signal transduction protein, activated by TGFB-signaling	Cerebral AVMs, ICH, SAH	Telangiectases; epistaxis; pulmonary, liver, gastrointestinal AMVs	56,57
Capillary malformation-arteriovenous malformation (AD)	RASA1 (608354) 5q14.3	Ras GTPase-Activating Protein 1	Cytoplasm, GTP-ase activating protein	Cerebral AVMs, Vein of Galen aneurysmal malformation	Cutaeous capillary malformations, spinal, facial and extremity AVMs	58,59

Cerebral Cavernous Malformations (CCMs)

Syndrome (inheritance)	Gene (OMIM) Chomosome	Protein	Protein function	Cerebrovascular phenotype	Additional features	Refs
Cerebral Cavernous Malformations (AD)	KRIT1 (116860) 7q21.2	Krev Interaction Trapped 1	Parts of the CCM complex, associated with cytoskeletal elements, signal transduction components, and cell junctions	CCMs, ICH, calcifications	Retinal, hepatic and cutaneous vascular lesions	60,61
	MGC4607/CCM2 (603284) 7p13	Malcavernin				
	PDCD10 (603285) 3q26.1	Programmed Cell Death Protein 102				

(continued on next page)

Table 2. Genetic causes of cerebrovascular disease

Cerebral Artery Aneurysms (CAA)

Syndrome (inheritance)	Gene (OMIM) Chomosome	Protein	Protein function	Cerebrovascular phenotype	Additional features	Refs
Loeys-Dietz syndrome (AD)	TGFβR1 (609192, 608967) 9q22.33	Transforming Growth Factor, Beta Receptor I	TGFBR1 and TGFBR2 form complex, role in TGFB-signaling transduction	CAA	Facial features (hypertelorism, micrognatia, bifid uvula, blue sclerea), pectus deformities, congenital heart defects, generalized arterial tortuosity and aneurysms, joint laxity, scoliosis, arachnodactyly	62,63
	TGFβR2 (610168, 610380) 3p24.1	Transforming Growth Factor, Beta Receptor 2				
	TGFβ2 (614816) 1q42	Transforming Growth Factor, Beta				
Ehlers Danlos syndrome type IV (AD)	COL3A1 (130050) 2q31	Collagen, Type III, Alpha 1	Extracellular matrix protein	CAA	Facial features (Sunken eyes, pinched nose, thin lips), mitral valve prolaps, arterial dissections, gastrointestinal or uterine rupture, fragile skin	64,65
Aneurysm Osteoarthritis syndrome (AD)	SMAD3 (613795) 15q22.33	Mothers Against Decapentaplegic Homolog 3	Signal transduction protein, activated by TGFB-signaling	CAA, cerebral arterial tortuosity	Facial features (hypertelorism, bifid uvula, high-arched/ cleft palate, dental malocclusion), aneurysms, arterial tortuosity, osteoarthritis, arachnodactyly, joint laxity, easy bruising, velvety skin	66,67
Familial Thoracic Aortic Aneurysm with Dissection syndrome (AD)	ACTA2 (611788) 10q23.31	Actin, Alpha 2	Globular protein with a role in cell motility, structure and integrity	Stenosis of ICA, small vessel disease with microinfarcts and CAA, corpus callosum and gyral abnormalities	Mydriasis, patent ductus arteriosus, aortic aneurysms, pulmonary hypertension, malrotation, livedo reticularis	39,40,68

(continued on next page)

Table 2. Genetic causes of cerebrovascular disease *(continued from previous page)*

Lysyl hydroxylase-3 (LH3) deficiency (AR)	PLOD3 (612394) 7q22	Procollagen-Lysine, 2-Oxoglutarate 5-Dioxygenase 3	Enzyme that catalyzes the hydroxylation of lysyl residues in collagen-like peptides, essential for collagen cross-linkking	CAA, ICH	Facial features (flat facial profile, shallow orbits, simple, low set ears), deafness, cataract, arterial rupture, aneurysms, scoliosis, contractures, muscle atrophy, fractures, easy bruising, developmental delay	69
Hereditary porencephaly (AD)	COL4A1 (175780, 611773) 13q34	Collagen, Type IV, Alpha 1	Extracellular matrix proteins, major component of basement membranes	CAA, porencephaly, small vessel disease, ICH, schizencephaly, intracranial calcifications	Retinal arterial tortuosity, cataract, microphthalmia, glaucoma, muscle cramps, myopathy, renal cysts, hematuria, Raynaud phenomenon	29,33,70
	COL4A2 (614483) 13q34	Collagen, Type IV, Alpha 2				
Autosomal Dominant Polycystic Kidney Disease (AD)	PKD1 (173900) 16p13.3	Polycystic Kidney Disease 1	Transmembrane proteins, involved in renal tubulogenesis and cilium regulation	CAA	Bilateral renal cysts, renal failure, liver cysts	71,72
	PKD2 (613095) 4q22.1					
Neurofibromatosis type 1 (AD)	NF1 (162200) 17q11.2	Neurofibromin-1	Cytoplasmatic protein, stimulates the GTPase activity of Ras	CAA	macrocephaly, caf'e-au-lait spots, axillary freckling, Lisch nodules, (plexiform) neurofibromas, skeletal abnormalities, learning difficulties	73,74
Microcephalic osteodysplastic primordial dwarfism, type II (AR)	PCNT (210720) 21q22.3	Pericentrin	Integral component of the centrosome	CAA, moyamoya disease, cerebral infarcts	Severe growth retardation, microcephaly, skeletal abnormalities, high-pitched voice, café-au-lait spots, normal intelligence/developmental delay	75,76
Alagille syndrome (AD)	JAG1 (118450) 20p12	Jagged-1	Ligand for NOTCH receptors	CAA	Facial features (broad forehead, hypertelorism, posterior embryotoxon and other opthalmological abnormalities, intrahepatic bile duct paucity, congenital heart defects, renal disease, abnormal vertebral segmentation	77,78

(continued on next page)

Table 2. Genetic causes of cerebrovascular disease *(continued from previous page)*

Moyamoya syndrome

Syndrome (inheritance)	Gene (OMIM) Chomosome	Protein	Protein function	Cerebrovascular phenotype	Additional features	Refs
Moyamoya disease type 5 (AD)	ACTA2 (611788) 10q23.31	Actin, Alpha 2	Globular protein with a role in cell motility, structure and integrity	Stenosis of ICA, small vessel disease with microinfarcts and CAA, corpus callosum and gyral abnormalities	Mydriasis, patent ductus arteriosus, aortic aneurysms, pulmonary hypertension, malrotation, livedo reticularis	39,40,68
Down syndrome (CHR)	Trisomy 21			moyamoya, cerebral infarction	Facial features, gastroenterologic, ophthalmologic, endocrine, immunologic and ortopedic abnormalities	80
Neurofibromatosis type 1 (AD)	NF1 (162200) 17q11.2	Neurofibromin-1	Cytoplasmatic protein, stimulates the GTPase activity of Ras	CAA, moyamoya disease	macrocephaly, caf'e-au-lait spots, axillary freckling, Lisch nodules, (plexiform) neurofibromas, skeletal abnormalities, learning difficulties	73,74
Microcephalic osteodysplastic primordial dwarfism, type II (AR)	PCNT (210720) 21q22.3	Pericentrin	Integral component of the centrosome	CAA, moyamoya disease, cerebral infarcts	Severe growth retardation, microcephaly, skeletal abnormalities, high-pitched voice, café-au-lait spots, normal intelligence/developmental delay	75,76
Alagille syndrome (AD)	JAG1 (118450) 20p12	Jagged-1	Ligand for NOTCH receptors	CAA, moyamoya disease	Facial features (broad forehead, hypertelorism, posterior embryotoxon and other opthalmological abnormalities, intrahepatic bile duct paucity, congenital heart defects, renal disease, abnormal vertebral segmentation	77,78
Sickle cell anemia (AR)	HBB (603903) 11p15.4	Hemoglobin, Beta	Determines toghether with HBA the structure of the 2 types of polypeptide chains in adult hemoglobin	ICH, moyamoya disease, cerebral infarcts	Generilized vasoocclusive disease, chronic anemia, hemolysis	81
Susceptibility to moyamoya disease-2 (MF)	RNF213 (607151)	Ring Finger Protein 213	E3 ubiquitin-protein ligase that may play a role in angiogenesis. May also have an ATPase activity	Moyamoya, ischemic stroke		82

(continued on next page)

Table 2. Genetic causes of cerebrovascular disease *(continued from previous page)*

Metabolic causes of cerebral vascular disease

Syndrome (inheritance)	Gene (OMIM) Chomosome	Protein	Protein function	Cerebrovascular phenotype	Additional features	Refs
Glutaric aciduria type 1 (AR)	GCDH (231670) 19p13.2	Glutayl-CoA dehydrogenase	Catalyzes a step in the degradative pathway of lysine, hydroxylysine, and tryptophan metabolism	SDH	Cerebral atrophy, subependymal pseudocysts, white matter and basal ganglia changes, macrocephaly, metabolic encephalopathy	83,84
Menkes disease (X-L)	ATP7A Xq13.3	Copper-Transporting ATPase 1	Transmembrane protein that functions in copper transport across membranes.	Bilateral SDH, SAH	Delayed cerebral myelination, cerebral and cerebellar atrophy, short stature, microcephaly, kinky hair, joint and skin hyperlaxity, wormian bones, metaphyseal widening with spurs	85,86
Molybdenum cofactor deficiency (AR)	MOCS1 (252150) 6p21.2	Molybdenum cofactor synthesis step 1	Enzymes responsible for the formation of molybdenum cofactor. Molybdenum cofactor is essential for the function of sulfite oxidase, xanthine dehydrogenase and aldehyde oxidase and mitochondrial amidoxime reducing component	ICH, IVH	Neonatal intractable seizures, progressive encephalopathy, feeding difficulties, lens dislocation, cerebral oedema, cystic encephalomalacia, atrophy, basal gangliachanges, corpus callosum dysgenesis, ventriculomegaly	87-89
	MOCS2 (252150) 5q11.2	Molybdenum cofactor synthesis step 2				
	GPHN (252150) 14q23.3	Gephyrin				

(continued on next page)

Table 2. Genetic causes of cerebrovascular disease *(continued from previous page)*

Fabry (X-L)	GLA (301500) Xq22.1	Alpha-galactosidase A	Role in glycosphingolipid catabolism	Transient ischemic attacks, ischemic strokes, microbleeds	Cardiac left ventricular hypertrophy, progressive renal failure, neuropathy, skin lesions	90
Dihydropyrimidine dehydrogenase deficiency (AR)	DPYD (274270) 1p21.3	Dihydropyrimidine dehydrogenase	Initial and rate-limiting enzyme in the catabolism of the pyrimidine bases uracil and thymine	Cerebral infarction, white matter abnormalities	Microcephaly, mental retardation, opthalmological abnormalities, autism, seizures, hyperactivity	91
Congenital disorders of glycosylation, type 1a (AR)	PMM2 (212065) 16p13.2	Phosphomannomutase-2	Enzyme necessary for the synthesis of GDP-mannose	Stroke-like episodes, cerebral infarction	Failure to thrive, retinitis pigmentosa, liver fibrosis and steatosis, cardiomyopathy, inverted nipples, abnormal fat tissue distribution, renal problems, coagulopathy, neuropathy	92.93

(continued on next page)

Table 2. Genetic causes of cerebrovascular disease *(continued from previous page)*

Hemophilia/ Coagulopathies

Syndrome (inheritance)	Gene (OMIM) Chomosome	Protein	Protein function	Cerebrovascular phenotype	Additional features	Refs
Hemophilia (X-L)	F8 Xq28 F9 Xq27.1	Coagulation Factor VIII Coagulation Factor IX	Participate in the blood coagulation pathway	ICH, IVH, SDH	Hemarthrosis, degenerative joint disease, ecchymoses	94,95
Sickle cell anemia (AR)	HBB (603903) 11p15.4	Hemoglobin, Beta	Determines toghether with HBA the structure of the 2 types of polypeptide chains in adult hemoglobin	ICH, moyamoya, cerebral infarcts	Generilized vasoocclusive disease, chronic anemia, hemolysis	81
Factor VII deficiency (AR)	F7 (227500) 13q34	Coagulation Factor VIII	Participate in the blood coagulation pathway	ICH	Hemarthrosis, epistaxis, intramuscular hemorrhage, bleeding diathesis	96
Protein S deficiency (AD/AR)	PROS1 (176880) 3q11.1	Protein S	Inhibits blood clotting by serving as a cofactor for activated protein C in the inactivation of procoagulant factors V and VIII	Cerebral venous thrombosis, arterial thrombosis	Recurrent venous thombosis, thromboflebitis, arterial thrombosis, pulmonary embolism	97
Protein C deficiency (AD/AR)	PROC (612283) 2q14.3	Protein C	Vitamin K-dependent serine protease that regulates blood coagulation by inactivating factors Va and VIIIa	Cerebral thrombosis	Recurrent venous thombosis, thromboflebitis, arterial thrombosis, pulmonary embolism	97
Factor V Leiden (AD/AR)	F5 (188055) 1q24.2	Factor V	Essential cofactor of the blood coagulation cascade.	Cerebral venous thrombosis, cerebral arterial thrombosis	Venous thrombosis	97
MTHFR deficiency (AR)	MTHFR (188050) 1p36.22	Methylenetetrahydrofolate reductase	Catalyzes the conversion of 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate, a cosubstrate for homocysteine remethylation to methionine	Cerebral thrombosis	Recurrent venous thombosis, pulmonary embolism	97
Prothrombin deficiency (AD/ AR)	F2 (188050) 11p11.2	Coagulation Factor II	Is proteolytically cleaved to form thrombin in the first step of the coagulation cascade which ultimately results in the stemming of blood loss. F2 also plays a role in maintaining vascular integrity	Intracranial bleeding	Hemarthrosis, epistaxis, gastrointestinal bleeding, bleeding diathesis	97

(continued on next page)

Table 2. Genetic causes of cerebrovascular disease *(continued from previous page)*

Antenatal- infantile hemorrhage/ Pseudo-TORCH disease

Syndrome (inheritance)	Gene (OMIM) Chomosome	Protein	Protein function	Cerebrovascular phenotype	Additional features	Refs
Hemorrhagic destruction of the brain, subependymal calcification, and cataracts (AR)	JAM3 (613730) 11q25	Junctional Adhesion Molecule 3	Tight junction molecule	ICH, cystic white matter degeneration, porencephaly, ventrulomegaly	Subependymal calcifications, cataracts, microphthalmia, hepatomegaly, renal cystic dysplasia, ectopic kidney	36,98
Aicardi-Goutieres syndrome (AR)	TREX1 3p21.31	Three Prime Repair Exonuclease 1	May play a role in DNA repair and serve as a proofreading function for DNA polymerase	Intracranial calcifications	Progressive microcephaly, white matter destruction, brain atrophy, PMR, thrombocytopenia, hepatosplenomegaly, elevated hepatic transaminases, intermittent fever, glaucoma, cataract, chilblain lupus, arthropathy	49-51,99
	RNASEH2B 13q14.3	Ribonuclease H2, subunit B	Catalytic subunit of RNase HII, an endonuclease that specifically degrades the RNA of RNA:DNA hybrids	Intracranial calcifications	Idem	
	RNASEH2C 11q13.1	Ribonuclease H2, subunit C	Catalytic subunit of RNase HII, an endonuclease that specifically degrades the RNA of RNA:DNA hybrids	Intracranial calcifications	Idem	
	RNASEH2A 19p13.2	Ribonuclease H2, subunit A	Catalytic subunit of RNase HII, an endonuclease that specifically degrades the RNA of RNA:DNA hybrids	Intracranial calcifications	Idem	

(continued on next page)

Table 2. Genetic causes of cerebrovascular disease *(continued from previous page)*

	SAMHD1 20q11.23	SAM Domain And HD Domain 1	Role in mediation of immune response	moyamoya syndrome, CAA, ICH	Idem	
	ADAR1 1q21.3	Adenosine Deaminase, RNA-Specific	This enzyme destabilizes double-stranded RNA through conversion of adenosine to inosine	Intracranial calcifications	Idem	
Band-like calcification with simplified gyration and polymicrogyria (AR)	OCLN (251290) 5q13.2	Occludin	Plays a role in the formation and regulation of the tight junction (TJ) paracellular permeability barrier	Band-like intracranial calcifications	Polymicrogyria, simplified gyral pattern, progressive microcephaly	35
Incontinentia Pigmenti (X-L)	NEMO (308300) Xq28	NF-KappaB Essential Modulator	Regulatory subunit of the inhibitor of kappaB kinase (IKK) complex, which activates NF-kappaB, resulting in activation of genes involved in inflammation, immunity, cell survival, and other pathways	ICH, periventricular and subcortical white matter disease, cerebral infarcts	Corpus callosum hypoplasia, cerebral atrophy, hair, nail and dental abnormalities, ophthalmological abnormailies, pathognomonic skin inflammation/ hyper- and hypopigmentation	100, 101
Cerebroretinal microangiopathy with calcifications and cysts (AR)	CTC1 (612199) 17p13.1	CTS Telomere Maintenance Complex Component 1	Subunits of an alpha accessory factor (AAF), that stimulates the activity of DNA polymerase-alpha-primase, the enzyme that initiates DNA replication	Leukoencephalopathy, brain cysts, intracranial calcifications	Retinal telangiectasia and exudates , osteopenia, vasculature ectasias in the stomach, small intestine and liver, anemia, thrombocytopenia	109
Leukoencephalopathy, cystic, without megalencephaly (AR)	RNASET2 (612951) 6q27	Ribonuclease T2	Enxyme with ribonuclease activity. Probably involved in lysosomal degradationof ribosomal RNA.	Cystic leukoencephalopathy, multifocal white matter lesions, ventricular enlargement, intracranial calcifications	Microcephaly, hearing loss	110

(continued on next page)

Table 2. Genetic causes of cerebrovascular disease *(continued from previous page)*

Hereditary porencephaly (AD)	COL4A1 (175780, 611773) 13q34	Collagen, Type IV, Alpha 1	Extracellular matrix proteins, major component of basement membranes	CAA, porencephaly, small vessel disease, ICH, schizencephaly, intracranial calcifications	Retinal arterial tortuosity, cataract, microphthalmia, glaucoma, muscle cramps, myopathy, renal cysts, hematuria, Raynaud phenomenon	29,33,70
	COL4A2 (614483) 13q34	Collagen, Type IV, Alpha 2				
Pseudo-TORCH syndrome with cerebral hemorrhage	USP18 22q11.2	Ubiquitin Specific Protein 18	Upregulated upon IFN type I signalling, functions in deISGylation and is an inhibitor of IFNAR receptor	Cerebral and cerebellar hemorrhage, intracranial calcifications, white matter abnormalities, necrosis	Thrombocytopenia, liver failure with ascitis, respiratory distress	

(continued on next page)

Table 2. Genetic causes of cerebrovascular disease *(continued from previous page)*

Arterial ischemic stroke/ angiopathy

Syndrome (inheritance)	Gene (OMIM) Chomosome	Protein	Protein function	Cerebrovascular phenotype	Additional features	Refs
CADASIL (AD)	NOTCH3 19p13.12	NOTCH3	Functions as a receptor for membrane-bound ligands Jagged1, Jagged2 and Delta1 to regulate cell-fate determination	Lacunar infarcts, microbleeds, leukoencephalopathy	Seizures, migraine, dementia	106
CARASIL (AR)	HTRA1 (600142) 10q26.13	HtrA Serine Peptidase 1	Member of the trypsin family of serine proteases	Subcortical lacunar infarcts, diffuse white matter anomalies, acute stroke	White matter demyelination, ataxia, dementia, progressive encephalopathy, low back pain, premature baldness	107
Mitochondrial myopathy, encephalopathy, lactic acidosis, and stroke-like episodes (MELAS)(Mit)	Mitochondrial genes MTTL1, MTTQ, MTTH, MTTK, MTTC, MTTS1, MTTS2, MTND1, MTND5, MTND6 (540000)			Stroke-like episodes, encephalopathy	Cataract, hearing loss, myopathy, migraine, seizures, dementia, lactic acidosis	113
Arterial Tortuosity Syndrome (AR)	SLC2A10 (208050) 20q13.12	Glucose transporter GLUT10	Plays a role in regulation of glucose homeostasis	Arterial tortuosity, cerebral infarction, arterial aneurysm	Arterial tortuosity, arterial aneurysms, downslanting palpebral fissures, hypertelorism, pectus deformity, joint laxity, diaphragmatic hernia	114
Williams-Beuren syndrome (CHR)	7q11.23 deletion (194050)			Cerebral infarction, ICH	Intellectual disability, facial dysmorphisms, growth retardation, congenital heart disease, iris stellata, renal anomalies, hearing loss	115,116
Cerebral Amyloid Arteriopathy (AD)	APP (605714) 21q21.3	Amyloid beta (A4) precursor protein	transmembrane precursor protein that is cleaved by secretases to form a number of peptides. Some of these peptides are secreted and promote transcriptional activation, while others form the protein basis of the amyloid plaques.	Cerebral amyloid angiopathy, cerebral infarction, recurrent strokes, microbleeds, cerebral and cerebellar hemorrhage	Dementia	117
	CST3 (105150) 20p11.21	Cystatin C	An inhibitor of lysosomal cysteine proteinases	Cerebral hemorrhage	Dementia, amyloid deposition	118

ICH, intracerebral hemorrhage; SAH, subarachnoidal hemorrhage; ICA, internal carotid artery; SDH, subdural hemorrhage; IVH, intraventricular hemorrhage
AD, autosomal dominant; AR, autosomal recessive; X-L, X-linked; CHR, chromosomal; MIT, mitochondrial; MF, multifactorial

PART I

GENETIC CAUSES OF ISCHEMIC STROKE

Chapter 2.1

ACTA2 MUTATION WITH CHILDHOOD CARDIOVASCULAR, AUTONOMIC AND BRAIN ANOMALIES AND SEVERE OUTCOME

Marije E.C. Meuwissen[1], Maarten H. Lequin[2], Karen Bindels-de Heus[3], Hennie T. Bruggenwirth[1], Maarten F.C.M. Knapen[4], Michiel Dalinghaus[5], Rene de Coo[6], Yolande van Bever[1], Beerend H.J. Winkelman[7], and Grazia M.S. Mancini[1*]

European Journal of Human Genetics Part A 161A:1376–1380.

1. *Dept. of Clinical Genetics, Erasmus University Medical Center, Rotterdam.*
2. *Dept. of Radiology, Erasmus University Medical Center, Rotterdam.*
3. *Dept. of Pediatrics, Erasmus University Medical Center, Rotterdam.*
4. *Dep. of Obstetrics and Prenatal Medicine, Erasmus University Medical Center, Rotterdam.*
5. *Dept. of Cardiology, Erasmus University Medical Center, Rotterdam.*
6. *Dept. of Neurology, Erasmus University Medical Center, Rotterdam.*
7. *Netherlands Institute for Neuroscience, Amsterdam.*

* *These authors equally contributed to this manuscript.*

ABSTRACT

Thoracic aortic aneurysm and dissection (TAAD) are associated with connective tissue disorders likeMarfan syndrome and Loeys–Dietz syndrome, caused by mutations in the fibrillin-1, the TGFb-receptor 1- and -2 genes, the *SMAD3* and *TGFB2* genes, but have also been ascribed to *ACTA2* gene mutations in adults, spread throughout the gene. We report on a novel *de novo* c.535C>T in exon 6 leading to p.R179C aminoacid substitution in *ACTA2* in a toddler girl with primary pulmonary hypertension, persistent ductus arteriosus, extensive cerebral white matter lesions, fixed dilated pupils, intestinal malrotation, and hypotonic bladder. Recently, *de novo ACTA2* R179H substitutions have been associated with a similar phenotype and additional cerebral developmental defects including underdeveloped corpus callosum and vermis hypoplasia in a single patient. The patient here shows previously undescribed abnormal lobulation of the frontal lobes and position of the gyrus cinguli and rostral dysplasis of the corpus callosum; she died at the age of 3 years during surgery due to vascular fragility and rupture of the ductus arteriosus. Altogether these observations support a role of *ACTA2* in brain development, especially related to the arginine at position 179. Although all previously reported patients with R179H substitution successfully underwent the same surgery at younger ages, the severe outcome of our patient warns against the devastating effects of the R179C substitution on vasculature.

INTRODUCTION

ACTA2 is a major component of actin filaments of the contraction units of vascular smooth muscle cells (SMC), which is also expressed in other organs including brain with highest levels of expression in late pregnancy and neonatal period. Actin filaments are organised in polymerized fibers across the cell, they interact with myosin and mediate anchoring of the cytoskeleton to the plasma membrane, all processes necessary for cell motility and maintenance of cell shape[1]. Autosomal dominant mutations in the *ACTA2* gene were first described in adult thoracic aortic aneurysms and dissection (TAAD) patients. Additional features are livedo reticularis, iris flocculi, patent ductus arteriosus (DA) and bicuspid aortic valves, early onset coronary artery disease, ischemic stroke, cerebral aneurysms and Moyamoya disease[2-4].

Recently, the *de novo* heterozygous R179H substitution in *ACTA2* was associated with a severe phenotype in childhood, including ascending aorta aneurysms, patent ductus arteriosus, and disruption of SMC-dependent organs leading to congenital mydriasis, hypotonic bladder and congenital intestinal malrotation, as well as dilated pulmonary arteries, cystic lung disease and pulmonary hypertension[5,6]. Gastrointestinal prune belly was present in one patient[6]. Also tortuosity and dilatation of retinal arterioles in childhood are reported[23]. Childhood-onset cerebrovascular disease including fusiform dilatation of the internal carotid artery (ICA) and tapering of the terminal ICA, indicative of stenosis (Moyamoya-like) are described[5,7]. Brain MRI findings are (progressive) periventricular leucomalacia and incidentally a thin corpus callosum, colpocephaly, small cerebellar vermis or Chiari I malformation[5,7].

We report on a patient with a novel *de novo ACTA2* missense leading to substitution of arginine with cysteine at position 179 (R179C), presenting with pulmonary hypertension, patent DA, mydriasis, intestinal malrotation, bladder dysfunction and cerebral developmental anomalies of the corpus callosum and cortical gyri.

PATIENT DESCRIPTION

The girl was born at 36+3 weeks gestation from Caucasian, non-consanguineous parents. Pregnancy was complicated by recurrent vaginal blood loss in the first trimester. Labour was induced prematurely because of maternal pre-eclampsia. Antenatal ultrasound at 20 weeks gestation was normal. At 32 weeks gestation an enlarged bladder, a right sided hydro-

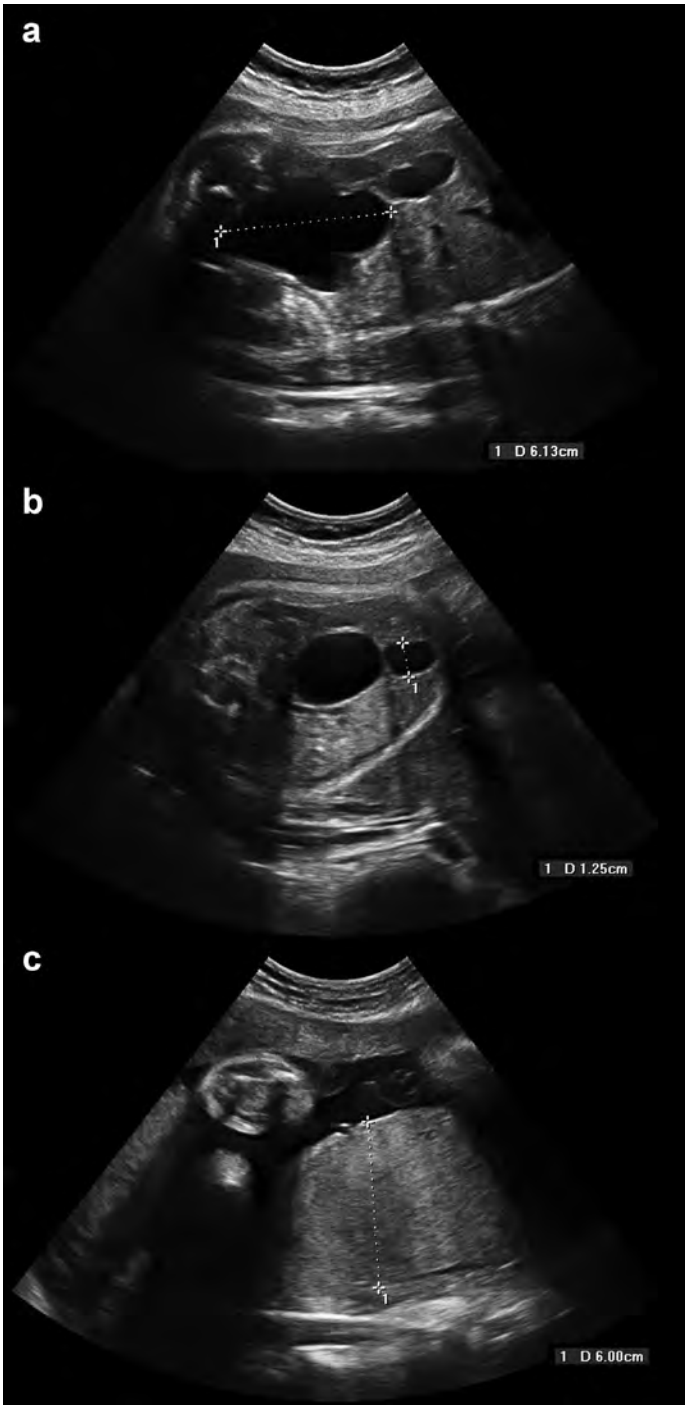


Figure 1. Prenatal ultrasound images (at 32 weeks gestation) showing an enlarged bladder, 6.13 cm in diameter (**a**), a umbilical vein varix, 1.25 cm in diameter (**b**) and a thickened placenta, 6.00 cmin diameter (**c**).

nephrosis, an umbilical vein varix and placental thickening were noted (Figure 1a,b,c). Birth weight was 2925 grams (+1 SD), OFC was 33 cm (0SD) and Apgar scores were 9 and 9 after 1 and 5 minutes. Postpartum she became oxygen dependent for three days with spontaneous recovery. In contrast to prenatal findings, abdominal ultrasound was normal. Cerebral ultrasound showed prominent striate vessels and a small choroid plexus cyst. TORCHES screen was negative. Ophthalmological examination showed wide, non-responsive pupils with partial aniridia, iris/ lens adhesions and hypermetropia. At the age of 10 months, she was admitted with a RS-bronchiolitis. At 16 months she was readmitted with an airway infection and quickly developed respiratory insufficiency with ventilation difficulties due to extreme bronchospasm. Pulmonary hypertension was seen, requiring temporary mechanical ventilation. Thoracic CT-scans showed pulmonary emphysema. A pulmonary perfusion scan at the age of 1.5 years showed a diminished perfusion. Echocardiography showed a patent DA. Cardiac catheterisation at this stage revealed an increased pulmonary vascular resistance (PVR) with bidirectional ductal shunt; PVR was reactive to inhaled oxygen and NO with abolishment of the right-to-left shunt across the DA. Before deciding to close the patent DA, treatment was started with continuous oxygen and Bosentan.

She had feeding difficulties necessitating intermittent gastric tube feeding and an intestinal malrotation was suspected.

Her motor, cognitive and speech development were normal. At age 2 years, height was at -1 SD, weight at -2 SD and head circumference at -1.8 SD. Facial features included a high forehead, full eyelids with downslanting palpebral fissures, epicanthic folds, a full nasal tip and she had a soft, doughy skin. Neurologic examination was normal. Brain MRI at the age of 2 years revealed an abnormal corpus callosum with a small genu, absent rostrum and lamina terminalis but a normal anterior commissure. The gyrus cinguli was abnormally positioned, the rostral dorsal cingulum looked absent. The medial cerebral hemispheric sulcation was very abnormal resulting in symmetrically distorted architecture of the frontal lobes (Figure 2a,b,c). Vasculature was also abnormal, with straight arteries radiating from the A2 segment of the anterior cerebral arteries and M2, M3 and M4 segments of the medial cerebral arteries (Figure 2b). No Moyamoya-like occlusive lesions were seen. Additionally, extensive confluent periventricular white matter lesions (leukoencephalopathy) were present (Figure 2c). A repeat funduscopy at the age of two years revealed retinal arterial tortuosity, raising the suspicion of a vascular connective tissue disorder (Figure 3). During the diagnostic work up of our patient the paper from Milevicz *et al* was published[5],

which triggered us to test *ACTA2*. At the age of 3 years, shortly after identifying the *ACTA2* mutation, the pulmonary situation appeared stable, leading to the assumption that is was safe to close the patent DA. However, during surgery the DA appeared to be too wide to clip or ligate and lead, together with unexpected extremely fragile vasculature, to rupture and massive bleeding, resulting in patient's demise.

MOLECULAR STUDIES

Sequencing analysis of *PAX6* and *COL4A1*, FISH analysis of the *PAX6*/*WT1* locus, and genomic Affymetrix 250k SNP microarray CNV analysis were normal. Sequencing of the coding exons and exon/intron boundaries of *ACTA2* (NM_001613.1) identified the het-

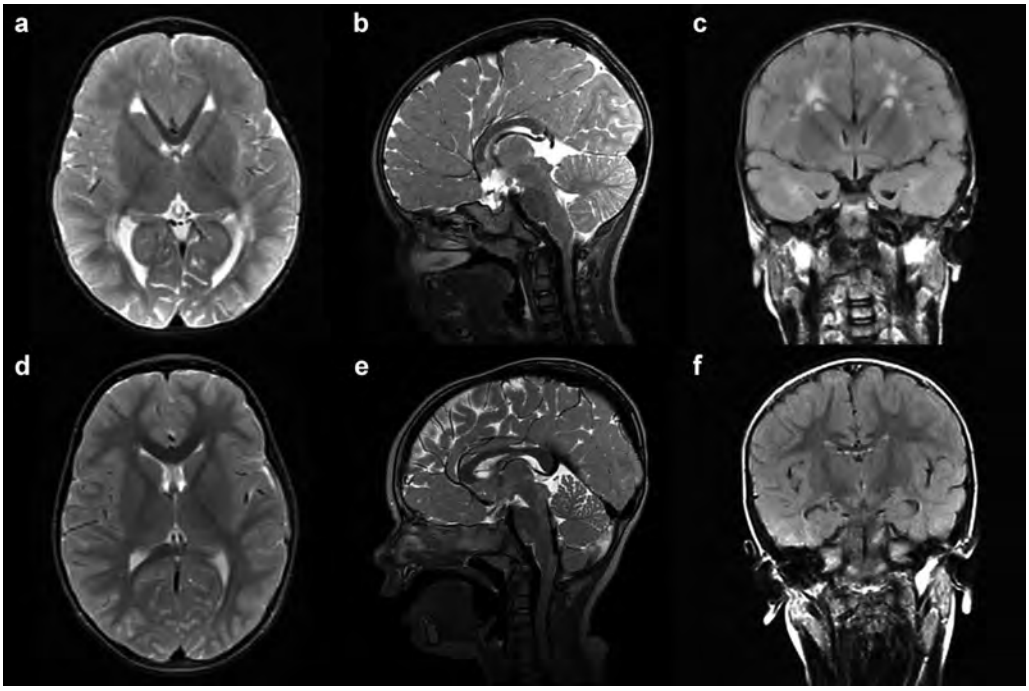


Figure 2. T2-weighed (**a,b**) and FLAIR (**c**) patient images showing a small genu, absent rostrum, and lamina terminalis (**b**) and abnormal "steep" course (**a,c**) of the corpus callosum. An abnormal fornix and aberrant orientation of the cingulum gyrus, due to absence of the rostral dorsal part can be noted (**b**). An abnormal, straightened course of the frontal branches of the anterior cerebral artery, including the pericallosal arteries (**b**) is seen. On FLAIR image, extensive confluent hyperintensities of the periventricular white matter, which can be interpreted as confluent leukoencephalopathy, can be appreciated (**c**). Also, bilateral underdevelopment of the hippocampal region is present (**c**). T2-weighed axial and sagittal (**d,e**) and coronal FLAIR (**f**) image of age-matched control.

erozygous sequence variant c.535C>T in exon 6, substituting an arginine with a cysteine (p.R179C). The sequence variant was absent in WBC DNA from both parents, suggesting a *de novo* event. The mutation affects the same codon as the previously reported p.R179H substitution[5].

DISCUSSION

We identified a previously unreported *de novo* ACTA2 substitution changing the arginine at position 179 (R179C). Since the phenotype in our patient is similar to that of R179H substitutions, a genotype-phenotype correlation involving Arginine 179 changes is supported. Arginine 179 is located at the macromolecular surface of the alpha-actin near a key protein-protein interaction site[5]. Substitutions at this amino acid causes a severe systemic disorder in early childhood, different from the adult presentation. One of the characteristic features of this disorder is the extensive cerebral white matter lesion[5-7]. A distinctive vascular phenotype has been associated with Arg179 substitution in a large scale study [Munot *et al*., 2012].

Based on the cerebral white matter abnormalities and the extensive vascular phenotype a connective tissue disorder was considered in our patient. Disorders like Arterial Tortuosity

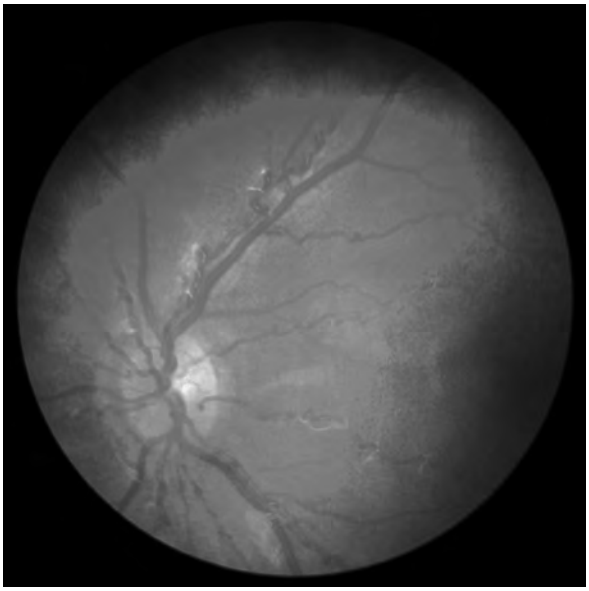


Figure 3. An image of the retina shows tortuosity of the retinal vessels.

Syndrome (OMIM 208050) and Loeys-Dietz syndrome (OMIM 609192) have overlapping features such as arterial tortuosity, pulmonary artery dysfunction with stenosis or aneurysm formation[8,9].

The cerebral vasculopathy and white matter lesions of the Arginine 179 substitutions, although distinctive, resemble to a certain extent those of COL4A1, COL4A2 and are possibly based on silent microbleeds. In *COL4A1* and *COL4A2* mutations, cerebral aneurysms, cerebral hemorrhage, porencephaly and leukoencephalopathy are described[10-13], as well as retinal arterial tortuosity[12].

One of the major proteins responsible for the cross-linking of actin filaments in F-actin networks and to cellular membranes, by binding to transmembrane receptor or ion channels, is filamin A. The F-actin/ filamin A complex hereby regulates cell morphology, membrane integrity and cell locomotion[14,15]. Phenotypic features in *FLNA* and *ACTA2* mutations show similarities, such as patent DA, vascular anomalies such as (cerebral) aneurysms and stroke, aortic stenosis and coarctation[16]. Also severe congenital cystic lung disease with pulmonary arterial hypertension[17,18] and intestinal pseudo-obstruction, resembling SMC-related malrotation or hypoperistalsis[19] were reported in *FLNA* patients.

The brain MRI in our patient shows, besides severe leukoencephalopathy, a developmental defect of the frontal lobes and rostral part of the corpus callosum. A thin corpus callosum, colpocephaly, small cerebellar vermis and Chiari I malformation were previously reported in sporadic *ACTA2* patients[5-7]. Although a distinctive straight and steely appearance of intracerebral arteries has previously been reported, the associated rostral and gyral malformation have not been appreciated [Munot *et al*., 2012]. Since the callosal and gyral abnormalities follow the straight pattern of the anterior cerebral artery (most evident in Figure 2b), we suggest that both vascular and structural abnormalities in this region share a common pathogenesis and might be more common than previously reported in Arg179 substitutions, This MRI presentation can be a diagnostic clue in children with isolated pulmonary hypertension or other non-specific symptomatology, lacking neurological symptoms. This observation underscores the role of *ACTA2* in brain development[20] and broadens the cerebral phenotype related to *ACTA2* mutations. It is possible that the Arginine 179 resides in an actin-2 domain primary involved in protein interactions during brain development[21]. The role of other actins in brain development is demonstrated by the identification of *de novo* mutations in *ACTB* and *ACTG1* in Baraitser Winter syndrome

(OMIM 243310), associated with anterior-predominant brain developmental defects (lissencephaly)[22].

At the age of 3 years our patient died during surgical intervention of the patent DA due to extreme vascular fragility and rupture. The previously reported cases with the ACTA2 R179H substitution all successfully underwent this operation, on ages varying from 20 days to 4 months. Later on, three patients underwent surgery for thoracic aorta aneurysms, at reported ages of 10-12 years[5-7]. In striking contrast to our experience, no complications of surgery were reported. It is possible that the vasculature becomes more damaged due to the disease process, and that surgical intervention for patent DA should be performed as early as possible. However, surgeons should be cautious of possible severe complications of vascular interventions of *ACTA2* patients at young age.

In conclusion, we describe a patient with a novel heterozygous p.R179C substitution in *ACTA2*. The severe phenotype including brain developmental defects and the severely affected vasculature, suggests an additional function of this specific amino acid and/or protein domain. Furthermore, our experience recommends caution regarding surgical intervention, since there is an increased risk of bleeding and vascular rupture.

Acknowledgements
We thank Prof. Dr. I.K.M. Reiss, neonatologist-intensivist and Prof. Dr. H.A.W.M. Tiddens, pulmonologist, both at the Erasmus University Medical Center Rotterdam, and Prof. Dr. R.M.F. Berger, paediatric cardiologist at the University Medical Center Groningen, for their contribution in patient care.

References

1. **Fletcher DA, Mullins RD.** Cell mechanics and the cytoskeleton. Nature 2010;463:485-92.

2. **Guo DC, Pannu H, Tran-Fadulu V, et al.** Mutations in smooth muscle alpha-actin (ACTA2) lead to thoracic aortic aneurysms and dissections. Nat Genet 2007;39:1488-93.

3. **Hoffjan S, Waldmuller S, Blankenfeldt W, et al.** Three novel mutations in the ACTA2 gene in German patients with thoracic aortic aneurysms and dissections. Eur J Hum Genet 2011.

4. **Guo DC, Papke CL, Tran-Fadulu V, et al.** Mutations in smooth muscle alpha-actin (ACTA2) cause coronary artery disease, stroke, and Moyamoya disease, along with thoracic aortic disease. Am J Hum Genet 2009;84:617-27.

5. **Milewicz DM, Ostergaard JR, Ala-Kokko LM, et al.** De novo ACTA2 mutation causes a novel syndrome of multisystemic smooth muscle dysfunction. Am J Med Genet A 2010;152A:2437-43.

6. **Richer J, Milewicz DM, Gow R, et al.** R179H mutation in ACTA2 expanding the phenotype to include prune-belly sequence and skin manifestations. Am J Med Genet A;158A:664-8.

7. **Moosa AN, Traboulsi EI, Reid J, Prieto L, Moran R, Friedman NR.** Neonatal Stroke and Progressive Leukoencephalopathy in a Child With an ACTA2 Mutation. Journal of child neurology.

8. **Coucke PJ, Willaert A, Wessels MW, et al.** Mutations in the facilitative glucose transporter GLUT10 alter angiogenesis and cause arterial tortuosity syndrome. Nat Genet 2006;38:452-7.

9. **Callewaert BL, Willaert A, Kerstjens-Frederikse WS, et al.** Arterial tortuosity syndrome: clinical and molecular findings in 12 newly identified families. Hum Mutat 2008;29:150-8.

10. **Breedveld G, de Coo IF, Lequin MH, et al.** Novel mutations in three families confirm a major role of COL4A1 in hereditary porencephaly. J Med Genet 2006;43:490-5.

11. **Gould DB, Phalan FC, van Mil SE, et al.** Role of COL4A1 in small-vessel disease and hemorrhagic stroke. N Engl J Med 2006;354:1489-96.

12. **van der Knaap MS, Smit LM, Barkhof F, et al.** Neonatal porencephaly and adult stroke related to mutations in collagen IV A1. Annals of neurology 2006;59:504-11.

13. **Verbeek E, Meuwissen ME, Verheijen FW, et al.** COL4A2 mutation associated with familial porencephaly and small-vessel disease. Eur J Hum Genet 2012.

14. **Zhou AX, Hartwig JH, Akyurek LM.** Filamins in cell signaling, transcription and organ development. Trends Cell Biol 2010;20:113-23.

15. **Nakamura F, Stossel TP, Hartwig JH.** The filamins: Organizers of cell structure and function. Cell Adh Migr 2011;5.

16. **de Wit MC, de Coo IF, Lequin MH, Halley DJ, Roos-Hesselink JW, Mancini GM.** Combined cardiological and neurological abnormalities due to filamin A gene mutation. Clin Res Cardiol 2011;100:45-50.

17. **Masurel-Paulet A, Haan E, Thompson EM, et al.** Lung disease associated with periventricular nodular heterotopia and an FLNA mutation. Eur J Med Genet 2010.

18. **de Wit MC, Tiddens HA, de Coo IF, Mancini GM.** Lung disease in FLNA mutation: Confirmatory report. Eur J Med Genet 2010.

19. **Kapur RP, Robertson SP, Hannibal MC, et al.** Diffuse abnormal layering of small intestinal smooth muscle is present in patients with FLNA mutations and x-linked intestinal pseudo-obstruction. Am J Surg Pathol 2010;34:1528-43.

20. **Robinson R.** Loss of microtubule-to-actin linkage disrupts cortical development. PLoS Biol 2011;9:e1001175.

21. **Pawlisz AS, Feng Y.** Three-dimensional regulation of radial glial functions by Lis1-Nde1 and dystrophin glycoprotein complexes. PLoS Biol 2011;9:e1001172.

22. **Riviere JB, van Bon BW, Hoischen A, et al.** De novo mutations in the actin genes ACTB and ACTG1 cause Baraitser-Winter syndrome. Nat Genet 2012;44:440-4, S1-2.

23 Moller HA, Fledelius HC, Milewicz DM, Regalado ES, Ostergaard JR. Eye features in three Danish patients with multisystem smooth muscle dysfunction syndrome. Br J Ophthalmol 2012; 96(9):1227-1231.

Chapter 2.2

NEUROLOGICAL FINDINGS IN INCONTINENTIA PIGMENTI; A REVIEW

Marije E.C. Meuwissen, MD, Grazia M.S. Mancini, MD, PhD

European Journal of Medical Genetics 2012 (55): 323-331

Dept. Of Clinical Genetics, Erasmus University Medical Center, Rotterdam

ABSTRACT

Incontinentia Pigmenti is a rare X-linked multisystem disorder with well described and pathognomonic skin manifestations. Neurological manifestations are found in 30% of IP patients, forming one of the major causes of morbidity and mortality of the condition. In this review, clinical and brain imaging data of 45 IP patients with a neurological phenotype are reviewed. Several clinical presentations could be identified, comprising seizures, infantile encephalopathy, acute disseminated encephalomyelitis and ischaemic stroke. Most neurological features presented during the neonatal period. No patients presented during adolescence or at adult age. Seizures of different type are reported in about 20% of the patients at young age and seem to correlate with the degree of cerebrovascular damage. Brain MRI findings include periventricular and subcortical white matter disease, haemorrhagic changes, corpus callosum hypoplasia, cerebral atrophy and cerebellar hypoplasia. Ocular findings comprise a range of retinal vascular changes and optic atrophy, but also developmental defects like microphthalmia and cataract. Most findings may reflect changes following brain injury. Both (ischaemic) vascular and inflammatory components may play a role in the cerebral and ocular phenotype. However, a role of disturbed apoptosis during development may also be a contributing factor.

INTRODUCTION

Incontinentia Pigmenti (IP) (also known as Bloch-Sulzberger syndrome, OMIM 308300) is an uncommon X-linked multisystem disorder, affecting the skin in all patients, but also other ectodermal tissues comprising teeth, hair and nails, the eyes and the central nervous system. The inheritance pattern is X-linked dominant, with usually male lethality. Some affected males, however, have been reported; some have a concomitant diagnosis of Klinefelter syndrome, in others somatic mosaicism is demonstrated[1,2]. The dermatological features are well defined, occurring in four successive stages, comprising of erythema and vesicles in a linear distribution in the first stage, verrucous, hyperkeratotic papules and plaques in the second stage, linear hyperpigmentation (not necessarily in the areas affected in the first two stages) in stage 3 and pallor and atrophy in the last stage. In 1993, Landy and Donnai defined criteria for IP. Although not truly validated, these criteria still seem very useful in establishing a clinical diagnosis of IP[3-5] (Table 1). In 2000, the molecular defect underlying IP was identified; mutations in the *NEMO* gene on chromosome Xq28 (officially *IKBKG* (inhibitor of κB kinase gamma), also known as *IKKγ*) were shown to cause IP[6]. It was demonstrated that, with rare exceptions, mutations causing IP severely truncate the NEMO protein, leading to a loss of function[6]. In approximately 70% of IP patients, *NEMO* mutations can be identified[7]. In 60-88% of IP patients harboring a mutation, a common deletion of exon 4-10 is found[6,7]. Male lethality is assumed, however, some affected males have been reported; some have a concomitant diagnosis of Klinefelter syndrome, in others somatic mosaicism is demonstrated[1,2]. However, hypomorphic *NEMO* mutations in males lead to a different phenotype of X-linked hypohydrotic ectodermal dysplasia and immunodeficiency (OMIM 300291).

Although not admitted in the clinical criteria of Landy and Donnai, the central nervous system manifestations in IP patients may have a great impact on the quality of life. Overall, the prevalence of central nervous system manifestations is approximately 30%[4,8]. Ophthalmological abnormalities are another major cause of disability in IP patients. Ocular defects, comprising strabismus, retinopathy, congenital cataract and microphthalmia, are present in approximately 20%-37% of IP patients, with severe presentation in 8%[4,9]. A relation is postulated between the severity of the ophthalmological findings and the neurological phenotype[9]. In this review, we want to give an overview of the various central nervous system presentations associated with IP and reflect on possible pathogenic mechanisms.

MATERIALS AND METHODS; SEARCH STRATEGY AND SELECTION CRITERIA

Literature references were identified through a search on Pubmed with the terms "Incontinentia pigmenti and seizures", Incontinentia pigmenti and epilepsy", "incontinentia pigmenti and neurological", "Incontinentia pigmenti and MRI". Reference lists of relevant articles were also reviewed. Cases were selected when the diagnosis IP was documented by skin biopsy or DNA-analysis of the NEMO-gene, or when the clinical findings were documented well enough to establish a clinical diagnosis, and when the neurological disease was documented by brain imaging (CT/ MRI). Only articles in the English language were

Table 1. Diagnostic criteria for Incontinentia Pigmenti (IP) (Landy and Donnai)

No incidence of I in at least 1 first degree Female Relative	**Evidence of IP in a first degree female relative**
Major criteria	
Typical neonatal rash	Suggestive history or evidence of typical rash
Erythema, vesicles, eosinophilia	Skin manifestations of IP
Typical truncal hyperpigmentation	Hyperpigmentation
Mainly on trunk	Scarring
Following Blaschko's lines	Hairless streaks
Fading in adolescence	Alopecia at vortex
Linear, atrophic, hairless lesions	Anomalous dentition
	Woolly hair
	Retinal disease
	Multiple male miscarriages
Minor criteria (supportive evidence)	
Dental involvement	
Alopecia	
Woolly hair/abnormal nails	
Retinal disease	

At least one major criterion is necessary to make a firm diagnosis of sporadic incontinentia pigmenti. The minor criteria, if present, will support the diagnosis but because of their high incidence complete absence should induce a degree of uncertainty

selected. Using these criteria, we selected 29 reports, describing 44 IP patients with neurological features. Of these patients, 43 are female, one is male.

RESULTS

Neurological findings associated with IP are variable, ranging from a single seizure episode to psychomotor delay, intellectual disability, hemiplegia, epilepsy, cerebellar ataxia, microcephaly, neonatal encephalopathy, childhood encephalomyelitis and neonatal and childhood ischaemic stroke. There are only a few studies reporting the neurological phenotype in groups of IP patients. In 1976, Carney reviewed 653 patients with at that time a clinical diagnosis of IP. In 142/465 cases with sufficient information on neurological status, CNS involvement was present (30.5%)[8]. Landy and Donnai (1993) questioned this number, suggesting that it may be too high due to misdiagnosis of e.g. hypomelanosis of Ito. From their experience in their series of over 100 subjects, the overall incidence of intellectual or physical disability is less than 10%, with a higher incidence of intellectual disability in the sporadic cases (15%) compared to the familial cases (3%)[5]. Hadj-Rabia performed a clinical study in 40 IP patients fitting the Landy and Donnai criteria. In 13 of the 40 (32.5%) patients CNS involvement was noted[4]. Seizures were present in 10 of the 13 patients with neurological deficits; 2 patients eventually died due to vascular cerebral damage. Chronic sequelae of IP are incompletely reported. Delayed psychomotor retardation was present in 7 of the 13 patients, intellectual disability in 3 and spastic hemiplegia in 2[4]. Phan *et al*

Table 2. Incidence of neurological manifestations

	Carney	**Landy and Donnai**	**Hadj-Rabia** *et al*	**Phan** *et al*
Spastic paralysis/ hemiplegia	53/465 (11.4%)	ND	3/40 (7.5%)	5/47 (11.6%)
(Psycho)motor delay	35/465 (7.5%)	<10%	7/40 (17.5%)	ND
Mental retardation	57/ 465 (12.3%)	3% (familial cases) 15%(sporadic cases)	3/40 (7.5%)	4/48 (8.3%)
Microcephaly	22/465 (4.7%)	ND	ND	ND
Seizure disorder	62/465 (13.3%)	ND	10/40 (25%)	11/47 (23.4%)
Cerebellar ataxia	2/465 (0.4%)	ND	ND	1/47 (2%)
Overall incidence of neurological features	142/465 (30.5%)	ND	13/40 (32.5%)	ND

ND = Not documented

reported a retrospective case series in which they describe 25 probands and an additional 28 female family members fulfilling the Landy and Donnai criteria of IP. Of the patients with sufficient data on neurological status, 23% had a history of at least one seizure, 8% had intellectual disability and 11% had significant physical disability such as spastic hemiplegia. The same patients with intellectual disability also showed ophthalmological abnormalities. Interestingly, none of the relatives with IP had a history of seizures, intellectual or physical disability, suggesting that there may be an underrecognition of IP in patients with a milder phenotype lacking neurological manifestations[9] (Table 2). Since data on outcome of IP patients in the case reports or case series that we identified in our Pubmed search are greatly lacking, we will mainly discuss data on initial neurological presentations in the following sections. Variable degrees of vascular insufficiency, documented on brain imaging, seem to underly the neurological presentations in IP patients.

Initial CNS clinical manifestation

Many authors reports neurological symptoms of "pseudo-encephalitis", with acute neurological manifestations, at times associated with a comatose state and apnoea, mimicking encephalitis. Evidence of brain necrosis and multiple infarcts was reported[10-13].

Ischemic stokes seems therefore to underlie the neurological manifestation in the neonatal period. In 5 of the 7 cases in which ischaemic stroke was documented, the onset was in the first week of life[14-17] (Table 3). One of these patients had recurrent strokes, at the age of 5 days, 10 days and 3 months[14]. The other 2 cases experienced ischemic stroke at the age of respectively 2 months[18] and 4,5 years[19]. The infarcts affected the deep and subcortical white matter in 4 cases[4,15,20]. In 3 cases, ischaemic stroke affecting larger cerebral arteries (middle cerebral artery, anterior cerebral artery)[17-19] and in one case also affecting the left cerebellar hemisphere[17], were documented.

Acute disseminated encephalomyelitis (ADEM), which represents an acute inflammatory and demyelinative disease involving brain and spinal cord, has also been reported in two IP patients, with an onset at the ages of 6 and 7 months and subsequent recovery[21,22] (Table 3). In one of the patients, serum and CSF were positive for *M. Pneumoniae*[22]. Both patients were unresponsive to all stimuli in the acute phase, except for deep pain. One of the patients presented with seizures. CSF myelin basic protein was elevated in both patients, indicative for a CNS pathology characterized by demyelination. In one patient, nerve conduction

defects were detected[21]. Brain MRI in one patient revealed scattered lesions, hyperintense on T2- and diffusion-weighed images, mainly in the subcortical and periventricular white matter. After treatment with high-dosage corticosteroids and antimicrobial therapy against *M. Pneumoniae*, the patient responded well. Brain MRI revealed an almost complete disappearance of the initially abnormal signals. The patient did not have any neurological sequelae. In the other patient, CT-scan revealed areas of hypodensity in the central white matter and ventricular enlargement at the age of 4 years and symptoms were not responsive to ACTH-therapy, but spontaneously improved later. Retrospectively, the child had experienced a similar episode of lethargy, antedated by diarrhea and minor head trauma[21] at the age of 6 months. Based on these reports, ADEM should be considered early in the differential diagnosis of IP patients with neurological symptoms such as seizures[22].

Seizures

Seizure disorder is reported in 13.3-25% of IP patients[4,8,9]. In reports of 44 IP patients with CNS involvement[10-39], seizures were reported in 37, with information about age of onset in 35. Onset of seizures varied from 12 hours postpartum to 10 years. However, in the majority of patients (23 patients), seizures presented in the first week postpartum, an additional 5 presented in the first two months and another 4 in the first year. Two patients presented at ages 4 (after brain infarction), and 10 years. In this last patient a concomitant diagnosis of sarcoidosis was made at the age of 20 years, although no signs of neurosarcoidosis were found. Focal clonic seizures were most frequently reported, in 22 of the 29 cases in which description of the seizure type was present. Partial complex or generalized seizures were also reported. In 3 children with early onset seizures (day 2-3), the diagnosis of hypsarrhythmia at EEG or the clinical diagnosis of West syndrome was made (Table 3). In 16 cases, information on the duration of the first seizure episode was documented, which varied from 12 hours to 2 weeks. In only 11 cases of the 25 cases recurrence of seizures was reported. The remaining 14 cases only experienced one seizure episode. Treatment of seizures is most often initialized using phenobarbital as mono- or polytherapy. This is not surprising, considering that phenobarbital has long been the treatment of choice in infantile seizures and most patients experienced seizures in infancy[40].

In 25 of the 37 patients described with seizures, EEG registration results were documented. EEG patterns however, are not specific and could reflect multiple types of brain damage (Table 3).

Brain imaging data were present in 37 patients with seizures. In 36 patients, brain imaging showed abnormalities compatible with variable degrees of vascular insufficiency due to ischaemia or necrosis. In only 1 patient brain MRI was reported normal[16].

In general we can conclude that in most cases seizures are a manifestation of cerebrovascular damage, thus symptomatic, and are mostly present in childhood i.e. in the most severely affected patients. The seizure disorder can be self-limiting but in a few cases a severe epilepsy (e.g. West syndrome) can occur.

Ophthalmological finding

Ophthalmological findings are speculated to increase the chance of neurological abnormalities in IP patients. Also, the pathogenic mechanism leading to ophthalmological abnormalities is also thought to be implicated in the neurological phenotype op IP. Therefore, we provide an overview of the documented findings (Table 3). In 29 cases, ophthalmological findings were documented[10,11,13,15-17,20,21,23,24,26,33,36-38]. Of these, 9 patients showed no ophthalmological abnormalities[10,13,16,24,26,36-38]. In 4 patients, retinal haemorrhage was noted[16-18,21,27]. In four patient, retinal vascular abnormalities without haemorrhage were seen, two of them showed arteriovenous anastomoses, one showed vessel dilatation[11,16,18,20]. In 6 patients, ischaemic retinal lesions were seen[15-17,20], in 2 patients, (peripheral) avascularity of the retina was documented[27,30]. Microphthalmia was a finding in 2 patients[3,34]. In 6 patients, retinal detachment was noted[16,23,34]. In two patients, irregularities of the retinal pigment epithelium were appreciated[16,21]. Optic atrophy was a finding in 3 patients[16]. Strabismus was documented in three patients[11,15,23]. Signs of conjunctivitis were found in 2 patients[29,33]. Finally, cataract was described in 1 patient[34]. Most patients with ophthalmological manifestations also have brain abnormalities at MRI (Table 3). However, abnormalities at brain MRI were regularly seen also in patients without reported ophthalmological abnormalities[16]. There is only one patient reported with a normal brain MRI , who showed a small chorioretinal scar on ophthalmological examination[16]. Most of the reported ophthalmological manifestations reflect vascular dysfunction. Since a reasonable proportion of patients have both opthalmological as well as central nervous system lesions, it is likely that a similar pathological mechanism is involved.

Brain imaging

Literature findings about brain imaging are summarized in Table 3. We will briefly discuss the major abnormalities.

White matter and corpus callosum abnormalities and cerebral atrophy.

The white matter seems to be especially vulnerable in patients with IP. In 27 of the 43 patients of which brain MRI data were documented, abnormalities of the white matter could be identified[10-16,20,22-25,28,32-34,36,37,39]. Periventricular white matter changes, consistent with periventricular leukomalacia, were most often present, but subcortical white matter changes were also frequent findings. In one patients, a white matter abnormality, comprising of a small lesion in the semioval center, was present without any neurological abnormalities[39]. In some patients, the white matter changes eventually led to cyst or cavity formation in the white matter, suggesting tissue destruction, possibly due to microbleeds or microinfarctions[10,13,32,33,39]. Prominent Virchow Robin spaces were seen in one patient[36], a finding suggestive of small-vessel disease[41]. A delay in myelination was present in two patients[13,16]. Ventricular dilatation was seen unilaterally in 5 patients[10,16,23]and bilaterally in 6 patients[10,16,21,23,33,34] . Cerebral atrophy was documented in 6 patients[10,16,30,33,35], in 4 of them located unilaterally. Both ventricular dilatation and cerebral atrophy can result from generalized tissue loss following a vascular event.

In patients with seizures, on brain imaging, findings compatible with vascular insufficiency, including periventricular leukomalacia, patchy gliotic white matter changes, white matter cavitations, basal ganglia damage, diffuse haemorrhagic necrosis with tissue destruction were seen[10-13,20,21,23,25,28,30,32-34,36-38]. Also infarction affecting both small vessels affected the deep and subcortical white matter[14,15,20] as well as larger cerebral arteries (middle cerebral artery, anterior cerebral artery)[17-19] and in one case also the left cerebellar hemisphere[17], were documented. One of these patients had recurrent strokes, at the age of 5 days, 10 days and 3 months[14].

Hypoplasia of the corpus callosum was a finding in 8 patients[10,16,20,23,24,34]. In addition, all these patients exhibited hemisphere white matter abnormalities and in 5 patients uni- or bilateral ventricular enlargement, with reduced volume of the cerebral white matter was seen. Therefore, it is likely that the corpus callosum hypoplasia is secondary to white matter loss, rather than a primary developmental defect . However,, corpus callosum agenesis was

reported in 1 patient[26]. This patient had, besides the corpus callosum agenesis, a second unusual presentation with a midline posterior skull defect and a protruding encephalocele. Cutaneous findings, including a histological examination of a skin biopsy were consistent with the diagnosis of IP. Since it is only described once, it remains to be elucidated whether the encephalocele and corpus callosum agenesis, both developmental defects, are part of the IP phenotype or unrelated concomitant findings in this patient.

Cerebral haemorrhage

In 8 patients, a haemorrhagic component was seen on brain imaging. In most of these patients however, haemorrhagic necrosis was present, most likely secondary to an ischaemic event. Encephalomalacia with bilateral haemorrhagic necrosis and widespread tissue destruction was noted in 5 patients[12,13,16,25,28]. Another patient presented 3 days postpartum with bilateral encephalomalacia, but in addition a moderate subarachnoidal haemorrhage was seen[29]. Also, more subtle microvascular haemorrhagic infarcts of the periventricular white matter are described[11]. One patient presented during the first day with a right sided grade I intraventricular haemorrhage with a germinolytic cyst[30]. The pregnancy of this last patient, however, was complicated by maternal drug and alcohol abuse. Germinal matrix cysts and haemorrhage are also described in association with maternal cocaine abuse during pregnancy, making it difficult to correlate these findings in the patient with IP[42,43].

A supraclinoid right internal carotid artery aneurysm was demonstrated in a 56-year-old woman with classical dermatological features consistent of IP without neurological symptoms[16]. It is unclear whether carotid aneurysms are part of the phenotypic spectrum of IP cerebrovascular lesions , or whether this is an occasional finding in this patient.

Cerebellar abnormalities

Only one of the reported patients had cerebellar hemispheres and vermis atrophy, which was already seen on brain MRI at the age of 6 weeks[17]. Additional findings on neonatal brain ultrasound in the same patient were striatal arteriopathy, suggesting inflammation, and on MRI gliosis of the white matter of the centrum semi-ovale and a thin corpus callosum. Cerebellar underdevelopment might be therefore secondary to intrauterine cerebrovascular damage[4,45].

Cortical malformation

In one of the patients, brain MRI in the first week of life showed polymicrogyria in the perisylvian area with cortical dysplasia[7]. Cranial ultrasound on day 5 in this patient already demonstrated bilateral multiple echogenic foci in temporal and thalamic areas. Polymicrogyria was alo described in neuropathology data by O'Doherty *et al* (see section 3.5)[46]. Known etiologies involved in the development of polymicrogyria, a brain developmental disorder that arises from the second trimester of pregnancy, are, beside genetic, acquired brain injury due to abnormal perfusion and/ or oxygenation of the fetal brain, and infection during fetal life[47]. Both polymicrogyria and cerebellar hypoplasia implicate that the cerebral pathology in IP can develop even antenatally.

Neuronal heterotopias of the left parietal lobe were documented, although not very clear in the presented data, in one male patient on brain MRI at the age of 3 and 5 years, which was performed because of seizures at the age of 3 and 5 years[34]. Although the diagnosis in this patient was based on clinical criteria, such as suggestive skin lesions and unilateral cataract with microphthalmia, no DNA analysis was performed and the report remains until now isolated.

Neuropathology findings

Only a few authors report on neuropathological findings in IP. O'Doherty and Norman report on the postmortem pathology in a 7 weeks-old infant with IP. Structural abnormalities such as polymicrogyria of the left hemisphere were seen. Calcification of nerve fibers, Purkinje cells and microglia cells were present. In addition, focal areas of cerebral and cerebellar necrosis and neuronal loss were seen, indicative of vascular insufficiency. No pathological changes were seen in blood vessels[46]. In 1977, Hauw *et al* reported neuropathological findings in a 3 month-old infant with IP confirmed in a skin biopsy [48]. They found signs of a destructive process secondary to vascular insufficiency, comprising of ulegyria, white matter cavities and patchy scar softening of the cerebellar cortex with loss of Purkinje fibers and granule cells. Interestingly, signs of a diffuse inflammatory process involving pia and brain parenchyma were found, with perivascular cuffs of lymfocytes, histiocytes and eosinophilic polymorphs, and mononuclear nodules, indicating that inflammation, in addition to ischemia, may contribute to the vascular insuficiency. Hence, the interpretation of the MRI findings confirms this hypothesis.

Genetics and pathophysiology

In 11 of the 44 patients with neurological involvement, sequence analysis of the *NEMO* gene was performed. In 10/11 patients, the common deletion of exon 4-10 was identified [14,17,22,24,27,28,32,35,37,38]. One patient did not harbor a mutation[17]. In one additional patient, DNA analysis in the (asymptomatic) mother was performed, without identifying a mutation[15].

The NEMO (IKK-γ, NFκB essential modulator) protein is an important regulator of the NF-κB signaling pathway. The NF-κB transcription factor family is important in many signal transduction pathways, regulating genes involved in critical developmental processes, innate and adaptive immune responses, cell adhesion and protection against apoptosis. In most resting cell types, NF-κB is kept inactive in the cytoplasm through interaction with inhibitory IκB-molecules. Phosphorylation of these inhibitory molecules leads to IκB ubiquitination and degradation by the proteasome pathway, leading to a release of active NF-κB. NEMO is an important regulatory subunit of the IκB kinase (IKK) complex, that can phosphorylate IκB[49]. This IκB phosphorylation, and subsequent activation of NF-κB can be initiated in response to multiple stimuli, including tumor necrosis factor α (TNFα), bacterial lipopolysaccharide (LPS) and genotoxic agents. Active NF-κB can translocate to the nucleus and activate transcription of its target genes[6,49-52]. Absence of NEMO leads to a complete inhibition of NF-κB signaling, reflecting its major role in the activation of the IKK-complex[6]. Recently, an additional role has been ascribed to NEMO as the principle molecule signaling the presence of double strand DNA breaks (DSB) in the nucleus to the NF-κB complex in the cytoplasm, leading to transport of NFκB to the nucleus to stimulate transcription of anti-apoptotic genes[53,54],

Functional NF-κB complexes are present in essentially all cell types. In the nervous system, it is present in neurons, astrocytes, microglia and oligodendrocytes. In the nervous system, besides TNFα, additional neuron-specific NF-κB-activating signals exist, including nerve growth factor (NGF), and the secreted form of β-amyloid precursor protein (βAPP), sAPPα. Also electrical activity within neurons and synaptic transmission between neurons possibly lead to a relatively high constitutive activity of NF-κB in brain tissue[55]. An important role of NF-κB is postulated in the control of neuronal death during development of the nervous system and in the modulation of synaptic function following glutamate receptor stimulation[55]. Bradykinin, an inflammatory mediator produced in the brain in response to ischemia and trauma, is also a potent stimulator or NF-κB-activation, inducing the produc-

tion of several inflammation-related cytokines by microglia. This is counterbalanced by the production of anti-inflammatory cytokines, including TGF-β and IL-10.

In general, injury to the brain induces a cascade of signaling events that stimulate NF-κB-activation in injured neurons and injury-responsive glial cells. After ischemic brain injury, NF-κB activation promotes ischemic neuronal degeneration in glial cells, but has a neuroprotective function in neurons, leading to an increased survival of neurons[55]. After (kainite-induced) seizures, an increase of NF-κB activity protects neurons from excitotoxicity, by reducing neuronal sensitivity to glutamate[55].

In summary, NEMO plays an important role in the activation of NF-κB. In the nervous system, active NF-κB is important in processes like immune responses and protection against neuronal damage and apoptosis caused by brain injury, hypoxia and excitotoxicity induces by seizures. Therefore, it is likely that dysfunction of these processes in IP patients contribute to the neurological phenotype and seizure disorder in IP patients. Studies investigating NF-κB activation and its role in the cerebral phenotype in IP-patients are lacking. Even in mouse models, neuropathology data are not present. In female NEMO+/- mice findings suggestive of neurological dysfunction were reported by day 8, comprising of spasm, tremors and locomotor difficulties[56]. Many female mice die within the first 6-10 days. Some of the female NEMO+/- mice also contained high numbers of apoptotic cells[56], confirming the hypothesis that cells lacking NEMO-protein are more prone to apoptosis. Since apoptosis is a major mechanism during brain development, this may also lead to cerebral damage before birth.

This has also been demonstrated in skin lesions in IP. Due to lyonisation, a mosaic pattern of NEMO-deficient and wild-type cells are present in IP-patients. It has been postulated that the skin phenotype originates when NEMO-deficient cells start to produce pro-inflammatory interleukins, mostly IL-2. As a response, the wild-type cells start an inflammatory reaction, producing TNF, a known stimulus of IκB phosphorylation leading to NF-κB signaling and the transcription of anti-apoptotic genes. The NEMO-deficient cells, however, undergo apoptosis. This leads to a transient inflammatory reaction of the wild-type cells, leading to an eradication of the NEMO-deficient cells. If a few NEMO-deficient cells survive, in time, a second episode of inflammation can be expected[3,57].

Besides data on skin histopathology, the ocular histopathology in heterozygous NEMO-deficient mice was recently described. These data are interesting, since, although studies confirming this are lacking, similar processes may play a role in the cerebral vasculature in IP patients. Although the ocular phenotype in mice seems to be somewhat milder than in humans, without complete vaso-occlusion or neovascularisation, the findings are similar to findings in human IP patients. By 3 months of age, arteriolar tortuosity and irregular configurations were seen, associated with cellular changes such as thickened and irregular walls with highly irregular lumens. This thickening appeared to be caused by a redundancy of hypertrophic vascular endothelial cells and reduplication of their basement membrane. This eventually led to impingement of the vascular lumen, in some cases cross sections of arterioles revealed only the remaining of a slit-like lumen. The retinal venules appeared normal[58].

Management

No specific treatment is available for neurological symptoms of IP. Treatment of seizures is symptomatic. Anecdotal treatment with antiplatelet medication in one patient did not protect from recurrent stroke[13]. Administration of corticosteroids instead led to improvement in one patient with acute disseminated encephalomyelitis[30]. Spontaneous improvement has been documented in patients with seizures and the two ADEM patients.

DISCUSSION

Since the NF-κB signaling pathway, and therefore the *NEMO* gene, is of great importance in preventing neurons from undergoing apoptosis following (ischaemic, inflammatory or excitotoxic) brain injury, NEMO-deficient cells probably are more prone to inflammation and apoptosis. The neurological and brain MRI features can reflect tissue damage due to small vessel disease, but also the larger cerebral arteries can be involved. Ischaemia due to vascular changes, as described in mouse retinal vasculature, may underly this damage. However, in some cases the lesions are more diffuse and do not seem to follow a certain vascular pattern. An inflammatory component, as seen in skin lesions of IP patients, may also contribute to the cerebral damage. This was demonstrated in the neuropathology findings described by Hauw *et al*[48], who demonstated signs of an inflammatory process of pia and brain parenchyma. Many of the described abnormalities, such as cerebral, cerebellar and corpus callosum atrophy, optic nerve hypoplasia and polymicrogyria may represent the

endstage of (ischaemic and/ or inflammatory) brain injury. However, in addition, developmental defects comprising microcephaly, microphthalmia, cataract and heterotopia may also be the consequence of aberrant apoptosis in NEMO-deficient cells. Data supporting this in IP patients are lacking. Increased apoptosis, however, has previously been described as a mechanism underlying neuronal heterotopia and microcephaly[59-61]. Polymicrogyria, heterotopia, microphthalmia and microcephaly are indicative of a disease process that already started in the antenatal period.

In conclusion, neurological findings are frequently reported in IP patients, in some cases leading to major morbidity and even mortality. The symptoms mostly manifest in infancy, only rarely in late childhood and are not reported in adolescence or adulthood. Seizures usually correlate with the severity of the neurological sympoms and mostly occur in childhood. Inflammatory mechanisms, vascular injury and possibly a disturbed apoptosis during development apparently contribute to pathogenesis of cerebral manifestations. The cells with NEMO mutation are destroyed by an inflammatory mechanism, which might explain why the disease is self-limiting and symptoms occur between the fetal period and childhood. The prognosis and outcome is dependent on the damage to the brain and other organs in this period and is mostly favorable if no neurological problems occur until childhood. Magnetic resonance imaging is recommended in the clinical evaluation of infants with IP and neurological symptoms. Since the neurological manifestations are polymorphic and can occur at or soon after birth, while initial skin lesions can be mild, it is recommended to carefully search for dermatological signs of IP in any newborn with unexplained neurologic deterioration, stroke or seizures. Further studies involving neuropathology and the role of inflammation and apoptosis in the neurological phenotype in IP patients are needed to further elucidate the precise pathophysiological mechanism of the central nervous system manifestations in IP patients.

Table 3. Summary of literature on neurological and ocular findings in IP.

Patient	Reference	Seizures	Other	Age of onset	Type of seizures	EEG	Brain imaging (MRI unless indicated otherwise)	Ophthalmological findings	ID	*NEMO* sequencing
1	Maingay	+		Day 5	Clonic	Multifocal discharges	Non-haemorrhagic infarction left cerebral and cerebellar hemispheres	Minor haemorrhagic spots in left macula	ND	del ex 4-10, de novo
2	Maingay	+		Postpartum	Focal	Normal	Cerebellar vermis atrophy, PV gliosis, thin CC	Peripheral ischaemic lesions of retina	ND	Normal
3	Aydingoz	+		Day 5	ND	ND	CC hypoplasia, left-sided ventricular enlargement, PVL	Left-sided microphthalmia and retinal detachment	+	NP
4	Aydingoz	+		5 months	ND	ND	CC hypoplasia, bilateral ventricular enlargement, PVL	Squint	+	NP
5	Lou	+		Day 15	Focal clonic	ND	Patchy PV and subcortical WM changes, cystic lesions, ventricular enlargement	Bilateral conjunctivitis	+	NP
6	Loh	+		Day 3	ND	Hypsarrhytmia	Basal ganglia, thalamic and internal capsule damage, bilateral PV WM changes and cavitation	Visually impaired	+	del ex 4-10, de novo
7	Cartwright	+		Day 5	Focal clonic	Bilat continuous discharges	Multiple bilateral infarcts, cystic encephalomalacia	ND	ND	del ex 4-10
8	Hart	+		Day 2	Rightsided clonic-> West	ND	Haemorrhagic necrosis, poor filling of left cerebral arteries on angiogram	ND	died at 11 m	del ex 4-10
9	Shuper	+		Day 3	ND	Left-sided sharp waves and spikes	Whidespread tissue destruction and haemorrhagic necrosis	ND	ND	NP
10	Brunquell	-	ADEM	6 months, 4 yr	-	Diffuse slowing left > right	(CT) Low-absorbtion areas of central white matter, ventricular enlargement	Irregular pigment epithelium, retinal haemorrhage	ND	NP
11	Hennel	+		Day 4	Focal clonic	Normal (day 14)	Microvascular haemorrhagic infarcts and necrosis in PV WM	Retinal vascular abnormalities	ND	NP
12	Lee	-	-	56 yr	-	ND	Carotid artery aneurysm	Normal	-	NP
13	Lee	-	-	32 yr	-	ND	Moderate ventricular enlargement	Normal	-	NP
14	Lee	+		ND	ND	ND	WM infarcts	Retinal detachment and pigmentary changes, optic atrophy, foveal hypoplasia	+	NP
15	Lee	+		ND	ND	ND	Normal	Small chorioretinal scar	-	NP
16	Lee	+		Day 3	ND	ND	Cerebral atrophy, ventricular dilatation, WM infarcts and haemorrhage, CC hypoplasia	Retinal ischaemia, haemorrhage and detachment	ND	NP
17	Lee	-		ND	-	-	Small foci of hyperintensities in right centrum semiovale	Right-sided epipapillary membrane	-	NP

(continued on next page)

Table 3. Summary of literature on neurological and ocular findings in IP. *(continued from previous page)*

Patient	Reference	Seizures	Other	Age of onset	Type of seizures	EEG	Brain imaging (MRI unless indicated otherwise)	Ophthalmological findings	ID	*NEMO* sequencing
18	Lee	+		At birth	Tonic clonic	ND	Cerebral and cerebellar infarcts with encephalomalacia, PVL	Retinal vascular abnormalities with detachment	ND	NP
19	Shah	+		Day 4	Focal -> generalized	ND	PVL	Normal	-	del ex 4-10, de novo
20	Matsumoto	+	ADEM	7 months	Generalized	High voltage slow waves	scattered PV and subcortical WM lesions	ND	-	del ex 4-10, inherited
21	Bryant	+		Day 3	Focal and generalized tonic clonic	ND	CC hypoplasia, PVL and subcortical WM lesions	Normal	-	del ex 4-10
22	Lee '08	+		Day 9	Focal clonic	ND	CC hypoplasia, PVL	Ischaemic lesions, arteriovenous anastomosis	ND	NP
23	Yoshikawa	-		-	-	-	Cavitation semioval center	NP	-	NP
24	Fiorillo	+		Day 3	Partial complex	Multifocal discharges, disorganized slow background	Bilateral haemorrhagic WM infarctions, PVL	Ischaemic lesions, strabism	+	In mother normal
25	Abe	+		Day 44	Generalized, focal clonic	right frontal slowing of background activity	WM infarcts, cystic encaphalomalacia, basal ganglia damage, ventricular dilatation	Normal	+	NP
26	Abe	+		Day 5	Focal clonic	Ictal discharges	Patchy gliotic changes, basal ganglia damage	ND	ND	NP
27	Abe	+		6 months	Focal clonic	ND	Cerebral atrophy of left hemisphere	ND	ND	NP
28	Abe	+		Day 58	Focal clonic -> generalized	Mild low voltage	CC hypoplasia, PVL and subcortical WM lesions, basal ganglia damage	ND	ND	NP
29	Abe	+		Day 44	Focal clonic	Widespread spikes	Unilateral venticular dilatation, patchy gliotic WM changes	ND	ND	NP
30	Abe	+		Day 1	Focal clonic	Ictal discharges	Cerebral atrophy of left hemisphere	ND	ND	NP
31	Hubert	+		12 hours	Generalized	Multifocal spikes	(CT) Scattered ischaemia, subarachnoïdal haemorrhage	Conjunctivitis	+	NP
32	Wolf	+		Day 2	Apnoeic spells	Multifocal-> burst suppression-> hypsarrhytmia	Extensive cortical (hemorrhagic) necrosis, subcortical haemorrhage, myelination delay	Normal	+	NP
33	Demirel	-		-	-	Normal	CC agenesis, porencephaly, colpocephaly, posterion parietal encephalocele	Normal	ND	NP

(continued on next page)

Table 3. Summary of literature on neurological and ocular findings in IP. *(continued from previous page)*

Patient	Reference	Seizures	Other	Age of onset	Type of seizures	EEG	Brain imaging (MRI unless indicated otherwise)	Ophthalmological findings	ID	*NEMO* sequencing
34	Chatkupt	+		52 hours	Focal seizures	ND	(CT/ MRI) Haemorrhagic necrosis and edema, leucomalacia	ND	ND	NP
35	Kasai	+		2 months	Focal clonic -> generalized	Multifocal spikes and sharp waves	Left hemisphere, right occipital and parietal infarction	Small retinal haemorrhage and dilated vessels	+	NP
36	Pörksen	+		Day 5	Focal -> generalized	Intermittent signs of tiredness	Small cortical and subcortical infarctions, PVL	ND	-	NP
37	Pörksen	+		Day 5	Focal	Some steep waves	PVL, prominent Virchow Robin spaces	Normal	-	NP
38	Kaczala	+		21 hours	Focal, apneas	Multifocal spikes and sharp waves	Grade I IVH, germinolytic cyst, white and gray matter destruction, decreased flow MCA	Peripheral avascularity	+	NP
39	Godambe	-		-	-	-	(Ultrasound/ MRI) Temporal and thalamic hyperechogenic foci, perisylvian PMG with cortical dysplasia	Avascular retina	died day 26	del ex 4-10
40	Obermann	+		10 years	Complex partial -> generalized	inconsistent temporoparietal theta and sharp-slow-waves	Gray matter decrease right temporal lobe	ND	-	del ex 4-10
41	Türkmen	+		Day 4	Focal clonic, apnea	Multifocal spikes	Increase T2 in occipitotemporal cortex, hypoxic-ischemic encephalopathy	Normal	-	del ex 4-10, inherited
42	Pellegrino	+		4 years	Focal	ND	Infarct left frontoparietal, occlusion left middle cerebral artery	ND	ND	NP
43	Mangano	+		Day 4	Generalized	focal spike-wave complex discharges -> generalized	CC hypoplasia, bilateral ventricular dilatation, PVL	ND	+	NP
44	Mangano	+		1 year	Clonic	Sharp waves	PVL, neuronal heterotopia left parietal lobe	Microphthalmia, retinal detachment, cataract	-	NP

CC = corpus callosum
PVL = periventricular leukomalacia
PV = periventricular
IVH = intraventricular haemorrhage
WM = white matter
PMG = polymicrogyria
ND = not documented
NP = not performed

References

1. **Buinauskaite E, Buinauskiene J, Kucinskiene V, Strazdiene D, Valiukeviciene S.** Incontinentia pigmenti in a male infant with Klinefelter syndrome: a case report and review of the literature. Pediatr Dermatol 2010;27:492-5.

2. **Fusco F, Fimiani G, Tadini G, Michele D, Ursini MV.** Clinical diagnosis of incontinentia pigmenti in a cohort of male patients. J Am Acad Dermatol 2007;56:264-7.

3. **Berlin AL, Paller AS, Chan LS.** Incontinentia pigmenti: a review and update on the molecular basis of pathophysiology. J Am Acad Dermatol 2002;47:169-87; quiz 88-90.

4. **Hadj-Rabia S, Froidevaux D, Bodak N, *et al*.** Clinical study of 40 cases of incontinentia pigmenti. Arch Dermatol 2003;139:1163-70.

5. **Landy SJ, Donnai D.** Incontinentia pigmenti (Bloch-Sulzberger syndrome). J Med Genet 1993;30:53-9.

6. **Smahi A, Courtois G, Vabres P,** *et al*. Genomic rearrangement in NEMO impairs NF-kappaB activation and is a cause of incontinentia pigmenti. The International Incontinentia Pigmenti (IP) Consortium. Nature 2000;405:466-72.

7. **Fusco F, Bardaro T, Fimiani G,** *et al*. Molecular analysis of the genetic defect in a large cohort of IP patients and identification of novel NEMO mutations interfering with NF-kappaB activation. Hum Mol Genet 2004;13:1763-73.

8. **Carney RG.** Incontinentia pigmenti. A world statistical analysis. Arch Dermatol 1976;112:535-42.

9. **Phan TA, Wargon O, Turner AM.** Incontinentia pigmenti case series: clinical spectrum of incontinentia pigmenti in 53 female patients and their relatives. Clin Exp Dermatol 2005;30:474-80.

10. **Abe S, Okumura A, Hamano S, *et al*.** Early infantile manifestations of incontinentia pigmenti mimicking acute encephalopathy. Brain Dev 2011;33:28-34.

11. **Hennel SJ, Ekert PG, Volpe JJ, Inder TE.** Insights into the pathogenesis of cerebral lesions in incontinentia pigmenti. Pediatr Neurol 2003;29:148-50.

12. **Shuper A, Bryan RN, Singer HS.** Destructive encephalopathy in incontinentia pigmenti: a primary disorder? Pediatr Neurol 1990;6:137-40.

13. **Wolf NI, Kramer N, Harting I,** *et al*. Diffuse cortical necrosis in a neonate with incontinentia pigmenti and an encephalitis-like presentation. AJNR Am J Neuroradiol 2005;26:1580-2.

14. **Cartwright MS, White DL, Miller LM, 3rd, Roach ES.** Recurrent stroke in a child with incontinentia pigmenti. J Child Neurol 2009;24:603-5.

15. **Fiorillo L, Sinclair DB, O'Byrne ML, Krol AL.** Bilateral cerebrovascular accidents in incontinentia pigmenti. Pediatr Neurol 2003;29:66-8.

16. **Lee AG, Goldberg MF, Gillard JH, Barker PB, Bryan RN.** Intracranial assessment of incontinentia pigmenti using magnetic resonance imaging, angiography, and spectroscopic imaging. Arch Pediatr Adolesc Med 1995;149:573-80.

17. **Maingay-de Groof F, Lequin MH, Roofthooft DW,** *et al*. Extensive cerebral infarction in the newborn due to incontinentia pigmenti. Eur J Paediatr Neurol 2008;12:284-9.

18. **Kasai T, Kato Z, Matsui E,** *et al*. Cerebral infarction in incontinentia pigmenti: the first report of a case evaluated by single photon emission computed tomography. Acta Paediatr 1997;86:665-7.

19. **Pellegrino RJ, Shah AJ.** Vascular occlusion associated with incontinentia pigmenti. Pediatr Neurol 1994;10:73-4.

20. **Lee JH, Im SA, Chun JS.** Serial changes in white matter lesions in a neonate with incontinentia pigmenti. Childs Nerv Syst 2008;24:525-8.

21. **Brunquell PJ.** Recurrent encephalomyelitis associated with incontinentia pigmenti. Pediatr Neurol 1987;3:174-7.

22. **Matsumoto N, Takahashi S, Toriumi N,** *et al***.** Acute disseminated encephalomyelitis in an infant with incontinentia pigmenti. Brain Dev 2009;31:625-8.

23. **Aydingoz U, Midia M.** Central nervous system involvement in incontinentia pigmenti: cranial MRI of two siblings. Neuroradiology 1998;40:364-6.

24. **Bryant SA, Rutledge SL.** Abnormal white matter in a neurologically intact child with incontinentia pigmenti. Pediatr Neurol 2007;36:199-201.

25. **Chatkupt S, Gozo AO, Wolansky LJ, Sun S.** Characteristic MR findings in a neonate with incontinentia pigmenti. AJR Am J Roentgenol 1993;160:372-4.

26. **Demirel N, Aydin M, Zenciroglu A,** *et al***.** Incontinentia pigmenti with encephalocele in a neonate: a rare association. J Child Neurol 2009;24:495-9.

27. **Godambe S, McNamara P, Rajguru M, Hellmann J.** Unusual neonatal presentation of incontinentia pigmenti with persistent pulmonary hypertension of the newborn: a case report. J Perinatol 2005;25:289-92.

28. **Hart AR, Edwards C, Mahajan J, Wood ML, Griffiths PD.** Destructive encephalopathy in incontinentia pigmenti. Dev Med Child Neurol 2009;51:162-3.

29. **Hubert JN, Callen JP.** Incontinentia pigmenti presenting as seizures. Pediatr Dermatol 2002;19:550-2.

30. **Kaczala GW, Messer MA, Poskitt KJ, Prendiville JS, Gardiner J, Senger C.** Therapy resistant neonatal seizures, linear vesicular rash, and unusually early neuroradiological changes: incontinentia pigmenti: a case report, literature review and insight into pathogenesis. Eur J Pediatr 2008;167:979-83.

31. **Leung AK, Kao CP, Robson WL.** Incontinentia pigmenti in an infant. J Pediatr 2006;149:138.

32. **Loh NR, Jadresic LP, Whitelaw A.** A genetic cause for neonatal encephalopathy: incontinentia pigmenti with NEMO mutation. Acta Paediatr 2008;97:379-81.

33. **Lou H, Zhang L, Xiao W, Zhang J, Zhang M.** Nearly completely reversible brain abnormalities in a patient with incontinentia pigmenti. AJNR Am J Neuroradiol 2008;29:431-3.

34. **Mangano S, Barbagallo A.** Incontinentia pigmenti: clinical and neuroradiologic features. Brain Dev 1993;15:362-6.

35. **Obermann M, Weber R.** Concomitant diagnosis of sarcoidosis and incontinentia pigmenti in an epileptic patient. Eur J Neurol 2008;15:e36-7.

36. **Porksen G, Pfeiffer C, Hahn G,** *et al***.** Neonatal seizures in two sisters with incontinentia pigmenti. Neuropediatrics 2004;35:139-42.

37. **Shah SN, Gibbs S, Upton CJ, Pickworth FE, Garioch JJ.** Incontinentia pigmenti associated with cerebral palsy and cerebral leukomalacia: a case report and literature review. Pediatr Dermatol 2003;20:491-4.

38. **Turkmen M, Eliacik K, Temocin K, Savk E, Tosun A, Dikicioglu E.** A rare cause of neonatal seizure: incontinentia pigmenti. Turk J Pediatr 2007;49:327-30.

39. **Yoshikawa H, Uehara Y, Abe T, Oda Y.** Disappearance of a white matter lesion in incontinentia pigmenti. Pediatr Neurol 2000;23:364-7.

40. **Vento M, de Vries LS, Alberola A,** *et al***.** Approach to seizures in the neonatal period: a European perspective. Acta Paediatr 2010;99:497-501.

41. **Rouhl RP, van Oostenbrugge RJ, Knottnerus IL, Staals JE, Lodder J.** Virchow-Robin spaces relate to cerebral small vessel disease severity. Journal of neurology 2008;255:692-6.

42. **Cohen HL, Sloves JH, Laungani S, Glass L, DeMarinis P.** Neurosonographic findings in full-term infants born to maternal cocaine abusers: visualization of subependymal and periventricular cysts. J Clin Ultrasound 1994;22:327-33.

43. **Kapur RP, Shaw CM, Shepard TH.** Brain hemorrhages in cocaine-exposed human fetuses. Teratology 1991;44:11-8.

44. **Govaert P.** Prenatal stroke. Semin Fetal Neonatal Med 2009;14:250-66.

45. **Volpe JJ.** Brain injury in premature infants: a complex amalgam of destructive and developmental disturbances. Lancet neurology 2009;8:110-24.

46. **O'Doherty NJ, Norman RM.** Incontinentia pigmenti (Bloch-Sulzberger syndrome) with cerebral malformation. Dev Med Child Neurol 1968;10:168-74.

47. **Golden JA, Harding BN.** Cortical malformations: unfolding polymicrogyria. Nat Rev Neurol 2010;6:471-2.

48. **Hauw JJ, Perie G, Bonnette J, Escourolle R.** [Neuropathological study of incontinentia pigmenti. Anatomical case report (author's transl)] Les lesions cerebrales de l'incontinentia pigmenti. A propos d'un cas anatomique. Acta Neuropathol 1977;38:159-62.

49. **Schmidt-Supprian M, Bloch W, Courtois G,** *et al***.** NEMO/IKK gamma-deficient mice model incontinentia pigmenti. Mol Cell 2000;5:981-92.

50. **Nelson DL.** NEMO, NFkappaB signaling and incontinentia pigmenti. Curr Opin Genet Dev 2006;16:282-8.

51. **Sebban H, Courtois G.** NF-kappaB and inflammation in genetic disease. Biochem Pharmacol 2006;72:1153-60.

52. **Wu ZH, Shi Y, Tibbetts RS, Miyamoto S.** Molecular linkage between the kinase ATM and NF-kappaB signaling in response to genotoxic stimuli. Science 2006;311:1141-6.

53. **Biton S, Barzilai A, Shiloh Y.** The neurological phenotype of ataxia-telangiectasia: solving a persistent puzzle. DNA repair 2008;7:1028-38.

54. **Mavrou A, Tsangaris GT, Roma E, Kolialexi A.** The ATM gene and ataxia telangiectasia. Anticancer research 2008;28:401-5.

55. **Mattson MP, Camandola S.** NF-kappaB in neuronal plasticity and neurodegenerative disorders. The Journal of clinical investigation 2001;107:247-54.

56. **Makris C, Godfrey VL, Krahn-Senftleben G,** *et al***.** Female mice heterozygous for IKK gamma/NEMO deficiencies develop a dermatopathy similar to the human X-linked disorder incontinentia pigmenti. Mol Cell 2000;5:969-79.

57. **Courtois G, Smahi A.** NF-kappaB-related genetic diseases. Cell Death Differ 2006;13:843-51.

58. **Oster SF, McLeod DS, Otsuji T, Goldberg MF, Lutty GA.** Preliminary ocular histopathological observations on heterozygous NEMO-deficient mice. Exp Eye Res 2009;88:613-6.

59. **Takano T, Akahori S, Takeuchi Y, Ohno M.** Neuronal apoptosis and gray matter heterotopia in microcephaly produced by cytosine arabinoside in mice. Brain Res 2006;1089:55-66.

60. **Fitzgerald MP, Covio M, Lee KS.** Disturbances in the positioning, proliferation and apoptosis of neural progenitors contribute to subcortical band heterotopia formation. Neuroscience 2011;176:455-71.

61. **Poulton CJ, Schot R, Kia SK, *et al*.** Microcephaly with simplified gyration, epilepsy, and infantile diabetes linked to inappropriate apoptosis of neural progenitors. Am J Hum Genet 2011;89:265-76.

PART II

GENETIC CAUSES OF HEMORRHAGIC STROKE

Chapter 3

THE EXPANDING PHENOTYPE OF *COL4A1* AND *COL4A2* MUTATIONS; CLINICAL DATA ON 13 NEWLY IDENTIFIED FAMILIES AND REVIEW OF LITERATURE

Marije E.C. Meuwissen,MD[1], Dicky J.J.Halley,PhD[1], Liesbeth S. Smit, MD[2], Maarten H. Lequin[3], MD, PhD[3], Jan M. Cobben,MD, PhD[4], René de Coo, MD, PhD[2], Jeske van Harssel, MD[5], Tom Letteboer, MD, PhD[5], Suzanne Sallevelt, MD[6], Gwendolyn Woldringh, MD, PhD[7], Marjo S. van der Knaap, MD, PhD[8], Linda S. de Vries, MD, PhD[9], Grazia M.S. Mancini, MD, PhD[1]

Manuscript Submitted

1. *Dept. of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands*
2. *Dept. of Neurology, Division of Pediatric Neurology, Child Neurology, Erasmus University Medical Center, Rotterdam, The Netherlands*
3. *Dept. of Radiology, Erasmus University Medical Center, Rotterdam, The Netherlands*
4. *Dept. of Pediatric Genetics, Academic Medical Center, Amsterdam, The Netherlands*
5. *Dept. of Clinical Genetics, University Medical Center Utrecht, Utrecht, The Netherlands*
6. *Dept. of Clinical Genetics, Maastricht University Medical Center, Maastricht, The Netherlands*
7. *Dept. of Clinical Genetics, University Medical Center Nijmegen, Nijmegen, The Netherlands*
8. *Dept. of Child Neurology, VU Medical Center, Amsterdam, The Netherlands*
9. *Dept. of Neonatology, University Medical Center, University of Utrecht, Utrecht, The Netherlands*

ABSTRACT

Collagen IV alpha-1 protein (COL4A1) filaments form an triple helix together with collagen IV alpha-2 (COL4A2) and are constituents of the main mesh-type collagen of the extracellular matrix in many tissues. Since 2005, *COL4A1* mutations are known as an autosomal dominant cause of hereditary porencephaly. *COL4A1* and *COL4A2* mutations have been reported with a much broader spectrum of cerebrovascular, renal, ophthalmological, cardiac and muscular abnormalities, which prompted the term "*COL4A1* mutation related disorders". Genetic counseling is challenging, due to the broad phenotypic variation and reduced penetrance.

In the Erasmus University Medical Center, diagnostic DNA analysis of both *COL4A1* and *COL4A2* in 183 index patients has been performed between 2005 and 2013. In total, 21 *COL4A1* and 3 *COL4A2* mutations were identified, mostly in children with porencephaly or other patterns of parenchymal hemorrhagic involvement. The observations in 13 novel families harboring either *COL4A1* or *COL4A2* mutations prompted us to review the clinical spectrum. We observed several recognizable phenotypic patterns and propose a screening protocol at diagnosis.

Our data show a relatively high rate of *de novo* mutations and underscore the importance of *COL4A1* and *COL4A2* mutations as causes of cerebrovascular disease, also in sporadic patients. Follow-up data on symptomatic and asymptomatic mutation carriers are still needed for prognosis and appropriate surveillance.

INTRODUCTION

Already from the 1980s, investigators described recurrence of hemorrhagic stroke within families, manifesting as porencephalic cavities on CT-scan or MRI[1-6]. Before these observations, the presence of congenital porencephaly, a cyst which commuticates with the lateral ventricle and is (usually) seen following parenchymal hemorrhage, had often been considered the result of an external insult, e.g. postanoxic perinatal bleeding, without genetic substrate and, in the absence a coagulopathy, with low recurrence risk. Only in 2005, after observations in mouse models, *COL4A1* mutations were discovered as a cause of porencephaly, with an apparent autosomal dominant inheritance[7-9]. Over the years, it became clear that disorders of additional organs can result from mutations in this gene. The disorder related to *COL4A1* mutations is now known as a systemic disease, including a broad spectrum of cerebrovascular lesions including porencephaly and transmantle lesions, causing hydranencephaly or schizencephaly; lesions of the kidneys, leading to nephrosis and hematuria; lesions of the eyes, causing cataract, microphthalmia and blindness; of the heart, causing arrhythmia and of the skeletal muscles, causing dystrophic changes, weakness and myoglobinuria[10-22].

COL4A1 and *COL4A2* encode procollagen IV alpha-1 and IV alpha-2 chains, respectively, which assemble to form a heterotrimeric helix with a constant 2:1 ratio [alpha1(IV)]$_2$[alpha2(IV)]. This type IV collagen is a component of the non-fibrillary collagen, as main constituent of extracellular matrix of many tissues, among them vascular endothelia. Mice harbouring heterozygous mutations in *Col4a1* or *Col4a2* suffer from hemorrhage in the eye, brain, and skin, already occurring during gestation and sometimes leading to developmental defects of eye and brain. Therefore, similarities in phenotypes between *COL4A1* and *COL4A2* mutations were to be expected. In 2012, several parallel genetic, epidemiologic and functional studies revealed *COL4A2* mutations in both familial and sporadic porencephaly[23-25].

The disease resulting from both *COL4A1* and *COL4A2* mutations is extremely variable, with broad intra-and interfamilial variation and evidence for reduced penetrance. Sporadic individuals with severe presentation may harbor a *de novo* mutation.

At the department of Clinical Genetics of the Erasmus University Medical Center in Rotterdam, sequence analysis of both *COL4A1* and *COL4A2* has been offered since 2005 in a diagnostic setting. In this review, the clinical and genetic data of newly identified mutations

in the past 8 years are summarized. Although many reports have been published on the mutation spectrum, no clear recent guidelines regarding genetic counselling and management of affected patients exists. We here provide an up to date overview on the genotypic and phenotypic spectrum in order to improve clinical management and surveillance guidelines.

PATIENTS AND METHODS

At the Clinical Genetics laboratory of the Erasmus University Medical Center in Rotterdam, 183 index patients (and whenever available both parents), mostly with cerebral hemorrhage or porencephaly, were referred for testing of *COL4A1* (NM_001845.4) and *COL4A2* (NM_001846.2). This was done by Sanger sequencing or by application of a next generation sequencing panel encompassing 87 genes for brain developmental disorders including genes for porencephaly and Aicardi-Goutières syndrome (capturing of exons and intron-exon boundaries by eArray Sure Select (Agilent) and sequencing on the MiSeq platform (paired-end, 150bp, Illumina) followed by conformation of mutations with Sanger sequenencing). A total of 21 *COL4A1* and 3 *COL4A2* putative pathogenic genomic variants were identified between 2005 and June 2013. We previously reported on 2 of the *COL4A2* and 9 of the *COL4A1* mutations[7,12,15,24,26]. Of the remaining 13 novel mutation families, clinical data were collected using a questionnaire sent to the referring physicians according to IRB regulations of our center.

Furthermore, we reviewed the literature, focussing on the clinical phenotypes of *COL4A1* and *COL4A2* mutations. For this, a PubMed search was performed, identifying 27 manuscripts with clinical and mutation data on *COL4A1*[7,8,10-22,26-37] and 3 manuscripts with data on *COL4A2* mutations[23-25]. A total of 137 individuals with a *COL4A1* mutation and 15 with a *COL4A2* mutation have been reported. Several clinical phenotypes were identified and described in more detail. In addition, both neurologic and systemic features and brain MRI findings were available for review.

RESULTS

Mutation data on 13 newly identified families

In the literature, most reported *COL4A1* and *COL4A2* mutations are missense changes leading to a substitution of a glycine in the G-X-Y triple helix domain. In analogy to

other collagenopathies, these mutations are predicted to be pathogenic and are assumed to have a dominant-negative effect. In addition, splice site and frameshift mutations have been reported, indicating that haploinsufficiency of either *COL4A1* or *COL4A2* is another pathogenic mechanism[24,35] (Fig 1). *COL4A1* and *COL4A2* sequence variants in our cohort were considered pathogenic when *de novo*, when truncating or when previously described and proven to be pathogenic by *de novo* occurrence or functional studies. Most missense changes that were either predicted (ALAMUT package, http://www.interactive-biosoftware.com) or proven to be pathogenic disrupted the Gly-X-Y repeat of the triple helix.

In total, 21 *COL4A1* mutations, 12 of them novel, and 3 *COL4A2* mutations were identified. All the known *COL4A1* and *COL4A2* mutations, including those reported here, are depicted in Figure 1. The pedigrees of the newly identified families are shown in Figure 2.

Of the 13 cases associated with novel *COL4A1* mutations, 5 were sporadic and shown to *de novo* (Fig 2; family D, E, H, I and K) and 7 were familial. Of the 7 familial mutations, the one in family B occurred *de novo* in patient I.1. Of the mutations in Fig 2, only the *COL4A2* mutation in family L was previously reported in a sporadic adult patient with intracerebral hemorrhage[23]. It introduces a glycine at the triple helix domain of the protein and was shown to be pathogenic using functional studies[23]. All but one of the *COL4A1* mutations missense changes led to substitution of glycine in the triple helix domain of the protein (family A, B, C, G, H, I, K and M). The changes in families A, C, G and M have an official status of "variant of unknown clinical significance", although they are likely pathogenic in view of their predicted effect on the protein. In addition, segregation with the phenotype has been observed in families A and C. In family E, a *de novo* missense mutation was present affecting the NC1-domain. In family D, a *de novo* splice site mutation was identified, family G harboured an inherited frameshift mutation.

Identified clinical phenotypes in our cohort and in the literature

A total number of 183 index patients were tested for *COL4A1* and *COL4A2* mutations. A diagnosis was possible in 24/183 (13%) of index patients (published and unpublished), of which 10/24 (42%) were *de novo*. The 12 novel *COL4A1* families and 1 *COL4A2* family comprise 21 individuals with a (suspected) pathogenic mutation. Of these, 5 (24%) developed symptoms antenatally, leading to a termination of pregnancy in 1, 8 (38%) presented with symptoms soon after birth, 3 (14%) developed symptoms at a later age and 3 (14%) are clin-

ically asymptomatic until now. (Of 2 patients (family G), clinical data on onset are lacking. However, brain MRI has not been performed in these asymptomatic carriers till now and brain damage cannot be fully excluded. Seizures were the most common clinical symptom, present in 9 patients (43%). Also, a high percentage of motor dysfunction was present: hemiparesis in 6 (29%) and tetraparesis in 3 (14%). Developmental delay was present in 8 cases (38%). The clinical phenotypes and mutations of the newly identified families are summarized in Table 1. A summary of the brain MRI findings, associated ophthalmological, renal, cardiac and muscular findings, as well as incidentally reported findings in both our patients and literature is provided in Table 2. We provide an overview of several observed phenotypes in our cohort. The reported data on these phenotypes in literature are discussed later, together with additionally reported phenotypes.

Prenatal and neonatal intracerebral hemorrhage and porencephaly

Porencephaly has been reported as a result of both *COL4A1* and *COL4A2* mutations, most often due to germinal matrix hemorrhage leading to deep venous infarction with subsequent tissue necrosis and porencephalic cavitation. The first reports suggested an onset around birth[8], later reports also describe patients with an onset in late pregnancy[12,21,26].

In our cohort, 12 patients from 10 families (A, B, D, E, G, H, I, J, K, M) presented with early intracerebral hemorrhage and porencephaly; in 5 patients (family A-II.1, D-II.1, E-II.1, G-II.1, H-II.1) this was already seen prenatally (in 2 of them, the *COL4A1* mutation was also identified prenatally), 6 patients were diagnosed after birth (family B-I.1 and B-II.1,J-II.1, J-II.1, K-II.1, M-II.1) and showed hemi- or tetraplegia and/ or seizures. Although most patients with an antenatal diagnosis presented after 30 weeks of pregnancy, one patient (family D-II.1) presented at 25 weeks.

Of interest, in patient A-II.1 the porencephalic cyst showed enlargement on MRI scanning of the brain in his first year of life. Patient D-II.1 developed severe hydrocephalus secondary to the hemorrhage. Recurrence of cerebral hemorrhage was documented in patient E-II.1. This patient presented with antenatal cerebral hemorrhage and porencephaly on antenatal brain MRI. She developed a severe hypoxic-ischemic encephalopathy, for which she received cooling therapy; she died soon after birth. Postmortem examination showed a novel parenchymal hemorrhage (Fig 3M,N). Patient II.2 from family C had a congenital tetraplegia, however, data on brain imaging are lacking.

Sporadic extensive bilateral porencephaly resembling hydranencephaly

Hydranencephaly is defined as the end result of massive hemispheric necrosis and extreme ventricular dilation, with most of the hemispheres replaced by a CSF-filled membranous sac, and relative preservation of the diencephalic and posterior cranial fossa brain structures, with a variable onset, even starting in the first trimester[38]. In our cohort, patient II.1 from family H showed severe brain destruction with undetectable medial cerebral arteries, resembling hydranencephaly, at 33 gestation weeks (GW). The child was stillborn with 34 weeks of gestational age.

Periventricular leukomalacia with intracranial calcification

Periventricular leukomalacia (PVL) is defined as post-hypoxic-ischemic leukoencephalopathy resulting from a pre-or perinatal hypoxic-ischemic insult[39]. PVL is characterized by focal periventricular necrosis and gliosis in the surrounding white matter[40]. Also, intracranial calcifications are reported in this context[14]. In our cohort, patients A-I.2 and C-II.1 both show PVL without porencephaly (Fig 3B, E). Brain calcifications were not present. Additional features in patient A-I.2 were congenital cataracts. Patient C-II.1 also showed ophthalmological features comprising microcornea, congenital cataract and posterior embryotoxon. In both patients, this combination, together with the family history, suggested the diagnosis.

Axenfeld Rieger anomaly with leukoencephalopathy

Axenfeld Rieger anomaly comprises a constellation of ocular findings affecting the anterior chamber, including the anterior chamber angle and aqueous drainage structures (iridogoniodysgenesis), iris hypoplasia, eccentric pupil, iris tears and iridocorneal tissue adhesions traversing the anterior chamber. A frequent association consists with a posterior embryotoxon and a high risk of glaucoma and secondary blindness. In our newly reported family C, the 3 individuals with the *COL4A1* change (C-I.2, C-II.2, C-II.2) were affected with similar ophthalmological findings from the Axenfeld Rieger spectrum, cataract and microcornea. The neurological symptoms, however, varied greatly, from absence in patient C-I.2, to PVL and normal cognition in patient C-II.1, to severe mental retardation and spastic hemiplegia in patient C-II.2.

Cortical malformations; schizencephaly

Although cortical lesions secondary to tissue necrosis have been described in *COL4A1* mutations [15], however, malformations of the cortex, in particular schizencephaly, have only

been appreciated recently to be associated with *COL4A1* mutations[34,36], Schizencephaly is defined as a cleft extending from the pial surface to the lateral ventricle, lined by heterotopic gray matter[41].

In our cohort, we observed two patients with focal cortical malformations. The first has a maternally inherited c.1964G>A (p.G655E) *COL4A1* mutation and presented with schizencephaly, porencephaly and intraventricular hemorrhage (family H-II.1, Fig 3H). The mother has a severe hypermetropia of +10 Dpt. The second patient (family L-II.1) has a paternally inherited c.3368A>G (p.E1123G) *COL4A2* mutation and presented with porencephaly of the left frontal ventricle and overlying dysplastic cortex resembling transmantle heterotopia on brain MRI (Fig 3L). The father is asymptomatic, except for ptosis. The same *COL4A2* mutation was previously reported in two single adult patients with intraparenchymal hemorrhage; the mutation was proven to be pathogenic in *in vitro* functional studies[23].

Previously unreported findings in our cohort

Patient II.1 from family K had a severe neonatal neurological presentation with seizures due to intracerebral hemorrhages. At the age of 6 months, he was diagnosed with neuroblastoma, which has not been previously associated with *COL4A1* mutations, but is a relatively frequent tumor in childhood. We mention this because of the occurrence of other neurological tumors in this cohort (meningioma in patient A-1.2 and in another *COL4A1* carrier[7]). Whether neural tumors are part of the phenotypic spectrum does not seem likely from these data, but remains unclear.

The index patient from family J presented with a left-sided germinal matrix and intraventricular hemorrhage and posthemorrhagic venous infarction after birth. Interestingly, also supraventricular tachycardia was noted at this young age, requiring sotalol treatment. It is unclear whether the tachycardia is causally related to the *COL4A1* mutation, however it has also been reported in HANAC syndrome[16].

DISCUSSION

In a cohort of 183 index patients, mostly porencephaly patients or patients with infantile hemorrhage, we identified 21 *COL4A1* and 3 *COL4A2* pathogenic or likely pathogenic mutations. This suggests a high prevalence (13%) of mutations in this patient population. The clinical data of 13 families are novel, and 12 *COL4A1* mutations have not been described

previously. A high percentage of *de novo* mutations (38%) was found in our cohort; 5 index patients harbour a *de novo* mutation (family D,E,H,I,K). One other mutation appeared *de novo* in a carrier father of an index patient (family B). No clear genotype-phenotype correlation is present. A high percentage (12/20, 60%) of severe perinatal presentation was observed. The clinical phenotypes of our patients greatly overlap the phenotype previously reported in the literature. We will discuss the reported phenotypes in the literature in the following sections.

Prenatal and neonatal intracerebral hemorrhage and porencephaly

Porencephaly is one of the most frequently reported findings, described in 53 *COL4A1*[7,8,12,14,15,17,18,21,34-36] and 6 *COL4A2* patients[24,25] (Table 2). However, since porencephaly was the first reported associated phenotype, there may be a bias in the inclusion of patients for *COL4A1* and *COL4A2* testing. Intraventricular hemorrhage (IVH) is a common complication of very low birth weight preterm infants and a group of 40 preterm infants was tested for the presence of *COL4A1* mutations. Only one pathogenic mutation was identified[10]. This result indicates that *COL4A1* mutations are probably only minor contributors to IVH in very low birth weight preterm infants. A recent report of *COL4A1* screening in a group of 61 porencephaly patients revealed mutations in 10 patients (16%) aged 3 months to 14 years. All these patients had additional features including focal cortical dysplasia, intracranial calcification, hemolytic anemia, elevated CK, myopathy, ophthalmological features or hematuria[36]. A complication of the hemorrhage can be the development of secondary hydrocephalus, as seen in patient D-II.1 and previously reported[13,15,42].

Sporadic extensive bilateral porencephaly resembling hydranencephaly

We previously described a subset of patients with extensive prenatal porencephaly and gray and white matter loss, cortical destruction and cerebellar and brain stem atrophy, resembling hydranencephaly. A pathogenic mechanism was suggested, consisting of massive germinal matrix hemorrhage followed by extensive venous infarction with compression, edema and secondary ischemia of larger parenchymal areas leading to white matter and cortical destruction. Interestingly, we found *de novo COL4A1* mutations in all these patients, including a case of germline mosaicism in the mother of one patient[15].

PVL with intracranial calcification

Subtle periventricular, basal ganglia and/ or deep white matter calcifications are reported in *COL4A1* mutations, together with PVL, also in the absence of porencephaly[14,30,34,36,43].

The diagnosis in patients with this PVL phenotype without a contributing family history or additional findings could easily be missed. Associated features, such as elevated CK concentration or microbleeds may help in suspecting the diagnosis in the absence of other major findings such as porencephaly[14,30,34,36]. These associated findings may also help in discriminating *COL4A1* related calcifications and leukoencephalopathy from disorders with partially overlapping manifestations, such as Aicardi-Goutières syndrome, CMV infection or cystic leukoencephalopathy without megalencephaly[14,44].

Axenfeld Rieger anomaly with leukoencephalopathy

Axenfeld Rieger anomaly, as well as cataracts, microcornea and retinal detachment have been reported as consistent findings in several other families harbouring *COL4A1* mutations. Although major cerebral findings, such as porencephaly or hemorrhagic stroke can be present these patients, all patients showed leukoencephalopathy and small vessel disease, even in the absence of neurological symptoms. This indicates that brain MRI in Axenfeld Rieger patients may provide a clue for the diagnosis of a *COL4A1* related disorder[11,18].

HANAC (Hereditary Angiopathy with Nephropathy, Aneurysm and muscle Cramps) syndrome

The HANAC syndrome comprises a specific combination of features, and is attributed to mutations in *COL4A1* affecting glycine residues in close proximity of exons 24 and 25, later reported to be the triple helical CB3[IV] domain, encompassing major integrin binding sites[16,29,32]. The angiopathy in HANAC syndrome comprises retinal vessel arterial tortuosity and cerebral small and large vessel disease with aneurysms of the carotid syphon. The nephropathy consists of persistent hematuria and/ or proteinuria with or without bilateral, large renal cysts. Other associated findings are muscle cramps with elevated CK levels, Raynaud's phenomenon and cardiac arrhytmia. Interestingly, HANAC syndrome patients do not present with infantile hemiplegia or porencephaly. Also, the risk of major (hemorrhagic) stroke is thought to be lower than in other *COL4A1* mutations[16,29,32]. In our cohort, no patients displaying the HANAC phenotype were identified, possibly due to referral bias, as our cohort comprises mostly neurological patients. Possibly, the clinical picture of HANAC is insufficiently known to be associated with *COL4A1* mutations among referring physicians.

Stroke in childhood and young adulthood

Besides prenatal hemorrhage leading to porencephaly, strokes in the form of cerebral bleeding at later age can occur with *COL4A1* and *COL4A2* mutations[17,20,28,31]. In our cohort, stroke in later childhood and young adulthood was not reported. In the literature, cerebral hemorrhages in childhood or in young adulthood have been documented in *COL4A1* mutations. Differential diagnostic considerations in these cases comprise other genetic cerebrovascular malformation syndromes, such as cerebral cavernous hemangiomas or hereditary hemorrhagic telangiectasia. The hemorrhagic strokes typically affect the deep white matter, in combination with diffuse leukoencephalopathy and microbleeds[17,20,28,31]. Recurrence of strokes has been reported[20,28]. Two of the documented cases were sporadic[20,31], the others were familial[17,28].

Sporadic late-onset hemorrhagic stroke

Sporadic intracerebral hemorrhage generally occurs in the elderly with a worldwide incidence of 24.6 per 100.000 person-years[45], frequently in the setting of risk factors such as cerebral amyloid angiopathy or hypertensive vasculopathy, alcohol consumption or cigarette smoking.

In our cohort, patients with adult onset cerebral hemorrhage are lacking. This may very well be due to selection bias, probably due to the fact that most patients were referred by clinical geneticists and neonatologists because of pre- and perinatal hemorrhage or porencephaly.

In a cohort of 96 sporadic patients with intracerebral hemorrhage not caused by arteriovenous malformations, tumors or impaired coagulation, *COL4A1* and *COL4A2* were tested, leading to the detection of 2 *COL4A1* mutations in 2 patients and 3 *COL4A2* mutations in 4 patients[22,23]. These findings indicate that *COL4A1* and *COL4A2* mutations contribute to approximately 6% of sporadic late onset intracerebral hemorrhage. Since intracerebral hemorrhage constitutes approximately 15% of all intracranial hemorrhages in this group of patients, the contribution of *COL4A1* and *COL4A2* mutations in the total group of intracranial hemorrhage is approximately 1%. Although the portion may seem small, general prevalence of *COL4A1* and *-2* mutations may be quite high, since intracerebral hemorrhage in the elderly is not a rare event. The mutations identified in the patients are missense mutations, that probably have a milder effect on collagen IV function, and not the frequently identified triple helical domain glycine changes[22,23]. However, pathogenicity of these milder missense mutations has only been tested in an *in vitro* expression system.

Focal cortical dysplasia and schizencephaly

In the literature, *COL4A1* mutations were identified as the first genetic cause of schizencephaly[34,36] ; mutations were found in 5 out of 10 schizencephaly patients tested. We describe the first patient with cortical dysplasia harbouring a *COL4A2* mutation (L-II.1). The localization of the cortical dysplasia and the association with an underlying porencephalic enlargement of the frontal part of the lateral ventricle in our patient suggest a causal relation between the *COL4A2* mutation and the cortical malformation (Fig 3L). Interestingly, the father also carries the mutation and is asymptomatic, but no MRI has been performed. Probably, the pathogenesis of this malformation is similar to that of the schizencephaly and hydranencephaly reported in *COL4A1* mutations. Our findings indicate that *COL4A2* mutations can also lead to dysplastic cortex, hereby broadening the *COL4A2* phenotypic spectrum. The role of *COL4A2* mutations in cortical dysplasia associated with porencephaly needs to be investigated in a larger patient cohort.

Issues in genetic counselling and management of affected families

There are several plausible, non-mutually exclusive pathogenic mechanisms of various *COL4A1* and *COL4A2* mutations, which are summarized in a recent review[46]. The pathogenic mechanism, is still not elucidated completely, and may be different depending on mutation type.

Another unresolved question is the matter of the reduced penetrance. The same mutation can lead to a severely affected infant, while the carrier parent is hardly affected. This leads to the hypothesis that *COL4A1* and *COL4A2* mutations must be regarded as risk factors, which, together with additional modifying factors, lead to a phenotype. Mouse models support this hypothesis, showing that penetrance and disease severity is related to the genetic context[13,47].

Due to the reduced penetrance with possible modifying factors and the variable phenotype, the counselling of affected patients and their families remains a challenge. We suggest an initial workup in mutation families, including neurological, ophthalmological, renal and cardiac screening in mutation carriers and first degree relatives with a 50% chance of harbouring the mutation (the latter taking into account the social consequences of presymptomatic DNA testing) (Table 3). Neurological screening should include a medical history and physical examination, followed by brain MRI if abnormalities are found. We recommend adding susceptibility-weighted imaging, since this improves the detection of

microbleeds[48]. Performing MRA to identify cerebral aneurysms is still under debate; up till now no ruptured aneurysms have been reported in mutation carriers, suggesting a slow progression rate and discouraging the use of MRA. Follow-up data on the course of identified aneurysms in this specific group of patients, however, are lacking.

Another subject under debate is the perinatal management of pregnancies in which the child or the mother harbours a *COL4A1* or *COL4A2* mutation. Prenatal testing can be offered in high risk pregnancies after genetic counselling, with special attention for the variable phenotypic expression and reduced penetrance. Delivery by caesarean section has been proposed in order to prevent brain vascular injury attributable to birth trauma[27]. However, evidence that this will lead to prevention of cerebral hemorrhage is lacking and several patients have now been reported who had established lesions long before delivery. Further studies on the clinical course of individuals with *COL4A1* or *COL4A2* mutations are needed in order to determine the follow-up in more detail.

CONCLUSIONS

In conclusion, our data confirm that *COL4A1* mutations and *COL4A2* mutations are important causes of cerebrovascular disease with a high mutation detection rate in porencephaly and childhood cerebral hemorrhage with a relatively high rate of *de novo* mutations. Although also present in (sporadic) adult onset intracerebral hemorrhage with an incidence of 6%[22,23], the role of mutations in this patient cohort seems less prominent. It seems important to increase awareness of this disorder among neurologists and internists coping with the adult population. Besides the cerebrovascular phenotype, systemic involvement, with ocular, renal, muscular and cardiac features must not be underestimated and needs screening at diagnosis, even outside the formal HANAC syndrome. The precise role of *COL4A1* and *COL4A2* mutations in cortical malformations needs to be elucidated, but it seems to contribute to those malformations that are most likely a result of a vascular insult during fetal development[49]. Follow-up data on *COL4A1* and *COL4A2* mutation carriers are important in order to develop appropriate surveillance protocols and adapt treatment.

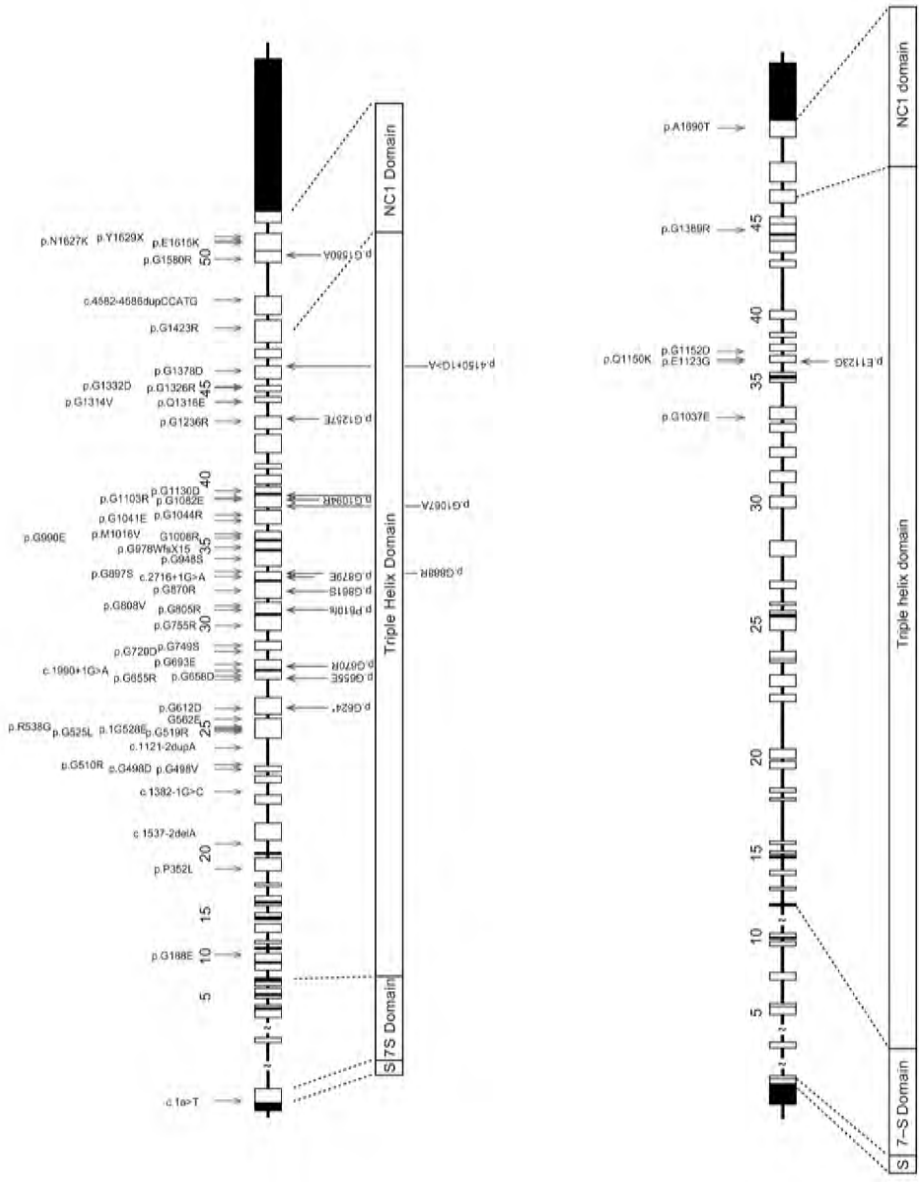


Figure 1. In this figure, the *COL4A1* mutations (A) and *COL4A2* mutation (B) in our novel families are depicted on the bottom (red arrows), the mutations reported in literature are depicted on the top (blue arrows).

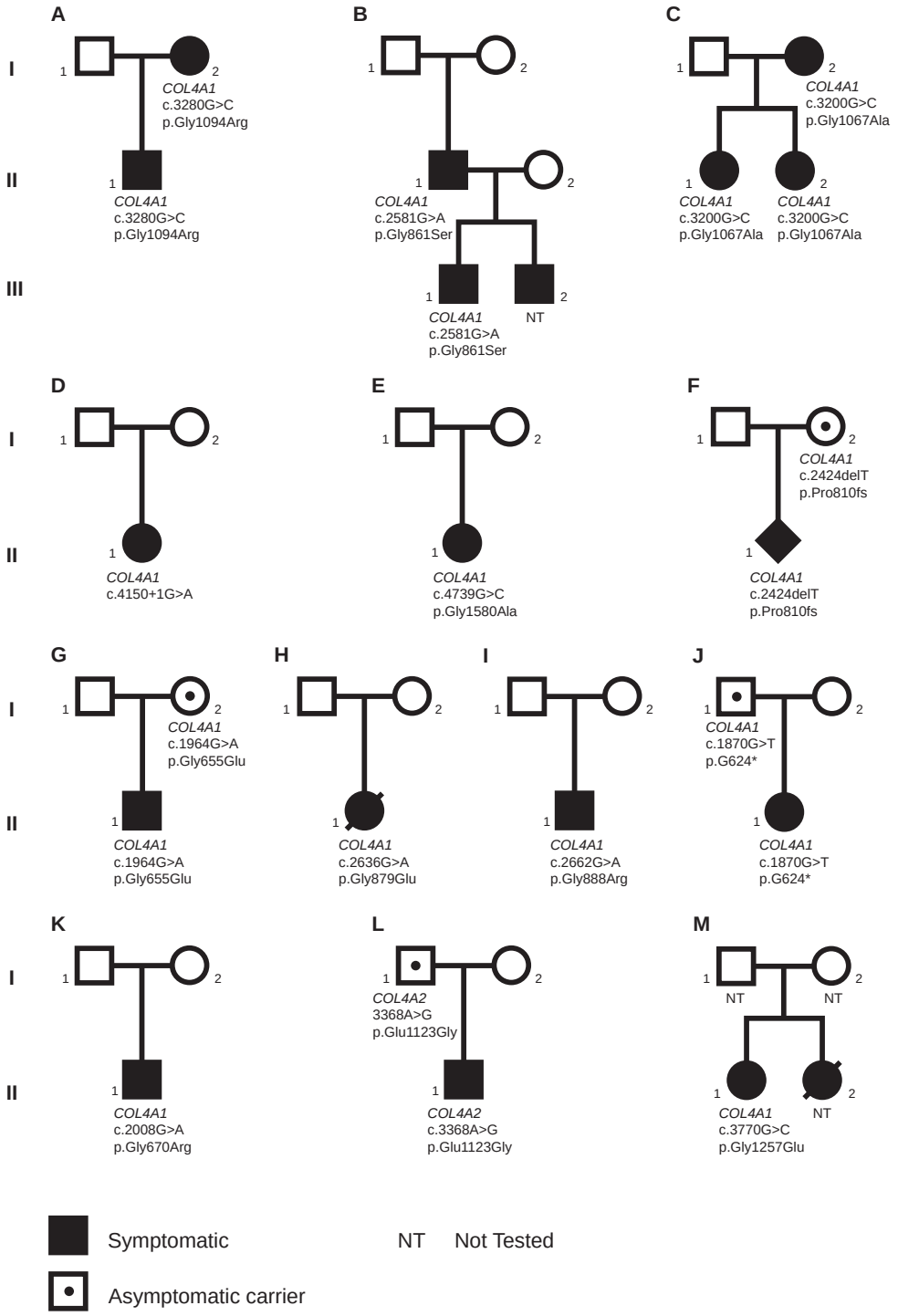


Figure 2. Pedigrees of the 13 novel families

Figure 3.
Selection of brain MRIs of novel patients

A. T1-weighted image of A-II.1 showing porencephaly with cortical destruction and white matter hyperintensities suggestive of calcifications and/or hemorrhage.
B. T2-weighted image of A-I.2 showing enlarged perivascular spaces.
C and D. T2-weighed image of B-III.1 and B-II.1, with classic left-sided unilateral porencephaly.
E. FLAIR image of C-II.1, indicating PVL.
F. FLAIR image of D-II.1 depicting severe expansive porencephaly and severe white matter loss.
G. T2-weighed image of E-II.1 with left-sided hemorrhage with ipsilateral volume loss of white matter, thalamus and basal ganglia.
H. T2-weighted image of H-II.1 showing right-sided schizencephaly and porencephaly and a small hemorrhage in the left frontal lobe.
I. T2-weighted image of J-II.1with left-sided porencephaly with bilateral ventricular enlargement and leukoencephalopathy.
J. T2-weighted image of K-II.1 with left-sided germinal matrix hemorrhage leading to intraventricular hemorrhage and a left-sided venous infarction.
K. T2-weighted image of L-II.2 depicting bilateral severe cerebellar haemorrhage in both hemispheres.
L. FLAIR image of M-II.2 showing closed-lip schizencephaly and focal cortical dysplasia.
M and N depict post-mortem images of patient E-II., showing ventricular dilatation and a large intraparenchymal hemorrhage.

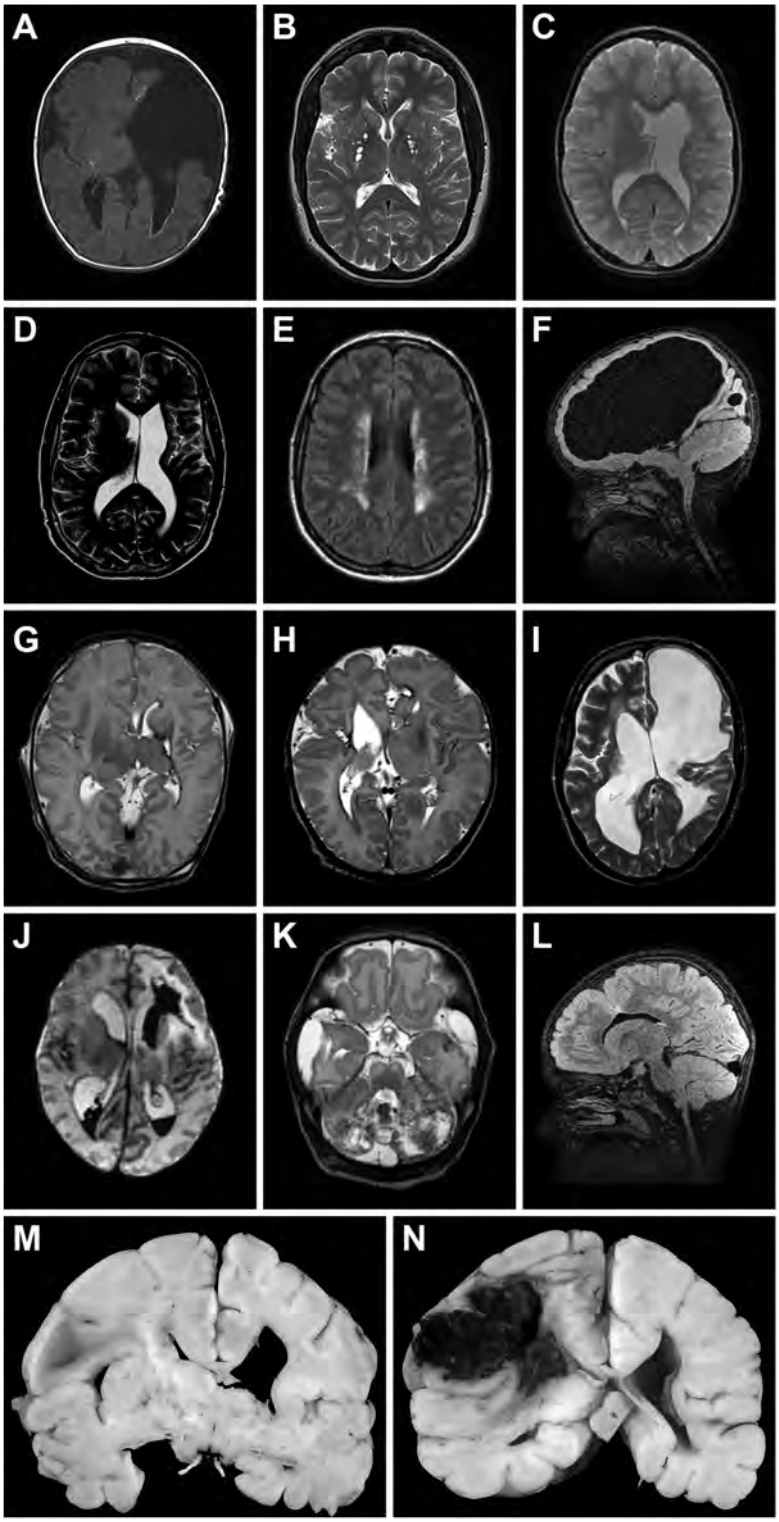


Table 1. Clinical phenotypes and mutations of novel identified families

FAM	Pt	MUTATION			SYMPTOMS							
		COL4A1	*COL4A2*	INH/DN	Onset	Neurological	Other	Brain MRI	Eyes	Kidneys	Muscles	Heart
A	II.1	c.3280G>C, p.Gly1094Arg	-	INH	Prenatal	TP, SEIZ, DEV		POR, CORT, CEBL, CAL	CAT	-	-	-
	I.2	c.3280G>C, p.Gly1094Arg	-	Unk	Adult	MIGR	Meningeoma	LEU, LAC	CAT	-	-	-
B	II.1	c.2581G>A, p.Gly861Ser	-	INH	Postnatal	HP, DEV, SEIZ		POR	HYPM	-	-	-
	II.2	Not tested			Postnatal							
	I.1	c.2581G>A, p.Gly861Ser	-	DN	Postnatal	HP	Autism	POR	RAT	-	-	-
C	II.1	c.3200G>C, p.Gly1067Ala	-	INH	Adult	-		LEU	CAT, MICRC, PE, VFD	-	-	-
	II.2	c.3200G>C, p.Gly1067Ala	-	INH	Postnatal	HP, SEIZ, DEV		Unk	CAT, MICRC	-	-	-
	I.2	c.3321G>C, p.Gly1067Ala	-	Unk	Adult	-		Unk	CAT, GLAU, MICRO	-	-	-
D	II.1	c.4150+1G>A	-	DN	Prenatal	TP, SEIZ, DEV, HYDR		POR, CEBL, BGA	MICRC, OPTA, HYPM	Dilated pyelum	-	-
E	II.1	c.4739G>C, p.Gly1580Ala	-	DN	Prenatal, postnatal	SEIZ	MC	POR, LEU, LAC, CAL, BGA	-	-	-	-
F	II.1	c.2424delT, p.Pro810fs	-	INH	Unk	Unk		Unk	Unk	Unk	Unk	Unk
	I.2	c.2424delT, p.Pro810fs	-	Unk	Unk	Unk		Unk	Unk	Unk	Unk	Unk
G	II.1	c.1964G>A, p.Gly655Glu	-	INH	Prenatal	HP, DEV, SEIZ	MC	POR, IVH, SCHIZ, CEBL	HYPM	Mild hydronephrosis	-	-
	I.2	c.1964G>A, p.Gly655Glu	-	Unk	-	-		Unk	HYPM	-	-	-
H	II.1	c.2636G>A, p.Gly879Glu	-	DN	Prenatal	NA	SPFM	HYDR, CEBL	Unk	Unk	Unk	Unk

(continued on next page)

Table 1. Clinical phenotypes and mutations of novel identified families *(continued from previous page)*

FAM	Pt	MUTATION			SYMPTOMS							
		COL4A1	*COL4A2*	INH/DN	Onset	Neurological	Other	Brain MRI	Eyes	Kidneys	Muscles	Heart
I	II.1	c.2662G>A, p.Gly888Arg	-	DN	Postnatal	TP, DEV, SEIZ	Contractures	POR, LEU	CAT, uveitis	-	MYOP, MA	-
J	II.1	c.1870G>T, p.G624*	-	INH	Postnatal	HP		POR, IVH	-	-	-	SVA
	I.1	c.1870G>T, p.G624*	-	Unk	-	-		Unk	Unk	Unk	Unk	Unk
K	II.1	c.2008G>A, p.Gly670Arg	-	DN	Postnatal	SEIZ		POR, IVH, LEU, CBLH	-	-	-	-
L	II.1	-	c.3368A>G, p.Glu1123Gly	INH	Postnatal	DEV	ADHD	SCHIZ	-	-	-	-
	I.1	-	c.3368A>G, p.Glu1123Gly	Unk	-	-	-	Unk	Unk	Unk	Unk	Unk
M	II.1	c.3770G>A, p.Gly1257Glu		INH?	Postnatal	DEV, HP, SEIZ	-	POR, LEU, BGA	HYPM, VFD	-	MYOP, MUC	-
	II.2	Not tested			Postnatal	DEV, TP, SEIZ	MC	POR, CATR	Unk	Unk	Unk	Unk

Pt, patient; **INH**, inherited; **DN**, de novo; **Unk**, unknown; **TP**, tetraparesis; **HP**, hemiparesis; **SEIZ**, seizures; **DEV**, developmental delay; **MIGR**, migraine; **MC**, microcephaly; **NA**, not applicable;

POR, porencephaly; **IVH**, intraventricular hemorrhage; **CEBL**, cerebellar atrophy; **LEU**, leukoencephalopathy, **LAC**, lacunar infarctions; **SCHIZ**, schizencephaly, **CORT**, cortical destruction; **CAL**, calcifications;

BGA, basal ganglia abnormalities; **HYDRO**, hydrocephalus; **HYDRA**, hydranencephaly; **CBLH**, cerebellar hemorrhage; **CATR**, cerebral atrophy

CAT, cataract; **RAT**, retinal arterial tortuosity; **GLAU**, glaucoma; **MICRO**, microphthalmia; **MICRC**, microcornea; **PE**, posterior embryotoxon; **OPTA**, optic atrophy; **HYPM**, hypermetropy; **VFD**, visual field defect

SPFM, sporadic fetal movements; **MYOP**, myopathy; **MA**, muscular atrophy; **MUC**, muscle cramps; **SVA**, supraventricular arrhythmia

Table 2. Brain MRI findings and Systemic manifestations in *COL4A1* and *COL4A2* mutations

	COL4A1 **mutations*** (137 carriers)	***COL4A2*** **mutations*** (15 carriers)	**Novel** ***COL4A1*** **mutations** (20 carriers)	**Novel** ***COL4A2*** **mutation** (2 carriers)
Brain MRI findings				
Periventricular leukoencephalopathy/ small vessel disease	54	3	5	
Porencephaly	53	6	11	1
Cerebral calcification	16		2	
Microbleeds	16			
Intracerebral hemorrhage	15	5		
Cerebellar atrophy	12	1	4	
Intracranial aneursym	8	1		
Lacunar infarct	7		2	
Schizencephaly	6		1	
Intraventricular hemorrhage (without porencephaly)	5			
Dysplastic brain stem	5			
Hydrocephalus	4	1		
Hydranencephaly	4		1	
Mild ventriculomegaly	3			
Abnormal basal ganglia	3		3	
Gyral abnormalities	2			
Multicystic encephalomalacia	2			
Lissecephaly	1			
Traumatic subarachnoidal hemorrhage	1			
Tortuosity of infra-and supratentorial vessels	1			
Dandy Walker malformation	1			
Focal cortical dysplasia	1			1

(continued on next page)

Table 2. Brain MRI findings and Systemic manifestations in *COL4A1* and *COL4A2* mutations

	COL4A1 **mutations*** (137 carriers)	***COL4A2*** **mutations*** (15 carriers)	**Novel** ***COL4A1*** **mutations** (20 carriers)	**Novel** ***COL4A2*** **mutation** (2 carriers)
Ophthalmological findings				
Cataract	29		6	
Retinal arterial tortuosity	26		1	
Strabism	10			
Iris hypoplasia	10			
Posterior embryotoxon	9		1	
Corneal opacities	8			
Retinal hemorrhage	7			
Anterior segment	7			
Optic atrophy	6	2	1	
Microcornea	5		3	
microphthalmia	5		1	
Glaucoma	2		1	
High myopia	2	1		
Reduced cone and rod responses	1			
nystagmus	1			
Optic coloboma	1			
Retinal detachment	1			
Hypermetropia			2	
Renal findings				
Renal cysts	4			
Hematuria	4			
Renal agenesis	1			
Hyperechogenicity of renal pyramids	1			
Dilated pyelum			1	

(continued on next page)

Table 2. Brain MRI findings and Systemic manifestations in *COL4A1* and *COL4A2* mutations

	COL4A1 **mutations*** (137 carriers)	***COL4A2*** **mutations*** (15 carriers)	**Novel *COL4A1* mutations** (20 carriers)	**Novel *COL4A2* mutation** (2 carriers)
Muscular abnormailties				
Elevated CK	25		1	
Muscle cramps	18		2	
Myopathy	2		2	
Muscular atrophy	1		1	
Cardiac abnormalities				
Raynaud	6			
Cardiac (supraventricular) arrhytmia	4		1	
Mitral valve prolaps	4			
VSD	1			
Other findings				
Hemolytic anemia	5			
Thymus, liver and adrenal hemorrhage	1			
Sensorineural deafness	1			

Number indicates reported patients in literature

Table 3. Screening protocol

Screening protocol
Neurological examination
Brain MRI (upon indication)
Ophthalmological examination
Ultrasound of kidneys
Urine analysis for the presence of hematuria
Renal function test (serum creatinine, estimation of glomerular filtration rate)
Serum CK measurement
Electrocardiogram (arrhythmias)

References

1. **Zonana J, Adornato BT, Glass ST, Webb MJ.** Familial porencephaly and congenital hemiplegia. *The Journal of pediatrics.* Oct 1986;109(4):671-674.

2. **Sensi A, Cerruti S, Calzolari E, Vesce F.** Familial porencephaly. *Clinical genetics.* Nov 1990;38(5):396-397.

3. **Berg RA, Aleck KA, Kaplan AM.** Familial porencephaly. *Archives of neurology.* Sep 1983;40(9):567-569.

4. **Shastri NJ, Bharani SA, Modi UJ, Trivedi C.** Familial porencephaly. *Indian journal of pediatrics.* May-Jun 1993;60(3):459-463.

5. **Mancini GM, de Coo IF, Lequin MH, Arts WF.** Hereditary porencephaly: clinical and MRI findings in two Dutch families. *Eur J Paediatr Neurol.* 2004;8(1):45-54.

6. **Vilain C, Van Regemorter N, Verloes A, David P, Van Bogaert P.** Neuroimaging fails to identify asymptomatic carriers of familial porencephaly. *American journal of medical genetics.* Oct 1 2002;112(2):198-202.

7. **Breedveld G, de Coo IF, Lequin MH, et al.** Novel mutations in three families confirm a major role of COL4A1 in hereditary porencephaly. *J Med Genet.* Jun 2006;43(6):490-495.

8. **Gould DB, Phalan FC, Breedveld GJ, et al.** Mutations in Col4a1 cause perinatal cerebral hemorrhage and porencephaly. *Science.* May 20 2005;308(5725):1167-1171.

9. **Aguglia U, Gambardella A, Breedveld GJ, et al.** Suggestive evidence for linkage to chromosome 13qter for autosomal dominant type 1 porencephaly. *Neurology.* May 11 2004;62(9):1613-1615.

10. **Bilguvar K, DiLuna ML, Bizzarro MJ, et al.** COL4A1 mutation in preterm intraventricular hemorrhage. *The Journal of pediatrics.* Nov 2009;155(5):743-745.

11. **Coupry I, Sibon I, Mortemousque B, Rouanet F, Mine M, Goizet C.** Ophthalmological features associated with COL4A1 mutations. *Arch Ophthalmol.* Apr 2010;128(4):483-489.

12. **de Vries LS, Koopman C, Groenendaal F, et al.** COL4A1 mutation in two preterm siblings with antenatal onset of parenchymal hemorrhage. *Annals of neurology.* Jan 2009;65(1):12-18.

13. **Labelle-Dumais C, Dilworth DJ, Harrington EP, et al.** COL4A1 mutations cause ocular dysgenesis, neuronal localization defects, and myopathy in mice and Walker-Warburg syndrome in humans. *PLoS Genet.* May 2011;7(5):e1002062.

14. **Livingston J, Doherty D, Orcesi S, et al.** COL4A1 mutations associated with a characteristic pattern of intracranial calcification. *Neuropediatrics.* Dec 2011;42(6):227-233.

15. **Meuwissen ME, de Vries LS, Verbeek HA, et al.** Sporadic COL4A1 mutations with extensive prenatal porencephaly resembling hydranencephaly. *Neurology.* Mar 1 2011;76(9):844-846.

16. **Plaisier E, Gribouval O, Alamowitch S, et al.** COL4A1 mutations and hereditary angiopathy, nephropathy, aneurysms, and muscle cramps. *N Engl J Med.* Dec 27 2007;357(26):2687-2695.

17. **Shah S, Ellard S, Kneen R, et al.** Childhood presentation of COL4A1 mutations. *Dev Med Child Neurol.* Jun 2012;54(6):569-574.

18. **Sibon I, Coupry I, Menegon P, et al.** COL4A1 mutation in Axenfeld-Rieger anomaly with leukoencephalopathy and stroke. *Annals of neurology.* Aug 2007;62(2):177-184.

19. **Vahedi K, Boukobza M, Massin P, Gould DB, Tournier-Lasserve E, Bousser MG.** Clinical and brain MRI follow-up study of a family with COL4A1 mutation. *Neurology.* Oct 16 2007;69(16):1564-1568.

20. **Vahedi K, Kubis N, Boukobza M, et al.** COL4A1 mutation in a patient with sporadic, recurrent intracerebral hemorrhage. *Stroke.* May 2007;38(5):1461-1464.

21. **Vermeulen RJ, Peeters-Scholte C, Van Vugt JJ, et al.** Fetal origin of brain damage in 2 infants with a COL4A1 mutation: fetal and neonatal MRI. *Neuropediatrics.* Feb 2012;42(1):1-3.

22. **Weng YC, Sonni A, Labelle-Dumais C, et al.** COL4A1 mutations in patients with sporadic late-

onset intracerebral hemorrhage. *Annals of neurology.* Apr 2012;71(4):470-477.

23. Jeanne M, Labelle-Dumais C, Jorgensen J, et al. COL4A2 mutations impair COL4A1 and COL4A2 secretion and cause hemorrhagic stroke. *Am J Hum Genet.* Jan 13 2012;90(1):91-101.

24. Verbeek E, Meuwissen ME, Verheijen FW, et al. COL4A2 mutation associated with familial porencephaly and small-vessel disease. *Eur J Hum Genet.* Aug 2012;20(8):844-851.

25. Yoneda Y, Haginoya K, Arai H, et al. De novo and inherited mutations in COL4A2, encoding the type IV collagen alpha2 chain cause porencephaly. *Am J Hum Genet.* Jan 13 2012;90(1):86-90.

26. Lichtenbelt KD, Pistorius LR, De Tollenaer SM, Mancini GM, De Vries LS. Prenatal genetic confirmation of a COL4A1 mutation presenting with sonographic fetal intracranial hemorrhage. *Ultrasound Obstet Gynecol.* Jun 2012;39(6):726-727.

27. Gould DB, Phalan FC, van Mil SE, et al. Role of COL4A1 in small-vessel disease and hemorrhagic stroke. *N Engl J Med.* Apr 6 2006;354(14):1489-1496.

28. Shah S, Kumar Y, McLean B, et al. A dominantly inherited mutation in collagen IV A1 (COL4A1) causing childhood onset stroke without porencephaly. *Eur J Paediatr Neurol.* Mar 2010;14(2):182-187.

29. Alamowitch S, Plaisier E, Favrole P, et al. Cerebrovascular disease related to COL4A1 mutations in HANAC syndrome. *Neurology.* Dec 1 2009;73(22):1873-1882.

30. Rouaud T, Labauge P, Tournier Lasserve E, et al. Acute urinary retention due to a novel collagen COL4A1 mutation. *Neurology.* Aug 24 2010;75(8):747-749.

31. Coutts SB, Matysiak-Scholze U, Kohlhase J, Innes AM. Intracerebral hemorrhage in a young man. *CMAJ.* Jan 11 2011;183(1):E61-64.

32. Plaisier E, Chen Z, Gekeler F, et al. Novel COL4A1 mutations associated with HANAC syndrome: A role for the triple helical CB3[IV] domain. *Am J Med Genet A.* Sep 3 2010;152A(10):2550-2555.

33. Garel C, Rosenblatt J, Moutard ML, et al. Fetal intracerebral hemorrhage and COL4A1 mutation: promise and uncertainty. *Ultrasound Obstet Gynecol.* Feb 2012;41(2):228-230.

34. Tonduti D, Pichiecchio A, La Piana R, et al. COL4A1-related disease: raised creatine kinase and cerebral calcification as useful pointers. *Neuropediatrics.* Oct 2012;43(5):283-288.

35. Lemmens R, Maugeri A, Niessen HW, et al. Novel COL4A1 mutations cause cerebral small vessel disease by haploinsufficiency. *Hum Mol Genet.* Jan 15 2013;22(2):391-397.

36. Yoneda Y, Haginoya K, Kato M, et al. Phenotypic Spectrum of COL4A1 Mutations: Porencephaly to Schizencephaly. *Annals of neurology.* Jan 2012;73(1):48-57.

37. Rodahl E, Knappskog PM, Majewski J, et al. Variants of anterior segment dysgenesis and cerebral involvement in a large family with a novel COL4A1 mutation. *American journal of ophthalmology.* May 2013;155(5):946-953.

38. Cecchetto G, Milanese L, Giordano R, Viero A, Suma V, Manara R. Looking at the missing brain: hydranencephaly case series and literature review. *Pediatr Neurol.* Feb 2013;48(2):152-158.

39. van der Knaap MS, Valk J, de Neeling N, Nauta JJ. Pattern recognition in magnetic resonance imaging of white matter disorders in children and young adults. *Neuroradiology.* 1991;33(6):478-493.

40. Nagasunder AC, Kinney HC, Bluml S, et al. Abnormal microstructure of the atrophic thalamus in preterm survivors with periventricular leukomalacia. *AJNR. American journal of neuroradiology.* Jan 2011;32(1):185-191.

41. Dies KA, Bodell A, Hisama FM, et al. Schizencephaly: association with young maternal age, alcohol use, and lack of prenatal care. *Journal of child neurology.* Feb 2013;28(2):198-203.

42. van der Knaap MS, Smit LM, Barkhof F, et al. Neonatal porencephaly and adult stroke related to mutations in collagen IV A1. *Annals of neurology.* Mar 2006;59(3):504-511.

43. Livingston JH, Stivaros S, van der Knaap MS, Crow YJ. Recognizable phenotypes associated with intracranial calcification. *Dev Med Child Neurol.* Jan 2012;55(1):46-57.

44. Henneke M, Diekmann S, Ohlenbusch A, et al. RNASET2-deficient cystic leukoencephalopathy resembles congenital cytomegalovirus brain infection. *Nature genetics.* Jul 2009;41(7):773-775.

45. van Asch CJ, Luitse MJ, Rinkel GJ, van der Tweel I, Algra A, Klijn CJ. Incidence, case fatality, and functional outcome of intracerebral haemorrhage over time, according to age, sex, and ethnic origin: a systematic review and meta-analysis. *Lancet Neurol.* Feb 2010;9(2):167-176.

46. Kuo DS, Labelle-Dumais C, Gould DB. COL4A1 and COL4A2 mutations and disease: insights into pathogenic mechanisms and potential therapeutic targets. *Hum Mol Genet.* Oct 15 2012;21(R1):R97-110.

47. Gould DB, Marchant JK, Savinova OV, Smith RS, John SW. Col4a1 mutation causes endoplasmic reticulum stress and genetically modifiable ocular dysgenesis. *Hum Mol Genet.* Apr 1 2007;16(7):798-807.

48. Cheng AL, Batool S, McCreary CR, et al. Susceptibility-weighted imaging is more reliable than T2*-weighted gradient-recalled echo MRI for detecting microbleeds. *Stroke.* Oct 2013;44(10):2782-2786.

49. Robin NH, Taylor CJ, McDonald-McGinn DM, et al. Polymicrogyria and deletion 22q11.2 syndrome: window to the etiology of a common cortical malformation. *Am J Med Genet A.* Nov 15 2006;140(22):2416-2425.

Chapter 4.1

SPORADIC *COL4A1* MUTATIONS WITH EXTENSIVE PRENATAL PORENCEPHALY RESEMBLING HYDRANENCEPHALY

M.E.C. Meuwissen, MD[1*], L.S. de Vries MD, PhD[2*], H.A. Verbeek, BSc[1], M.H. Lequin, MD, PhD[3], P.P. Govaert, MD, PhD[4], R. Schot BSc[1], F.M. Cowan, MD, PhD[5], R. Hennekam, MD, PhD[6], P. Rizzu, PhD[7] , F.W. Verheijen, PhD[1], M.W. Wessels, MD, PhD[1*], and G.M.S. Mancini, MD, PhD[1*]

Neurology 2011 (76): 844-846

1. *Department of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands*
2. *Department of Neonatology, Wilhelmina Children's Hospital, University Medical Centre Utrecht, The Netherlands*
3. *Department of Radiology, Erasmus University Medical Center, Rotterdam, The Netherlands*
4. *Department of Radiology and Neonatology, Erasmus University Medical Center, Rotterdam, The Netherlands*
5. *Department of Paediatrics, Imperial College Healthcare Trust , London, United Kingdom*
6. *Great Ormond Street Hospital for Children, UCL Institute of Child Health, London, UK and Department of Paediatrics, Amsterdam Medical Center, The Netherlands*
7. *Department of Clinical Genetics, section of Medical Genomics, VU Medical Center, Amsterdam, The Netherlands*

* *These authors equally contributed to this manuscript.*

INTRODUCTION

COL4A1 mutations were initially described in familial porencephaly (OMIM 175780)[1-3]. The phenotypic spectrum of *COL4A1* mutations is however wide and includes cerebral white matter small vessels disease, cerebral aneurysms, cataract, anterior segment dysgenesis, microcornea, nephropathy, muscle cramps and cardiac arrhythmia[4-6]. Prenatal onset has been documented[7]. Phenotype-genotype correlation has been proposed for the HANAC phenotype (OMIM 611773)[5].

We report on novel *COL4A1* mutations occurring *de novo* in four Caucasian patients with extensive prenatal brain destruction, adding to the wide phenotypic spectrum.

METHODS

COL4A1 sequencing was performed according to the Dutch regulations for genetic diagnosis[3]. Written informed consent for publication was obtained from the parents.

CASE REPORTS

Case 1. This patient (male) presented antenatally at 27 weeks gestation with asymmetric ventriculomegaly and extensive cerebral infarction seen on fetal ultrasound and MRI (figure 1A,B). At birth (37 weeks' gestation), apnea, poor feeding and seizures developed, leading to death at age 10 days. Postnatal cerebral ultrasound (CUS) and post-mortem MRI confirmed severe bilateral ventriculomegaly and tissue atrophy following extensive, putatively deep venous infarctions. Absence of the rostral part of the corpus callosum, a poorly gyrated cortex, atrophy of the basal ganglia, thalami, brainstem and cerebellum with an enlarged fourth ventricle were noted (Figure 1, C and D). Tortuosity of infra- and supratentorial vessels (Figure 1C), agenesis of the right kidney, renal artery and femoral vein and left-sided renal cystic lesions were also seen (Figure 1E).

Case 2. This patient (female), born at 40 weeks gestation, had severe ventriculomegaly and severe cerebellar atrophy seen on fetal ultrasound at 37 weeks gestation and fetal MRI. Postnatal CUS and MRI confirmed the findings (Figure 1, F,G). Susceptibility-weighted imaging (SWI) showed low signal intensity on the ventricular margins suggestive of antenatal hemorrhage (Figure 1, F-H). Fundoscopy revealed optic atrophy soon after birth. Abdominal ultrasound was normal. Progressive high-pressure hydrocephalus developed. She died at 11 weeks.

Case 3. This patient (male) presented with asymmetric ventriculomegaly seen on fetal ultrasound at 26 weeks gestation. Fetal MRI showed massive cerebral infarction. At birth (40 weeks' gestation) there were apneas and poor feeding; bilateral small corneas and focal central cataracts were noted. Postnatal CUS and MRI showed severe ventriculomegaly and a small cerebellum (figure 1I). MRI 2 years later showed no change in the degree of tissue loss (figure 1J). Renal ultrasound was normal. He developed cerebral palsy and seizures.

Case 4. This patient (male), born at 37 weeks' gestation, was very growth restricted (weight 1460 gram <<0.4thC and head circumference 27 cm <0.4thC). CUS on postnatal day 1 and MRI showed right-sided ventricular dilatation with a large cyst adjacent to the right lateral ventricle, an area of cavitation in the left temporal lobe and a small cerebellum (figure 1K). Bilateral cataracts were noted.

Results of screening for thrombophilia, maternal infection, and anti-rhesus D antibodies in all patients and their mothers were normal.

RESULTS

In patient 1, a heterozygous c.2545G>T mutation (p.G808V) in exon 31 of *COL4A1* was found. Patient 2 harbors a c.2716 +1G>A splice site mutation in exon 33. In patient 3 a c.3022G>A mutation (p.G1008R) in exon 36 was detected. Patient 4 showed a c.3130G>C mutation (p.G1044R) in exon 37. None of these mutations have been described previously, and they were absent in a panel of 200 ethnically matched control subjects. The three glycine substitutions affect highly conserved G-X-Y collagen repeats[1,2]. All mutations were absent in the parents, suggesting *de novo* events, except for the mother of patient 3, who was found to have a mosaic mutation in the blood. The mutations are located in exons 31, 33, 36 and 37 and are in the same area covering one fifth of the triple helix domain of the collagen IVα1 protein (Figure 2).

DISCUSSION

The *COL4A1* mutations of our patients are associated with severe encephaloclastic lesions, at first sight resembling hydranencephaly. The type and localization of the lesions might suggest a massive MCA territory ischemic stroke, mostly in patients 1 and 3. However, hemosiderin deposits on the ventricular margins from SWI data are more specific for venous

infarction and suggest a venous component to the damage in all patients. The pathogenesis could be a massive germinal matrix and intraventricular hemorrhage followed by extensive venous infarction, with compression, edema and secondary ischemia of large parenchymal areas leading to destruction of the cerebral white matter and cortex and secondary changes in the central gray matter. A similar mechanism could underlie the secondary cerebellar atrophy. However, primary arterial involvement is suggested by the renal agenesis of patient 1 and the previously reported retinal arterial tortuosity and cerebral arterial aneurysms[2,5,6]. Our data show that *COL4A1* testing should also be considered in sporadic severe antenatal hemorrhagic stroke, resembling hydranencephaly.

Acknowledgments

We thank the patients' families for their collaboration. We thank Ruud Koppenol for his help with figure 1 and Ronald Grefhorst for providing us with figure 2.

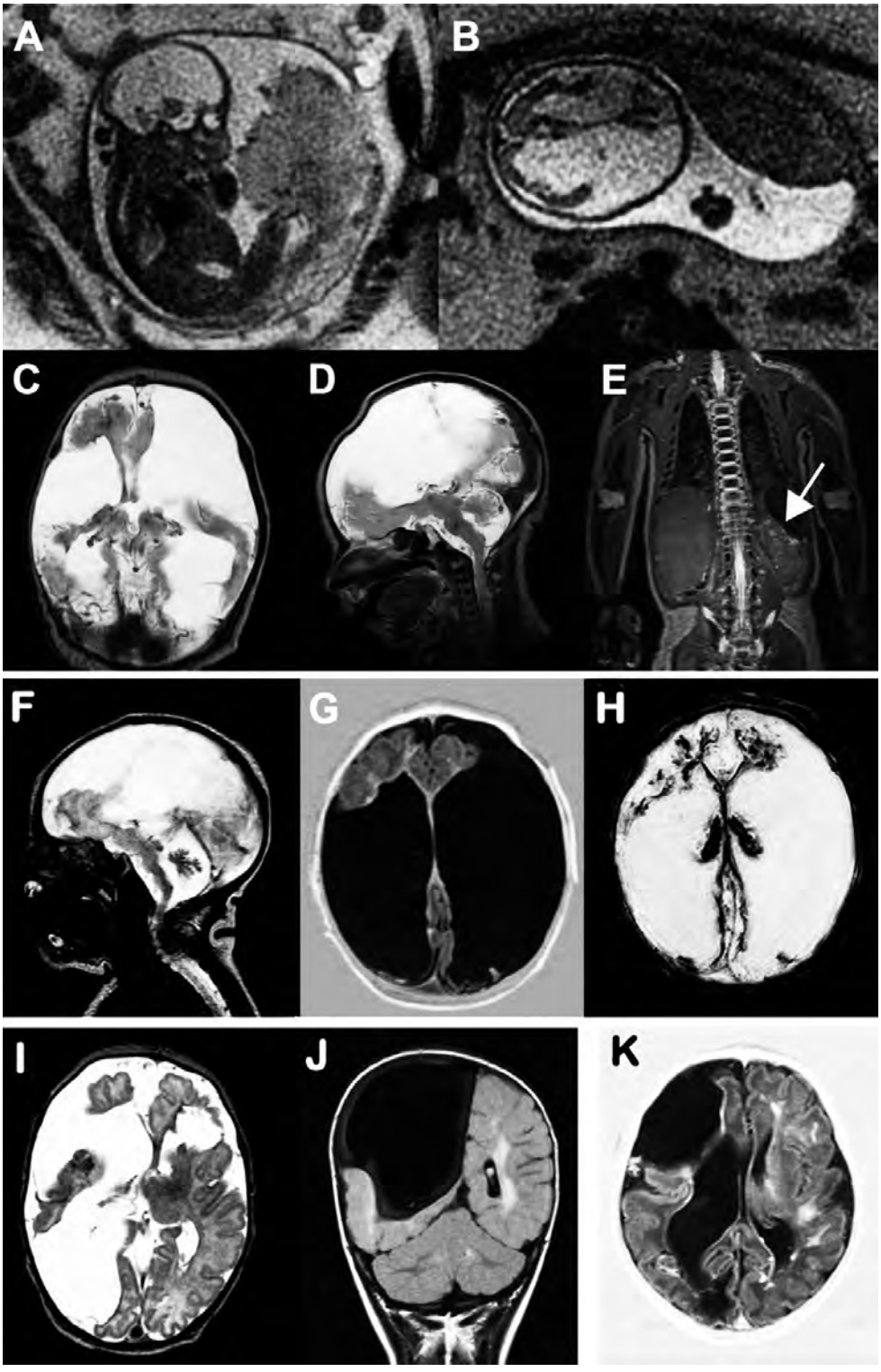

A
B
C
D
E
F
G
H
I
J
K

Figure 1. Magnetic resonance imaging *(opposite page)*

Case 1: Fetal MRI at the 28th gestation week. Sagittal (**A**) and transverse (**B**) T2-weighed image showing massive cerebral infarction extending to the cortex with loss of gray and white matter. (**C, D**) T2-weighed images of post-mortem brain MRI showing severe bilateral ventriculomegaly with extensive gray and white matter loss, tortuosity of the cerebral arteries, and cerebellar and pontine atrophy. (**E**) Thoracic and abdominal imaging showing right-sided renal agenesis and multiple small cystic lesions in the left kidney (arrow).

Case 2: postnatal sagittal (**F**) and transverse (**G**) T1-weighted images showing extensive bilateral infarction as well as severe atrophy of the vermis, pons and brainstem. The areas of low signal intensity on the susceptibility-weighed image (**H**) show remnants of old hemorrhage following the venous distribution of the periventricular white matter.

Case 3: neonatal transverse T2-weighted images (**I**) and coronal fluid-attenuated inversion recovery sequence at age 2 years (**J**), both showing extensive tissue destruction, which is more marked on the right.

Case 4: postnatal transverse inversion recovery images (**K**) showing bilateral porencephaly with cortical destruction. Note the high signal intensity lesions adjacent to the porencephaly and to the lateral ventricles, suggestive of previous hemorrhage.

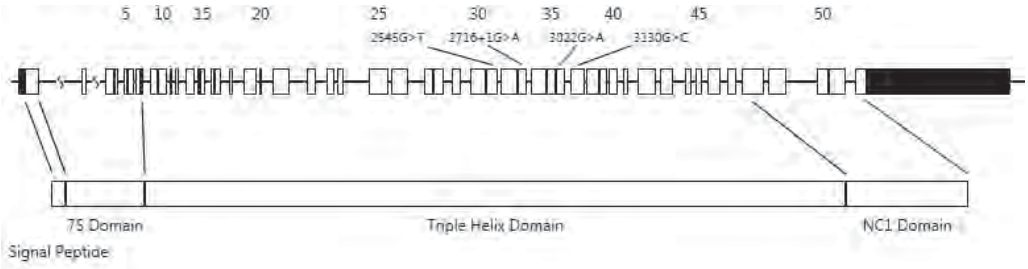


Figure 2. *COL4A1* gene and patient mutations

Schematic representation of *COL4A1* exons (with numbering of the exons), introns and functional domains, including the location of the mutations in our four patients.

References

1. **Gould DB, Phalan FC, Breedveld GJ, et al.** Mutations in COL4A1 cause perinatal cerebral hemorrhage and porencephaly. Science 2005;308:1167-1171.

2. **Gould DB, Phalan FC, van Mil SE, et al.** Role of COL4A1 in small-vessel disease and hemorrhagic stroke. N Engl J Med 2006;354:1489-1496.

3. **Breedveld G, de Coo IF, Lequin MH, et al.** Novel mutations in three families confirm a major role of COL4A1 in hereditary porencephaly. J Med Genet 2006;43:490-495.

4. **Coupry I, Sibon I, Mortemousque B, Rouanet F, Mine M, Goizet C.** Ophthalmological features associated with COL4A1 mutations. Arch Ophthalmol 2010;128:483-489.

5. **Plaisier E, Gribouval O, Alamowitch S, et al.** COL4A1 mutations and hereditary angiopathy, nephropathy, aneurysms, and muscle cramps. N Engl J Med 2007;357:2687-2695.

6. **van der Knaap MS, Smit LM, Barkhof F, et al.** Neonatal porencephaly and adult stroke related to mutations in collagen IV A1. Ann Neurol 2006;59:504-511.

7. **de Vries LS, Koopman C, Groenendaal F, et al.** COL4A1 mutation in two preterm siblings with antenatal onset of parenchymal hemorrhage. Ann Neurol 2009;65:12-18.

Chapter 4.2

NOVEL *COL4A1* MUTATION IN FAMILIAL ANTERIOR SEGMENT DYSGENESIS

Marije E.C. Meuwissen, MD[1], Marjo van der Knaap, MD, PhD[2], L. Ingeborgh van den Born, MD, PhD[3], Rogier Q. Hintzen, MD, PhD[4], Grazia M.S. Mancini, MD, PhD[1]

Manuscript Submitted

1. *Dept. Of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands*
2. *Dept. of Child Neurology, VU Medical Center, Amsterdam, The Netherlands*
3. *The Rotterdam Eye Hospital, Rotterdam, The Netherlands*
4. *Dept. of Neurology, Erasmus University Medical Center, Rotterdam, The Netherlands*

TO THE EDITOR

Anterior segment dysgenesis disorders (ASDD) broadly affect the cornea, lens and iris. Specific clinical entities are Axenfeld-Rieger anomaly and Peters anomaly. Axenfeld Rieger comprises iris hypoplasia, eccentric pupils (corectopia), iris tears (polycoria), posterior embryotoxon and/ or iridocorneal adhesions and a risk of glaucoma Peters anomaly comprises a central absence of the corneal endothelium and Descemet's membrane, leading to congenital corneal opacities[1]. ASDD can be isolated, or associated with systemic problems as part of a syndrome. Both autosomal dominant and autosomal recessive inheritance have been described[1]. We present a family with autosomal dominant ASDD associated with a *COL4A1* mutation, and underscore the importance of the additional neurological investigations in reaching a diagnosis.

We observed a non-consanguineous Dutch family with three affected individuals. The index individual was born after an uneventful pregnancy and had a slightly delayed motor development. She had congenital cataracts with strabismus. Ophthalmological evaluation at the age of 5 years also showed amblyopia and microcornea. At the age of 14 years an embryotoxon posterior and a deep anterior chamber were noted with a nystagmus and exotropia of the left eye. Fundoscopy was normal. At the age of 15 years she underwent cataract extraction of both eyes. Because of visual field loss with normal funduscopy additional investigations were performed. Goldmann perimetry showed tunnel vision, whereas visual evoked potentials and electroretinogram were normal. Due to this discrepancy, cerebral MRI was performed, which showed periventricular leukomalacia (Figure 1a,b,c). Physical examination at the age of 36 years showed joint hyperlaxity, mild pyramidal tract abnormalities comprising generalized brisk deep tendon reflexes with a left-sided Babinski sign and bilaterally decreased coordination skills. Her sister had ophthalmological abnormalities comprising congenital cataract, microcornea, nystagmus but intact peripheral visual fields. She had a psychomotor retardation with congenital left-sided spastic hemiplegia, left-sided hip dyplasia with coxa valga, scoliosis and seizures. In adulthood she developed contractures impairing ambulation. Physical examination at the age of 42 years showed small joint hyperlaxity and a left-sided hemiplegia. The mother is known to have microphthalmia, congenital cataract and secondary glaucoma, for which she received numerous operations (Figure 1d). Later she developed visual field defects with tunnel vision. Physical examination at the age of 74 years showed a soft skin with hyperlaxity of skin and hands, without neurological problems.

Since this combination was previously reported in *COL4A1* mutations, the opthalmological and neurological abnormalities[2], especially the MRI picture of periventricular leukomalacia, prompted us to perform Sanger sequencing of the *COL4A1* gene (NM_001845.4) on DNA isolated from peripheral blood. Written informed consent was obtained. In all three patients, the novel heterozygous *COL4A1* missense mutation c.3200G>C in exon 38 was found, leading to a substitution of glycine into alanine (p.Gly1067Ala). This mutation, located in the conserved triple helix Gly-X-Y repeat domain of the gene, was absent in 100 ethnically matched controls and was predicted to have a damaging effect on the protein function by Polyphen2 and SIFT prediction algorithms.

The COL4A1 and COL4A2 proteins form a heterotrimeric helix that is a major component conferring structural integrity to basement membranes. In the eye it is widely expressed in the basement membranes of the conjunctiva, corneal epithelium and endothelium, Schlemm's canal, lens, retina and vascular endothelia[2,3].

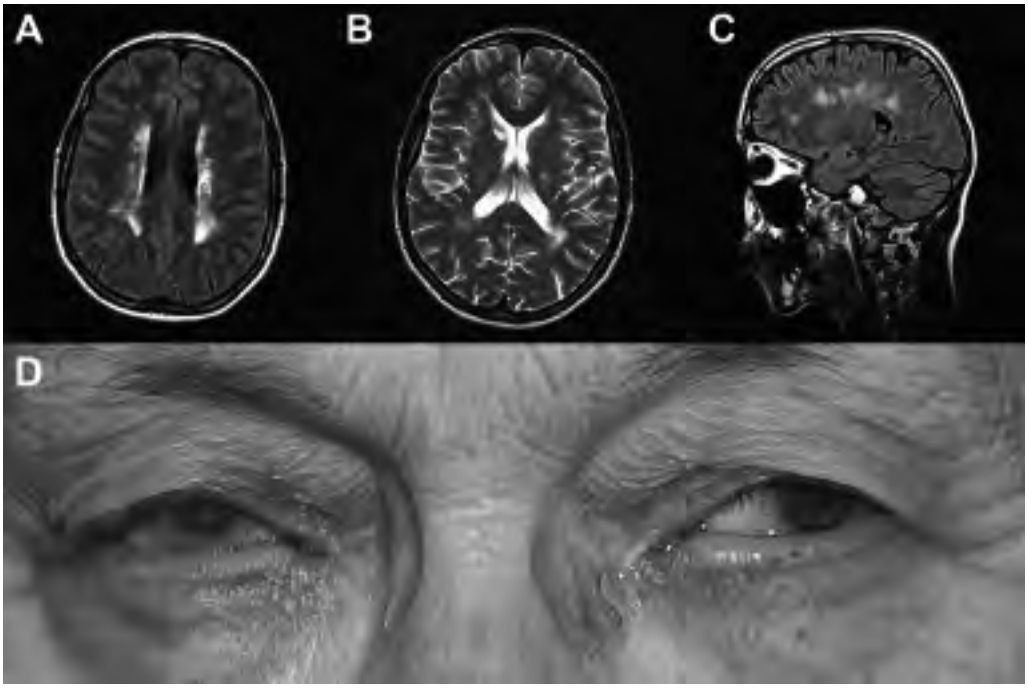


Figure 1. Brain MRI of the index patient (**A,B,C**). FLAIR (**A,C**) and T2- weighed (**B**) images show periventricular leukomalacia. Picture of ocular features of the mother (**D**), with microphthalmia and status after many ocular operations because of congenital cataract and secondary glaucoma.

COL4A1 and *COL4A2* mutations were initially identified in familial hemiplegic porencephaly, but can be associated with different cerebrovascular phenotypes including small vessel disease and stroke. *COL4A1*, and possibly *COL4A2* mutations, can also lead to involvement of other organs, including eye, kidneys, heart and muscles[3]. The presentation can vary even within one family. Ocular findings include ASDD, congenital cataracts, posterior embryotoxon, microcornea and glaucoma, as well as retinal arterial tortuosity and optic nerve hypoplasia. The ocular abnormalities can also vary within one family since *COL4A1* mutations can show a reduced penetrance[2,3]. Our patients represent the severe end of the ocular spectrum with a consistent phenotype among the affected family members.

In this family, the combination of ASDD, neurological disease and brain MRI findings of periventricular leukomalacia is typical of *COL4A1* mutations. It may therefore be useful in the clinical workup of patients with ASDD to perform a neurological examination and possibly a brain MRI. Brain MRI may help in the diagnosis of *COL4A1* mutations, especially since white matter changes and small strokes can be present in absence of neurological symptoms.

The frequency of *COL4A1* and *COL4A2* mutations among patients with ASDD associated with neurological abnormalities and/ or hemorrhagic stroke might be much higher than appreciated up till now. For instance, it has been suggested that up to 6% of sporadic hemorrhagic stroke is related to *COL4A1* and *COL4A2* mutations[4,5]. Therefore, we recommend a thorough family history with special attention for neurological and cerebrovascular disorders in the diagnostic workup of ASDD, in order to find clues for this disorder.

Acknowledgements
This work was financially supported by Fonds NutsOhra.

References

1. **Reis LM, Semina EV.** Genetics of anterior segment dysgenesis disorders. *Curr Opin Ophthalmol* 2011; 22: 314-24.

2. **Coupry I, Sibon I, Mortemousque B, Rouanet F, Mine M, Goizet C.** Ophthalmological features associated with *COL4A1* mutations. *Arch Ophthalmol* 2010; 128: 483-9.

3. **Vahedi K, Alamowitch S.** Clinical spectrum of type IV collagen (COL4A1) mutations: a novel genetic multisystem disease. *Curr Opin Neurol* 2011; 24: 63-8.

4. **Jeanne M, Labelle-Dumais C, Jorgensen J, Kauffman WB, Mancini GM, Favor J, Valant V, Greenberg SM, Rosand J, Gould DB.** COL4A2 mutations impair COL4A1 and COL4A2 secretion and cause hemorrhagic stroke. *Am J Hum Genet* 2012; 90: 91-101.

5. **Weng YC, Sonni A, Labelle-Dumais C, de Leau M, Kauffman WB, Jeanne M, Biffi A, Greenberg SM, Rosand J, Gould DB.** *COL4A1* mutations in patients with sporadic late-onset intracerebral hemorrhage. *Annals of neurology* 2012; 71: 470-7.

Chapter 4.3

IS AUTOSOMAL RECESSIVE INHERITANCE POSSIBLE IN HEREDITARY PORENCEPHALY DUE TO *COL4A1* MUTATIONS?

Marije E.C. Meuwissen[1], Liesbeth S. Smit[2], Atti T. Go[3], Maarten H. Lequin[4], Dicky J. Halley[1], Grazia M.S. Mancini[1]

Manuscript in preparation

1. *Dept. of Clinical Genetics, Erasmus University Medical Center, Rotterdam, The Netherlands*
2. *Dept. of Child Neurology, Erasmus University Medical Center, Rotterdam, The Netherlands*
3. *Dept. of Gynaecology and Prenatal Diagnosis, Erasmus University Medical Center, Rotterdam, The Netherlands*
4. *Dept. of Radiology, Erasmus University Medical Center, Rotterdam, The Netherlands*

TO THE EDITOR

Heterozygous mutations in *COL4A1* have previously been associated with a broad neurological spectrum, ranging from absence of symptoms to severe prenatal disruption as a result of cerebral hemorrhage leading to porencephaly or hydranencephaly[1-4]. Other organs may be affected, including eyes, kidneys, heart and muscles. Ophthalmological findings include anterior segment dysgenesis, retinal arterial tortuosity and cataract[5]. In antenatal porencephaly, most reported *COL4A1* mutations are located in the collagenous triple helix domain, although some affect the non-collagenous NC1 domain[2,6]. The inheritance pattern is autosomal dominant, with reduced phenotypic expression and penetrance. Recently we encountered a patient of consanguineous parents with a phenotype compatible with the severe-end spectrum of *COL4A1* mutations.

The patient is the third child of healthy, consanguineous parents (second degree cousins) of Moroccan ancestry. During pregnancy, ultrasound performed at 22 and 23 gestational weeks (GW) was normal. However, a routine ultrasound at 26 GW showed intracerebral cysts and microcephaly (head circumference <p5). At 28 GW, an increase in cysts was noted, together with an irregular right cerebellar hemisphere and hypercoiling of the umbilical cord. Prenatal MRI at 28 GW confirmed the finding of multiple cysts, including in the cerebellum. At 32 GW, polyhydramnios developed. The girl was born at 40 6/7 GW, and had Apgar scores of 7, 8 and 8 after 1, 5 and 10 minutes. She was microcephalic (-3.5 SD). Brain MRI postpartum showed multiple cerebral and cerebellar (porencephalic) cysts, polymicrogyria, periventricular calcifications and a ventricular dilatation (Figure 1). Opthalmological examination showed a leftsided corneal clouding, a rightsided conjunctival hemorrhage and Rieger anomaly with microcornea, microphthalmus and adhesions between lens and iris. Cardiological evaluation, including EEG and echocardiogram were normal, as well as abdominal ultrasound. She developed a severe psychomotor retardation with bilateral spasticity. Metabolic screening, screening for TORCH and Parvo-B19 infections and anti-thrombocyte antibodies were normal. The porencephaly and opthalmological findings prompted us to test for *COL4A1* or *COL4A2* mutations.

A novel bi-allelic c.4901C>A missense change in exon 51 of *COL4A1* (13q34) was identified. The variant predicts a pAla1634Asp substitution in the noncollagenous NC1 domain, specifically in the VR3 docking site[7]. No mutations at this specific locus have previously been reported, although one (heterozygous) mutation is located just outside the VR3 docking site (c,4881C>G, Asn1627Lys)[8]. The identified mutation was not present in the dbSNP database,

the 1000 Genomes Project or the NHLBI Exome Sequencing Project (ESP), is present in a highly conserved region of the gene among species and is predicted to be pathogenic in Polyphen2, SIFT and Mutation Taster. The parents refused any additional investigation to study segregation. SNP array (Illumina Human CytoSNP 12) showed a female genotype with several large regions of homozygosity (ROH), including a 18.4 Mb region on chromosome 13q31.3q34 encompassing *COL4A1*. The ROH and absence of a deletion strongly suggest a homozygous state of the mutation (the approximately 100 Kb large *COL4A1* locus is covered by multiple SNPs on the used platform). Other genes known to cause resembling phenotypes (*OCLN*, *RNASET2*, *SAMHD1*, *JAM3*) were not located in the other ROH regions. The parents are apparently healthy, the family history is negative for cerebrovascular, ocular or renal disorders.

The patient's phenotype, comprising severe cerebral and cerebellar multicystic porencephaly, microcephaly and calcifications, together with corneal clouding and Rieger anomaly, fit within the severe end of the clinical spectrum associated with *COL4A1* mutations. Polymicrogyria, as seen in our patient, is a developmental disorder of the cerebral cortex of presumable vascular origin and belongs to the spectrum of schizencephaly observed in *COL4A1* mutations[9,10].

In addition, the reported mutation is the first *COL4A1* mutation located in the VR3 docking site of the *COL4A1* NC1 domain. The collagen NC1 domain has an important function in the initiation of triple helix formation. In this process, three NC1 domains (two *COL4A1* and one *COL4A2*) are stabilized through tight and selective interactions between two sites, comprising a beta-hairpin motif and its docking site with a variable region (VR3). This VR3 is a 15-residue-long variable region with sequence hypervariability across all the chains of collagen IV, which is thought to be essential in directing chain recognition in the trimerization process[11,12], allowing the specific binding of i.e. *COL4A1* and *COL4A2*. Since no mutations in this VR3 docking site have been reported[7], also not in the highly homologous genes *COL4A3*, *COL4A4*, *COL4A5* or *COL4A6*, nothing is known about the pathogenic effect of VR3 mutations and the mode of inheritance. It can be postulated that a VR3 mutation, especially in a homozygous form, will disturb chain recognition, trimerization and NC1 trimer stabilization of *COL4A1* and *COL4A2*.

Both autosomal dominant and autosomal recessive inheritance has been described for *COL4A3* and *COL4A4* mutations involved in Alport syndrome. Although mutations in

the collagenous triple helix domain are the most frequent cause of (both autosomal recessive and autosomal dominant) Alport disease caused by *COL4A3* and *COL4A4* mutations, mutations in the NC1 domain, however not in its VR3 docking site, have also been associated with both recessive and dominant forms. In the recessive cases, homozygous mutation carriers display a severe phenotype, with early onset renal failure and deafness, while heterozygous mutation carriers can be mildly affected , or even be asymptomatic. The lack of phenotype in the parents of our patient is in line with these observations[13-16].

In conclusion, we observed a putatively pathogenic homozygous *COL4A1* variant affecting the VR3 docking site of the *COL4A1* NC1 domain in a patient with severe porencephaly, polymicrogyria, corneal clouding, Rieger anomaly and microcephaly. The open question remains whether this is the cause of the disease and whether autosomal recessive inheritance is a general mechanism in *COL4A1*, besides *COL4A3* and *COL4A4* mutations. Additional cases are needed to make a definite statement regarding this question, which is very important regarding genetic counselling of families.

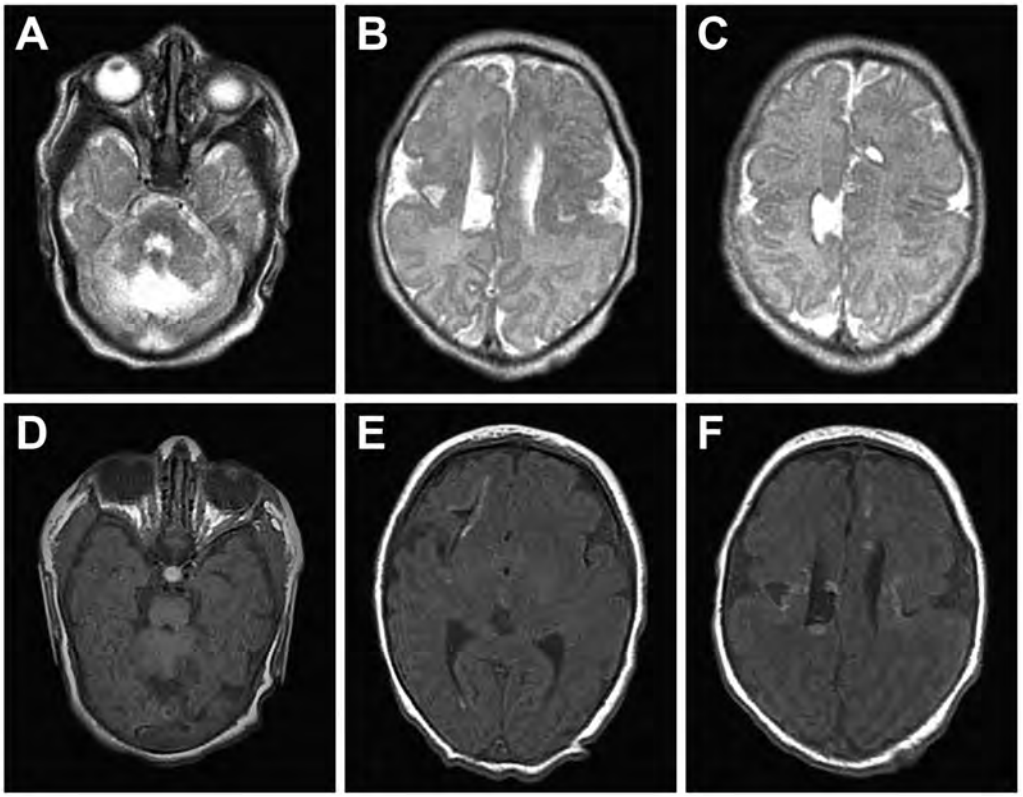


Figure 1.

A. T2-weighed image, showing cerebellar cysts and destruction.

B and C. T2-weighed images depicting multiple cerebral (porencephalic) cysts and leftsided polymicrogyria (arrow). **D.** T1-weighed image demonstrating a normal pons.

E and F. T1-weighed image with multiple cerebral cysts, surrounded by calcification, and white matter calcifications.

References

1. **Breedveld G, de Coo IF, Lequin MH, et al.** Novel mutations in three families confirm a major role of COL4A1 in hereditary porencephaly. *J Med Genet.* Jun 2006;43(6):490-495.

2. **de Vries LS, Koopman C, Groenendaal F, et al.** COL4A1 mutation in two preterm siblings with antenatal onset of parenchymal hemorrhage. *Annals of neurology.* Jan 2009;65(1):12-18.

3. **Gould DB, Phalan FC, Breedveld GJ, et al.** Mutations in COL4A1 cause perinatal cerebral hemorrhage and porencephaly. *Science.* May 20 2005;308(5725):1167-1171.

4. **Meuwissen ME, de Vries LS, Verbeek HA, et al.** Sporadic COL4A1 mutations with extensive prenatal porencephaly resembling hydranencephaly. *Neurology.* Mar 1 2011;76(9):844-846.

5. **Coupry I, Sibon I, Mortemousque B, Rouanet F, Mine M, Goizet C.** Ophthalmological features associated with COL4A1 mutations. *Arch Ophthalmol.* Apr 2010;128(4):483-489.

6. **Bilguvar K, DiLuna ML, Bizzarro MJ, et al.** COL4A1 mutation in preterm intraventricular hemorrhage. *The Journal of pediatrics.* Nov 2009;155(5):743-745.

7. **Parkin JD, San Antonio JD, Pedchenko V, Hudson B, Jensen ST, Savige J.** Mapping structural landmarks, ligand binding sites, and missense mutations to the collagen IV heterotrimers predicts major functional domains, novel interactions, and variation in phenotypes in inherited diseases affecting basement membranes. *Hum Mutat.* Feb 2011;32(2):127-143.

8. **Rodahl E, Knappskog PM, Majewski J, et al.** Variants of anterior segment dysgenesis and cerebral involvement in a large family with a novel COL4A1 mutation. *American journal of ophthalmology.* May 2013;155(5):946-953.

9. **Barkovich AJ, Guerrini R, Kuzniecky RI, Jackson GD, Dobyns WB.** A developmental and genetic classification for malformations of cortical development: update 2012. *Brain : a journal of neurology.* May 2012;135:1348-1369.

10. **Yoneda Y, Haginoya K, Kato M, et al.** Phenotypic Spectrum of COL4A1 Mutations: Porencephaly to Schizencephaly. *Annals of neurology.* Jan 2012;73(1):48-57.

11. **Khoshnoodi J, Cartailler JP, Alvares K, Veis A, Hudson BG.** Molecular recognition in the assembly of collagens: terminal noncollagenous domains are key recognition modules in the formation of triple helical protomers. *J Biol Chem.* Dec 15 2006;281(50):38117-38121.

12. **Khoshnoodi J, Pedchenko V, Hudson BG.** Mammalian collagen IV. *Microsc Res Tech.* May 2008;71(5):357-370.

13. **Boye E, Mollet G, Forestier L, et al.** Determination of the genomic structure of the COL4A4 gene and of novel mutations causing autosomal recessive Alport syndrome. *Am J Hum Genet.* Nov 1998;63(5):1329-1340.

14. **Cook C, Friedrich CA, Baliga R.** Novel COL4A3 mutations in African American siblings with autosomal recessive Alport syndrome. *Am J Kidney Dis.* May 2008;51(5):e25-28.

15. **Zhang Y, Wang F, Ding J, et al.** Genotype-phenotype correlations in 17 Chinese patients with autosomal recessive Alport syndrome. *Am J Med Genet A.* Sep 2012;158A(9):2188-2193.

16. **Fallerini C, Dosa L, Tita R, et al.** Unbiased next generation sequencing analysis confirms the existence of autosomal dominant Alport syndrome in a relevant fraction of cases. *Clinical genetics.* Sep 4 2013.

Chapter 5

COL4A2 MUTATION ASSOCIATED WITH FAMILIAL PORENCEPHALY AND SMALL-VESSEL DISEASE

Elly Verbeek[1,8], Marije EC Meuwissen[1*], Frans W Verheijen[1*], Paul P Govaert[2], Daniel J Licht[3], Debbie S Kuo[4], Cathryn J Poulton[1], Rachel Schot[1], Maarten H Lequin[5], Jeroen Dudink[2], Dicky J Halley[1], René IF de Coo[6], Jan C den Hollander[7], Renske Oegema[1], Douglas B Gould[4] and Grazia MS Mancini[1*]

European Journal of Human Genetics 2012; 20 (8): 844-851

1. *Dept. of Clinical Genetics, Erasmus University Medical Center, Rotterdam.*
2. *Dept. of Neonatology, Erasmus University Medical Center, Rotterdam.*
3. *Children's Hospital and University of Pennsylvania Medical School, Philadelphia*
4. *Departments of Ophthalmology and Anatomy, Institute for Human Genetics University of California, San Francisco*
5. *Dept. of Radiology, Erasmus University Medical Center, Rotterdam.*
6. *Dept. of Child neurology, Erasmus University Medical Center, Rotterdam.*
7. *Dept. of Pathology, Erasmus University Medical Center, Rotterdam.*
8. *This work is dedicated to Elly Verbeek who sadly passed away during the preparation of the manuscript.*

* *These authors equally contributed to this manuscript.*

ABSTRACT

BACKGROUND. Familial porencephaly, leukoencephalopathy and small-vessel disease belong to the spectrum of disorders ascribed to dominant mutations in the gene encoding for type IV collagen alpha-1 (*COL4A1*). Mice harbouring mutations in either *Col4a1* or *Col4a2* suffer from porencephaly, hydrocephalus, cerebral and ocular bleeding and developmental defects. We observed porencephaly and white matter lesions in members from two families that lack *COL4A1* mutations.

METHODS. We hypothesized that *COL4A2* mutations confer genetic predisposition to porencephaly, therefore we sequenced *COL4A2* in the family members and characterized clinical, neuroradiological and biochemical phenotypes.

RESULTS. Genomic sequencing of *COL4A2* identified the heterozygous missense G1389R in exon 44 in one family and the c.3206delC change in exon 34 leading to frame shift and premature stop, in the second family.

Fragmentation and duplication of epidermal basement membranes were observed by electron microscopy in a c.3206delC patient skin biopsy, consistent with abnormal collagen IV network. Collagen chain accumulation and ER stress have been proposed as cellular mechanism in *COL4A1* mutations. In $COL4A2^{3206delC}$ fibroblasts we detected increased rates of apoptosis under stress conditions. Mutation phenotypes varied including porencephaly, white matter lesions, cerebellar and optic nerve hypoplasia and unruptured carotid aneurysm. In the second family however, we found evidence for additional factors contributing to the phenotype.

CONCLUSION. We conclude that dominant *COL4A2* mutations are a novel major risk factor for cerebrovascular disease, including porencephaly and small-vessel disease with reduced penetrance and variable phenotype, which might be also modified by other contributing factors.

INTRODUCTION

Porencephaly is defined as a cavity in the cerebral parenchyma communicating with the lateral ventricles, which results from destruction of the parenchyma after a developmental defect or an intracerebral haemorrhage. Porencephaly has both genetic and non-genetic causes. Mutations in the *COL4A1* gene, coding for the procollagen alpha-1 chain of collagen IV, have been associated with variable phenotypes. *COL4A1* mutations predispose to germinal matrix haemorrhage in late pregnancy or around birth, which can lead to deep venous infarction with subsequent tissue necrosis and porencephalic cavitation. When the haemorrhage occurs during gestation or around birth it usually manifests as congenital hemiplegia[1-5]. Familial porencephaly with congenital hemiplegia has been ascribed to autosomal dominantly inherited mutations in the *COL4A1* gene. Although a number of rare monogenetic disorders may predispose to intracerebral haemorrhage, such as CADASIL, Fabry's disease, autosomal dominant amyloid angiopathy, MELAS, homocystinuria, Ehlers-Danlos syndrome type IV, Marfan and Maeda syndrome[1-2,5], only mutations of *COL4A1* have been associated with familial porencephaly[3]. The phenotypic manifestation of *COL4A1* mutations is however quite variable both within and among families, with different presentations in children or adults. As well as porencephaly, *COL4A1* mutations predispose to a variety of vascular lesions in the brain and other organs, leading to the conclusion that *COL4A*1 mutation causes a multisystem disease[6]. The cerebral lesions vary from deep intracerebral haemorrhage, to diffuse leukoencephalopathy, or small-vessel disease with deep lacunar infarcts, dilated perivascular spaces, silent microbleeds or (unruptured) intracranial aneurysms. In mutation carriers symptoms are variable, including congenital hemiplegia, transient ischaemic attack, adult onset haemorrhagic stroke, rarely brain infarction, and may even be absent[7-10]. At the severe end of the spectrum, massive prenatal bleeding leading to apparent hydranencephaly, renal agenesis and early demise have been described[11]. Other organs can also be affected. Ocular features can be associated with *COL4A1* mutations such as retinal arteriolar tortuosity, ocular cataract, anterior segment dysgenesis and microcornea[9,12-15]. Other manifestations are cardiac arrhythmia, renal cysts, haematuria, hepatic cysts, muscle cramps and elevated creatine kinases. The HANAC syndrome (hereditary angiopathy, nephropathy, aneurysms and muscle cramps) has been specifically associated with mutations in exons 24 and 25 of the *COL4A1* gene[8,16]. *COL4A1* mutations are often described within a familial context, but can also occur *de novo*, without a family history of bleeding[11,17]. Although heterogeneous, the common association of the

symptoms has led to the definition of general criteria for consideration of *COL4A1* genetic testing[6]. The identification of a familial *COL4A1* mutation helps to monitor complications and supports genetic counselling.

Non-fibrillary collagen IV is a major component of the extracellular matrix and basement membranes and consists of six alpha chains, encoded by distinct and tandemly located genes respectively on chromosome 13q (*COL4A1* and *COL4A2*), 2q (*COL4A3* and *COL4A4*) and Xq (*COL4A5* and *COL4A6*). *COL4A3*- to *6* mutations all cause Alport syndrome and benign familial haematuria. *COL4A1* and *COL4A2* are under regulation of the same promoter and encode for the procollagen IV alpha-1 and IV alpha-2 chains, which assemble to form a heterotrimeric helix with a constant 2:1 ratio, [alpha1(IV)]$_2$[alpha2(IV)]. Among collagen IV genes, only *COL4A1* and *COL4A2* are ubiquitously expressed[18]. This explains why the basement membranes of several epithelia and endothelia can be affected by a *COL4A1* mutation. Mutations in the human *COL4A1* gene were originally identified after description of the prenatal cerebral and ocular bleeding in mice bearing mutations in the homolog *Col4a1* gene[3]. Because of the common function, mutations in the collagen IV alpha-2 gene were supposed to cause similar manifestations and in fact, mice bearing heterozygous *Col4a2* missense mutations survive the postnatal period but show haemorrhage in the eye, brain, and skin and developmental defects of eye and brain[19] similar to *Col4a1* mutants. We observed porencephaly, white matter lesions suggestive of small-vessel disease and aneurysms in members of two families, fulfilling the criteria for *COL4A1* testing, but without evidence for *COL4A1* mutation. We therefore explored by a candidate gene approach the involvement of *COL4A2* and its potential role in human disease.

SUBJECTS/MATERIALS AND METHODS

Patient study

All tested individuals and legal caretakers gave their informed consent for the study, according to the Dutch local ethical committee requirements. MRI (magnetic resonance imaging) and analysis of DTI (diffusion tensor imaging) data were performed according to Lobel *et al*[20-22]. Transmission electron microscopy of the skin biopsies was performed in double blind experiments and independently scored by two investigators[8].

Genomic *COL4A1* sequence analysis was according to Breedveld *et al*[7]. Primers were designed and analysis performed for the genomic sequence of *COL4A2*. RT-PCR was performed as described[21]. RT-qPCR was performed using KAPA sybr fast qPCR mix (ABI prism) and the CFX96 Real Time System (Biorad). Skin fibroblast culture and test for ER stress was conducted by Western Blot and RT-qPCR according to Lin *et al*[23]. Staining for the collagen specific chaperon HSP47 and the ER stress marker KDEL[26] were performed under standard culture condition and after stress induced by DTT in control and patient fibroblasts. A fluorescent kit for multiple active caspases was used (FlicaTM apoptosis detection kit, Immunochemistry Technologies, LLC). For additional details see the Supplementary data.

RESULTS

Patient description (Clinical details are summarized in Table 1.)

Family A (Fig. 1A) The proband III-2 was the first of this Caucasian family to present at the age of 6 months with a right-sided hemiparesis. Her prenatal course was complicated by oligohydramnion. Brain MRI at the age of 3 years and 8 months showed left-sided porencephaly and right-sided periventricular white matter hyperintense lesions on T2 weighted images. At the age of 4.5 years she has a moderate learning disability. Eye fundoscopy is normal. (Fig. 2A, B).

Subject III-1 was born at 38 weeks of a normal gestation and developed normally. A brain MRI at the age of 8 years revealed patchy white matter lesions. Eye fundoscopy is normal (Fig. 2C).

The mother (II-2) is asymptomatic at the age of 31 years. A brain MRI was normal, while MRA revealed bilateral internal carotid artery aneurysms at the level of the cavernous sinus (Fig. 2D). Subjects II-4, II-5 and I-1 are all reported to have a right-sided hemiparesis. No medical records are available.

Family B (Fig. 1B) This consanguineous family of Afghani ancestry includes two probands (II-2 and II-5).

Proband II-2 was born at term of a normal gestation with a birth weight of 2500 g (-3 SD).

At the age of six months a right-sided hemiparesis was noted. At the age of 16 years he has an intelligence quotient of 48, a dystrophic appearance with microcephaly and a right-sided dystonic paresis. Brain MRI revealed left-sided cerebral atrophy with porencephaly and Wallerian degeneration of the brain stem (Fig. 2E, K, L, M). Ophthalmologic examination showed severe myopia with bilateral amblyopia and small optic discs, but no arteriolar tortuosity. Proband II-5's prenatal course was complicated by maternal diabetes and intrauterine growth retardation. A caesarian section was performed at 31 weeks of gestation because of fetal distress. Cerebral ultrasound at birth showed a right-sided subependymal bleeding with venous infarction. At 6 months brain MRI revealed a porencephalic cyst and hypoplastic left cerebellar hemisphere (Fig. 2F, G). Physical examination at the age of 1 year showed a left-sided hemiparesis and borderline microcephaly (-2 SD). Ophthalmologic examination, renal ultrasound and echocardiogram were normal. Screening for coagulopathies in the neonatal period showed a suspected protein S deficiency, but no analysis was repeated afterwards. The mother (I-2) has hypothyroidism. She was small at birth and had feeding difficulties. Ophthalmologic examination showed high myopia, astigmatism and tilted optic discs with some peripapillary atrophy. Permission for brain imaging was denied. She also delivered two daughters who died of complicated congenital hydrocephalus (subjects II-1 and II-4). Brain MRI of one daughter (II-4) at the age of 2 months showed extreme hydrocephalus with periventricular cystic lesions, stenosis of the Sylvius aqueduct and periventricular, subcortical and cerebellar calcifications (supplementary Fig. S1). Another son (subject II-3) is mildly intellectually disabled with a total intelligence quotient of 62, a normal head circumference and normal brain MRI. Her last pregnancy was uneventful, was monitored by ultrasound and ended with the birth of a third daughter (subject II-6) in the 38th week. At birth cerebral ultrasound revealed a small intraparenchymal bleeding. The girl developed a mild hemiparesis, milder than the probands; her brain MRI revealed a small right-sided cerebral intraparenchymal cyst (supplementary Fig. S2). Extensive coagulopathy screening at age of 2 years revealed a heterozygote protein S deficiency.

Sequence analysis and expression data

COL4A1

DNA sequence of the 52 coding exons and intron-exon boundaries of *COL4A1* in subjects III-1 and III-2 of family A and subjects II-5 and I-1 of family B was normal[7]. A maximum number of 13 heterozygote SNPs were observed in the sequence of each individual, which excludes the presence of large deletions in one of the *COL4A1* alleles.

COL4A2

Family A. DNA sequencing of part of the 5' UTR and the 48 exons and intron-exon boundaries of *COL4A2* was performed in subjects III-1, III-2, III-3 and II-2. In patient III-2, III-1 and II-2 a heterozygous missense change, c.4165G>A in exon 44, leading to an amino acid substitution of glycine by arginine at position 1389 (p.G1389R) was found (Fig. 1C). *COL4A2* was normal in patient III-3. Glycine 1389 is part of the Gly-Xaa-Yaa repeat, which is essential for the proper folding of the triple helix[18,24], and is highly conserved through species and among all the six collagen IV genes. In silico analysis by Polyphen-2 and SIFT prediction programs indicated this change as probably damaging.

Family B. Subjects II-2, II-3, II-5, II-6, I-1 and I-2 were tested. In probands II-2, II-5 and subject I-2 *COL4A2* sequencing showed a heterozygous c.3206delC deletion in exon 34 leading to a frameshift and premature stop at position +27 (Fig. 1D). The *COL4A2* sequence was normal in subjects I-1, II-3 and II-6. No DNA was available from patient II-1 and II-4. RT-PCR of the *COL4A2* transcript in patient II-2's fibroblasts was performed. Only a transcript of the expected wild-type sequence was observed, compatible with degradation of the mutant mRNA by nonsense-mediated decay. Quantitative RT-PCR (RT-qPCR) of *COL4A2* and *COL4A1* transcript in fibroblasts from the same patient was performed and confirmed diminished amount of *COL4A2* compared to *COL4A1* mRNA (Fig.1E).

Skin transmission electron microscopy

Electron microscopy of the skin of patient II-2 from family B showed abnormalities of the epidermis-dermis junction with scattered thickening, blurring and duplications of the basement membrane (Fig. 3 B,C). These abnormalities were not observed in control skin (Fig. 3A) and are similar to those seen in *COL4A1* patients[8].

Test for ER stress response and apoptosis in cells with *COL4A2*^{3206delC} mutation

The mouse *Col4a1*$^{+/\Delta\ ex40}$ mutation leads to synthesis of abnormal Col4a1 chains, which accumulate in the endoplasmic reticulum (ER) of lens epithelia. This accumulation activates the unfolded protein response (UPR), a well-known ER stress response preceding apoptosis[20,25-26]. Since the c.3206delC *COL4A2* mutation predicts a null-allele and no such mutations have been described in human *COL4A1* patients, we investigated the pathogenic mechanism of this mutation in cultured skin fibroblasts from proband II-2. We wondered

whether ER stress could be induced by abnormal stoichiometry of COL4A1 and COL4A2 chains, because of reduced COL4A2 synthesis.

ER stress response was analysed by staining fibroblasts with antibodies anti HSP47, a collagen-specific chaperon protein, and anti KDEL, an ER stress marker specific for ER-retained proteins. If collagen chains accumulate in patient cells, then increased storage of HSP47 can be expected[25,26] . Under standard culture conditions, HSP47 staining was comparable in patient and control cells, suggesting no accumulation of unfolded collagen chains (Supplementary Fig. S3) . Subsequently, we analyzed whether KDEL staining under standard and stress conditions, induced by DTT, would detect ER stress in patient cells. Under the DTT condition, we know that normal cells slightly increase susceptibility to apoptosis from 10 to 18% Fig 4C and Supplementary Fig S5)[37]. No clear signs of ER stress were detected by KDEL staining under DTT in the patient cells (Supplementary Fig S4)[26]. We then tested whether signs of expression of ER stress markers could be detected by western blot analysis using antibodies specific for IRE1, cleaved form of ATF6, CHOP, BiP and by real time-PCR for XBP-1 splicing[23]. We did not detect expression of the markers, in the patient cells, similarly to a control cell line (Fig. 4A,B). As positive control, tunicamycin-treated control cells showed increased expression of markers, which means that the stress response was negative rather than below detection level. Since ER stress is supposed to trigger apoptotic cell death, we studied sensitivity of the cells to apoptosis under standard conditions and stress stimuli. Under stress induced by DTT, we observed an abnormally high percentage (>60%) of apoptotic cells in the patient compared to five control cell lines (Fig. 4C). No difference was observed in the absence of DTT treatment (Supplementary Fig S5). We conclude that *COL4A2*^{3206delC} fibroblasts are more susceptible to apoptosis under stress condition, *in vitro*.

DISCUSSION

Pathogenesis of *COL4A2* mutations

Using the knowledge of the *Col4a2* mouse model, we have used a candidate gene approach in our porencephaly families, and we provide evidence that *COL4A2* mutations represent a novel genetic risk factor for haemorrhagic porencephaly and small vessel disease.

We describe cerebral abnormalities including porencephaly, small-vessel disease in the form of scattered white matter lesions, carotid aneurysm, cerebellar and eye abnormalities

in two families, associated with two dominantly inherited changes in *COL4A2* (G1389R, c.3206delC), all in the triple helix domain of the procollagen IV alpha-2 protein. Carotid aneurysm and cerebellar hypoplasia have been observed in a single mutation carrier respectively in family A and B (Table 1), making a causal relationship possible but not definitive.

Extensive data from several types of collagens have shown that missense and splice site mutations in the triple helix domain are highly pathogenic and particularly substitutions of the glycine at each third position (G-Xaa-Yaa) disrupt the triple helix structure, with a dominant negative effect. These missense mutations lead to abnormal assembly of the triple helix with intracellular accumulation of procollagen chains[26]. The stereotypical nature of pathogenic glycine missense mutations in collagens makes therefore mutation recognition relatively easy compared to other genes. Considering the cosegregation of the G1389R mutation with the cerebrovascular disease we conclude that this mutation is involved in the pathogenesis of the porencephaly and white matter lesions observed in family A.

The c.3206delC frame-shift mutation found in family B leads to loss of COL4A2 chain synthesis from one allele, however the pathogenic mechanism might be different from the G-Xaa-Yaa type of mutation, as it is predicted to cause a loss of function and null-alleles have not been described in patients harbouring *COL4A1*-mutations. In support of a pathogenic effect of the c.3206delC mutation, we observed an increased susceptibility to apoptosis and, in addition, EM of patient skin showed abnormalities of the epidermal basement membranes, suggesting abnormal extracellular collagen-IV synthesis. Heterozygote truncating mutations have been already observed in collagen IV-related Alport syndrome, but null-alleles for either the C.elegans *COL4A1* homologue, *emb-9* and *COL4A2* homologue *let2*, cause disruption of the basement membrane and embryonic lethality although at a later stage than the G-Xaa-Yaa missense mutations[27].

The non-collagen (NC1) domain of collagen IV alpha-2, but not alpha-1 chain, promotes cell adhesion, outgrowth of embryonic neurons and is responsible for strict regulation of the 2:1 stoichiometry of the triple helix[18,28-30].

This could indicate that a disturbed ratio of procollagen alpha-1 and procollagen alpha-2 chains due to a heterozygous null allele of either *COL4A1* or *COL4A2* may lead to a disturbed or insufficient heterotrimer assembly and secretion.

The mechanism leading to stroke and porencephaly is presumably a decreased resistance of the vascular wall to increased mechanic stimuli. In the lens epithelia of *Col4a1*$^{+/\Delta ex40}$ mice accumulation of COL4A1 and COL4A2 in the endoplasmic reticulum (ER) induces an ER stress response, a known trigger of apoptosis[26]. Nevertheless, studies supporting this mechanism in human *COL4A1* mutations are lacking. We observed increased susceptibility to apoptosis of c.3206delC patient fibroblasts, possibly related to ER stress, which anyhow could not be detected under our experimental conditions. Future studies are needed to address this discrepancy, which might be caused for example by the culture conditions compared to experiments performed in mouse tissues[20,25]. Apoptosis is an important mechanism regulating brain development and is involved in the onset of microcephaly[31]. Some of the *COL4A2* patients are born microcephalic and the reason is not apparent at the moment, but it could be related to susceptibility of the developing brain to apoptosis[37]. It is also possible that, independently of the ER stress, *COL4A2* and the apoptotic pathways are linked since they are developmentally co-regulated. In fact, it has been shown that micro RNA mir-29b represses expression of both *COL4A2* and antiapoptotic Bcl-2 family member Mcl-1 during skeletal development[32-33].

COL4A2 mutation: phenotypic variation and reduced penetrance

COL4A1 and *COL4A2* are ubiquitously co-expressed in the basement membrane of epithelia and endothelia and from the observation of mouse *Col4a1* and *Col4a2* mutations, human *COL4A1* and *COL4A2* mutations could be predicted to result in cerebral bleeding. However, only *COL4A1* mutations have been identified after linkage in large pedigrees[3]. One of the reasons could be the difficulty to recruit large families with similarly affected patients, because of phenotypic variability or reduced penetrance of *COL4A2* mutations, similarly to other causes of stroke and aneurysm[34], which makes linkage analysis quite difficult.

We can assume reduced penetrance of the *COL4A2* mutations in both our families. In family A subject II-2 has the G1389R mutation and is asymptomatic, but MRI revealed a carotid aneurysm, similar to the findings in an obligate carrier of *COL4A1* mutation that we previously described[7]. In family B, we also observed an asymptomatic carrier (subject I-2) of the c.3206delC *COL4A2* mutation, who presents ocular anomalies but denied an MRI. Asymptomatic carriers have been observed also in *COL4A1* mutation and although this has been interpreted as reduced penetrance of the mutation, extensive investigation has

often revealed hidden signs of the disease[3, 7]. However, considering the wide heterogeneity of symptoms, the contribution of other factors, besides the *COL4A1* mutation, has been suggested as trigger of the pathogenic event[3].

In both our families, a wide intra-familial phenotypic variation has been observed, similar to *COL4A1* mutations[7]. In *Col4a1* mutant mice it has been shown that the genetic background is important to determine the phenotype, probably through the effect of modifying loci[26]. *Col4a2* mutant mice present with cerebral porencephaly, microphthalmia, lens opacity, buphthalmos and renal anomalies, but in general with a milder phenotype compared to *Col4a1* deficient mice[19].

Interestingly, in family B we observed an individual (subject II-6), affected by a perinatal intraparenchymal bleeding leading to a cyst, who lacks the familial *COL4A2* mutation (Supplemental figure S2) and additional children died at young age with severe cerebral hemorrhage and hydrocephalus, who were not tested for *COL4A2* mutations. Subject II-6 is heterozygote for protein S deficiency, which in itself has not been the cause of the bleeding but might have contributed to the onset of the germinal matrix haemorrhage. As for *COL4A1* mutation carriers, it is therefore possible that in this family additional factors have summed up and contribute to the phenotypic manifestation.

The particular fragility of the cerebral vessels in patients with either *COL4A1* or *COL4A2* mutations remains unexplained in light of the ubiquitously expression of *COL4A1* and *COL4A2*. However, it could reflect selective expression in subtypes of brain vascular endothelia[4]. It has been shown that in the kidney expression of *COL4A3*, *COL4A4* and *COL4A5* genes can be cell-type specific[35-36].

In summary, we observed that familial *COL4A2* mutations could lead to a wide spectrum of cerebrovascular disorders including porencephaly, white matter lesions and possibly aneurysm and cerebellar hypoplasia, which might go undiagnosed because of reduced penetrance and variable expression. Additional risk factors may influence the course of cerebrovascular diseases in families with *COL4A2* mutations.

Acknowledgments
The authors thank Dr. Hajo Wildschut for prenatal patient care, Dr. Marion Smit, Ton de Jong, Ronald Grefhorst, Tom de Vries-Lentsch and Ruud Koppenol for logistic and technical support.

Table 1. Summary of clinical features

	Family A			Family B		
Clinical features	**III.1**	**III.2**	**II.2**	**II.2**	**II.5**	**I.2**
Sex	F	F	F	M	M	F
Birth (gestational week)	38	40	u	40	31	u
Birth weight	u	u	u	2500 g	1045 g	low
Age at investigation	8 years	4.3 years	31 years	16 years	1 year	36 years
Growth parameters						
Head circumference (cm)	0 SD	−1 SD	u	−3 SD	−2 SD	−1.5 SD
Height (cm)	−1.5 SD	0 SD	u	−2.3 SD	−1.5 SD	−2.5SD
Weight (kg)	−0.5 SD	0 SD	u	−4 SD	−3 SD	u
Developmental delay	−	+	−	+	+, mild	−
Feeding problems	−	u	−	+	+	+
Neurological signs	−	hemiparesis	−	dystonic hemiparesis	hemiparesis	−
Brain MRI findings						np
Porencephaly	−	+	−	+	+	
Periventricular leukoencephalopathy	+	+	−	−	−	
Cerebellar hypoplasia	−	−	−	−	unilateral	
Cerebral atrophy	−	−	−	unilateral	unilateral	
Vascular	−	−	bilateral ICA aneurysms	−	−	
Ophthalmological signs			np			
Small / tilted optic discs	−	−		+	−	+
Myopia	−	−		+	−	+
Amblyopia	−	−		+	−	−
Renal ultrasound	np	np	np	np	normal	np

u = unknown, np = not performed, + present, − absent, ICA = internal carotid artery

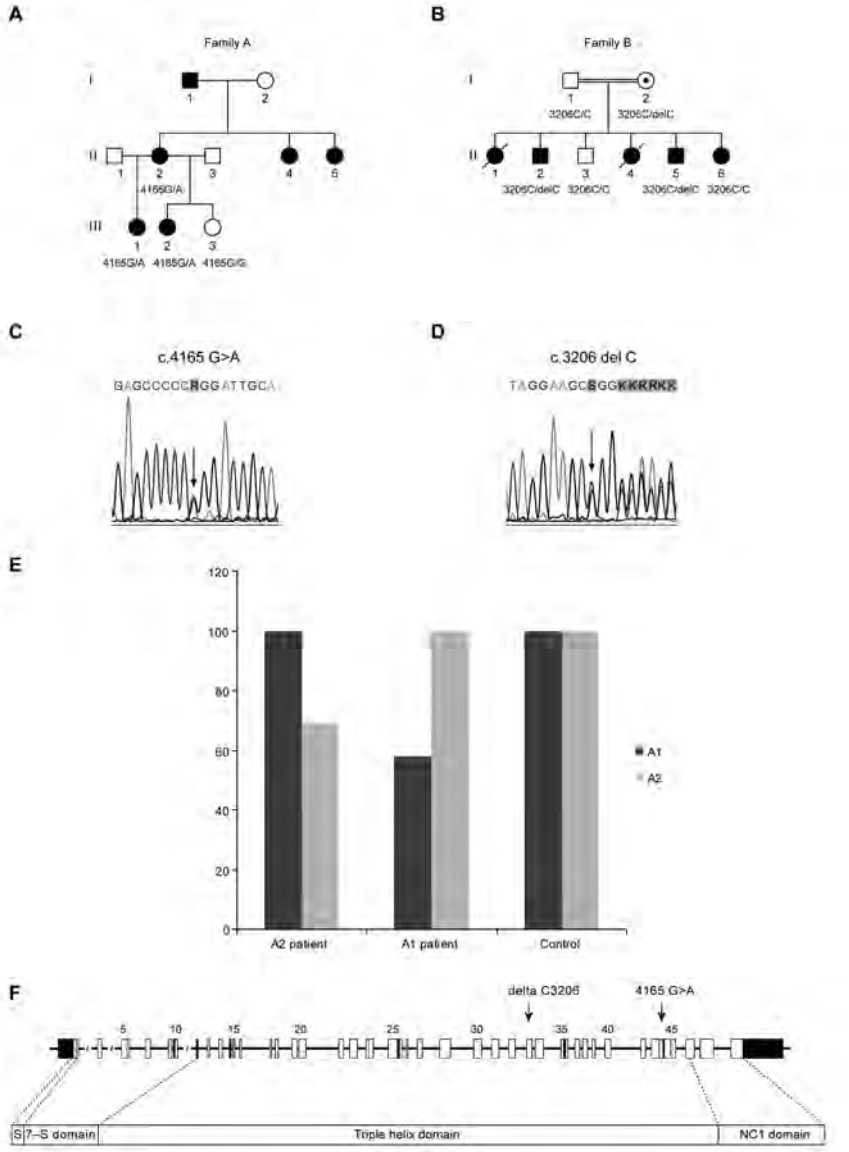


Figure 1. Pedigrees of families A and B.

Different symbols indicate individuals affected with various cerebral vascular disease as indicated in the legend. The genotype of the tested individuals is indicated: (**A**) 4165G/G = wild type sequence; 4165G/A heterozygous mutation; (**B**) 3206C/C = wild type sequence; 3206C/delC = heterozygous mutation. Electroferograms indicate the heterozygous mutation in the *COL4A2* sequence of family A c.4165G>A (p.G1389R) (**C**) and family B c.3206delC leading to frame shift (**D**). Quantitative, real time PCR analysis showing relative expression of *COL4A1* and *COL4A2* mRNA in fibroblasts from patient II.2 (family B) with c.3206delC mutation (**A2 patient**) and a patient bearing a pathogenic c.3321G>C (p.G1067A) mutation in exon 38 of *COL4A1* (**A1 patient**), compared to a control cell line. Results are average of two separate experiments. For each cell line the highest expression is set as 100% on the Y axis (**E**). Position of the mutations within the schematic representation of the *COL4A2* genomic organization (**F**).

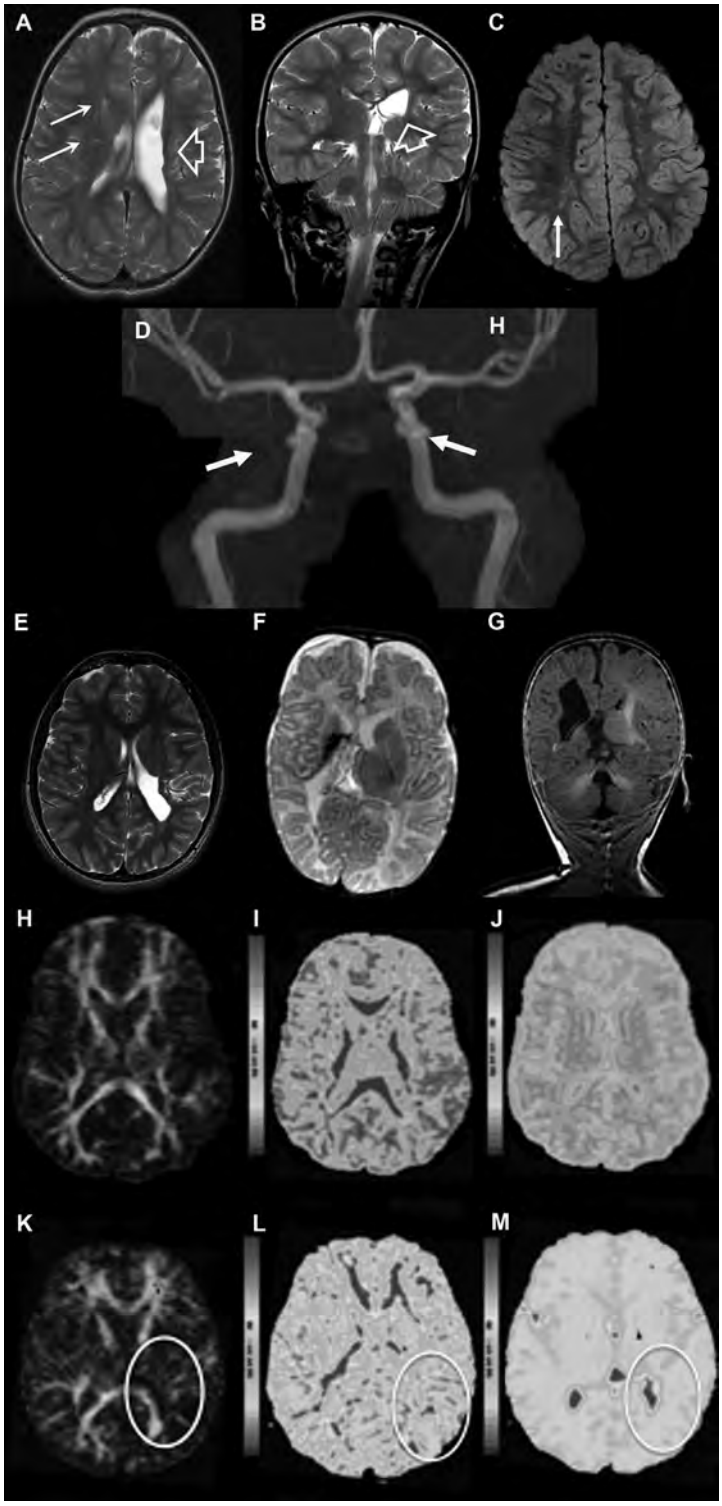


Figure 2. Brain MRI of patients with *COL4A2* mutations.

A, B. Axial and coronal T2 of patient III.2 from family A at the age of 2 years indicate ex-vacuo dilation of the left lateral ventricle (porencephaly) (open arrow) and periventricular white matter lesions (solid arrows) resulting from presumed perinatal stroke.

C. FLAIR image illustrates T2 prolongation in the white matter – in patient III.1 from family A at the age of 8 years, suggesting gliosis (arrow).

D. MR angiography of their mother (subject II.1, family A) at adult age shows bilateral internal carotid aneurysms (at the level of cavernous sinus).

E. Axial T2 weighted images of Patient II.2, family B, at the age of 15 years show a porencephalic dilatation of the left occipital ventricle.

F, G. Axial T2 weighted and coronal FLAIR images of patient II.5 from family B at the age of 5 months show porencephaly of the right ventricle with hypoplastic left cerebellar hemisphere.

H-M. Reconstruction of the white matter tracts obtained from magnetic resonance diffusion tensor imaging (MR-DTI) data.

K,L. Patient II-2 (family B) reveals reduced fractional anisotropy in the left radiation optica and tractus corticospinalis at the side of the porencephalic lesion (encircled), compared with an age-matched control

H,I. A restriction of the total ADC was observed in the whole cerebral white matter of the patient (**M**), compared to an age-matched control (**J**).

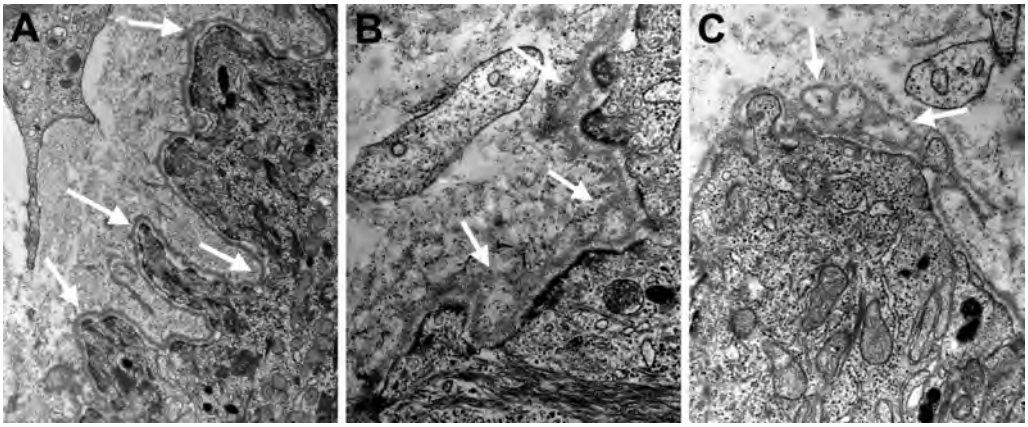


Figure 3. Skin transmission electron microscopy.

Upper arm skin biopsy of subject II-2 of family B was analysed by transmission electron microscopy according to Plaisier et al[8] in double blind experiments by two investigators and independently scored.

A. Control skin biopsy showing illustrative digitations (arrows) of the epidermis-dermis junction with normal basement membrane structure.

B, C. Illustrative areas of the basement membrane of the epidermis-dermis junction in the patient shows areas of thickening, blurring, fragmentation and duplication, giving it at times a blebby appearance (arrows).

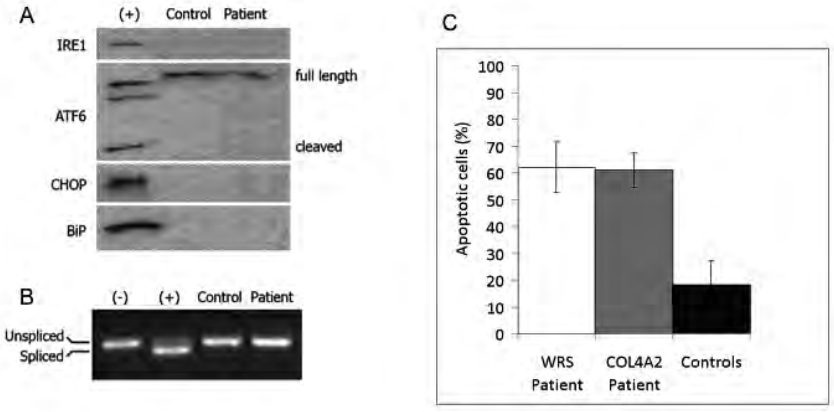


Figure 4. *COL4A2* c.3206delC mutation does not cause endoplasmic reticulum stress but reveals susceptibility to apoptosis.

A. Western blot analysis for endoplasmic reticulum stress markers (IRE-1, cleaved form of ATF6, CHOP and BiP) was negative in patient II-2 (family B) and control skin fibroblasts.

B. XBP-1 mRNA, which is spliced in response to endoplasmic reticulum stress, was evaluated by RT-PCR. Patient and control samples did not show detectable levels of spliced XBP-1. Tunicamycin treated cells were included as a positive control (+) in A and B. Untreated cells were used as a negative control (-).

C. Mean percentage of apoptotic cultured fibroblasts after stress induction by dithiothreitol. WRS: fibroblasts from patient with mutation in *EIF2AK3* gene with increased susceptibility to apoptosis[37]. *COL4A2*: cells from patient with c.3206delC mutation. Controls: fibroblast cell lines from 5 healthy individuals. Data are average of triplicate experiments.

References

1. **Dichgans M:** Genetics of ischaemic stroke. *Lancet Neurol* 2007; **6:** 149-161.

2. **Domingues-Montanari S, Mendioroz M, del Rio-Espinola A, Fernandez-Cadenas I, Montaner J:** Genetics of stroke: a review of recent advances. *Expert Rev Mol Diagn* 2008; **8:** 495-513.

3. **Gould DB, Phalan FC, Breedveld GJ** *et al***:** Mutations in Col4a1 cause perinatal cerebral hemorrhage and porencephaly. *Science* 2005; **308:** 1167-1171.

4. **Govaert P:** Prenatal stroke. *Seminars in fetal & neonatal medicine* 2009; **14:** 250-266.

5. **Tonk M, Haan J:** A review of genetic causes of ischemic and hemorrhagic stroke. *J Neurol Sci* 2007; **257:** 273-279.

6. **Vahedi K, Alamowitch S:** Clinical spectrum of type IV collagen (COL4A1) mutations: a novel genetic multisystem disease. *Curr Opin Neurol* 2011; **24:** 63-68.

7. **Breedveld G, de Coo IF, Lequin MH** *et al***:** Novel mutations in three families confirm a major role of COL4A1 in hereditary porencephaly. *J Med Genet* 2006; **43:** 490-495.

8. **Plaisier E, Gribouval O, Alamowitch S** *et al***:** COL4A1 mutations and hereditary angiopathy, nephropathy, aneurysms, and muscle cramps. *N Engl J Med* 2007; **357:** 2687-2695.

9. **van der Knaap MS, Smit LM, Barkhof F** *et al***:** Neonatal porencephaly and adult stroke related to mutations in collagen IV A1. *Annals of neurology* 2006; **59:** 504-511.

10. **Volonghi I, Pezzini A, Del Zotto E** *et al***:** Role of COL4A1 in basement-membrane integrity and cerebral small-vessel disease. The COL4A1 stroke syndrome. *Curr Med Chem* 2010; **17:** 1317-1324.

11. **Meuwissen ME, de Vries LS, Verbeek HA** *et al***:** Sporadic COL4A1 mutations with extensive prenatal porencephaly resembling hydranencephaly. *Neurology* 2011; **76:** 844-846.

12. **Coupry I, Sibon I, Mortemousque B, Rouanet F, Mine M, Goizet C:** Ophthalmological features associated with COL4A1 mutations. *Arch Ophthalmol* 2010; **128:** 483-489.

13. **Gould DB, Phalan FC, van Mil SE** *et al***:** Role of COL4A1 in small-vessel disease and hemorrhagic stroke. *N Engl J Med* 2006; **354:** 1489-1496.

14. **Sibon I, Coupry I, Menegon P** *et al***:** COL4A1 mutation in Axenfeld-Rieger anomaly with leukoencephalopathy and stroke. *Annals of neurology* 2007; **62:** 177-184.

15. **Vahedi K, Boukobza M, Massin P, Gould DB, Tournier-Lasserve E, Bousser MG:** Clinical and brain MRI follow-up study of a family with COL4A1 mutation. *Neurology* 2007; **69:** 1564-1568.

16. **Plaisier E, Chen Z, Gekeler F** *et al***:** Novel COL4A1 mutations associated with HANAC syndrome: A role for the triple helical CB3[IV] domain. *Am J Med Genet A* 2010.

17. **Vahedi K, Kubis N, Boukobza M** *et al***:** COL4A1 mutation in a patient with sporadic, recurrent intracerebral hemorrhage. *Stroke* 2007; **38:** 1461-1464.

18. **Khoshnoodi J, Pedchenko V, Hudson BG:** Mammalian collagen IV. *Microsc Res Tech* 2008; **71:** 357-370.

19. **Favor J, Gloeckner CJ, Janik D** *et al***:** Type IV procollagen missense mutations associated with defects of the eye, vascular stability, the brain, kidney function and embryonic or postnatal viability in the mouse, Mus musculus: an extension of the Col4a1 allelic series and the identification of the first two Col4a2 mutant alleles. *Genetics* 2007; **175:** 725-736.

20. **Marutani T, Yamamoto A, Nagai N, Kubota H, Nagata K:** Accumulation of type IV collagen in dilated ER leads to apoptosis in Hsp47-knockout mouse embryos via induction of CHOP. *Journal of cell science* 2004; **117:** 5913-5922.

21. **Verkerk AJ, Schot R, Dumee B** *et al***:** Mutation in the AP4M1 gene provides a model for neuroaxonal injury in cerebral palsy. *Am J Hum Genet* 2009; **85:** 40-52.

22. **Lobel U, Sedlacik J, Gullmar D, Kaiser WA, Reichenbach JR, Mentzel HJ:** Diffusion tensor imaging: the normal evolution of ADC, RA, FA, and eigenvalues studied in multiple anatomical regions

of the brain. *Neuroradiology* 2009; **51:** 253-263.

23. **Lin JH, Li H, Yasumura D** ***et al*****:** IRE1 signaling affects cell fate during the unfolded protein response. *Science* 2007; **318:** 944-949.

24. **Engel J, Prockop DJ:** The zipper-like folding of collagen triple helices and the effects of mutations that disrupt the zipper. *Annual review of biophysics and biophysical chemistry* 1991; **20:** 137-152.

25. **Firtina Z, Danysh BP, Bai X, Gould DB, Kobayashi T, Duncan MK:** Abnormal expression of collagen IV in lens activates unfolded protein response resulting in cataract. *J Biol Chem* 2009; **284:** 35872-35884.

26. **Gould DB, Marchant JK, Savinova OV, Smith RS, John SW:** Col4a1 mutation causes endoplasmic reticulum stress and genetically modifiable ocular dysgenesis. *Hum Mol Genet* 2007; **16:** 798-807.

27. **Gupta MC, Graham PL, Kramer JM:** Characterization of alpha1(IV) collagen mutations in Caenorhabditis elegans and the effects of alpha1 and alpha2(IV) mutations on type IV collagen distribution. *The Journal of cell biology* 1997; **137:** 1185-1196.

28. **Herbst TJ, McCarthy JB, Tsilibary EC, Furcht LT:** Differential effects of laminin, intact type IV collagen, and specific domains of type IV collagen on endothelial cell adhesion and migration. *The Journal of cell biology* 1988; **106:** 1365-1373.

29. **Petitclerc E, Boutaud A, Prestayko A** ***et al*****:** New functions for non-collagenous domains of human collagen type IV. Novel integrin ligands inhibiting angiogenesis and tumor growth in vivo. *J Biol Chem* 2000; **275:** 8051-8061.

30. **Lein PJ, Higgins D, Turner DC, Flier LA, Terranova VP:** The NC1 domain of type IV collagen promotes axonal growth in sympathetic neurons through interaction with the alpha 1 beta 1 integrin. *The Journal of cell biology* 1991; **113:** 417-428.

31. **Barkovich AJ, Kuzniecky RI, Jackson GD, Guerrini R, Dobyns WB:** A developmental and genetic classification for malformations of cortical development. *Neurology* 2005; **65:** 1873-1887.

32. **Li Z, Hassan MQ, Jafferji M** ***et al*****:** Biological functions of miR-29b contribute to positive regulation of osteoblast differentiation. *J Biol Chem* 2009; **284:** 15676-15684.

33. **Mott JL, Kobayashi S, Bronk SF, Gores GJ:** mir-29 regulates Mcl-1 protein expression and apoptosis. *Oncogene* 2007; **26:** 6133-6140.

34. **Guo DC, Papke CL, Tran-Fadulu V** ***et al*****:** Mutations in smooth muscle alpha-actin (ACTA2) cause coronary artery disease, stroke, and Moyamoya disease, along with thoracic aortic disease. *Am J Hum Genet* 2009; **84:** 617-627.

35. **Abrahamson DR, Hudson BG, Stroganova L, Borza DB, St John PL:** Cellular origins of type IV collagen networks in developing glomeruli. *J Am Soc Nephrol* 2009; **20:** 1471-1479.

36. **Heidet L, Cai Y, Guicharnaud L, Antignac C, Gubler MC:** Glomerular expression of type IV collagen chains in normal and X-linked Alport syndrome kidneys. *The American journal of pathology* 2000; **156:** 1901-1910.

37. **Poulton CJ, Schot R, Kia SK** ***et al*****:** Microcephaly with simplified gyration, epilepsy, and infantile diabetes linked to inappropriate apoptosis of neural progenitors. *Am J Hum Genet* 2011; **89:** 265-276.

SUPPLEMENTARY DATA

Methods of genomic analysis

Bioinformatics

COL4A1 accession numbers: NM_001845.4; OMIM *120130[6].

COL4A2 accession numbers: CCDS41907; Entrez Gene ID 1284; OMIM *120090. According to sequence CCDS 41907, the starting codon is in exon 2 and we used this numbering of the exons in the text. Prediction of pathogenicity of the non-synonymous G1389R substitution was performed by Polyphen-2 (http://genetics.bwh.harvard.edu/pph/), SNAP (snap@rostlab.org) and SIFT (http://sift.jcvi.org/) programs. All the programs reported the change as non-tolerated.

Here the Polyphen-2 scores are reported as probably damaging.

Sanger sequencing

Genomic DNA was isolated from peripheral blood by standard methods. All exons including surrounding boundaries of *COL4A1* and *COL4A2* were amplified by PCR and sequenced in both directions.

PCR reactions were carried out in 25 µl containing 1x Invitrogen PCR buffer, 1,5 mM MgCl2, 0,01% W1 detergent, 0,2mM of each dNTP, 1µM both forward and reserve primer, 0,1 units

of Platinum Taq DNA polymerase (Invitrogen) and 50 ng genomic DNA. Primer sequences and annealing conditions are available upon request.

Direct sequencing of both strands was performed using Big Dye Terminator chemistry version 3.1, electrophoresis on a ABI 3130 Genetic Analyzer and analysing with DNA Sequencing Analysis version 5.2 and SeqScape version 2.1 software (Applied Biosystems).

Fibroblasts from patients and controls were cultured in Dulbecco's modified Eagle medium, supplemented with 10% Fetal calf serum and antibiotics. Total RNA was extracted and purified using Trizol reagent (Gibco BRL) according to manufacturer

RT-PCR was performed with 2µg RNA using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen), followed by PCR using overlapping primer-pairs (primer sequences and PCR conditions on request).

RT-qPCR was performed using KAPA sybr fast qPCR mix (ABI prism) and the CFX96 Real Time System (Biorad), using *ACTB* and *UBE2D2* as control housekeeping genes according to Verkerk *et al* 2009[21].

RTqPCR primers

COL4A1

Primerpair 1;	GAAGGGTTTCCCCGGATTC	CCCGACTGTCCCGTTATGC
Primerpair 2;	GAGGAGTTTAGAAGTGCGCCATT	GGTGGCGAGCCAAAAGCT

COL4A2

Primerpair 1;	CCCTGTGGGCATGAAAGGT	TCCTTTAAATCCAGGGCTTCCT
Primerpair 2;	TGGGACAGATGGGTCCAGTT	CAAGTCCTCTGTTGCCTTGCT

Primer sequences used for *COL4A2* sequences

Oligoname	Oligo length	Oligo sequence
COL4A2 - 1F	36	tgtaaaacgacggccagtccaagcggagacctgagc
COL4A2 - 1R	38	caggaaacagctatgaccctactcccaagcaggagagc
COL4A2 - 2-3F	38	tgtaaaacgacggccagtgggaggctctccttctttcc
COL4A2 - 2-3R	37	caggaaacagctatgaccaacgtcccaaccactctcg
COL4A2 - 4F	40	tgtaaaacgacggccagtttggaaggattctcaacagatg
COL4A2 - 4R	38	caggaaacagctatgaccacagccgggtgtgtagtagg
COL4A2 - 5-6F	44	tgtaaaacgacggccagtccgtaactgatcatgagtatgtattg
COL4A2 - 5-6R	36	caggaaacagctatgacccctaggatgcacgcaatg
COL4A2 - 7F	45	tgtaaaacgacggccagttctcacagttacatgacaactagaagc
COL4A2 - 7R	38	caggaaacagctatgacccagttatgcttccgttctgg
COL4A2 - 8F	38	tgtaaaacgacggccagtgctgcaccgaatgttaatgg
COL4A2 - 8R	38	caggaaacagctatgaccgattatgccgccattctagg
COL4A2 - 9-10F	39	tgtaaaacgacggccagtgggctgatctgtttgatatgc
COL4A2 - 9-10R	38	caggaaacagctatgaccaagaagtggcatctggaagg
COL4A2 - 11F	38	tgtaaaacgacggccagttcagaaacctccatgcatcc
COL4A2 - 11R	40	caggaaacagctatgacctttgcaaacaacaaatcattcc
COL4A2 - 12F	37	tgtaaaacgacggccagtgccgataaataggccttgg
COL4A2 - 12R	39	caggaaacagctatgaccggtgacaacctagcactttgc
COL4A2 - 13F	40	tgtaaaacgacggccagtggccaggttgtatttgtattcg
COL4A2 - 13R	36	caggaaacagctatgaccgccaagttggtggtgagg
COL4A2 - 14F	42	tgtaaaacgacggccagtgaggattgattcagtactttcagc
COL4A2 - 14R	37	caggaaacagctatgacccatctccatgctcccttcc
COL4A2 - 15F	38	tgtaaaacgacggccagtgggctgagaacacagagtcc
COL4A2 - 15R	38	caggaaacagctatgaccatggttcacggattgtagcc
COL4A2 - 16F	40	tgtaaaacgacggccagtgacttgttcaatctgtccatcg
COL4A2 - 16R	38	caggaaacagctatgacctctttcctgagatgccaagg
COL4A2 - 17F	38	tgtaaaacgacggccagtgctcctggttctagcaaagc
COL4A2 - 17R	38	caggaaacagctatgaccgtcagaggccgtgtatttgg
COL4A2 - 18F	37	tgtaaaacgacggccagtgctcgggtttcttctttgg
COL4A2 - 18R	38	caggaaacagctatgaccaggactgtctcaggcacagg
COL4A2 - 19F	38	tgtaaaacgacggccagtacagcatatggagcatttgg
COL4A2 - 19R	38	caggaaacagctatgaccagggttctgtgaaggtgtcc

Oligoname	Oligo length	Oligo sequence
COL4A2 - 20F	38	tgtaaaacgacggccagtacccatcggagttattgacg
COL4A2 - 20R	38	caggaaacagctatgacctcactggcctactggattcg
COL4A2 - 21F	36	tgtaaaacgacggccagtatgccctgcatctgtggt
COL4A2 - 21R	38	caggaaacagctatgaccggggatggatttcaccttct
COL4A2 - 22F	38	tgtaaaacgacggccagtcatcagaacaggcagtgtcc
COL4A2 - 22R	36	caggaaacagctatgaccgtgttgccggtctgaagc
COL4A2 - 23-24F	36	tgtaaaacgacggccagtttggtgacgggtgactgc
COL4A2 - 23-24R	38	caggaaacagctatgacctgcacaggttggtttagtgc
COL4A2 - 25F	38	tgtaaaacgacggccagtgaaaggaaacagggaagtcg
COL4A2 - 25R	41	caggaaacagctatgaccggaaacgtctacactgacaaagg
COL4A2 - 26F	38	tgtaaaacgacggccagtacgacacaccttcacacacg
COL4A2 - 26R	37	caggaaacagctatgaccccacgcatactgcaagagc
COL4A2 - 27F	37	tgtaaaacgacggccagtagaatggtagccggtttgc
COL4A2 - 27R	37	caggaaacagctatgacctggacacgaccatgtctgg
COL4A2 - 28F	38	tgtaaaacgacggccagtgtcagatgccaagcatgacc
COL4A2 - 28R	38	caggaaacagctatgaccaatctcccaaggacaaatgc
COL4A2 - 29F	42	tgtaaaacgacggccagttgactcttctcttagaacgtcacc
COL4A2 - 29R	38	caggaaacagctatgacccgctgcttcctaccaaatcc
COL4A2 - 30F	38	tgtaaaacgacggccagtgagtgtgtggagggagatgc
COL4A2 - 30R	39	caggaaacagctatgaccagctccacaagaagacagtgc
COL4A2 - 31F	38	tgtaaaacgacggccagtccacactttggaaactcacc
COL4A2 - 31R	37	caggaaacagctatgaccccagcagagctgtctcagg
COL4A2 - 32F	37	tgtaaaacgacggccagtccgaaatgttacggagacg
COL4A2 - 32R	38	caggaaacagctatgaccgccaccaagaaagggtaagg
COL4A2 - 33F	38	tgtaaaacgacggccagtggccttcacctgtgttctcc
COL4A2 - 33R	38	caggaaacagctatgaccggaaaccgaggttactcagg
COL4A2 - 34F	38	tgtaaaacgacggccagtttcacagcacgtaggacagc
COL4A2 - 34R	38	caggaaacagctatgacctgtccattcacaagcaaagg
COL4A2 - 35F	42	tgtaaaacgacggccagtcaaaccttgagtattgtcgttagc
COL4A2 - 35R	38	caggaaacagctatgacctctgaaattggctcacatgc
COL4A2 - 36F	39	tgtaaaacgacggccagtagacctgcaagtgctgttagg
COL4A2 - 36R	38	caggaaacagctatgaccttgatctgtttggcaagtcg
COL4A2 - 37F	39	tgtaaaacgacggccagttggttctgcacatcctagagc
COL4A2 - 37R	36	caggaaacagctatgaccagggttgggtgtgattgg

Oligoname	Oligo length	Oligo sequence
COL4A2 - 38F	40	tgtaaaacgacggccagtcctgtgtgctcagacttaatgc
COL4A2 - 38R	38	caggaaacagctatgaccagaagtcgccctgagagagg
COL4A2 - 39F	36	tgtaaaacgacggccagttttctcccacccagaacc
COL4A2 - 39R	37	caggaaacagctatgaccctctgagaccctccattcc
COL4A2 - 40F	37	tgtaaaacgacggccagtgctgcctctgtttctttgc
COL4A2 - 40R	38	caggaaacagctatgaccgctctttcctgcacttctgc
COL4A2 - 41F	37	tgtaaaacgacggccagtcactgtctcgctcgctagg
COL4A2 - 41R	39	caggaaacagctatgaccaggtgatggaaatgacacagg
COL4A2 - 42F	38	tgtaaaacgacggccagtagagactgtcgcctgaatgg
COL4A2 - 42R	38	caggaaacagctatgaccagggacacgaaagtctgtgg
COL4A2 - 43F	36	tgtaaaacgacggccagtcctggccacagtgagagg
COL4A2 - 43R	37	caggaaacagctatgaccgtgacccatgccagagagg
COL4A2 - 44F	38	tgtaaaacgacggccagtagcacagttgtctgggaagc
COL4A2 - 44R	40	caggaaacagctatgacctccacaaagagaacacaagagg
COL4A2 - 45F	40	tgtaaaacgacggccagtcttgtgttctctttgtggatcg
COL4A2 - 45R	38	caggaaacagctatgaccgctgggcataggagtgtagc
COL4A2 - 46F	38	tgtaaaacgacggccagtggggctgctctctctctctt
COL4A2 - 46R	38	caggaaacagctatgacccctttgatcttggccacttcc
COL4A2 - 47F	39	tgtaaaacgacggccagttccagtaggtggctaaactcc
COL4A2 - 47R	38	caggaaacagctatgacccagctgttcttgctgtgtcc
COL4A2 - 48F	38	tgtaaaacgacggccagttgcacagaagagggctatgc
COL4A2 - 48R	38	caggaaacagctatgaccCTGGATGCAAATTCCTTTGG

Immunofluorescence staining for ER stress detection

Immunofluorescence using antibodies directed against cytochrome P450, HSP47 and KDEL was performed on fibroblasts (3 control and 1 patient). The cells from patient II-2 family B with *COL4A2*^{3206delC} and 3 control cell lines were seeded onto glass coverslips in a 6 well plate, approximately 150 000 cells per well and maintained with Dulbecco's modified Eagle essential medium (DMEM) plus 10% fetal calf serum (FCS). After 24 hours of serum deprivation followed by 24 hours exposure to 5mM DTT the cells were fixed in cytoskelfix-20 (Cytoskeleton) for 10 minutes and washed three times in PBS. The cells were then blocked blocking buffer (0.05 M Tris; 0.9 NaCl; 0.25% gelatine;0.5% Triton X100, PH 7.4) and probed overnight at 4 degrees with primary antibodies, KDEL Mouse Monoclonal antibody raised

against a 6 residues synthetic peptide(Stressgen 10C3 1/100) and Cytochrome P450 Rabbit polyclonal antibody raised against the full length P450 1A1 fusion protein (abcam ab3568, 1/100), or Mouse Monoclonal Antibody Hsp47 raised against full length Hsp47 (stressgen M16.10A1 1/50). The samples were then incubated with Cy3-coupled secondary anti-mouse antibodies and Cy2-coupled anti-rabbit antibodies (Jackson ImmunoResearch, 1/200) for 1 hour at room temperature. Cells were mounted on slides with fluorescent mounting medium (DAKO). Fluorescent images were collected using AXip-Axioplan2 imaging microscope (Zeiss) with COOLSNAP-pro camera (Zeiss).

ER stress tests and susceptibility to apoptosis in cultured fibroblasts

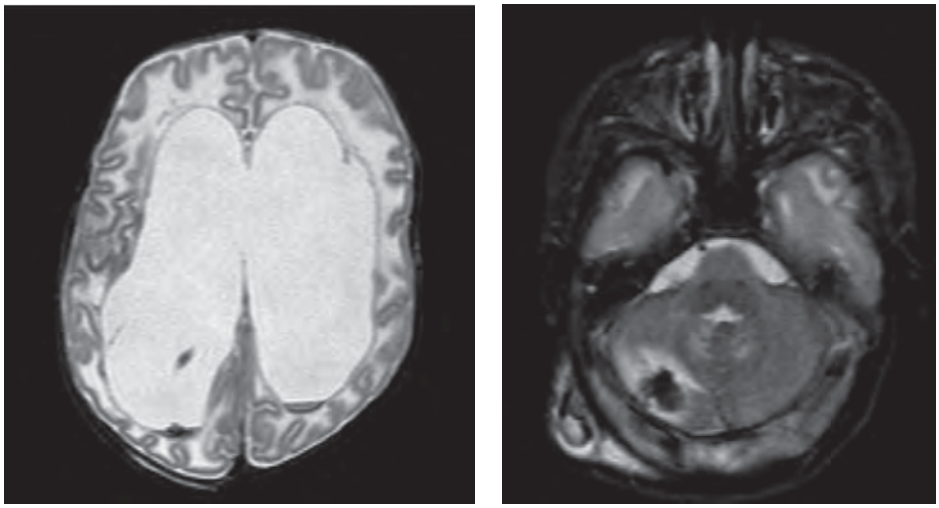
Western blot analysis for endoplasmic reticulum stress markers (IRE-1, cleaved form of ATF6, CHOP and BiP) was performed in skin fibroblast cultures of patient II-2 (family B) and control skin fibroblasts. . For Western blot, protein lysates from skin fibroblast cells (70 μg) were separated by SDS-PAGE and transferred to PVDF membranes. Membranes were blocked with 5% non-fat milk for 1 h at room temperature and incubated with anti-IRE1 (1:2000) (Abcam), anti-ATF6 (1:1000) (Abcam), anti-CHOP (1:250) (Abcam), or anti-BiP (1:2000) (Cell Signalling) antibodies in blocking buffer overnight at 4°C. After incubation with secondary antibodies conjugated to horseradish peroxidase (Jackson ImmunoResearch) for 1 h at room temperature, the signals were visualized using an ECL detection kit (Thermo Scientific). XBP-1 mRNA, which is spliced in response to endoplasmic reticulum stress, was evaluated by RT-PCR, before and after treatment with 5 microgram/mL tunicamycin. RT-PCR XBP-1 splicing analysis was according to Lin *et al*, 2007[23].

Susceptibility to apoptosis of cultured skin fibroblasts was measured by fluorescent staining of active caspases using the Flica™ (Fluorescent-Labeled Inhibitor of CAspases) apoptosis multi-caspase detection kit (Immunochemistry Technologies, LLC), which makes use of an inhibitor sequence of active caspases, according to the manufacturer instruction. Fluorescent cells were scored before and after stress induction by 24 hours serum deprivation and then a further 24 hours exposure to 5 mM dithiothreitol (DTT) by two blinded investigators (CP and RS) as percentage of apoptotic fibroblasts in separate triplicate cultures derived from one patient bearing the c.3206delC mutation (II-2, family B), one patient affected by Wolcott-Rallison syndrome (OMIM 226980) bearing an *EIF2AK3* mutation and 5 healthy individuals. Wolcott-Rallison mutations are a known to cause increased susceptibility to apoptosis. Necrosis vs apoptosis was tested by vital staining exclusion with PI or 7 AAD. The patient with the *EIF2AK3* mutation has been described by Poulton *et al*, 2011[37].

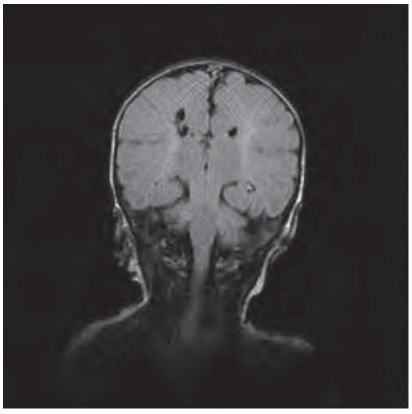
Electronmicroscopy of the skin

For routine transmission electron microscopy the material was fixed in 1% v/v and 4% v/v formaldehyde. After complete dehydration in acetone and embedding in Epon the sections of 50 nm were made on a Leica Supercut UCT.

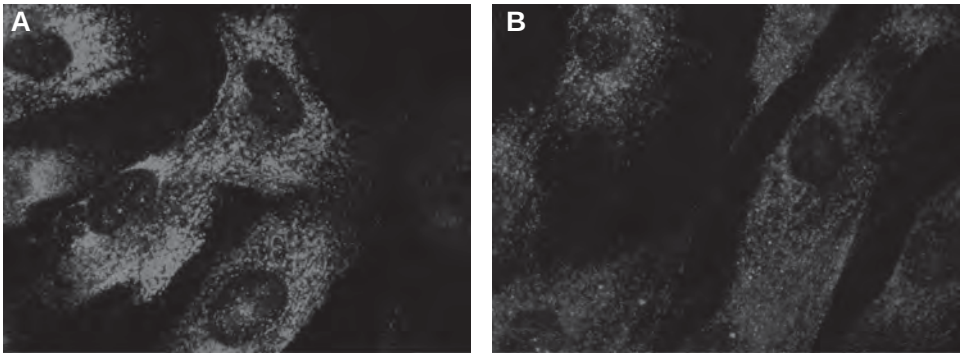
The ultra thin sections were collected on 200 mesh formfar filmed copper grids and stained with uranyl acetate and lead citrate and examined with a Fei Morgagni 286 electron microscope. Two investigators JdH and GMM independently scored the preparations in double blind against three control skin preparations, according to Plaisier *et al*[8].



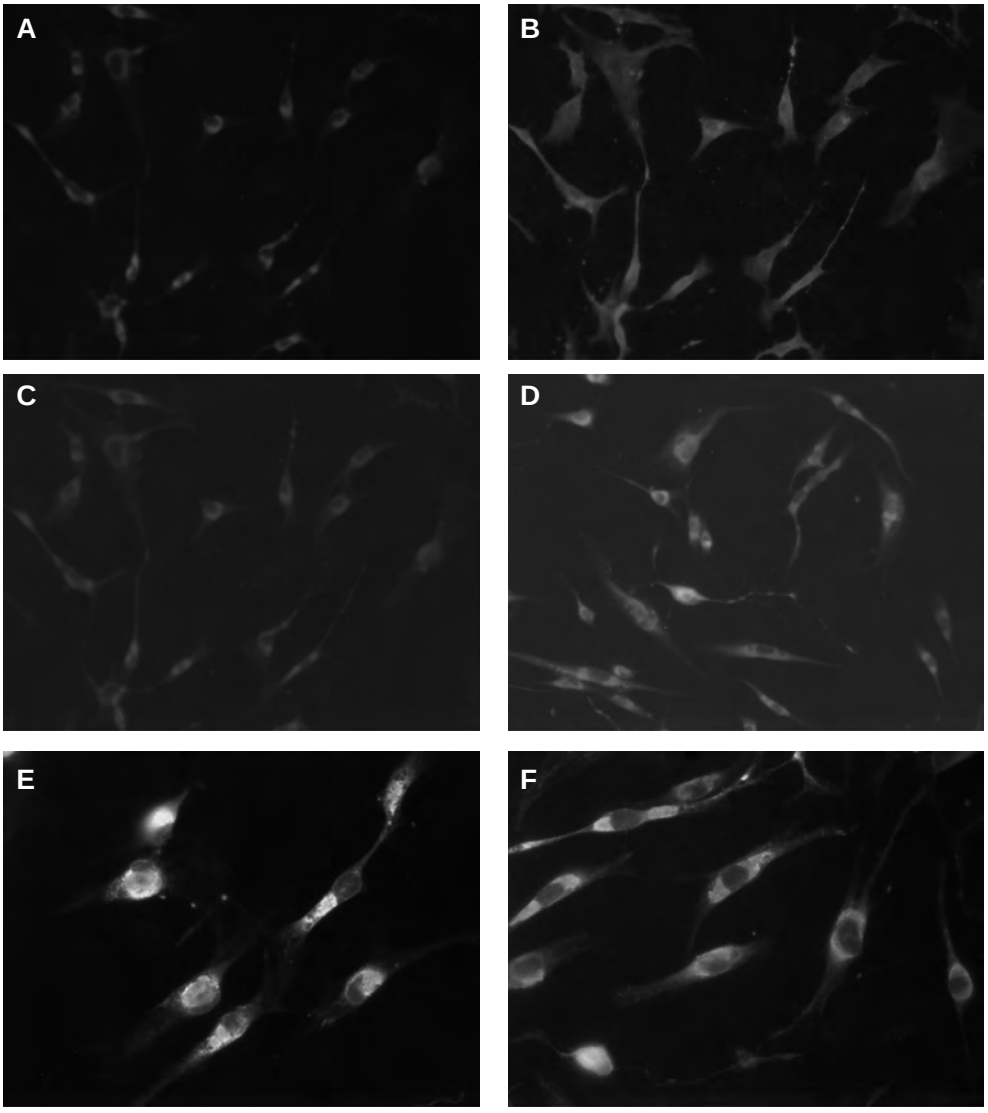
Supplementary figure 1. Axial T2 weighted MRI images of the brain and cerebellum of patient II-4 of family A at the age of 3 months, showing (left panel) hydrocephalus with hemosiderine residues in the occipital ventricles and (right panel) subcortical calcification in the right cerebellar hemisphere suggestive of massive bleeding. No DNA was available for testing.



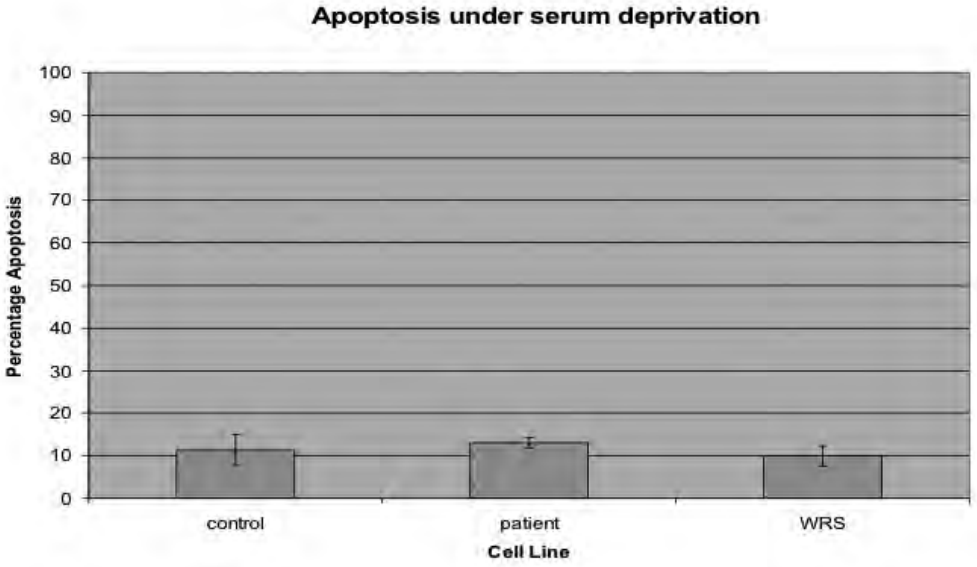
Supplementary Figure 2. FLAIR Coronal MRI section of patient II-6 from family A at birth, showing a small cyst in the right parietal lobe adjacent to the lateral ventricle. In her DNA the familial c.3206delC *COL4A2* mutation was absent.



Supplementary Figure 3. Cultured fibroblasts grown under standard conditions . Immunofluorescent staining for HSP47 (red). **A**: control; **B**: patient with *COL4A2*^{3206delC} mutation.



Supplementary Figure 4. Cultured fibroblasts after treatment with 5 mM DTT. Immunofluorescent staining for P450 (red), KDEL (green). **A and B**: control 1. **C:** merged picture of A and B. **D,E.F:** merged pictures of double staining with KDEL and P450. **D:** control 2; **E:** control 3; **F:** patient with *COL4A2*$^{3206\text{delC}}$ mutation.



Supplementary figure 5. Skin fibroblasts were cultured in 175 cm^2 culture flasks in Dulbecco's modified Eagle medium (DMEM, Lonza Biowhittaker) plus 10% FCS until 80% confluence. Cultures were obtained from 5 healthy individuals (control), one patient with *COL4A2*^{3206delC} mutation (patient) and one individual with confirmed Wolcott-Rallison syndrome bearing an *EIF2AK3* mutation (WRS) . Before testing apoptosis rates by the Flica multicaspase detection kit, the cells were exposed 24 hours to serum deprivation. Data are average of multiple experiments plus SD, as indicated in the methods.

DISCUSSION AND SUMMARY

Chapter 7

GENERAL DISCUSSION

GENERAL DISCUSSION

In 2007, when this project started, the *COL4A1* gene had just been identified as a cause of familial porencephaly; it was the first time that isolated prenatal or infantile cerebral intraparenchymal hemorrhage, independently of metabolic diseases and coagulopathies, was proven to result from a single gene mutation. Before the identification of *COL4A1*, cases of prenatal or infantile isolated cerebral hemorrhage were attributed to extrinsic factors, such as prematurity, very low birth weight or infection. The work in this thesis contributes to the concept that, besides external factors, several monogenetic factors can underly prenatal or infantile/ childhood cerebral hemorrhage. It started by expanding the phenotypic spectrum and inheritance mode in *COL4A1* mutations, and continued by the identification of *COL4A2* mutations as a novel genetic cause of cerebral hemorrhage and porencephaly. The description of cerebrovascular pathology of a well-known syndrome, Incontinentia Pigmenti, and of a newly identified syndrome, which is caused by *ACTA2* mutations changing the R179 amino acid adds to the phenotypic spectrum of childhood cerebrovascular disease. Finally, an extensive study in five young patients with cerebral hemorrhage, a pseudo-TORCH syndrome and early demise led to the identification of *USP18* mutations as a novel monogenetic cause of cerebrovascular disease with systemic manifestations.

Crucial for our identification of new genetic factors in prenatal or infantile/childhood stroke, such as *COL4A2* and *USP18*, was the multidisciplinary cooperation between the Departments of Clinical Genetics, Child Neurology, Neonatology and Neuroradiology. An approach of early (genetic) consultation of a baby born with cerebral hemorrhage together with adequate classification of the type of cerebral pathology, followed by follow-up in a multidisciplinary outpatient clinic proved very efficient in enrolling patients in our research project in the diagnostic process of prenatal and/ or neonatal cerebral hemorrhage. Multicentre cooperation with other University Medical Centers in The Netherlands was essential for our work.

COL4A1 and *COL4A2* mutations

Incidence of COL4A1 and COL4A2 mutations

Our results show the identification of 21 *COL4A1* and 3 *COL4A2* mutations in a group of 183 index patients tested at the Dept. of Clinical Genetics of the Erasmus University Medical Center Rotterdam, mostly referred for porencephaly or cerebral hemorrhage. This correlates with *COL4A1* or *COL4A2* mutations in 13% of index patients. A recent study did not

show *COL4A1* mutations in a cohort of 224 infants with low birth weights (500-1250 g) and grade 3-4 intraventricular hemorrhage[1]. A previous study in a group of 41 preterm infants presenting with intraventricular hemorrhage showed one *COL4A1* mutation in dizygotic twins, born at 24 weeks' gestation. This was a 6 bp duplication in the NC1-domain, carried by an asymptomatic mother and grandmother. In a cohort of 96 sporadic patients with intracerebral hemorrhage not caused by arteriovenous malformations, tumors or impaired coagulation, *COL4A1* and *COL4A2* were tested, leading to the detection of 2 *COL4A1* pathogenic mutations in 2 patients and 3 *COL4A2* mutations in 4 patients[2,3]. These findings suggest that *COL4A1* and *COL4A2* mutations contribute to approximately 6% of sporadic late onset intracerebral hemorrhage. These cases may reflect "milder" mutations, since the "classic" glycine changes in the adult onset patients have not been identified in this group.

One of the main conclusions from our review is that the mutations with onset in the perinatal period can be associated with developmental defects, such as microphthalmia, schizencephaly, hydranencephaly and renal agenesis. Whether these developmental defects should be considered as disruptions or malformations is a matter of discussion.

In summary, the chance of identifying *COL4A1* and *COL4A2* mutations in extremely premature infants and infants with very low birth weight seems small. We don't suggest testing until 30 weeks' gestation, unless the cerebral hemorrhage occurred prenatally without identifiable causative factors. *COL4A1* and *COL4A2* testing in infants with porencephaly and/or intracerebral hemorrhage is recommended, since our data show *COL4A1* or *COL4A2* mutations in 13% of index patients. Routine testing of *COL4A1* and *COL4A2* in adult onset stroke seems reasonable; based on the current (limited) data approximately 6% of sporadic intracerebral hemorrhage cases might be explained by *COL4A1* or *COL4A2* mutations.

Pathogenic mechanisms of COL4A1 and COL4A2 mutations

The general pathogenic mechanism in *COL4A1* and *COL4A2* mutations is presumed to be basement membrane dysfunction. Both *COL4A1* and *COL4A2* are ubiquitously expressed, and can be found in the basement membranes throughout the body. Abnormal basement membranes are demonstrated also in tissues that seem unaffected by the mutation, i.e. in oesophagus in mice[4] or in skin in human patients[5-7]. The latter is also observed in asymptomatic mutation carriers[7]. COL4A1 and COL4A2 are major components of all vascular basement membranes. One question that remains unanswered is why the vascular phenotype of *COL4A1* and *COL4A2* mutations is most often present in the cerebral vasculature

and eye. Of course, one explanation may be a biased testing for *COL4A1* and *COL4A2* mutations, limited to patients with cerebrovascular disease. However, to date, family histories of index patients are not suggestive of other vascular pathologies associated with mutations. This suggests an additional function and/ or a different interacting environment of COL4A1 and COL4A2 in cerebral vasculature or differences in anatomic structures.

One factor that likely contributes to the predominance of the cerebral phenotype is the fragility of the germinal matrix. Most cases of congenital porencephaly originate from a germinal matrix hemorrhage with subsequent venous infarction and tissue destruction, eventually followed by cyst formation. At term gestation, the germinal matrix has a subependymal location and contains immature vascular structures. A pathology study investigating the origin of germinal matrix hemorrhage indicates that the hemorrhagic foci within the germinal matrix tissue were in proximity to venous vessels. The venous vessels were all distorted with a loss of structural integrity[8]. Since the germinal matrix is a fragile structure in itself, with immature basal lamina, incomplete glial support and poor matrix support, prematurity is an important risk factors for germinal matrix hemorrhage[8,9]. One can postulate, however, that the basement membrane changes in *COL4A1* and *COL4A2* mutations increase the loss of structural integrity of the germinal matrix veins, leading to an increased risk of hemorrhage in this specific location.

Another theory may be that *COL4A1* mutations lead to an altered expression of occludin, a major component of tight junctions. COL4A1 plays a regulating role in the formation of the tight junctions and the expression of occludin[10]. The tight junctions play an important role in the formation of the blood-brain barrier. Dysfunction of the blood-brain barrier has been identified as a cause of cerebral hemorrhage and cerebrovascular disease, i.e. as seen in homozygous mutations in occludin itself and in *JAM3* mutations[11,12]. Thus, *COL4A1* mutations may lead to less occludin expression, leading to weakening of the tight junctions in the blood-brain barrier and the cerebrovascular phenotype of hemorrhage and small vessel disease.

Counseling issue

Another important issue regarding *COL4A1* and *COL4A2* mutations to date is the phenotypic variability with reduced penetrance. Besides the HANAC phenotype, with mutations in exons 24 and 25 of the *COL4A1* gene, which affect the CB3[IV] fragment of COL4A1 and encompasses a specific integrin-binding site[5,13], no genotype-phenotype correlations can be made. A high percentage of *de novo* mutations is identified, and *de novo* mutations appear to have phenotypes in the severe end of the spectrum[14]. However, severe phenotypes can also be appreciated in familial cases. It is therefore not possible to predict the phenotype based on the type and location of the mutation. A recent study on an affected child with porencephaly and asymptomatic father both carrying a G702D mutation in *COL4A2* interestingly shows basement membrane defects in both carriers, but retention of COL4A2 in the ER was unique to cells of the affected child. In the child, ER stress and an increased apoptosis was present, which were both absent in cells of the asymptomatic father. This implicates that, theoretically, the ability of cells to cope with mutant collagen folding and ER-retention is dependent on critical, yet unknown modifiers[7]. The variable phenotypes and reduced penetrance are factors that complicate genetic counselling of affected families. Future studies on the role of additional modifying factors are therefore needed.

Possible therapeutic options in COL4A1 and COL4A2 mutations

Treatment of the cultured cells from the same child harbouring the *COL4A2* G702D mutation with 4-phenylbutyrate (PBA), a chemical chaperone that can reduce ER stress levels, led to a reduced intracellular accumulation of COL4A2 and a decrease in ER volume, as well as a decrease in ER stress and UPR markers and reduced levels of apoptosis[7]. This may be due to an increased protein folding in the ER. Also ascorbic acid (Vitamin C precursor) has been shown to improve protein folding, secretion and extracellular deposition of type IV collagen in cell cultures[15,16]. These are interesting findings, however, these results were only obtained in an *in vitro* system. The question whether this would be a future treatment option suitable in human disease caused by mutations leading to protein misfolding will need further investigation. The problem will mainly be to design an elective chemical chaperone that does not interfere with overt post-translational modification.

USP18 mutations

USP18 mutation phenotype

We identified a new autosomal recessive syndrome due to *USP18* mutations, associated with severe cerebral hemorrhage and "pseudo-TORCH" syndrome. The family in which we identified the first *USP18* mutations came to our attention due to the presence of severe cerebral hemorrhage in the absence of a *COL4A1* or *COL4A2* mutation. Essential features of the affected individuals were extensive cerebral hemorrhage, presenting either prenatally or soon after birth, and early demise. The hemorrhage was predominantly located in the area of the basal ganglia, brain stem, cerebellar peduncles and lateral ventricles. Additional features were cerebral calcifications, extensive periventricular white matter abnormalities and cortical necrosis. It was not until the identification of the second family that we appreciated additional features as clear parts of the phenotypic spectrum. Besides the cerebral phenotype, thrombocytopenia, liver failure with ascites and severe respiratory problems were overlapping features. Novel findings in the second family were unilateral diaphragmatic paresis, thin ribs and cervical and axillary calcifications. These findings indicate that, although the cerebrovascular phenotype is most prominent, *USP18* mutations cause a systemic disorder. This is in line with the wide expression of *USP18*, with expression in heart, liver, lung, heart, kidney, thymus, spleen, pancreas, bone marrow and brain. However, the brain seems to be particularly susceptible. The identification of USP18 mutations with high recurrence risk provides the possibility of counselling to the families and prenatal diagnosis.

Both mouse *Usp18* knockout models and findings in a patient from our first family indicate ependymal pathology underlying at least part of the cerebral phenotype. Ependymal pathology and alterations of the subventricular zone have been described in several mouse models with hydrocephalus, cerebral hemorrhage and/ or neuronal migration defects[17-24] and were also identified in human foetuses with a communicating hydrocephalus[25], sudden infant death syndrome[26] and foetal spina bifida aperta[27]. However, to our knowledge, this is the first example of a human gene mutation where the ependymal layer is primary involved. It is possible that milder mutations leading to reduced USP18 expression may lead to prolonged survival, or, in line with the mouse models, to ependymal dysfunction with hydrocephalus due to Silvii aqueduct stenosis. Screening of hydrocephalus patient cohorts for *USP18* mutations might therefore be warranted.

Pathogenic mechanisms of USP18 mutations

USP18 is expressed after IFN type I signalling and acts an inhibitor of the IFNAR-2 receptor. It has an important function in the suppression of the activation of the innate immune response, probably in order to reduce toxic effects secondary to this activation in the vulnerable brain tissue.

Differential diagnostic considerations in our patients were a congenital viral infection or Aicardi-Goutières syndrome, a known cause of " pseudo-TORCH" syndrome. The IFN type I signalling pathway is also a common pathway involved in these groups of disorders. Upon viral infection, an increased IFN type I signalling is observed, leading to an inhibition viral replication within the cells. In Aicardi-Goutières syndrome, an inadequate processing of cellular nucleic acid debris eventually triggers the innate immune response, leading to induction of IFNα expression and again to an upregulation of the IFN type I signalling pathway that in turn leads to cerebrovascular damage[28,29]. In Aicardi-Goutières syndrome, this effect is indirect and the exact mechanism that leads to the upregulated IFN type I signalling is not clear.

USP18 mutations underly a new cerebrovascular disorder with a direct link to the IFN type I signalling pathway. USP18 normally functions as a sort of feedback regulator of the IFN signalling pathway. As a consequence, in the absence of USP18 the IFN type I signalling pathway might be up-regulated. *Usp18* mouse knockouts die of hydrocephalus and cerebral hemorrhage because of the loss of inhibition of the IFN type I signalling. This mechanism, rather than the lack of deISGylation, seems to be the underlying cause of cerebral phenotype. However, further studies in humans are needed to address this question. It will be interesting to test whether USP18 interacts with the known genes for Aicardi-Goutières, which are known to regulate RNA turnover. This question could be approached by testing double knock-out animal models or by *in vitro* proteomics and protein interaction analysis.

Incidence of USP18 mutations

The incidence of *USP18* mutations is yet unknown. A review of brain imaging data of 555 patients admitted to the Dept. of Neonatology with infantile cerebrovascular disease failed to identify additional patients with brain phenotypes resembling our patients harbouring *USP18* mutations. In addition, although the number is small, sequencing of *USP18* in a cohort of 38 patients with isolated prenatal or infantile cerebral hemorrhage patients did not show mutations.

Possible therapeutic options in USP18 mutations

For *USP18* mutations, interfering in the IFN type I signalling pathway may be a theoretically interesting therapeutic approach. IFN type I signalling is also abnormal in Aicardi-Goutières syndrome and pathways abnormalities are reflected in specific "interferon signatures" in patient blood[30]. Mutations in *TREX1* can cause both AGS and systemic lupus erythematosus (SLE), a common auto-immune disorder with an upregulated IFN type I signalling. For SLE, one of the therapeutic options under development is specific targeting of the IFN type I signalling pathway. Anti-IFNα monoclonal antibody and an IFNα vaccine are used in therapeutical trials[31]. Another option under development for SLE is to use an inhibitor of the IFNAR receptor[31]. Recently, the BRISC-SHMT complex was identified, that localizes to the IFNAR1 and deubiquinates actively engaged IFNAR1. This limits its internalization and lysosomal degradation. BRISC deficient cells and mice show decreased (but not absent) responses to IFN and seem protected from IFN-associated immunopathology[32]. In case of a similar dysregulation of the IFN type I signalling in *USP18* mutations, a similar therapeutic approach may be justified.

The question remains whether interference in the IFN type I signalling pathway in the *USP18* group of patients is a realistic treatment option. Due to the early, even prenatal, onset of symptoms, it seems unlikely that the occurrence of symptoms can be prevented. A possible slowing of disease progression might be expected, however, whether this will influence the quality of life of patients in a positive manner remains to be elucidated. However, our patients are likely to represent the severe end of the *USP18* mutation spectrum. When milder phenotypes related to *USP18* mutations might be identified, these possible therapeutic options may become more realistic. The first therapeutic trials might therefore be performed in animal models, for example a transgenic mouse with a milder *Usp18* mutation. When treatment options would appear to be present, it is to be expected that a lifelong treatment is warranted.

Genetics of perinatal hemorrhagic stroke and in general cerebrovascular disease: Future perspectives

A proper clinical stratification has been instrumental and will be essential in order to find genetic factors in childhood cerebral hemorrhage. This requires a multidisciplinary approach involving clinical genetics, radiology, neonatology and child neurology.

Next generation sequencing (NGS) will increasingly be applied as first tool to identify novel genes for these disorders. The challenge remains to prove pathogenicity of identified variants. Since the mouse model of *USP18* mutations proved crucial in the interpretation of the identified mutations in our family, the use of known mouse models with cerebral hemorrhage may help in data analysis of future patients. Several mouse models exist that show cerebral hemorrhage, with knockout of genes that have no matching human phenotypes to date (Table 1).

When mutations in novel candidate genes of unknown function are identified, animal models in order to prove pathogenicity will prove invaluable. For cerebral hemorrhage, zebrafish models have already proven to be efficient in this respect. A high similarity exists between the anatomical form of the developing vasculature and molecular mechanisms underlying vessel formation of the zebrafish and humans. By using morpholinos, synthetic oligonucleotides of 25 basepairs that can disrupt translation of a specific gene, the effect of knockdown of the candidate gene on cerebral vasculature can be studied. The zebrafish has been used as an animal model for cerebral hemorrhage, e.g. due to mutations in hereditary cerebral cavernous malformations[33,34].

In the ideal setting, facilities for high throughput functional studies need to complement high throughput genomic analysis tools in order to accelerate the discovery of genetic factors in childhood cerebrovascular disorders and ultimately to improve patient care.

Concluding remarks

In summary, our findings provide additional evidence that genetic mutations can cause a broad range of cerebrovascular diseases at young age.

The aim of this study was to describe the spectrum of collagen IV related disease and to identify the genetic cause of cerebrovascular disorders at young age, particularly of the poorly understood perinatal hemorrhagic stroke. After starting the work, it soon became clear that the phenotypic spectrum of the collagen IV disease is much wider than porencephaly, that the genetic cause of hemorrhagic stroke at young age is highly heterogeneous and largely undiscovered, that in the perinatal period developmental defects often intermingle with vascular disorders and that genetic causes can underly phenotypes leading to both arterial hemorrhagic and ischemic stroke.

It can therefore be difficult to classify these disorders when they affect foetuses, newborns and young children according to criteria used for adult stroke. I will support this conclusion by some examples.

1. The spectrum of *COL4A1* and *-2* mutation indicates that the disorders are not restricted to congenital porencephaly but can include a wider cerebrovascular disorder, with diffuse white matter involvement and functional deficits that go beyond the focal lesions of the hemorrhage and even including developmental defects in multiple organs. As shown in chapter 4.1, practically the whole brain can be destroyed in the process, or, as described in chapter 4.2, the brain can be relatively spared while other organs, such the eyes, can totally lack normal structure and lose their function.

2. In porencephaly caused by *COL4A1* and *-2* mutations, a presumably early (pre- peri- or early postnatal) hemorrhage leads to secundary venous infarction: the occurrence of these two events indicates that the border between arterial versus venous and hemorhage versus infarct can be blurred. The same can be postulated for the disease caused by *ACTA2* mutations (chapter 2.1). Originally *ACTA2* mutations have been linked to thoracic aneurysms. We show, confirming earlier literature data that mutations in specific amino acids (R179) in the protein lead to a totally different systemic disorder with vascular and central and peripheral nervous system involvement and no evidence for aneuryms, hereby underscoring the extreme variability of the disorder.

3. In animal models, *Usp18* null-mutations cause stenosis of the aqueduct of Sylvius, ependymal necrosis and hydrocephalus, while in our family 1 the disorder appears to be systemic and to cause essentially early intrauterine haemorrhage and severe cerebral destruction, while in the second family, besides destructive lesions, also cerebellar hypoplasia and cortical gyral abnormalities were found. The best classification of the human USP18 "null-disorder" is within pseudo-TORCH syndrome, with a clear hemorrhagic component, but we expect that milder mutations might be found among patient with aqueduct stenosis and or cerebellar hypoplasia, with or without hemorrhage.

In Figure 1, the known causes of perinatal, infantile and childhood cerebral hemorrhage are summarized. Considering the overlap between the clinical phenotypes of the different disorders, it may be useful to develop a targeted NGS panel for perinatal, infantile and childhood cerebral hemorrhage.

In clinical practice, we still encounter neonates with cerebral hemorrhage, with similar brain findings to e.g. *COL4A1* and *COL4A2* mutations, without clear cause. We therefore presume that more, probably rare, genetic contributing factors are to be found. The identification of these factors is a challenge, since they may have reduced penetrance and variable expression, and may interact with additional, non-genetic factors. Next generation sequencing techniques used in large patient cohorts will provide possibilities to identify novel genetic factors. The broad knowledge of molecular pathways linked to specific symptoms can sustain the clinician in his/her search for an aetiological diagnosis. In view of the "genotyping first" approach that will characterize the near future of clinical genetics, the modern geneticist might need to subspecialize in molecular mechanisms and become a pathway expert that interprets the molecular data in view of this knowledge. In order to prove pathogenicity of identified variants, support of these techniques by facilities for high throughput functional studies, for example in animal models, are essential.

References

1. **Aden U, Lin A, Carlo W, et al.** Candidate gene analysis: severe intraventricular hemorrhage in inborn preterm neonates. The Journal of pediatrics 2013;163:1503-6 e1.

2. **Jeanne M, Labelle-Dumais C, Jorgensen J, et al.** COL4A2 mutations impair COL4A1 and COL4A2 secretion and cause hemorrhagic stroke. Am J Hum Genet 2012;90:91-101.

3. **Weng YC, Sonni A, Labelle-Dumais C, et al.** COL4A1 mutations in patients with sporadic late-onset intracerebral hemorrhage. Annals of neurology 2012;71:470-7.

4. **Van Agtmael T, Schlotzer-Schrehardt U, McKie L, et al.** Dominant mutations of Col4a1 result in basement membrane defects which lead to anterior segment dysgenesis and glomerulopathy. Hum Mol Genet 2005;14:3161-8.

5. **Plaisier E, Gribouval O, Alamowitch S, et al.** COL4A1 mutations and hereditary angiopathy, nephropathy, aneurysms, and muscle cramps. N Engl J Med 2007;357:2687-95.

6. **Verbeek E, Meuwissen ME, Verheijen FW, et al.** COL4A2 mutation associated with familial porencephaly and small-vessel disease. Eur J Hum Genet 2012.

7. **Murray LS, Lu Y, Taggart A, et al.** Chemical chaperone treatment reduces intracellular accumulation of mutant collagen IV and ameliorates the cellular phenotype of a COL4A2 mutation that causes haemorrhagic stroke. Hum Mol Genet 2013.

8. **Ghazi-Birry HS, Brown WR, Moody DM, Challa VR, Block SM, Reboussin DM.** Human germinal matrix: venous origin of hemorrhage and vascular characteristics. AJNR American journal of neuroradiology 1997;18:219-29.

9. **Raets MM, Dudink J, Govaert P.** Neonatal disorders of germinal matrix. The journal of maternal-fetal & neonatal medicine : the official journal of the European Association of Perinatal Medicine, the Federation of Asia and Oceania Perinatal Societies, the International Society of Perinatal Obstet 2013.

10. **Wolburg H, Lippoldt A.** Tight junctions of the blood-brain barrier: development, composition and regulation. Vascular pharmacology 2002;38:323-37.

11. **Mochida GH, Ganesh VS, Felie JM, et al.** A homozygous mutation in the tight-junction protein JAM3 causes hemorrhagic destruction of the brain, subependymal calcification, and congenital cataracts. Am J Hum Genet 2010;87:882-9.

12. **O'Driscoll MC, Daly SB, Urquhart JE, et al.** Recessive mutations in the gene encoding the tight junction protein occludin cause band-like calcification with simplified gyration and polymicrogyria. Am J Hum Genet 2010;87:354-64.

13. **Plaisier E, Chen Z, Gekeler F, et al.** Novel COL4A1 mutations associated with HANAC syndrome: A role for the triple helical CB3[IV] domain. Am J Med Genet A 2010.

14. **Meuwissen ME, de Vries LS, Verbeek HA, et al.** Sporadic COL4A1 mutations with extensive prenatal porencephaly resembling hydranencephaly. Neurology 2011;76:844-6.

15. **Kishimoto Y, Saito N, Kurita K, Shimokado K, Maruyama N, Ishigami A.** Ascorbic acid enhances the expression of type 1 and type 4 collagen and SVCT2 in cultured human skin fibroblasts. Biochemical and biophysical research communications 2013;430:579-84.

16. **Fichard A, Tillet E, Delacoux F, Garrone R, Ruggiero F.** Human recombinant alpha1(V) collagen chain. Homotrimeric assembly and subsequent processing. J Biol Chem 1997;272:30083-7.

17. **Mori N, Kuwamura M, Tanaka N, et al.** Ccdc85c encoding a protein at apical junctions of radial glia is disrupted in hemorrhagic hydrocephalus (hhy) mice. The American journal of pathology 2012;180:314-27.

18. **Paez P, Batiz LF, Roales-Bujan R, et al.** Patterned neuropathologic events occurring in hyh congenital hydrocephalic mutant mice. Journal of neuropathology and experimental neurology 2007;66:1082-92.

19. **Peng X, Lin Q, Liu Y, et al.** Inactivation of Cdc42 in embryonic brain results in hydrocephalus with ependymal cell defects in mice. Protein & cell 2013;4:231-42.

20. **Fredriksson L, Nilsson I, Su EJ, et al.** Platelet-derived growth factor C deficiency in C57BL/6 mice leads to abnormal cerebral vascularization, loss of neuroependymal integrity, and ventricular abnormalities. The American journal of pathology 2012;180:1136-44.

21. **Qin S, Liu M, Niu W, Zhang CL.** Dysregulation of Kruppel-like factor 4 during brain development leads to hydrocephalus in mice. Proc Natl Acad Sci U S A 2011;108:21117-21.

22. **Lavado A, Oliver G.** Six3 is required for ependymal cell maturation. Development 2011;138:5291-300.

23. **Baas D, Meiniel A, Benadiba C, et al.** A deficiency in RFX3 causes hydrocephalus associated with abnormal differentiation of ependymal cells. The European journal of neuroscience 2006;24:1020-30.

24. **Davy BE, Robinson ML.** Congenital hydrocephalus in hy3 mice is caused by a frameshift mutation in Hydin, a large novel gene. Hum Mol Genet 2003;12:1163-70.

25. **Dominguez-Pinos MD, Paez P, Jimenez AJ, et al.** Ependymal denudation and alterations of the subventricular zone occur in human fetuses with a moderate communicating hydrocephalus. Journal of neuropathology and experimental neurology 2005;64:595-604.

26. **Lavezzi AM, Corna MF, Matturri L.** Ependymal alterations in sudden intrauterine unexplained death and sudden infant death syndrome: possible primary consequence of prenatal exposure to cigarette smoking. Neural development 2010;5:17.

27. **Sival DA, Guerra M, den Dunnen WF, et al.** Neuroependymal denudation is in progress in full-term human foetal spina bifida aperta. Brain Pathol 2011;21:163-79.

28. **Chahwan C, Chahwan R.** Aicardi-Goutieres syndrome: from patients to genes and beyond. Clin Genet 2012;81:413-20.

29. **Crow YJ, Rehwinkel J.** Aicardi-Goutieres syndrome and related phenotypes: linking nucleic acid metabolism with autoimmunity. Hum Mol Genet 2009;18:R130-6.

30. **Rice GI, Forte GM, Szynkiewicz M, et al.** Assessment of interferon-related biomarkers in Aicardi-Goutieres syndrome associated with mutations in TREX1, RNASEH2A, RNASEH2B, RNASEH2C, SAMHD1, and ADAR: a case-control study. Lancet neurology 2013;12:1159-69.

31. **Kirou KA, Gkrouzman E.** Anti-interferon alpha treatment in SLE. Clin Immunol 2013;148:303-12.

32. **Zheng H, Gupta V, Patterson-Fortin J, et al.** A BRISC-SHMT complex deubiquitinates IFNAR1 and regulates interferon responses. Cell reports 2013;5:180-93.

33. **Gore AV, Monzo K, Cha YR, Pan W, Weinstein BM.** Vascular development in the zebrafish. Cold Spring Harb Perspect Med 2012;2:a006684.

34. **Butler MG, Gore AV, Weinstein BM.** Zebrafish as a model for hemorrhagic stroke. Methods Cell Biol 2011;105:137-61.

35. **Srivastava M, Atwater I, Glasman M, et al.** Defects in inositol 1,4,5-trisphosphate receptor expression, Ca(2+) signaling, and insulin secretion in the anx7(+/-) knockout mouse. Proc Natl Acad Sci U S A 1999;96:13783-8.

36. **Meyer C, Zizioli D, Lausmann S, et al.** mu1A-adaptin-deficient mice: lethality, loss of AP-1 binding and rerouting of mannose 6-phosphate receptors. The EMBO journal 2000;19:2193-203.

37. **Kwon YT, Kashina AS, Davydov IV, et al.** An essential role of N-terminal arginylation in cardiovascular development. Science 2002;297:96-9.

38. **Lotz K, Pyrowolakis G, Jentsch S.** BRUCE, a giant E2/E3 ubiquitin ligase and inhibitor of apoptosis protein of the trans-Golgi network, is required for normal placenta development and mouse survival. Molecular and cellular biology 2004;24:9339-50.

39. **Kostka G, Giltay R, Bloch W, et al.** Perinatal lethality and endothelial cell abnormalities in several vessel compartments of fibulin-1-deficient mice. Molecular and cellular biology 2001;21:7025-34.

40. **Spyropoulos DD, Pharr PN, Lavenburg KR, et al.** Hemorrhage, impaired hematopoiesis, and

lethality in mouse embryos carrying a targeted disruption of the Fli1 transcription factor. Molecular and cellular biology 2000;20:5643-52.

41. **McCarty JH, Monahan-Earley RA, Brown LF, et al.** Defective associations between blood vessels and brain parenchyma lead to cerebral hemorrhage in mice lacking alphav integrins. Molecular and cellular biology 2002;22:7667-77.

42. **Vogel P, Read RW, Hansen GM, et al.** Congenital hydrocephalus in genetically engineered mice. Veterinary pathology 2012;49:166-81.

43. **Krebs DL, Metcalf D, Merson TD, et al.** Development of hydrocephalus in mice lacking SOCS7. Proc Natl Acad Sci U S A 2004;101:15446-51.

44. **Yang A, Walker N, Bronson R, et al.** p73-deficient mice have neurological, pheromonal and inflammatory defects but lack spontaneous tumours. Nature 2000;404:99-103.

45. **Kaneko-Oshikawa C, Nakagawa T, Yamada M, et al.** Mammalian E4 is required for cardiac development and maintenance of the nervous system. Molecular and cellular biology 2005;25:10953-64.

46. **Whitehead KJ, Smith MC, Li DY.** Arteriovenous malformations and other vascular malformation syndromes. Cold Spring Harbor perspectives in medicine 2013;3:a006635.

47. **Revencu N, Vikkula M.** Cerebral cavernous malformation: new molecular and clinical insights. J Med Genet 2006;43:716-21.

48. **Kronenburg A, Braun KP, van der Zwan A, Klijn CJ.** Recent advances in moyamoya disease: pathophysiology and treatment. Current neurology and neuroscience reports 2014;14:423.

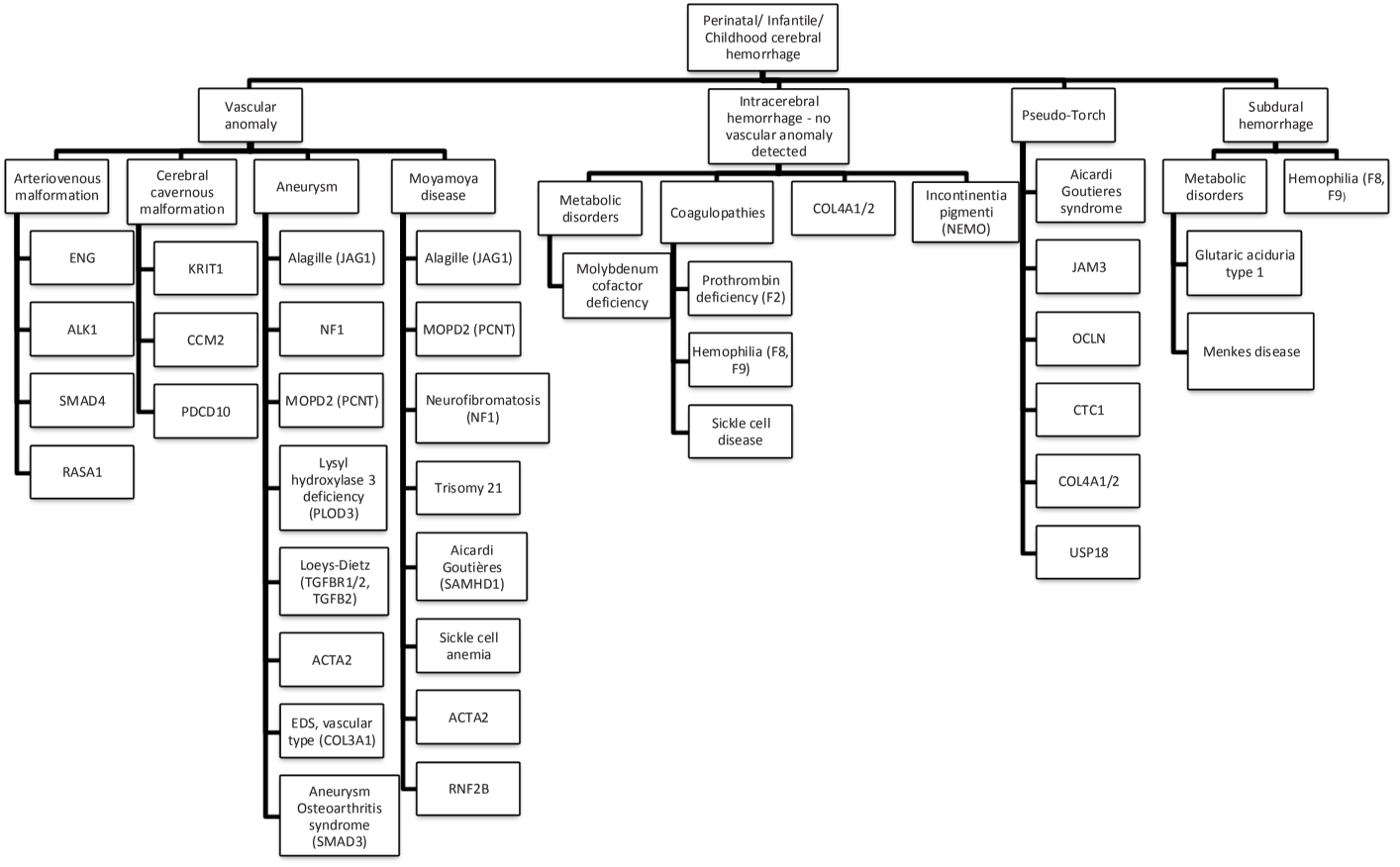


Figure 1. Differential diagnostic considerations in perinatal, infantile and childhood cerebral hemorrhage.

Arteriovenous malformation is defined as an abnormal connection between arteries and veins, bypassing the capillary system[46]. Cerebral cavernous malformations are vascular malformation characterised by closely clustered enlarged capillary-like channels with a single layer of endothelium without intervening brain parenchyma[47]. Moyamoya disease is defined as progressive bilateral stenosis of the distal portion of the internal carotid artery and the proximal anterior and middle cerebral arteries[48]. Intracerebral hemorrhage is defined as intraparenchymal and intraventricular hemorrhage.

Table 1. Mouse models of cerebral hemorrhage without associated human disease

Gene (OMIM)	Mutation type	Protein	Protein function	Cerebrovascular phenotype	Additional features	Refs
Anxa7 (186360)	Knock-out	Annexin A7	Ca2+/ GTP-dependent membrane fusion and ion channel protein. Role in regulating exocytic secretion in neuroendocrine cells.	Lethality at E10 due to cerebral intraventricular hemorrhage	No	32
Ap1m1 (603535)	Knock-out	Adaptor-related protein complex 1, MU-1 subunit	Subunit of clathrin-associated adaptor complex 1 that plays a role in protein sorting in the trans-Golgi network and endosomes	Hemorrhage in ventricles and spincal canal at E10.5	Embryonic lethality at E13.5	33
Ate1 (607103)	Knock-out	Arginyltransferase 1	Arginylation of the N-terminal part of proteins	Hemorrhages, including cerebral	Pale, thinner blood vessels, skin edema, VSD, ASD, hypoplasia of myocardium, truncus arteriosus	34
Birc6 (605638)	Knock-out	Baculoviral IAP repeat-containing protein 6	E2/E3 Ubiquitin ligase and inhibitor of apoptosis protein of the*trans*-Golghi network	Hemorrhage in ventricles and neural tube	Perinatal lethality after E14, growth retardation, pale skin, subcuatneous hemorrhage, edema	35
Ccdc85c (-)	Knock-out	Coiled-coil domain containing 85C	May play an important role in cortical development, especially in the mainainance of radial glia. Expressed in wall of lateral ventricles	Hydrocephalus, brain hemorrhage, subcortical heterotopia, agenesis of ependymal layer		15
Fbln1 (135820)	Knock-out	Fibulin 1	Extracellular matrix protein in vessel walls	Hemorrhages in telencephalon and spinal cord	Postnatal lethality, growth retardation, hemorrhages in snout and limbs, petechiae, delayed lung development, abnormal renal glomeruli	36
Fli1 (193067)	Knock-out	Friend leukemia virus integration 1	Transcription factor that binds consensus sequence GGA(A/T), role in embryogenesis, vascular development and megakaryopoiesis	Hemorrhages in ventricles and neural tube at E11	Lethality at E12.5, abnormal liver hematopoiesis	37

(Continued on next page)

Table 1. Mouse models of cerebral hemorrhage without associated human disease *(continued from previous page)*

Gene (OMIM)	Mutation type	Protein	Protein function	Cerebrovascular phenotype	Additional features	Refs
Itga5 (135620)	Knock-out	Integrin alpha-5	Fibronectin receptor, mediates binding of cells to fibronectin substrates	Cerebral blood vessel dilatation and hemorrhage from ganglionic eminences, hydrocephalus	Lethality within hours after birth	38
Kif27 (611253)	Knock-out	Kinesin family member 27	Member of the kinesin family. Plays an essential role in motile ciliogenesis	Hydrocephalus, intraventricular hemorrhage	Lethality at P56, growth retardation	39
Nme (603575)	Knock-out	Nonmetastatic cells 5, protein expressed in	Major role in synthesis of nucleoside triphosphates other than ATP. Required for neural development including neural patterning and cell-fate determination	Hydrocephalus, intraventricular hemorrhage	Doming of skull, impaired spermatogenesis	39
Socs7 (608788)	Knock-out	Suppressor of cytokine signaling 7	Regulates signaling cascades through protein ubiquitination and/ or sequestration. Prevents STAT3 and STAT5 activation.	Hydrocephalus, cerebral hemorrhage, thin cerebral cortex	Decreased survival, mild growth retardation	40
TP73 (601990)	Knock-out	Tumor protein 73	Member of the p53 family of transcription factors involved in cellular responses to stress and development. Participates in apoptotic responses to DNA damage	Intracranial and intraventricular hemorrhage, hydrocephalus, abnormal hippocampus	Reduced postnatal survival, chronic inflammation, gastrointestinal hemorrhage, decreased body size, cachexia, carcinoma	41
Ube4b (613565)	Knock-out	Ubiquitination factor E4B	Role in ubiquitination, catalyzes ubiquitin chain assembly	Massive ventricular hemorrhage at E 12.5-E13.5	Intrauterine lethality, hemorrhage in cervical and abdominal subcutaneous tissue, congestive heart failure and cardiac apoptosis	42
Ulk4 (-)	Knock-out	Unc-51-like kinase 4	Unknown	Hydrocephalus, intraventricular hemorrhage, fibrosis and neovascularization of meninges and choroid	Reduced postnatal survival	39

SUMMARY

In this thesis, our work on genetic causes of childhood cerebrovascular disease is described, with a main focus on infantile cerebral hemorrhage. Our primary goal was to improve the diagnostics in this group of children, in order to provide better counselling of families. Since little was known, we wanted to gain insight in the contribution of genetic factors in this group of patients. In addition, when known causes of cerebral hemorrhage were excluded, we wanted to identify novel genetic causes for childhood cerebral hemorrhage.

In **chapter 1**, the known genetic causes of childhood cerebral hemorrhage are provided. The two main pathophysiological processes described in this thesis are introduced, comprising structural changes affecting the blood-brain barrier integrity and dysregulation of inflammation. Also, the diseases related to these pathophysiological processes are summarized. The outline of the thesis is presented.

Chapter 2 includes descriptions of other causes of childhood cerebrovascular disease.

In **chapter 2.1** a case report is provided on a toddler girl with a systemic disorder comprising primary pulmonary hypertension, persistent ductus arteriosus, extensive cerebral white matter lesions, fixed dilated pupils, intestinal malrotation, and hypotonic bladder. Recently, de novo ACTA2 R179H substitutions were associated with a similar phenotype and additional cerebral developmental defects. In our patient, an *ACTA2* R179C substitution was identified. She showed previously undescribed abnormal lobulation of the frontal lobes and position of the gyrus cinguli and rostral dysplasis of the corpus callosum together with cerebrovascular developmental defects; she died at the age of 3 years during surgery due to vascular fragility and rupture of the ductus arteriosus. Altogether these observations support a role of *ACTA2* in brain development, especially related to the arginine at position 179.

Chapter 2.2 comprises a review of literature about neurological complications of Incontinentia Pigmenti, is a rare X-linked multisystem disorder with well described and

pathognomonic skin manifestations. Neurological manifestations are found in 30% of IP patients, forming one of the major causes of morbidity and mortality of the condition. The clinical and brain imaging data of 45 IP patients described in literature with a neurological phenotype were reviewed. Several clinical presentations could be identified, comprising seizures, infantile encephalopathy, acute disseminated encephalomyelitis and ischemic stroke. Most neurological features presented during the neonatal period. No patients presented during adolescence or at adult age. Brain MRI findings included periventricular and subcortical white matter disease, hemorrhagic changes, corpus callosum hypoplasia, cerebral atrophy and cerebellar hypoplasia. Most findings may reflect changes following brain injury. Both (ischemic) vascular and inflammatory components may play a role in the cerebral and ocular phenotype. However, a role of disturbed apoptosis during development may also be a contributing factor.

In **chapter 3**, an overview is provided of the results of *COL4A1* and *COL4A2* testing of 183 index patients at the Dept. of clinical genetics of the Erasmus University Medical Center since 2006. In 13%, a *COL4A1* or *COL4A2* mutation was detected (21 *COL4A1* and 3 *COL4A2* mutations). The clinical data in 13 novel families harbouring *COL4A1* or *COL4A2* mutations are provided. A high percentage of de novo mutations (38%) was found in our cohort. Several clinical phenotypes were identified. Novel findings included the first case of focal cortical dysplasia in a patient with a *COL4A2* mutation. In addition a review of the clinical spectrum of *COL4A1* and *COL4A2* mutations in literature is provided, and several clinical phenotypes are described in more detail. Our cohort did not include cases of stroke in young adulthood or sporadic late-onset hemorrhagic stroke, which probably reflects a referral bias in our cohort. A protocol for initial screening of *COL4A1* and *COL4A2* patients is provided, giving an outline for clinical management. However, clinical follow-up data, also of apparently unaffected mutation carriers, are needed in order to develop appropriate screening protocols and adapt treatment.

In **chapter 4**, our experience with several specific *COL4A1* mutation phenotypes is described.

Chapter 4.1 describes the most severe end of the *COL4A1* mutation spectrum, comprising of severe brain destruction resembling hydranencephaly due to *de novo* mutations in four affected infants .

Chapter 4.2 gives a description of the severe ophthalmological phenotype of anterior segment dysgenesis disorder (ASDD) with cataract, microcornea, microphthalmia and tunnel vision in a family with three affected patients. Interestingly, the combination of ASDD, and additional neurological disease in one and brain MRI findings of periventricular leukomalacia in a second patient prompted us to test *COL4A1*. Neurological examination and brain MRI are suggested as useful tools in the diagnostic workup of ASDD patients in order to suspect the diagnosis of a *COL4A1* or *COL4A2* mutation.

Chapter 4.3 provides the description of the first putatively pathogenic homozygous *COL4A1* variant affecting the VR3 docking site of the *COL4A1* NC1 domain in a patient with severe porencephaly, polymicrogyria, corneal clouding, Rieger anomaly and microcephaly. Austosomal recessive inheritance has been reported in the very homologous *COL4A3* and *COL4A4* gene, related to Alport syndrome. The open question remains whether this is the cause of the disease and whether autosomal recessive inheritance is a general mechanism in *COL4A1*. Additional cases are needed to make a definite statement regarding this question, which is very important regarding genetic counselling of families.

In **chapter 5**, we describe the identification of *COL4A2* mutations in two families, using a candidate gene approach. Mutation phenotypes varied, including porencephaly, white matter lesions, cerebellar and optic nerve hypoplasia and unruptured carotid aneurysm. In the second family however, we found evidence for additional factors contributing to the phenotype. Fragmentation and duplication of epidermal basement membranes were observed by electron microscopy in a c.3206delC patient skin biopsy, consistent with abnormal collagen IV network. Collagen chain accumulation and endoplasmic reticulum (ER) stress have been proposed as cellular mechanism in *COL4A1* mutations. In *COL4A2* 3206delC fibroblasts we detected increased rates of apoptosis but no signs of ER stress. Based on our findings, we conclude that dominant *COL4A2* mutations are a novel major risk factor for familial cerebrovascular disease, including porencephaly and small-vessel disease with reduced penetrance and a variable phenotype, which might also be modified by other contributing factors.

In **chapter 6**, the clinical and molecular findings of two families with pseudo-TORCH syndrome and in addition severe cerebral hemorrhage and early demise, who harbour autosomal recessive mutations in *USP18*. *Usp18* mouse knockout models have already showed cerebral hemorrhage and hydrocephalus with ependymal necrosis. Immunohistochemistry

on patient brain tissue shows a very abnormal brain ependyme. Our findings hereby represent the first genetic disorder linked to ependymal cell dysfunction. USP18, an interferon (IFN) stimulated gene, plays a role in immune response, i.e. by inhibiting IFN-type I signalling in cells. The mutation is likely to cause upregulated intracellular IFN type I signalling, similar to findings in viral infections or Aicardi Goutières syndrome, both conditions that can present with infantile cerebrovascular disease. In Aicardi Goutières syndrome, however, this upregulation is an indirect effect triggered by an inadequate processing of cellular nucleic acid debris through mechanisms not clarified to date. *USP18* mutations form the first genetic disease that directly links the increased IFN type I signalling to the (cerebrovascular) phenotype of the affected patients.

Chapter 7 comprises the general discussion of the thesis.

SAMENVATTING

Dit proefschrift is een beschrijving van ons werk betreffende genetische oorzaken van cerebrovasculaire aandoeningen op de kinderleeftijd, met de nadruk op infantiele hersenbloedingen. Het belangrijkste doel was om de diagnostiek voor deze patiëntengroep te verbeteren, om een betere counseling te kunnen geven aan de betreffende families. Omdat nog niet veel bekend was over dit onderwerp, wilden we een beter inzicht krijgen in de bijdrage van genetische factoren in deze patiëntengroep. Bovendien wilden we nieuwe genetische factoren betrokken bij bloedingen op de kinderleeftijd identificeren, nadat bekende oorzaken werden uitgesloten.

In **hoofdstuk 1** worden de bekende oorzaken van cerebrale bloedingen op de kinderleeftijd samengevat. De twee belangrijkste pathofysiologische processen die in dit proefschrift worden beschreven worden geïntroduceerd, namelijk ten eerste structurele veranderingen die de integriteit van de bloed-hersen barrière-aantasten, en ten tweede ontregeling van de van de immuunrespons. De bekende cerebrovasculaire aandoeningen die worden veroorzaakt door deze processen worden beschreven. Ook wordt een overzicht van de inhoud van het proefschrift gegeven.

Hoofdstuk 2 bevat beschrijvingen van een tweetal voorbeelden van ischemische arteriopathieën op de kinderleeftijd. Hoofdstuk 6.1 bevat een beschrijving van een peuter met een systemische aandoening met primaire pulmonale hypertensie, persisterende ductus arteriosus, uitgebreide afwijkingen van de witte stof van de hersenen, verwijde pupillen, malrotatie van de darm en een hypotone blaas. Recent werden nieuw ontstane R179H mutaties in *ACTA2* geassocieerd met een vergelijkbaar ziektebeeld met hersenontwikkelingsstoornissen. In onze patiënt werd een R179C verandering aangetoond. Ze had een afwijkende lobulatie van de frontaalkwabben en afwijkende positie van de gyrus cingulus en een afwijkende aanleg van de hersenbalk, ook had ze afwijkingen van de hersenvaten. Ze overleed op de leeftijd van 3 jaar tijdens een operatie door extreme zwakte van de vaten en scheur van de ductus arteriosus. Deze bevindingen suggereren dat *ACTA2* een rol speelt in de hersen(vaat)ontwikkeling. **Hoofdstuk 2.2** is een review van de literatuur over de neuro-

logische complicaties van incontinentia pigmenti (IP), een zeldzame geslachtsgebonden multisysteemaandoening met goed gedocumenteerde en kenmerkende huidverschijnselen. Neurologische verschijnselen worden gevonden in 30% van de patiënten en vormen een van de belangrijkste oorzaken van morbiditeit en mortaliteit. De klinische kenmerken, data van de beeldvorming van de hersenen en de oogafwijkingen in 45 patiënten met IP met een neurologisch beeld die in de literatuur werden beschreven werden bekeken. Verschillende klinische presentaties konden worden geïdentificeerd, waaronder epilepsie, infantiele encefalopathie, acute disseminerende encefalomyopathie en ischemische beroerte. De meeste neurologische kenmerken begonnen vlak na de geboorte. Bij geen enkele patiënt begonnen de verschijnselen tijdens de puberteit of op volwassen leeftijd. Afwijkingen op MRI-scans van de hersenen waren periventriculaire en subcorticale wittestofafwijkingen, hemorrhagische veranderingen, corpus callosum hypoplasie, atrofie van de grote hersenen en onderontwikkeling van de kleine hersenen. De meeste bevindingen zijn waarschijnlijk het gevolg van hersenschade. Zowel (ischemische) vasculaire als ontstekingscomponenten kunnen een rol spelen in het ontstaan van de hersenafwijkingen en oogafwijkingen. Mogelijk speelt een verstoorde apoptose tijdens de ontwikkeling echter ook een rol.

In **hoofdstuk 3** wordt een overzicht gegeven van de resultaten van het testen van het *COL4A1* en *COL4A2* gen in 183 indexpatiënten, verricht op de afdeling Klinische Genetica van het Erasmus MC vanaf 2006. In 13% van de patiënten werd een *COL4A1* of een *COL4A2* mutatie aangetoond (21 COL4A1 en 3 COL4A2 mutaties). De klinische data van 13 nieuwe families met een *COL4A1* of *COL4A2* mutatie worden beschreven. In ons cohort werd een hoog percentage (38%) de novo mutaties gevonden. Verschillende klinische fenotypes werden geïdentificeerd. Nieuwe bevindingen waren onder andere de identificatie van een eerste patiënt met een *COL4A2* mutatie en focale corticale dysplasie. Aanvullend wordt een overzicht van het klinische spectrum van *COL4A1* en *COL4A2* mutaties vanuit de literatuur beschreven, en verschillende klinische fenotypes worden apart beschreven. In ons cohort werden geen patiënten opgenomen met hersenbloedingen op jong volwassen leeftijd of op oudere leeftijd, waarschijnlijk omdat de meeste verwijzingen kwamen vanuit de kinderneurologie en neonatologie. Een protocol met screenende onderzoeken die na de diagnose geadviseerd worden is beschreven. Het is van groot belang om meer klinische gegevens met betrekking tot het beloop van de aandoening te verkrijgen, ook van de personen die drager zijn van een mutatie zonder dat ze evidente verschijnselen hebben. Op basis hiervan is het mogelijk om geschikte protocollen te ontwikkelen voor screenings en om de behandeling eventueel aan te passen.

Hoofdstuk 4 bevat een beschrijving van onze ervaring met enkele bijzondere fenotypes geassocieerd met *COL4A1* mutaties. **Hoofdstuk 4.1** beschrijft de meest ernstige uitingsvorm van *COL4A1* mutaties in vier baby's, bestaande uit ernstige destructie van de hersenen, wat eruit ziet als hydranencefalie. Dit beeld wordt veroorzaakt door nieuw ontstane mutaties. **Hoofdstuk 4.2** geeft een beschrijving van een familie met drie aangedane familieleden die allen ernstige oogproblemen hebben bestaande uit een afwijkende aanleg van de voorste oogkamer met staar, te kleine ogen en tunnelvisie. Door de combinatie van de oogafwijkingen, samen met de aanwezigheid van neurologische afwijkingen bij een van de patiënten, en de aanwezigheid van periventriculaire leukomalacie op een MRI-scan bij haar zus, werd het *COL4A1* gen getest. In patiënten met ontwikkelingsstoornissen van de voorste oogkamer is het daarom zinvol om een neurologisch onderzoek en MRI-scan van de hersenen te verrichten, om een aanwijzingen voor de aanwezigheid van een *COL4A1* of *COL4A2* mutatie te vinden. **Hoofdstuk 4.3** bevat de eerste beschrijving van een mogelijk ziekteveroorzakende homozygote variant in de VR3 docking site van het NC1 domein van het *COL4A1* gen in een patiënt met ernstige porencefalie, polymicrogyrie, sluiering van het hoornvlies, Rieger anomalie en een te kleine hoofdomtrek. Autosomaal recessieve overerving is beschreven in de zeer homologe genen *COL4A3* en *COL4A4*, betrokken bij het Alport syndroom. De vraag blijft vooralsnog of de mutatie de oorzaak is van het ziektebeeld in deze patiënt en of autosomaal recessieve overerving van *COL4A1* mutaties mogelijk is. Aanvullende casus zijn nodig om deze vraag met meer zekerheid te beantwoorden , hetgeen van belang zou zijn voor de counseling van families.

In **hoofdstuk 5** wordt de identificatie van *COL4A2* mutaties in twee families beschreven als nieuwe oorzaak van erfelijke porencefalie. De ziektebeelden van de aangedane patiënten waren variabel en bevatten porencefalie, wittestofafwijkingen, een aneurysma van de halsslagader en onderontwikkeling van de kleine hersenen en van de oogzenuwen. In de tweede familie vonden we aanwijzingen voor aanvullende factoren die een bijdrage geleverd kunnen hebben aan het ziektebeeld. In een huidbiopt van een van de patiënten werd met electronenmicroscopie afwijkingen gezien in de basale membranen van de huid, passend bij een afwijkend collageen IV netwerk. Accumulatie van collageen ketens en endoplasmatisch reticulum stress werden eerder beschreven als mogelijk cellulair mechanisme in *COL4A1* mutaties. In fibroblasten van een van de patiënten met een 3206delC mutatie werd een toegenomen celdood (apoptose) gezien, maar tekenen van ER stress werden niet waargenomen. Op basis van onze bevindingen concluderen we dat *COL4A2* mutaties nieuwe belangrijke risicofactoren zijn voor familiale cerebrovasculaire aandoeningen, waaronder

porencefalie en small vessel disease, met een verminderde penetrantie en variabele uitingsvormen, waarbij andere bijdragende factoren mogelijk ook een rol spelen.

In **hoofdstuk 6** worden de klinische en moleculaire bevindingen beschreven in twee families met ernstige hersenbloedingen en een ernstig pseudo-TORCH syndroom, waarbij de aangedane baby's vroeg overlijden. Autosomaal recessieve mutaties in het *USP18* gen werden aangetoond. In muismodellen met een uitschakeling van het *USP18* gen werden al eerder hersenbloedingen en hydrocefalie gezien met necrose van de ependymlaag van de hersenventrikels. Immunohistochemie van hersenweefsel van een patiënt toonde een zeer afwijkende opbouw van het ependym. *USP18* speelt een rol in de immuunrespons en wordt gestimuleerd door interferon (IFN) en remt de type I IFN signalling. Door de mutatie is er waarschijnlijk een toegenomen IFN type I signalling. Dit is ook het geval in virusinfecties en in het Aicardi Goutières syndroom, met overlappende kenmerken. In Aicardi Goutières syndroom is deze upregulatie echter een indirect effect dat wordt veroorzaakt door de afwijkend verlopende verwerking van nucleïnezuren. Waarom dit leidt tot een upregulatie van de IFN type I signalling is vooralsnog niet duidelijk. *USP18* mutaties vormen de eerste genetische aandoening die het verband laat zien tussen een verhoogde IFN type I signalling en het (cerebrovasculaire) ziektebeeld van de aangedane patiënten.

Hoofdstuk 7 bevat de algemene discussie van het proefschrift.

CURRICULUM VITAE

Marije Meuwissen werd op 16 juli 1980 geboren te Leidschendam. Zij behaalde haar VWO diploma aan het Bisschoppelijk College Echt in 1998, waarna zij Geneeskunde studeerde aan de Universiteit Maastricht. Haar doctoraal Geneeskunde behaalde zij cum laude in 2002, gevolgd door het artsexamen in 2004. Tijdens haar coschappen volgde ze een keuze-coschap Klinische Genetica in het Erasmus MC te Rotterdam. Na het artsexamen ging zij werken als ANIOS (arts niet in opleiding tot specialist) neurologie in het Reinier de Graaf Gasthuis. In 2006 begon ze als ANIOS Klinische Genetica in het Erasmus MC te Rotterdam. In 2007 begon hier haar werk in het aandachtsgebied neurogenetica onder supervisie van Dr. G.M.S. Mancini. Deze samenwerking resulteerde uiteindelijk in dit proefschrift. In september 2008 begon zij aan haar opleiding tot klinisch geneticus in het Erasmus MC met Dr. J.A. Maat-Kievit als opleider. In december 2012 vond haar registratie als klinisch geneticus plaats. In 2012 ontving zij een onderzoeksbeurs van Fonds Nuts-Ohra tezamen met Dr. G.M.S. Mancini voor het ROSEN project (Rotterdam Onderzoek naar Stroke en Erfelijkheid bij Neonaten). Op dit project werkte zij in 2013 als arts-onderzoeker en was eveneens in deeltijd aangesteld als klinisch geneticus op de afdeling Klinische Genetica van het Erasmus MC. Sinds januari 2014 is zij werkzaam als klinisch geneticus op de afdeling Medische Genetica in het UZ Brussel. Zij is getrouwd met Ronald Grefhorst.

PHD PORTFOLIO

SUMMARY OF PHD TRAINING AND TEACHING ACTIVITIES

Name PhD Student:	Marije Meuwissen
Erasmus MC Department:	Clinical Genetics
PhD period:	2007-2014
Promotor:	Prof. Dr. R. Hofstra
Copromotor:	Dr. G.M.S. Mancini

PhD Training		Year	Workload (ECTS/ Hours)
General courses			
	Training course Regulation and Organisation for Clinical Researchers (BROK course) and exam, Rotterdam	2013	2
	Writing course "Write an article and get it published", Utrecht	2013	0.3
	CPO Minicourse "Methodologie van patiëntgebonden onderzoek en subsidieaanvragen", Rotterdam	2010	0.3
	Training (Discipline Overstijgend Onderwijs) Evidence Based Medicine, Rotterdam	2010	0.8
In-depth courses			
	MGC course Technological Facilities, Leiden	2013	0.3
	Training course "Grasduinen in Genome Browsers", Rotterdam	2011	0.3
	Fourth European course in Clinical Dysmorphology "What I know best", Rome, Italy	2011	0.6
	23th Course in Medical Genetics, Bologna, Italy	2010	1.4
	Course "Rekenen aan genen", Amsterdam	2009	2
	Course "Brain ultrasound: Asphyxia and congenital brain anomalies", Rotterdam	2007	0.6

Presentations			
	See "(Inter)national conferences"	2007-2013	
	2 oral presentations at the weekly Clinial Genetics Work Discussion		4
(Inter)national conferences			
	NVHG najaarssymposium, Arnhem (poster presentation)	2013	0.6
	European Human Genetics Conference, Paris, France (poster presentation)	2013	1.3
	Joint UK-Dutch meeting 2012, Newcastle, UK (oral presentation)	2012	0.6
	Second European Dysmorphology club, Rome, Italy (case presentation)	2011	0.4
	VKGN Assistenten Voordrachtendag, Rotterdam (oral presentation)	2011	0.3
	European Human Genetics Conference, Amsterdam (poster presentation)	2011	1.3
	11th international Congress European Society of Magnetic Resonance in Neuropediatrics (ESMRN), Amsterdam	2011	0.6
	Joint UK-Dutch meeting 2010, Amsterdam (oral presentation)	2010	0.6
	VKGN Assistenten Voordrachtendag, Maastricht (oral presentation)	2009	0.3
	19th European Meeting on Dysmorphology, Strasbourg, France	2008	0.6
	Inaugural Joint UK-Dutch Genetics Meeting, Liverpool, UK	2008	0.6
Teaching			
	Tutor Internship Camilla Heckmatt, biomedical student Dublin, Ireland	2013	2.5
	Minor Clinical Genetics, Rotterdam	2012	0.6
	Obstetric nurses, Albeda College, Rotterdam, "Clinical genetics and prenatal diagnostics"	2011	0.6
	Pediatrians, UMC Utrecht, Wilhelmina Kinderziekenhuis, "COL4A1 mutations"	2008	0.3

Other			
	Writing grant application Cerebral Palsy Alliance Research Foundation	2013	2
	Writing grant application Fonds NutsOhra (grant assigned of €100.000)	2012	2
	Writing grant application Cerebral Palsy International Research Foundation	2011	2
	Clinical Genetics Work Discussion (weekly)	2010-2013	160 hours
	Research group work discussion (weekly)	2008-2013	400 hours
	Journal clubs	2008-2013	45 hours

LIST OF PUBLICATIONS

Glucose transporter-1 deficiency syndrome: the expanding clinical and genetic spectrum of a treatable disorder. *Leen WG, Klepper J, Verbeek MM, Leferink M, Hofste T, van Engelen BG, Wevers RA, Arthur T, Bahi-Buisson N, Ballhausen D, Bekhof J, van Bogaert P, Carrilho I, Chabrol B, Champion MP, Coldwell J, Clayton P, Donner E, Evangeliou A, Ebinger F, Farrell K, Forsyth RJ, de Goede CG, Gross S, Grunewald S, Holthausen H, Jayawant S, Lachlan K, Laugel V, Leppig K, Lim MJ, Mancini G, Marina AD, Martorell L, McMenamin J,* ***Meuwissen ME****, Mundy H, Nilsson NO, Panzer A, Poll-The BT, Rauscher C, Rouselle CM, Sandvig I, Scheffner T, Sheridan E, Simpson N, Sykora P, Tomlinson R, Trounce J, Webb D, Weschke B, Scheffer H, Willemsen MA*. Brain (2010) 133:655-670.

Sporadic *COL4A1* mutations with extensive prenatal porencephaly resembling hydranencephaly. ***Meuwissen ME****, de Vries LS, Verbeek HA, Lequin MH, Govaert PP, Schot R, Cowan FM, Hennekam R, Rizzu P, Verheijen FW, Wessels MW, Mancini GM*. Neurology (2011) 76(9): 844-846.

COL4A2 mutation associated with familial porencephaly and small-vessel disease. *Verbeek E,* ***Meuwissen ME****, Verheijen FW, Govaert PP, Licht DJ, Kuo DS, Poulton CJ, Schot R, Lequin MH, Dudink J, Halley DJ, de Coo IR, den Hollander JC, Oegema R, Gould DB, Mancini GM*. Eur J Hum Genet (2012) 20(8): 844-851

Neurological findings in incontinentia pigmenti; a review. ***Meuwissen ME****, Mancini GM*. Eur J Med Genet (2012) 55(5): 323-331.

ACTA2 mutation with Childhood Cardiovascular, Autonomic and Brain anomalies and Severe Outcome. ***Meuwissen ME****, Lequin MH, Bindels- de Heus K, Bruggenwirth H, Knapen M, Dalinghaus M, De Coo IR, Van Bever Y, Winkelman B, Mancini GM*. Am J Med Genet Part A (2013) 161A:1376–1380

The expanding phenotype of *COL4A1* and *COL4A2* mutations; clinical data on 14 newly identified families and review of literature. ***Meuwissen ME**, Halley DJ, Smit LS, Lequin MH, Cobben JM, De Coo IR, Van Harssel J, Letteboer T, Sallevelt S, Woldringh G, Van der Knaap MS, De Vries LS, Mancini GM*. Article submitted.

Novel *COL4A1* mutation in familial anterior segment dysgenesis. ***Meuwissen ME**, Van der Knaap M, Van den Born LI, Hintzen RQ, Mancini GM*. Article submitted.

Is Autosomal Recessive inheritance possible in Hereditary Porencephaly due to *COL4A1* mutations? ***Meuwissen ME**, Smit LS, Go AT, Lequin MH, Halley DJ, Mancini GM*. Manuscript in preparation.

Interferon type 1 response regulator *USP18* is mutated in severe pseudo-TORCH syndrome. ***Meuwissen ME**, Schot R, Oudesluijs G, Tinchert S, Van Unen L, Heijsman D, Lequin MH, Kros M, Willemsen R, Brouwer R, Van IJcken W, De Coo IR, Dudink J, MD, Bertoli Avella A, Verheijen F, Mancini GM*. Manuscript in preparation.

DANKWOORD

Het is zover… ik hoef alleen het dankwoord nog te schrijven. Er zijn zoveel mensen die ik wil bedanken, dat de kans dat ik iemand vergeet heel reëel is, waarvoor alvast mijn excuses.

Mijn promotor, Prof. Dr. Robert Hofstra, ik wil je bedanken voor het in mij gestelde vertrouwen en je kritische blik bij het beoordelen van mijn proefschrift. Prof. Dr. Irwin Reiss, Prof. Dr. Kees Braun, Prof. Dr. Raoul Hennekam, bedankt voor jullie deelname in mijn leescommissie. Ook mijn overige commissieleden wil ik van harte bedanken voor hun deelname. Prof. Dr. Linda de Vries, we hebben in de afgelopen jaren op een heel prettige manier kunnen samenwerken, je goede klinische blik was zeer waardevol voor ons onderzoek. Dr. Dicky Halley, bedankt voor de samenwerking in de afgelopen jaren, en zeker ook voor je scherpe commentaar betreffende de pathogeniciteit van COL4A1 mutaties. Dr. Maarten Lequin, telefonisch ben je onbereikbaar, maar ik kon altijd bij je binnenlopen voor een medebeoordeling van MRI's. Ik heb veel van je geleerd, bedankt. Prof. Dr. An Jansen, we hebben nog niet echt samengewerkt, maar hopelijk gaan we met mijn nieuwe baan in het UZ Brussel een prettige samenwerking tegemoet.

Woorden schieten tekort om mij copromotor, Dr. Grazia Mancini, te bedanken. Lieve Grazia, toen je me in 2007 vroeg welk type onderzoek ik zou willen doen, had ik hiervan eigenlijk nog geen idee. Onder jouw begeleiding heb ik echter geleerd om onderzoek te doen geïnspireerd door en volgend uit de patiëntenzorg. Ik heb ontzettend veel van je geleerd, zowel van jou als geweldig klinisch geneticus en onderzoeker, maar ook van jou als mens. Bedankt voor je vertrouwen en ik hoop dat we nog lang zullen blijven samenwerken.

Ook de andere leden van onze onderzoeksgroep wil ik bedanken. Frans, je nuchtere kijk op dingen helpt om de zaken in perspectief te blijven zien, wat heel zinvol is wanneer je onderzoek doet. Leontine, bedankt voor je hulp, zeker voor het doen van al die Westerns, dit is nooit mijn ding geworden. Daarnaast bedankt dat je lekker 'down to earth' in het leven staat en voor de cappuchino's (waarvoor ook mijn dank aan Rachel...). Rachel, jij introduceerde me in de wondere wereld van het exome sequencen (en Sanger sequencen en qPCR

en celkweken en apoptosis testen en zelfs immuunhistochemie). We hebben samen heel wat theorieën bedacht, getest en vervolgens weer verworpen. Gelukkig was er uiteindelijk toch een feestje! Daarnaast bedankt voor de gezelligheid, je bent een geweldig mens. Cathryn, it was nice and useful for me to have an example since you finished your thesis last year. All the best for you, your family and your work in Australia. Dan tot slot onze stagaire Wendy. Je stage begon spetterend toen je de USP18 mutatie vond met Sanger sequencing. Je vraag "Wat betekent een sterretje? (stopmutatie)" zal ik nooit vergeten. Heel erg bedankt voor je inzet.

Ruud Koppenol en Tom de Vries-Lentsch, bedankt voor jullie enorme hulp bij de figuren. Ondanks de vaak lage resolutie van mijn plaatjes wisten jullie er altijd wel weer iets moois van te maken. Ook bedankt voor de gezelligheid.

Rob Willemsen en Max Kros, bedankt voor jullie hulp met de immuunhistochemieproeven. Herma, heel erg bedankt voor je hulp bij de zebravissen, dankzij jouw rust en geduld is het zelfs mij gelukt om een aantal morfolinoinjecties te doen. Helaas bleek het het verkeerde gen… Adriana, I also want to thank you for your help with the zebrafish and for our nice conversations. I wish you good luck with finishing your own thesis next year. Lies-Anne, bedankt voor je hulp met de antilichamen, de paraffinecoupes en de immuunhistochemie. Alan and Danny, thank you for your interest and our nice conversations. Good luck to you both. Ook de andere collega's op het lab wil bedanken voor al jullie hulp, en voor de regelmatig langskomende taarten.

De mensen van Biomics Core, met name Wilfred van IJcken en Rutger Brouwer, en de bioinformatica, met name Daphne Heijsman, bedankt voor jullie hulp met de exome data en jullie bijdrage aan het USP18 artikel.

Mijn dank gaat ook uit naar mijn collega's van de afdelingen Neonatologie en Kinderneurologie van het Erasmus MC, locatie Sophia Kinderziekenhuis. Jeroen Dudink, Paul Govaert, Marlou Raets en Annemarie Plaisier; jullie waren de basis van het "neuroteam" binnen de neonatologie. Bedankt voor onze samenwerking betreffende de neonatale strokes, voor het gebruik van jullie databases en voor alle consultaanvragen die ik van jullie heb mogen ontvangen. René de Coo en Liesbeth Smit; René, bedankt voor de samenwerking, zeker in mijn eerste stappen in de neurogenetica. Liesbeth, de strokepoli bij het ROSEN project was "ons kindje". Heel erg bedankt voor je enthousiasme en succes met je verdere plannen.

Mijn collega's van de afdeling Klinische Genetica wil ik bedanken voor de mooie tijd die ik heb gehad in Rotterdam. Allereerst wil ik Fred Petrij bedanken, die ervoor heeft gezorgd dat ik na mijn werk als ANIOS neurologie kon komen werken op de afdeling. Ook Anneke Maat wil ik bedanken voor mijn opleiding tot klinisch geneticus. Om de lengte van mijn dankwoord te beperken zal ik de klinisch genetici alleen bij naam noemen, maar ik ga ervan uit dat jullie wel weten hoeveel jullie voor me hebben betekend; Yolande van Bever, Alice Brooks, Margriet Collee, Karin Diderich, Marieke van Dooren, Robert-Jan Galjaard, Lutgarde Govaerts, Jeannette Hoogeboom, Marieke Joosten, Ingrid van de Laar, Danielle Majoor, Rogier Oldenburg, Grétel Oudesluijs, Marleen Simon, Anja Wagner en Marja Wessels: heel erg bedankt. Ook iedereen van het secretariaat en de poli-assistentes, van harte bedankt voor jullie ondersteuning in de afgelopen jaren. Jeannette Lokker, bedankt voor je hulp met het administratieve geregel rondom het promoveren. De (ex)assistentengroep, alhoewel er al een aantal genoemd zijn, wil ik ook bedanken. We waren een echt team. Anja Kattentidt, Rogier Kersseboom, Yvonne Hoedemaekers, Judith Verhagen, Laura van Loon, Shimriet Zeidler en Iris Hollink, bedankt voor de gezelligheid en voor jullie steun afgelopen jaren. Anja en Rogier, heel veel succes als AVG. Myrthe van den Born, ik heb je maar kort gezien als co-assistent, maar ik wens je veel succes in de klinische genetica. Lieke Berger, lieve Lieke, we zijn kamergenootjes geweest vanaf 2007. We hebben samen heel wat lief en leed gedeeld. Je bent een heel fijn mens, ik ga je missen. Renske Oegema, lieve Renske, heel erg bedankt dat je vandaag mijn paranimf wil zijn. Je was afgelopen jaren mijn maatje, we deden beiden onderzoek met Grazia en delen dezelfde passie voor de neurogenetica. Met je nuchtere houding en relativeringsvermogen heb ik veel steun aan je gehad. Ik wens je heel veel geluk met alle nieuwe dingen die op je pad komen ;-)

Mijn lieve vriendinnen in Limburg; ondanks het feit dat ik veel te ver weg woon en veel te weinig bel en altijd vergeet kaartjes te sturen, zijn jullie er nog steeds altijd voor mij. Lieve Debby, Colette, Marjon, Mechteld en Monique, bedankt voor jullie interesse en vriendschap. Hopelijk ga ik nu wat meer tijd krijgen, waardoor er meer mogelijkheden ontstaan voor shoppen of weekendjes Brussel! Lieve Nicole, we zijn al vriendinnen zo lang als ik me kan herinneren. Ik was getuige op je huwelijk en ben peettante van Sven. Ik vind het heel leuk dat jij mij vandaag wilt bijstaan als paranimf.

En dan "Schranz" (the extended version). Ik was de eerste echte "aanhang" van deze vriendengroep, waarin ik liefdevol werd opgenomen. Gelukkig is er in de afgelopen jaren heel wat uitbreiding geweest, waardoor we nu zelfs geen singles meer hebben. Sonja en Roy, Tirza en Jaap, Vincent en Lotte (en Jesse), Jojanneke en Arnoud, Jeroen en Annelies, Arjan

en Eunice en Bart en Esther, het is altijd erg gezellig om, met zijn allen of in gedeelten, iets te drinken, te eten, carnaval te vieren, of op vakantie, een weekendje weg of naar Lowlands te gaan. Bedankt voor jullie vriendschap.

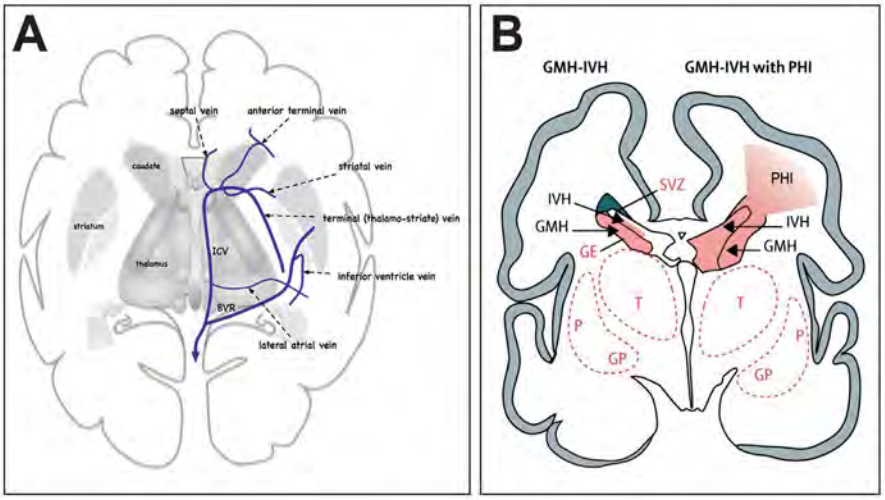
Jan en Ans, Aldo en Maaike en David, een betere schoonfamilie had ik niet kunnen wensen. Jullie staan altijd voor ons klaar, en steunen ons in de beslissingen die we nemen. Heel erg bedankt.

Jeroen, "broertje", en Sanne, ook jullie er altijd voor me. Heel erg bedankt voor jullie interesse. Samen gaan jullie dit jaar ook een belangrijke stap zetten. Ik ben vereerd getuige te zijn op jullie huwelijk in september.

Pap en mam, jullie hebben me altijd gestimuleerd om me te ontwikkelen, "leren doe je voor jezelf" was een gevleugelde uitspraak. Jullie hebben me een stevige basis gegeven, waardoor ik zo ver kon komen. Ook al zijn mijn keuzes wellicht niet altijd de gemakkelijkste, toch blijven jullie me steunen. Dank jullie wel.

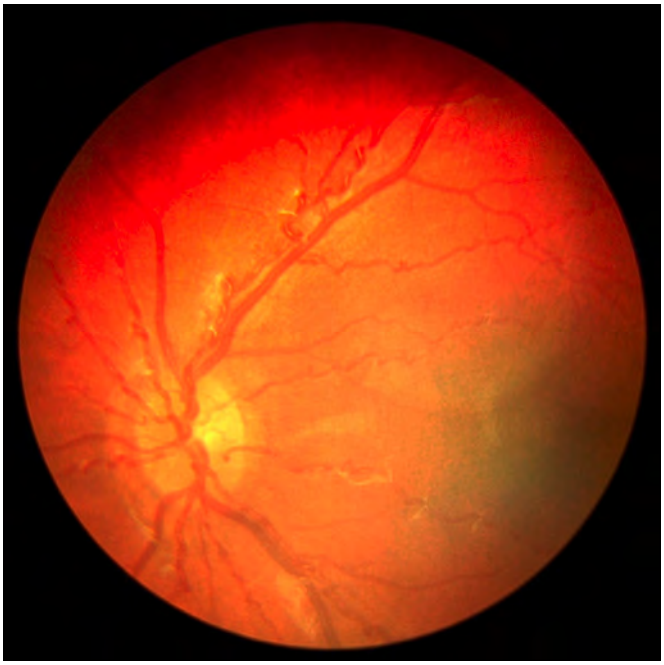
Lieve Ronald, waar zou ik zijn zonder jou. Jouw liefde en vertrouwen, maar ook je relativerend vermogen en rationele kijk op de wereld houden mij overeind. Er zijn veel onzekerheden geweest de afgelopen tijd, maar door jouw steun heb ik er altijd op vertrouwd dat het op de een of andere manier wel goed zou komen. Dit boekje is hier een voorbeeld van. Samen kunnen we de wereld aan…

COLOUR FIGURES



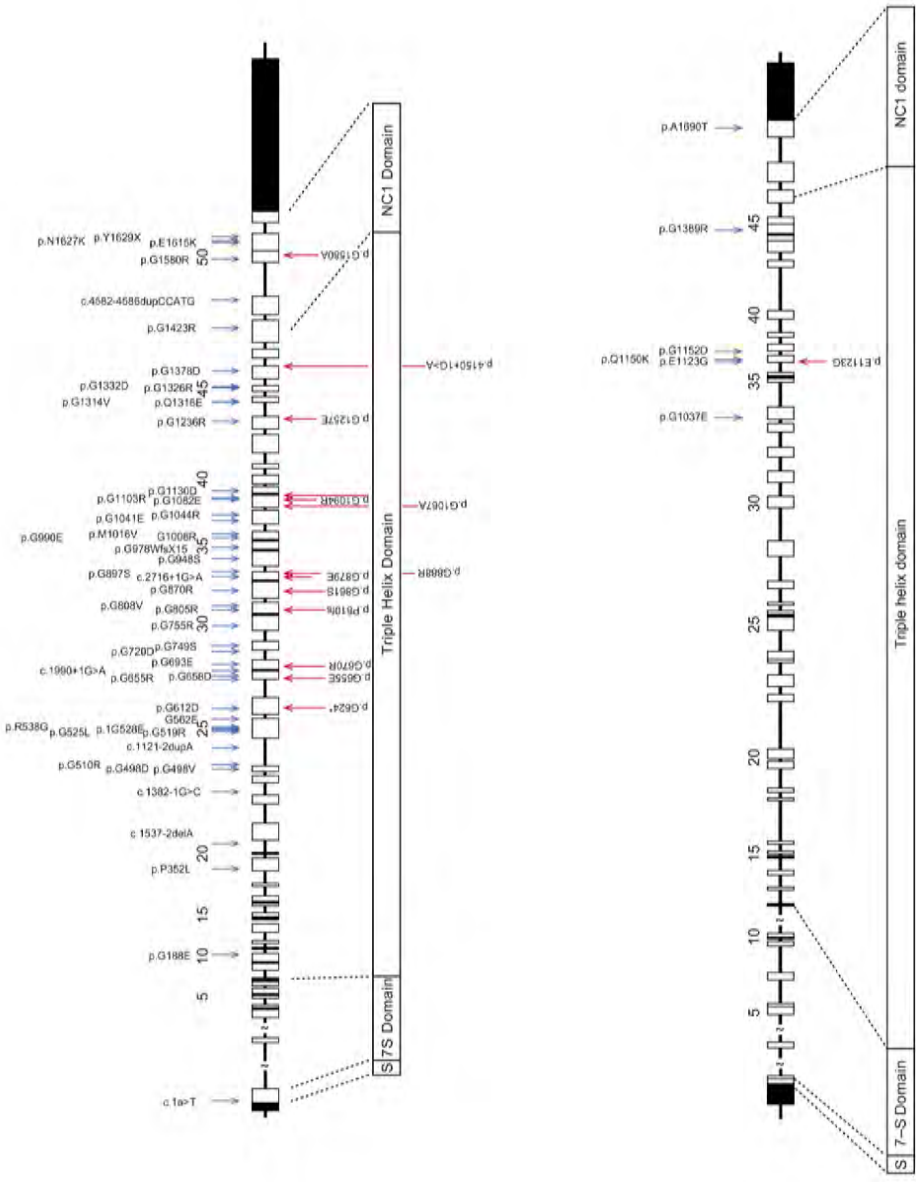
Chapter 1

Figure 1. Schematic overview of pathogenic mechanism leading to porencephaly[107,108]. The lateral ventricles with surrounding deep veins are depicted in **A**. Porencephaly originates from a germinal matrix hemorrhage, often followed by intraventricular hemorrhage. This leads to deep venous infarctions and destruction of white matter in that area, eventually leading to a cyst communicating with the ventricle (**B**)



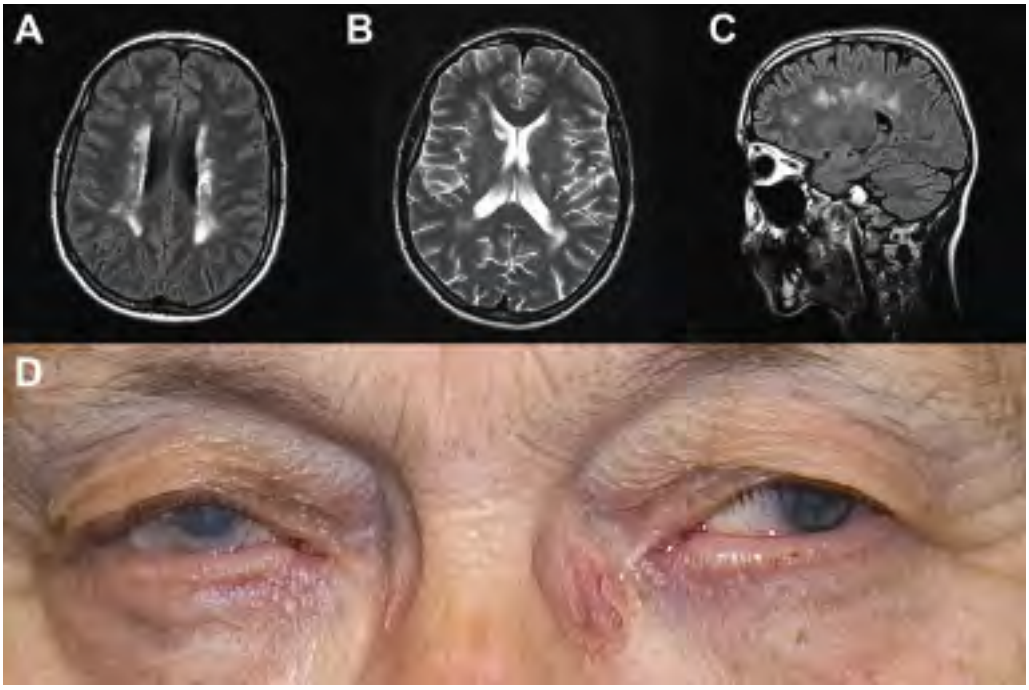
Chapter 2.1

Figure 3. An image of the retina shows tortuosity of the retinal vessels.



Chapter 3

Figure 1. In this figure, the *COL4A1* mutations (A) and *COL4A2* mutation (B) in our novel families are depicted on the bottom (red arrows), the mutations reported in literature are depicted on the top (blue arrows).



Chapter 4.2

Figure 1. Brain MRI of the index patient (**A,B,C**). FLAIR (**A,C**) and T2- weighed (**B**) images show periventricular leukomalacia. Picture of ocular features of the mother (**D**), with microphthalmia and status after many ocular operations because of congenital cataract and secondary glaucoma.

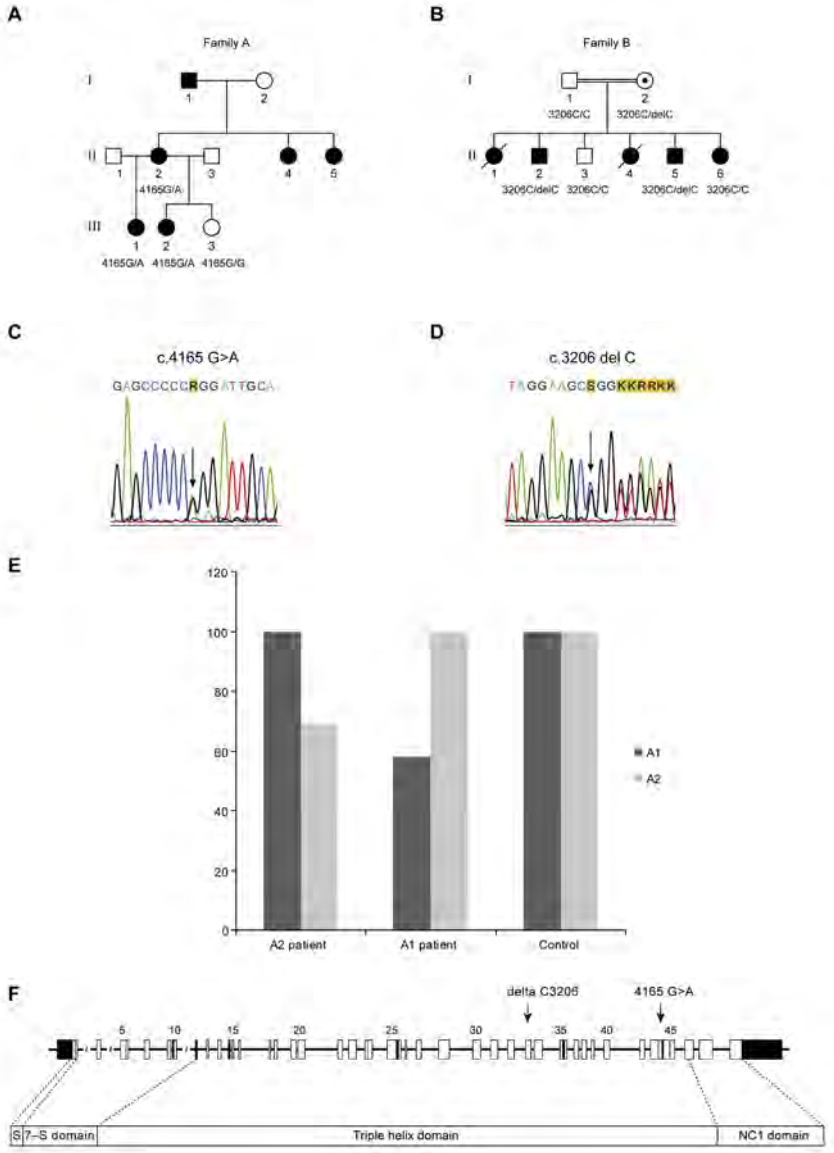
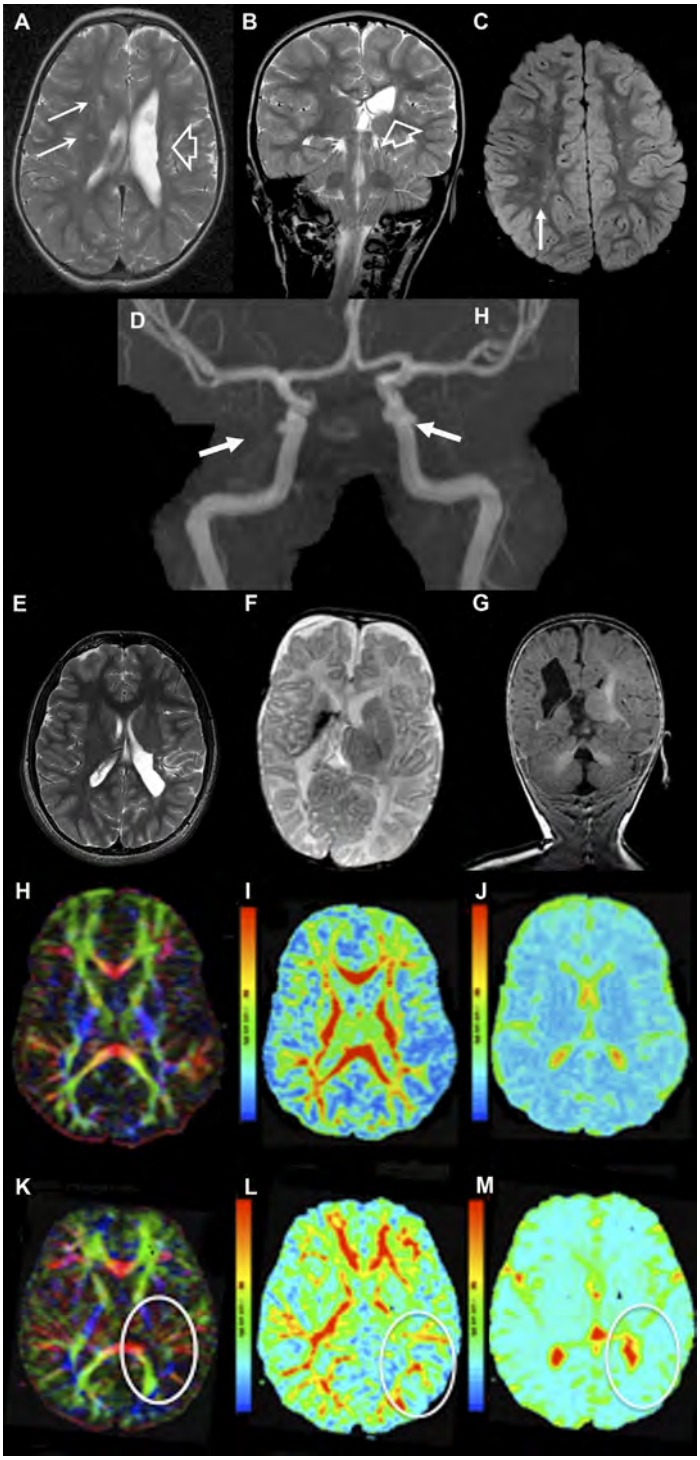


Figure 1. Pedigrees of families A and B.

Different symbols indicate individuals affected with various cerebral vascular disease as indicated in the legend. The genotype of the tested individuals is indicated: (**A**) 4165G/G = wild type sequence; 4165G/A heterozygous mutation; (**B**) 3206C/C = wild type sequence; 3206C/delC = heterozygous mutation. Electroferograms indicate the heterozygous mutation in the *COL4A2* sequence of family A c.4165G>A (p.G1389R) (**C**) and family B c.3206delC leading to frame shift (**D**). Quantitative, real time PCR analysis showing relative expression of *COL4A1* and *COL4A2* mRNA in fibroblasts from patient II.2 (family B) with c.3206delC mutation (**A2 patient**) and a patient bearing a pathogenic c.3321G>C (p.G1067A) mutation in exon 38 of *COL4A1* (**A1 patient**), compared to a control cell line. Results are average of two separate experiments. For each cell line the highest expression is set as 100% on the Y axis (**E**). Position of the mutations within the schematic representation of the *COL4A2* genomic organization (**F**).

Chapter 5

Figure 2. Brain MRI of patients with *COL4A2* mutations.



A, B. Axial and coronal T2 of patient III.2 from family A at the age of 2 years indicate ex-vacuo dilation of the left lateral ventricle (porencephaly) (open arrow) and periventricular white matter lesions (solid arrows) resulting from presumed perinatal stroke.

C. FLAIR image illustrates T2 prolongation in the white matter – in patient III.1 from family A at the age of 8 years, suggesting gliosis (arrow).

D. MR angiography of their mother (subject II.1, family A) at adult age shows bilateral internal carotid aneurysms (at the level of cavernous sinus).

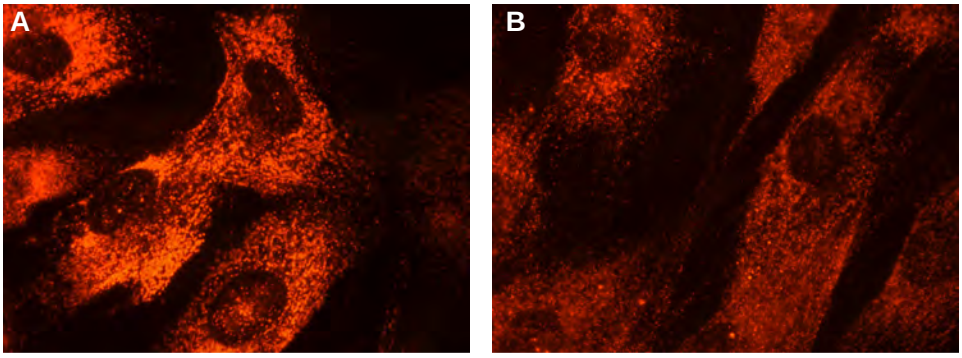
E. Axial T2 weighted images of Patient II.2, family B, at the age of 15 years show a porencephalic dilatation of the left occipital ventricle.

F, G. Axial T2 weighted and coronal FLAIR images of patient II.5 from family B at the age of 5 months show porencephaly of the right ventricle with hypoplastic left cerebellar hemisphere.

H-M. Reconstruction of the white matter tracts obtained from magnetic resonance diffusion tensor imaging (MR-DTI) data.

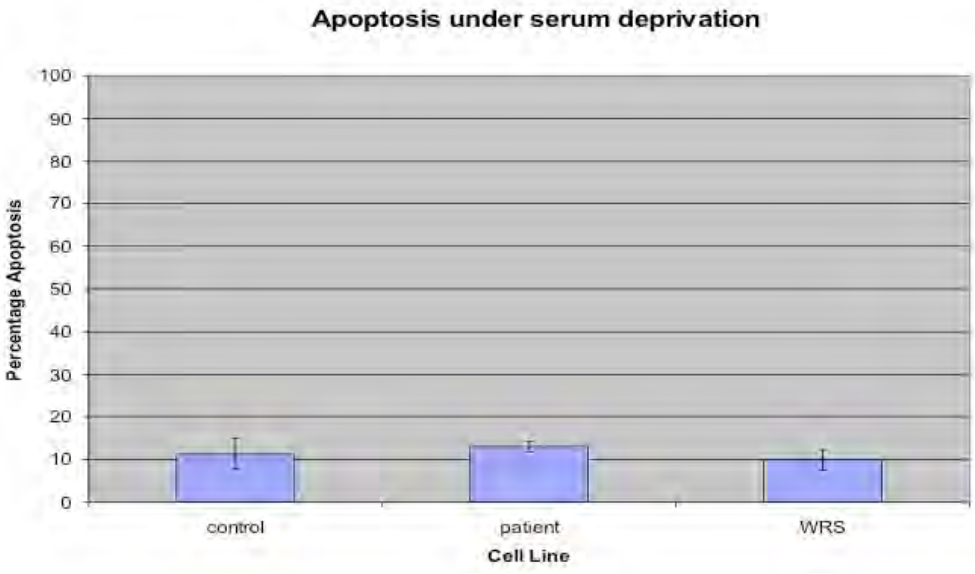
K,L. Patient II-2 (family B) reveals reduced fractional anisotropy in the left radiation optica and tractus corticospinalis at the side of the porencephalic lesion (encircled), compared with an age-matched control

H,I. A restriction of the total ADC was observed in the whole cerebral white matter of the patient (**M**), compared to an age-matched control (**J**).



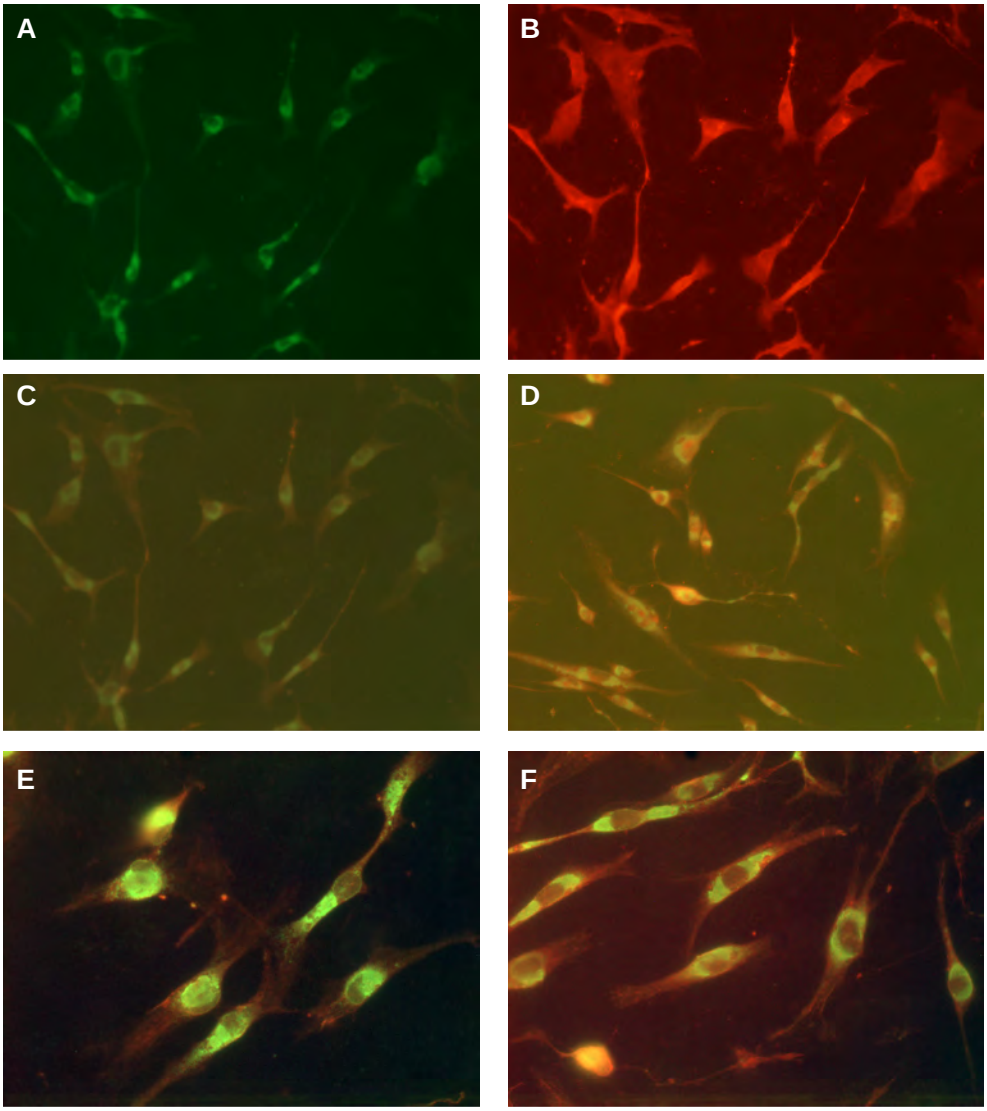
Chapter 5

Supplementary Figure 3. Cultured fibroblasts grown under standard conditions . Immunofluorescent staining for HSP47 (red). **A**: control; **B**: patient with *COL4A2*^{3206delC} mutation.



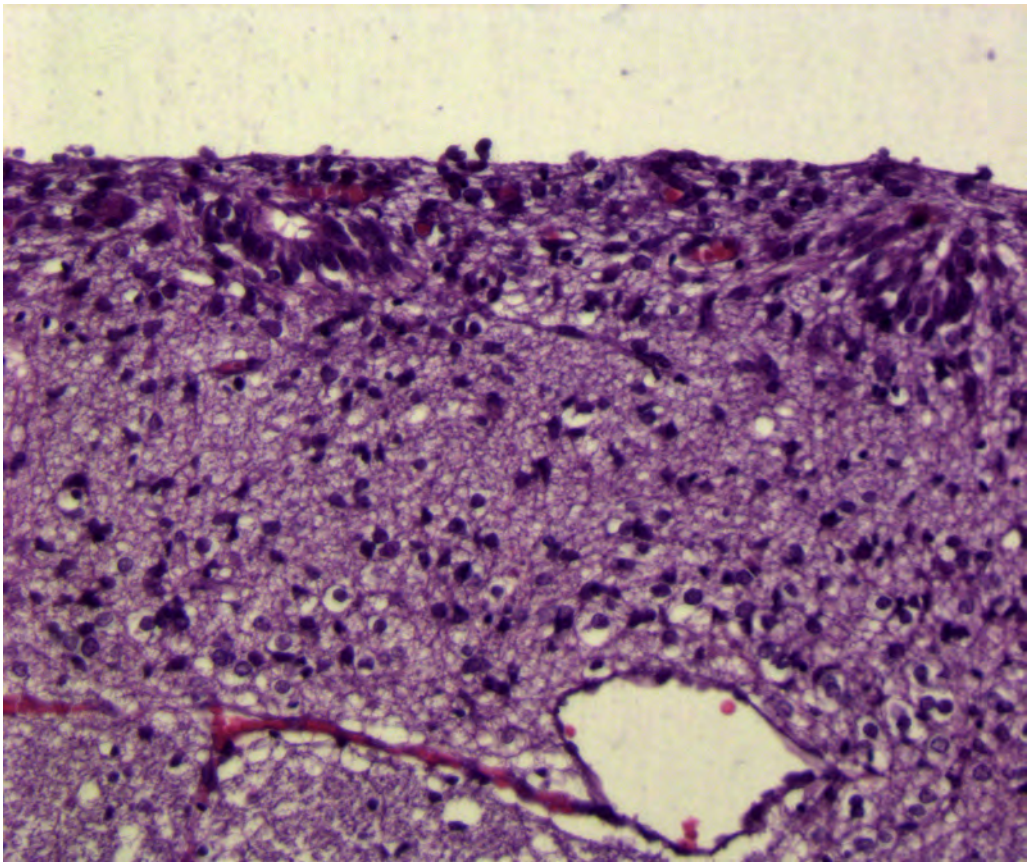
Chapter 5

Supplementary figure 5. Skin fibroblasts were cultured in 175 cm^2 culture flasks in Dulbecco's modified Eagle medium (DMEM, Lonza Biowhittaker) plus 10% FCS until 80% confluence. Cultures were obtained from 5 healthy individuals (control), one patient with *COL4A2*^{3206delC} mutation (patient) and one individual with confirmed Wolcott-Rallison syndrome bearing an *EIF2AK3* mutation (WRS). Before testing apoptosis rates by the Flica multicaspase detection kit, the cells were exposed 24 hours to serum deprivation. Data are average of multiple experiments plus SD, as indicated in the methods.



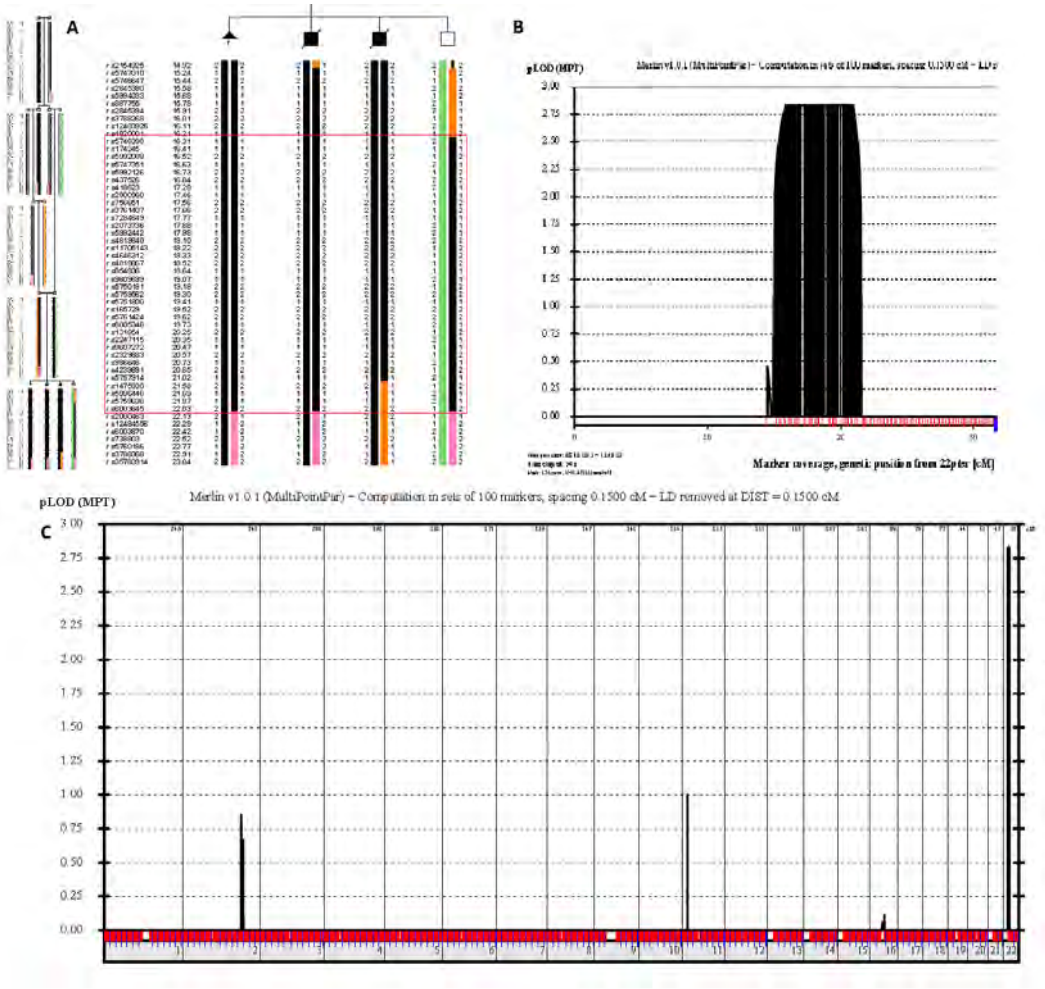
Chapter 5

Supplementary Figure 4. Cultured fibroblasts after treatment with 5 mM DTT. Immunofluorescent staining for P450 (red), KDEL (green). **A and B**: control 1. **C:** merged picture of A and B. **D,E.F:** merged pictures of double staining with KDEL and P450. **D:** control 2; **E:** control 3; **F:** patient with *COL4A2*$^{3206\text{delC}}$ mutation.



Chapter 6

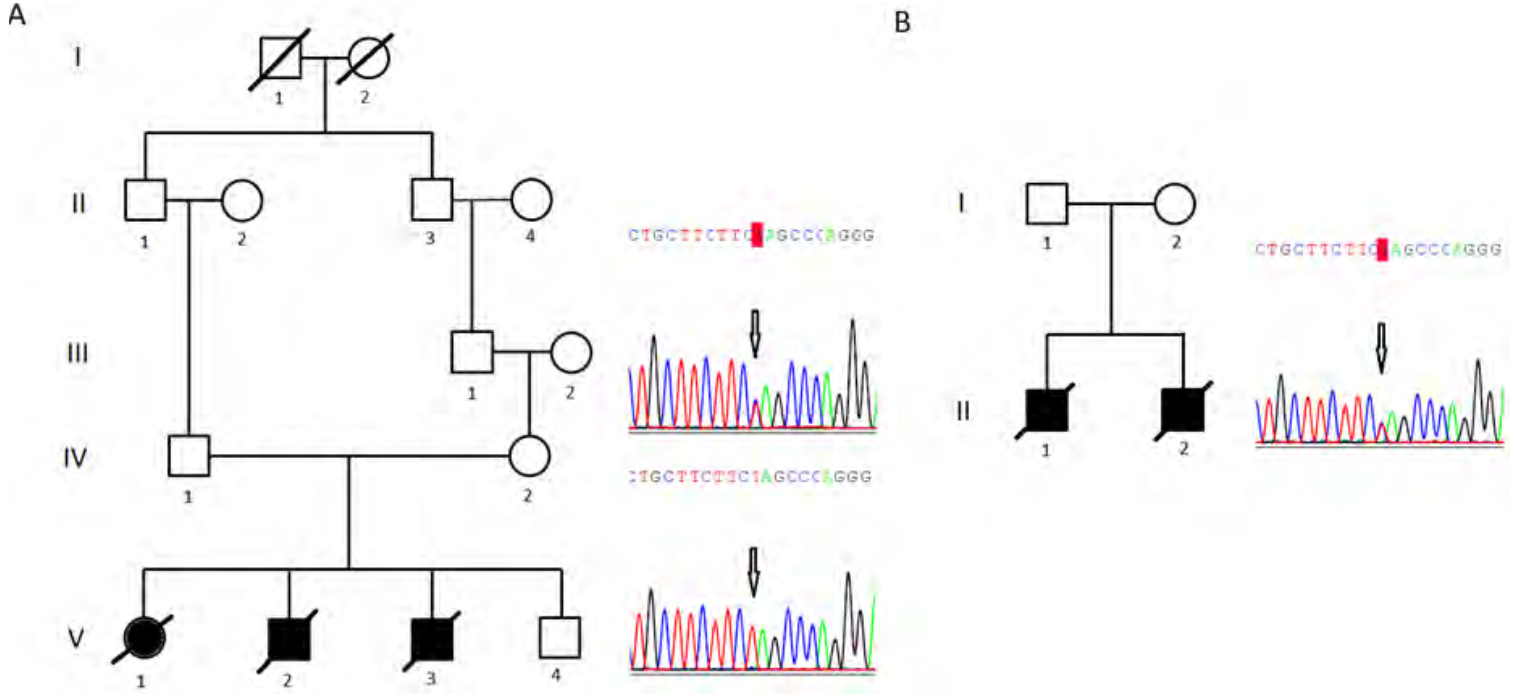
Figure 2. Patient ependyme of left lateral ventricle. Haematoxyline & Eosin (H&E) staining of patient autopsy material (patient A-V.1) showing abnormal, irregular ependyme with disorganisation of the ventricular zone.



Chapter 6

Supplementary figure 1. Linkage data and haplotyping of family A

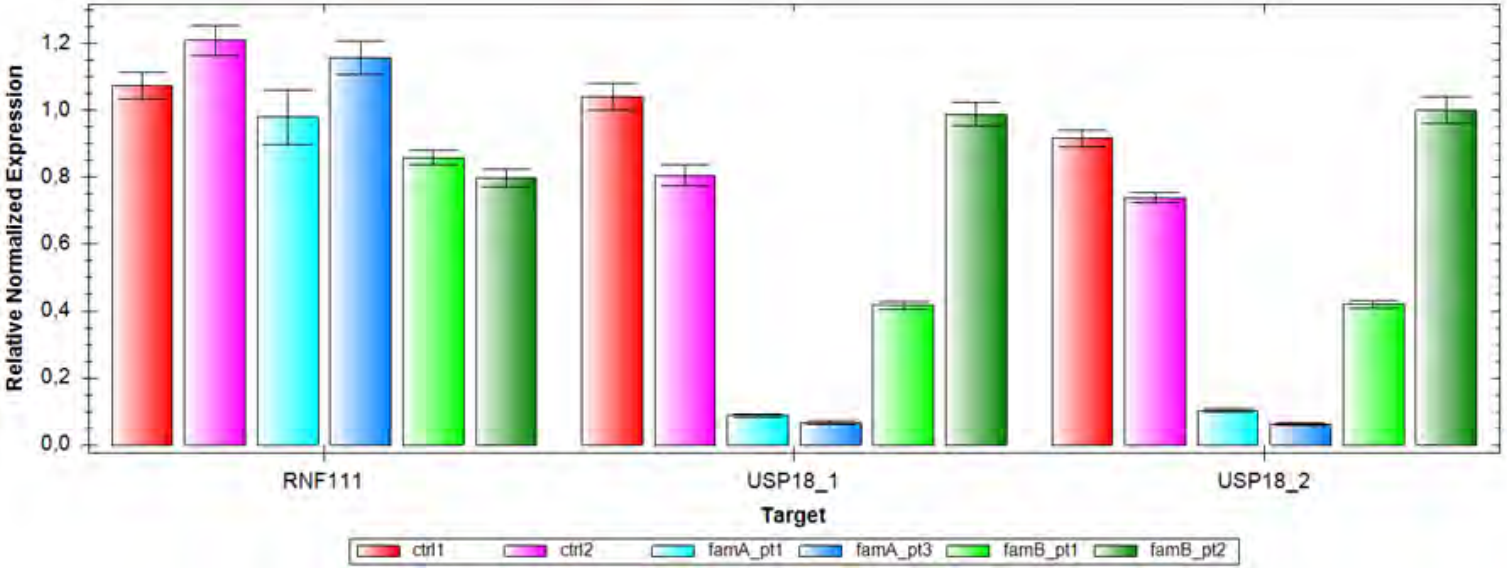
Haplotype analysis of family A, the shared haplotype between the affected individuals is located in the red box (**A**). **B** shows the linked area on chromosome 22 with LOD score of 2.83 in detail. **C** shows the genome wide linkage, confirming that the linked area on chromosome 22 is the only linkage area with a significant LOD score.



Chapter 6

Supplementary figure 2. *USP18* Mutations

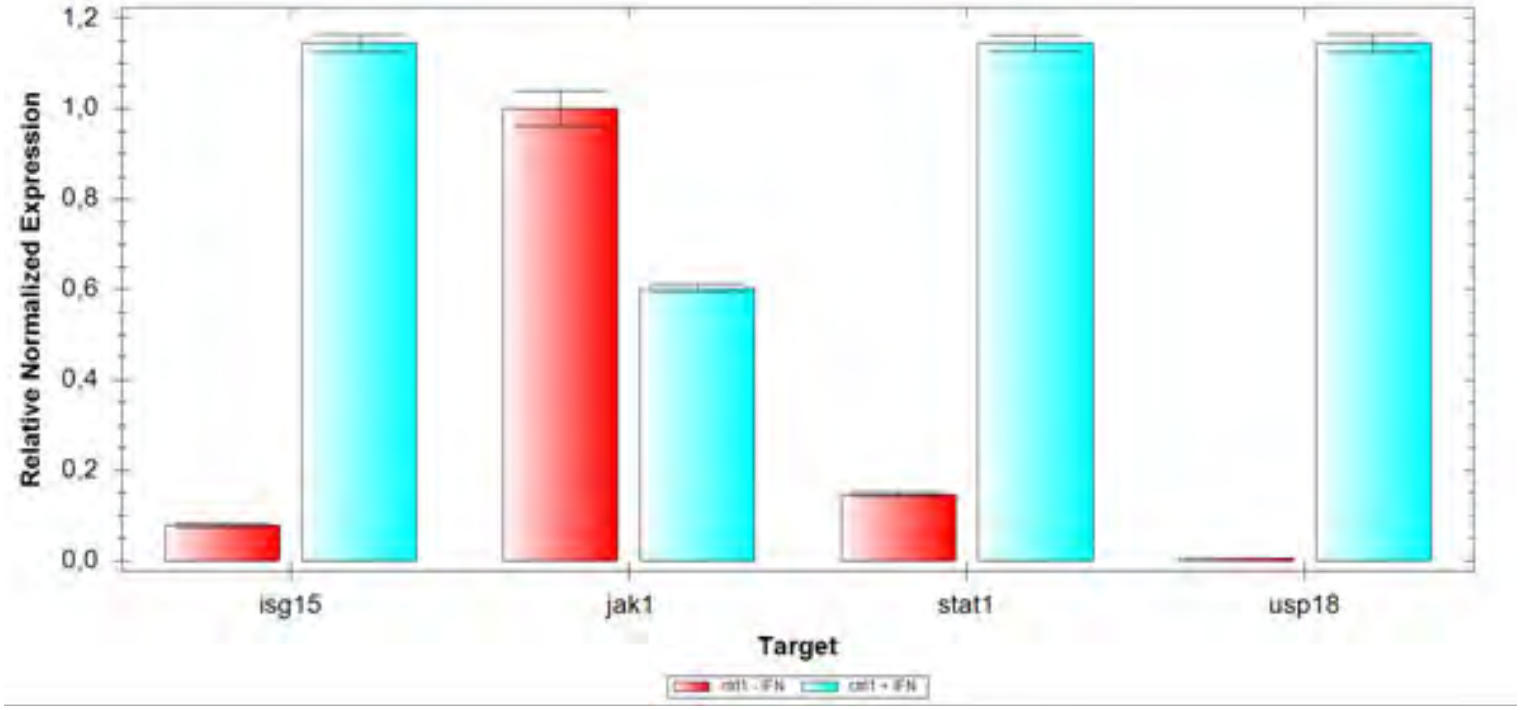
Pedigree of family A, with a representation of the *USP18* mutations, the heterozygous c.652C>T, p.Q218X mutation in IV.1, IV.2 and V.4 (upper panel) and the mutation in homozygous form in V.1, V.2 and V.3 (**A**). **B** shows the pedigree of family B, with the same USP18 c.652C>T, p.Q218X mutation, present in heterozygous form in II.1 and II.2.



Chapter 6

Supplementary figure 4. *USP18* qRT-PCR of family A and family B

RT-qPCR on cDNA of patient A-V.1, A-V.3, B-II.1, B-II.2 and two controls. USP18_1 correlates with primers located in exon 4-6, while USP18_2 correlates with primers located in exon 8-9. An almost absent expression of *USP18* mRNA is seen in both patients from family A, suggestive of nonsense mediated decay. The controls show variable USP18 expression. Family B shows *USP18* expression. Since the same stop mutation is present in heterozygous form in family B, these results indicate that there is mRNA of the second allele present that includes exons 4-6 and exons 8-9.



Chapter 6

Supplementary figure 6. Control fibroblast RT-qPCR *USP18, ISG15, STAT* after IFNβ treatment

qRT-PCR on cDNA isolated from control fibroblasts without and with overnight treatment with IFNβ (1000U/l)(Tebu-Bio, The Netherlands). These data show that IFNβ treatment leads to upregulation of USP18, ISG15 and STAT1. Tests in patient fibroblasts are pending.


9 789068 240474 >