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DOTTORATO DI RICERCA TOSCANO IN NEUROSCIENZE

CICLO XXXII

COORDINATORE Prof. Renato Corradetti

Screening of the *CTSA* gene in a population of *NOTCH3* and *HTRA1* negative patients with Small Vessel Disease.

Settore Scientifico Disciplinare MED/26

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SUMMARY

1. INTRODUCTION

1.1 Cerebral Small Vessel Disease

Cerebral small vessel disease (cSVD) is a term universally used to refer a group of pathological processes with various etiologies that involves the perforating cerebral arteries, arterioles, capillaries and venules, leading to progressive cognitive and motor disabilities (Pantoni L, 2010; Cuadrado-Godia E et al., 2018; Markus H et al., 2019). Generally, cSVD is used to describe a series of magnetic resonance imaging (MRI) changes in the white matter and subcortical grey matter, including recent small subcortical infarct, lacunes, white matter hyperintensities (WMHs), prominent perivascular spaces (PVS), cerebral microbleeds (CMBs) and atrophy. According to recent epidemiological data, (Feigin VL et al., 2017), the incidence is of 133/100,000 males-years and 99/100,000 females-years in American population (Feigin VL et al., 2017) with more than 5 million deaths each year (Hankey GJ et al., 2017). Age/hypertension-related cSVDs and cerebral amyloid angiopathy are the most common sporadic forms of SVD. Among a few genetic forms of SVD, CADASIL (cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy), caused by *NOTCH3* gene mutations, is the most frequent one, but recently, with the growing discoveries in the field of genetics, a greater number of monogenic cSVD has been identified (Pantoni L, 2010)

- **Sporadic cSVDs linked to risk factors** such as hypertension, smoking and diabetes are considered among the most commonly known neuropathological processes and has an important role in stroke, cognitive impairment, and functional loss in elderly (Charidimou A et al., 2016). Sporadic cSVDs are usually categorized in two main forms:

    - **Sporadic Cerebral Amyloid Angiopathies (CAAs)**, characterized by progressive β-amyloid deposition in the media and adventitia of small arteries, arterioles, and sometimes capillaries in the cerebral cortex and small arteries, arterioles, and sometimes venules in the overlying leptomeninges (Charidimou A et al., 2012; Charidimou A et al., 2014).

- **Sporadic cSVDs without amyloid depositions** that are more difficult to define and are indicated with the misleading term of “hypertensive arteriopathy”, because at this category belongs also sporadic cSVDs not necessarily related to hypertension although it can play a role in their evolution (Charidimou A et al., 2016). These forms of cSVDs typically affect the small perforating end arteries of the deep gray nuclei and deep white matter and are characterized by collagenous thickening of the vessel wall with narrowing of the lumen and progressive loss of smooth muscle cells and sometimes by exudation of fibrin and other serum proteins or by scanty mural deposition of lipid. At this category belongs arteriolosclerosis, fibrinoid necrosis, and lipohyalinosis (Charidimou A et al., 2016).

- **Genetic cSVDs** are essentially monogenic and with an earlier onset than sporadic forms (Pantoni L 2010; Tan R et al., 2017). The most common form of genetic cSVD is cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), caused by mutations in *NOTCH3* gene with an autosomal dominant inheritance (Joutel A et al., 1996). An autosomal recessive cSVD has been associated with *HTRA1* mutations mainly in Japanese and Chinese and is called cerebral autosomal recessive arteriopathy with subcortical infarcts and leukoencephalopathy (CARASIL) (Hara K et al., 2009). Heterozygous mutations in *HTRA1* gene are recently associated to a less severe disease respect to CARASIL in Caucasian and Japanese populations (Verdura E et al., 2015; Nozaki H et al., 2016). Mutations in collagen-IV α 1 chain (*COL4A1* gene) or *COL4A2* gene can cause a familial cSVD characterized by intracerebral hemorrhages (Markus H et al., 2019). In 2016, *Bugiani M et al*. identified a new form of dominant cSVD caused by mutations in the *CTSA* gene, named CARASAL (Bugiani M et al., 2016). Other rarer forms of genetic cSVDs such as *TREX1* and *GLA* genes related cSVDs are summarized in Table 1 (Cuadrado-Godia E et al., 2018; Giau VV et al., 2019). Thanks to Next Generation Sequencing (NGS) and Genome Wide Association Studies (GWAS), new genetic information about genetic cSVD have been added to our knowledge, and both new genes and new gene mutations are continuously associated to cSVDs, expanding the group of known genetic cSVDs.

Table 1: classification of the principal genetic cSVDs and their clinical features (Tan R et al., 2017; Cuadrado-Godia E et al., 2018).

Type of disease	Age at presentation	Presence of accumulated materials	Vessels involved	Known genetic alteration	Consequences
Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL)	Adults	Granular Osmiophilic material (GOM)	Plasma membrane of vascular muscle (smooth muscle cells)	Missense mutations in *NOTCH3* gene	Alters the number of cysteine residues in the Notch3 protein, leading to accumulation and deposition of Notch3 receptor in ECD
Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CARASIL)	Adults	Deposition of hyaline material in the wall	Splitting of the internal elastic membrane	Homozygous mutations in the *HTRA1* gene	Interferes the enzymatic property of the protease
Heterozygous HTRA1 associated Cerebral Small Vessel Disease	Adults	Deposition of hyaline material in the wall	Splitting of the internal elastic membrane	Heterozygous mutations in the *HTRA1* gene	Interferes the enzymatic property of the protease
Collagen IV-related cSVD	Adults	-	Impaired synthesis of the basement membrane and blood vessel fragility	Mutations in *COL4A1* and *COL4A2* genes	Impairing in the formation of the resulting collagen molecule
Retinal vasculopathy with cerebral leukodystrophy	Adults	Accumulation of genetic material in the cells	Vessel wall degeneration	Frameshift mutations in *TREX1* gene which encodes a 3'-5' exonu-clease	Impairment of this enzyme trigger immune system reactions
Fabry disease	Adults	Accumulation of glycolipids	Walls of small blood vessels, nerves, glomerular and tubular epithelial cells, and cardiomyocytes	Mutations in lysosomal *GLA* gene	Absent or deficient lysosomal GLA activity
Cathepsin A-related Arteriopathy with Strokes and Leukoencephalopathy (CARASAL)	Adults	-	Small vessels of the CNS	Missense mutations in *CTSA* gene	Not known

1.2 Pathogenesis of Cerebral Small Vessel Diseases

Consequences of small vessel diseases on the brain parenchyma are heterogeneous and not completely known (Pantoni L, 2010; Tan R et al., 2017). In general, pathologic changes of small vessels lead to a variety of pathophysiological changes resulting in reduced cerebral blood flow and cerebral autoregulation that lead to hypoperfusion, and increased blood-brain barrier (BBB) permeability due to loss of endothelial cells and pericytes (Tan R et al., 2017). Together, these changes lead to both ischaemic and haemorrhagic consequences (Pantoni L 2010). In ischaemic lesions, the chronic hypoperfusion of the white matter results in degeneration of myelinated fibers due to oligodendrocytes death. The principal consequences of this process are white matter lesions that appears has diffuse hyperintensities on T2-weighted MRI, so called leukoencephalopathies (Vanderver A et al., 2015; Ayrignac X et al., 2015; Van der Knaap MS et al., 2017). Alternatively, the chronic hypoperfusion lead to a focal and acute ischemia that lead to a tissue necrosis zones called lacunar infarcts (Pantoni L, 2010), that are hypointense foci on MRI T1-weighted sequences and typically seen in basal ganglia, thalamus, internal capsule, thalamus and pons (Pantoni L, 2010) (Figure 1).

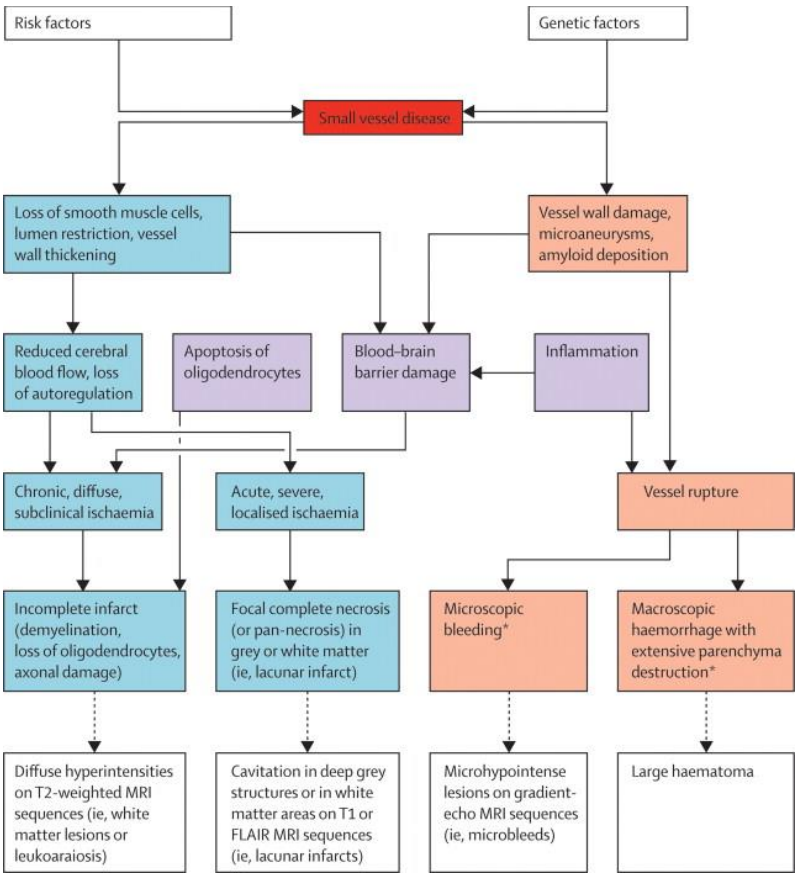

Figure 1: Pathogenetic pathways of the brain caused by cerebral small vessel diseases (Pantoni L, 2010).

Genetic studies on cSVDs have brought to light different molecular mechanisms that could be involved in cSVDs pathogenesis. Recent studies have provided evidence for the involvement of the extra-cellular matrix (ECM) proteins in their pathogenesis (Tan R et al., 2017). Typical feature of CADASIL is the deposition of granular osmiophilic material (GOM) in the extracellular space of small blood vessels and NOTCH3 ectodomain ($NOTCH3^{ECD}$) cleaved from mutant NOTCH3 receptors (Lorenzi T et al., 2017; Tan R et al., 2017). It is provided that $NOTCH3^{ECD}$ co-aggregates with many ECM proteins: Thrombospondin-2 (TSP2), Latent TGFβ-binding protein (LTBP-1), Tissue inhibitor of metalloproteinase 3 (TIMP3) and Vitronectin (Tan R et al., 2017). These aggregates may contribute to loss of pericytes and endothelial cells causing an alteration in the BBB permeability and blood vessels thickening (Müller K et al., 2018). The LTBP-1 protein is a substrate for Htra1 protease that is mutated in CARASIL. It is estimated that TGFβ signalling is enhanced in CARASIL, due to loss of function of Htra1 protein (Hara K et al., 2009; Müller K et al., 2018). However, the effect of increased TGFβ signalling is unknown. It has been assumed that increased TGFβ signalling could reduce pericyte proliferation and elevates the expression of proinflammatory cytokines, which may cause a BBB impaired function (Miyazaki T et al., 2014; Tan R et al., 2017). The majority of *COL4A1* and *COL4A2* gene mutations affect a glycine residue responsible for the correct conformation of Type IV collagen protein, the most abundant protein in ECM. This could lead to impaired synthesis of the basement membrane, leading to blood vessel fragility (Tan R et al., 2017).

1.3 Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CADASIL)

CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy, OMIM 125310) was the first genetic cSVD discovered (Di Donato I et al., 2017). Clinical features of CADASIL, despite a heterogeneous phenotype both between and within families, include migraine as the first symptom (onset at 30 years), followed by recurrent subcortical ischemic events like strokes that progressively lead to gait difficulty, urinary urgency, pseudobulbar palsy, and cognitive impairment (Nannucci S et al., 2014; Bersano A et al., 2017). Cognitive impairment is observed in nearly all patients by the fifth decade (Bersano A et al., 2017), is commonly progressive and worsens with recurrent strokes. It involves mostly executive functions and processing speed but may also be associated with memory and attention deficits. Despite the origin of these disturbances is unknown, it has been suggested a vascular damage of cortical-subcortical circuits as one of the leading causes (Valenti R et al., 2008).

Typical CADASIL neuroimaging findings on MRI show extensive WM lesions that are age- and disease stage- dependent: T2-hyperintense lesions occur during the first stage of the disease, followed by supratentorial lesions in frontal and temporal lobes. In the late stages of the disease, patients develop lacunar infarcts and lesions that involves basal ganglia, thalamus and periventricular regions (Aurer DP et al., 2001; Van den Boom R et al., 2003; Choi J et al., 2010; Nannucci S et al, 2014) (Figure 2). The main neuropathological feature of CADASIL is the deposition of granular osmiophilic material (GOM) close to vascular smooth muscle cells, seen on electron microscopy. GOM can also be detected in vessels of extracerebral tissues, including skin and muscle (Ruchoux MM et al., 1994; Ishiko A et al., 2006; Lorenzi T et al., 2017).

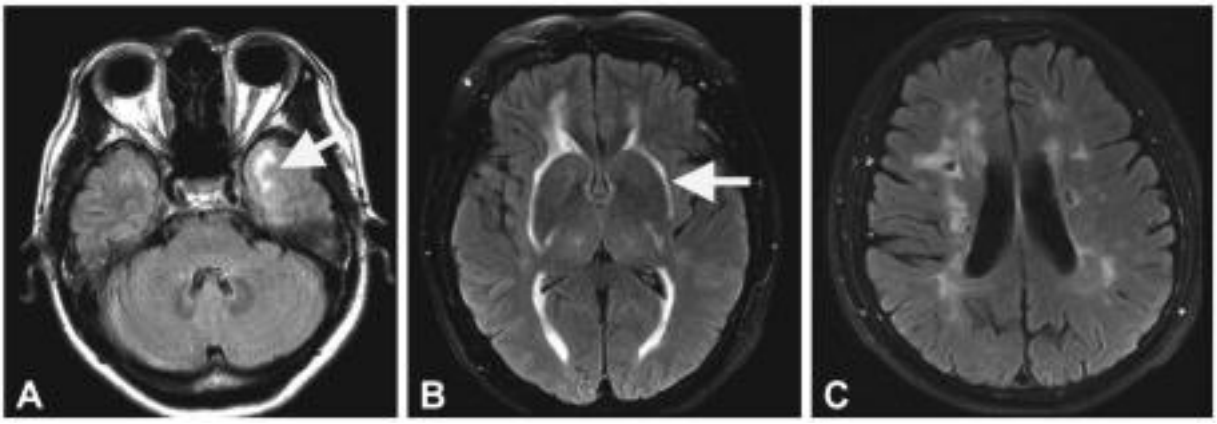

Figure 2: Axial fluid-attenuated inversion recovery (FLAIR) MRI in CADASIL patient. High-signal-intensity (HSI) lesions are present in the anterior temporal lobe (A) and bilateral external capsules (B). Multiple lacunar infarctions (C) are noted in bilateral periventricular and deep white matter with punctate HSI lesions. (Choi J at al., 2010).

Heterozygous mutations in *NOTCH3* gene are recognized as the principal genetic cause for CADASIL (Joutel A et al., 1996; Joutel A et al., 1997). The NOTCH3 gene encodes for an EGF-like (Epidermal Growth Factor-like) transmembrane receptor protein, constituted by 34 Tandem Epidermal Growth Factor-like Repeat (EGFRs) extracellular domains and 3 Lin-12/Notch repeats (LNR), a transmembrane and an intracellular domain containing Ankyrin-repeat Motifs and a Proline (P), Glutamic Acid (E), Serine (S), and Threonine (T) (PEST) domain (Larsson C et al., 1994). There are more than 200 identified NOTCH3 CADASIL-associated gene mutations nowadays, most of these consisting of missense substitutions of a single base or base deletions, duplications and splice site mutations. In CADASIL disease, NOTCH3 gene mutations lead to an unpaired cysteine residue within one of the EGFRs of Notch3 extracellular domain, leading to deposition of NOTCH3 receptor in extracellular domain (Rutten JW et al., 2014).

1.4 Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy (CARASIL)

CARASIL (Cerebral Autosomal Recessive Arteriopathy with Subcortical Infarcts and Leukoencephalopathy, OMIM 600142) is a very rare and early onset form of cSVD characterized by recurrent ischemic strokes, brain functions deterioration and cognitive dysfunction (Di Donato I et al. 2014). The epidemiology of CARASIL is not well known, but it has been reported in less than 50 patients worldwide, mostly from Japan and China (Nozaki H et al., 2014) and only some Caucasian (Mendioroz M et al., 2010; Menezes Cordeiro I et al., 2015), one of which has been diagnosed in our department (Bianchi S et al., 2014). The ischemic strokes in CARASIL resemble typical acute lacunar strokes, manifesting both pyramidal and extrapyramidal signs. Typical brainstem dysfunction includes vestibular symptoms, oculomotor abnormalities and facial weakness. Non-neurological manifestations include early onset hair loss and chronic back pain, that is very common in CARASIL patients (about 80% of cases), with onset between 10 and 40 years of age (Federico A et al., 2012). MRI shows a pattern of white matter involvement like those with CADASIL disease: confluent WM lesions in both internal and external capsules and temporal lobes, including temporal poles. Small lesions of the basal ganglia, thalami, and brainstem have been observed. Degenerative changes in the cervical and lumbar spine have been observed on Spine imaging (Ito J et al., 2018) (Figure 3). Neuropathological examination shows typical findings that resemble those seen in patients with nonhereditary ischemic cerebral small-vessel disease: arteriosclerosis associated with intimal thickening and dense collagen fibers, loss of vascular smooth-muscle cells, and hyaline degeneration of the tunica media (Hara K et al., 2010). No pathognomonic histological features have been observed.

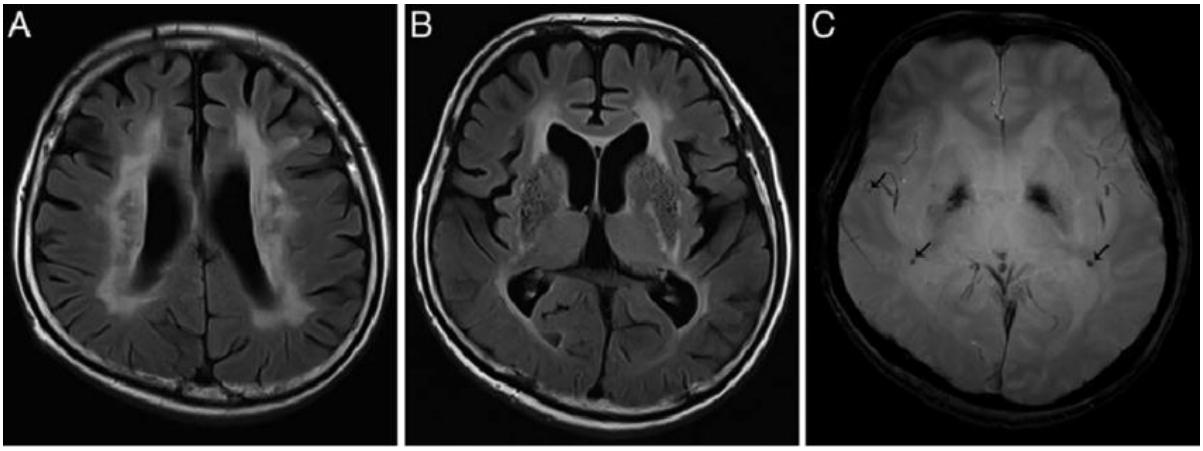

Figure 3: Brain MRI of CARASIL patient. Axial FLAIR images demonstrate hyperintensity in the deep and periventricular WM (A, B). T2-star gradient echo image showing several foci of microbleeding in the bilateral periventricular white matter (C). (Ito J et al., 2018).

Biallelic mutations in the *HTRA1* gene are the only known cause of CARASIL. *HTRA1* gene is localized on chromosome 10 (10q26) and codifies for a serine protease, which mediates cell signaling and protein degradation, playing an important role in vascular integrity, skeletal development and osteogenesis (Khaleeli Z et al., 2015). HTRA1 serine protease also modulates transforming growth factor (TGF)-β, a cytokine that promotes cell differentiation and fibrous proliferation, in response to the tissue damage. Mutations in *HTRA1* result in the loss of Htra1 protease activity, leading to an increase in TGF-β signaling, causing the degeneration of smooth muscle cells in the cerebral small vessels and angiopathy and subsequently cerebral small-vessel arteriopathy (Ibrahimi M et al., 2017). The upregulation of TGF-β may cause non-neurological manifestations of CARASIL, including alopecia and degenerative spine disease (Ibrahimi M et al., 2017).

1.4.1 Heterozygous HTRA1-associated cerebral SVD

Recent studies have demonstrated that individuals with heterozygous mutations in *HTRA1* may develop a cSVD (Verdura E et al., 2015; Di Donato I et al., 2017; Ito J et al., 2018). The histopathologic and neuroimaging of a patient carrying heterozygous *HTRA1* mutation displayed features of the small vessels were resembling those seen in CARASIL patient, but less severe (Ito J et al., 2018). Clinical features of this condition overlap with common sporadic SVD and other genetic cerebrovascular disorders like CADASIL and CARASIL. In heterozygous *HTRA1* carriers have a later age of onset, compared to CARASIL patients, a milder phenotype and the lack of the extra-neurological signs, such as early-onset spondylosis and alopecia (Di Donato I et al., 2017).

1.5 Cathepsin A–related Arteriopathy with Strokes and Leukoencephalopathy (CARASAL)

Cathepsin A–related arteriopathy with strokes and leukoencephalopathy (CARASAL) is a new identified form of adult leukodystrophy caused by heterozygous mutations in the Cathepsin-A (*CTSA*) gene (Hervé D et al., 2012; Bugiani M et al., 2016; Lynch DS et al., 2017). Patients affected by CARASAL show migraine and cognitive impairment, accompanied by therapy-resistant hypertension and ischemic and hemorrhagic strokes, starting from the third to fifth decade (Bugiani M et al. 2016; Haffner et al., 2016).

MRI shows multifocal signal changes in the cerebral WM and basal nuclei, thalami, and brainstem, typical of SVD. Over time, the leukoencephalopathy becomes diffuse and confluent, followed by strokes and signal changes in the frontoparietal periventricular and deep WM that were patchy in younger patients (Bugiani M et al., 2016; Hwang YT et al., 2017). Temporal poles are generally spared with microbleeds that are also described (Bugiani M et al., 2016; Hwang YT et al., 2017) (Figure 4).

Clinical feature of CARASAL disease is vessel wall fibrous thickening which appear asymmetric, involving distal arteriolar branches and vasa vasorum, and lumen occlusion accompanied by loss of smooth muscle cells (Hwang YT et al., 2017).

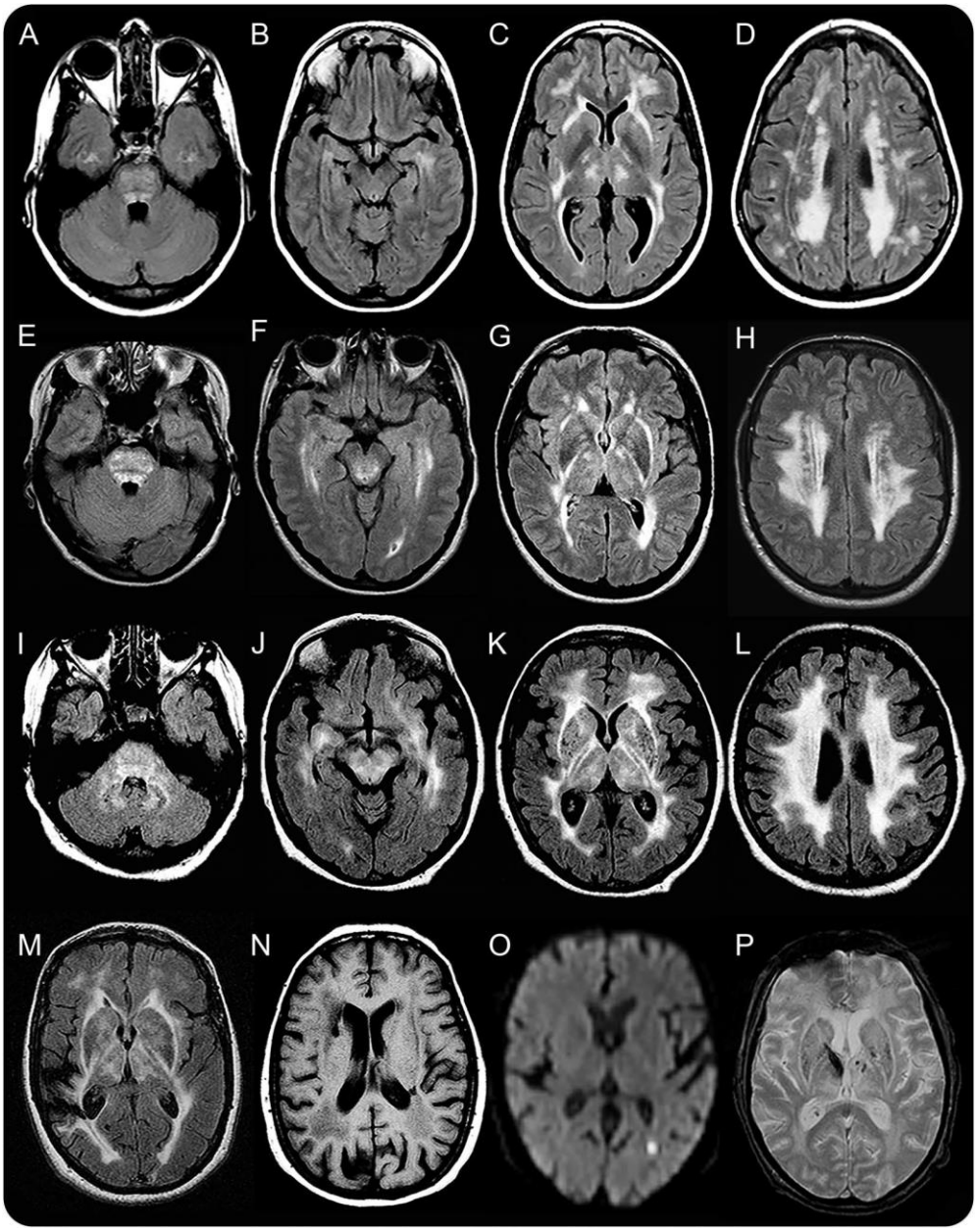

Figure 4: Axial images of 39 years-old patient (A–D), patient of 46 years (E–H), patient of 67 years (I–L), and patient of 69 years (M) FLAIR; T1-weighted image of patient of 73 years (N); diffusion weigthed imaging (DWI) of patient of 53 years (O); gradient echo image of another patient (P). MRIs show the multifocal to confluent WM abnormalities located predominantly in the frontoparietal deep and periventricular WM (B–D, F–H, J–L), the basal nuclei, thalamus, and the internal and external capsule (C, G, K). The pons shows multifocal T2-hyperintensities, even at an early age (A and E). The WM abnormalities are more extensive in the elder patients (compare figures A–H and I–L). Patient of 69 years had a large infarct located in the right temporoparietal region (M). Small cystic infarcts are seen in the periventricular WM (N). Some infarcts are acute, as indicated by a high signal on DWI (O). Microbleeds and a small hemorrhage are seen in the basal nuclei and thalamus (P). (Bugiani, M et al., 2016).

Heterozygous *CTSA* gene mutations have been recognized as the leading cause of CARASAL disease in some Caucasian families (Bugiani M et al., 2016). The *CTSA* gene is located within the chromosome 20q13 and encodes for the Cathepsin-A (CathA) protein, a ubiquitous lysosomal enzyme which stabilizes the multienzyme complex with lysosomal β-galactosidase (*GLB1*) and neuraminidase-1 (*NEU1*). Cathepsin A has a protective and catalytic function: it activates and stabilizes both β-galactosidase and neuraminidase-1 and inactivates many endogenous proteins including endothelin-1, bradykinin, substance P, oxytocin and angiotensin I (Timur ZK et al., 2016; Calhan O.Y et al., 2016).

Biallelic mutations in the *CTSA* gene are found to cause the galactosialidosis (GS) disease, whose pathogenesis is well understood (D'azzo A et al., 1982; Caciotti A et al., 2013) unlike the CARASAL, where the functional role of the *CTSA* mutation in CARASAL is currently unknown.

Bugiani M et al. observing an overexpression of Cathepsin A in a patient's WM astrocytes, speculated that that heterozygous mutation in *CTSA* gene might lead to an alternative Cathepsin A function or could interfere with Cathepsin A folding, provoking a neomorphic effect. *Bugiani M et al*. (2016) also observed a higher abundance of endothelin-1-stimulated astrocytes widespread in the brain of CARASAL patients probably a consequence of a reduced Cathepsin A activity (Bugiani M et al., 2016). Increased endothelin-1 levels, a potent regulator of blood vessels tone, might result in a general impairment of myelination, leading to a widespread leukoencephalopathy independently of vascular lesions, and could contribute to arterial hypertension in these patients (Bugiani M et al., 2016).

1.6 Overview of the Cathepsin-A Protein and *CTSA* gene

The human *CTSA* gene is located on chromosome 20q13 and codify for Cathepsin A protein, also known as Protective Protein/ Cathepsin A (PPCA) (Annunziata I & d'Azzo A, 2017). Cathepsin A is synthesized as a 480-amino acid pre-pro-form of 54 kDa, containing a canonical hydrophobic signal peptide of ~46 residues and nine cysteines. The signal peptide contains a stretch of leucine repeats, that varies in number among different individuals (Annunziata I & d'Azzo A, 2017).

Maturation of the Cathepsin A protein starts with removal of the signal peptide. After this process, the precursor protein is N-glycosylated in the endoplasmic reticulum at Asn163 and Asn351 positions, leading to the formation of a homodimer. Subsequently, the addition of the mannose-6-phosphate on the Asn117 allows the Cathepsin A precursor to migrate to the lysosomes where it undergoes to endoproteolytic cleavage that generates an intermediate with two chains of 34- and 20-kDa molecular weight. The last step is the excision of a 2 kDa peptide from the C-terminus of the 34 kDa chain, which gives rise to the mature and fully active enzyme (Bonten EJ et al., 1995).

Cathepsin A belongs to the serine carboxypeptidase family, with two highly conserved domains, and a catalytic site composed by Ser196, His475 and Aps418 residues (Annunziata I & d'Azzo A, 2017). The protein has both a protective (in this case is referred as PPCA) and catalytic (referred as CathA) function on many endogenous peptides, including beta-galactosidase (β-gal), neuraminidase-1 (Neu1), endothelin-1, oxytocin, bradykinin and substance P (Galjart NJ et al., 1991; Annunziata I & d'Azzo A, 2017). Recent studies demonstrated that the principal function of Cathepsin A is to protect and stabilize both beta-galactosidase and neuraminidase into lysosomes, preventing their degradation (Galjart NJ et al., 1991; Bonten EJ et al., 2014). Cathepsin A is also implicated in endothelin-1 degradation in human tissues (Itoh K et al., 1995), and in autophagy of peptides that contain a KFERQ domain in their structure (Annunziata I & d'Azzo A, 2017). The Cathepsin A activity on endothelin-1 gives rise that it plays an important role in blood pressure regulation and elastic fibers development (Seyrantepe V et al., 2008; Annunziata I & d'Azzo A, 2017).

Homozygous *CTSA* gene mutations are associated with galactosialidosis (GS) that belongs to a group of diseases called lysosomal storage diseases (LSDs), linked to lysosomal enzymes deficiency and not degraded macromolecules accumulation (Ferreira CR et al., 2017).

1.7 Cathepsin A regulation of Endothelin-1 homeostasis

One of the functions of Cathepsin A is the rapidly inactivation of endothelin-1 in human tissues by converting it into biologically inactive des-Trp21-endothelin-1 through its carboxypeptidase activity, leading to a reduction of circulating endothelin-1 (Hanna WL et al., 1994; Itoh K et al., 1995). ET-1 peptide overexpression is documented in patients with the acute peripheral ischemia, myocardial infarction and in patients with other chronic conditions as congestive heart failure, atherosclerosis, hypercholesterolemia, and systemic and pulmonary hypertension (Goraca A, 2002; Pshezhetsky AV et al., 2009). Overexpression of endothelin-1 has been reported both in galactosialidosis and CARASAL due to alterations in Cathepsin A function (Pshezhetsky AV et al., 2009; Bugiani M et al., 2016), suggesting the inactivation of Cathepsin A as one of the primary factors in endothelin-1 level alteration.

1.8 Overview of the Endothelin-1 peptide and *EDN1* gene

The peptide hormone endothelin-1 (ET-1) is a 21 amino acid protein that plays multiple and complex roles in cardiovascular, neural, pulmonary and renal physiology in a paracrine or autocrine function (Stow LR et al., 2011). ET-1 belongs to the isopeptides family (ETs: ET-1, ET-2, ET-3, ET-4), which have a similar protein structure and vasoactive function on blood vessels (Sandoval YH et al., 2014; Houde M et al., 2016; D'Orleans-Juste Pet al., 2019). ETs are synthetized starting from a precursor (Pre-proendothelin) of 212 amino acids, that is processed by carboxypeptidases to form the proendothelin. Subsequently, the proendotelin is cleaved by furin (a protein that belongs to the subtilisin family) to generate an inactive form of ET-1 that is further cleaved by a group of enzymes called "endothelin converting enzymes" (ECEs) on Trp21 and Val22 residues to generate active ET-1 (Blais V et al., 2002; D'Orleans-Juste P et al., 2019) (Figure 5).

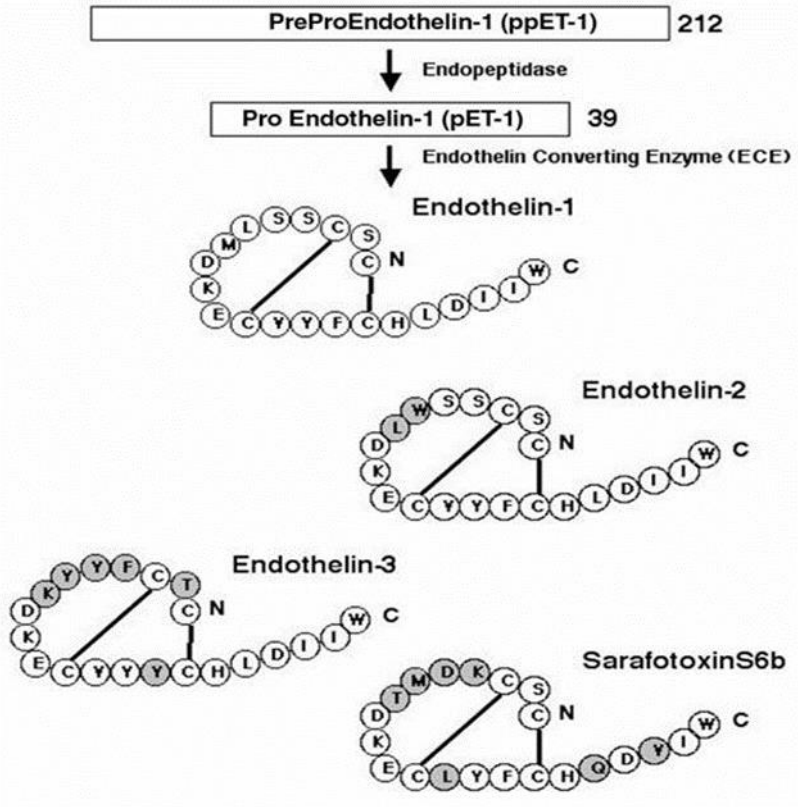

Figure 5: Biosynthesis and amino acid sequence and structure of endothelin-1, endothelin-2, and endothelin-3 and related sarafotoxins, that differs for some amino acids each other (Fagan K et al., 2001).

All isoforms of ETs bind in active form to transmembrane G-coupled receptors called ET receptors subtype A (ET-A) and B (ET-B), which have a specific tissue localization that define their function (Li JJ et al., 2010; Zhang & Sui, 2014; D'Orleans-Juste P et al., 2019). ET-1 binds ET-A with the highest affinity among ET receptors, ET-A is mainly expressed on vascular smooth muscle in the brain (Hori S et al., 1992).

ET-1 activation of ETA leads to vasoconstriction through Phospholipase C activation, inositol triphosphate (IP3) release that stimulates Ca2+ release, resulting in vascular smooth cells contraction (Khimji & Rockey et al., 2010; D'Orleans-Juste P et al. 2019). Conversely, the stimulation of ETB mediates vasodilatation through the activation of IP3/Akt pathway, with release of Nitric Oxide (NO) causing relaxation of smooth muscle cells (D'Orleans-Juste P et al., 2019). ETB is mainly expressed in astrocytes (Li JJ et al., 2010). It has been assumed that ET-1 modulates astrocytes activation and proliferation (Li JJ et al., 2010) (Figure 6).

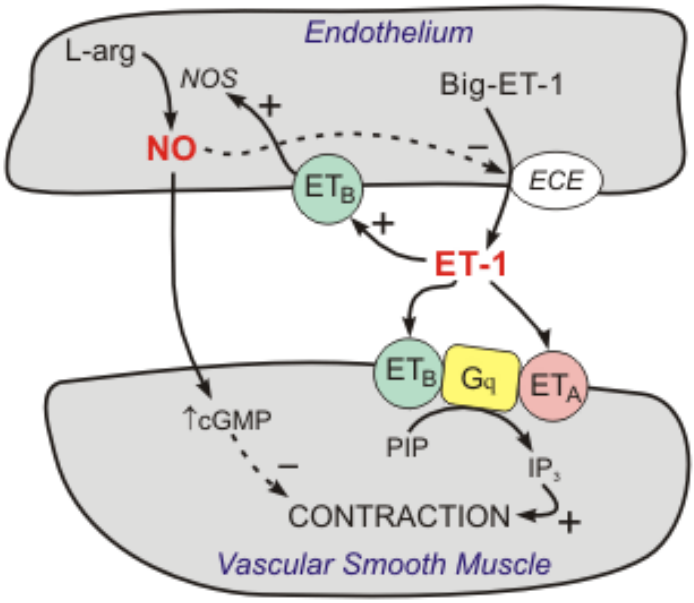

Figure 6: Endothelin-1 interaction with ET receptors and generated pathways in the vascular smooth muscle cells homeostasis (Klaubunde R et al., 2011).

There is accumulating evidence that ET-1 may play a role in the pathophysiology of many vascular disease, including hypertension and in ischemic/hypoxic injuries in the adult brain (Feldstein & Romero et al., 2007; Li JJ et al., 2010; Zhang L et al., 2014; D'Orleans-Juste P et al., 2019) (Figure 7). ET-1 both enhances glutamate-induced neuronal toxicity and the clearance of glutamate, resulting in an increase of intensity of the ischemic brain damage (Matsuura S et al., 2002; D'Orleans-Juste P et al., 2019). Recent studies also demonstrate the importance of ET-1 for oligodendrocytes precursor cells (OPC) differentiation and remyelination processes (Hammond TR et al., 2014). It has been demonstrated that ET-R antagonists promote remyelination, at least in part, by preventing Notch activation (Hammond TR et al., 2014). These findings demonstrate the importance of ET-1 signaling in myelin pathology and as a potential therapeutic target to promote remyelination and prevent OPC differentiation failure (Hammond TR et al., 2014).

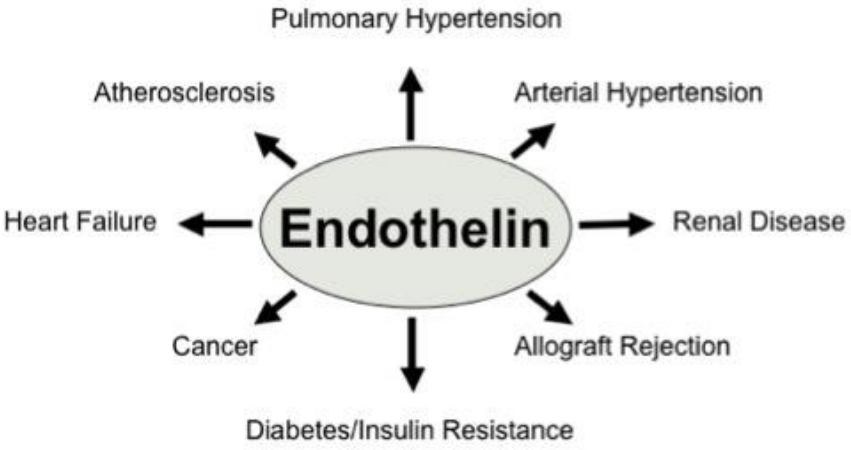

Figure 7: Disease processes in which endothelin-1 / endothelin ETA-receptor signaling has been demonstrated to play a role (Matthias Barton & Masashi Yanagisawa, 2008).

ET-1 precursor is codified by *EDN1* gene, consisting of 5 exons and it is localized on chromosome 6 (6p24.1), spanning -6.8 kb of genomic DNA (Stow LR et al., 2011). Transcriptional regulation of *EDN1* gene, despite other regulatory factors exist, is supposed to be the major mechanism controlling ET-1 bioavailability being the first regulation level of ET-1 (Stow LR et al., 2011). *EDN1* regulation is also controlled by histone modifications (Stow LR et al., 2011). It is possible that unregulated *EDN1* expression could be due to the progressive loss of epigenetic markers, either histone modifications or DNA methylation, that progressively lead to an altered level of ET-1 observed in some late-onset diseases such as diabetes or hypertension. (Stow LR et al., 2011).

The *EDN1* gene can respond to various stimuli, most of them are not well known (Stow LR et al., 2011). The *EDN1* promoter is regulated by both proximal and distal cis-acting elements (Stow LR et al., 2011). Alteration of *EDN1* gene expression have been largely documented in the various human disease, including left ventricular hypertrophy, asthma and atherosclerosis (Stow LR et al., 2011). In general, increased ET-1 production and have been seen in various pathogenic mechanisms related to diabetes (Biswas S et al., 2018), and neuron necrosis after stroke (Armstead WM et al., 2018). Moreover, ET-1 appears to be closely associated with loss of integrity of endothelial wall and of the blood brain barrier (BBB) (D'Orleans-Juste P et al., 2019). Finally, recent studies demonstrated the close link between ET-1 upregulation with the Alzheimer's Disease and other brain pathologies pathogenesis (Palmer, Tayler & Love, 2013; Hammond TR et al., 2014).

1.9 Cathepsin A and Galactosialidosis

Galactosialidosis (GS, OMIM # 256540), also known as Goldberg syndrome, is a rare form of autosomal recessive lysosomal storage disease caused by homozygous mutations in the *CTSA* gene, characterized by secondary combined deficiency of β-galactosidase (*GLB1*) and neuraminidase 1 (*NEU1*), due to loss of function of the PPCA protein (d'Azzo AAG et al., 2001; Caciotti A et al., 2013; Ferreira CR et al., 2017). The disease is rare, although its prevalence is currently unknown. GS is usually classified as early infantile, late infantile and juvenile-adult type, which differ in age of onset and severity of symptoms (Annunziata I & d'Azzo A, 2017). Most patients have a juvenile/adult form of the disease, characterized by a more severe clinical presentation.

Principal symptoms that are common among all clinical forms include: coarse facies, vertebral changes, cherry-red spots and corneal clouding, accompanied by severe neurologic manifestations, including myoclonus, cerebellar ataxia, generalized seizures, progressive cognitive impairment and mental retardation (d'Azzo, A et al., 2013; Annunziata I & d'Azzo A, 2017). The GS patients show and arterial hypertension and a cardiomyopathy that cannot be mechanistically linked to the lysosomal storage of glycoconjugate (Pshezhetsky AV et al., 2009) and develop a severe vacuolization and swelling of endothelial cells in central nervous system vessels (Kyllerman M et al. 1993, Nordborg C et al. 1997), that are linked to altered levels of ET-1 (Pshezhetsky AV et al., 2009).There is no cure for Galactosialidosis. Treatment is symptomatic and supportive. However, some therapeutic approaches are in development using the knockout mouse model of GS (Annunziata I & d'Azzo A 2017). The strategy consisted on transplantation of knockout (PPCA-/-) mice with the transgenic bone marrow containing erythroid precursor overexpressing PPCA enough in the serum of transgenic mice to correct NEU1 and β-GAL activities when taken up by deficient patient's fibroblasts (Annunziata I & d'Azzo A 2017). However, there is no evidence of efficacy on human cells nowadays.

A total of more than 30 biallelic mutations associated with Galactosialidosis have been identified in the *CTSA* gene, including small deletions/insertions, missense mutations, splicing variants and nonsense mutations, mostly identified in Japanese patients. (http://www.hgmd.cf.ac.uk/ac/index.php and http://www.ncbi.nlm.nih.gov/clinvar). These mutations are strongly related to the severity of symptoms and the onset of the disease. However, not only *CTSA* mutations are linked to the clinical outcome of the disease, but other genetic, environmental and dietary factors could play a crucial role in the expression and penetrance of specific clinical manifestations in GS patients (Annunziata I & d'Azzo A, 2017). However, specific secondary factors involved in GS pathogenesis are still unknown.

2. AIM OF THESIS

CARASAL seems to be an ultra-rare form of cSVD in middle age patients. However, the frequency of CARASAL could be underestimated, due to the only recent association of the *CTSA* gene to a dominant form of SVD. To the best of our knowledge, only 19 CARASAL patients, belonging to 4 families (two Dutch, one French and one British family) have been reported worldwide. All of them carry the c.973C>T (p. Arg325Cys) (Bugiani M et al., 2016). Since the last report of 2017 (Lynch DS et al., 2017), no further patients have been reported.

The principal aim of this thesis is to perform a genetic analysis of *CTSA* gene in a group of selected patients with adult onset cSVD who screened negative for mutations in the causative genes of CADASIL and CARASIL, the more common forms of familial SVD.

Furthermore, we aimed to analyze the *EDN1* promoter site in order to exclude alterations of the transcriptional processes as primary cause of endothelin-1 levels increase, given that transcriptional regulation of *EDN1* gene is supposed to be the major mechanism controlling ET-1 bioavailability (Stow LR et al., 2011). Previous evidences have shown that endothelin-1 peptide may have a role in the pathophysiology of many vascular diseases, including hypertension and in ischemic/hypoxic injuries in adults, and to be a possible link with CARASAL pathogenesis (Bugiani M et al., 2016; D'Orleans-Juste P et al., 2019, Zhang L et al., 2014; Li JJ et al., 2010; Hammond TR et al., 2014; Palmer, Tayler & Love, 2013).

3. MATERIALS AND METHODS

3.1 Patient selection

In a period ranging from 2016 to 2019, a population of 130 patients (60 females with age ranging from 30 to 80 years) has been recruited by the Neurology and Neurometabolic diseases Department of Policlinico Santa Maria Le Scotte Hospital with cSVD with the following inclusion criteria:

1) Ischemic and hemorrhagic strokes and MRI evidence of symmetrical and confluent WM hyperintensities on T2-weighted MRI.

2) Exclusion of acquired causes such as infections or autoimmune syndromes and other classical leukodystrophies through laboratory examinations (white cell enzyme studies, very long chain fatty acids, amino/organic acids and bile alcohols).

3) Absence of mutations in *NOTCH3* and *HTRA1* by conventional Sanger sequencing.

Moreover, about 20 patients with uncertain familiarity and no other explained vascular leukoencephalopathy were also included in the study. We defined family history, when available, with direct examination or with interview of the probands and family members. Previously to participation, participants signed informed consents.

3.2 Gene Amplification and Sequencing

Genomic DNA samples were obtained from the peripheral blood leukocytes, according to standard methods. Quantitative and qualitative controls have been performed through NanoDrop-1000 spectrophotometer (Thermo Scientific, Wilmington, USA). The DNA samples of the patients were amplified using Polymerase Chain Reaction (PCR). All coding portion of the CTSA gene (from exon 1 to exon 15, NM_000308) was analyzed using primers designed on intronic sequences (Table 3). The PCR amplifications were carried out in 25 µl of reaction volume containing about 100 ng of genomic DNA mixed with 10× buffer containing MgCl2, 0.4 µM forward and reverse primers, 0,2 mM of each dNTPs and 0.2 U FastStart Taq DNA Polymerase (Roche Diagnostics Gmbh, Mannheim, Germany). Amplification consisted in 40 cycles comprising of 30 seconds denaturation at 94 °C, 40 seconds annealing at 58 °C or 60 °C and 1 minute and 30 seconds extension at 72 °C. A 2 minutes initial denaturation at 94 °C and a 5 minutes final extension at 72 °C were performed. The products of individual PCR reactions were separated electrophoretically in 1.5% agarose gels and revealed with GelRed Nucleic Acid Staining (Biotium Inc., 46117 Landing Parkway Fremont, CA 94538). PCR products were sequenced on both strands by use of the same PCR primers, and the Big Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) on ABI-PRISM 3100 genetic analyzer automated sequencer (Applied Biosystems, Foster City, CA, USA) according to manifacturer's protocol. Obtained results were analyzed using ChromasPro software (Technelysium Pty Ltd, South Brisbane QLD 4101 Australia) and compared with the standard human *CTSA* gene (NM_000308) found on Ensembl browser genome database (EMBL-EBI, Hinxton, Cambridgeshire, CB10 1SD, United Kingdom). The same procedure of gene amplification and sequencing have been performed for *EDN1* gene promoter analysis, with primers summarized in Table 4.

Table 3: PCR primers used for exon amplification and sequencing of the *CTSA* gene.

Forward primer sequence [5′→3′]	Reverse primer sequence [5′→3′]	Fragment length (bp)	Tann (°C)
CTSA_1-2fw AATGATGGTGACCGCAAGG	CTSA_1-2rv GCTCCCAGGCATCCTCAG	811	60
CTSA_3-4-5fw GTAGTTCTGCAGACCCCTGAG	CTSA_3-4-5rv TGGCCACCATACCACAATAG	791	58
CTSA_6-7fw TGGCCAGTCACTTCCTCTTG	CTSA_6-7rv GGGCCTGTCATATCATGTGC	793	60
CTSA_8fw AGGAGGCCTGTCTGTATGACC	CTSA_8rv TGTCCCAGAGAACACTAAATTGC	419	60
CTSA_9-10-11fw CAGTAAATCTTGGGACAACTTGG	CTSA_9-10-11rv TCAATCCACTGAGGGGTCA	718	60
CTSA_12fw CTTCTGGGTTGGAGCTTGG	CTSA_12rv TCCTATAGCAGGACAGCATGG	372	62
CTSA_13-14fw TTGGCCCCTTGAATTTCC	CTSA_13-14rv AAGCTTTTATTCTTCCCAGAACC	663	60
CTSA_15fw TGGGAAGAATAAAGGGTTTGC	CTSA_15rv CTGTGGGCTGTCCTGAGC	494	62

Table 4: PCR primers used for exon amplification and sequencing of the *EDN1* gene promoter.

Forward primer sequence [5′→3′]	Reverse primer sequence [5′→3′]	Fragment length (bp)	Tann (°C)
P3F TAGAATATTCTGAGGTGCATTCCT	P3R TGCGGGTCTTTTTTGCTG	400	62
P2F CCAATCCCTCACGGATCTTTC	P1R TTCGGGGGCAGGGCTAAGAAAAA	718	62
E1F GACGCGCCTCTGCATCTG	E1R GGTGGATAAGAACACACTAACTTG	345	58

3.3 *In Silico* Prediction Analysis

Prediction of the functional effects of novel missense variants was performed by Poliphen-2, SIFT, Mutation Taster and VarSome prediction tools.

Poliphen-2 (*Polymorphism Phenotyping v2*) is a prediction software that evaluates the aminoacidic substitution impact on the protein structure or function through structural and comparative parameters (http://genetics.bwh.harvard.edu/pph/; Adzhubei IA et al., 2010).

SIFT (*Sort Intolerant from Tolerant*) prediction is based on the conservation rate of specific aminoacidic residues, calculated through alignment among correlated sequences, grouped by PSI-BLAST (http://sift.jcvi.org/www/SIFT_enst_submit.html; Matsuki Y et al., 2009).

Mutation Taster is a prediction software able to distinguish between polymorphisms or disease-causing mutations thanks to statistical models (http://www.mutationtaster.org/; Schwarz JM et al., 2010).

Varsome platform prediction results by bringing together the statistical results of more than nine in silico predictor tools (DANN, SIFT, PolyPhen-2, GERP++, MutationTaster, Mutation Assessor, FATHMM, LRT, SiPhy and PhyloP) (Kopanos C et al., 2019).

Clinical information of databases (Ensembl, HGMD, ClinVar), were used to classify reported variants as polymorphisms or mutations. We used the default settings for all tools, and we take the binary prediction (pathogenic/neutral) into account for the prediction results.

Predictions of possible effects of novel missense variants on protein translation and maturation were performed by SignalP-5.0 Server (DTU Bioinformatics, Kemitorvet Building 208 DK-2800 Kgs. Lyngby) and ProtScale bioinformatic tools (ExPASy, University of Geneva, Switzerland) (http://web.expasy.org/translate/).

The SignalP 5.0 server is based on a deep convolutional and recurrent neural network architecture including a conditional random field and predicts the presence of signal peptides and the location of their cleavage sites in proteins from Archaea, Gram-positive Bacteria, Gram-negative Bacteria and Eukarya (Almagro Armenteros JJ et al., 2019).

ProtScale allows to compute and represent (in the form of a two-dimensional plot) the profile produced by any amino acid scale on a selected protein. The most frequently used scales are hydrophobicity scales, most of which were derived from experimental studies on partitioning of peptides in apolar and polar solvents, with the goal of predicting membrane-spanning segments that are highly hydrophobic, and secondary structure conformational parameter scales. In addition, many other scales exist which are based on different chemical and physical properties of the amino acids (Gasteiger E et al., 2005).

3.4 Enzymatic Activity Assay

Lysosomal Beta-Galactosidase activity has been measured by fluorometric assay by using 4-Methylumbelliferone ≥98% (4-MU, Sigma Aldrich) derived artificial substrate. Briefly, leukocytes derived from 2 mL peripheral blood mixed with 150 µl H2O are sonicated (10 seconds) and diluted 21 times with a solution containing 0,2% BSA denaturized at 50 °C and 0,9% NaCl. Subsequently, 10 µl homogenate is mixed and incubated 1 hour at 37°C with 20 µl substrate solution containing 1 mM MU-β-D-galactopyranoside monohydrate, 0,1 M NaAc pH 4.3 and 0,1 M NaCl, dissolved at 60 °C. After incubation, 500 µl stop reaction at pH 10.7 has been added. Result is the release of 4-MU by processed substrates, highly fluorogenic at pH 10-11, that is read by fluorometer at λex 372 nm; λem 445 nm in ethanol. The amount of released 4-MU is proportional to the emitted fluorescence and to enzyme activity. The method is suitable for each other lysosomal enzyme by using their specific artificial substrates. The Table 5 shows each enzyme with its respective substrate:

Table 5: All lysosomal enzymes analysed with their respective substrate.

Lysosomal enzyme	**Substrate**
α-L-Fucosidase	4 MU-α-L-Fucopyranoside
α-L-Mannosidase	4 MU-α-D-Mannopyranoside
α-D-Galactosidase	α-D-Galactopyranoside
β-D-Galactosidase	MU-β-D-Galactopyranoside
N-Acetyl-β-Glucosaminidase	N-acetyl-β-D-glucosaminide
N-Acetyl-β-Glucosaminidase-isoA	MU-β-GluNac-6S
β-Glucuronidase	MU-β-D-Glucoronide
N-Acetyl-α-Galactosaminidase	4-Nitrophenyl N-acetyl-α-D-galactosaminide
P-Nitrocatechol-Sulphatase A	P-Nitrocathecol-Sulphate (PNCS)
P-Nitrocatechol-Sulphatase B	P-Nitrocathecol-Sulphate (PNCS)

4. RESULTS

Sanger sequencing of 130 probands revealed a high prevalence of known polymorphisms, both localized in intronic and exonic portions of the *CTSA* gene. All these variants are reported on Ensembl database, showing high frequency (MAF > 0.1). All probands had one or more of these variants at a heterozygous state. None of them affects protein structure, function or expression (Table 6). The same genetic analysis revealed a novel missense variant (c.84C>A, p. Phe28Leu) localized on exon 2 (NM_000308), corresponding to the portion of the gene codifying for the signal peptide (SP) (Table 6). The novel mutation is absent in GnomAD, ExAC and dbSNP databases and is predicted with Uncertain Significance of pathogenicity by Varsome, whereas is predicted as polymorphism by MutationTaster, PolyPhen-2 and SIFT bioinformatic tools. This variant is absent in proband's healthy family members. The *EDN1* promoter analysis did not show any variant or mutation that could interfere with the normal protein expression.

Table 6: List of variants found on proband's *CTSA* gene.

Location	SNP	Frequency (MAF)
Exon 2	c.108_110delGCT	0.35
Exon 2	c.118_110insGCT	0.48
Exon 3	c.273C>G	0.13
Splice region Intron 10	c.1002+7G>A	0.49
Intron 6-7	c.746+55A>G	0.49
Intron 7-8	c.747-33A>G	0.49
Intron 9-10	c.924-15delC	0.49
Intron 11-12	c.1002+7G>A	0.49
Intron 11-12	c.1142+10C>T	0.49
Intron 14-15	c.1413+35G>A	0.49
Intron 14-15	c.1413+107T>C	<0.01
NEW IDENTIFIED		
Exon 2	c.84C>A	

Table 7: List of variants found on proband's EDN1 gene promoter.

Location	SNP	Frequency (MAF)
Promoter	c. -131delA	0.35

Patient presentation (c.84C>A, p.F28L): the proband was a 78-years old male who was referred to our department after an acute episode of confusion and speech disorder. He suffered from hypertension and mild cognitive impairment. He had an ischemic stroke one year before. No family history of dementia, but both his parents had an ischemic stroke in elderly. Neurological examination of the patient showed spatial-temporal disorientation and difficulties in comprehension and speech. MRI showed diffuse leukoencephalopathy and cortical atrophy more prevalent on anterior left hemisphere. Small multifocal areas of signal abnormality were seen in basal nuclei, thalami, internal and external capsules but the brain stem were not affected, and temporal poles and U-fibers were relatively spared. Subcortical microbleeds and cortical hemosiderosis were present only in the left hemisphere. Cortical laminar necrosis was present but never described in literature before and could testify the neurovascular impairment as well as the perfusion MR imaging. Indeed, arterial spin labelling acquisition confirmed the large cerebral hypoperfusion in early and late phases of disease progression. (Figure 8).

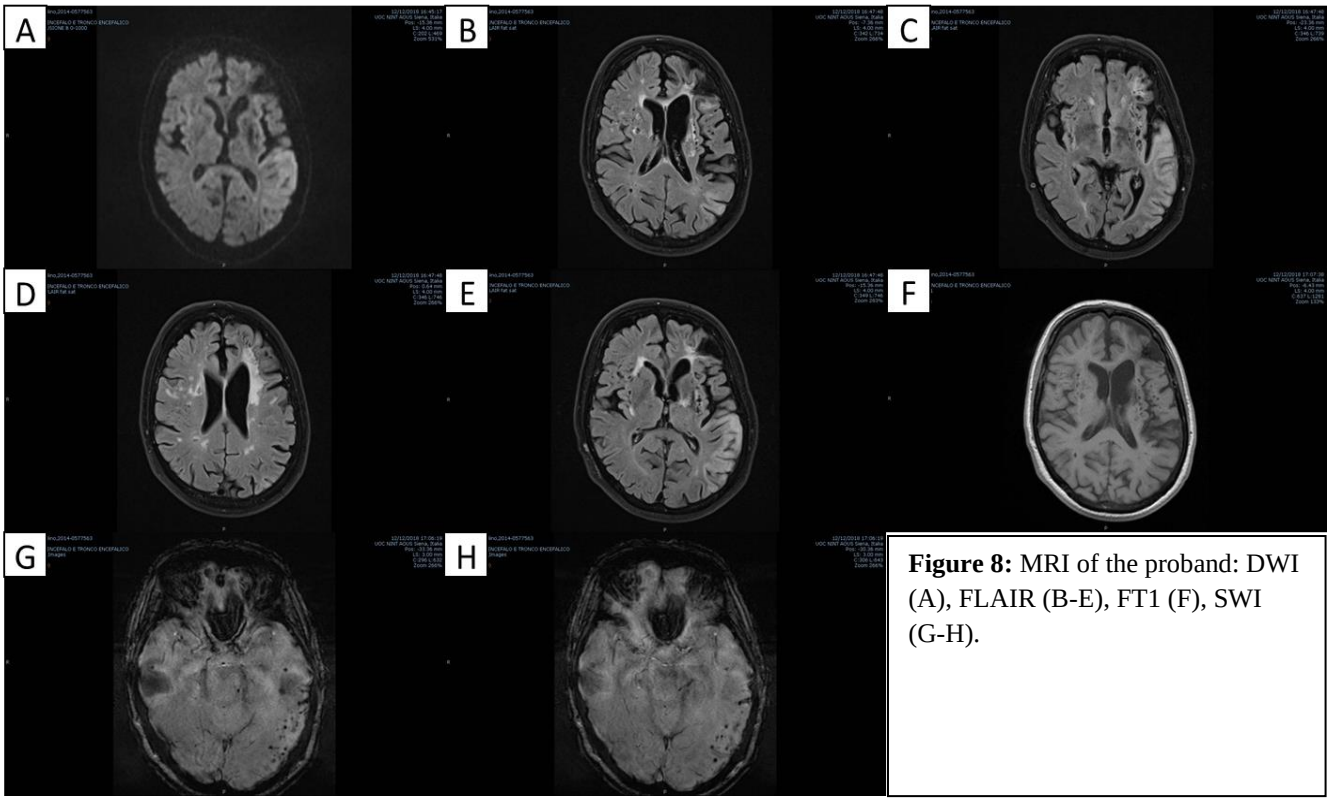

Figure 8: MRI of the proband: DWI (A), FLAIR (B-E), FT1 (F), SWI (G-H).

Lysosomal enzymes in the proband, measured with standard fluorometric assay, resulted in normal ranges. No other patients have been subjected to this type of analysis (Table 8).

Table 8: Value rates of the proband's lysosomal enzymes activity.

Lysosomal enzyme	Proband's value (nmol/mg prot)	Normal range (nmol/mg prot)
α-L-Fucosidase	81	40-100
α-L-Mannosidase	342	110-325
α-D-Galactosidase	45	30-100
β-D-Galactosidase	126	100-200
N-Acetyl-β-Glucosaminidase	2172	1100-3600
N-Acetyl-β-Glucosaminidase-isoA	179	90-240
β-Glucuronidase	315	250-650
N-Acetyl-α-Galactosaminidase	21	9-30
P-Nitrocatechol-Sulphatase A	83	36-100
P-Nitrocatechol-Sulphatase B	146	105-240

We identified the c.84C>A (p. Phe28Leu) variant in the proband at a heterozygous state (Figure 9 A), which is absent in his healthy brother (Figure 9 B-C). However, we had no other information on other brothers or sisters. By comparing the Sanger results among his brother and his son, the mutation seems to be on the same allele of another exonic variant (c.108_110delGCT, p. Leu37del), previously reported as benign and with high frequency (Figure 9 A-C). However, no further information about allele segregation have been reached. Orthologous sequence alignment shows that the mutation involves a phenylalanine into a not conserved domain of the protein from an evolutionary point of view. (Figure 9 D). The *EDN1* gene promoter analysis did not show any variant or mutation.

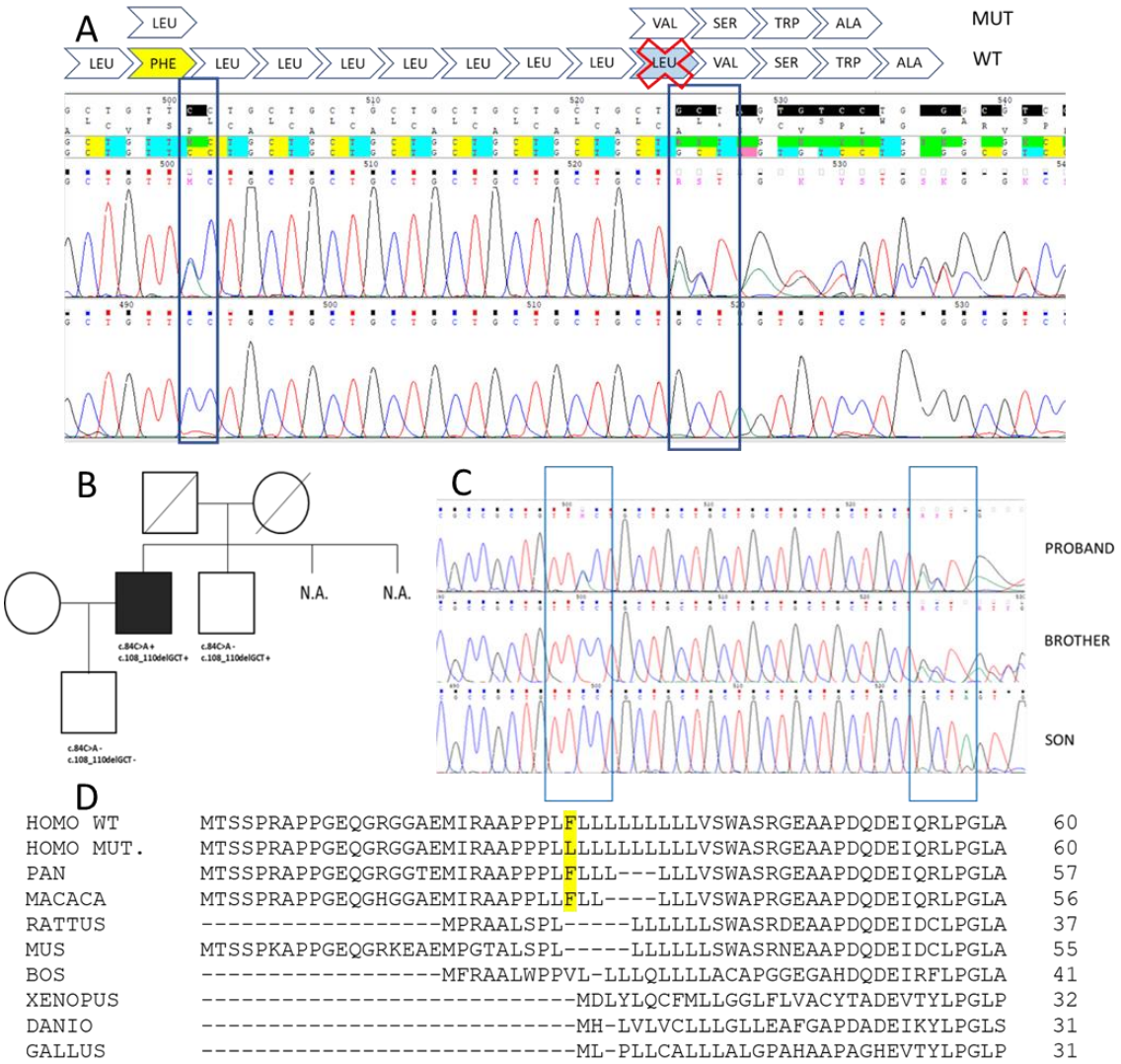

Figure 9: The c.84C>A variant is reported at a heterozygous state and seems to be on the same allele of another exonic variant (c.108_110delGCT) reported as common polymorphism, because proband's son has neither (A-B-C). The mutation is absent in the proband's healthy brother and affect a not conserved domain of the protein (D).

In *silico* analysis of the protein structure by UniProtKB/Swiss-Prot has revealed that the mutation occurs in the portion codifying for the signal peptide (SP) of the 54-kDa Cathepsin A protein. SignalIP-5.0 showed no significant changes on the cutting site of the SP respect to the wild-type (Figure 10), whereas ProtScale showed little changes in its hydrophobicity and secondary alpha-helix structure value rates respect to controls (Figure 11 A-B). However, *in vitro* analysis of the effect of proband's mutation on protein structure has not been performed yet.

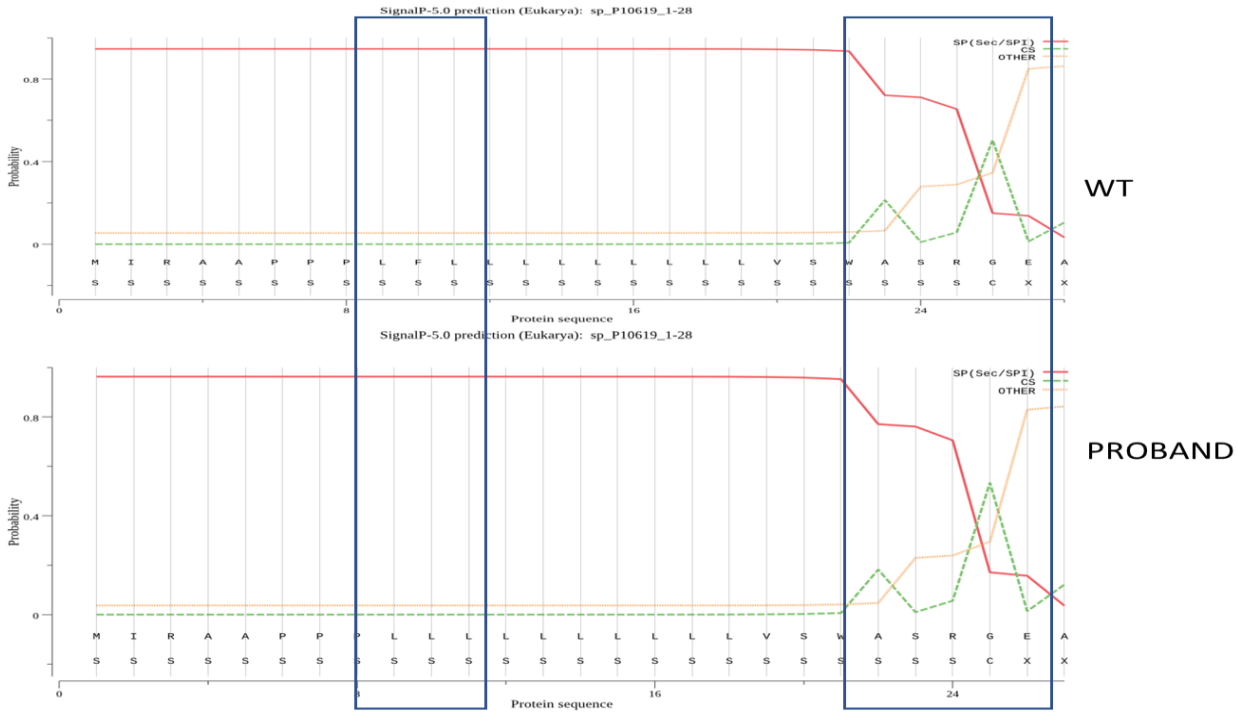

Figure 10: The c.84 C>A variant occurs into the highly hydrophobic chain of the signal peptide. However, *in silico* analysis did not show no changes in signal peptide structure or cutting site.

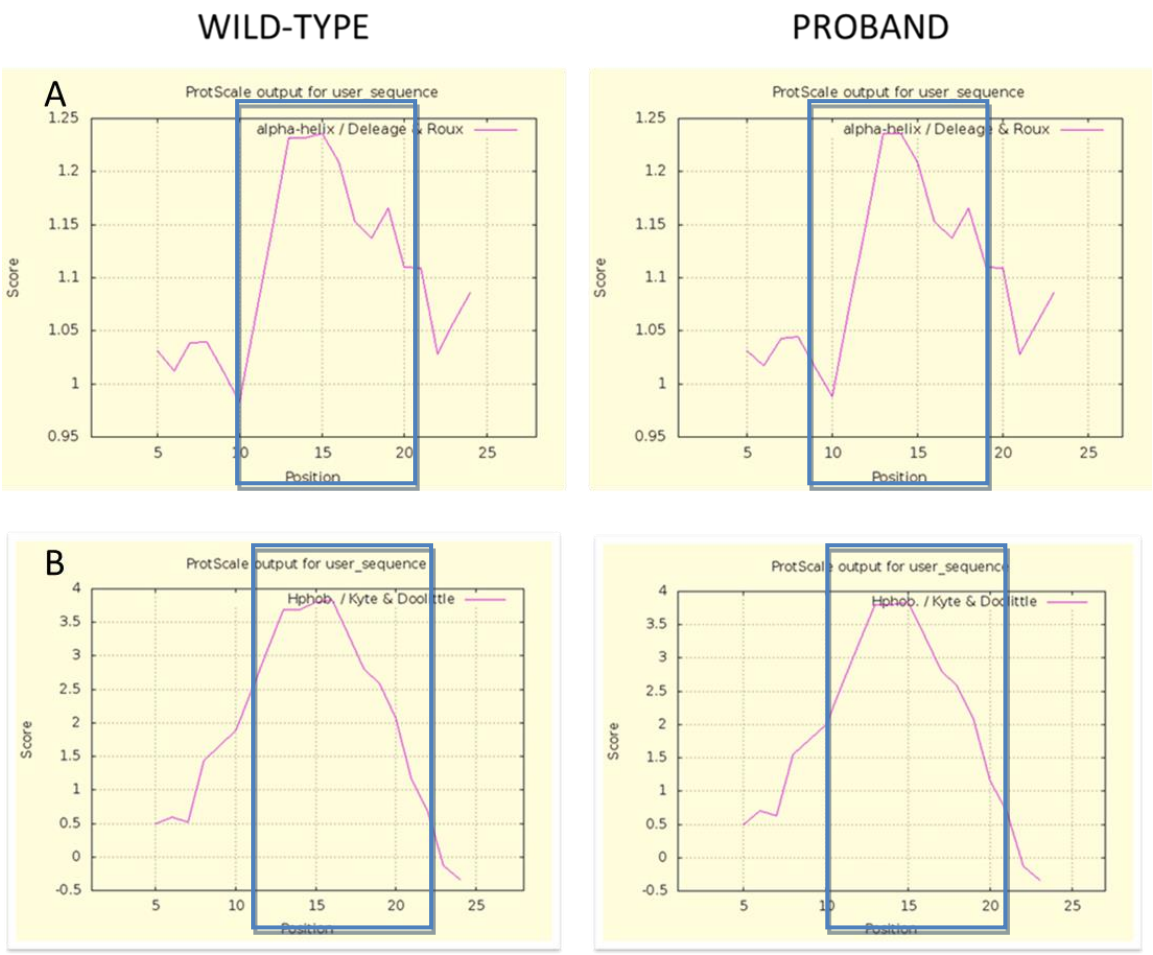

Figure 11: ProtScale analysis reveals little changes in alpha-helix turns (A) and hydrophobicity (B) scores in proband's signal peptide structure respect to controls. ProtScale has been imposted with default settings.

5. DISCUSSION

The pathological processes involved in cSVDs are complex and not fully understood. Genetic studies in both monogenic and sporadic forms of SVDs are expected to help in clarifying their pathomechanism. Most forms of cSVDs share similar pathological findings and clinical phenotypes, therefore the identification of a specific cSVD can be challenging. Genetic studies [Next Generation Sequencing (NGS) and Genome Wide Association Studies (GWAS)] will provide further information in our understanding of this disorder and the molecular mechanisms, also helping to discriminate different SVDs leading to a specific diagnosis.

The aim of this study was to evaluate the frequency of *CTSA* gene mutations in a selected pool of patients presenting clinical and neuroimaging features of cSVD and eventually compatible with CARASAL. These patients showed a family history of ischemic stroke, and a heterogeneous clinical phenotype including cognitive decline, behavioral changes, gait impairment, and stroke. All patients showed brain MRI evidence of signal alterations compatible with cSVD. In all cases the analysis for *NOTCH3* and *HTRA1* gene mutations was negative.

The screening of *CTSA* gene in our population revealed a novel mutation at a heterozygous state in a single patient, that involves a different portion of the gene with respect to the mutation previously reported in the CARASAL patients (Bugiani M et al., 2016; Lynch DS et al., 2017). The segregation analysis was limited to the available family members: a brother and a child clinically unaffected. In both cases the mutation was not found. Our mutation involves the exon 2, codifying for the hydrophobic core of the signal peptide (SP), responsible for the correct ribosome migration to the endoplasmic reticulum (ER) membrane and the mRNA translation on its surface. *In silico* analysis revealed that the mutation leads to small changes on SP secondary structure and hydrophobicity. As previously reported (Lodish H et al., 2000), the hydrophobic “core” of ER signal peptide is essential for its function, and alterations of its structure or hydrophobicity rate can abolish the ability of the protein to cross the ER membrane into the lumen. However, further investigations on the protein structure are needed to confirm the pathogenicity of this variant and to understand how it could interfere with the normal protein synthesis and localization. In conclusion, the effect of the new variant and its pathogenicity remains unclear.

The patient also carried the c.108_110delGCT (p. Leu37del) polymorphism. The combination between the c.108_110delGCT (p. Leu37del) polymorphism and our c.84 C>A (p. Phe28Leu) missense variant lead to an alteration in aminoacidic count of the SP respect to the wild-type, specifically a deletion of Phenylalanine, that could alter the correct conformation and hydrophobicity of the signal peptide. For this reason, the possible effect generated by the combination between the c.84 C>A (p. Phe28Leu) *CTSA* mutation and the c.108_110delGCT (p. Leu37del) polymorphism would be investigated. According to the results obtained by the lysosomal activity assay, the new variant does not lead to secondary deficiency of lysosomal enzymes as reported with other *CTSA* gene mutations linked to galactosialidosis (Caciotti A et al., 2013). Unfortunately, it has not been possible to analyze the *NEU1* activity, due to the unavailability of the kit, and further investigations will evaluate it specifically. However, the result obtained by performing enzymatic tests suggest a new and not known pathogenic pathway involved in CARASAL disease, as reported in literature (Bugiani M et al., 2016).

Bugiani M et al. (2016) observed a high amount of endothelin-1 reactive astrocytes around the WM lesions of CARASAL patients. They speculated that increased endothelin-1 levels might result in a general impairment of myelination, leading to a widespread leukoencephalopathy independently of vascular lesions, and this increase could be consequence of a reduced Cathepsin A activity. There are many factors that could increase the levels of endothelin-1, but as reported in literature (Stow LR et al., 2011), the major one seems to be the transcriptional regulation of *EDN1* gene, whose alterations have been demonstrated in various pathogenic mechanisms in which endothelin-1 is involved (Feldstein & Romero et al., 2007; Li JJ et al., 2010; Zhang L et al., 2014; D'Orleans-Juste P et al. 2019).

We therefore decided to perform a genetic screening of *EDN1* promoter in a subset of our patients, including the case with *CTSA* mutation and other cases with drug resistant hypertension in order to exclude alterations of endothelin-1 expression as co-factor of cSVDs pathogenesis. Our results are preliminary, due to the small number of patients analyzed, but they seem to confirm the previous hypothesis (Bugiani M et al., 2016) of increased endothelin-1 levels due to the impairment of Cathepsin A degradation function. However other factors, such as epigenetic mechanisms, could be involved in endothelin-1 levels increase (Stow LR et al., 2011) and are worth of further investigation.

Among cSVDs, CARASAL seems to be a very rare form with autosomal dominant inheritance and SVD-linked leukoencephalopathy. The only 19 cases from 4 families reported up to now (no further case has been reported since 2017) (Finsterer J et al., 2019), all carrying the *CTSA* gene mutation (c.973C>T), suggest that we are facing with a very rare cause of cSVD. However, this disease could be underestimated, due to the not well-defined clinical phenotype and the only recent association with *CTSA* gene. For this reason, genetic screening including *CTSA* gene on of larger population of patients with cSVD is crucial. In this respect, this study represents one of the first genetic screening of *CTSA* gene in a significant number of selected patients with cSVD.

Similarly to what happen with the *GBA* gene, whose homozygous mutations cause Gaucher's disease, whereas heterozygous mutations are responsible for late-onset Parkinson's disease (Ferreira M et al., 2017; Deng H et al., 2018), homozygous *CTSA* gene mutations are responsible for galactosialidosis whereas a heterozygous mutation is linked to a different cerebrovascular phenotype. It would be interesting to characterize the clinical and neuroimaging phenotype of homozygous patients and above all obligate heterozygous carriers in order to identify possible cerebrovascular alterations.

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