

Review

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Molecular genetic analysis of cerebral cavernous malformations: an update

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Abstract

Cerebral cavernous malformations (CCM) can occur either as sporadic or familial form with autosomal dominant inheritance. Three CCM genes have been identified: *CCM1* (*KRIT1*), *CCM2* (*MGC4607*), and *CCM3* (*PDCD10*). In this review, we provide an overall update on genetics of cerebral cavernous malformations. We discuss the main features of these three genes and provide an updated listing of the mutations identified so far. Most of them lead to a premature stop codon regardless of the nature of the variation, including nonsense mutations, small deletions/insertions, and intronic/exonic substitutions causing an altered splicing and a frame-shift. In addition, deletions or duplications of one or more exons of CCM genes can be responsible for the disease. We examine the use of different mutation screening methods to identify all these mutations, providing a comprehensive approach to CCM genetic diagnosis. We also report the main strategies to evaluate the actual impact of the mutations on the protein function. Moreover, we recapitulate the available data on penetrance, phenotype-genotype correlations, and founder effect. Finally, we discuss the main aspects of genetic counseling, including genetic risk assessment in family members, in sporadic patients with multiple CCMs, and in the case of *de novo* mutations.

Keywords: *KRIT1*, *CCM2*, *PDCD10* gene variants, *de novo* mutation, penetrance, phenotype-genotype correlation, genetic counseling, screening methods

INTRODUCTION

Cerebral cavernous malformations (CCM) are vascular lesions that can occur as a sporadic (80% of cases) or



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a familial autosomal dominant disorder (FCCM) (20% of cases), with incomplete clinical and neuroradiological penetrance and great inter-individual variability^[1].

Sporadic forms usually present with a single lesion on MRI^[2,3], although multiple lesions have been reported in some cases^[4,5]. In contrast, familial forms typically exhibit multiple lesions, which increase in number and size over time^[6]. CCM result in a variety of clinical manifestations, including recurrent headaches, seizures, focal neurological deficit, and hemorrhage, with onset usually during adult life, but symptoms can also start in early infancy or in old age^[1].

In addition to neural lesions, extraneural cavernous malformations have been described in the familial form, in particular cutaneous and retinal vascular malformations^[7,8].

Three CCM genes have been identified so far: *CCM1/KRIT1*^[9], *CCM2/MGC4607*^[10], and *CCM3/PDCD10*^[11]. Almost 80% of CCM patients affected with a genetic form of the disease carry a heterozygous germline mutation in one of these genes^[1].

The purpose of this review is to provide an update on recent advances in molecular genetics of cerebral cavernous malformations. We provide the main features of the three genes and an updated listing of CCM pathogenic variants published so far in the peer-reviewed literature. We summarize the available data on penetrance, phenotype-genotype correlations, and founder effect for the variants described in the three CCM genes. Moreover, we briefly examine the different mutation screening methods in the genetic diagnostic approach to CCM and discuss the main aspects of genetic counseling.

CCM GENES

Mutations in the *KRIT1* gene have been found in 53%-65% of the familial forms of CCM. Mutations in the *CCM2* gene account for approximately 20% of the familial CCM cases, whereas 10%-16% of CCM families harbor mutations in the *PDCD10* gene^[12]. The existence of a potential fourth gene linked to CCM has long been postulated, since 5%-15% of familial cases cannot be explained by mutations in the three known CCM genes. It is however rather unlikely now, more than 15 years after the identification of the *PDCD10* gene^[11]. It is probable that the very few CCM families apparently negative for mutations in *KRIT1*, *CCM2*, and *PDCD10* genes harbor a pathogenic variant not identified by the routinely used techniques (e.g., a variant outside the screened exonic regions or a copy number neutral genomic rearrangement in one of the three genes^[13]).

Thus far, more than 350 different *KRIT1/CCM2/PDCD10* germline mutations have been identified and included in the Human Gene Mutation Database (HGMD)^[14]. These variants are highly stereotyped. Almost all of them introduce a premature termination codon in the protein, through various mechanisms, such as nonsense, splice-site, and frameshift mutations, as well as larger genomic rearrangements. Of note, even though the consequences are highly stereotyped, germline CCM mutations are usually present in only one or few families and are rarely recurrent^[1].

The molecular mechanisms responsible for the formation of CCM lesions in the presence of CCM gene mutations remain unclear. The proteins encoded by these genes are essential for regulating the angiogenesis during embryonic development and vascular postnatal stages of development^[9,15-17]. They are involved in the maintenance of junctional integrity between adjacent vascular endothelial cells^[18,19] and are expressed in endothelium, neurons, and astrocytes^[20,21]. Endothelial cells seem to be the cell of origin for CCM^[22,23].

CCM1/KRIT1

The *KRIT1* gene (OMIM #604214) is located on chromosome 7q21.2 and contains 16 coding exons^[24] that encode for the Krev interaction trapped 1 (KRIT1) protein (UniProt #O00522). KRIT1 is a 736-amino acid microtubule-associated protein, containing a NUDIX domain, multiple NPxY/F motifs, four ankyrin repeat domains at the N-terminus, and one C-terminal FERM domain (band 4.1 ezrin radixin moesin domain)^[25,26]. Gene and protein structures are shown in [Figure 1](#).

The NPxY/F motifs may be involved in dimerization and intramolecular folding of the KRIT1 protein^[26] and are recognized by phosphotyrosine binding (PTB) domains. PTB domains are present on several proteins, including the α -isoform of β 1-integrin regulator integrin cytoplasmic adaptor protein 1 (ICAP1 α)^[27]. Through the same PTB domain, ICAP1 α can interact with KRIT1 or with integrins. In the latter case, the interaction causes the activation of β -integrin signaling and stimulates angiogenesis. Thus, KRIT1 may act as a modulator of ICAP1 α activity, by competing with β -integrin interaction^[28]. The NpxY/F motifs also interact with other known proteins: sorting nexin 17 (SNX17), a modulator of endocytosis and intracellular trafficking^[29]; the Kelch family protein Nd1-L, showing a role in ROS (Reactive Oxygen Species) homeostasis^[30]; and the PTB domain present in Malcavernin, which in turn binds to PDCD10 and acts as a bridge between KRIT1 and PDCD10^[26].

The ankyrin repeats in KRIT1, present in many proteins, mediate inter- and intra-molecular interactions. They are involved in different cellular processes, such as gene transcription, cell cycle control, and organization of the cytoskeleton^[31]. Finally, the FERM domain of KRIT1, composed of three subdomains F1-F3, interacts with the NPxY/F motif on the cytoplasmic face of transmembrane receptors^[24] and with Rap1. Association with Rap1 relocalizes KRIT1 from microtubules to cell junction membranes^[32].

KRIT1 pathogenic variants

More than 300 pathogenic variants have been reported so far in the HGMD^[14]. These variants are present across the whole gene, with no evidence of hot spot regions [[Supplementary Table 1](#)]. The majority of them are substitutions, deletions, and insertions [[Figure 2A](#)], mainly located in the coding and splicing regions [[Figure 2B](#)]. They are splice junctions, frameshift, nonsense, and missense variants, often affecting the splicing process [[Figure 2C](#)]^[12]. Gross deletions, involving one or more exons, until the complete lack of the gene, have also been reported.

Regardless of the type of mutation, pathogenic variants result in a premature termination of translation, introducing an early termination codon, generating an unstable mRNA, or truncated KRIT1 proteins totally or partially lacking in the putative Rap1-interacting region^[33]. This evidence supports the hypothesis of a loss-of-function mechanism^[34], which can lead to CCM lesion genesis. Loss of KRIT1 alters cellular signaling and behavior. Moreover, endothelial cells acquire stem cell-like features and become more proliferative and invasive^[35].

CCM2/MGC4607

The *CCM2/MGC4607* gene (OMIM #607929) is located on chromosome 7p13 and contains 10 coding exons^[24]. *CCM2* encodes for CCM2/Malcavernin (UniProt #Q9BSQ5), a 444-amino acid protein that contains a predicted N-terminal PTB domain and a C-terminal Harmonin homology domain (HHD) [[Figure 3](#)].

Through the PTB domain, Malcavernin binds to KRIT1 and regulates its cellular localization^[26,36]. The HHD domain is involved in the interaction with the protein kinase MEKK3 (MAP3K3)^[37]. As a result of this

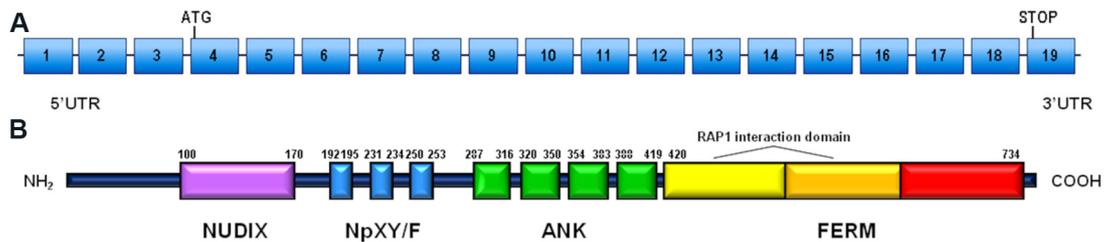


Figure 1. Schematic representation of *KRIT1* gene and KRIT1 protein: (A) *KRIT1* gene structure including 19 coding exons (rectangles) and introns (horizontal lines) (reference sequence: NM_004912.3); and (B) KRIT1 protein structure, including NUDIX domain, NPXY/F motifs, ankyrin repeat domains (ANK), and FERM domain (band 4.1 ezrin radixin moesin domain), composed of three subdomains. The position of the amino acid at the beginning and at the end is indicated for each domain.

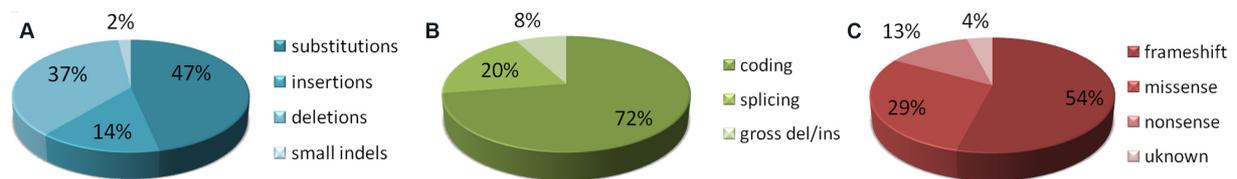


Figure 2. Mutation distribution of pathogenic *KRIT1* variants, as reported so far in the HGMD^[14]: (A) distribution by type at DNA level; (B) distribution by DNA location; and (C) distribution by type at protein level (please note that in this case the term “missense” is used to define variants so classified in the original studies, without information regarding their impact on splicing process). The classification of variants has not been independently verified by the authors.

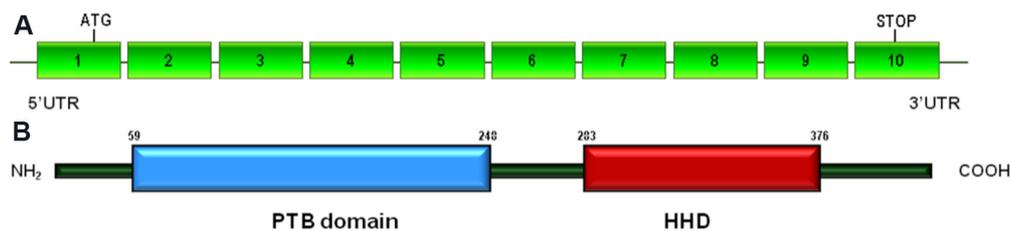


Figure 3. Schematic representation of *CCM2* gene and Malcavernin protein: (A) *CCM2* gene structure including 10 coding exons (rectangles) and introns (horizontal lines) (reference sequence: NM_031443.3); and (B) Malcavernin protein structure, including the N-terminal phosphotyrosine binding (PTB) domain and the C-terminal Harmonin homology domain (HHD). The position of the amino acid at the beginning and at the end is indicated for each domain.

interaction, Malcavernin acts as a scaffold protein in the signaling cascade that controls the activation of p38 MAPK^[33]. A linker region, located between the PTB and HHD domains, binds to PDCD10 through its N-terminal portion^[38].

Due to all these interactions, Malcavernin plays a pivotal role in signal transduction pathways that regulate adhesion, cytoskeleton remodeling, proliferation, migration of cells, and, ultimately, maintenance of vascular integrity^[33].

CCM2 pathogenic variants

To date, more than 90 mutations in the *CCM2* gene have been identified and listed in HGMD [Supplementary Table 2]. Most of them are deletions, substitutions, and insertions [Figure 4A]. They are prevalently located in the coding region and are nonsense, missense, frameshift, and splice site variants [Figure 4B and C]. They lead to a premature stop codon, frequently causing partial or total deletion of the PTB domain. The majority of missense mutations actually activate cryptic splice sites and lead to an

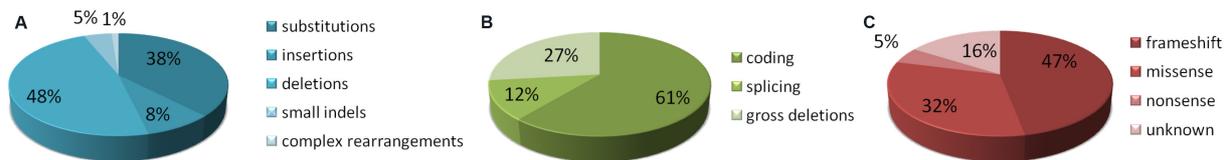


Figure 4. Mutation distribution of pathogenic *CCM2* variants as reported so far in the HGMD^[14]: (A) distribution by type at DNA level; (B) distribution by DNA location; and (C) distribution by type at protein level (please note that in this case the term “missense” is used to define variants so classified in the original studies, without information regarding their impact on splicing process). The classification of variants has not been independently verified by the authors.

aberrant splicing with a consequent frameshift. The only pathogenic missense variants that do not alter the splicing process have been identified within the PTB domain^[39]. These variants abolish the interaction between Malcavernin and KRIT1, strongly suggesting a causative role in CCM disease^[40,41].

As in the case of the *KRIT1* gene, deletions involving whole gene or a large part of it have been described. A 77.6-kb deletion spanning from exon 2 to exon 10 is a common founder mutation in the United States population^[42].

CCM3/PDCD10

The *PDCD10/CCM3* gene (OMIM #609118), located on chromosome 3q26.1, is a highly conserved gene containing seven coding and three non-coding exons^[24]. It encodes the programmed cell death protein 10 (PDCD10), a 212-amino acid protein (UniProt #Q9BUL8) that contains a dimerization domain at the N-terminus, and a C-terminal focal adhesion targeting (FAT)-homology domain [Figure 5].

The dimerization domain allows PDCD10 to form a dimer. The FAT-homology domain stabilizes the PDCD10 protein and interacts with Malcavernin^[38] and with several other signaling proteins^[43,44]. Among these are molecules involved in VEGF signaling, which is fundamental for vascular development^[45,46]. In addition, PDCD10 plays a critical role in the regulation of the angiogenesis through DLL4-Notch signaling^[45,47].

It is known that PDCD10 is involved in apoptosis: its overexpression induces the activation of caspase 3 and increases cell death^[48,49]. Thus, in the presence of *PDCD10* mutations, CCM disease may originate from a modification of the apoptotic process, which alters the equilibrium between neural cells and endothelium^[50].

PDCD10 pathogenic variants

More than 70 pathogenic variants of the *PDCD10* gene are present in the HGMD [Supplementary Table 3], including small and gross deletions, substitutions, insertions, and duplications [Figure 6A]. They are mainly located in the coding region, often between exon 5 and exon 7. In this case as well, frameshift, nonsense, and missense variants have been identified, all leading to a premature stop codon. Moreover, the presence of large deletions may result in the lack of protein production [Figure 6B and C].

Considering that the pathogenic variants detected so far are loss of function variants, haploinsufficiency and somatic loss of heterozygosity have been proposed as pathogenic mechanisms for CCM3^[23,51].

PHENOTYPE CORRELATIONS BY GENE

An important feature of CCM is the heterogeneity of the phenotypes. Variable expressivity is observed within affected individuals belonging to the same family and between families linked to the same mutation.

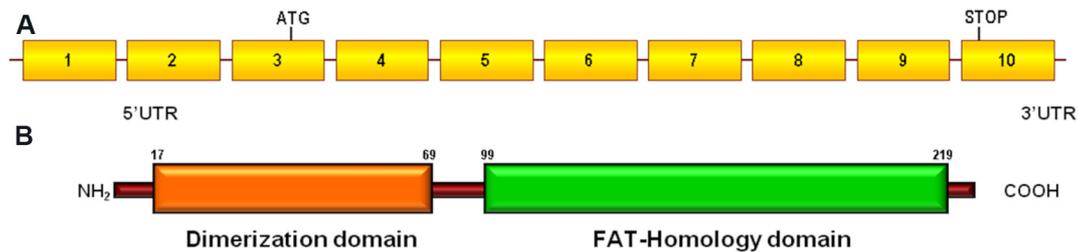


Figure 5. Schematic representation of *PDCD10* gene and *PDCD10* protein: (A) *PDCD10* gene structure including 10 exons (rectangles) and introns (horizontal lines) (reference sequence: NM_007217.3); and (B) *PDCD10* protein structure, including the N-terminal dimerization domain and the C-terminal focal adhesion targeting (FAT)-homology domain. The position of the amino acid at the beginning and at the end is indicated for each domain.

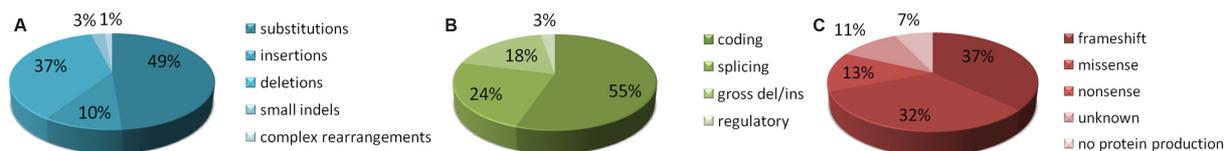


Figure 6. Mutation distribution of pathogenic *PDCD10* variants as reported so far in the HGMD^[14]: (A) distribution by type at DNA level; (B) distribution by DNA location; and (C) distribution by type at protein level (please note that in this case the term “missense” is used to define variants so classified in the original studies, without information regarding their impact on splicing process). The classification of variants has not been independently verified by the authors.

To date, only few data are available on genotype-phenotype correlation. Several studies have established some potential phenotype correlations by gene in FCCM.

Familial cases with *KRIT1* mutations have been demonstrated to have less severe clinical manifestations than the other familial forms^[52]. Up to 60% of patients with FCCM caused by a pathogenic variant in *KRIT1* gene ultimately become symptomatic^[53]. Moreover, cutaneous vascular malformations have been identified in 9% of FCCM patients. They have been reported more commonly in familial patients with *KRIT1* gene variants^[7] than in patients with pathogenic variant in *CCM2* or *PDCD10*. Three distinct major cutaneous vascular malformation phenotypes have been identified, namely hyperkeratotic cutaneous capillary-venous malformation (HCCVM), capillary malformation (CM), and venous malformation (VM), accounting for 39%, 34%, and 21% of FCCM cases, respectively^[7].

In contrast to most patients with *KRIT1* and *CCM2* genotypes, who often live normal lives with rarely disabling clinical manifestations, patients with *PDCD10* mutations appear to be related to more specific and severe phenotype. They have greater lesion burden, a higher risk for cerebral hemorrhage, and an earlier onset before the age of 15 years^[54-56]. The difference in hemorrhage risk, compared to patients with *KRIT1* and *CCM2* mutations, seems apparently not related to the size or number of lesions.

There is also a significant association with other clinical features, which include skin lesions, scoliosis, spinal cord cavernous malformations, brain tumor (meningioma, astrocytoma, and acoustic neuroma), and cognitive disability, unrelated to lesion burden or hemorrhage. Interestingly, an association with skin lesions had been reported primarily in familial patients with *KRIT1* variants. However, in patients with *PDCD10* mutations, lesions are different (more café-au-lait lesions rather than hyperkeratotic angiomas)^[56]. In addition, *PDCD10* mutation carriers have a greater likelihood of *de novo* mutational event than *KRIT1* and *CCM2* mutation carriers^[13].

Comparison of the MRI features of *KRIT1*, *CCM2*, and *PDCD10* mutation carriers suggested that the increment with age of the number of lesions on MRI gradient echo sequence (GRE) varies according to the mutated *CCM* gene. Fewer brain lesions on GRE MRI and a slower rate of lesion development have been observed in *CCM2* mutation carriers compared to patients with *KRIT1* gene variants^[54].

PENETRANCE

Clinical penetrance varies among the *CCM* genes and may be specific to the pathogenic variant^[57]. Clinical penetrance is estimated to be 60%-88% in *CCM1*, 100% in *CCM2*, and 63% in *CCM3* families^[53,58,59]. Within *KRIT1* positive families, both clinical and neuroradiological penetrance are incomplete and age dependent. Neuroradiological penetrance of *CCM* was previously considered to be complete or almost complete. Nevertheless, molecular screening of asymptomatic individuals revealed that cerebral MRI penetrance is also incomplete, even using the highly sensitive GRE sequences, and age dependent^[53,60].

Among the three *CCM* genes, *CCM2* is the only one reported with 100% of clinical and neuroradiological penetrance^[59]. However, in 2018, Scimone *et al.*^[61] found seven members of a family with a novel mutation, c.1555-1G>A (published as IVS10-1G>A), in whom only five of them showed lesions on MRI scan. Therefore, for the first time, a penetrance < 100%, equivalent to 70%, was reported for the *CCM2* gene.

FOUNDER PATHOGENIC VARIANTS

To date, more than 350 different mutations in the three *CCM* genes have been reported in the HGMD. Although most of them are family specific, four founder pathogenic variants have been identified so far that may be useful for stratifying genetic analysis in specific populations.

A founder effect has been shown for the pathogenic variant c.1363C>T (p.Gln455Ter) in the *KRIT1* gene, referred to as “the common Hispanic variant”, identified in about 70% of affected families with ancestry from north Mexico and the American Southwest^[62,63].

A 77.6-kb deletion spanning exons 2-10 of the *CCM2* gene resulted a common founder deletion with a high prevalence (up to 22%) in the United States population^[42,64], while it was rare in the Italian population^[65].

In the Italian population, a founder effect in four Sardinian families was observed for the mutation c.987C>A (p.Cys329Ter) in exon 10 of the *KRIT1* gene^[66].

More recently, a two-base pair change, c.30+5_30+6delinsTT, affecting messenger RNA splicing of the *CCM2* gene, was identified in seven apparently unrelated probands from 10 different kindreds of Ashkenazi Jewish descent, resulting a founder mutation in the Ashkenazi Jewish population^[67].

Finally, a redundant 14-bp deletion (c.554_567del) in exon 5 of *CCM2* gene has been described in patients from the Iberian Peninsula^[68,69]. However, in this case, a haplotype study has not been performed to determine a founder effect.

The main features of the founder pathogenic variants are summarized in [Table 1](#).

GENETIC TESTING

The molecular testing approach for *CCM* includes several methods to ensure the best diagnostic accuracy. A stepwise protocol may provide a high screening sensitivity, rationalizing costs and times of the analysis.

Table 1. Founder pathogenic variants identified so far

Gene	DNA nucleotide change	Predicted protein change	Reference sequences	Population	Ref.
<i>KRIT1</i>	c.987C>A	p.Cys329Ter	NM_194456.1	Sardinian	[62]
<i>KRIT1</i>	c.1363C>T	p.Gln455Ter	NP_919438.1	North Mexican and American Southwest ancestry	[58,59]
<i>CCM2</i>	c.30+5_30+6delGCinsTT	--	NM_031443.3	Ashkenazi Jewish	[63]
<i>CCM2</i>	77.6-kb deletion (exons 2-10)	--	NP_113631.1	United States	[38,60]

Nomenclature follows the standard naming conventions of the Human Genomic Variation Society (varnomen.hgvs.org).

The first step is usually the sequencing of all coding exons and exon-intron boundaries of the three *CCM* genes, on genomic DNA obtained from blood cells. A serial single gene testing, using Sanger sequencing, or a multigene panel in Next Generation Sequencing (NGS) can be performed. Both techniques allow the identification of point mutations with a very high sensitivity. NGS is a faster and more time-effective method, since it allows analyzing all genes in the same session. However, sequence variants identified by NGS still have to be confirmed by Sanger sequencing.

When no mutation is identified by sequencing, the next step is a quantitative analysis of exon copy number, to evaluate the presence of deletions or duplications affecting one or multiple exons in the three *CCM* genes. This analysis can be performed by several approaches: Quantitative Multiplex PCR Short Fragments (QMPSF), gene-targeted microarray designed to detect exon deletions or duplications, or Multiplex Ligation-dependent Probe Amplification (MLPA). The last method is in general less expensive; ensures high reproducibility, since it is supplied as a tested and proven commercial kit; and shows a very low coefficient of variation when compared to quantitative PCR^[70]. NGS can also be used to identify copy number variations with algorithms based on sequencing reads depth analysis. However, at the moment, this approach is less sensitive and less specific than MLPA, which remains the best solution for detecting exon deletion/duplication^[71].

If a variant is identified in the first two steps, its role has to be evaluated. If the variant introduces a premature stop codon (nonsense or frameshift variant, caused by point mutations or exon deletions) or is located in an invariant splicing region, its pathogenic role is easy to define. The pathogenic features of missense mutations are more difficult to establish. Indeed, it has been demonstrated that some “missense” mutations actually activate cryptic splice sites and cause an aberrant splicing of *CCM* mRNA, resulting in frameshift and introduction of a premature stop codon^[1,72,73]. In these cases, a predictive in silico analysis, using tools such as NetGene2 server (<http://www.cbs.dtu.dk/services/NetGene2>)^[74], may be useful to evaluate the impact of the variant on mRNA. However, an analysis at the cDNA level is always highly recommended to verify the presence of aberrant splicing and correctly define the role of the variants. The same approach is suggested also in the case of variants located in the splicing regions. This can be easily performed, since mRNA can be extracted from peripheral leukocytes, where *CCM* genes are expressed, reverse transcribed into cDNA, and analyzed by PCR.

In rare cases, some missense variants may impair the interactions between *KRIT1* and *CCM2* and undermine the stability of the *CCM* complex^[39]. It has been demonstrated that some mutations located in the PTB domain of *CCM2* are able to destroy the interaction with *KRIT1*^[38,40,41]. Thus, missense variants affecting PTB domain of *CCM2* may be considered pathogenic, and their role should be verified by performing functional studies, to evaluate whether they damage the *CCM2*-*KRIT1* interaction, as previously described^[39].

The genomic DNA sequencing followed by copy number analysis allows identifying pathogenic variants in 87%-98% of all familial CCM cases^[13]. In a minority of patients with a positive family history of CCM or with multiple CCM lesions, however, this approach does not allow detecting any mutations. For these cases, pathogenic variants outside of the standard diagnostic target regions may be considered. In addition, in that event, cDNA analysis is advisable. It can reveal splicing anomalies produced by deep intronic variants, not revealed by standard sequencing, or a loss of heterozygosity causing the lack of expression of one of the CCM alleles. Some CCM deep intronic variants have been identified by using this approach. An example is a deep intronic *KRIT1* gene deletion (c.262+132_262+133delAA), which leads to the insertion of a 99-bp pseudo-exon causing a premature stop codon in the open reading frame^[75].

Finally, rare structural anomalies, such as inversions, can be detected using whole genome sequencing (WGS) and bioinformatics analysis. An example is the recent identification of a 24-kb inversion involving exon 1 of *CCM2* gene^[76]. However, this kind of approach is currently performed in a research context and is not part of the standard diagnostic process.

A flow-chart summarizing the approach to CCM genetic screening is shown in [Figure 7](#).

GENETIC COUNSELING

Genetic testing for *KRIT1*, *CCM2*, and *PDCD10* genes can confirm the clinical diagnosis in patients and guide the genetic counseling. However, to correctly evaluate the genetic risk of CCM, it is necessary to assess whether the patient has a family history of disease and presents with single or multiple CCM lesions, in the absence of developmental venous anomaly or history of brain radiation. Thus, a detailed three-generation pedigree providing information about probable CCM symptoms, such as seizures, recurrent headaches, cerebral hemorrhages, focal neurological deficits, and a proband's brain MRI, including gradient echo (GRE) or susceptibility-weighted imaging (SWI) sequences, should be obtained.

It is necessary to take into account that the family history can be negative because the disease is not recognized in other family members. This could be due to parents' death before symptom onset, reduced penetrance, or phenotypic variability. Thus, in the presence of an apparently negative family history, it is still advisable to perform the appropriate evaluations in the proband's parents.

In the case of familial forms of CCM, the genetic screening sensitivity for the three CCM genes in a proband with an affected relative is more than 90%. When the proband carries a mutation, the sensitivity of screening in his/her relatives reaches 100%^[77]. If a mutation is identified in a proband, genetic testing of at-risk family members can be offered. Genetic counseling is fundamental to give patients and relatives all details necessary to make an informed choice. In particular, asymptomatic individuals should be carefully informed about the possible psychological implications of a positive test before they take a decision. Moreover, proband's family members should be informed that about 40% of the CCM mutation carriers remain asymptomatic^[54] and intrafamilial phenotypic variability may be high. Predictive testing in minors raises ethical issues and should not be carried out^[24].

In the case of sporadic forms of CCM, in individuals with a single lesion, the presence of CCM gene mutations is exceedingly rare. A study on *KRIT1* gene mutations in sporadic cases showed the presence of mutations in 29% of sporadic cases with multiple lesions, while no mutation was identified in patients with single lesions^[5]. For this reason, genetic testing is usually not indicated in sporadic forms with a single lesion.

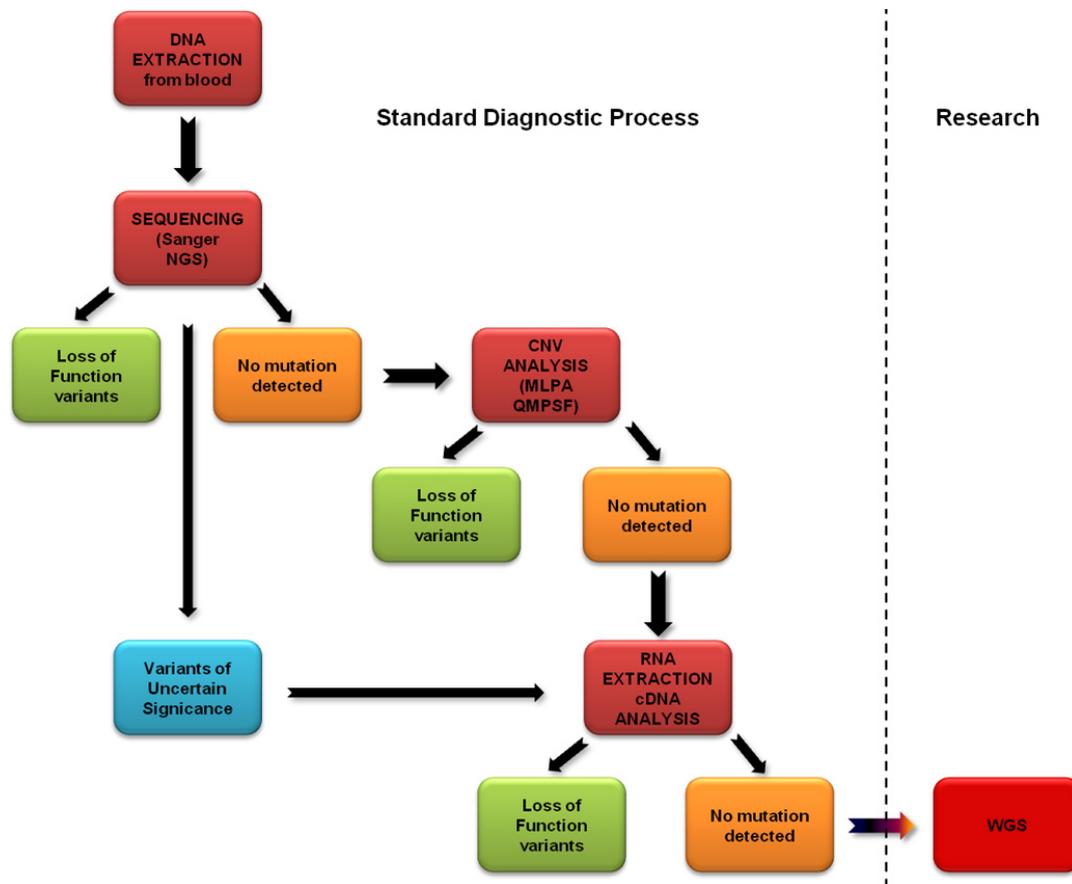


Figure 7. Stepwise protocol of CCM gene screening in familial CCM cases (proband) and sporadic CCM cases with multiple lesions. After genomic DNA extraction from white blood cells, sequencing of all exons and intron-exon boundaries of the three CCM genes is performed, using serial single gene testing by Sanger sequencing or multigene panels by Next Generation Sequencing (NGS). If no mutation is detected, the analysis is extended to exon copy number variations (CNV) using quantitative MLPA or QMPFS. If negative, RNA extracted from blood leukocytes is reverse transcribed and sequenced by Sanger sequencing to evaluate the presence of aberrant splicing. This approach is also used in the case of variants of uncertain significance identified in the first step of the analysis. These approaches are routinely performed in diagnostic labs. These steps are part of the standard diagnostic process. If no alteration is detected, whole genome sequencing (WGS) may be performed to assess the presence of rare structural anomalies (usually in a research context). CCM: Cerebral cavernous malformations.

On the other hand, sporadic cases with multiple CCM lesions likely carry a pathogenic variant and should be managed as familial cases. Thus, genetic screening of all three CCM genes is recommended. However, in these cases, the mutation detection rate is close to 60%, a value much lower than in familial cases^[13,78]. The patient should be informed that, even in the presence of a negative test, a genetic cause cannot be ruled out. There can be several explanations for a negative test: it may be because somatic mosaicism of *de novo* mutations occurred during gestation and therefore are not detectable in DNA from peripheral leukocytes; mutations may not be detectable because they are located outside the regions usually analyzed by conventional methods (i.e., in regulatory regions far from coding exons); and epigenetic modifications able to alter the expression of CCM proteins may occur.

When a pathogenic variant is detected in the proband, the analysis may be extended to the parents, in the same way as described for familial cases. Data reported so far indeed show that most sporadic patients with multiple lesions are cases with mutations inherited from an asymptomatic parent. Alternatively, they may be affected by CCM as the result of a *de novo* mutation^[1,77]. In the latter case, the mutation is not found in

proband's parents.

The real percentage of cases caused by *de novo* pathogenic variants is unknown, because genetic analysis in parents is not always possible^[79]. Individuals carrying a *de novo* germline pathogenic variant in all three CCM genes, most frequently in *PDCD10*, have been reported^[10,11,53,54,56,64,65,80]. These mutations may occur randomly at any stage of embryonic development or at the germline level in the parents' gametes. However, other possible explanations, including incorrect attribution of paternity or maternity (in the case of assisted reproduction), could be considered. All these aspects should be considered for a correct genetic counseling.

CONCLUSIONS

In the last years, great progress has been obtained in understanding the genetic bases of CCM and in CCM patients' characterization. The advances of the molecular biology technologies, as well as a combined use of different methods, may now unravel the genetic cause of the majority of CCM cases. Genetic data should always be associated with a detailed clinical characterization of patients and, when possible, their affected family members. This may allow better defining several aspects of the disease, such as the relationship between phenotypes and genotypes and the penetrance of the variants identified in the three CCM genes. In this respect, it is of fundamental importance for the scientific community to have access to updated databases reporting genetic and phenotypic data derived from the literature. This approach may allow reaching a deeper knowledge of the disease, to better understand CCM pathophysiology, guide genetic counseling, and eventually improve clinical care of patients.

DECLARATIONS

Authors' contributions

Made substantial contributions to conception and design of the study: Battistini S, Ricci C
Performed graphical design, as well as provided technical, and material support: Riolo G

Availability of data and materials

Not applicable.

Financial support and sponsorship

None.

Conflicts of interest

All authors declared that there are no conflicts of interest.

Ethical approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

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