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Clinical Classification of Variants in the Valosin-Containing Protein Gene Associated With Multisystem Proteinopathy

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Abstract

Background and Objectives

Pathogenic variants in the valosin-containing protein (*VCP*) gene cause a phenotypically heterogeneous disorder that includes myopathy, motor neuron disease, Paget disease of the bone, frontotemporal dementia, and parkinsonism termed multisystem proteinopathy. This hallmark pleiotropy makes the classification of novel *VCP* variants challenging. This retrospective study describes and assesses the effect of 19 novel or nonpreviously clinically characterized *VCP* variants identified in 28 patients (26 unrelated families) in the retrospective VCP International Multicenter Study.

Methods

A 6-item clinical score was developed to evaluate the phenotypic level of evidence to support the pathogenicity of the novel variants. Each item is allocated a value, a score ranging from 0.5 to 5.5 points. A receiver-operating characteristic curve was used to identify a cutoff value of 3 to consider a variant as high likelihood disease associated. The scoring system results were confronted with results of in vitro ATPase activity assays and with in silico analysis.

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Glossary

ALA = age at last assessment; **ALS** = amyotrophic lateral sclerosis; **ACMG** = American College of Medical and Genomic Genetics; **AOO** = age of onset; **CADD score** = combined annotation dependent depletion; **CM** = cardiomyopathy; **D** = dementia; **Dys** = dysautonomia; **ED** = extrapyramidal disorder; **FTD** = fronto temporal dementia; **IBMPFD** = Hereditary Inclusion-Body Myopathy with Paget's Disease of the Bone and Frontotemporal Dementia; **LL** = lower limbs; **LMN** = lower motor neuron signs; **LP** = likely pathogenic; **MND** = motor neuron disease; **MSP** = multisystem proteinopathy; **Myo** = myopathic pattern; **Neu** = neurogenic pattern; **NGS** = next generation sequencing; **PDB** = Paget disease of the bone; **RA** = regional atrophy; **RV** = rimmed vacuoles; **SIFT** = sorting intolerant from tolerant; **SW** = scapular winging; **UL** = upper limbs; **UMN** = upper motor neuron signs; **VCP** = Valosin-containing protein; **VUS** = variant of unknown significance.

Results

All variants were missense, except for one small deletion-insertion, 18 led to amino acid changes within the N and D1 domains, and 13 increased the enzymatic activity. The clinical score coincided with the functional studies in 17 of 19 variants and with the in silico analysis in 12 of 19. For 12 variants, the 3 predictive tools agreed, and for 7 variants, the predictive tools disagreed. The pooled data supported the pathogenicity of 13 of 19 novel VCP variants identified in the study.

Discussion

This study provides data to support pathogenicity of 14 of 19 novel *VCP* variants and provides guidance for clinicians in the evaluation of novel variants in the *VCP* gene.

Introduction

Valosin-containing protein (VCP), or p97, is an hexameric protein from the AAA+ (ATPases Associated with diverse cellular Activities) family involved in the remodeling of molecules using the energy of ATP hydrolysis.[1] VCP is encoded by the *VCP* gene, a 17-exon gene on chromosome 9.[2] Variants in the *VCP* gene were initially described in patients with inclusion body myopathy, Paget disease of the bone, and frontotemporal dementia (IBMPFD).[2] However, this acronym is insufficient to capture the expanding phenotypic spectrum of VCP patients, and currently, this disease is more accurately considered a member of a group of conditions known as multisystem proteinopathy (MSP).[3,4] In fact, only 3–12% of patients with VCP-MSP show the typical triad of IBMPFD[5]; there is heterogeneous phenotypes within families[6-8]; and variants in *VCP* can lead to a plethora of clinical presentations making the diagnosis a complicated task.[6,9-19]

To date, only missense variants have been described in patients with VCP-MSP.[3,7,20] In all cases, the pattern of inheritance is dominant. The mechanism of VCP dysfunction is likely context-dependent because assays studying VCP mutant function in vitro and in vivo support a gain and loss of function mechanism. In vitro, most pathogenic variants have an increase in ATPase activity, what reflects an induced structural change allowing for an increase in ATP accessibility and ADP release. By contrast, cells and animals expressing VCP-MSP variants behave similarly to VCP chemical or genetic inhibition suggesting that VCP pathogenic variants are dysfunctional.[21] How the apparent increase in ATPase activity in vitro correlates with a loss of VCP function in vivo remains to be explored but is likely due to the complex interactions of adaptor proteins mediated by the ATPase cycle.

Challenges in asserting the pathogenicity to novel *VCP* variants are due to the diverse phenotypic presentations, the possible varied gene penetrance, and the fact that ancillary tests may support the diagnosis but do not show pathognomonic features. In addition, patients could be seen by clinicians with different backgrounds; neurologists specialized in dementia, movement disorders, or muscle diseases; endocrinologists; or rheumatologists, which could lead to fragmented and siloed care making difficult the recognition that there is a monogenic disease segregating in the family.

Current strategies to evaluate the pathogenicity of novel variants often rest solely on genetic evidence such as variant rarity in population databases or segregation of the variant with phenotype in families. This approach does not consider that in multisystem diseases, such as in VCP-MSP, sometimes only limited phenotypic information is available for the ordering clinician. In addition, there is no clear consensus on functional assays that may help define pathogenic function of VCP variants.

In this study, we report 19 novel variants in the *VCP* gene identified in the collaborative VCP International Multicenter Study.[8] We have developed a scoring system to help in the assessment of the potential pathogenicity of novel *VCP* variants and have confronted the results of our score with results of functional testing.

Methods

Patients

Patients included in this report are part of the descriptive retrospective VCP International Multicenter Study that collected

Table 1 Scoring System

Item	Description	Points allocated
Item 1	Presence of a VCP-MSP core phenotype in the patient: myopathy, dementia,[a] PDB, motor neuron disease, and/or extrapyramidal disorder	1
Item 2	Positive family segregation following an autosomal dominant pattern of inheritance	1
Item 3	First-degree relative showing any of the VCP-MSP core phenotypes (even without genetic confirmation)	1
Item 4	Novel variant reported in an unrelated individual (from an unrelated family) with a VCP-MSP core phenotype[b]	1
Item 5	Muscle biopsy of the patient showing rimmed vacuoles or protein inclusions/aggregates	1
Item 6	Presence of "fat pockets" on axial T1-w MRI of the tight and/or legs.	0.5

Abbreviations: MSP = multisystem proteinopathy; PDB = Paget disease of the bone.
The scoring system aids in evaluating the phenotypic level of evidence supporting the pathogenicity of a novel and/or nonpreviously clinically characterized variant in the VCP gene using clinical, family history, and ancillary test data available on clinical practice. For each variant, the points of the items present are added, and based on the total score, the variant is classified as follows:
•High likelihood disease associated variant: >3 points.
•Probable disease associated variant: 2–3 points.
•Variant with undetermined association: 0.5–1.5 points.
This scoring system is suggested to be applied after a novel/VUS variant in the VCP gene was identified and other genetic diagnosis are less likely to explain the clinical phenotype of the patient.
This scoring should be reapplied on each regular patient's clinical follow-up to build up the phenotypic level of evidence longitudinally. This may require collecting additional data on sign/symptoms, family history, ancillary test results, and published literature evidence.
[a] Preferably, but not exclusively, frontotemporal dementia.
[b] This is the case for variant p.Ile216Met, p.Ile369Thr, p.Ile241Ser, or p.Met158Thr in this study.

clinical, genetic, and ancillary test data from 255 patients seen in 52 centers from 24 countries.[8] "To standardize variant interpretation, which was performed differently within individual countries, all genetic variants were centrally reviewed by an experienced geneticist from the John Walton Muscular Dystrophy Centre using the criteria suggested by the American College of Medical and Genomic Genetics (ACMG) as a guide.[22] All variants were analyzed using larger databases including 1,000 genomes, dbSNP, ExAC and Exome Variant Server. Variant nomenclature was based on transcript reference NM_007216.3. Inclusion criteria for the present report were adapted from the one used in the VCP International Multicenter Study and included: (1) patients $\geq$18 years old heterozygous for a novel or a previously reported but not thoroughly clinically characterized as pathogenic (P) or Likely Pathogenic (LP) variant in the *VCP* gene and (2) enough data available in the clinical notes to answer questions about age of disease onset, symptoms and clinical signs at onset and/or during disease's progression, family segregation analysis, and ancillary test results.[8]" Two additional patients with novel pathogenic variants who were not part of the VCP International Multicenter Study were also included in this study.

Standard Protocol Approvals, Registrations, and Patient Consents

The VCP International Multicenter Study obtained Caldicott approval from The Newcastle upon Tyne Hospitals Register Audit (project number 10833, Caldicott Approval: 7918) and institutional review board approvals from the LMU Klinikum at Ludwig-Maximilians University in Munich (project 21-0071); Washington University School of Medicine Institutional Review Board, USA (no 201103416); and the Johns Hopkins Hospital Institutional Review Board, Baltimore, USA (no 00288171). The novel variants in the VCP gene associated with the VCP-MSP article was approved by the human studies review committee at Washington University School of Medicine in St. Louis (201903027). These ethics committees cataloged this study as an audit because it collected deidentified retrospective data of patients with VCP. This study has been performed in accordance with the ethical standards laid down in the 1964 Declaration of Helsinki and its later amendments.

Development of a Pathogenicity Score

To evaluate the phenotypic level of evidence to support the pathogenicity of the novel and/or nonpreviously clinically characterized variants, a score was developed (Table 1). The score consists of 6 items assessing information that can be obtained on daily clinical practice. These items were chosen based on the authors' clinical experience and because they are commonly used to support a diagnosis of VCP-MSP in the literature. Item 6, which refers to muscle MRI, requires the presence of the so-called fat pockets in the skeletal muscle of the patients. This is a feature that can be found in patients with VCP-MSP as previously reported[23] and as shown in the muscle MRI images in eAppendix 1 (links.lww.com/NXG/A625). Each item is allocated a value to support their contribution to the variant association with the clinical phenotype. The sum of all the items leads to a total score, with a minimum of 0.5 and maximum of 5.5 points. We considered a variant to be high likelihood disease associated if its total score was greater than 3 points, probably associated if the total score was between 2 and 3 points, and undetermined association if the score was less than 2 points.

Table 2 Classification of the Novel Variants Based on the Scoring System

DNA m (Protein m)	Highest score in the variant
Scoring system applied to the 4 most frequent variants identified in the VCP International Multicenter Study	
c.464G>A, (p.Arg155His)	5.5
c.463C>T (p.Arg155Cys)	5.5
c.476G>A (p.Arg159His)	5.5
c.277C>T (p.Arg93Cys)	5.5
High likelihood disease associated variant	
c.648A>G (p.Ile216Met)	5.5
c.722T>G (p.Ile241Ser)	5
c.1105A>T (p.Ile369Phe)	4.5
c.431_432delGAinsAC (p.Arg144His)	4.5
c.473T>C (p.Met158Thr)	4.5
c.1106T>C (p.Ile369Thr)	4.5
Probable disease associated variant	
c.268A>G (p.Asn90Asp)	3
c.367G>A (p.Val123Met)	3
c.196G>A (p.Glu66Lys)	2.5
c.463C>G (p.Arg155Gly)	2
c.490A>C (p.Lys164Gln)	2
c.286C>G (p.Leu96Val)	2
c.625T>G (p.Cys209Gly)	2
Variant with undetermined association	
c.80T>C (p.Ile27Thr)	1.5
c.1988A>G (p.Lys663Arg)	1.5
c.265 C>G (p.Arg89Gly)	1
c.1057A>G (p.Ile353Val)	1
c.697A>G (p.Ile233Val)	1
c.335A>G (p.Lys112Arg)	1

For a detailed description of the clinical scoring system applied to each novel variants, please refer to eAppendix 2 (links.lww.com/NXG/A626).

To assess the validity of the scoring system, we first tested the score using several patients harboring the 4 most frequent pathogenic variants included in the VCP International Multicenter Study: p.Arg155His, p.Arg155Cys, p.Arg159His, and p.Arg93Cys, and then, we scored the novel nonpreviously clinically characterized variants and all the variants included in the VCP International Multicenter Study (eAppendix 2, links.lww.com/NXG/A626, 58 variants in total).

In Silico Analysis

The deleteriousness of the novel variants was predicted by 3 independent in silico tools commonly used in daily routine: Mutation Taster (mutationtaster.org/),[24] SIFT (sorting intolerant from tolerant, sift.bii.a-star.edu.sg/),[25] and CADD (combined annotation-dependent depletion, cadd.gs.washington.edu/).[26] These tools use predictive algorithms, based on different variables such as sequence homology, physical properties of amino acids, or evolutionary conservation of the protein sequence to establish whether an amino acidic change could affect protein function and, therefore, its potential pathogenicity.

In vitro ATPase Assay

Human VCP plasmid (TCB197) was subjected to site-directed mutagenesis with primers containing mutations to create each of the indicated variants. Proteins were purified as described.[27] Purified VCP (12.5 μL of 50 μM; final concentration in the reaction was 25 nM) was diluted in 20 mL of assay buffer [5 mL of 5× assay buffer A (1× = 50 mM Tris pH 7.4, 20 mM MgCl2, 1 mM EDTA) mixed with 15 mL of water and 25 μL 0.5M TCEP, 25 μL 10% Triton] to make the enzyme solution. 40 μL of the enzyme solution was dispensed into each well of a 96-well plate. The ATPase assay was prepared by adding 10 μL of 1,000 μM ATP (Roche, pH 7.5) to each well and by incubating the reaction at room temperature for 25 minutes. Reactions were stopped by adding 50 μL of BIOMOL Green reagent (Enzo Life Sciences). Absorbance at 635 nm was measured after 4 minutes on the Synergy Neo Microplate Reader (Bio-Tek). All assays were performed in triplicate, and the activity was averaged from independent experiments.

Statistical Analysis

Data were expressed as number and percentage for categorical variables and as mean ± SD for quantitative ones. For VCP ATPase assays, statistical significance was defined using a 2-way analysis of variance across all samples compared with the wild-type control.

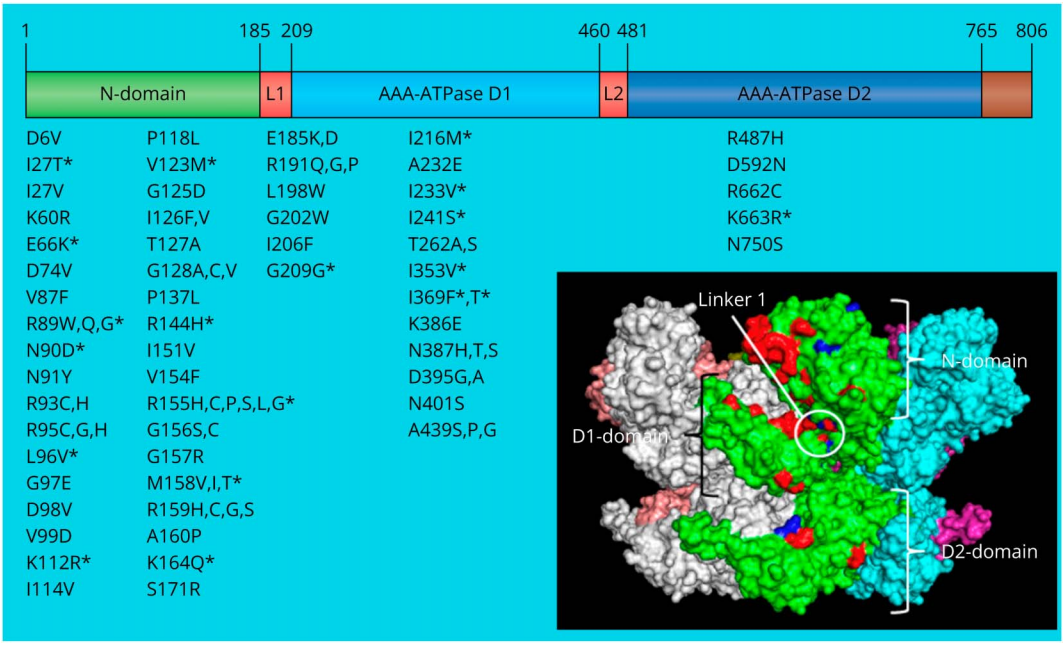
Mean difference in the 6-item score between pathogenic/likely pathogenic variants and variants of unknown significance, of the 58 variants included in the VCP International Study, was explored using independent sample T tests. To identify which score predicted with the highest sensitivity and specificity whether a variant was pathogenic/likely pathogenic based on the ACGM criteria, a receiver-operating characteristic curve was performed (eAppendix 3, links.lww.com/NXG/A627) and its area under the curve and the optimal cutoff point (Youden index) were calculated.

A level of significance of 0.05 was used for hypothesis testing. Statistical analysis was performed using the program IBM SPSS statistics, version 28.

Data Availability

The clinical, genetic, and enzymatic test data reported in this study are available on reasonable request to the corresponding authors.

Figure 1 Location of the Novel Mutations in the VCP Gene and Protein



Scheme of the location of all the variants described in the VCP gene and protein structure. The VCP protein contains 806 amino acids and is constituted by an N-terminal domain involved in the cofactor and ubiquitin-binding function, a D1 domain involved in the assembly of VCP homohexamer, a D2 domain responsible for the major ATPase activity, and the C-terminal domain involved in nuclear localization by interacting with other proteins. The N-domain and D1 domain are connected by N-D1 linker (L1), and the D1and D2 domains are connected by flexible D1-D2 linker (L2). All variants are listed underneath the domain affected; those with an asterisk are considered novel. The black square contains a 3D render of a VCP hexamer. Each subunit is independently colored. The positions of previously reported pathogenic residues are denoted in red and the novel reported variant residues in blue within a single green monomer. VCP = valosin-containing protein.

Results

We identified 19 previously uncharacterized variants in the *VCP* gene from 28 patients with presumed VCP-MSP from 26 unrelated families (Table 2, eAppendix 2, links.lww.com/NXG/A626, and eAppendix 4, links.lww.com/NXG/A628). Eighteen of the 19 variants had not been previously reported in GNOMAD. One variant, p.Ile233Val, had been reported twice, and GNOMAD provided with a minor allele frequency of 7.95E-06. Six of the 19 variants had been reported in ClinVar, and one of these 6 were additionally reported in LOVD. These 7 previously reported variants were not thoroughly clinically characterized in the literature though. Figure 1 shows the localization of the novel variants in the *VCP* gene.

Phenotypic Scoring

As shown in eAppendix 2 (links.lww.com/NXG/A626), at least one patient carrying the novel variants on the *VCP* gene manifested with one or more of the VCP-MSP core phenotypes (e.g., myopathy, PDB, FTD, ALS or parkinsonism), except for one patient (Family 26 III-1), harboring the c.335A>G (p.Lys112Arg) variant, who presented with signs of upper motor neuron involvement without muscle weakness. In all cases, pathogenic variants in other genes were excluded by exome or panel-based sequencing. Notably, muscle weakness was reported in 85.7% (24/28) of the cases, FTD in 21.4% (6/28), and PDB in 18.0% (5/28). No patient showed the classical IBMPFD triad. Fifteen of the 28 patients had a first-degree relative with an VCP-MSP core phenotype, dementia being the most common symptom in the relatives (10/28).

All patients were heterozygous for their *VCP* variant, compatible with a dominant inheritance. All variants were missense, except for one small deletion-insertion that created the missense variant p.Arg144His. Eighteen of the 19 variants led to amino acid changes within the N and D1 domains, where 64 of the 68 previously reported VCP-MSP variants were located (Figure 1).

To establish the validity of our scoring system, we first evaluated the score obtained by the 4 most common *VCP* variants—p.Arg155His, p.Arg155Cys, p.Arg159His, and p.Arg93Cys. These 4 variants achieved the highest score possible of 5.5 points. Then, we applied the scoring system to the 28 patients with the 19 novel variants described here, obtaining a range of scores from 0.5 to 5.5 (Table 2). Examples of the classical clinical and ancillary test findings seen in patients with VCP-MSP found in our cohort are described in eAppendix 1 (links.lww.com/NXG/A625).

In Silico Analysis

The in silico predictions on the 4 most common pathogenic mutations predicted them to be probably deleterious. Table 3

Table 3 In Silico Analysis

DNA change	Protein change	CADD score	Mutation taster	POLYPHEN-2
More prevalent pathogenic variants				
c.464G>A	p.Arg155His	24.5	DC	PrD 0.989
c.463C>T	p.Arg155Cys	33	DC	PrD 1
c.476G>A	p.Arg159His	24.1	DC	PsD 0.517
c.277C>T	p.Arg93Cys	31	DC	PrD 0.998
Novel variants				
c.648A>G	p.Ile216Met	23.6	DC	PrD 1
c.722T>G	p.Ile241Ser	29.9	DC	PrD 1
c.1105A>T	p.Ile369Phe	27.2	DC	PrD 1
c.431_432delGAinsAC	p.Arg144His	n/a	DC	PrD 1
c.473T>C	p.Met158Thr	24.2	DC	PB 0.988
c.1106T>C	p.Ile369Thr	26.7	DC	PrD 1
c.268A>G	p.Asn90Asp	23.1	DC	PsD 0.602
c.367G>A	p.Val123Met	26.9	DC	PsD 0.696
c.196G>A	p.Glu66Lys	23.9	DC	B 0.002
c.463C>G	p.Arg155Gly	25.8	DC	PrD 1
c.490A>C	p.Lys164Gln	25.2	DC	PrD 1
c.286C>G	p.Leu96Val	23.2	DC	B 0.028
c.625T>G	p.Cys209Gly	24.1	DC	PsD 0.704
c.80T>C	p.Ile27Thr	23	DC	PrD 0.968
c.1988A>G	p.Lys663Arg	23.7	DC	B 0.005
c.335A>G	p.Lys112Arg	22.9	DC	B 0.066
c.265C>G	p.Arg89Gly	23.2	DC	PrD 1
c.1057A>G	p.Ile353Val	20.3	DC	B 0.008
c.697A>G	p.Ile233Val	22.6	DC	PsD 0.810

Abbreviations: B = benign; DC = disease-causing; PB = possible benign; PrD = probable deleterious; PrD = probable deleterious; PsD possible deleterious.
In silico scores for the 4 prevalent variants identified in the VCP International Multicenter Study and the 19 variants described in this study.
CADD scores >20: top 1% deleterious variants in the genome.

shows the results of the predictive algorithms for each of the 19 novel variants that ranged from benign to probably deleterious.

In Vitro ATPase Assays

To further understand whether the variants identified lead to a functional change in the VCP protein, we purified a recombinant wild-type VCP, the 4 most common pathogenic VCP mutants (VCP-Arg155His, VCP-Arg155Cys, VCP-Arg159His, and VCP-Arg93Cys), a previously reported benign variant (VCP-Ile27Val), and the new 19 VCP variants. VCP hydrolyzes ATP, and the rate of hydrolysis is enhanced by most of the previously described VCP pathogenic variants. VCP-WT ATPase activity was arbitrarily set to 100%. The common 4 pathogenic variants demonstrated a 4 to 5-fold increase in ATPase activity consistent with previous studies, whereas the VCP- p.Ile27Val benign variant had only a 1.6-fold increase in ATPase activity. Using these data points, we selected a 3-fold increase as being consistent with a dysfunctional variant. Thirteen of the novel variants met this threshold (Figure 2).

A summary of the evidence obtained for each variant using the scoring system, in silico analysis, and enzymatic activity is provided in Table 4. For 12 variants, there was agreement between the 3 predictive tools, including 9 variants predicted to be deleterious and 3 variants predicted to be nondeleterious.

For 7 variants, the predictive tools disagreed. For 3 of them (p.Met158Thr, p.Glu66Lys and p.Leu96Val), clinical score and enzymatic activity supported pathogenicity while in silico studies did not. Conversely, in other 2 variants (p.Ile27Thr and p.Ile233Val), clinical score and enzymatic activity supported nonpathogenicity while in silico studies did.

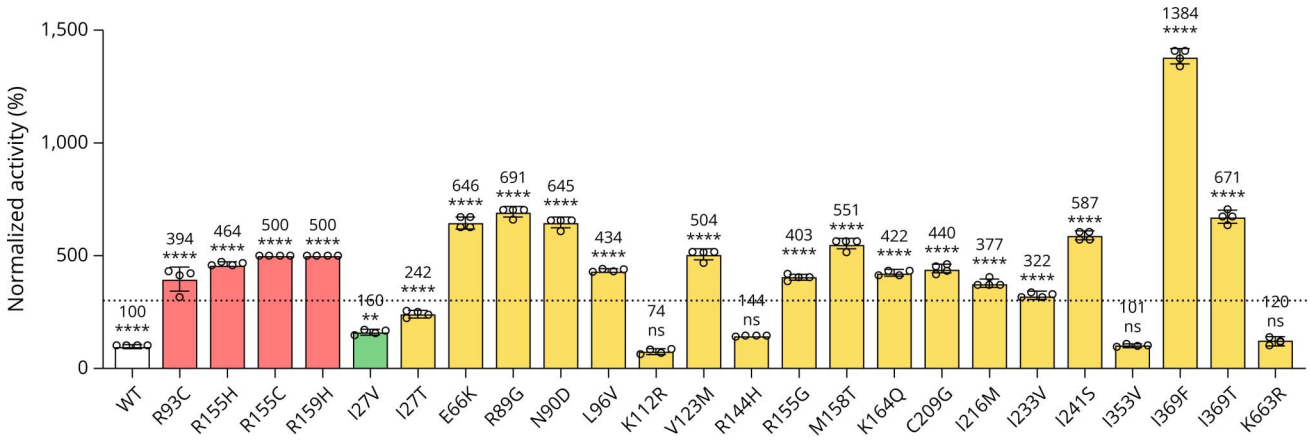
For the variant p.Arg144His, both the scoring system and the in silico algorithms predicted pathogenicity, but the enzymatic activity did not. This variant was found in a patient with isolated muscle disease with a muscle biopsy showing rimmed vacuoles and a MRI showing patchy fat replacement in the quadriceps and who had several relatives with dementia, ALS, and muscle disease. The variant obtained 4.5 of 5.5 points in the scoring system, but the enzymatic activity was normal.

For the variant p.Arg89Gly, the in silico studies and enzymatic activity suggested pathogenicity, but the clinical score was very low (1 point). This variant was found in a patient with isolated muscle weakness, with no relevant family history, who had just fiber atrophy in the muscle biopsy but not rimmed vacuoles or protein aggregates and who did not have an MRI.

Discussion

The increasing availability of next-generation sequencing panels and whole-exome/genome sequencing is leading to the identification of new variants in the *VCP* gene.[28] The evaluation of these novel variants is challenging because of the intrafamilial and interfamilial phenotype variability[7] and because of the progressive nature of the condition, implying that patients might not show the whole clinical spectrum when the results of the genetic tests are obtained. As a result, VCP-MSP diagnosis can be missed in early stages of the disease or clinical features could be attributed to other diagnosis.[20] This situation can be even more complex if thorough family history or segregation studies are not obtained. Moreover, patients present with a variety of clinical, ancillary tests or family history findings and allocating them a hierarchical weight in

Figure 2 Enzymatic in Vitro Test



Basal ATPase activity was determined from purified recombinant VCP protein. Values are normalized to VCP-wild type activity and presented as percent change from VCP-wild type. Red bars represent known pathogenic variants. Green bars represent known benign variants. Yellow bars represent the novel variants investigated. The dotted line represents the 3-fold increase threshold in ATPase activity consistent with a dysfunctional variant. ns: nonsignificant difference. $^{**}p < 0.05$; $^{****}p < 0.0001$. VCP = valosin-containing protein.

the analysis of the potential pathogenicity of a new variant is challenging. It is crucial to estimate adequately the pathogenicity of new variants to avoid overestimating variants that could lead to a wrong diagnosis and stop the surveillance of other etiologies behind the patients' phenotype. In addition, when it comes to interpreting novel variants, information about its rarity based on their frequency in population databases is not enough to confirm pathogenicity. Even if a variant is labelled as VUS following the ACGM criteria, clinicians should consider other information such as patient phenotype, family history, segregation in the family, and ancillary test results to support the potential pathogenicity of the variant.

The scoring system proposed here intends to be a tool to support clinicians when facing a novel variant in the VCP gene in clinical practice. The current standard of care in VCP suggests that symptomatic patients should be assessed every 6–12 months or more frequently if required.[29] The evolving nature of this disease implies that a novel variant that scored low early in disease progression can end up having a higher score. This could be the case of the p.Arg89Gly variant, for which the in silico and enzymatic studies suggested pathogenicity, but it was found in a young patient with isolated weakness, therefore, leading to a low clinical score. Updating the scoring system on each clinical visit is encouraged in these cases because new symptoms can appear later in disease progression further supporting the diagnosis of the disease. The clinical score coincided with the functional studies in 17 of 19 new variants and with the in silico prediction in 12 of 19 new variants, suggesting that functional studies, which assess enzymatic function, may provide a more reliable prediction of the potential pathogenicity of variants.

VCP has 2 ATPase domains, D1 and D2, which are organized as 2 stacked rings with a central channel, whereas its regulatory N domain is situated at the periphery of the D1 ring.[30] Reported pathogenic variants do not seem to alter VCP oligomerization but increase basal ATP hydrolysis activity, which is mediated through the D2 domain.[30] Pathogenic variant residues are commonly found in the interface between the N and D1 domains, suggesting that communication between these 2 regions is important for disease pathogenesis.[1] Other variants might affect the association of VCP with cofactors.[31] All the novel mutations identified in this study change an amino acid in the N terminal or D1 domain, except for the variant p.Lys663Arg located in the D2 domain. Being an ATPase enzyme, VCP hydrolyzes ATP releasing an inorganic phosphate. We have performed an enzymatic test comparing the intrinsic ATPase activity of VCP-wild type with several previously reported MSP mutations and the novel mutations, as reported in other studies.[13] Most of the novel variants reported here increased ATP activity compared with VCP-wild type. For those variants in which the ATPase activity did not differ from the VCP-wild type, clinical score and/or in silico analysis suggested nonpathogenicity. The only exception to this is the p.Arg144His variant that was suggested as pathogenic by the in silico studies and found in a patient with clinical data highly suggestive of the disease, but with a normal enzymatic activity. An elevation in basal ATPase activity has been described for most VCP variants, with the exception of 2 previously reported variants. Specifically, the p.Glu185Lys variant described in a large family with axonal CMT and p.Asp395Gly in 2 families with a pathologically distinct dementia (vacuolar tauopathy) had unchanged and reduced ATPase activity, respectively.[32,33] These variants suggest that VCP mutations could alter VCP functionality by an ATPase-independent manner. Thus, a reliance exclusively on in vitro ATPase assays of VCP function may be misleading.

Table 4 Summary of the Study Results

Variant	Scoring system	In silico analysis	Enzymatic analysis	Interpretation
c.648A>G; p.Ile216Met	5.5	Deleterious	+	Agreement in the 3 tools, deleterious
c.722T>G; p.Ile241Ser	5	Deleterious	+	Agreement in the 3 tools, deleterious
c.1105A>T; p.Ile369Phe	4.5	Deleterious	+	Agreement in the 3 tools, deleterious
c.431_432delGAinsAC; p.Arg144His	4.5	Deleterious	−	Enzymatic analysis disagrees
c.473T>C; p.Met158Thr	4.5	Inconclusive	+	In silico analysis disagrees, Polyphen-2 PB
c.1106T>C; p.Ile369Thr	4.5	Deleterious	+	Agreement in the 3 tools, deleterious
c.268A>G; p.Asn90Asp	3	Deleterious	+	Agreement in the 3 tools, deleterious
c.367G>A; p.Val123Met	3	Deleterious	+	Agreement in the 3 tools, deleterious
c.196G>A; p.Glu66Lys	2.5	Inconclusive	+	In silico analysis disagrees, Polyphen-2 B
c.463C>G; p.Arg155Gly	2	Deleterious	+	Agreement in the 3 tools, deleterious
c.490A>C; p.Lys164Gln	2	Deleterious	+	Agreement in the 3 tools, deleterious
c.286C>G; p.Leu96Val	2	Inconclusive	+	In silico analysis disagrees, Polyphen-2 B
c.625T>G; p.Cys209Gly	2	Deleterious	+	Agreement in the 3 tools, deleterious
c.80T>C; p.Ile27Thr	1.5	Deleterious	−	In silico analysis disagrees, Polyphen-2 PrD
c.1988A>G; p.Lys663Arg	1.5	Inconclusive	−	Agreement in the 3 tools, nondeleterious
c.335A>G; p.Lys112Arg	1	Inconclusive	−	Agreement in the 3 tools, nondeleterious
c.265 C>G; p.Arg89Gly	1	Deleterious	+	Scoring system disagrees
c.1057A>G; p.Ile353Val	1	Inconclusive	−	Agreement in the 3 tools, nondeleterious
c.697A>G; p.Ile233Val	1	Deleterious	−	In silico analysis disagrees, Polyphen-2 PrD

The table shows the evidence obtained for each variant using the scoring system, the in silico analysis, and the in vitro ATPase assay (enzymatic activity) and the variant effect interpretation based on the agreement of the 3 tools.

The results of muscle biopsy and MRI in patients with VCP-MSP are not specific[34] and can overlap with conditions such as myofibrillar myopathies, other multisystem proteinopathies, or myopathies with rimmed vacuoles.[4] In this context, an enzymatic test that measures the effect of a variant in the ATPase activity can aid in diagnosis.[13,35] However, assessing the intrinsic ATPase enzymatic activity in VCP requires purified recombinant protein. Moreover, there is not an easier assay that uses blood, muscle, or even cerebrospinal fluid samples. Other blood biomarkers, such as tumor necrosis factor alpha and epidermal growth factor, have been suggested to differentiate patients with VCP-MSP from healthy controls,[36] but these biomarkers are not disease-specific and can be found in other inflammatory conditions.[37,38]

Three of the variants described here affected amino acidic residues that have been previously identified in patients with VCP-MSP. One variant affected the hot spot amino acid position Arginine 155 codified by exon 5, creating the p.Arg155Gly variant. A second variant was identified at amino acid residue Arginine at position 89, creating a p.Arg89Gly missense variant. Notably, p.Arg89Gln and p.Arg89Trp variants have been reported in a patient with sporadic ALS and distal myopathy with cognitive impairment without family history, respectively.[39,40] Another variant created a p.Ile27Thr missense change which affected the same amino acid that the already reported p.Ile27Val variant. Although this later variant has been found in cohorts of sporadic inclusion body myositis and Parkinson disease, it has a MAF of 0.005 to 0.0006 depending on population ethnicity and was equally represented in healthy controls, suggesting that it may be benign.[29,41]

Limitations of this study include the retrospective nature of the VCP International study, from which the novel variants were obtained. Missing data could affect the score system result leading to underestimation of the variant pathogenicity. The sensitivity of the clinical scoring system to label the variants can progress over time because the clinical, muscle MRI and biopsy results can be modified along disease progression, reinforcing the idea that the scoring system should be applied on a regular basis. A greater than 3-fold increase in VCP-variant ATPase activity is helpful to support MSP-VCP mutant pathogenicity but could miss potential pathogenic variants because VCP mutation dysfunction may occur

independent of ATPase activity. Other assays of VCP function may need to be used. These could include assays of cellular autophagic or proteosomal activity.

In conclusion, we have developed a clinical score able to predict pathogenicity of new variants in the *VCP* gene with high accuracy that highly correlates with the results of in silico and enzymatic activity studies. This score could be of great help in the diagnostic process of patients with novel variants in daily clinics. This study provides data to support pathogenicity of 13 of 19 new *VCP* variants based on the fact that (1) patients affected had clinical and family history data highly suggestive of the VCP-MSP; (2) most of the new variants reported resulted in an increase in enzymatic activity in the in vitro assays; (3) novel variants are not present in public databases of controls (dbSNP); (4) these variants affected highly conserved residues across species in the N-terminal and D1 domain, in which most pathogenic VCP pathogenic variants have been reported; (5) no variants were found in other genes which were reported in the patients with MSP, FTD, or ALS previously; and (6) these variants were predicted to be deleterious by 3 prediction in silico assays.

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Continued

Appendix *(continued)*

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