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Next-generation sequencing approach to hyperCKemia

A 2-year cohort study

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Abstract

Objective

Next-generation sequencing (NGS) was applied in molecularly undiagnosed asymptomatic or paucisymptomatic hyperCKemia to investigate whether this technique might allow detection of the genetic basis of the condition.

Methods

Sixty-six patients with undiagnosed asymptomatic or paucisymptomatic hyperCKemia, referred to tertiary neuromuscular centers over an approximately 2-year period, were analyzed using a customized, targeted sequencing panel able to investigate the coding exons and flanking intronic regions of 78 genes associated with limb-girdle muscular dystrophies, rhabdomyolysis, and metabolic and distal myopathies.

Results

A molecular diagnosis was reached in 33 cases, corresponding to a positive diagnostic yield of 50%. Variants of unknown significance were found in 17 patients (26%), whereas 16 cases (24%) remained molecularly undefined. The major features of the diagnosed cases were mild proximal muscle weakness (found in 27%) and myalgia (in 24%). Fourteen patients with a molecular diagnosis and mild myopathic features on muscle biopsy remained asymptomatic at a 24-month follow-up.

Conclusions

This study of patients with undiagnosed hyperCKemia, highlighting the advantages of NGS used as a first-tier diagnostic approach in genetically heterogeneous conditions, illustrates the ongoing evolution of molecular diagnosis in the field of clinical neurology. Isolated hyperCKemia can be the sole feature alerting to a progressive muscular disorder requiring careful surveillance.

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Glossary

CK = creatine kinase; **EFNS** = European Federation of Neurologic Societies; **HPO** = Human Phenotype Ontology; **LGMD** = limb-girdle muscular dystrophy; **NGS** = next-generation sequencing; **ULN** = upper limit of normal; **VUS** = variants of unknown significance.

Creatine kinase (CK) levels can be mildly and transiently increased as a result of muscle injury or exercise, even in healthy individuals. Persistent elevation of serum CK (termed hyperCKemia) is defined, according to European Federation of Neurological Societies (EFNS) guidelines (ean.org/Guideline-Reference-Center.2699.0.html), as the presence of serum CK values beyond 1.5 times the upper limit of normal (ULN) in least 2 measurements,[1] and it is a common reason for referrals to specialized neuromuscular centers.

Increased serum CK can be present in the absence of obvious clinical signs.[2] In individuals with a normal neurologic examination, the condition is termed asymptomatic or isolated hyperCKemia, and it can signal the presence of several unsuspected metabolic, cardiac, rheumatic, or endocrine conditions. When no underlying cause is found, it is referred to as idiopathic hyperCKemia. The clinical management of idiopathic hyperCKemia is unclear; although clinically asymptomatic, affected patients are potentially susceptible to malignant hyperthermia.[3]

In asymptomatic hyperCKemia, definition of the correct diagnosis may be time consuming, and success is not guaranteed. Next-generation sequencing (NGS) has recently been proposed as a cost-effective strategy for the molecular diagnosis of inherited neuromuscular disorders.[4] The efforts to define the molecular etiology in hyperCKemia come from the need to refine follow-up avoiding unnecessary examinations and to improve counseling in the family. We set out to explore whether NGS might allow detection of the molecular basis of hyperCKemia, addressing this question in a consecutive series of adults and children recruited at 7 Italian tertiary neuromuscular centers over an approximately 2-year period.

Methods

Standard protocol approvals, registrations, and patient consents

This study was approved by the Tuscany Regional Pediatric Ethics committee. All the procedures complied with the Helsinki Declaration of 1975. Genetic studies and muscle biopsies were performed with written informed consent. All participants (including parents or legal guardians in case of minor patients) were provided pre- and post-test genetic counseling as routine in our neurogenetic clinics.

Patients and study design

Over an approximately 2-year period (May 2016–August 2018), 66 patients presenting with hyperCKemia (meeting the EFNS criteria) were consecutively referred to the neurology, pediatric, or neuropediatric units of 7 Italian tertiary neuromuscular centers for clinical and diagnostic purposes. All 66 met the inclusion criteria for our study: (1) persistent serum CK elevation at rest and (2) values higher than twice the ULN on at least 2 occasions after refraining from muscular exercise for at least 72 hours before CK measurement. For each patient, we collected clinical and laboratory data and the results of familial segregation analyses and previous genetic tests. MRI scans of thigh and calf muscles were performed in 26 patients, EMG in 26, and muscle biopsy in 57, in all cases using routine clinical methods.[5] Before this study, we had performed multiplex ligation-dependent probe amplification analysis in all the patients to exclude multiexon rearrangements in the *DMD* gene and tested the levels of acid alpha-glucosidase from dried blood spots[6] to detect possible undiagnosed late-onset Pompe disease. Patients with borderline enzyme values or biallelic mutations in the *GAA* gene were not included in this study. DNA samples were analyzed at a single center.

None of the 66 individuals had a medical history of anesthesia-related complications or a family history of cardiovascular events. Any family history of neuromuscular disorders or mild muscle complaints was carefully recorded. Occasional causes of hyperCKemia, such as malignancies, drug and alcohol abuse, rheumatic, thyroid, and parathyroid disorders, infections, and hematologic diseases, were all excluded. All patients underwent routine serum chemistry, including serum myoglobin measurement. No participant was under statin treatment or taking other drugs potentially capable of inducing hyperCKemia.

NGS workflow and sequencing analyses

We used the SureSelect technology (Agilent, Santa Clara, CA) and SureDesign software (earray.chem.agilent.com/suredesign/) to design a multiexon amplicon panel containing a total of 78 genes known to be associated with limb-girdle muscular dystrophies (LGMDs), rhabdomyolysis, and metabolic and distal myopathies[7]; the panel spanned more than 259 Mbp, with gene coverage >99%. To analyze the data obtained from our study, we used a routine bioinformatic pipeline[8] that adopts the Ingenuity Variant analysis suite (Qiagen, apps.ingenuity.com). To assign pathogenicity, we set up a precise Alissa (Agilent) pipeline using the following criteria: a sequence quality score greater than 30, a read depth greater than 30, and rare occurrence in publicly available polymorphic data sets (with a minor allele frequency <0.01% for autosomal dominant and <0.1% for autosomal recessive genes) with less than 1 occurrence in homozygosity in

gnomADv2.1 (gnomad.broadinstitute.org/; macarthurlab.org/2018/10/17/gnomad-v2-1). As reported previously, we determined predictably or probably deleterious scores using an in silico pipeline using a set of 10 prediction software packages.[8] Putatively deleterious variants were validated by PCR-based standard capillary Sanger sequencing, both in patients and in relatives whose DNA was available for segregation studies, also to determine inheritance and phases of multiple gene variants and to establish whether variants had occurred de novo. Segregation in affected and unaffected relatives made it possible to better define pathogenic variants once we had identified those more likely to be disease causative.

Routine morphology and immunofluorescence analysis of muscle proteins were performed on vastus lateralis biopsy samples according to standard protocols.

Data availability

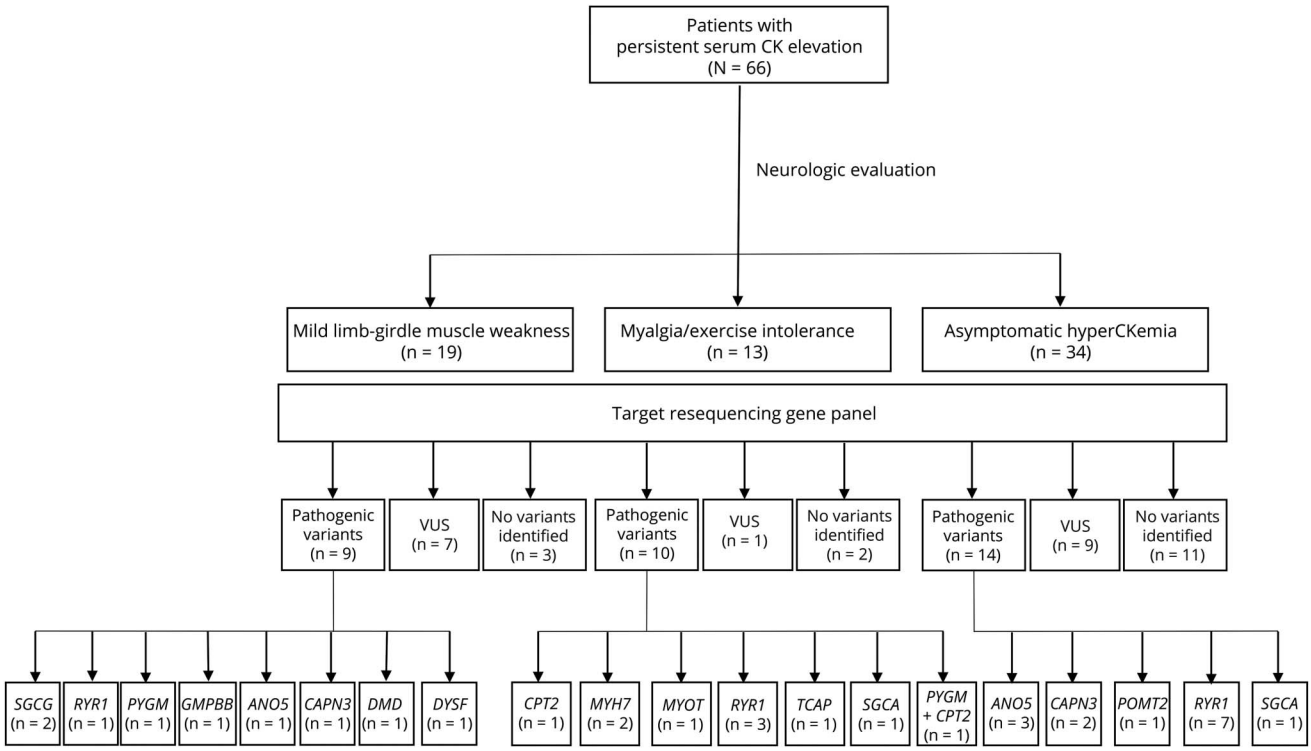
The data set used and analyzed during the current study is available from the corresponding author on reasonable request.

Results

We evaluated 66 patients with hyperCKemia (44 men and 22 women, age range 5–73 years), referred over an approximately 2-year period to tertiary neuromuscular centers. Most of the patients were Italian, adults (≥16 years), and apparently sporadic cases. Although none of them had obvious neuromuscular disorders, 16 had a positive family history of muscle weakness or fatigue compatible with autosomal dominant inheritance in 13 and a recessive pattern of inheritance in 3. Their mean age at onset (i.e., at the time of the first documented evidence of hyperCKemia) was 26.7 ± 19.2 years, when they had serum CK levels ranging from 300 to 103,000 UI/L (normal <190 UI/L). Their disease duration ranged from 3 months to 30 years. Clinical examination revealed mild limb-girdle muscle weakness (median Medical Research Council scale score of 5 in upper girdle and 4+ in lower girdle muscle) in 19 patients (figures 1–2). The distribution of the muscle weakness in these symptomatic patients is presented in figure 2 using the Human Phenotype Ontology (HPO) ID codes and nomenclature.[9] Thirteen participants reported occasional exercise intolerance and myalgia, but showed no clear evidence of muscle weakness. Thirty-four patients remained completely asymptomatic over a 2-year observational period (figure 1). The patients' serum CK levels at their latest neurologic examination ranged from 150 to 64,610 UI/L.

Cardiac examination, performed at some point during the course of the clinical follow-up, was unremarkable in all the patients except 4 whose ECG showed arrhythmias of uncertain significance. Pulmonary and functional respiratory tests were within normal limits in all the patients and remained so throughout the study.

Figure 1 Flowchart of patients' enrollment and results of genetic testing



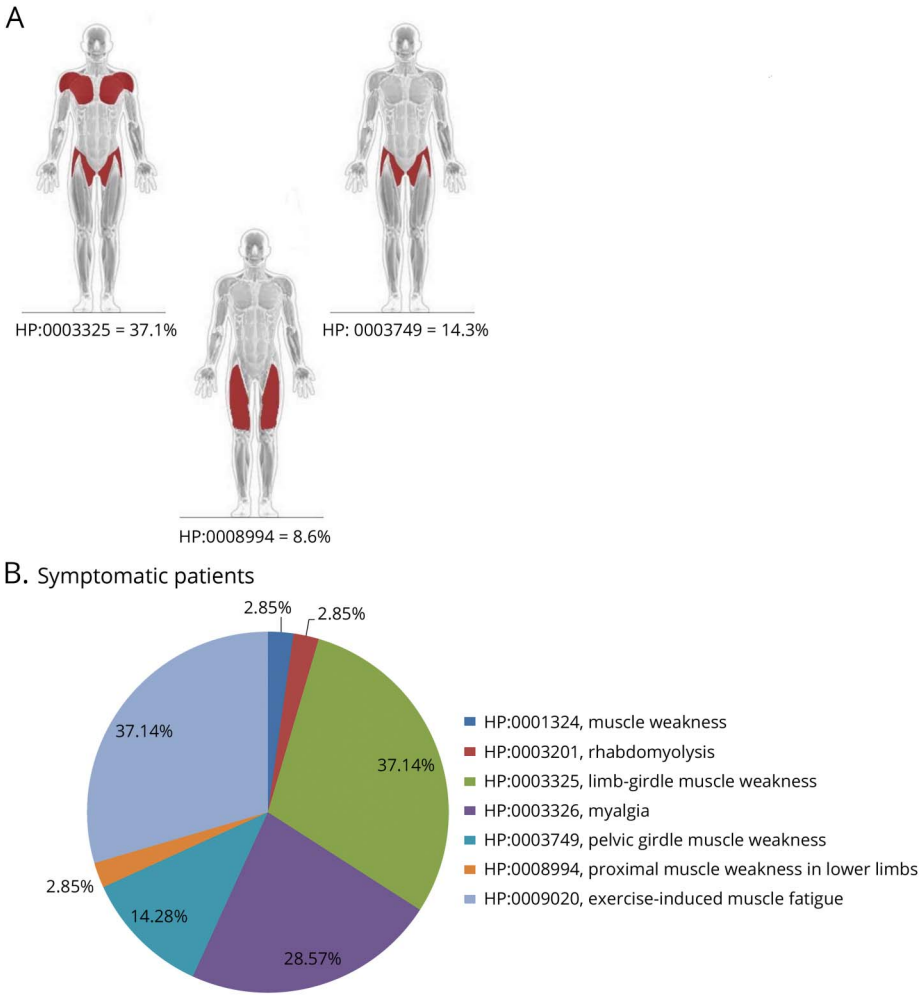
VUS = variants of unknown significance.

EMG, performed on average 1 year after hyperCKemia onset, showed myopathic changes in 17 patients. Few patients underwent repeated neurophysiologic follow-up, and none of these showed substantial modifications. Twenty-six patients underwent muscle MRI scans at least once during the study, and these scans were unremarkable in 17 and not informative in the other 9. Fifty-seven patients underwent a skeletal muscle biopsy. In 8 cases (14%), this showed clear dystrophic changes, with fat and connective tissue replacement suggesting a possible diagnosis of muscular dystrophy, whereas the vast majority (38/57, 67%) showed only mild, nonspecific myopathic changes, with normal muscle protein staining and no abnormal metabolic features (figure 3 and supplementary figure e-1, links.lww.com/NXG/A175). Muscle biopsy was normal in 11 patients.

Through multigene targeted NGS analysis, we identified an average of 50 rare variants in each patient. To facilitate our "needle in a haystack" search, we used a stringent set of bioinformatics filters, including effects on the protein, scores for predicting pathogenicity, allele frequencies in public and internal databases, and annotation as disease-associated variants with Alissa, a customizable pipeline tool.[10] Presence of the specific variant in the Human Genome Mutation Database (hgmd.cf.ac.uk) was not considered a mandatory criterion for attributing pathogenic significance. Bioinformatics data were combined and critically reevaluated taking into consideration clinical presentation with the relative HPO definition, age at onset, and segregation studies in familial cases. Moreover, in cases submitted to muscle biopsy, we integrated histologic and immunofluorescence features.

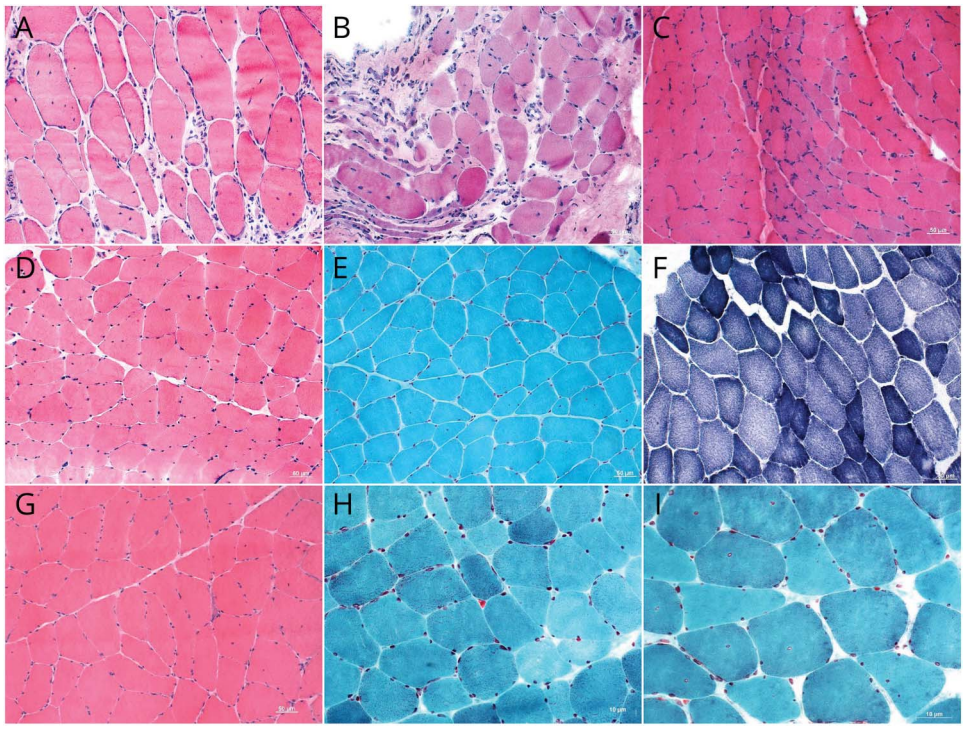
On the basis of these procedural steps and criteria, already used by us in previous research,[7] we assigned a confirmatory genetic diagnosis to 19 patients presenting pathogenic, disease-associated (n = 13), or likely pathogenic (n = 6) variants. We also identified 14 patients harboring changes predicted to affect function in genes corresponding to the clinical suspicions in these cases, thereby increasing group of individuals with a molecular diagnosis to 33 patients. We confirmed the molecular diagnosis using segregation analysis or immunofluorescence, or Western blotting on muscle

Figure 2 Clinical features of symptomatic patients presented using HPO ID codes and nomenclature



(A) Muscle weakness pattern frequencies expressed as percentage rates. Clinical phenotypes are indicated using the HPO ID codes. HP:0003325 = limb-girdle muscle weakness; HP:0008994 = proximal muscle weakness in lower limbs; HP:0003749 = pelvic girdle muscle weakness. (B) Frequencies of clinical phenotypes associated with hyperCKemia expressed as percentage rates. Clinical phenotypes are indicated using HPO codes and nomenclature. HPO = human phenotype ontology.

Figure 3 Myopathologic changes in patients with limb-girdle muscle weakness (top row, A–C), myalgia, and exercise intolerance (middle row, D–F) and in asymptomatic patients (bottom row, G–I)



(A and B) Hematoxylin and eosin (HE) staining demonstrating marked variation in fiber size, several central nuclei, and increased connective tissue staining in P4 and P16, respectively. (C) HE staining showing several degenerating fibers in P29. (D) HE staining revealing slight variation in fiber size and some central nuclei in P3. (E) Gomori trichrome staining showing some hypotrophic fibers and increased internal nuclei in P28. (F) NADH-TR staining showing moth-eaten fibers in P33. (G) HE staining demonstrating mild variation in fiber size and a few central nuclei in P7. (H and I) Gomori trichrome staining showing slight variation in fiber size and some internal nuclei in P19 and P22, respectively. Magnification 200×.

biopsies, or their combination. Detailed information on these patients, including clinical characteristics, CK levels, muscle biopsy, and gene mutations, is shown in tables 1 and 2, whereas the genetic data are summarized in figure 4 and further detailed in supplementary web data (tables e-1 to e-4, links.lww.com/NXG/A176, links.lww.com/NXG/A177, links.lww.com/NXG/A178, and links.lww.com/NXG/A179).

Of the aforementioned 33 patients, 9 showed mild limb-girdle muscle weakness, 10 showed myalgia and/or occasional exercise intolerance, and 1 case rhabdomyolysis, whereas 14 were asymptomatic despite having muscle biopsies showing low immunofluorescence for specific proteins (Figure 1, figure e-1, links.lww.com/NXG/A175). Immunofluorescence analysis was performed to corroborate loss-of-function variants in 7 cases. With regard to the genetic analyses, pathogenic variants in *RYR1* were found in 11 patients, in *ANO5* in 4. Four patients harbored pathogenic variants in genes encoding sarcoglycans, whereas 3 in *CAPN3* (figure 4). Serum CK levels in the group of molecularly defined patients ranged from 600 to 67,490 UI/L, whereas muscle biopsy (performed in 31/33 individuals) showed evidence of dystrophic processes in 6 (figure 3, figure e-1), mild nonspecific muscle changes in 21, and no noteworthy alterations in 4 patients.

Variants of unknown significance (VUS) with a potential causative role were found in 17 additional patients (supplementary table e-3, links.lww.com/NXG/A178). In these cases, either there was only a partial correspondence with the clinical phenotype or we discovered a single truncating variant (or a known disease-associated variant) in a recessive candidate gene. Most VUS occurred in 3 genes, namely *CAPN3*, *CPT2*, and *ANO5* (figure 1 and supplementary table e-3, links.lww.com/NXG/A178). Although it is possible that the low number of genes in our panel limited its informativeness on the patients' genotypes, it is also conceivable that in the VUS subgroup, the second allele may carry a deep intronic change leading to a mRNA splicing defect or large scale gene deletions/duplications, or a variation in genomic regulatory regions not covered in our study. Our study explored none of these possibilities.

Sixteen patients harbored molecular findings not compatible with their phenotypes. Although we cannot exclude a possible role for some of the variants detected in this subgroup, other additional and undetected genetic changes could well be responsible for their phenotypes.

Discussion

Both the EFNS guidelines[1] and the authors of a more recent review article[35] recommend conducting further investigations in individuals with apparently idiopathic hyperCKemia if the CK level is repeatedly more than 3 times higher than the ULN, EMG reveals myopathic changes, and the patient is younger than 25 years. However, it is anticipated that advancing knowledge in the

Table 1 Clinical and genetic features in 13 patients with pathogenic variants

ID	Sex/ age (y)	Serum CK (UI/L)	Clinical findings	HPO ID code	Muscle biopsy	Mutated gene	Mutation cDNA level	Mutation protein level	Inheritance	Reference
P9	F/54 y	1,310	Asymptomatic	NA	NA	*ANO5*[b]	c.1733T>C/c.2295C>G	p.Phe578Ser/p.Tyr765*	AR	[11]
P10	F/61 y	2000	Limb-girdle muscle weakness	HP:0003325	Mild myopathic signs	*CAPN3*[b]	c.633G>T/ c.1537insCCCCATCTCTCAG	p.Lys211Asn/ Met513Thrfs*68	AR	[12]
P13	M/32 y	1800	Myalgia and exercise intolerance	HP:0003326; HP: 0009020	Normal	*TCAP*[c]	c.33_35del/-	p.Glu12del/-	AD	This work
P15	M/7 y	7,348	Asymptomatic	NA	NA	*CAPN3*[b]	c.549delA/c.2115+2T>A	p.Thr184Argfs*35/splice site[a]	AR	[13,14]
P16	M/14 y	2,800	Limb-girdle muscle weakness	HP:0003325	Dystrophic signs	*SGCG*[c]	c.195+1G>A/c.195+1G>A	Splice site[a]/splice site[a]	AR	This work
P22	F/48 y	2000	Asymptomatic	NA	Mild myopathic signs	*ANO5*[b]	c.1356C>G/c.1356C>G	p.Tyr452*/p.Tyr452*	AR	This work
P25	M/11 y	19,000	Exercise intolerance and pelvic girdle muscle weakness	HP:0009020; HP: 0003749	Dystrophic signs	*DMD*[c]	c.151_153del	p.Leu51del	XL	This work
P27	F/67 y	900	Pelvic girdle muscle weakness	HP:0003749	Mild myopathic signs	*PYGM*[c]	c.2262delA/c.406G>A	p.Lys754Asnfs*49/ p.Gly136Ser	AR	[14]
P28	F/32 y	4500	Exercise intolerance and myalgia	HP:0009020; HP: 0003326	Mild myopathic signs	*SGCA*[c]	c.739G>A/c.850C>T	p.Val247Met/p.Arg284Cys	AR	[15, 16]
P29	F/6 y	16,340	Limb-girdle muscle weakness	HP:0003325	Dystrophic signs	*SGCG*[c]	c.521delT/c.521delT	p.Leu174Leufs*21/ p.Leu174Leufs*21	AR	This work
P30	M/66 y	1800	Asymptomatic	NA	Mild myopathic signs	*ANO5*[b]	c.629C>T/c.726dupT	p.Ser210Leu/ p.Ser243Phefs*2	AR	[17]
P32	M/53 y	780	Myalgia and exercise intolerance	HP:0003326; HP: 0009020	Mild myopathic signs	*PYGM*/ *CPT2*[b]	*PYGM:* c.[148C>T]; *CPT2:* c.[1348A>T]	*PYGM*:p.[Arg50*]; *CPT2*: p.[Arg450*]	NA	[18–20]
P33	M/18	2,937	Myalgia and exercise intolerance	HP:0003326; HP: 0009020	Mild myopathic signs	*MYH7*[b]	c.3790delG/-	p.Glu1264Argfs*34/-	AD	This work

Abbreviations: AD = autosomal dominant; AR = autosomal recessive; HPO = Human Phenotype Ontology; NA = not available; XL = X-linked.
[a] Predicting splice site loss and exon skipping (fruitfly.org/seq_tools/splice.html). Functional validation was derived by [b]immunofluorescence/protein studies combined with predictive early protein truncation or [c]immunofluorescence studies only.

Table 2 Clinical and genetic features in 20 patients with "likely" pathogenic variants

ID	Sex/ age (y)	Serum CK (UI/ L)	Clinical findings	HPO ID code	Muscle biopsy	Mutated gene	Mutation cDNA level	Mutation protein level	Inheritance	Reference
P1	M/ 19 y	3,000	Asymptomatic	NA	Mild myopathic signs	*RYR1*	c.8888T>C/-	p.Leu2963Pro/-	AD	[21]
P2	M/ 56 y	800	Asymptomatic	NA	Mild myopathic signs	*RYR1*[b]	c.7373G>A/-	p.Arg2458His/-	AD	[22]
P3	M/ 68 y	816	Exercise intolerance	HP: 0009020	Mild myopathic signs	*RYR1*	c.1163C>T/-	p.Ser388Leu/-	NA	This work
P4[d]	M/ 48 y	1,044	Limb-girdle muscle weakness	HP: 0003325	Dystrophic signs	*GMPPB*[b]	c.95C>T/ c.727C>T	p.Pro32Leu/ p.Arg243Trp	AR	[8, 23]
P5	F/65 y	600	Myalgia	HP: 0003326	Mild myopathic signs	*MYH7*	c.2009T>C/-	p.Val670Ala/-	NA	This work
P6	M/ 17 y	865	Asymptomatic	NA	Mild myopathic signs	*RYR1*[b]	c.7291G>A/-	p.Asp2431Asn/-	NA	[24]
P7	M/ 32 y	1,000	Asymptomatic	NA	Mild myopathic signs	*RYR1*[b]	c.5036G>A/-	p.Arg1679His/-	AD	[25]
P8	M/ 40 y	1,000	Myalgia	HP: 0003326	Mild myopathic signs	*RYR1*[b]	c.7048G>A/-	p.Ala2350Thr/-	AD	[26]
P11[d]	F/51 y	3,000	Limb-girdle muscle weakness	HP: 0003325	Dystrophic signs	*DYSF*[c]	c.862G>T/ c.2875C>T	p.Asp288Tyr/ p.Arg959Trp	AR	[27]
P12	M/ 11 y	728	Myalgia and exercise intolerance	HP: 0003326; HP: 0009020	Mild myopathic signs	*RYR1*[b]	c.6599C>T/-	p.Ala2200Val/-	AD	[28]
P14	F/65 y	3,300	Limb-girdle muscle weakness	HP: 0003325	Dystrophic signs	*ANO5*	c.580C>T/ c.2219C>T	p.Arg194Trp/ p.Ser740Phe	AR	This work
P17[d]	M/5 y	67,490	Rhabdomyolysis	HP: 0003201	Mild myopathic signs	*CPT2*[b]	c.338C>T/ c.338C>T	p.Ser113Leu/ p.Ser113Leu	AR	[29]
P18	M/ 29 y	600	Asymptomatic	NA	Normal	*RYR1*[b]	c.[4711A>G; 10097G>A]/ c.11798A > G	p.[(Ile1571Val; Arg3366His)]/ p.Tyr3933Cys	NA	[30–32]
P19	M/ 39 y	2000	Asymptomatic	NA	Normal	*CAPN3*	c.[1,395_ 1397del; 2257G>A]/ 1453A>G	p.[(Leu465_ Glu466del; Asp753Asn)]/ p.Met485Val	AR	[12, 32, 33]
P20[d]	M/ 24 y	12,000	Asymptomatic	NA	Mild myopathic signs	*SGCA*	c.242G>A/ c.739G>A	p.Arg81His/ p.Val247Met	NA	[15]
P21	F/53 y	500	Limb-girdle muscle weakness	HP: 0003325	Mild myopathic signs	*RYR1*	c.10923-8C>A/-	Splice site[a]/-	NA	This work
P23	M/ 64 y	1,000	Asymptomatic	NA	Mild myopathic signs	*RYR1*	c.14812A>G/-	p.Ile4938Val/-	NA	This work
P24[d]	M/ 67 y	1,103	Myalgia	HP: 0003326	Mild myopathic signs	*MYOT*	c.179C>T/-	p.Ser60Phe/-	AD	[34]

Continued

Table 2 Clinical and genetic features in 20 patients with "likely" pathogenic variants *(continued)*

ID	Sex/ age (y)	Serum CK (UI/ L)	Clinical findings	HPO ID code	Muscle biopsy	Mutated gene	Mutation cDNA level	Mutation protein level	Inheritance	Reference
P26[d]	M/ 21 y	1929	Asymptomatic	NA	Normal	*POMT2*[c]	c.[1733G>A; 239C>T]/ c.707T>G	p.[(Arg578His; Pro80Leu)]/ p.Leu236Arg	AR	This work
P31	M/ 26 y	40,000	Asymptomatic	NA	Mild myopathic signs	*RYR1*[c]	c.5288C>T/ c.7681C>T	p.Pro1763Leu/ p.Leu2561Phe	NA	This work

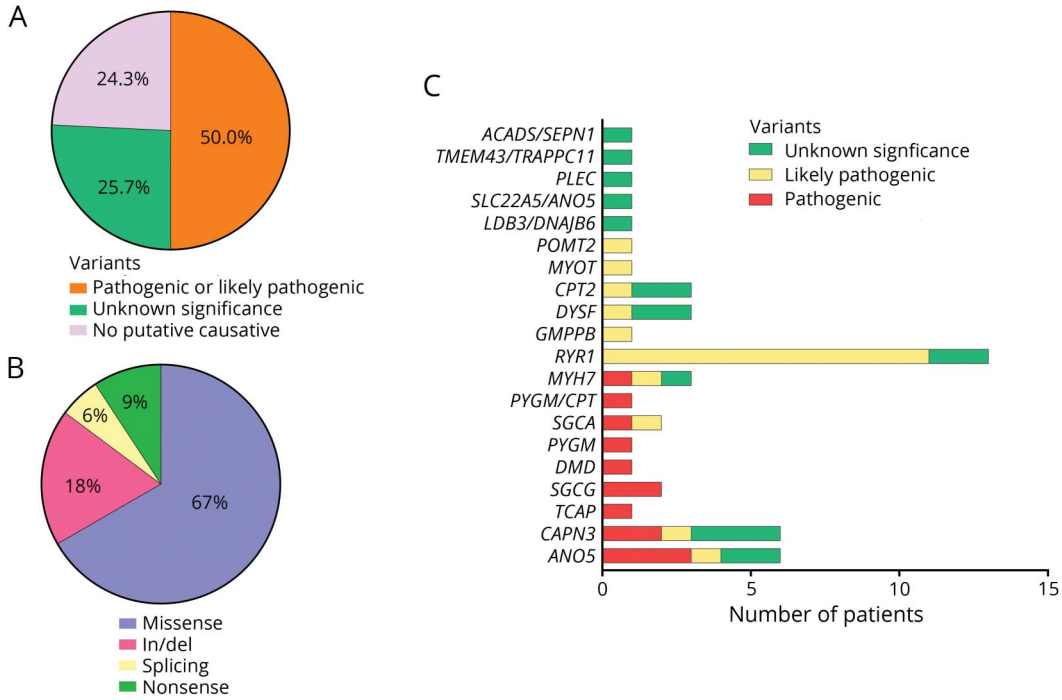
Abbreviations: AD = autosomal dominant; AR = autosomal recessive; HPO = human phenotype ontology; NA = not available.
[a]Predicting splice site loss and exon skipping (fruitfly.org/seq_tools/splice.html).
[b]Functional validation derived by immunofluorescence/protein studies combined with predictive early protein truncation/previous published work with evidences of pathogenicity.
[c]Functional validation derived through immunofluorescence studies only.
[d] Patients with "likely" pathogenic variants confirmed by immunofluorescence analysis or previously published evidence of pathogenicity.

field of muscle disorders will lead to the identification of previously unrecognized causes of hyperCKemia, thereby reducing the proportion of idiopathic cases. This would be an important development, as it would allow more precise clinical monitoring and follow-up and help to clarify the natural history of the different forms.

Studies conducted before NGS entered the clinical arena considered the diagnostic impact of muscle biopsy in the evaluation of patients with asymptomatic hyperCKemia[2,3,36–38] and the relative frequency of common genes such as *DMD*.[38] In recent years, NGS, which has involved the development of different gene panels and seen the focus switch to peculiar muscle phenotypes, has transformed the approach to the study of neuromuscular disorders (the relevant data are for the most part reviewed in reference 4). Some studies have also addressed, among other aspects, the genotype of patients with isolated hyperCKemia, reporting a limited diagnostic yield (21%) in fewer than 40 patients.[39,40]

Herein, we report the results obtained in a cohort of 66 patients with hyperCKemia, half of whom were asymptomatic on evaluation. Our use of massive parallel sequencing with

Figure 4 Diagnostic rates and molecular results



(A) Pathogenic or likely pathogenic variants were found in 50% of the patients, whereas 25.7% showed variants that required further characterization, and 24.3% did not present pathogenic variants. (B) Type of causative mutations identified in diagnosed patients: missense (67%), small indels (18%), splice site variants (6%), and nonsense mutations (9%). (C) Bar chart showing number of patients with "pathogenic" (red), "likely pathogenic" variants (yellow), and variants of unknown significance (green) for each gene.

a multigene panel combined with the application of stringent bioinformatics filters, and integration of muscle phenotypes (both clinical and morphological), produced a 50% diagnostic yield, a rate higher than those previously reported.[39,40] This difference is likely related to the combination of genotype with integrated clinico-morphological phenotype data in the present study, to its more homogeneous population (mostly adults in our cohort as opposed to the mixed populations studied by others), to technical improvements implicit in more recent gene panel technologies, or to a combination of these factors. There were no straightforward differences between the solved and unsolved participants, with clinical features and serum CK values being roughly the same in the 2 subgroups. Our data confirm that main challenges in NGS data analysis are the clinical interpretation of molecular findings and the distinction of causative mutations from the plethora of not clinically significant DNA variations. The low frequency of variants, or their absence in reference polymorphic databases, is recognized as a necessary but not sufficient criterion to assign pathogenicity. Moreover, in silico predictions present often unanticipated discordance between different predicting algorithms.[4] All this make more complex to reach a final molecular diagnosis. Considering our results overall, there emerge several points worth underlining. First, of the 33 patients who received a molecular diagnosis, 11 harbored mutations in *RYR1*, and all of these presented normal muscle MRI scans and subtle muscle involvement on skeletal muscle biopsy. Only 1 presented episodes of rhabdomyolysis, 2 presented myalgia, and 1 showed mild limb-girdle muscle weakness. Conversely, 7 patients were fully asymptomatic with a normal clinical evaluation associated with serum steady CK levels ranging from 500 to 1,000 UI/L. Although we did not seek to further corroborate our genomic findings by evaluating their functional impact on intracellular calcium homeostasis through complex studies or by performing immunoblotting with commercial antibodies, all the detected *RYR1* variants were rare ones, namely those inherited segregated in familial cases with clinical manifestations, and all are likely to perturb secondary ryanodine-1 receptor structure in protein modeling (not shown). Overall, our data imply that mutations in *RYR1* may be a common in hyperCKemia, even when CK levels are <1,000 UI/L. Of interest, we observed that the *RYR1* participants presented pseudometabolic features more frequently than patients harboring other genotypes but did not stand up as a clinically different subgroup. Second, we identified biallelic mutations in *ANO5* in 4 patients and mutations in *SGCA* in 2 adults with long-lasting hyperCKemia without muscle weakness. These data support previous impressions suggesting that analysis of known LGMD genes is justified even when isolated hyperCKemia or minimal muscle weakness is the sole clinical manifestation.[41,42] Third, we frequently observed mild, nonspecific muscle abnormalities consisting of variation in fiber size, nuclear internalization, or these 2 features combined; these alterations were always unrelated to serum CK levels, age at onset, clinical features, disease duration, and mutated gene. Nevertheless, muscle biopsy remains an important part of the diagnostic process in hyperCKemia, as it can also fulfill a "functional" role assisting in the clarification of uncertain cases. This is illustrated by case P28 in our study where 2 changes in *SGCA* were substantiated by reduced α-sarcoglycan labeling in skeletal muscle (figure e-1, links.lww.com/NXG/A175, A-B). Fourth, 26% of the patients were found to harbor VUS. Nonetheless, we frequently identified single deleterious variants in *CAPN3* and *CPT2*, 2 autosomal recessive genes where manifesting heterozygosity reportedly occurs.[43–45] The existence, in hyperCKemia, of symptomatic carriers, various degrees of clinical severity, and large intrafamilial heterogeneity of the phenotype (ranging from asymptomatic to fatal) could be explained by factors other than the genotype. In the case of CPT2-related myopathy, for example, one might consider different degrees of exposure to triggering/environmental factors (e.g., temperature, nutrition, fitness level) capable of increasing dependency on fat oxidation and further impairing exercise performance.[46] Taking these factors into consideration, as well as the possibility of synergistic heterozygosity[34] (as in our case P32) and digenic inheritance, it is imperative to combine thorough expert clinical and myopathologic evaluation with NGS as a prelude to a higher diagnostic rate in patients with paucisymptomatic hyperCKemia. Finally, isolated hyperCKemia can be the sole feature alerting to a progressive muscular disorder requiring careful surveillance and may open new prospective in future therapeutic opportunities.

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Continued

Appendix *(continued)*

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Next-generation sequencing approach to hyperCKemia: A 2-year cohort study

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