

UNIVERSITY OF GENOVA

Department of Neuroscience, Rehabilitation, Ophthalmology,
Genetics, and Maternal and Children's Sciences (DINOEMI)

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Genetic approach to neuromuscular disorders in the NGS era

Tutor

Prof. Marina Grandis

Ph.D. candidate

Chiara Gemelli

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2 ABSTRACT

Introduction/aims. Currently, there are no straightforward guidelines for the clinical and diagnostic management of neuromuscular disorders. Therefore, I have aimed to describe the diagnostic workflow which is used in my neuromuscular clinic for evaluating patients with this condition. The neuromuscular clinic is situated in IRCCS Policlinico San Martino in Genova and is a neuromuscular university centre in Northwest Italy.

Methods. I describe our diagnostic approach to two frequent neuromuscular disorders: hyperCKemia and CMT neuropathy.

The first work is an Italian multicentre study evaluating our diagnostic workflow for isolated hyperCKemia, which is based on electrodiagnostic data, biochemical screening and first-line genetic investigations, followed by successive targeted sequencing panels. Using this approach, we established a definitive diagnosis in one third of the patients. The detection rate was higher in patients with severe hyperCKemia and abnormal electromyographic findings.

The second work includes patients affected by CMT with regular follow-ups in our CMT clinic. I describe the genetic distribution of CMT subtypes in our cohort and report a peculiar phenotype. Moreover, I define our diagnostic experiences as a multidisciplinary outpatient clinic, combining a gene-by-gene approach or targeted gene panels based on clinical presentation.

Discussion/conclusion. Taking as a model our experience, I generalise the genetic approach to neuromuscular disorders: the diagnosis strategy should be flexible and tuned to the clinical features of the patient in order to select the best molecular approach for each patient.

3 INTRODUCTION

Neuromuscular disorders (NMd) affect motor neurons, sensory neurons in dorsal root ganglia, spinal roots, peripheral nerves, neuromuscular junctions and muscles. NMd include heterogeneous diseases, varying for the age of onset, severity and clinical manifestations. One of the main clinical manifestations is the impairment to perform voluntary movements, with the possible involvement of bulbar and respiratory muscles leading to swallowing and respiratory problems. Frequently, muscle weakness is associated with wasting, fasciculations, cramps and pain.

Historical information regarding the onset, duration and evolution of symptoms provides important clues to diagnosis. Knowledge about the time course of disease (acute, subacute or chronic) and the course (monophasic, progressive or relapsing) narrows the diagnostic possibilities. Family history is aimed at identifying similar symptoms and bony deformities (such as pes cavus or specific muscular hypertrophy or atrophy) in relatives that point to a familial disorder. The presence of additional symptoms may suggest an underlying systemic disorder. Pathological history should enquire about preceding or concurrent associated medical conditions (diabetes mellitus, hypothyroidism, chronic renal failure, liver disease, intestinal malabsorption, malignancy, connective tissue diseases, human immunodeficiency virus [HIV] seropositivity); drug use, including over-the-counter vitamin preparations (vitamin B6); alcohol and dietary habits and exposure to solvents, pesticides or heavy metals¹.

Clinical examination and medical investigations, such as biochemical blood tests, neurophysiological assessments, lumbar puncture, imaging techniques such as MRI or ultrasound, nuclear medicine imaging as well as muscle and/or nerve biopsies help clinicians to define the anatomical localisation and type of damage in order to suspect an acquired or genetic NMd. Acquired NMd are associated with autoimmune disorder, toxic damage and compressive damage. Inflammatory NMd could be isolated or frequently associated with systemic involvement as a

part of an autoimmune disorder or secondary to hematologic or paraneoplastic disorder. Other time specific toxic substances or metabolic disorders are at the basis of damage.

However, the vast majority of NMD has a genetic cause². Genetic NMD can be listed as follows based on the location of the damage: Skeletal muscle [muscular dystrophies (MD), myotonic dystrophies type 1 and 2 (DM1 and DM2), congenital DM (CDM), congenital myopathies (CMs) and metabolic myopathies], skeletal muscle voltage-gated ion channels (periodic paralysis, congenital myotonia), neuromuscular junctions (congenital myasthenic syndromes), nerves/motor neurons [Charcot–Marie–Tooth neuropathies (CMTs), familial amyotrophic lateral sclerosis (fALS), hereditary spastic paraplegias (HSPs) and spinal muscular atrophies (SMA)]. Onset may occur, in the basis of the disease, throughout life, from birth to old age. Progression also varies amongst the different types and amongst patients: it can be rapidly progressive or it may be slower over time².

Clinical examination allows to identify the specific phenotype and detect any sign suspected of a specific disorder. Some disorders present easily recognisable phenotypes as Duchenne or Becker MD (DMD, BMD), Emery-Dreifuss MD, Facioscapulohumeral MD (FSHD), Myotonic Dystrophy, CMT and SMA. Other time disorders have a hardly recognisable phenotype as in myasthenia, mitochondrial myopathy, distal myopathy, limb girdle muscular dystrophy (LGMD), congenital myopathy or MD and distal SMA. A simple inspection allows the observation of focal or diffuse muscle wasting, or the focal enlargement of muscles as with the “pseudohypertrophy”, which may be typical of specific NMD. Pseudohypertrophy could be non-specific but suggestive of a dystrophic process as calf hypertrophy or macroglossia. In DMD, an increase in fat and connective tissue in the gastrocnemius causes calf pseudohypertrophy that is associated with a reduced bulk of the quadriceps caused by more severe fibre loss³. Facial features of myotonic muscular dystrophy may be noted on inspection as the long thin face with temporal and masseter and frontal balding or a tent-shaped mouth³. Most weakness in neuromuscular disorders is associated with focal atrophy. Focal atrophy of the muscle groups may provide diagnostic clues to specific neuromuscular disorders. SMA gives diffuse muscle atrophy or focal atrophy in more

slowly progressive subtypes. Emery-Dreifuss may present with striking wasting of the biceps, accentuated by sparing of the deltoids and forearm muscles. FSHD patients presents a characteristic appearance of the shoulders that are forward-sloped, with a profound posterior and lateral winging scapula that could be asymmetric³. FSHD patients, moreover, present facial weakness with horizontal smile, hyperlordosis and humeral and peroneal wasting. CMT demonstrate distal atrophy or “stork leg appearance” relatively early in the disease course with foot drop and pes cavus.

The family history is based upon establishing a pedigree, searching for any consanguineous links and to then characterise them and decipher the geographical and ethnic background. With all this information, clinicians are able to define if the patient could be affected by a familial NMD and which inheritance could be present: X-linked recessive, autosomal dominant or autosomal recessive.

There are some features which can suggest a hereditary process: a slowly progressive deficit, an early age of onset, clinical signs out of proportion to the symptom of the patient especially in neuropathies which present discrepancy between severe electrophysiological data and a milder clinical examination.

Over 600 genes have already been identified (see <http://www.muscle.genetable.fr/index.html>) and the molecular diagnostic yield progressively increases due to technology development: moving from PCR in the 1980s to target gene panels, exome (protein-coding sequences) in 2009/10 and to the emerging use of genome sequencing starting from 2015.⁴ Next generation sequencing can be used to analyse a set of genes associated with specific clinical manifestations (gene panel sequencing, GPS), the exome (whole-exome sequencing, WES) or the whole genome of a patient (whole-genome sequencing, WGS). These new technologies have improved genetic diagnosis. In less than 10 years, NGS has resulted in a near doubling of the number of genes recognised which are implicated in neuromuscular diseases from 290 in 2010 to 535 in 2019⁵.

NMD pathogenic mutations are different and include single nucleotide variants, deletions and duplications, expansion repeats, alterations occurring in regulatory regions as promoters or

intergenic segments⁶. 85% of disease-causing variants are believed to be in the coding region⁴ while it is estimated that 15% of variants potentially causative of mendelian traits are localised in the non-coding regions of the genome⁷. WGS covers up to 98% of the whole human genome, and WES covers nearly 95% of the coding regions but only 1–2% of the genome⁸.

With so many opportunities, the choice of the molecular approach is highly debated. In general, the larger the genomic region is which needs to be investigated (from GPS to WGS), the smaller the average sequence depth will be and the greater the number of variants identified². Target gene panels allow the simultaneous analysis of different genes associated with a specific phenotype. All coding regions of target genes are sufficiently high covered. It is indicated in clinical practice, as it is less expensive and faster compared to over NGS and it avoids the risk of disclosing unrelated pathogenic variants to the phenotype being investigated⁹. However, GPS are frequently custom-made and require periodically update due to the frequent identification of novel causative genes². WES sequences the entire coding regions of the genome. It is often performed in unsolved cases or in research because it is potentially able to find novel disease causative genes. Thuriot et al.¹⁰ recently compared the use of GPS and WES: WES resulted in a lower risk of missed diagnoses, while potentially increasing the diagnostic yield. At the same time, they found that almost half of the diagnoses were based on a few genes, suggesting a role for GPS as a first-line approach. Alternatively, whole exome sequencing, followed by filtering for defined genes, could be a valid strategy^{11,12}. WES presents higher diagnostic rates when the trio-WES rather than a patient-only strategy was applied. Trio-WES consists in simultaneous sequencing of a patient and their biological parents⁹. However, WES presents some limits: copy number variations (CNVs), expansions or contractions in repetitive regions, chromosomal rearrangements and deep intronic variants are not detect². Those limitations are overcome by WGS that can detect CNVs, chromosomal abnormalities, deep intronic variants¹³ and analysis mitochondrial genome¹⁴. Nevertheless, the use of WGS is limited by its costs and the time needed to analyse a huge amount of data, but it requires a lot of expertise for data analysis and interpretation. Alfares et al.⁸ compared WGS and WES diagnostic yield and found that WGS presented a detection rate only 7% higher than WES, so they recommended the reanalysis of WES raw data before performing

WGS. Compared with WGS, WES has a lower cost, a greater depth of coverage in target regions, fewer storage requirements and the data analysis is easier to perform⁸. In clinical practice, patients with a phenotype that allows to identify the disorder in a specific NMD category are firstly evaluated with GPS implemented with some single gene analysis. WES and WGS are only used in selected patients, frequently within research.

Regardless of the technique used, a definitive molecular diagnosis is important and useful for patients not only to achieve a correct diagnosis with a specific prognosis, but also for familial counselling in order to identify subjects at risk, and eventually offer prenatal diagnosis to prevent the recurrence of the disease. Moreover, a specific diagnosis can allow access to causative therapies as in the cases of Pompe disease, hereditary transthyretin-mediated amyloidosis and SMA, and it could permit access to a clinical trial with new gene therapies.

4 AIM:

The purpose of the project was to critically revise the diagnostic yield of two common inherited neuromuscular conditions: hyperCKemia, a frequent and nonspecific presentation in muscle diseases, and Charcot-Marie-Tooth disease, the most frequent inherited neuromuscular disorder.

For both conditions, the advent of NGS has increased the possibility to obtain a molecular diagnosis. However, the diagnosis is frequently achieved after a careful clinical examination and subsequent tests which combine old and new technologies.

For hyperCKemia, guidelines are under revision, since the previous ones precede the advent of NGS which has completely changed the scenario, while for CMT, the approach is more standardised, even if many cases remain unsolved.

During my PhD, I coordinated a multicentric Italian study applying a novel diagnostic algorithm for hyperCKemia and I have evaluated the diagnostic yield of the different steps.

I have also revised the data of a wide cohort of CMT patients visited at the CMT Clinic in Genoa where we observed a peculiar phenotype frequently associated with the MPZ and HSP variants.

The specific aims are:

- 1) To define the role of NGS, in particular the clinical use of GPS, which could be a first-tier diagnostic approach to different disorders and how the diagnostic rate could be implemented by electrodiagnostic data, biochemical screening and first-line genetic investigations.
- 2) define when the use of GPS is indicated or when a single gene analysis is preferable in order to reach a genetic diagnosis with a maximum yield and minimum expenses of both time and money.
- 3) evaluate the detection rate of a different genetic strategy.
- 4) evaluate the limits of NGS and clarify why a consistent number of cases remain undiagnosed.

5) evaluate the ethical and familial consequences of a genetic diagnosis.

The chapters 3¹⁵ and 4¹⁶ contain sections including the background, aims, methods and discussion specific for each study.

5 AN INTEGRATED APPROACH TO THE EVALUATION OF PATIENTS WITH ASYMPTOMATIC OR MINIMALLY SYMPTOMATIC HYPERCKEMIA.

5.1 INTRODUCTION:

Muscle diseases encompass a broad range of disorders, and in most cases, they lead to motor impairment, often due to a genetic defect¹⁷. In some patients, the clinical features are straightforward, making it possible to establish an accurate diagnosis immediately. In most cases, however, the clinical clues are limited due to overlapping and nonspecific presentations, making it challenging to establish a precise diagnosis¹⁸. In cases of milder signs or symptoms, such as isolated hyperCKemia, the clinical presentation might be even less specific, hampering the diagnostic process.

With the advent of next-generation sequencing (NGS) technology, the limitations of the previous gene-by-gene approach have largely been overcome. The simultaneous analysis of numerous genes is time-saving and allows for the analysis of rare genes associated with heterogeneous phenotypes¹⁹. Therefore, compared with Sanger sequencing, targeted gene panels are more cost-effective and result in a larger number of diagnoses²⁰. Overall, massively parallel sequencing has improved the diagnostic approach in genetic disorders, as confirmed by several reports describing its diagnostic efficacy worldwide^{19,21–36}.

Accordingly, the reported diagnostic rate of a massively parallel sequencing-based approach in muscle diseases is higher than that obtained using traditional strategies, such as Sanger sequencing³⁷, and an increasing number of studies using targeted sequencing panels for the molecular characterization of muscular disorders have reported detection rates ranging from 15% to 65%^{10,32}. Target gene panels are frequently used in clinical practice, but a consensus has not yet been reached regarding when they should be used during the diagnostic process. For instance, it has been proposed that massively parallel sequencing should be performed before other investigations³², thereby endorsing target gene panels or genome sequencing as the universal first-

tier test for these heterogeneous genetic conditions^{27,38}. However, the massively parallel sequencing approach presents some limitations because the large volume of generated data requires time for data analysis and management, and the process involves some risk of errors, including false negative results owing to missed repeat expansions or false positive results³⁹. Additionally, gene panels may lead to incidental findings in unrelated genes⁹. Therefore, we aimed to describe our diagnostic approach, which includes a combination of different steps for overcoming the limitations of NGS, in patients with asymptomatic or minimally symptomatic hyperCKemia.

5.2 METHODS:

Patients were recruited from neuromuscular clinics at the IRCCS Polyclinic San Martino in Genoa, IRCCS Giannina Gaslini Institute in Genoa, AOU Hospital Federico II in Naples, and Molinette Hospital in Turin during a period of approximately 3 years from March 2017 to January 2020. All the patients were older than 16 years of age. The inclusion criteria were the presence of hyperCKemia (confirmed in two independent examinations) alone or in association with mild signs or symptoms of muscle disease. HyperCKemia was defined as a creatine kinase (CK) level greater than 1.5 times the upper limit of normal (ULN), as defined in the European Federation of the Neurological Societies (EFNS) guidelines^{40,41}.

All patients were evaluated by taking a clinical history and performing a neurological examination that included the Medical Research Council (MRC) scale for assessing muscle strength.

We included minimally symptomatic patients who presented with mild signs of muscle disease, such as mild muscle weakness ($MRC \geq 4$) present in fewer than four muscles, rippling phenomena, and episodes of rhabdomyolysis. We also considered minimally symptomatic patients who presented with only vague symptoms, such as myalgia, undue fatigue, exercise intolerance, cramps, and stiffness⁴⁰. Patients with a family history of diagnosed myopathy or with a clear phenotype suggestive of myotonic dystrophy type 1 or facioscapulohumeral muscular dystrophy, which could easily lead to a diagnosis, as well as patients with predominant distal weakness or congenital onset myopathy were excluded. Patients exhibiting the acute or subacute

onset of muscle weakness or a rapid disease course were suspected of having inflammatory myopathy and were required to undergo a specific workup⁴². The medical causes of hyperCKemia, such as statins or other drug-based treatment associated with CK elevation, abnormalities in thyroid function, and other endocrine or metabolic causes, were ruled out⁴³. Furthermore, we excluded patients with primary involvement of the respiratory or cardiac system. Figure 1 summarizes the diagnostic workflow. All the patients underwent a confirmatory laboratory test to evaluate the levels of CK, aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase, and lactic acid and a urine analysis. We classified hyperCKemia as follows: mild, $< 5 \times$ the ULN; moderate, $5-10 \times$ the ULN; and severe, $> 10 \times$ the ULN. Electrodiagnostic studies, consisting of both nerve conduction studies and electromyography (EMG), were part of the routine evaluation. Electrodiagnostic data were used to stratify patients into different groups: patients with normal EMG findings, patients with EMG findings compatible with a muscular disorder, and patients with neurogenic signs. In some patients, a muscle biopsy was previously performed and did not aid in establishing a diagnosis. Based on clinical data and electrodiagnostic results, the patients were examined using our diagnostic workflow, and the previous muscle biopsy was not an exclusion criterion. In these cases, the electrodiagnostic studies were retrospective.

After obtaining written consent for genetic testing, we performed a dried blood spot (DBS) test for evaluating alpha-glucosidase (GAA) activity. We then tested our patients for myotonic dystrophy type II (DM2) and copy number variations in the *DMD* gene, which is responsible for most of the Duchenne and Becker forms of muscular dystrophy. An analysis through a target gene panel was performed in patients without a diagnosis after these initial steps. The target gene panel was performed in the Laboratory of Neurogenetics and Neuroscience of Giannina Gaslini Institute (Genova) and included 20 different genes associated with metabolic myopathies or with limb-girdle muscular dystrophy (LGMD) (*AGL*, *ENO3*, *GAA*, *LAMP2*, *LDHA*, *PFKM*, *PGAM2*, *PGK1*, *PGM1*, *PYGM*, *ACADVL*, *CPT2*, *LPIN1*, *ANO5*, *LMNA*, *CAPN3*, *FKRP*, *FKTN*, *CAV3*, and *RYR1*).

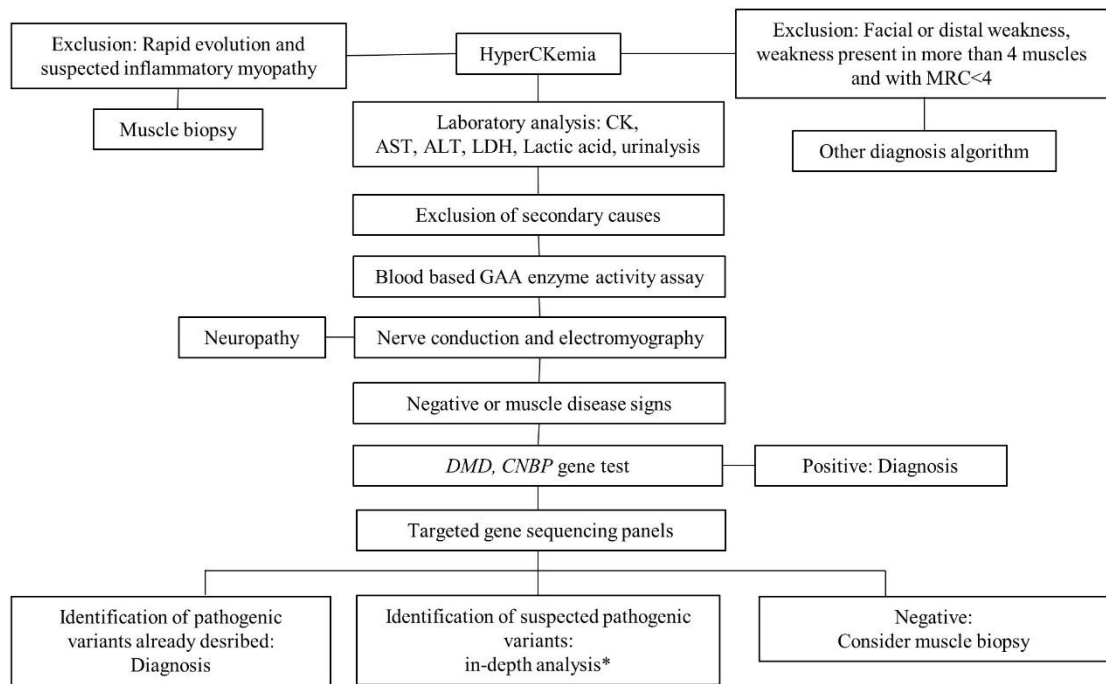


Fig. 1 Diagnostic algorithm for hyperCKemia.

MRC = Medical Research Council scale, CK = creatine kinase, AST = aspartate aminotransferase, ALT = alanine aminotransferase, LDH = lactate dehydrogenase, GAA = α -1,4-glucosidase, MLPA = multiplex ligation probe amplification, cDNA = complementary deoxyribose nucleic acid. *evaluation of segregation study, MLPA and cDNA analysis, and muscle biopsy results to establish the pathogenicity of a VUS

The Ampliseq/Ion Torrent PGM technology was used to perform the NGS study, with the minimum fraction of targeted regions set at 95% to be covered by at least 20 \times . The median coverage was 300 \times . After the removal of duplicates, the paired-end reads were mapped to the reference human genome sequence (GRCh37/hg19) using the IOn Reporter and CLC Bio Genomics Workbench 7.5.1 software (CLC Bio, Aarhus, Denmark). Single-nucleotide polymorphisms (SNPs) and short deletion or insertion (indels) variants were called with the CLC Bio Workbench software using a specific variant calling plugin and dbSNP147 databases. The validation of variants was performed using Sanger sequencing. In selected cases, to exclude genic micro-deletions/duplications, multiplex ligation probe amplification (MLPA) was performed for specific genes (*CAPN3* and *GAA*). Standards and guidelines for the interpretation of sequence variants were used⁴⁴.

Based on the results, we reevaluated the muscle biopsy slides of patients who had previously undergone muscle biopsy. The specimens were examined with routine staining (hematoxylin and

eosin; modified Gomori trichrome; cytochrome c oxidase; succinate dehydrogenase; reduced nicotinamide adenine dinucleotide [NADH] dehydrogenase; adenosine triphosphatase at pH 10.4, 4.6, and 4.3; periodic acid–Schiff; and Sudan) and reactions to myoadenylate deaminase, myophosphorylase, and phosphofructokinase. An immunofluorescence analysis was performed using antibodies against dystrophin (Dys 1, 2, and 3), sarcoglycans, alpha-dystroglycan, collagen VI, caveolin, dysferlin, and merosin.

All the patients or their parents or guardians provided written informed consent, and the study was approved by the local ethics committee.

5.3 RESULTS:

This study included 83 patients: 28 (34%) female. All the patients were Italian (except for a man of African origin) and were aged between 16-71 years (median age, 38.5 ± 16.8 years).

Forty-five patients (54%) had previously undergone a muscle biopsy, which did not lead to a definite diagnosis. Thirty-eight patients (46%) were new patients who underwent examinations based on the established protocol after being referred.

We found that 36% of the patients were affected by isolated hyperCKemia ($n = 30$), while others had hyperCKemia associated with mild signs of muscle involvement, including mild muscle weakness ($n = 9$, 11%), rhabdomyolysis ($n = 7$, 8%), rippling ($n = 1$, 1%), or other symptoms, such as myalgia, cramps, exercise intolerance, or “second-wind” phenomenon ($n = 36$, 43%). Further, the hyperCKemia was mild in 35%, moderate in 39% and severe in 26%. On average, our patients had hyperCKemia for at least 10 years. Patient demographics, clinical phenotypes, muscle biopsy results, and genetic findings are presented in Table 1.

Through the electrodiagnostic studies, one patient who was affected by axonal neuropathy was identified and excluded from further investigations, leaving our cohort with 82 patients. Needle EMG revealed alterations suggestive of myopathy in 31 patients (38%), while the findings were normal in the remaining patients. A blood-based GAA enzyme assay showed reduced activity in

two patients, which was later confirmed by *GAA* gene sequencing, leading to a diagnosis of late-onset Pompe disease in both these patients. Through the first set of genetic tests, we identified a female patient carrying the Duchenne/Becker muscular dystrophy gene, three male patients carrying pathogenic variants in the *DMD* gene, and a patient with DM2. Thus, our first step in the investigation allowed for the diagnosis of seven patients with well-known muscle diseases.

In the remaining 75 patients, we performed a massively parallel sequencing analysis with our target panel. A conclusive genetic diagnosis was reached in 18 patients (24%): *ANO5* pathogenic variants (n = 8) including isolated hyperCKemia (n = 2), myalgia (n = 2), rhabdomyolysis (n = 2), exercise intolerance (n = 1), and mild muscle weakness causing anoctamin 5-related LGMD (LGMDR12) (n = 1); rippling muscle disease due to a *CAV3* pathogenic variant (n = 1); McArdle disease (n = 3); carnitine palmitoyltransferase 2 (*CPT2*) deficiency (n = 1); very long-chain acyl-CoA dehydrogenase (*VLCAD*) deficiency (n = 2); and *RYR1* pathogenic variants (n = 3). One of the patients with a *VLCAD* deficiency was diagnosed because he was found to be a carrier of two variants of the *ACADVL* gene: one pathogenic variant and one variant of uncertain significance (VUS) predicted to be likely pathogenic. As the patient's clinical history was consistent with a *VLCAD* deficiency, the patient was considered to have been diagnosed.

No variants were found in 24 patients, and at least one VUS was found in 33 patients. Among these, 12 patients were found to be carriers of a heterozygous pathogenic variant in a recessive gene (6 *CAPN3*, 1 *ACADVL*, 1 *ANO5*, 1 *GAA*, and 1 *FKTN*), and two were carriers of two pathogenic variants in two different recessive genes (*CPT2* and *ANO5* and *ENO3* and *ANO5*). The variants are listed in Table 1. Therefore, our protocol enabled us to reach a diagnosis in 25 patients, with a global detection rate (DR) of 30%. The distribution of positive diagnostic results is graphically summarized in Figure 2.

We found no differences in the DR (29% versus 31%) between patients who developed hyperCKemia during childhood to adolescence (< 18 years, n = 31) and those who developed hyperCKemia during adulthood (> 18 years, n = 51).

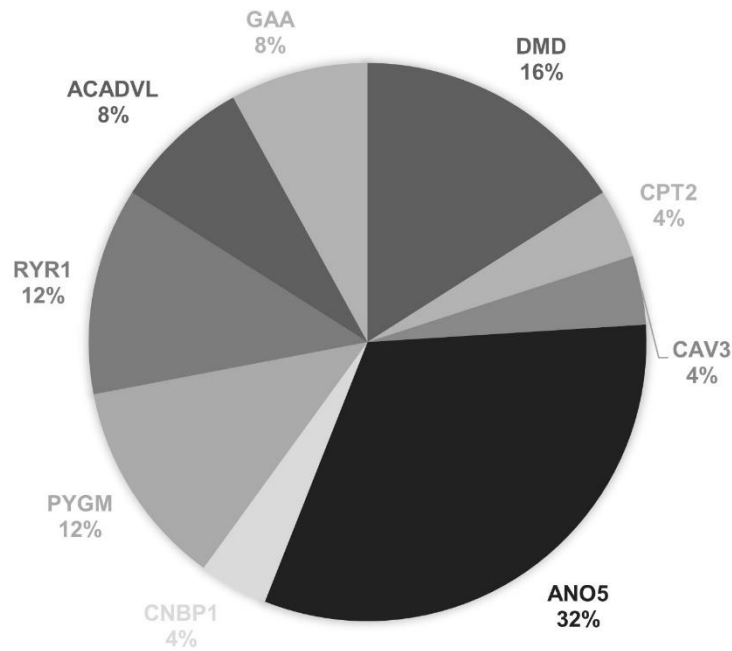


Fig. 2 Distribution of diagnostic results.

A diagnosis was achieved in 25 patients (30%): Pompe disease (n = 2); pathogenic variants of *DMD* (n = 4); a female patient who was a carrier and three patients with Becker muscular dystrophy), *DM2* (n = 1), *ANO5* pathogenic variants (n = 8), rippling muscle disease (n = 1), McArdle disease (n = 3), *CPT2* deficiency (n = 1), *VLCAD* deficiency (n = 2), and *RYR1* pathogenic variants (n = 3). *DM2* = myotonic dystrophy type II, *CPT2* = carnitine palmitoyltransferase 2, *VLCAD* = very long-chain acyl-CoA dehydrogenase

When we evaluated the DR in relation to the severity of hyperCKemia, we observed a low diagnostic yield in patients with mild hyperCKemia ($CK < 5 \times ULN$, n = 29, DR = 14%). No differences in the DR were observed in patients with either severely (n = 21, DR = 48%) or moderately elevated CK (n = 32, DR = 34%) levels. DR was higher in patients with altered EMG (n=31, DR=42%) compared with patients with normal EMG (n=51, DR=24%). Among these patients, the DR was higher in those with severe hyperCKemia (Figure 3). No difference in the DR was evident in patients who presented with mild muscular signs or symptoms compared with in those who presented with isolated hyperCKemia (33% versus 27%). However, in the subgroup of patients with mild muscle weakness, the DR increased to 44%.

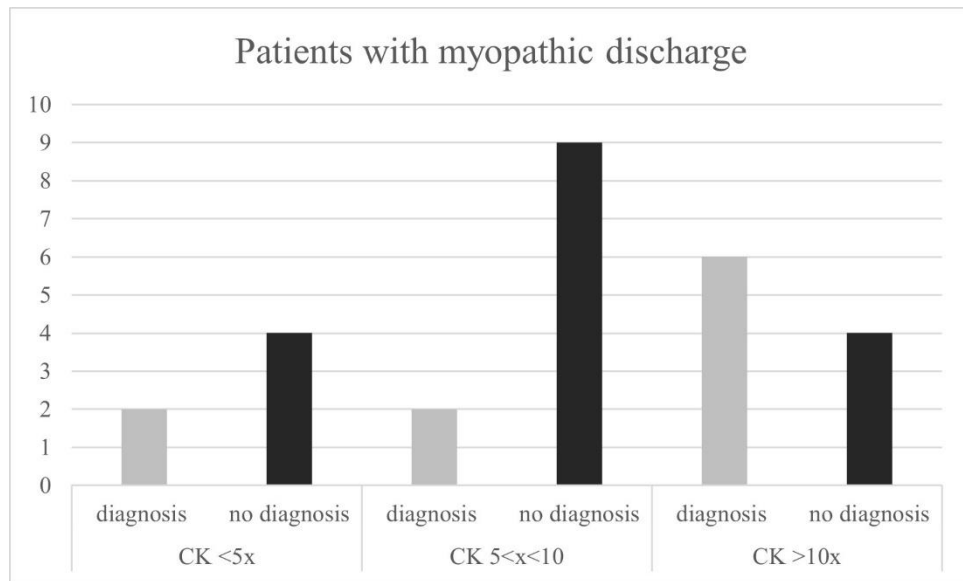


Fig. 3 Diagnosis of patients with myopathic discharges and evaluation of the different severity levels of hyperCKemia. The abscissa represents patients with myopathic discharges subdivided based on the hyperCKemia severity. Ordinates represent the number of patients. The black columns represent patients without a diagnosis, and the gray columns represent the diagnosed patients.

Previous muscle biopsies were performed in 45 patients and revealed nonspecific signs of myopathy (fiber size variability, degenerating fibers, intra-fibrillar vacuoles, and increased connective tissue) in 32 patients and normal findings in the remaining 13 patients. Among the patients who previously underwent a muscle biopsy, a final diagnosis was established in 11 patients through genetic analysis: *ANO5* pathogenic variants (n = 3) and *VLCAD* deficiency (n = 1) among patients with normal muscle biopsy findings, and *ANO5*-related myopathies (n = 2), Pompe disease (n = 1), *RYR1* pathogenic variants (n = 2), *VLCAD* deficiency (n = 1), and *DMD* pathogenic variants (n = 1) among patients whose biopsy revealed nonspecific signs of muscle damage. In the last patient, the muscle biopsy was performed in 2012 and showed only nonspecific signs of myopathy. After analyzing the genetic results, the specimens were reevaluated using more specific antibodies, and a western blot revealed a reduction in the dystrophin-related protein complex levels. The diagnosis of a patient affected by late-onset Pompe disease was missed despite having undergone a previous muscle biopsy. The reevaluation of the biopsy specimens in the two patients carrying pathogenic variants in *RYR1* revealed the presence

of core-like areas with NADH staining. Cores are typical of *RYR1* mutations but are not a specific and exclusive finding; thus, the muscle biopsy in these cases was not considered diagnostic.

Among the 38 patients who did not undergo a muscle biopsy, we established a diagnosis in 14 patients (36%): pathogenic variants in dystrophin (n = 2, male patients), *DMD* mutation (n = 1, female patient), DM2 (n = 1), CPT2 deficiency (n = 1), *ANO5* pathogenic variants (n = 3), ring muscle disease due to *CAV3* mutations (n = 1), McArdle disease (n = 3), Pompe disease (n = 1), and *RYR1* pathogenic variant (n = 1). In these patients, we could easily establish a diagnosis with the help of a muscle biopsy (for example, McArdle disease or *DMD* mutations). At the time of writing this paper, none of the patients who were undiagnosed underwent a subsequent muscle biopsy.

5.4 DISCUSSION:

Our study introduces an algorithm for diagnosing hyperCKemia with the aim of improving the diagnostic yield in patients with this condition.

The initial steps of our protocol were focused on excluding Pompe disease, pathogenic deletions in *DMD*, and DM2. These conditions are relatively common, easy to diagnose with DBS in the case of Pompe disease, and most importantly, potentially missed during the massively parallel sequencing analysis, which does not reliably detect large deletions/duplications or nucleotide repeat expansions²³. In addition, the most common *GAA* pathogenic variant is the splice site mutation, c.-32-13T>G⁴⁵, which can also be missed in a routine NGS analysis. Vacuoles and glycogen accumulation in both juveniles and adults with Pompe disease may not be prominent and could easily be missed⁴⁶ in a muscle biopsy, as happened in one of our patients. Indeed, with our screening process, we identified two patients with an alpha-glucosidase defect successively confirmed by polymerase chain reaction (PCR) Sanger sequencing of the *GAA* gene, three patients with a *DMD* pathogenic variant, one female patient who was a *DMD* carrier, and one patient with DM2. Interestingly, this last patient had normal EMG findings, thus highlighting that this disease is probably under-diagnosed^{47,48} and should be evaluated even when myotonia is not evident.

Overall, the combination of targeted PCR sequencing, MLPA, and massively parallel sequencing in the entire cohort enabled us to reach a molecular diagnosis in a third of our patients within the first year of follow-up (25 diagnoses in 82 patients). It is important to highlight that the target panel approach alone would have been less effective, only enabling a diagnosis in 18 patients (24%).

Two studies evaluated the role of a target panel in the diagnosis of hyperCKemia. The first study by Wu³² evaluated the diagnostic value of a target panel in a population of patients with muscle weakness (n = 135), rhabdomyolysis (n = 18), or asymptomatic hyperCKemia (n = 16). Both pathogenic and likely pathogenic variants were considered in the calculation of a DR of 36.09%, which is similar to our results. *ANO5*-related myopathies were the most common diagnoses. More recently, a report by Rubegni et al.³³ described the role of NGS in patients with undiagnosed asymptomatic hyperCKemia (n = 34) or mildly symptomatic hyperCKemia (mild limb-girdle muscle weakness [n = 19] and occasional exercise intolerance and myalgia [n = 13]). A diagnosis was reached in 33 patients (50%), among whom 11 harbored a pathogenic variant in the *RYR1* gene. Both these studies have highlighted the efficacy of a target panel in establishing a diagnosis, whereas our report describes its role in combination with other genetic techniques as part of a diagnostic algorithm.

The size of our target panel, comprising 20 genes, is a limitation that could be overcome by a larger target gene panel, but cost-effectiveness and time efficiency make targeted sequencing panels an effective approach as the first-line screening process for studying heterogeneous disorders, such as muscle diseases³⁵. Interestingly, Thuriot et al.¹⁰ recently compared the use of target gene panel testing and exome sequencing in patients with suspected muscle disorders from outpatient clinics. They stated that, in comparison with gene panel testing, exome sequencing resulted in a lower risk of missed diagnoses, while potentially increasing the diagnostic yield. At the same time, they found that almost half of the diagnoses were based on a few genes (*DMD*, *RYR1*, *CAPN3*, *PYGM*, *DYSF*, and *FKRP*), suggesting a role for target gene panels as a first-line approach. Alternatively, whole exome sequencing, followed by filtering for defined genes, could be a valid strategy^{11,12}. Indeed, an important limitation is that the *DMD* gene was not included in

our target gene panel, potentially missing rare *DMD* point mutations causing isolated hyperCKemia^{49,50}. Moreover, it must be noted that gene panels must be periodically updated due to the frequent identification of novel causative genes².

In our analysis, the most frequent pathogenic variants were in the *ANO5* gene, which were identified in eight patients. This finding is in line with those in previous reports, according to which *ANO5*-related muscle diseases frequently manifest in patients with a long-standing history of hyperCKemia without muscle weakness and commonly present in association with exercise intolerance, myalgia, and more rarely, episodes of rhabdomyolysis^{51,52}, in addition to the muscle biopsy not showing specific pathological signs⁵³.

Three patients presented with a pathogenic *RYR1* variant, further confirming the frequency of this disorder in hyperCKemia patients³³. All the *RYR1* pathogenic variants identified in our study have been associated with malignant hyperthermia⁵⁴⁻⁵⁶. None of our patients developed malignant hyperthermia, but they are still at risk.

We found single causative variants in recessive genes in 16% of the patients (12 of 75 patients who underwent panel analysis), which is also a common finding²⁷. In such patients, further genetic investigations are warranted to exclude the presence of a hidden variant in the second allele. In this group, *CAPN3* was the most frequently mutated gene (42%), as seen in six patients. MLPA was performed for the *CAPN3* gene in all the patients with a single variant and yielded negative results. Autosomal dominant transmission has been described for *CAPN3*-related myopathy⁵⁷⁻⁶⁰. Interestingly, one of the pathogenic variants identified (c.1706 T>C), has been recently described by Gonzalez-Mera et.al⁶⁰ as a cause for dominant calpainopathy. However, our patient did not have a family history compatible with autosomal dominant transmission and had not yet undergone a muscle biopsy analysis to investigate calpain-3 expression; therefore, at present, they remain undiagnosed. Even if several studies suggest that carriers of single heterozygous pathogenic variants in genes associated with autosomal recessive (AR) disorders can present with milder forms of the disease, further epidemiological and genetic studies on larger populations are required⁶¹.

One of the pitfalls of the massively parallel sequencing approach is the high prevalence of VUSs. Most VUSs are variants that have not been previously reported or have been reported less frequently but without established pathogenicity studies. VUSs were found in 28% (n = 21) of the patients evaluated with parallel sequencing tests (n = 75) and were mostly in the *RYR1* gene (40%). This gene is highly polymorphic and the list of associated variants continues to grow⁶²; these variants could be common in hyperCKemia cohorts³³. However, given that the role of these variants is still unclear, all cases involving VUSs were considered unsolved cases.

The high prevalence of VUSs and single heterozygous pathogenic variants in AR genes highlights the importance of further investigations. Of note is that two of the three patients with pathogenic variants of *RYR1* were actually found to have core areas in the muscle biopsy specimens after careful reanalysis of the muscle sections. In this context, muscle biopsy could be used to establish the pathogenicity of new variants²⁹.

Historically, muscle biopsies have played a fundamental role in the diagnostic algorithm for elevated CK levels. According to Morandi et al.⁶³, patients with asymptomatic hyperCKemia should be evaluated by first excluding systemic disorders and performing an electrodiagnostic study. Muscle biopsy is recommended after these steps. In our protocol, we performed a target genetic analysis before muscle biopsy. However, our protocol could lead to missed mitochondrial myopathies, which can present with isolated hyperCKemia^{64,65}, or atypical inflammatory myopathies, such as anti-3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) myopathy, which can be present with an asymptomatic elevation of CK for several years before the appearance of weakness⁶⁶. Therefore, we believe that a muscle biopsy should always be performed in unsolved cases or to confirm new pathogenic variants or validate candidate genes⁶⁷. In our cohort of patients, there were no differences in the DR with regard to the age of onset of hyperCKemia (childhood or adolescence vs adulthood); this finding is different from the expected finding according to the EFNS guidelines that suggest a higher probability of diagnosis in younger patients⁴⁰. However, the fact that the two groups were not equally represented, as the number of adults was larger, could have influenced our results.

According to Prella et al.⁶⁸, the probability of establishing a diagnosis in patients with hyperCKemia is positively associated with needle EMG results ($p < 0.05$; odds ratio, 2.9). Similarly, in our study, the DR in patients with EMG signs of myopathy was higher than that in patients with normal EMG test results. Moreover, among these patients, the DR was higher in those with severe hyperCKemia (Figure 3). This observation supports the application of extensive genetic analyses, particularly in patients with a severe increase in CK levels and EMG abnormalities. Nerve conduction study was as important as a screening investigation, since it enabled us to identify a patient with neuropathy, who was required to undergo a different diagnostic process.

5.5 CONCLUSION:

In this study, we have described our diagnostic algorithm for asymptomatic or mildly symptomatic hyperCKemia, which enabled us to establish a diagnosis in approximately one third of our patients. The steps presented in the flow chart improved the efficacy of focused massively parallel sequencing.

Table 1. Individual Patient data

N	Sex	age (years)	age of onset (years)	sign or symptoms	CK range	hereditary	EMG/NCS	Biopsy	DIAGNOSIS
1	F	16	5	hyperCKemia	>10x		normal	nonspecific alteration	
2	F	17	15	hyperCKemia	>10x		normal	normal	pathogenic variants in <i>ANO5</i>
3	F	60	20	myalgia, second wind	>10x		myopathic changes		McArdle disease
4	M	25	22	exercise intolerance	>10x		myopathic changes	moderate muscle damage with dysferlin reduction	
5	M	47	40	cramps, myalgia	<5x		normal	nonspecific alteration	
6	M	45	23	cramps	5x<X<10x		normal	nonspecific alteration	
7	F	44	41	myalgia	>10x		myopathic changes		McArdle disease
8	M	54	54	muscle weakness	5x<X<10x		myopathic changes	muscle degeneration	
9	M	17	11	myalgia	<5x		myopathic changes		pathogenic variant in <i>RYR1</i>
10	M	36	36	hyperCKemia	5x<X<10x		normal		
11	M	60	54	myalgia	5x<X<10x		myopathic changes	muscle degeneration	
12	F	56	45	muscle weakness	5x<X<10x		normal		Pompe disease
13	M	59	51	exercise intolerance	>10x	AR	myopathic changes	mild myopathic features	
14	M	21	21	hyperCKemia	5x<X<10x	AD	myopathic changes		
15	M	71	48	hyperCKemia	5x<X<10x		normal		
16	M	53	50	cramps and myalgia	<5x		normal	nonspecific alteration	
17	M	49	40	hyperCKemia	5x<X<10x		normal		
18	M	60	60	hyperCKemia	<5x	AD	normal		
19	M	38	34	cramps, myalgia, myoglobinuria	5x<X<10x		normal	nonspecific alteration	pathogenic variants in <i>ANO5</i>

20	M	44	40	cramps, myalgia	5x<X<10x	myopathic changes	nonspecific alteration	
21	M	32	16	myalgia	<5x	normal		
22	M	26	16	hyperCKemia	5x<X<10x	normal		
23	F	23	21	hyperCKemia	5x<X<10x	normal	normal	
24	M	20	13	hyperCKemia	>10x	myopathic changes	myopathic features	pathogenic variant in <i>DMD</i>
25	M	25	12	myalgia	>10x	normal	nonspecific alteration	
26	M	25	11	hyperCKemia	<5x	normal	normal	
27	M	21	18	cramps, myalgia, myoglobinuria	<5x	normal	nonspecific alteration	
28	M	51	48	cramps and myalgia	5x<X<10x	myopathic changes	nonspecific alteration	
29	F	43	30	rippling	<5x	myopathic changes		rippling muscle disease due to <i>CAV3</i>
30	F	53	46	muscle weakness	>10x	myopathic changes	muscle damage and reduction of dysferlin expression	
31	M	54	40	hyperCKemia	<5x	normal		Myotonic dystrophy type II
32	M	27	16	hyperCKemia	<5x	normal	myofiber hypertrophy	pathogenic variant in <i>RYR</i>
33	M	18	13	hyperCKemia	5x<X<10x	myopathic changes		
34	F	21	10	hyperCKemia	<5x	normal	normal	
35	F	55	51	hyperCKemia	<5x	myopathic changes	moderate muscle damage	
36	M	24	16	hyperCKemia	>10x	normal		
37	M	22	18	myalgia	5x<X<10x	normal	nonspecific alteration	
38	M	36	14	myalgia	5x<X<10x	myopathic changes	myopathic features	
39	M	68	4	myalgia	>10x	normal		CPT II deficiency
40	F	34	24	muscle weakness	>10x	normal	normal	
41	M	43	32	myalgia	5x<X<10x	normal	normal	pathogenic variants in <i>ANO5</i>
42	M	30	30	myalgia	<5x	normal		
43	M	54	10	myalgia	>10x	myopathic changes		

44	F	34	13	myalgia	5x<X<10x	myopathic changes	vacuoles	
45	F	64	59	myalgia	<5x	myopathic changes		
46	M	31	8	myalgia	5x<X<10x	normal	normal	
47	F	17	15	myoglobinuria, second wind	>10x	myopathic changes		McArdle disease
48	M	28	21	hyperCKemia	>10x	nonspecific alteration	myopathic features	VLCAD deficiency
49	F	56	54	exercise intolerance	5x<X<10x	nonspecific alteration	normal	pathogenic variants in <i>ANO5</i>
50	F	27	27	myalgia	5x<X<10x	myopathic changes		
51	M	67	45	myalgia	<5x	normal	normal	
52	M	24	8	hyperCKemia	>10x	normal	normal	
53	F	69	60	muscle weakness	5x<X<10x	myopathic changes		DMD pathogenic variant female carrier
54	F	19	11	myoglobinuria	>10x	normal	myopathic features	
55	M	56	48	myoglobinuria	5x<X<10x	normal		pathogenic variants in <i>ANO5</i>
56	F	69	58	muscle weakness	<5x	normal		
57	M	17	11	hyperCKemia	<5x	normal		
58	M	60	57	cramps	5x<X<10x	normal	myopathic features	
59	M	58	40	muscle weakness	<5x	myopathic changes		
60	M	20	20	exercise intolerance	>10x	normal		
61	F	18	11	exercise intolerance	<5x	normal		
62	M	54	45	muscle weakness	>10x	myopathic changes		pathogenic variant in <i>DMD</i>

63	M	44	15	cramps, myalgia, myoglobinuria	<5x		normal	nonspecific alteration	
64	F	48	46	hyperCKemia	5x<X<10x		normal		pathogenic variants in <i>ANO5</i>
65	M	21	8	hyperCKemia	5x<X<10x		normal	nonspecific alteration	Pompe disease
66	M	18	18	myalgia	<5x		nonspecific alteration		
67	M	18	13	hyperCKemia	<5x	AD	normal	normal	
68	M	16	11	hyperCKemia	<5x		normal	normal	
69	F	41	18	myoglobinuria	>10x		nonspecific alteration	normal	VLCAD deficiency
70	M	57	52	hyperCKemia	>10x		normal		pathogenic variant in <i>DMD</i>
71	M	29	14	hyperCKemia	<5x		myopathic changes	mild myopathic features	
72	M	41	30	myalgia	<5x		normal		
73	F	47	10	myalgia	>10x		myopathic changes		pathogenic variants in <i>ANO5</i>
74	F	47	40	exercise intolerance	5x<X<10x		normal	moderate muscle damage	
75	F	22	20	muscle weakness	5x<X<10x		normal	nonspecific alteration	LGMDR12
76	F	49	45	myalgia	5x<X<10x		normal		
77	F	21	14	hyperCKemia	5x<X<10x		normal	myopathic features	
78	M	55	49	hyperCKemia	<5x		normal		
79	M	40	33	hyperCKemia	5x<X<10x		myopathic changes	muscle degeneration, reduction of dysferlin expression	pathogenic variant in <i>RYR1</i>
80	M	26	20	cramps, myalgia, myoglobinuria	<5x	AD	normal	nonspecific alteration	
81	M	17	11	myalgia	<5x		normal	myopathic features	

82	F	18	8	hyperCKemia	<5x	normal
83	M	56	56	cramps	<5x	axonal neuropathy

N	DIAGNOSIS	Pathogenic variants	Likely pathogenic variants	Variants of uncertain significance
1				
2	pathogenic variants in <i>ANO5</i>	<i>ANO5</i> (c.172C>T; p.Arg58Trp) <i>ANO5</i> (c.191dupA;Asn64Lysfs*15)		
3	McArdle disease	<i>PYGM</i> (c.2262delA; p.Lys754Asnfs*49) <i>PYGM</i> (c.2262delA; p.Lys754Asnfs*49)		
4				<i>LDHA</i> (c.681G>C; p.Val227=) <i>FKRP</i> (c.235G>A ; p.Val79Met)
5				<i>GAA</i> (c.2092G>A; p.Ala698Thr)
6				
7	McArdle disease	<i>PYGM</i> (c.148 C>T ; p.Arg50*) <i>PYGM</i> (c.148 C>T ; p.Arg50*)		
8				
9	pathogenic variant in <i>RYR1</i>	<i>RYR1</i> (c.5036 G>A p.Arg1679His)		
10				<i>RYR1</i> (c.10619A>T; p.Tyr3540Phe)
11				
12	Pompe disease	<i>GAA</i> (c.-32-13T>G;IVS1) <i>GAA</i> (c.1064T>C; p.Leu355Pro)		

13			
14			
15			<i>RYR1</i> (c.7025A>G; p.Asn2342Ser)
16		<i>ACADLV</i> (c.1700G>A;p.Arg567Gln)	
17			<i>ANO5</i> (c.155A>G; p.Asn52Ser)
18			<i>CAPN3</i> (c.1478G>A; p.Arg493Gln)
19	pathogenic variants in <i>ANO5</i>	<i>ANO5</i> (c.41-1G>C) <i>ANO5</i> (c.2141C>G; p.Thr714Ser)	<i>GAA</i> (c.1123C>T; p.Arg375Cys)
20			<i>LPIN1</i> (c.1744G>A;p.Gly582Arg) <i>RYR1</i> (c.11590+7C>T)
21			<i>LPIN1</i> (c.1526C>T; p.Ala424Val)
22		<i>FKTN</i> (c.42delG; p.Thr14*)	
23			<i>AGL</i> (c.980G>A; p.Arg327His)
24	pathogenic variant in <i>DMD</i>	<i>DMD</i> (ex24del)	
25			
26			<i>RYR1</i> (c.6384C>T; p.Tyr2128=)
27			
28			
29	rippling muscle disease due to <i>CAV3</i>	<i>CAV3</i> (c.233C>A; p.Thr78Lys)	
30		<i>CAPN3</i> (c.C1324T; p.Arg442Asp)	
31	Myotonic dystrophy type II	<i>CNBP</i> (CCTG expansion)	
32	pathogenic variant in <i>RYR1</i>	<i>RYR1</i> (c.7042_7044delGAA; p.Glu2348del)	
33			
34		<i>CAPN3</i> (c.964T>C; p.Tyr322His)	
35			<i>CPT2</i> (c.1436A>T; p.Tyr479Phe)
36		<i>CAPN3</i> (c.802-9G>A)	
37			
38			

39	CPT II deficiency	<i>CPT2</i> (c.149C>A; p.Pro50Hys) <i>CPT2</i> (c.149C>A; p.Pro50Hys)	
40			
41	pathogenic variants in <i>ANO5</i>	<i>ANO5</i> (c.1733T>C; p.Phe578Ser) <i>ANO5</i> (c.191dupA;Asn64Lysfs*15)	
42			
43			<i>FUKTN</i> (c.166-4A>G) <i>FUKTN</i> (c.877G>C;p.Val293Leu)
44		<i>ENO3</i> (c.467G>A; p.Gly156Asp) <i>ANO5</i> (c.428A>G; p.Tyr143Cys)	
45			<i>RYRI</i> (c.6444C>T;Ser2148=)
46		<i>CAPN3</i> (c.664G>A;p.Gly222Arg)	
47	McArdle disease	<i>PYGM</i> (c.558delC;p.Tyr186*) <i>PYGM</i> (c.558delC;p.Tyr186*)	
48	VLCAD deficiency	<i>ACADVL</i> (c.1259T>C; p.Ile420Thr)	<i>ACADVL</i> (c.896_898delAGA;p.Lys299del)
49	pathogenic variants in <i>ANO5</i>	<i>ANO5</i> (c.142_143insAA;p.ala48Glufs*9) <i>ANO5</i> (c.2060T>A;p.val687Glu)	
50			
51			<i>MURC</i> (c.425C>T;p.Pro142Leu)
52			
53	<i>DMD</i> pathogenic variant female carrier	<i>DMD</i> (ex8-43 del)	
54		<i>CPT2</i> (c.338C>T; p.Ser113Leu) <i>ANO5</i> (c.1640G>A; p.Arg547Gln)	
55	pathogenic variants in <i>ANO5</i>	<i>ANO5</i> (c.220C>T; p.Arg74*) <i>ANO5</i> (c.2141C>G; p.Thr714Ser)	
56			
57			
58			

59		<i>CAPN3</i> (c.1706T>C; p.Phe569Ser)	
60			<i>CAV3</i> (c.216C>G; p.Cys72Trp) <i>RYR1</i> (c.418G>A; p.Ala140Thr)
61			
62	pathogenic variant in <i>DMD</i>	<i>DMD</i> (ex45-47 del)	
63			<i>RYR1</i> (c.1882C>T; p.Arg628Cys)
64	pathogenic variants in <i>ANO5</i>	<i>ANO5</i> (c.2272C>T; p.Arg758Cys) <i>ANO5</i> (c.2498T>A; p.Met833Lys)	
65	Pompe disease	<i>GAA</i> (c.-32-13T>G;IVS1) <i>GAA</i> (c.1082C>G;p.Pro361Arg)	
66			<i>PGAM2</i> (c.596-7G>A)
67			<i>LPIN1</i> (c.1049C>A; p.Thr350Asn)
68			
69	VLCAD deficiency	<i>ACADVL</i> (c.728T>A;p.Leu243His) <i>ACADVL</i> (c.1097G>A; p.Arg366His)	
70	pathogenic variant in <i>DMD</i>	<i>DMD</i> (ex14-23 del)	
71			<i>RYR1</i> (c.2697C>A; p.Asn899Lys)
72			
73	pathogenic variants in <i>ANO5</i>	<i>ANO5</i> (c.762+5 G>A) <i>ANO5</i> (c.2521-1delG)	
74		<i>ANO5</i> (c.2235+1G>A)	<i>AGL</i> (c.1028G>A; p.Arg343Gln) <i>RYR1</i> (c.7025A>G; p.Asn2342Ser)
75	LGMDR12	<i>ANO5</i> (c.191dupA; p.Asn64fs) <i>ANO5</i> (c.817C>T; p.Leu273Phe)	
76		<i>GAA</i> (c.2105G>A; p.Arg702His)	
77			
78		<i>CAPN3</i> (c.133G>A; p.Ala45Thr)	<i>PFKM</i> (c.2300G>A; p.Arg767His)

			<i>CPT2</i> (c.236A>C; p.Lys79Thr)
79	pathogenic variant in <i>RYR1</i>	<i>RYR1</i> (c.7304 G>T; p.Arg2435Leu)	
80			<i>GAA</i> (c.2845G>A;p.Val949Ile)
81			<i>RYR1</i> (c.12827G>A;p.Gly4281Glu) <i>RYR1</i> (c.12864G>C;p.Ale4293=)
82			<i>CPT2</i> (c.1511C>T; p.Pro504Leu) <i>FKRP</i> (c.1136G>A; R379Q)
83			

Reference sequence: ACADLV-NM_000018.3, AGL-NM_000642.2, ANO5-NM_213599 , CAPN3-NM_000070, CAV3-NM_001234, CPT2-NM_000098, ENO3-NM_001996 , FKRP-NM_024301, FKTN-NM_006731,GAA-NM_000152, LAMP2-NM_013995, LDHA-NM_005566, LMNA-NM_170707, LPIN1-NM_001261428, PFKM-NM_000289, PGAM2-NM_000290 , PGK1-NM_000291, PGM1-NM_002633, PYGM-NM_005609, RYR1-NM_000540. (Assembly GRCh37/hg19).

Autosomal dominant (AD), autosomal recessive (AR), creatine kinase (CK), electromyography (EMG), nerve conduction studies (NCS), Very long-chain acyl-CoA dehydrogenase (VLCAD), Limb-girdle muscular dystrophy (LGMD), Very long-chain acyl-CoA dehydrogenase (VLCAD).

6 GENETIC WORKUP FOR CHARCOT–MARIE–TOOTH NEUROPATHY: A RETRO-SPECTIVE SINGLE-SITE EXPERIENCE COVERING 15 YEARS.

6.1 INTRODUCTION

Charcot–Marie–Tooth disease (CMT) is the most common inherited neuromuscular disorder, with a prevalence ranging from 9.7/100,000 in Serbia to 82.3/100,000 in Norway ⁶⁹.

CMT comprises a group of inherited motor and sensory neuropathies that are phenotypically and genetically heterogeneous, with more than 100 different disease-associated genes identified ⁷⁰.

Electrophysiological and neuropathological findings differentiate CMT forms into the demyelinating type, with motor nerve conduction velocities (mNCV) of <38 m/s from the ulnar or median nerve, and the axonal type with an mNCV of >38 m/s ⁷¹. This classification, somehow “didactic”, still helps to address genetic investigations or the interpretation of molecular results.

Genetic diagnosis in CMT has evolved rapidly in recent years with the introduction of next-generation sequencing (NGS) into routine diagnostic practice ⁷². Since the frequencies of gene pathogenic variants may vary considerably between different populations, data on patient cohorts from different countries are useful for improving the diagnostic molecular algorithms ⁷³.

Here we describe the clinical features as well as the distribution of genetic variants in patients evaluated at our neuromuscular center. Data presented here provide an overview on the frequencies of genetic subtypes of CMT patients in a neuromuscular center from northern Italy. Moreover, we describe a peculiar phenotype with the lower limbs predominantly involved.

6.2 MATERIALS AND METHODS

All patients evaluated in the neuromuscular center at the University of Genova between 2004 and 2020 were enrolled in this study. We selected patients affected by CMT based on:

a) The presence of a clinical motor-sensory neuropathy with or without positive family history;

b) A neurological and neurophysiological examination demonstrating peripheral neuropathy; and
c) The exclusion of primary acquired causes, such as inflammatory, toxic, metabolic and infectious neuropathies. Patients carrying *TTR* pathogenic variants were also excluded.

Pure motor or sensitive neuropathies were included as distal hereditary neuropathies.

Patients were evaluated in an outpatient setting with a multidisciplinary evaluation from a team including a neurologist, neurophysiologist, medical geneticist and physical medicine and rehabilitation (PM&R) physician. Our integrated approach takes into account the complexity of CMT for which a multidisciplinary approach improves long-term care ⁷⁴.

All patients, in the same day, were evaluated with an electrodiagnostic test in order to confirm the suspicion of hereditary neuropathy and categorize it. Patients were classified as CMT1 (demyelinating form) with a median mNCV below 38 m/s and CMT2 (axonal form) with a median mNCV above 38 m/s. Occasionally, clinical, electrophysiological and pathological features could not fit into this classification so a third group of CMT called intermediate CMT was identified ^{71,75}. This group presented a combination of axonal and demyelinating changes reflected in electrophysiological studies with a median mNCV different from CMT1 (usually <25 m/s) and CMT2 (usually >45 m/s). This phenotype was described for different genes with X-linked transmission, such as males with *GJB1* pathogenic variants or autosomal dominant or recessive transmission. This is a complex characterization that requires a specific electrophysiological protocol ⁷¹ that was not conducted every time. In order to avoid error, we prefer to simplify the classification using only axonal and demyelinating forms. Nerve conduction studies were also performed as a follow-up screening and to compute the CMT neuropathy score (CMTNS version 1 or 2) ^{76,77}. The CMTNS and CMT examination scores (CMTES version 1 or 2) were used to categorize cases into mild (CMTNS 0 to 10 or CMTES 0 to 7), moderate (CMTNS 11 to 20 or CMTES 8 to 16), and severe (CMTNS 21 to 36 or CMTES 17 to 28) ^{76,77}.

A neurologist and medical geneticist evaluated family history, clinical and neurophysiological features and planned the diagnostic flow chart recommended for the patients. The medical geneticist helped the patients understand the clinical, ethic, technical and familial implications

involved with the genetic tests. The neurologist offered the management of supportive therapies⁷⁸. Moreover, every two months, complex cases were discussed in a multidisciplinary team to decide the genetic workout. The PM&R physician's assessment was performed with the help of the orthopedic technician and physiotherapist in order to customize physical therapies, orthotics and prosthetics, and recommendations regarding exercise.

All the patients signed informed consent waivers in accordance with national laws and guidelines for genetic testing that are used in current clinical practice. This consent provides for the anonymous use of data for research and publication. Molecular analyses were performed at the Laboratory of Medical Genetics Unit, University of Genoa where genomic DNA was extracted from peripheral blood according to standard protocols.

Over the course of 15 years, different labs' approaches and technologies have been used to achieve the molecular definition.

The presence of the 17p11.2 duplication or deletion was excluded by multiplex ligation-dependent probe amplification (MLPA) and quantitative real-time PCR (qPCR).

Initially, subsequent genetic tests were performed using a gene-by-gene approach based on clinical and electrophysiological features suggesting specific genetic defects. Most of these analyses were performed through conventional Sanger sequencing. More recently, the gene-by-gene approach has been progressively replaced by next-generation sequencing analysis.

Direct sequencing was achieved on an ABI PRISM 3130XL Genetic Analyzer (Applied Biosystems, Thermo Fisher Scientific, Waltham, MA USA). Alignment on reference sequences and analysis was performed using the SeqScape software (Thermo Fisher Scientific).

For next-generation sequencing (NGS) studies, a 56 CMT custom AmpliSeq gene panel (Thermo Fisher Scientific) (full list available on request) was run on an Ion S5 GeneStudio (Thermo Fisher Scientific) sequencer and Ion Reporter (Thermo Fisher Scientific), and the ANNOVAR⁷⁹ software was used for data analysis.

6.3 RESULTS

In total, 585 patients (447 index cases; 99 familial and 348 isolated cases) were evaluated in our neuromuscular center since 2004, and received a diagnosis of CMT, according to clinical and neurophysiological features. The overall mean age of our patients was 53 years (± 16) and the median age was 53 years, with an age range of 13–94 years. The disease was nearly equally distributed between males and females (47% female, 53% male). Neurophysiology was consistent with a demyelinating phenotype in 290 patients (64.9%), and axonal phenotype in 157 patients (35.1%).

6.3.1 Genetically confirmed patients

Among the 585 patients, a genetic diagnosis was achieved in 391 patients (277 index cases; 79 familial and 198 isolated cases). The statistical analysis was based on index cases. Most patients were affected by a demyelinating neuropathy (86%), whereas axonal forms accounted for 14% of genetically identified cases. As already described in the literature, demyelinating cases achieved a positive genetic diagnosis more frequently than axonal and intermediate ones⁶⁹.

In familial cases, autosomal dominant inheritance was the most frequent pattern of inheritance, accounting for 82% of cases. X-linked inheritance was present in 14% of cases, and only a small percentage (4%) was characterized by a recessive inheritance.

The most common genetic diagnoses were CMT1A caused by *PMP22* duplication accounting for one half of all patients (48%); HNPP caused by *PMP22* deletion (14%); CMT1X caused by pathogenic variants in *GJB1* (13%); P0-related neuropathies caused by *MPZ* pathogenic variants (7%); and CMT2A due to *MFN2* pathogenic variants (5%). All together, these accounted for the 87% of all molecular defined neuropathies. Pathogenic variants in rarely mutated genes (*SH3TC2*, *LITAF*, *RAB7A*, *NEFL*, *AARS*, *MTMR2*, *NDRG1*, *PRPS1*, *INF2*, *PMP2*, *DNM2*, *FBLN5*, *HINT1*, *IGHMBP2*, *PMP22*) each accounted for less than 1% of the total, except for *HSPB1* pathogenic variants that were found in 3% of all index cases and *GDAP1* which accounted for 2% of all index cases. Figure 4 describes the genetic distribution of our cohort.

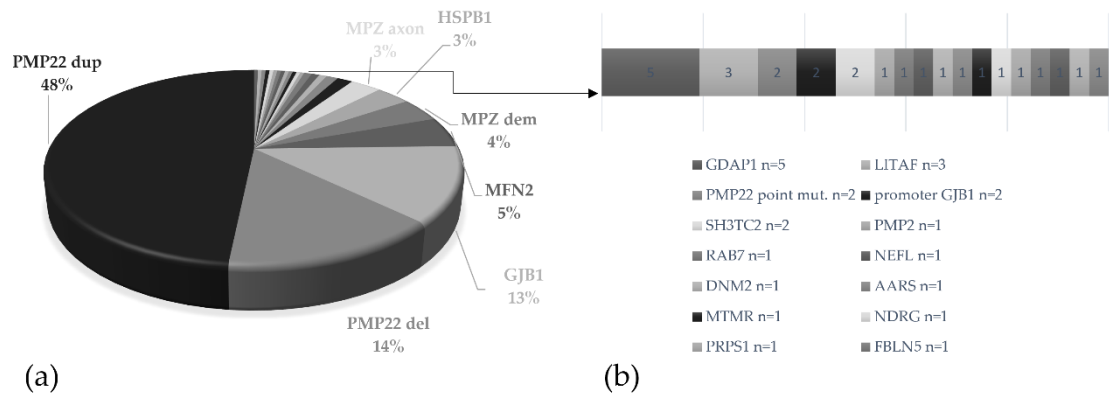


Fig. 4 Genetic spectrum of 277 cases with pathogenic variants. (a) The following genes are indicated: *PMP22* dup (n=134); *PMP22* del (n=40); *GJB1* (n=35); *MFN2* (n=14); *MPZ* causing demyelinating neuropathy (n=11); *MPZ* causing axonal neuropathy (n=8); *HSPB1* (n=9). (b) Other less frequent genes.

NGS genetic analysis was performed in a total of 44 patients and in 10 of them, we achieved a diagnosis (two *GJB1*, one *AARS*, one *PRPS1*, one *NDRG1*, one *DNM2*, one *LITAF*, one *MFN2*, one *HINT1* and one *FBLN5*). For seven of them, a family history of neuropathy was known.

The remaining 34 patients did not receive a genetic diagnosis despite NGS analysis.

6.3.2 Patients without genetic confirmation

For 194 patients, it was not possible to achieve a genetic diagnosis (33% of the whole population). Of these, index cases were 170 (20 familial and 150 isolated cases) and they more frequently presented with an axonal neuropathy (69%).

Since our database includes patients evaluated during a wide time span, different genetic approaches have followed one another. This implies that most of the undiagnosed cases had been studied with a gene-by-gene approach with a mean of 4 genes studied for each patient (minimum one, and maximum nine) on the basis of diagnostic algorithms. In 78 patients, three or fewer genes were analyzed. The most studied genes, after *PMP22* duplication or deletion, were *MPZ*, *HSPB1*, *GDAP1* and *MFN2*. *MPZ* was analyzed in 68.6% of patients, followed by *HSPB1* (60.4%), *GDAP1* (55.2%) and *MFN2* (46.3%). An NGS analysis was performed in only 34 patients.

6.3.3 Genotype–phenotype correlation: a new CMT phenotype

Our population displayed a relatively high frequency of pathogenic variants in *MPZ* and *HSPB1* genes. We identified fifteen patients (nine index cases and six relatives) affected by axonal CMT associated with the *MPZ* pathogenic variant CMT2I/2J) and nine patients (all isolated cases) affected by CMT (CMT2F n=three patients) or distal hereditary motor neuropathies (dHMN) (n=six patients). A clearly length-dependent phenotype with exclusive involvement of the lower limbs in the earlier stage was found in 60% of cases with a pathogenic variant in *HSPB1* (five out of nine patients) and 80% of patients with the late onset *MPZ* pathogenic variant (CMT2) (12 out of 15 patients). A complete electrophysiological study was not available for all patients. From evaluating the electrophysiological studies of patients with a clinical length-dependent phenotype, we confirmed a neuropathy confined in the lower limbs in almost 50% of *MPZ* and *HSPB1* pathogenic variants (five out of nine patients and two out of three patients, respectively) despite a long history of illness (the median number of years between the onset of neuropathy and the first evaluation with an electrophysiological study was 14; the minimum number was 7 and the maximum was 19), identifying a phenotype that was maintained over time. The age of onset was 43 ± 14 (minimum 14, maximum 64) for *MPZ* and 40 ± 20 for *HSPB1* patients (minimum 10, maximum 65). If we excluded patients with onset before the age of 40, the percentage of patients with length-dependent phenotypes rise to 83% in *HSPB1* patients and remained elevated (73%) for *MPZ*. This result could be associated with the lower illness duration in patients with adult onset at the time of the first evaluation in our center. Summarizing this exclusive involvement of the lower limbs was the first sign of the disease and was maintained over time as demonstrated by the long history of illness in our patients. A later progression with the extension of neuropathy in the upper limbs was nevertheless present.

None of the patients showed a severe neuropathy. Patients with the *MPZ* pathogenic variant presented a mild phenotype in 78% (11 out 14 patients), with a mean of 14.2 years of illness duration. The percentage decreased to 66% (six out nine patients) in patients affected by the *HSPB1* pathogenic variant with a mean 16 years of illness.

Clinical and electrophysiological data of *HSPB1* and *MPZ* patients are listed in Table 2.

Based on our findings, we were able to highlight a neuropathy phenotype that differs from the classical ones, whose features can be summarized as follows: 1) the onset of the disease during adulthood (fourth decade on average); 2) the early exclusive or prevalent involvement of the lower limbs; and 3) the mild to moderate severity of the disease. Although all CMT neuropathies cause length-dependent damage, the upper limbs are frequently clinically involved⁸⁰ in CMT1A⁸¹ or CMT1X⁸² and predominantly in some forms such as neuropathies caused by *GARS* and *BSCL2* pathogenic variants⁸³. Nerve conduction studies confirm polyneuropathy in all four limbs.

6.4 DISCUSSION

Genetic testing for CMT involved the sequencing of individual genes addressed by the mode of inheritance, clinical and electrophysiological phenotype and data about the prevalence of different genetic subtypes, as well as peculiar genotype–phenotype associations. This approach has been transformed by the advent of NGS, where several disease-associated genes are tested in parallel. Nevertheless, the diagnostic rate of massive parallel sequencing tests described in the literature ranges from 4.6% to 93%, according to the analyzed cohort^{84–104}. In routine clinical practice, the NGS approach, more realistically, allows us to reach a genetic diagnosis in 30% of genetically undetermined patients when *PMP22* duplication has been previously ruled out¹⁰⁰.

Our study evaluated the frequency of the genetic subtype of CMT patients in a population from a specialized clinical diagnostic setting in northern Italy. In our cohort, 66% of patients obtained a genetic diagnosis (including 17p11.2 duplication), a diagnostic rate that is comparable with previously described epidemiological studies. In 4% (10 out of 277 index cases), the diagnosis was achieved with an NGS approach. The phenotype distribution showed that 86% of diagnosed patients had a demyelinating neuropathy, whereas axonal CMT remained largely undiagnosed. These data confirm that copy number variations in *PMP22* or pathogenic variants in three genes (*GJB1*, *MPZ* and *MFN2*) were responsible for about 90% of genetically determined neuropathies. This genetic prevalence was similar to the prevalence in Europe and North America^{73,105–110},

whereas it differed from those found in Spain and southern Italy where *GDAP1* pathogenic variants were more frequent, due to the founder effect ^{111,112}. *SH3TC2* was described as a frequently mutated gene in different papers ^{85,91,103,112}, although it represented less than 1% of our cohort because of the adult age of the patients. The remaining genetically diagnosed cases include pathogenic variants in less common genes.

Interestingly, in our case series, *HSPB1* pathogenic variants were found in 3% of genetically determined neuropathies. A similar prevalence was described in a large cohort of Sicily ¹¹³ and Spain ¹¹² and even greater (4.6%) in Japan ⁹⁷, thus suggesting that the higher prevalence of these pathogenic variants could be more likely attributed to their epidemiological distribution rather than being caused by a specific bias, such as the adult population assessed in our study. *HSPB1* was described as the most common cause of dHMN ^{99,114}, but in our population, it also accounts for the 9% of the axonal motor-sensory neuropathies (3 of 33 axonal sensory-motor neuropathy). *MPZ* pathogenic variants were associated with an axonal phenotype in 42% of patients (8 out of 19 patients). Among patients carrying these variants, almost all (86%; 13 out of 15 patients) presented with adult onset. Frequently, *MPZ* variants associated with adult onset presented electrophysiological findings classified as CMT2, with intermediate or normal mNCV ^{115,116}. From the clinical data available in our cohort, we were able to establish an association between pathogenic variants in *MPZ* or *HSPB1* and a peculiar phenotype, characterized by clinical onset after the third decade, initial exclusive or highly prevalent lower limb involvement, and mild to moderate severity. This phenotype, which is strictly length dependent, is common in patients carrying *HSPB1* and late-onset *MPZ* pathogenic variants. Houlden et al. ¹¹⁷ described the predominant motor involvement in the lower limbs in *HSPB1* pathogenic variants, whereas a similar involvement, predominantly in the lower limbs, was noticed in *MPZ* pathogenic variants with adult onset by Sanmaneechai et al. ¹¹⁵.

The description of a distinct genotype–phenotype association may seem anachronistic in the era of massive parallel genetic testing through NGS. However, NGS requires time and expertise for data analysis and interpretation, although in cases with definite phenotypes, a gene-by-gene

approach might still be effective. Moreover, also in a NGS context, detailed phenotypic information can be used to guide the interpretation of molecular results ¹¹⁸. Finally, NGS panels can explore only a very limited part of the coding genomic DNA, which might represent a significant part of the missing heritability in neurologic diseases as well as CMT ¹⁰⁰. It is also important to note that a significant part of the genome is extremely resistant to the single-nucleotide variant (SNV)/small indel calling due to a repetitive sequence, causing poor variant detection in some clinically relevant genes ¹¹⁹. The contribution of these types of variants in the pathogenesis of neurological diseases is increasingly recognized, as in the case of the identification of the *RFC1* pentanucleotide repeat associated with cerebellar ataxia with neuropathy and vestibular areflexia syndrome (CANVAS) ¹²⁰ and idiopathic sensory neuropathy ¹²¹. Similarly, pathogenic variants in the *SORD* gene were recently identified as the most common recessive inherited neuropathy ¹⁰⁰. *SORD* was not described previously as a gene involved in hereditary neuropathies due to the inability of NGS analysis to call variants because of the presence of the *SORD2P* pseudogene. These findings underline the possibility that many novel genes involved in neuromuscular diseases remain to be identified. In general, genetic advances in DNA sequencing technologies have led to a continuous increase in genes related to neuromuscular diseases, and, in clinical practice, gene panels must be periodically updated. For this reason, we believe that whole-exome sequencing, followed by filtering for defined genes, could be a valid method ¹¹.

Therefore, in our experience, the diagnosis strategy should be flexible and tuned to the clinical features of the patient, in order to select the best molecular approach for each patient. Our study confirms that the collaboration of a multidisciplinary team provides better outcomes for patients

¹²².

Table 2

	CMT DIA GNO SIS	MUTATION	FAMILY	SEX	AGE OF ONSET	AGE OF FIRST EVALUATION	years from first symptom to first evaluation	clinic length dependent phenotype	SNAP velocity (m/s)	amplitude (mV)	cMAP velocity (m/s)	amplitude (mV)	CMTNS	CMTES	electrophysiological length dependent phenotype
1	CMT 2I	MPZ Ser70Pro	1	F	55	69	14	y	u normal	u normal	u normal	u normal	10/36	10	Y
2	CMT 2I	MPZ p.Ser70Pro	1	M	50	66	16	y	u normal		u normal	u normal	12/32	12	Y
3	CMT 2I	MPZ p.Ser51Phe	2	F	48	55	7	y	u 51	u 13	u 50 p 27.1	u 3 p 0.1	9/36	7	N
4	CMT 2J	MPZ p.Thr124Met		M	43	54	11	n					18/36	13	
5	CMT 2J	MPZ p.Thr124Met	3	F	14	48	34	y	u 45.4 m 37 s 52	u 4.4 m 17 s 12	u 57.9 m 48 p 36.7	u 9.3 m 4.1 p 4.8	9/36	7	N
6	CMT 2J	MPZ p.Thr124Met	3	M	40	49	9	y	u 46 s 47	u 9 s 37	u 46.6 p 37.5	u 8.8 p 2.6	2/36	2	Y
7	CMT 2I	MPZ p.Ser70Pro		M	50	71	21	n	u 37	u 3.7	u 43 m 49.1	u 4.5 m 9.4	9/36	6	
8	CMT 2I	MPZ p.Ser44Phe		M	30	44	14	y	u 55 r 54 s 54.6	u 16 r 24 s 2.5	u 50.5 m 49.1 p 32.9	u 11.1 m 9.4 p 1.5	5/36	5	Y
9	CMT 2J	MPZ p.Thr124Met	4	M	50	66	16	y	s 54.6 m 50	s 2.5 m 10	u 50 p 36.4	u 11.7 p 0.4	10/36	10	Y
10	CMT 2J	MPZ p.Thr124Met	4	M	64	64	0	y					5/28	5	X
11	CMT 2I	MPZ p.Thr124Ala		M	48	51	3	Y	r 50	r 1.5	p 25	p 0.2	8/36	4	N

										u 49	u 5.8				
12	CMT 2J	MPZ p.Thr124Met		M	35	49	14	y	u 52	u 5.4	u 53 p NE	u 15 p NE	19/36	14	N
13	CMT 2J	MPZ p.Thr124Met	4	F	46	53	7	Y					2/28	2	X
14	CMT 2J	MPZ p.Thr124Met	4	F	15	60	45	Y	s 43	s 22	p 37	p 4.2	7/36	7	X
15	CMT 2I	MPZ p.Ser51Phe	2	M	63	66	3	n	u 48	u 4.8	u 50 p 31	u 6 p 0.9	10/36	8	
	CMT DIA GNO SIS	MUTATION	FAMILY	SEX	AGE OF ONSET	AGE OF FIRST EVALUATION	years from first symptom to first evaluation	clinic length dependent phenotype	SAP velocity amplitude		cMAP velocity amplitude		CMTNS	CMTES	electrophysiological length dependent phenotype
1	CMT 2F	HSPB1 p.Arg136Leu		M	50	69	19	y	r normal s 37.5	r normal s 2.1	u normal p NE	u normal p NE	7/36	7	Y
2	dHM N	HSPB1 p.Ser135Phe		M	25	55	30	n					9/28	9	
3	CMT 2F	HSPB1 p.Arg188Trp		M	45	49	4	y	u 60 s 37 m 55	u 8.7 s 5.8 m 14.8	u 61 p 41	u 9.2 p 4.3	6/36	5	N
4	dHM N	HSPB1 p.Thr180Ile		F	12	32	20	n	u 56 s 49.3	u 8.5 s 15	u NE	u NE	16/36	11	
5	dHM N	HSPB1 p.Arg136Leu		M	65	72	7	y	u normal	u normal	u normal	u normal	3/36	3	Y
6	dHM N	HSPB1 p.Arg136Leu		M	60	63	3	y					5/28	5	X
7	CMT 2F	HSPB1 p.Arg136Leu		M	40	64	24	n	u 48 s 34	u 10 s 5	u 39 p NE	u 2 p NE	11/36	9	

8	dHM N	<i>HSPB1</i> p.Gly34Arg	F	54	61	7	y	s 45	s 24	p 45	p 1.4	2/28	2	X
9	dHM N	<i>HSPB1</i> p.Glu41Lys	F	10	40	30	n	u 53 s 49	u 13 s 15	u 54 p 35	u 5.5 p 0.8	5/36	4	

LEGEND:

Charcot-Marie-Tooth (CMT); distal Hereditary Motor Neuropathies (dHMN); sensory nerve action potential (SNAP); Compound muscle action potential (CMAP); compute CMT neuropathy score (CMTNS version 1 or 2); CMTNS and CMT examination score (CMTES version 1 or 2)

m=median nerve; p=peroneal nerve; r=radial nerve; s=sural nerve; u=ulnar nerve. NE=not evocable.

Family: relatives have the same family number

length dependent phenotype: y=yes; n=no

electrophysiological length dependent phenotype: Y=yes; N=no; X= not available

7 DISCUSSION

NGS has deeply changed the diagnostic process of inherited neuromuscular diseases, allowing the analysis of a large number of potential genes. Despite this, in many cases it is still a challenge to achieve a genetic diagnosis in NMD, due to multiple causes that can determine the same clinical manifestations¹³ or different presentations of the same genetic disorder in patients, including incomplete penetrance or heterogeneous phenotypes¹²³.

In the first study, I have described our diagnostic algorithm for asymptomatic or mildly symptomatic hyperCKemia, which enabled us to establish a diagnosis in approximately one third of our patients. The different steps, based on electrodiagnostic data, biochemical screening and first-line genetic investigations improved the efficacy of focused massively parallel sequencing.

In the second study, I have described our diagnostic experiences as a multidisciplinary outpatient clinic, combining a gene-by-gene approach or targeted gene panels based on clinical presentation in patients affected by CMT. The analysis of our cohort of patients allowed us to define a new phenotype that can help in defining diagnosis in CMT. The definition of a specific phenotype could indicate a gene-by-gene approach that might be effective. Moreover, also in an NGS context, detailed phenotypic information can be used to guide the interpretation of molecular results.

In the present PhD thesis, the analysis of the genetic workup of these two different and frequent NMD disorders allow me to generalise the genetic approach to NMD. I evaluated the clinical use of NGS techniques in clinical practice, the reasons of a still considerable number of unsolved cases and the ethical implication of the results.

7.1 USE OF NGS IN CLINICAL PRACTICE

There is an increasing consensus to apply NGS as a first-tier genetic approach to neuromuscular diseases⁵, but based on our experience and the literature data, there are cases in which single gene testing should be considered as a first test. This is the case when pathogenic variants are more

frequently quantitative rather than qualitative (i.e. a preponderance for deletions or duplications) or if the disease of interest is caused by repeat expansions. For example, for demyelinating hereditary neuropathy, it is firstly recommended to exclude PMP22 duplication or deletion with multiple ligation probe analysis (MLPA). The same is true for spinal muscular atrophy (SMA) or in limb girdle dystrophies in which dystrophin (DMD) duplication or deletion should be excluded before further analyses. Moreover, there are disorders associated with genomic repeats, for example trinucleotide repeat expansions (myotonic dystrophy type 1), tetranucleotide repeat expansions (myotonic dystrophy type 2), repeat retractions of the D4Z4 macrosatellite in facioscapulohumeral muscular dystrophy type 1, for which both GPS and WES present technical limitations¹²⁴. In general, traditional tests combined with NGS might increase the diagnostic yield as we demonstrate in patients affected by hyperCKemia¹⁵. In selected cases, a typical phenotype in combination with ethnic or geographic origin could indicate a specific genetic defect which can be searched before applying an extensive genetic approach⁵. The advice of analysing *HSPB1* and *MPZ* in the “length dependent phenotype” of CMT could be an example for this approach that might still be effective¹⁶.

Nonetheless, NGS has been demonstrated to be cheaper and faster compared to a gene-by-gene approach in NMD and is particularly useful for unspecific clinical phenotypes^{27,38,100}, such as we described in asymptomatic or minimally symptomatic hyperCKemia where it can represent a first line diagnostic approach¹⁵. Other lines of investigation such as histopathology, which were the cornerstones of diagnosis in the pre-NGS era, have become second-line approaches or, as is the case of inherited neuropathies, have been almost completely discontinued. In general, since they require highly specific expertise, equipment and tend to be costly, time-consuming and burdensome for patients⁵, they are less prescribed compared to the past.

However, muscle biopsy still plays a central role in the diagnostic approach of muscle disorders. For example, it remains a powerful and informative tool to prioritise and choose candidate variants, once NGS data have been analysed. VUS interpretation may need a muscle biopsy to search for specific molecular features in order to confirm or exclude the genetic results.

Moreover, when a first-line genetic approach does not result in a diagnosis, additional omics techniques might be used, including transcriptome sequencing, which is also known as RNA sequencing, proteomics, glycomics and metabolomics⁵ in order to solve complex cases³⁵.

Conversely, nerve biopsy is not required for most patients with hereditary neuropathies, especially after the advent of next generation sequencing¹²⁵. As a matter of fact, for neuropathies, the large number of candidate gene variants detected by NGS analysis could be evaluated with the genotype–phenotype correlation established or verified from the family history, clinical examination, electrophysiological data and peripheral nerve imaging using high resolution magnetic resonance imaging (MRI) and ultrasound (US). In rare and selected cases, a nerve biopsy can help establish this correlation, and electron microscopy is required to detect the characteristic and sometimes specific lesions induced by the mutated gene¹²⁶. Nerve biopsy presents some risks and complications as persistent numbness (72%-100%), persistent pain (0%-58%), wound infection (5%-20%), delayed wound healing (1%-12%), dysesthesia (11%-60%), paresthesia, hematoma and neuroma¹²⁵. Therefore, considering the risks and the limited diagnostic yield, nerve biopsies are rarely proposed to CMT patients.

7.2 POSSIBLE CAUSES OF LACKS IN MOLECULAR DIAGNOSIS

Despite next generation sequencing having profoundly changed the approach to genetic disorders, there are still patients with a probable NMD, based on the clinical and laboratory data, in whom there is no genetic confirmation. In our experience^{15,16}, NGS present a detection rate of 24% in a hyperCKemia cohort and 22% in a CMT cohort. These results, which are slightly below the average of similar papers, are probably due to the size of our target hyperCKemia panel, comprising 20 genes, and the small number (44) of CMT patients who were extensively analysed previously with a gene-by-gene approach for the most frequent gene associated with CMT. In general, the reason for cases not being molecularly defined can be summarised as the technical and interpretation limits of NGS sequencing analysis and the presence of non-mendelian inheritance.

7.2.1 Technical and interpretation limits of NGS sequencing:

NGS presents uneven coverage and structural variations, including copy number variation, repeat expansion and contraction, are difficult or impossible to detect with this technique⁵. CNVs are important contributors to a pathogenic variant burden of hereditary disorders and should be routinely assessed¹²⁷, and are estimated to explain approximately 10% of all inherited disorders¹²⁷. GPS is limited by the restricted number of genes that are possible to analyse. WES is not able to identify CNVs, expansions or contractions in repetitive regions, chromosomal rearrangements and deep intronic variants², while WGS is limited by the difficulties to interpret the huge amount of data obtained. The data generated are directly proportional to the extension of the analysed genomic regions and its interpretation is the limit and challenges of NGS analysis.

NGS generated data that are converted in short sequences of nucleotides. Reads are aligned to the reference genome and genetic variants are called, filtered and then subjected to interpretation². Variants are so subdivided in pathogenic, benign or of uncertain significance (VUS), based on previous literature data. Most VUSs are variants that have not been previously reported or have been reported less frequently but without established pathogenicity studies. VUS may be identified in genes in which the function is known or unknown and which may or may not be related to the original rationale for sequencing the patient. Often, new VUS remains VUS because the difficulty to demonstrate its pathogenicity in post transcriptional analysis and difficulties in family segregation or identification of similar patients². Interdisciplinary collaborations between molecular geneticists, clinicians and (neuro)pathologists are the bases for the interpretation of VUS⁹⁴. Winder et al.³⁶ describe the use of NGS in a large cohort of patients affected by NMD. Evaluation of VUS in this cohort showed that at least one VUS was present in just over half of patients. VUS were resolved in only 2% of cases and, among these, almost one half were in the AD genes. Most of VUS occurred in single heterozygous alleles in AR genes and were less likely to be disease-causing.

NGS data is known to be error-prone: “false positives” are rare and post-processing data analysis were focused on the removal of this error. Less efficacy was observed in missing mutations or

“false negatives” in which the rate varies ~6%-18%¹²⁸. It is important to note that a significant part of the genome (8.5%) is extremely resistant to the single-nucleotide variant (SNV)/small indel calling due to a repetitive sequence, causing poor variant detection in some clinically relevant genes¹¹⁹. The contribution of these types of variants in the pathogenesis of neurological diseases is increasingly recognised. Moreover, causative disease mutations present in GC-rich exons of coding genes will be missed and the presence of highly homologous regions could generate coverage deficiency and variants present in those regions may be missed⁹.

These findings underline the possibility that many novel genes involved in neuromuscular diseases remain to be identified. It is important to re-evaluate cases undiagnosed for which new genetic findings could define a diagnosis, as new gene discovered or as VUS that can be reclassified as benign or pathogenic in accordance with newer variant-disease associations.

7.2.2 Non-Mendelian inheritance

Evidence of non-Mendelian inheritance is growing in the genetics community, and it could be associated with different mechanisms. There are qualifying variants (in a candidate gene and in known disease genes) that influence disease risk, and mutational burden, which can modulate phenotypic severity¹²⁹. Reduced penetrance and phenotypic variability observed, for example, within inherited axonopathies support the possibility of multilocus inheritance or genetic modification¹²⁹. Imprinting may contribute to non-Mendelian disease manifestation: a pathogenic variant expression could depend on the fact that it is maternally or paternally inherited, due to imprinting at this locus¹³⁰. Uniparental disomy (UPD) is another mechanism of non-Mendelian inheritance: isodisomy (two identical copies of a chromosome inherited from one parent replace the allele from the other parent), and heterodisomy (two non-identical chromatids are inherited from one parent and none from the other). Moreover, mosaicism (variant present in some of the somatic cells) could be the basis of incomplete penetrance explaining, for example, a patient with an autosomal-dominant condition while not having an affected parent carrier of the mutation¹³¹.

7.2.3 Not all disorders are genetically determined.

In particular cases, the phenotype might be atypical leading to misinterpretation, for example in inflammatory myopathies. It has already been described that a patient with idiopathic inflammatory myopathies (IIM) may be evaluated in a neurology clinic and receive a diagnosis of genetic myopathy. Conversely, a patient with a condition mimicking IIM may present to a rheumatologist who will naturally be more likely to consider a diagnosis of IIM. This phenomenon may be referred to as ‘looking down the wrong end of the telescope’¹³². Moreover, some IMM might mimic a genetic disorder such as anti-HMGCR myopathy which may present with a slow and progressive muscle damage, frequently preceded by asymptomatic hyperCKemia⁶⁶, resembling LGMD.

Furthermore, in the case of neuropathies, the differential diagnosis between inherited and acquired disorders can be difficult and misleading. For example, early onset chronic inflammatory demyelinating polyneuropathy (CIDP) may provoke foot deformities with pes cavus, the disease course may be slow and the CSF protein levels may be normal and, therefore, it could be misdiagnosed as a demyelinating hereditary neuropathy (CMT1)¹³³. Other times, neurophysiological studies could also not help in DD as in CMT1X, for which the conduction slowing is often unevenly distributed¹³³. A more complex study could be on the DD in acquired neuropathies and axonal hereditary neuropathy (CMT2) with a late onset¹³³.

The knowledge of these conditions is the basis of a correct diagnostic approach aimed to not exclude the various possible causes of NMD and eventually re-evaluate all not genetically defined cases.

7.3 ETHICAL RELEVANCE IN GENETIC DIAGNOSIS

GPS use in clinical practice is favoured by the absence of the discovery of any variants in genes not associated to the disease for which the patients could be asymptomatic. A genetic test determines the role not only for the patient but also for the entire family. During my PhD period, I also collaborated in order to define recommendations for pre-symptomatic genetic testing for

hereditary transthyretin amyloidosis in the era of effective therapies¹³⁴. As a matter of fact, for different late-onset neurodegenerative diseases, such as Huntington's disease^{135,136}, familial frontotemporal dementia/amyotrophic lateral sclerosis¹³⁷, spinocerebellar ataxias¹³⁸ and hereditary transthyretin amyloidosis (ATTRv)¹³⁹, protocols for pre-symptomatic genetic testing (PST) have been available for many years. These counselling protocols govern the access to pre-symptomatic testing in order to protect participants against an unfavourable result, providing them with information about the disease and the psychosocial consequences of the test result. The presence of new valid therapeutic options for ATTRv, which are maximally effective in the early stages, lead us to re-evaluate the PST protocol in these cases. After an initial survey on the ongoing approaches to PST for ATTRv in Italy, two roundtable meetings were held by 24 experts from 16 Italian centres involved in the diagnosis and treatment of this disease. These experts agreed that PST should be offered only in the context of genetic counselling to at risk individuals aged 18 or older. The protocol should consist of several steps, including a preliminary clinical examination, a pre-test information session, an interval time (at least one month), the genetic test and a post-test session with the disclosure of the test results, in the context of an experienced multidisciplinary team. Protocols for PST in the context of ATTRv is refined to offer at risk individuals with the best chance for early diagnosis and the timely initiation of treatment, while respecting autonomous decisions and promoting safe psychological adjustment to the genetic result.

In general, genetic analysis and its results can identify a specific diagnosis that will affect not only the patient but probably also their family.

8 CONCLUSION

The approach to NMD and any diagnostic assessment must begin with a comprehensive clinical evaluation since clinical observation may highlight peculiar phenotypes that guide the subsequent diagnostic pathway⁵ and it allows for understanding the diagnostic results.

Once the suspicion of a genetically determined NMD is raised, the clinical presentation and patient history guide the genetic approach. In clinical practice, the presence of a specific phenotype easily recognised as FSHD, Myotonic dystrophy, Emery-Dreifuss MD and length dependent CMT suggests a direct analysis of the gene hypothesized as causative. Genes panels, associated with CNVs assessment, are useful for individuals for whom a single-gene test cannot be confidently selected because of a mild or uncharacteristic phenotype³⁶. In these cases, gene panels enable a rapid and cost-effective analysis resulting in a shortened time, cost saving and minimisation of the problem of incidental findings. Exome sequencing and genome sequencing can be evaluated as a second-tier exam in selected patients. In myopathies after a first-line genetic approach, a muscle biopsy should always be performed in unsolved cases or to confirm new pathogenic variants or validate candidate genes⁶⁷.

In undiagnosed cases, it is important to re-evaluate the genetic results overtime in order to periodically revise novel variant-disease associations and eventually consider alternative diagnoses without underestimating the technical limits of NGS analysis.

Therefore, in our experience, the diagnosis strategy should be flexible and tuned to the clinical features of the patient in order to select the best molecular approach for each patient. Collaboration of a multidisciplinary team (geneticists, clinicians and neuropathologists) provides better outcomes for the patients and is the bases for addressing genetic analyses and the interpretation of their results.

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