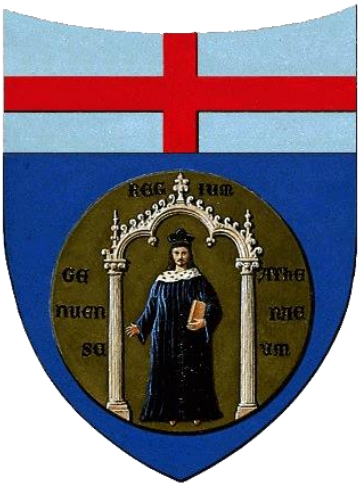

UNIVERSITY OF GENOVA

Department of Neuroscience, Rehabilitation, Ophthalmology,

Genetics, and Maternal and Children's Sciences (DINOGMI)

University of Genova

Ph.D. in NEUROSCIENCES XXXIV CYCLE

Genetic approach to neuromuscular disorders in the NGS era

Tutor

Prof. Marina Grandis

Ph.D. candidate

Chiara Gemelli

1 SUMMARY

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 o 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 metals[1].

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 cause[2]. 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 time[2].

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 loss[3]. Facial features of myotonic muscular dystrophy may be noted on inspection as the long thin face with temporal and masseter and frontal balding ore a tent-shaped mouth[3]. 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 asymmetric[3]. 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.musclegenetable.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 2019[5].

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

intergenic segments[6]. 85% of disease-causing variants are believed to be in the coding region[4] while it is estimated that 15% of variants potentially causative of mendelian traits are localised in the non-coding regions of the genome[7]. 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 genome[8].

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 identified[2]. 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 investigated[9]. However, GPS are frequently custom-made and require periodically update due to the frequent identification of novel causative genes[2]. 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 parents[9]. However, WES presents some limits: copy number variations (CNVs), expansions or contractions in repetitive regions, chromosomal rearrangements and deep intronic variants are not detect[2]. Those limitations are overcome by WGS that can detect CNVs, chromosomal abnormalities, deep intronic variants[13] and analysis mitochondrial genome[14]. 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 perform[8]. 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 3[15] and 4[16] 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 defect[17]. 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 diagnosis[18]. 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 phenotypes[19]. Therefore, compared with Sanger sequencing, targeted gene panels are more cost-effective and result in a larger number of diagnoses[20]. 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 sequencing[37], 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 investigations[32], 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 results[39]. Additionally, gene panels may lead to incidental findings in unrelated genes[9].

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 stiffness[40]. 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 workup[42]. 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 out[43]. 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 × the ULN; moderate, 5-10 × the ULN; and severe, > 10 × 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*).

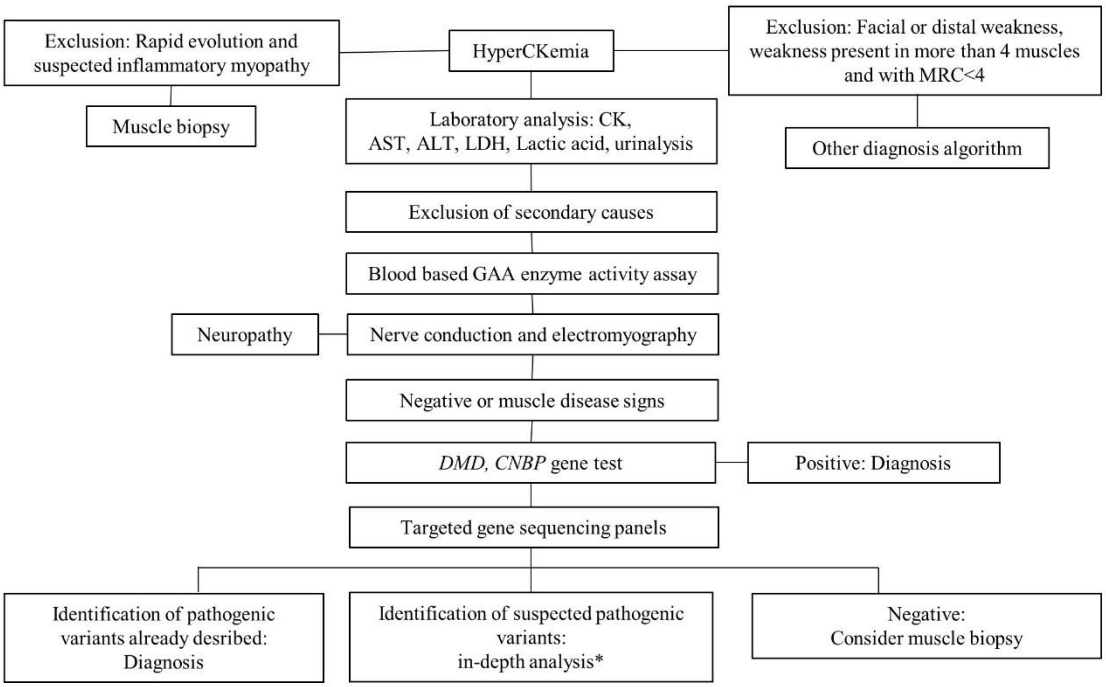

Fig. 1 Diagnostic algorithm for hyperCKemia.

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 ×. The median coverage was 300 ×. 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 used[44].

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).

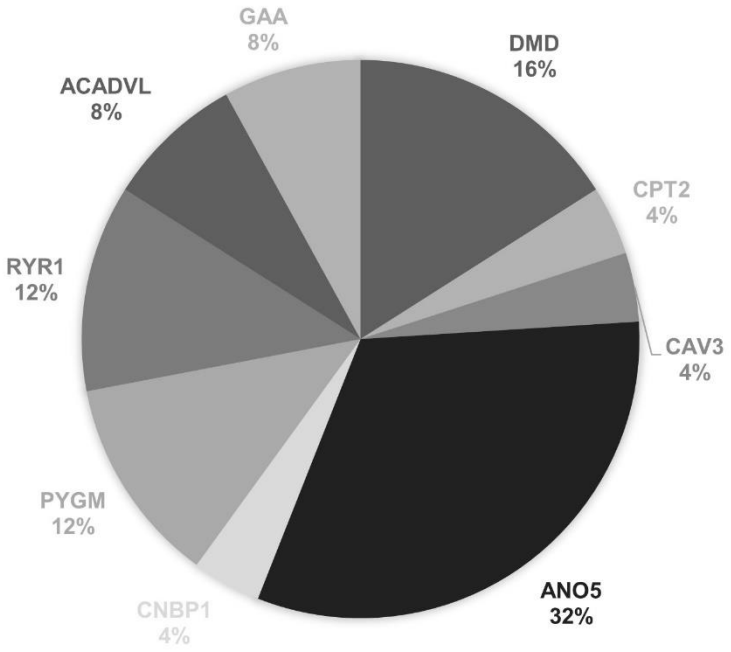

Fig. 2 Distribution of diagnostic results.

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 × 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%.

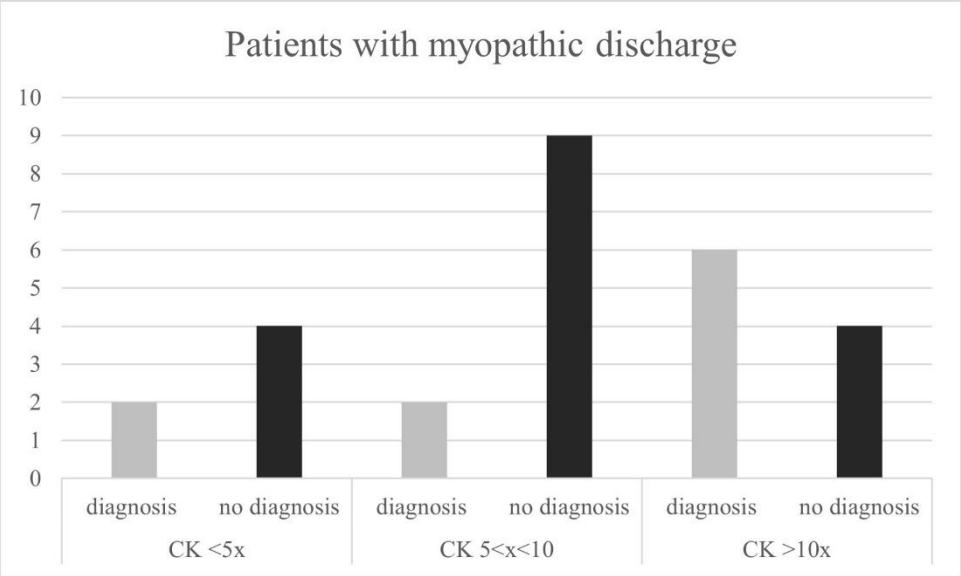

Fig. 3 Diagnosis of patients with myopathic discharges and evaluation of the different severity levels of hyperCKemia. The abscissa represents patients with myopathic discharges subdivided based on the hyperCKemia severity. Ordinates represent the number of patients. The black columns represent patients 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-fibral 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 expansions[23]. In addition, the most common *GAA* pathogenic variant is the splice site mutation, c.-32-13T>G[45], 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 missed[46] 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 Wu[32] 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 diseases[35]. 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 genes[2].

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 signs[53].

Three patients presented with a pathogenic *RYR1* variant, further confirming the frequency of this disorder in hyperCKemia patients[33]. All the *RYR1* pathogenic variants identified in our study have been associated with malignant hyperthermia[54–56]. 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 finding[27]. 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 myopathy [57–60]. Interestingly, one of the pathogenic variants identified (c.1706 T>C), has been recently described by Gonzalez-Mera et.al [60] 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 required[61].

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 grow[62]; these variants could be common in hyperCKemia cohorts[33]. 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 variants[29].

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 weakness[66]. Therefore, we believe that a muscle biopsy should always be performed in unsolved cases or to confirm new pathogenic variants or validate candidate genes[67].

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 patients[40]. 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 Prelle 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 symtoms	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 *ANO5*
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 *RYR1*
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 *ANO5*

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 *DMD*
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 *CAV3*
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 *RYR*
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 *ANO5*
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 *ANO5*
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 *ANO5*
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 *DMD*

63	M	44	15	cramps, myalgia, myoglobinuria	<5x		normal	nonspecific alteration	
64	F	48	46	hyperCKemia	5x<X<10x		normal		pathogenic variants in *ANO5*
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 *DMD*
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 *ANO5*
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 RYR1*
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 *ANO5*	*ANO5* (c.172C>T; p.Arg58Trp) *ANO5* (c.191dupA;Asn64Lysfs*15)		
3	McArdle disease	*PYGM* (c.2262delA; p.Lys754Asnfs*49) *PYGM* (c.2262delA; p.Lys754Asnfs*49)		
4				*LDHA* (c.681G>C; p.Val227=) *FKRP* (c.235G>A ; p.Val79Met)
5				*GAA* (c.2092G>A; p.Ala698Thr)
6				
7	McArdle disease	*PYGM* (c.148 C>T ; p.Arg50*) *PYGM* (c.148 C>T ; p.Arg50*)		
8				
9	pathogenic variant in *RYR1*	*RYR1* (c.5036 G>A p.Arg1679His)		
10				*RYR1* (c.10619A>T; p.Tyr3540Phe)
11				
12	Pompe disease	*GAA* (c.-32-13T>G;IVS1) *GAA* (c.1064T>C; p.Leu355Pro)		

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

39	CPT II deficiency	*CPT2* (c.149C>A; p.Pro50Hys) *CPT2* (c.149C>A; p.Pro50Hys)		
40				
41	pathogenic variants in *ANO5*	*ANO5* (c.1733T>C; p.Phe578Ser) ANO5 (c.191dupA;Asn64Lysfs*15)		
42				
43				*FUKTN* (c.166-4A>G) *FUKTN* (c.877G>C;p.Val293Leu)
44		*ENO3* (c.467G>A; p.Gly156Asp) *ANO5* (c.428A>G; p.Tyr143Cys)		
45				*RYR1* (c.6444C>T;Ser2148=)
46		*CAPN3* (c.664G>A;p.Gly222Arg)		
47	McArdle disease	*PYGM* (c.558delC;p.Tyr186*) *PYGM* (c.558delC;p.Tyr186*)		
48	VLCAD deficiency	*ACADVL* (c.1259T>C; p.Ile420Thr)	*ACADVL* (c.896_898delAGA;p.Lys299del)	
49	pathogenic variants in *ANO5*	*ANO5* (c.142_143insAA;p.ala48Glufs*9) ANO5 (c.2060T>A;p.val687Glu)		
50				
51				*MURC* (c.425C>T;p.Pro142Leu)
52				
53	*DMD* pathogenic variant female carrier	*DMD* (ex8-43 del)		
54		*CPT2* (c.338C>T; p.Ser113Leu) *ANO5* (c.1640G>A; p.Arg547Gln)		
55	pathogenic variants in *ANO5*	*ANO5* (c.220C>T; p.Arg74*) *ANO5* (c. 2141C>G; p.Thr714Ser)		
56				
57				
58				

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

			CPT2 (c.236A>C; p.Lys79Thr)
79	pathogenic variant in *RYR1*	*RYR1* (c.7304 G>T; p.Arg2435Leu)	
80			*GAA* (c.2845G>A;p.Val949Ile)
81			*RYR1* (c.12827G>A;p.Gly4281Glu)
			RYR1 (c.12864G>C;p.Ale4293=)
82			*CPT2* (c.1511C>T; p.Pro504Leu)
			FKRP (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 therapies [78]. 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 ANNOVAR [79] 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 ones [69].

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.

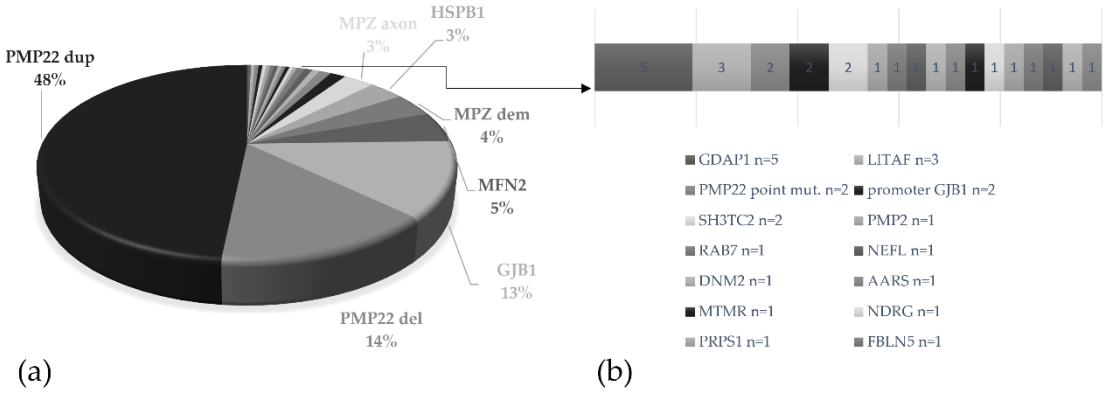

Fig. 4 Genetic spectrum of 277 cases with pathogenic variants. (a) The following genes are indicated: *PMP22* dup (n=134); *PMP22* del (n=40); *GJB1* (n=35); *MFN2* (n=14); *MPZ* causing demyelinating neuropathy (n=11); *MPZ* causing axonal neuropathy (n=8); *HSPB1* (n=9). (b) Other less frequent genes.

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 involved [80] in CMT1A [81] or CMT1X [82] and predominantly in some forms such as neuropathies caused by *GARS* and *BSCL2* pathogenic variants [83]. 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 out [100].

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 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 DIAGNOSIS	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)	SNAP amplitude (mV)	cMAP velocity (m/s)	cMAP 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 DIAGNOSIS	MUTATION	FAMILY	SEX	AGE OF ONSET	AGE OF FIRST EVALUATION	years from first symptom to first evaluation	clinic length dependent phenotype	SAP		cMAP		CMTNS	CMTES	electrophysiological length dependent phenotype
									velocity	amplitude	velocity	amplitude			
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	dHMN	*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	dHMN	*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	dHMN	*HSPB1* p.Arg136Leu		M	65	72	7	y	u normal	u normal	u normal	u normal	3/36	3	Y
6	dHMN	*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	dHMN	*HSPB1* p.Gly34Arg	F	54	61	7	y	s 45	s 24	p 45	p 1.4	2/28	2	X
9	dHMN	*HSPB1* 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 manifestations[13] or different presentations of the same genetic disorder in patients, including incomplete penetrance or heterogeneous phenotypes[123].

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 diseases[5], 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 limitations[124]. In general, traditional tests combined with NGS might increase the diagnostic yield as we demonstrate in patients affected by hyperCKemia[15]. 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 approach[5]. 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 effective[16].

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 approach[15]. 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 patients[5], 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 metabolomics[5] in order to solve complex cases[35].

Conversely, nerve biopsy is not required for most patients with hereditary neuropathies, especially after the advent of next generation sequencing[125]. 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 gene[126]. 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 neuroma[125]. 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 inherence.

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 technique[5]. CNVs are important contributors to a pathogenic variant burden of hereditary disorders and should be routinely assessed[127], and are estimated to explain approximately 10% of all inherited disorders[127]. 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 variants[2], 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 interpretation[2]. 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 patients[2]. Interdisciplinary collaborations between molecular geneticists, clinicians and (neuro)pathologists are the bases for the interpretation of VUS[94]. 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 genes[119]. 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 missed[9].

These findings underline the possibility that many novel genes involved in neuromuscular diseases remain to be identified. 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 inherence

Evidence of non-Mendelian inherence 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 severity[129]. Reduced penetrance and phenotypic variability observed, for example, within inherited axonopathies support the possibility of multilocus inheritance or genetic modification[129]. 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 locus[130]. 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 mutation[131].

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 hyperCKemia[66], 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 distributed[133]. A more complex study could be on the DD in acquired neuropathies and axonal hereditary neuropathy (CMT2) with a late onset[133].

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 therapies[134]. As a matter of fact, for different late-onset neurodegenerative diseases, such as Huntington's disease[135,136], familial frontotemporal dementia/amyotrophic lateral sclerosis[137], spinocerebellar ataxias[138] 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 pathway[5] 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 phenotype[36]. 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 genes[67].

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.

9 REFERENCES

1. Chokroverty, S. & Avidan, A. Y. *Bradley's Neurology in Clinical Practice, 2-Volume Set*. *Elsevier* vol. 01 (2016).

2. Barp, A., Mosca, L. & Sansone, V. A. Facilitations and Hurdles of Genetic Testing in Neuromuscular Disorders. *Diagnostics* **11**, (2021).

3. McDonald, C. M. Clinical approach to the diagnostic evaluation of hereditary and acquired neuromuscular diseases. *Physical Medicine and Rehabilitation Clinics of North America* vol. 23 (2012).

4. Efthymiou, S., Manole, A. & Houlden, H. Next-generation sequencing in neuromuscular diseases. *Current Opinion in Neurology* vol. 29 (2016).

5. Thompson, R. *et al.* Advances in the diagnosis of inherited neuromuscular diseases and implications for therapy development. *The Lancet Neurology* vol. 19 (2020).

6. Peterlin, B. *et al.* Genetic testing offer for inherited neuromuscular diseases within the EURO-NMD reference network: A European survey study. *PLoS ONE* **15**, (2020).

7. Mazzarotto, F., Olivotto, I. & Walsh, R. Advantages and Perils of Clinical Whole-Exome and Whole-Genome Sequencing in Cardiomyopathy. *Cardiovascular Drugs and Therapy* **34**, (2020).

8. Alfares, A. *et al.* Whole-genome sequencing offers additional but limited clinical utility compared with reanalysis of whole-exome sequencing. *Genetics in Medicine* **20**, (2018).

9. Fernandez-Marmiesse, A., Gouveia, S. & Couce, M. L. NGS Technologies as a Turning Point in Rare Disease Research , Diagnosis and Treatment. *Current Medicinal Chemistry* **25**, (2017).

10. Thuriot, F. *et al.* Molecular diagnosis of muscular diseases in outpatient clinics: A Canadian perspective. *Neurology: Genetics* (2020) doi:10.1212/NXG.0000000000000408.

11. Westra, D. *et al.* Panel-based exome sequencing for neuromuscular disorders as a diagnostic service. *Journal of Neuromuscular Diseases* (2019) doi:10.3233/JND-180376.

12. Töpf, A. *et al.* Sequential targeted exome sequencing of 1001 patients affected by unexplained limb-girdle weakness. *Genetics in Medicine* (2020) doi:10.1038/s41436-020-0840-3.

13. di Resta, C., Pipitone, G., Carrera, P. & Ferrari, M. Current scenario of the genetic testing for rare neurological disorders exploiting next generation sequencing. *Neural Regeneration Research* vol. 16 (2021).

14. Petrikin, J. E., Willig, L. K., Smith, L. D. & Kingsmore, S. F. Rapid whole genome sequencing and precision neonatology. *Seminars in Perinatology* vol. 39 (2015).

15. Gemelli, C. *et al.* An integrated approach to the evaluation of patients with asymptomatic or minimally symptomatic hyperCKemia. *Muscle and Nerve* **65**, (2022).

16. Gemelli, C. *et al.* Genetic Workup for Charcot–Marie–Tooth Neuropathy: A Retrospective Single-Site Experience Covering 15 Years. *Life* **12**, 402 (2022).

17. Laing, N. G. Genetics of neuromuscular disorders. *Critical Reviews in Clinical Laboratory Sciences* (2012) doi:10.3109/10408363.2012.658906.

18. Liewluck, T. & Milone, M. Untangling the complexity of limb-girdle muscular dystrophies. *Muscle and Nerve* (2018) doi:10.1002/mus.26077.

19. Evilä, A., Arumilli, M., Udd, B. & Hackman, P. Targeted next-generation sequencing assay for detection of mutations in primary myopathies. *Neuromuscular Disorders* (2016) doi:10.1016/j.nmd.2015.10.003.

20. Schofield, D. *et al.* Cost-effectiveness of massively parallel sequencing for diagnosis of paediatric muscle diseases. *npj Genomic Medicine* (2017) doi:10.1038/s41525-017-0006-7.

21. Dai, Y. *et al.* A comprehensive genetic diagnosis of Chinese muscular dystrophy and congenital myopathy patients by targeted next-generation sequencing. *Neuromuscular Disorders* (2015) doi:10.1016/j.nmd.2015.03.002.

22. Chae, J. H. *et al.* Utility of next generation sequencing in genetic diagnosis of early onset neuromuscular disorders. *Journal of Medical Genetics* (2015) doi:10.1136/jmedgenet-2014-102819.

23. Lévesque, S. *et al.* Diagnosis of late-onset Pompe disease and other muscle disorders by next-generation sequencing. *Orphanet Journal of Rare Diseases* (2016) doi:10.1186/s13023-016-0390-6.

24. Sevy, A. *et al.* Improving molecular diagnosis of distal myopathies by targeted next-generation sequencing. *Journal of Neurology, Neurosurgery and Psychiatry* (2016) doi:10.1136/jnnp-2014-309663.

25. Seong, M. W. *et al.* Clinical applications of next-generation sequencing-based gene panel in patients with muscular dystrophy: Korean experience. *Clinical Genetics* (2016) doi:10.1111/cge.12621.

26. O'Grady, G. L. *et al.* Diagnosis and etiology of congenital muscular dystrophy: We are halfway there. *Annals of Neurology* (2016) doi:10.1002/ana.24687.

27. Savarese, M. *et al.* The genetic basis of undiagnosed muscular dystrophies and myopathies. *Neurology* (2016) doi:10.1212/WNL.0000000000002800.

28. Kitamura, Y., Kondo, E., Urano, M., Aoki, R. & Saito, K. Target resequencing of neuromuscular disease-related genes using next-generation sequencing for patients with undiagnosed early-onset neuromuscular disorders. *Journal of Human Genetics* (2016) doi:10.1038/jhg.2016.79.

29. Kuhn, M. *et al.* Utility of a next-generation sequencing-based gene panel investigation in German patients with genetically unclassified limb-girdle muscular dystrophy. *Journal of Neurology* (2016) doi:10.1007/s00415-016-8036-0.

30. Yu, M. *et al.* Mutational spectrum of Chinese LGMD patients by targeted next-generation sequencing. *PLoS ONE* (2017) doi:10.1371/journal.pone.0175343.

31. Nallamilli, B. R. R. *et al.* Genetic landscape and novel disease mechanisms from a large LGMD cohort of 4656 patients. *Annals of Clinical and Translational Neurology* (2018) doi:10.1002/acn3.649.

32. Wu, L., Brady, L., Shoffner, J. & Tarnopolsky, M. A. Next-Generation Sequencing to Diagnose Muscular Dystrophy, Rhabdomyolysis, and HyperCKemia. *Canadian Journal of Neurological Sciences* (2018) doi:10.1017/cjn.2017.286.

33. Rubegni, A. *et al.* Next-generation sequencing approach to hyperCKemia. *Neurology Genetics* (2019) doi:10.1212/nxg.0000000000000352.

34. Beecroft, S. J. *et al.* Targeted gene panel use in 2249 neuromuscular patients: the Australasian referral center experience. *Annals of Clinical and Translational Neurology* (2020) doi:10.1002/acn3.51002.

35. Gonzalez-Quereda, L. *et al.* Targeted next-generation sequencing in a large cohort of genetically undiagnosed patients with neuromuscular disorders in Spain. *Genes (Basel)* (2020) doi:10.3390/genes11050539.

36. Winder, T. L. *et al.* Clinical utility of multigene analysis in over 25,000 patients with neuromuscular disorders. *Neurology: Genetics* (2020) doi:10.1212/NXG.0000000000000412.

37. Ankala, A. *et al.* A comprehensive genomic approach for neuromuscular diseases gives a high diagnostic yield. *Annals of Neurology* (2015) doi:10.1002/ana.24303.

38. Haskell, G. T. *et al.* Diagnostic utility of exome sequencing in the evaluation of neuromuscular disorders. *Neurology: Genetics* (2018) doi:10.1212/NXG.0000000000000212.

39. Vasli, N. & Laporte, J. Impacts of massively parallel sequencing for genetic diagnosis of neuromuscular disorders. *Acta Neuropathologica* (2013) doi:10.1007/s00401-012-1072-7.

40. Kyriakides, T. *et al.* EFNS guidelines on the diagnostic approach to pauci- or asymptomatic hyperCKemia. *European Journal of Neurology* (2010) doi:10.1111/j.1468-1331.2010.03012.x.

41. Kyriakides, T., Angelini, C., Vilchez, J. & Hilton-Jones, D. European Federation of the Neurological Societies guidelines on the diagnostic approach to paucisymptomatic or asymptomatic hyperCKemia. *Muscle and Nerve* (2020) doi:10.1002/mus.26777.

42. Schmidt, J. Current Classification and Management of Inflammatory Myopathies. *Journal of Neuromuscular Diseases* (2018) doi:10.3233/JND-180308.

43. Kley, R. A., Schmidt-Wilcke, T. & Vorgerd, M. Differential Diagnosis of HyperCKemia. *Aktuelle Neurologie* (2018) doi:10.1055/s-0043-124430.

44. Richards, S. *et al.* Standards and guidelines for the interpretation of sequence variants: A joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. *Genetics in Medicine* (2015) doi:10.1038/gim.2015.30.

45. Peruzzo, P., Pavan, E. & Dardis, A. Molecular genetics of Pompe disease: a comprehensive overview. *Annals of Translational Medicine* (2019) doi:10.21037/atm.2019.04.13.

46. Vissing, J., Lukacs, Z. & Straub, V. Diagnosis of pompe disease muscle biopsy vs blood-based assays. *JAMA Neurology* (2013) doi:10.1001/2013.jamaneurol.486.

47. Young, N. P., Daube, J. R., Sorenson, E. J. & Milone, M. Absent, unrecognized, and minimal myotonic discharges in myotonic dystrophy type 2. *Muscle and Nerve* (2010) doi:10.1002/mus.21615.

48. Hilbert, J. E. *et al.* Diagnostic odyssey of patients with myotonic dystrophy. *Journal of Neurology* (2013) doi:10.1007/s00415-013-6993-0.

49. Magri, F. *et al.* Clinical and molecular characterization of a cohort of patients with novel nucleotide alterations of the Dystrophin gene detected by direct sequencing. *BMC Medical Genetics* **12**, (2011).

50. Neri, M. *et al.* The Genetic Landscape of Dystrophin Mutations in Italy: A Nationwide Study. *Frontiers in Genetics* **11**, (2020).

51. Vázquez, J. *et al.* Phenotypic Spectrum of Myopathies with Recessive Anoctamin-5 Mutations. *J Neuromuscul Dis* (2020) doi:10.3233/JND-200515.

52. Papadopoulos, C. *et al.* Hyperckemia and myalgia are common presentations of anoctamin-5-related myopathy in French patients. *Muscle and Nerve* (2017) doi:10.1002/mus.25608.

53. Panadés-de Oliveira, L. *et al.* Persistent asymptomatic or mild symptomatic hyperCKemia due to mutations in ANO5: the mildest end of the anoctaminopathies spectrum. *Journal of Neurology* (2020) doi:10.1007/s00415-020-09872-7.

54. Vukcevic, M. *et al.* Functional properties of RYR1 mutations identified in Swedish patients with malignant hyperthermia and central core disease. *Anesthesia and Analgesia* (2010) doi:10.1213/ANE.0b013e3181cbd815.

55. Sambuughin, N., McWilliams, S., De Bantel, A., Sivakumar, K. & Nelson, T. E. Single-amino-acid deletion in the RYR1 gene, associated with malignant hyperthermia susceptibility and unusual contraction phenotype. *American Journal of Human Genetics* (2001) doi:10.1086/321270.

56. Galli, L. *et al.* Frequency and localization of mutations in the 106 exons of the RYR1 gene in 50 individuals with malignant hyperthermia. *Human Mutation* (2006) doi:10.1002/humu.9442.

57. Vissing, J. *et al.* A heterozygous 21-bp deletion in CAPN3 causes dominantly inherited limb girdle muscular dystrophy. *Brain* **139**, (2016).

58. Cerino, M. *et al.* Novel CAPN3 variant associated with an autosomal dominant calpainopathy. *Neuropathology and Applied Neurobiology* (2020) doi:10.1111/nan.12624.

59. Martinez-Thompson, J. M. *et al.* Autosomal dominant calpainopathy due to heterozygous CAPN3 C.643_663del21. *Muscle and Nerve* (2018) doi:10.1002/mus.25970.

60. González-Mera, L. *et al.* Heterozygous CAPN3 missense variants causing autosomal-dominant calpainopathy in seven unrelated families. *Neuropathology and Applied Neurobiology* **47**, (2021).

61. Savarese, M. *et al.* Next generation sequencing on patients with LGMD and nonspecific myopathies: Findings associated with ANO5 mutations. *Neuromuscular Disorders* (2015) doi:10.1016/j.nmd.2015.03.011.

62. Gonsalves, S. G. *et al.* Using exome data to identify malignant hyperthermia susceptibility mutations. *Anesthesiology* (2013) doi:10.1097/ALN.0b013e3182a8a8e7.

63. Morandi, L. *et al.* High plasma creatine kinase: Review of the literature and proposal for a diagnostic algorithm. *Neurological Sciences* (2006) doi:10.1007/s10072-006-0701-0.

64. Rubegni, A. *et al.* A case of 3243A>G mutation in mtDNA presenting as apparently idiopathic hyperCKemia. *Journal of the Neurological Sciences* (2014) doi:10.1016/j.jns.2014.01.010.

65. Finsterer, J., Kovacs, G. G., Rauschka, H. & Ahting, U. Adult, isolated respiratory chain complex IV deficiency with minimal manifestations. *Folia Neuropathologica* (2015) doi:10.5114/fn.2015.52412.

66. Mohassel, P. *et al.* Anti-HMGCR myopathy may resemble limb-girdle muscular dystrophy. *Neurology: Neuroimmunology and NeuroInflammation* (2019) doi:10.1212/NXI.0000000000000523.

67. Schoser, B. Diagnostic muscle biopsy: Is it still needed on the way to a liquid muscle pathology? *Current Opinion in Neurology* (2016) doi:10.1097/WCO.0000000000000366.

68. Prelle, A. *et al.* Retrospective study of a large population of patients with asymptomatic or minimally symptomatic raised serum creatine kinase levels. *Journal of Neurology* (2002) doi:10.1007/s004150200010.

69. Barreto, L. C. L. S. *et al.* Epidemiologic Study of Charcot-Marie-Tooth Disease: A Systematic Review. *Neuroepidemiology* (2016) doi:10.1159/000443706.

70. Rossor, A. M., Tomaselli, P. J. & Reilly, M. M. Recent advances in the genetic neuropathies. *Current Opinion in Neurology* vol. 29 (2016).

71. Berciano, J. *et al.* Intermediate Charcot–Marie–Tooth disease: an electrophysiological reappraisal and systematic review. *Journal of Neurology* (2017) doi:10.1007/s00415-017-8474-3.

72. Rossor, A. M., Polke, J. M., Houlden, H. & Reilly, M. M. Clinical implications of genetic advances in charcot-marie-tooth disease. *Nature Reviews Neurology* (2013) doi:10.1038/nrneurol.2013.179.

73. Gess, B., Schirmacher, A., Boentert, M. & Young, P. Charcot-Marie-Tooth disease: Frequency of genetic subtypes in a German neuromuscular center population. *Neuromuscular Disorders* (2013) doi:10.1016/j.nmd.2013.05.005.

74. McCorquodale, D., Pucillo, E. M. & Johnson, N. E. Management of Charcot–Marie–Tooth disease: Improving long-term care with a multidisciplinary approach. *Journal of Multidisciplinary Healthcare* vol. 9 (2016).

75. Nicholson, G. & Myers, S. Intermediate forms of charcot-marie-tooth neuropathy: A review. *NeuroMolecular Medicine* vol. 8 (2006).

76. Shy, M. E. *et al.* (1) Shy, M. E.; Blake, J.; Krajewski, K.; Fuerst, D. R.; Laura, M.; Hahn, A. F.; Li, J.; Lewis, R. A.; Reilly, M. Reliability and Validity of the CMT Neuropathy Score as a Measure of Disability. Neurology 2005, 64 (7). https://doi.org/10.1212/01.WNL.00001. *Neurology* **64**, (2005).

77. Murphy, S. M. *et al.* Reliability of the CMT neuropathy score (second version) in Charcot-Marie-Tooth disease. *Journal of the Peripheral Nervous System* **16**, (2011).

78. Jani-Acsadi, A., Krajewski, K. & Shy, M. E. Charcot-Marie-Tooth neuropathies: Diagnosis and management. *Seminars in Neurology* vol. 28 (2008).

79. Wang, K., Li, M. & Hakonarson, H. ANNOVAR: Functional annotation of genetic variants from high-throughput sequencing data. *Nucleic Acids Research* **38**, (2010).

80. Grandis, M. & Shy, M. E. Current therapy for Charcot-Marie-Tooth disease. *Current Treatment Options in Neurology* **7**, (2005).

81. Videler, A. J., Beelen, A., Van Schaik, I. N., De Visser, M. & Nollet, F. Limited upper limb functioning has impact on restrictions in participation and autonomy of patients with hereditary motor and sensory neuropathy 1A. *Journal of Rehabilitation Medicine* **41**, (2009).

82. Mandich, P. *et al.* Gap junction beta 1 (GJB1) gene mutations in Italian patients with X-linked Charcot-Marie-Tooth disease. *Journal of Human Genetics* **53**, (2008).

83. Pareyson, D. & Marchesi, C. Diagnosis, natural history, and management of Charcot-Marie-Tooth disease. *The Lancet Neurology* (2009) doi:10.1016/S1474-4422(09)70110-3.

84. Choi, B. O. *et al.* Exome sequencing is an efficient tool for genetic screening of Charcot-Marie-Tooth Disease. *Human Mutation* **33**, (2012).

85. Høyer, H. *et al.* Genetic diagnosis of Charcot-Marie-Tooth disease in a population by next-generation sequencing. *BioMed Research International* **2014**, (2014).

86. Antoniadi, T. *et al.* Application of targeted multi-gene panel testing for the diagnosis of inherited peripheral neuropathy provides a high diagnostic yield with unexpected phenotype-genotype variability. *BMC Medical Genetics* **16**, (2015).

87. Drew, A. P. *et al.* Improved inherited peripheral neuropathy genetic diagnosis by whole-exome sequencing. *Molecular Genetics and Genomic Medicine* **3**, (2015).

88. Lupo, V. *et al.* Assessment of Targeted Next-Generation Sequencing as a Tool for the Diagnosis of Charcot-Marie-Tooth Disease and Hereditary Motor Neuropathy. *Journal of Molecular Diagnostics* **18**, (2016).

89. Nam, S. H. *et al.* Identification of genetic causes of inherited peripheral neuropathies by targeted gene panel sequencing. *Molecules and Cells* **39**, (2016).

90. Laššuthová, P. *et al.* Improving diagnosis of inherited peripheral neuropathies through gene panel analysis. *Orphanet Journal of Rare Diseases* **11**, (2016).

91. Dohrn, M. F. *et al.* Frequent genes in rare diseases: panel-based next generation sequencing to disclose causal mutations in hereditary neuropathies. *Journal of Neurochemistry* **143**, (2017).

92. Bansagi, B. *et al.* Genetic heterogeneity of motor neuropathies. *Neurology* **88**, (2017).

93. He, J. *et al.* Clinical and genetic investigation in Chinese patients with demyelinating Charcot-Marie-Tooth disease. *Journal of the Peripheral Nervous System* **23**, (2018).

94. Bacquet, J. *et al.* Molecular diagnosis of inherited peripheral neuropathies by targeted next-generation sequencing: Molecular spectrum delineation. *BMJ Open* **8**, (2018).

95. Hartley, T. *et al.* Whole-exome sequencing is a valuable diagnostic tool for inherited peripheral neuropathies: Outcomes from a cohort of 50 families. *Clinical Genetics* **93**, (2018).

96. Vaeth, S. *et al.* Genetic analysis of Charcot-Marie-Tooth disease in Denmark and the implementation of a next generation sequencing platform. *European Journal of Medical Genetics* **62**, (2019).

97. Yoshimura, A. *et al.* Genetic profile and onset features of 1005 patients with Charcot-Marie-Tooth disease in Japan. *Journal of Neurology, Neurosurgery and Psychiatry* **90**, (2019).

98. Lee, D. C., Dankwa, L., Edmundson, C., Cornblath, D. R. & Scherer, S. S. Yield of next-generation neuropathy gene panels in axonal neuropathies. *Journal of the Peripheral Nervous System* **24**, (2019).

99. Liu, X., Duan, X., Zhang, Y., Sun, A. & Fan, D. Molecular analysis and clinical diversity of distal hereditary motor neuropathy. *European Journal of Neurology* **27**, (2020).

100. Cortese, A. *et al.* Targeted next-generation sequencing panels in the diagnosis of Charcot-Marie-Tooth disease. *Neurology* (2020) doi:10.1212/WNL.0000000000008672.

101. Lin, S. *et al.* Whole exome sequencing reveals a broader variant spectrum of Charcot-Marie-Tooth disease type 2. *Neurogenetics* **21**, (2020).

102. Ferese, R. *et al.* Cohort Analysis of 67 Charcot-Marie-Tooth Italian Patients: Identification of New Mutations and Broadening of Phenotype Expression Produced by Rare Variants. *Frontiers in Genetics* **12**, (2021).

103. Volodarsky, M. *et al.* Comprehensive genetic sequence and copy number analysis for Charcot-Marie-Tooth disease in a Canadian cohort of 2517 patients. *Journal of Medical Genetics* **58**, (2021).

104. Felice, K. J., Whitaker, C. H. & Khorasanizadeh, S. Diagnostic yield of advanced genetic testing in patients with hereditary neuropathies: A retrospective single-site study. *Muscle and Nerve* **64**, (2021).

105. Saporta, A. S. D. *et al.* Charcot-marie-tooth disease subtypes and genetic testing strategies. *Annals of Neurology* (2011) doi:10.1002/ana.22166.

106. Murphy, S. M. *et al.* Charcot-Marie-Tooth disease: Frequency of genetic subtypes and guidelines for genetic testing. *Journal of Neurology, Neurosurgery and Psychiatry* (2012) doi:10.1136/jnnp-2012-302451.

107. Divincenzo, C. *et al.* The allelic spectrum of charcot–marie–tooth disease in over 17,000 individuals with neuropathy. *Molecular Genetics and Genomic Medicine* **2**, (2014).

108. Fridman, V. *et al.* CMT subtypes and disease burden in patients enrolled in the Inherited Neuropathies Consortium natural history study: A cross-sectional analysis. *Journal of Neurology, Neurosurgery and Psychiatry* (2015) doi:10.1136/jnnp-2014-308826.

109. Lorefice, L. *et al.* Charcot–Marie–Tooth disease: genetic subtypes in the Sardinian population. *Neurological Sciences* (2017) doi:10.1007/s10072-017-2905-x.

110. Milley, G. M. *et al.* Genotypic and phenotypic spectrum of the most common causative genes of Charcot-Marie-Tooth disease in Hungarian patients. *Neuromuscular Disorders* (2018) doi:10.1016/j.nmd.2017.08.007.

111. Manganelli, F. *et al.* Charcot-Marie-Tooth disease: Frequency of genetic subtypes in a Southern Italy population. *Journal of the Peripheral Nervous System* (2014) doi:10.1111/jns.12092.

112. Sivera, R. *et al.* Charcot-Marie-Tooth disease: Genetic and clinical spectrum in a Spanish clinical series. *Neurology* (2013) doi:10.1212/WNL.0b013e3182a9f56a.

113. Gentile, L. *et al.* Charcot-Marie-Tooth disease: experience from a large Italian tertiary neuromuscular center. *Neurological Sciences* **41**, (2020).

114. Echaniz-Laguna, A. *et al.* Axonal Neuropathies due to Mutations in Small Heat Shock Proteins: Clinical, Genetic, and Functional Insights into Novel Mutations. *Human Mutation* **38**, (2017).

115. Sanmaneechai, O. *et al.* Genotype-phenotype characteristics and baseline natural history of heritable neuropathies caused by mutations in the MPZ gene. *Brain* (2015) doi:10.1093/brain/awv241.

116. Callegari, I. *et al.* Mutation update for myelin protein zero-related neuropathies and the increasing role of variants causing a late-onset phenotype. *Journal of Neurology* **266**, (2019).

117. Houlden, H. *et al.* Mutations in the HSP27 (HSPB1) gene cause dominant, recessive, and sporadic distal HMN/CMT type 2. *Neurology* (2008) doi:10.1212/01.wnl.0000319696.14225.67.

118. Pipis, M., Rossor, A. M., Laura, M. & Reilly, M. M. Next-generation sequencing in Charcot–Marie–Tooth disease: opportunities and challenges. *Nature Reviews Neurology* vol. 15 (2019).

119. Regier, A. A. *et al.* Functional equivalence of genome sequencing analysis pipelines enables harmonized variant calling across human genetics projects. *Nature Communications* **9**, (2018).

120. Cortese, A. *et al.* Mutation in RNF170 causes sensory ataxic neuropathy with vestibular areflexia: A CANVAS mimic. *Journal of Neurology, Neurosurgery and Psychiatry* vol. 91 (2020).

121. Cortese, A. *et al.* Cerebellar ataxia, neuropathy and vestibular areflexia syndrome (CANVAS): genetic and clinical aspects. *Practical Neurology* **22**, (2022).

122. Beecroft, S. J. *et al.* The Impact of Next-Generation Sequencing on the Diagnosis, Treatment, and Prevention of Hereditary Neuromuscular Disorders. *Molecular Diagnosis and Therapy* **24**, (2020).

123. Fogel, B. L. Genetic and genomic testing for neurologic disease in clinical practice. in *Handbook of Clinical Neurology* vol. 147 (2018).

124. Volk, A. E. & Kubisch, C. The rapid evolution of molecular genetic diagnostics in neuromuscular diseases. *Current Opinion in Neurology* vol. 30 (2017).

125. Nathani, D. *et al.* Nerve biopsy: Current indications and decision tools. *Muscle and Nerve* vol. 64 (2021).

126. Duchesne, M. *et al.* Nerve biopsy is still useful in some inherited neuropathies. *Journal of Neuropathology and Experimental Neurology* vol. 77 (2018).

127. Truty, R. *et al.* Prevalence and properties of intragenic copy-number variation in Mendelian disease genes. *Genetics in Medicine* **21**, (2019).

128. Dean Bobo, M. L. J. L. R.-F. A. A. B. M. H. False Negatives Are a Significant Feature of Next Generation Sequencing Callsets.

129. Bis-Brewer, D. M. *et al.* Assessing non-Mendelian inheritance in inherited axonopathies. *Genetics in Medicine* **22**, (2020).

130. Kadlubowska, M. K. & Schrauwen, I. Methods to Improve Molecular Diagnosis in Genomic Cold Cases in Pediatric Neurology. *Genes (Basel)* **13**, 333 (2022).

131. Shieh, P. B. Advances in the Genetic Testing of Neuromuscular Diseases. *Neurologic Clinics* **38**, 519–528 (2020).

132. Chinoy, H. & Lilleker, J. B. Pitfalls in the diagnosis of myositis. *Best Practice and Research: Clinical Rheumatology* vol. 34 (2020).

133. Pareyson, D. Differential diagnosis of Charcot-Marie-Tooth disease and related neuropathies. *Neurological Sciences* vol. 25 (2004).

134. Grandis, M. *et al.* Recommendations for pre-symptomatic genetic testing for hereditary transthyretin amyloidosis in the era of effective therapy: a multicenter Italian consensus. *Orphanet Journal of Rare Diseases* **15**, 348 (2020).

135. Mandich, P. *et al.* 1993–2014: two decades of predictive testing for Huntington's disease at the Medical Genetics Unit of the University of Genoa. *Molecular Genetics and Genomic Medicine* (2017) doi:10.1002/mgg3.238.

136. Macleod, R. *et al.* Recommendations for the predictive genetic test in Huntington's disease. *Clinical Genetics* (2013) doi:10.1111/j.1399-0004.2012.01900.x.

137. Chiò, A. *et al.* Genetic counselling in ALS: Facts, uncertainties and clinical suggestions. *Journal of Neurology, Neurosurgery and Psychiatry* (2014) doi:10.1136/jnnp-2013-305546.

138. Paneque, M. *et al.* Twenty years of a pre-symptomatic testing protocol for late-onset neurological diseases in Portugal. *Acta Medica Portuguesa* (2019) doi:10.20344/amp.10526.

139. Obici, L. *et al.* Recommendations for presymptomatic genetic testing and management of individuals at risk for hereditary transthyretin amyloidosis. *Current opinion in neurology* (2016) doi:10.1097/WCO.0000000000000290.

10 PUBLICATIONS DURING PHD

Gemelli C, Geroldi A, Massucco S, Trevisan L, Callegari I, Marinelli L, Ursino G, Hamedani M, Mennella G, Stara S, Maggi G, Mori L, Schenone C, Gotta F, Patrone S, Mammi A, Origone P, Prada V, Nobbio L, Mandich P, Schenone A, Bellone E, Grandis M. Genetic Workup for Charcot–Marie–Tooth Neuropathy: A Retrospective Single-Site Experience Covering 15 Years. Life. 2022; 12(3):402. https://doi.org/10.3390/life12030402

Gemelli C, Traverso M, Trevisan L, Fabbri S, Scarsi E, Carlini B, Prada V, Mongini T, Ruggiero L, Patrone S, Gallone S, Iodice R, Pisciotta L, Zara F, Origone P, Rota E, Minetti C, Bruno C, Schenone A, Mandich P, Fiorillo C, Grandis M. An integrated approach to the evaluation of patients with asymptomatic or minimally symptomatic hyperCKemia. Muscle Nerve. 2022 Jan;65(1):96-104. doi: 10.1002/mus.27448. Epub 2021 Nov 8. PMID: 34687219.

Salvalaggio A, Coraci D, Obici L, Cacciavillani M, Luigetti M, Mazzeo A, Pastorelli F, Grandis M, Cavallaro T, Bisogni G, Lozza A, **Gemelli C**, Gentile L, Russo M, Ermani M, Fabrizi GM, Plasmati R, De Napoli F, Campagnolo M, Castellani F, Salvi F, Fenu S, Devigili G, Pareyson D, Gasparotti R, Rapezzi C, Martinoli C, Padua L, Briani C. Progressive brachial plexus enlargement in hereditary transthyretin amyloidosis. J Neurol. 2021 Aug 19. doi: 10.1007/s00415-021-10754-9. Epub ahead of print. PMID: 34410494.

Currò R, Salvalaggio A, Tozza S, **Gemelli C**, Dominik N, Galassi Deforie V, Magrinelli F, Castellani F, Vegezzi E, Businaro P, Callegari I, Pichiecchio A, Cosentino G, Alfonsi E, Marchioni E, Colnaghi S, Gana S, Valente EM, Tassorelli C, Efthymiou S, Facchini S, Carr A, Laura M, Rossor AM, Manji H, Lunn MP, Pegoraro E, Santoro L, Grandis M, Bellone E, Beauchamp NJ, Hadjivassiliou M, Kaski D, Bronstein AM, Houlden H, Reilly MM, Mandich P, Schenone A, Manganelli F, Briani C, Cortese A. RFC1 expansions are a common cause of idiopathic sensory neuropathy. Brain. 2021 Jun 22;144(5):1542-1550. doi: 10.1093/brain/awab072. PMID: 33969391; PMCID: PMC8262986.

Magliano L, Obici L, Sforzini C, Mazzeo A, Russo M, Cappelli F, Fenu S, Luigetti M, Tagliapietra M, **Gemelli C**, Leonardi L, Tozza S, Pradotto LG, Citarelli G, Mauro A, Manganelli F, Antonini G, Grandis M, Fabrizi GM, Sabatelli M, Pareyson D, Perfetto F, Merlini G, Vita G; ATTRv Collaborators. Psychosocial burden and professional and social support in patients with hereditary transthyretin amyloidosis (ATTRv) and their relatives in Italy. Orphanet J Rare Dis. 2021 Apr 7;16(1):163. doi: 10.1186/s13023-021-01812-6. PMID: 33827635; PMCID: PMC8028211.

Zuppa A, De Michelis C, Meo G, Prada V, **Gemelli C**, Infantino M, Manfredi M, Pesce G, Tagliafico AS, Benedetti L, Fiorillo C, Schenone A, Quartuccio L, Grandis M. Maintenance treatment with subcutaneous immunoglobulins in the long-term management of anti-HMCGR myopathy. Neuromuscul Disord. 2021 Feb;31(2):134-138. doi: 10.1016/j.nmd.2020.12.012. Epub 2020 Dec 28. PMID: 33461845.

Grandis M, Obici L, Luigetti M, Briani C, Benedicenti F, Bisogni G, Canepa M, Cappelli F, Danesino C, Fabrizi GM, Fenu S, Ferrandes G, **Gemelli C**, Manganelli F, Mazzeo A, Melchiorri L, Perfetto F, Pradotto LG, Rimessi P, Tini G, Tozza S, Trevisan L, Pareyson D, Mandich P. Recommendations for pre-symptomatic genetic testing for hereditary transthyretin amyloidosis in the era of effective therapy: a multicenter Italian consensus. Orphanet J Rare Dis. 2020 Dec 14;15(1):348. doi: 10.1186/s13023-020-01633-z. PMID: 33317601; PMCID: PMC7734774.

Visigalli D, Capodivento G, Basit A, Fernández R, Hamid Z, Pencová B, **Gemelli C**, Marubbi D, Pastorino C, Luoma AM, Riekel C, Kirschner DA, Schenone A, Fernández JA, Armirotti A, Nobbio L. Exploiting Sphingo- and Glycerophospholipid Impairment to Select Effective Drugs and Biomarkers for CMT1A. Front Neurol. 2020 Aug 25;11:903. doi: 10.3389/fneur.2020.00903. PMID: 32982928; PMCID: PMC7477391.

Mauri E, Abati E, Musumeci O, Rodolico C, D'Angelo MG, Mirabella M, Lucchini M, Bello L, Pegoraro E, Maggi L, Manneschi L, **Gemelli C**, Grandis M, Zuppa A, Massucco S, Benedetti L, Caponnetto C, Schenone A, Prelle A, Previtali SC, Scarlato M, D'Amico A, Bertini E, Pennisi EM, De Giglio L, Pane M, Mercuri E, Mongini T, Ricci F, Berardinelli A, Astrea G, Lenzi S, Battini R, Ricci G, Torri F, Siciliano G, Santorelli FM, Ariatti A, Filosto M, Passamano L, Politano L, Scutifero M, Tonin P, Fossati B, Panicucci C, Bruno C, Ravaglia S, Monforte M, Tasca G, Ricci E, Petrucci A, Santoro L, Ruggiero L, Barp A, Albamonte E, Sansone V, Gagliardi D, Costamagna G, Govoni A, Magri F, Brusa R, Velardo D, Meneri M, Sciacco M, Corti S, Bresolin N, Moroni I, Messina S, Di Muzio A, Nigro V, Liguori R, Antonini G, Toscano A, Minetti C, Comi GP; Italian Association of Myology. Estimating the impact of COVID-19 pandemic on services provided by Italian Neuromuscular Centers: an Italian Association of Myology survey of the acute phase. Acta Myol. 2020 Jun 1;39(2):57-66. doi: 10.36185/2532-1900-008. PMID: 32904925; PMCID: PMC7460733.

Rota E, Grandis M, Di Sapio A, Ghiglione E, Fiorentino P, Repetto A, Giliberto C, **Gemelli C**, Morelli N, Schenone A, Cocito D. Screening for Fabry disease in unknown origin axonal polyneuropathy: to do or not to do, this is the question! Orphanet J Rare Dis. 2020 Aug 20;15(1):216. doi: 10.1186/s13023-020-01501-w. PMID: 32819406; PMCID: PMC7439676.

Salvalaggio A, Coraci D, Cacciavillani M, Obici L, Mazzeo A, Luigetti M, Pastorelli F, Grandis M, Cavallaro T, Bisogni G, Lozza A, **Gemelli C**, Gentile L, Ermani M, Fabrizi GM, Plasmati R, Campagnolo M, Castellani F, Gasparotti R, Martinoli C, Padua L, Briani C. Nerve ultrasound in hereditary transthyretin amyloidosis: red flags and possible progression biomarkers. J Neurol. 2021 Jan;268(1):189-198. doi: 10.1007/s00415-020-10127-8. Epub 2020 Aug 4. PMID: 32749600; PMCID: PMC7815618.

Russo M, Obici L, Bartolomei I, Cappelli F, Luigetti M, Fenu S, Cavallaro T, Chiappini MG, **Gemelli C**, Pradotto LG, Manganelli F, Leonardi L, My F, Sampaolo S, Briani C, Gentile L, Stancanelli C, Di Buduo E, Pacciolla P, Salvi F, Casagrande S, Bisogni G, Calabrese D, Vanoli F, Di Iorio G, Antonini G, Santoro L, Mauro A, Grandis M, Di Girolamo M, Fabrizi GM, Pareyson D, Sabatelli M, Perfetto F, Rapezzi C, Merlini G, Mazzeo A, Vita G. ATTRv amyloidosis Italian Registry: clinical and epidemiological data. Amyloid. 2020 Dec;27(4):259-265. doi: 10.1080/13506129.2020.1794807. Epub 2020 Jul 22. PMID: 32696671.

Geroldi A, Prada V, Veneri F, Trevisan L, Origone P, Grandis M, Schenone A, **Gemelli C**, Lanteri P, Fossa P, Mandich P, Bellone E. Early onset demyelinating Charcot-Marie-Tooth disease caused by a novel in-frame isoleucine deletion in peripheral myelin protein 2. J Peripher Nerv Syst. 2020 Jun;25(2):102-106. doi: 10.1111/jns.12375. Epub 2020 May 4. PMID: 32277537.

Panicucci C, Traverso M, Baratto S, Romeo C, Iacomino M, **Gemelli C**, Tagliafico A, Broda P, Zara F, Bruno C, Minetti C, Fiorillo C. Novel TRIM32 mutation in sarcotubular myopathy. Acta Myol. 2019 Mar 1;38(1):8-12. PMID: 31309175; PMCID: PMC6598407.

Callegari I, **Gemelli C**, Geroldi A, Veneri F, Mandich P, D'Antonio M, Pareyson D, Shy ME, Schenone A, Prada V, Grandis M. Mutation update for myelin protein zero-related neuropathies

and the increasing role of variants causing a late-onset phenotype. J Neurol. 2019 Nov;266(11):2629-2645. doi: 10.1007/s00415-019-09453-3. Epub 2019 Jul 5. PMID: 31278453.

Pareyson D, Stojkovic T, Reilly MM, Leonard-Louis S, Laurà M, Blake J, Parman Y, Battaloglu E, Tazir M, Bellatache M, Bonello-Palot N, Lévy N, Sacconi S, Guimarães-Costa R, Attarian S, Latour P, Solé G, Megarbane A, Horvath R, Ricci G, Choi BO, Schenone A, **Gemelli C**, Geroldi A, Sabatelli M, Luigetti M, Santoro L, Manganelli F, Quattrone A, Valentino P, Murakami T, Scherer SS, Dankwa L, Shy ME, Bacon CJ, Herrmann DN, Zambon A, Tramacere I, Pisciotta C, Magri S, Previtali SC, Bolino A. A multicenter retrospective study of charcot-marie-tooth disease type 4B (CMT4B) associated with mutations in myotubularin-related proteins (MTMRs). Ann Neurol. 2019 Jul;86(1):55-67. doi: 10.1002/ana.25500. Epub 2019 May 27. PMID: 31070812; PMCID: PMC6581441.

Gemelli C, Prada V, Fiorillo C, Fabbri S, Maggi L, Geroldi A, Gibertini S, Mandich P, Trevisan L, Fossa P, Tagliafico AS, Schenone A, Grandis M. A novel mutation in the N-terminal acting-binding domain of Filamin C protein causing a distal myofibrillar myopathy. J Neurol Sci. 2019 Mar 15;398:75-78. doi: 10.1016/j.jns.2019.01.019. Epub 2019 Jan 17. PMID: 30685713.

Tini G, Vianello PF, **Gemelli C**, Grandis M, Canepa M. Amyloid Cardiomyopathy in the Rare Transthyretin Tyr78Phe Mutation. J Cardiovasc Transl Res. 2019 Dec;12(6):514-516. doi: 10.1007/s12265-018-9859-0. Epub 2019 Jan 2. PMID: 30604309.

11 ACKNOWLEDGEMENTS

Several thanks are needed, because of the contributions and support I received during these years.

Thanks to Prof. Marina Grandis, for all precious advice and teaching, for giving me the chance to keep on my PhD program despite my job in hospital and for believing in me through these years.

Thanks to Prof. Angelo Schenone, for welcoming me in the Neurologic Clinic of Genova, where I learnt to be a neurologist, and for backing my job.

Thanks to Prof. Chiara Fiorillo for the introduction to the muscle biopsies.

Thanks to the laboratory of Medical Genetics of IRCCS G. Gaslini Institute and IRCCS Policlinico San Martino for all genetic analysis, to Prof. Paola Mandich and Emilia Bellone for the fundamental contribution to this work and, in particular, to Monica Traverso and Alessandro Geroldi for all explanation of genetic results.

Thanks to neuromuscular unit in AOU Federico II in Naples and Molinette Hospital in Turin for the collaboration to my study.