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INVESTIGATING MYOPATHIC CAUSES OF RHABDOMYOLYSIS

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SUMMARY

Rhabdomyolysis is an acute, and frequently severe, pathological event, characterized by rapid necrosis and destruction of striated muscle tissue. It is clinically characterized by muscle pain, weakness and emission of dark urine. The mechanisms that lead to rhabdomyolysis are various and different, and share common alterations such as dysfunction of the pumps Na + / K + and Ca2 + ATPase, and rupture of the sarcolemma, which in turn determine calcium homeostasis alterations, mitochondrial dysfunction, proteases activation, reduced availability of ATP and, overall, cell apoptosis and rhabdomyolysis. Several causes can initiate this vicious circle, both acquired and genetic. The acquired causes include the crush syndrome, numerous toxic, disendocrine diseases, but also intense exercise in favouring conditions (deconditioning, fasting, heat, etc.). Among the genetic causes we must include disorders of glucose metabolism (glycogen storage disease type V, VII, etc.), lipid metabolism (CPT2, VLCAD, etc.), mitochondrial diseases, some muscular dystrophies, diseases related to the metabolism of calcium (RYR1) and many others.

When facing an episode of rhabdomyolysis, the identification of the etiological cause can be extremely complex, long, and costly. Nevertheless, the correct identification of the underlying cause is of utmost importance for the correct information regarding prognosis (risk of recurrence) and for the family genetic counselling. The diagnostic tools currently in use are: profile of acylcarnitines (for the diagnosis of lipid myopathy); grip test (curve of lactate after forearm strain); neurophysiological tests (ENG and needle EMG); muscle biopsy with biochemical assays and analysis of muscle protein; and molecular analysis of candidate genes.

Despite the application of an extended diagnostic work up, however, frequently it is not easy to distinguish if the rhabdomyolysis episode is due to a genetic disorder or whether it is the result of an abnormal effort, of an infectious episode or effect of a toxic. In recent years, the evolution of molecular techniques allowed to develop methods for the analysis of exomes ("whole exome sequencing") or of several genes involved in specific diseases ("gene panels"). These techniques allowed us to increase the detection rate, reducing time and cost analysis, and have identified new

genes associated with myopathies. At the same time, it eased the identification of genetic variants whose pathogenicity is still doubtful and it is still far from identifying all molecular mechanisms underlying rhabdomyolysis. A careful analysis of the clinical picture of the patient, the correct choice of diagnostic tools and their careful evaluation is mandatory for the proper selection and interpretation of molecular investigations.

The main objective of this study is, therefore, the evaluation of the diagnostic process of cases of rhabdomyolysis, to propose an updated, comprehensive, rational and cost effective protocol based on the latest information and techniques.

The first objective of the study (aim 1) was the analysis of the clinical features and diagnostic procedures performed in patients with rhabdomyolysis. This retrospective study included all the patients referred for rhabdomyolysis to a Reference Centre for Neuromuscular Diseases (n = 208, 2000 - 2016). We verified that, despite an extensive work up (EMG, muscle biopsy, acylcarnitines, biochemical and genetic analyses), the diagnosis of genetic myopathy was done only in 41 patients (GSDV, CPT2, RYR1 and other) and of rhabdomyolysis of non-genetic origin only in 49 ("pure exertional rhabdomyolysis", toxic, infectious, etc.). In 118 patients, the cause could not be identified. Some diagnostic tests have shown high specificity and sensitivity for specific diseases: grip tests in the diagnosis of McArdle's disease and in phosphoglucomutase 1 deficit; profile of acylcarnitines in VLCAD and CPT2 deficiency; muscle biopsy in McArdle's disease and in some muscular dystrophies. Overall, however, the diagnostic tools were insufficient in guiding the diagnostic process to a defined etiologic diagnosis, as a molecular diagnosis was acquired only in 20% of patients.

The second aim of the study (aim 2) was the clinical, radiological, and molecular characterization of patients affected by a recently identified myopathy, the CASQ1 –related myopathy, that here we identified as potentially complicated by rhabdomyolysis. The disease has been identified in several Italian patients, all carrying the same mutation (p.Asp244Gly) because of a founder effect. Screening of the biobank and subsequent genetic analysis of the gene (with "ARMS-PCR", and sequencing) led us to identify the common mutation in 16 patients, and a novel mutation (p.Gly103Asp) in another. Clinical characteristics and evolution of the disease were evaluated by

medical record review, ad hoc structured questionnaires, multi-disciplinary clinical evaluation (neurological, cardiac and pulmonary clinic), and muscle MRI. In the present study, we show that this disease, which was considered benign, is a slowly progressive myopathy frequently manifesting iperCKemia, exertional myalgias and rhabdomyolysis. We also provide information about the clinical features, muscle imaging and histopathology that orient towards the diagnosis of CASQ1-related myopathy.

Aim 3 was to determine the clinical spectrum of limb-girdle muscular dystrophy 2E (LGMD2E), and to investigate the risk of rhabdomyolysis of this disease. We also investigated whether genetic or biochemical features can predict the phenotype of the disease. We included 32 European patients (16M-16F; age 7-67) in a multicentre study, with a specific multidisciplinary clinical protocol and *ad hoc* questionnaires. Molecular analysis of SGCB gene and biochemical features of muscle biopsies were also reviewed. All patients presented clinical features compatible with LGMD. We did not identify any episode of rhabdomyolysis. However, 75% of ambulant patients and no wheelchair-bound patients reported muscle pain. This suggests a membrane fragility that in certain cases could therefore also determine the more severe rhabdomyolysis. We also confirmed that cardiac involvement is frequent (63%) even before overt muscle involvement. We finally provide strong evidence that severity of clinical involvement may be predicted by SGCB gene mutation and sarcoglycan protein expression.

Aim 4 focused on diagnostic tools to be used in cases of rhabdomyolysis, and in particular on the role of EMG with provocative test: the Long Exercise Test (LET) for the diagnosis of McArdle disease (GSDV). We included 25 GSDV patients, 25 patients with rhabdomyolysis from other causes, and 14 healthy controls. All patients underwent to a complete neurophysiological protocol including standard ENG and EMG, repetitive stimulation, short exercise test and LET. LET in GSDV patients showed a marked and immediate reduction of compound action potential (CMAP) amplitude. This anomaly is indicative of membrane inexcitability that occurs after exertion in GSDV patients. We therefore demonstrated the role of the LET in the diagnosis of GSDV, to discriminate it from rhabdomyolysis due to other causes. LET can also be used as an outcome measure, because it is related to the severity of symptoms and is consistent with post-exercise symptoms

reported by patients. Furthermore, it is an in vivo evaluation of pathophysiological processes underlying the disease beyond the simple glycolytic blockade.

Aim 5 consisted in the creation of a multi gene panel that should be studied by Next Generation Sequencing (NGS) in patients presenting rhabdomyolysis. This should include at least the 32 genes involved in mechanisms that are known to be disrupted in rhabdomyolysis and in pathologies that are known to manifest with rhabdomyolysis, such as glycogen metabolism, lipid metabolism, and calcium homeostasis disorders.

In conclusion, the results of this study (the diagnostic process currently in use and the identification of rare causes of rhabdomyolysis; the identification of *CASQ1* as a new genetic cause of rhabdomyolysis; and the demonstration of the role of EMG with LET as a sensitive and specific diagnostic test in the work up of rhabdomyolysis), suggest a new diagnostic algorithm for rhabdomyolysis. In this algorithm, the clinical features and few first-line tests (blood tests, EMG, acylcarnitines, grip test) exclude the most frequent causes or the treatable ones. The subsequent muscle biopsy will identify certain myopathies and guide toward the use of specific genetic panels for metabolic myopathies, muscular dystrophies or mitochondrial diseases that should be studied by NGS. Any identified variation will be confirmed according to clinical and laboratory data. The “pure exertional rhabdomyolysis" remains a diagnosis of exclusion in most cases, and in these cases we cannot exclude genetic variations that affect the physiological response to exercise. The diagnostic algorithm that we propose will allow cost reduction and time optimization, and hopefully will increase the rate of etiological diagnosis of rhabdomyolysis, a serious event with major impact on patients' lives that, to date, remains poorly diagnosed.

Riassunto

La rabdomiolisi è un evento patologico acuto e talvolta grave, caratterizzato da rapida necrosi e distruzione del tessuto muscolare striato alla quale corrisponde clinicamente dolore e debolezza muscolare ed emissione di urine scure. I meccanismi che portano alla rabdomiolisi sono vari e differenti ma condividono alterazioni comuni quali la disfunzione delle pompe Na^+/K^+ e Ca^{2+} ATPAsi, e la rottura del sarcolemma, che a loro volta determinano alterazioni dell'omeostasi del calcio, disfunzione mitocondriale, attivazione di proteasi, riduzione della disponibilità di ATP e, complessivamente, apoptosi cellulare e rabdomiolisi. Numerose cause possono dare avvio a tale circolo vizioso, sia acquisite che genetiche. Le cause acquisite includono la sindrome da schiacciamento, numerosi tossici, malattie disendocrine, ma anche e soprattutto l'esercizio fisico intenso specie in condizioni favorenti (decondizionamento, digiuno, calore, etc.). Tra le cause genetiche si annoverano patologie del metabolismo glucidico (glicogenosi tipo V, VII, etc.) e lipidico (CPT2, VLCAD, etc.), malattie mitocondriali, alcune distrofie muscolari, patologie correlate al metabolismo del calcio (RYR1) e molte altre.

La corretta identificazione della causa sottostante è di estrema importanza per una corretta informazione al paziente a fini prognostici (rischio di ricorrenza) e per il counseling genetico familiare. Di fronte ad un episodio di rabdomiolisi, l'iter per determinare la causa etiologica può essere lungo, complesso e costoso. Tra gli strumenti diagnostici attualmente in uso vi sono il profilo delle acilcarnitine (per la diagnosi di miopatia lipidica); il test da sforzo anaerobico per il lattato detto "grip test" (curva del lattato dopo sforzo dell'avambraccio); l'EMG ad ago (per individuare eventuali segni di miopatia);la biopsia muscolare con eventuali dosaggi biochimici e analisi delle proteine muscolari; ed infine l' analisi molecolare di geni candidati.

Nonostante l'applicazione di questo complesso work up diagnostico, non è sempre facile determinare se alla base di un episodio di rabdomiolisi vi sia una patologia genetica o se sia esclusivamente conseguenza di uno sforzo, di un episodio infettivo o dell'effetto di un tossico. Negli ultimi anni, l'evoluzione delle tecniche molecolari ha permesso di mettere a punto metodiche per l'analisi dell'esoma ("whole exome sequencing") o per l'analisi simultanea di numerosi geni

coinvolti in malattie specifiche ("gene panels"). Tali tecniche hanno permesso di aumentare il numero delle diagnosi, riducendo i tempi e i costi dell'analisi ed hanno permesso di identificare nuovi geni associati a miopatie con rabdomiolisi. Contemporaneamente all'aumento della identificazione di geni causativi di rabdomiolisi è aumentata anche l'identificazione di varianti genetiche la cui patogenicità è dubbia e che pongono notevoli difficoltà nella consulenza genetica. Nonostante tutti questi avanzamenti diagnostico-molecolari si è ancora lontani dall'identificazione di tutti i meccanismi patogenetici ed al riconoscimento di tutti i geni alla base delle rabdomiolisi.

Un'attenta analisi del quadro clinico del paziente, la corretta scelta degli strumenti diagnostici ed una valutazione attenta dell'esito degli stessi è necessaria per indirizzare le indagini molecolari. L'obiettivo principale del presente studio è stato, pertanto, definire l'iter diagnostico ottimale in caso di rabdomiolisi, al fine di proporne uno aggiornato, completo, razionale ed economico che includa le più recenti nozioni e tecniche.

Il primo obiettivo dello studio (aim 1) è stato l'analisi delle caratteristiche cliniche e delle procedure diagnostiche eseguite in pazienti con rabdomiolisi. Lo studio retrospettivo ha incluso tutti i pazienti giunti per rabdomiolisi ad un Centro di Riferimento per Malattie Neuromuscolari (n = 208, periodo 2000 – 2016). Abbiamo verificato come, malgrado l'esteso work up al quale sono stati sottoposti i pazienti (EMG, acilcarnitine, biopsia muscolare, indagini biochimiche e genetiche), si è giunti alla diagnosi di miopatie genetiche solo in 41 pazienti (~20%) (GSDV, CPT2, RYR1 e altre) e di rabdomiolisi su base non-genetica solo in 49 (~23%) (rabdomiolisi da sforzo "pura", cause tossiche, infettive, etc.). In 118 pazienti la causa non è stata identificata. Alcuni test diagnostici hanno dimostrato elevata specificità e sensibilità in determinate patologie: "grip test" nella diagnosi di Malattia di McArdle e nel deficit di fosfoglucomutasi 1; profilo delle acilcarnitine nel deficit di VLCAD e CPT2; biopsia muscolare nella malattia di McArdle e in alcune distrofie muscolari. Complessivamente gli strumenti finora in uso si sono dimostrati insufficienti nel guidare l'iter diagnostico verso una diagnosi eziologica definita considerando che nel 57% dei pazienti non sono state identificate le cause degli episodi di rabdomiolisi.

Il secondo obiettivo dello studio (aim 2) è stata la caratterizzazione clinica, radiologica, e

molecolare dei pazienti affetti da una miopatia, talora associata a rabdomiolisi, che il nostro gruppo ha recentemente identificato, la miopatia da mutazioni nel gene *CASQ1.* Sulla base delle caratteristiche istologiche identificate nei primi pazienti descritti è stato condotto uno screening della nostra biobanca per l'individuazione di pazienti candidati. Questi sono stati sottoposti ad analisi del gene *CASQ1* con "ARMS-PCR" e sequenziamento. In In 16 pazienti è stata identificata la mutazione comune p.Asp244Gly ed in un singolo paziente una mutazione mai descritta,p.Gly103Asp. Lo studio degli aplotipi nei pazienti con la mutazione comune ha permesso di ipotizzare un effetto del portatore. Le caratteristiche cliniche e l'evoluzione della malattia sono state valutate mediante revisione delle cartelle cliniche, questionari strutturati creati ad hoc, valutazione clinica multidisciplinare (neurologica, cardiologica e pneumologica) e RM muscolare. Nel presente studio dimostriamo che la miopatia *CASQ1*-relatasia una miopatia lentamente evolutiva che si manifesta frequentemente con iperCKemia, mialgie da sforzo e rabdomiolisi. Descriviamo anche le caratteristiche cliniche, di imaging muscolare e di istopatologia, che possono orientare verso la diagnosi.

Il terzo obiettivo (aim 3) è stato lo studio, in un'ampia coorte di pazienti europei (Italia, Francia, Danimarca), delle caratteristiche cliniche della distrofia muscolare 2E (LGMD2E) ed in particolare dell'occorrenza di rabdomiolisi. Sono stati quindi raccolti dati anamnestici, mediante revisione delle cartelle cliniche e raccolta di questionari *ad hoc*, ed è stato proposto uno specifico protocollo di valutazione clinica, multidisciplinare. Sono stati anche valutati i fattori genetici o istopatologici possano essere predittivi del fenotipo Sono stati inclusi nello studio 32 pazienti (16M-16F; età 7-67 anni). Tutti i pazienti presentavano un quadro clinico tipico per una distrofia dei cingoli. Non sono stati evidenziati episodi di rabdomiolisi; tuttavia il 75% dei pazienti adulti riferiva dolore muscolare cronico, che invece non era mai presente nei pazienti non deambulanti. Cio' suggerisce la presenza di fragilità di membrana che potrebbe in talune circostanze condurre anche a rabdomiolisi. Nello studio è inoltre stato osservato un interessamento cardiaco in oltre la metà dei pazienti (63%), ed un'insufficienza respiratoria tale da richiedere ventilazione meccanica nel 19%. È stato inoltre possibile determinare una correlazione tra genotipo, espressione proteica e severità di malattia.

Il quarto obiettivo dello studio (aim 4) è stato centrato sugli strumenti diagnostici utilizzabili in caso di rabdomiolisi, ed in particolare sul ruolo dell'EMG con test provocativi come il test di esercizio prolungato per la diagnosi della malattia di McArdle (GSDV). Sono stati confrontati 25 pazienti GSDV, 25 pazienti con rabdomiolisi da altre cause e 14 controlli sani. Tutti sono stati sottoposti a protocollo neurofisiologico completo con EMG ed ENG standard, stimolazione ripetitiva, test di esercizio breve e prolungato. Il test di esercizio prolungato (Long Exercise Test, LET) ha evidenziato una netta e immediata riduzione del potenziale d'azione composto (CMAP) dopo esercizio nei pazienti GSDV. Tale anomalia è indicativa dell'ineccitabilità di membrana che si manifesta dopo sforzo in questi pazienti. Abbiamo quindi dimostrato il ruolo del LET nella diagnosi di GSDV, in particolare nel discriminarla dalla rabdomiolisi da altre cause. Abbiamo anche dimostrato come il test possa essere utilizzato come misura di outcome perché correlato alla gravità dei sintomi e coerente con i sintomi post-esercizio riferiti dai pazienti, e come strumento di valutazione *in vivo* dei processi fisiopatologici alla base della malattia. I risultati del test LET nella GSDV suggeriscono che anche in questa malattia l'ineccitabilità di membrana secondaria al blocco della produzione di ATP e alla disfunzione dei canali di membrana e del reticolo giochi un ruolo patogenetico significativo.

Il quinto obiettivo dello studio (aim 5) è stata la creazione di uno specifico pannello di geni da studiare mediante Next Generation Sequencing (NGS) nei pazienti che presentino rabdomiolisi. Sono stati selezionati pertanto i geni potenzialmente coinvolti nei meccanismi alla base delle rabdomiolisi, ovvero quelli principalmente coinvolti nel metabolismo lipidico, glucidico e nell'omeostasi del calcio intracellulare.

In conclusione, con le evidenze raccolte nel presente studio (la definizione dello stato dell'arte dell'iter diagnostico correntemente in uso nello studio dei pazienti con rabdomiolisi, l'identificazione di una causa rara di rabdomiolisi, la miopatia *CASQ1*-relata, e la dimostrazione del ruolo dell' EMG con test di esercizio prolungato nell'identificazione dei pazienti con GSDV), proponiamo un nuovo algoritmo diagnostico per le rabdomiolisi. In tale algoritmo le caratteristiche cliniche e pochi test di primo livello (esami ematochimici, EMG, profilo delle acilcarnitine, "grip test") permettono di escludere le cause più frequenti o trattabili. La biopsia muscolare, esame di

secondo livello, permetterà di identificare talune miopatie e guiderà il ricorso a specifici pannelli genetici per le miopatie metaboliche, le distrofie muscolari dei cingoli e le malattie mitocondriali. La rabdomiolisi da sforzo “pura” rimane una diagnosi di esclusione nella maggior parte dei casi. In questi casi lo studio di variazioni genetiche polimorfiche potrebbe portare all’identificazione di modificatori genetici della risposta all’esercizio. L’algoritmo diagnostico proposto permetterà però di contenere i costi ed ottimizzare i tempi della diagnosi, e auspicabilmente aumenterà il rate di diagnosi etiologiche della rabdomiolisi, un evento grave e con un notevole impatto sulla vita dei pazienti ma che ad oggi resta scarsamente diagnosticato.

INTRODUCTION

The word rhabdomyolysis uses the combining forms rhabdo- + myo- + -lysis, yielding a generic "striated muscle breakdown". Clinically, rhabdomyolysis is an acute, potentially life-threatening, syndrome characterised by the breakdown of skeletal muscle resulting in the subsequent release of intracellular contents into the circulatory system. These include enzymes such as creatine kinase (CK), glutamic oxalacetic transaminase, lactate dehydrogenase, aldolase, the haeme pigment myoglobin, electrolytes such as potassium and phosphates, and purines (Warren 2002; Huerta-Alardın et al, 2005; Khan et al, 2009; Zutt et al, 2014; Chavez 2016)

DEFINITION

The clinical definition of rhabdomyolysis is still debated. In fact, the normal physiological responses to strenuous exercise and clinically significant exertional rhabdomyolysis (ER) have considerable overlap in their major clinical and serological markers. The main clue in rhabdomyolysis is the observation of increased CK level. However, there is no agreement on which value of CK is significant to define a rhabdomyolysis. The most recent laboratory diagnostic guidelines and clinical studies (mostly proposed for internal medicine purposes, such as to evaluate statin side effects) define rhabdomyolysis with different CK levels. Rhabdomyolysis is usually defined when CK levels are 5 to 10 times the ULN (>1,000 U/L). These limits, however, have a too low specificity, including also physiological responses to exercise or effort, thus limiting its role in the diagnosis of exertional rhabdomyolysis, for example in military trainees or athletes. Other studies proposed CK levels of > 50 ULN (corresponding to a > 12,000 IU/L). This more stringent limit improves specificity, but would exclude several patients with potential subjacent muscle diseases. Futhermore, it is important to observe not only the peak of CK level, but also its relationship with rest (or inter-critical) level. In fact, CK levels very high, even greater than 15,000 U/L, can be observed in several muscular dystrophies (Harris et al, 2016). In such cases however these values are persistent (Pasternak et al, 2002; McKenney et al, 2006; Sewright et al, 2007)

It is not possible to define rhabdomyolysis only by its clinical features. In fact, muscle pain and soreness after effort are very frequent among healthy subjects . On the other hand, the

appearence of dark-colored urines is not always present also in episodes of overt rhabdomyolyis with very high CK levels. The combination of clinical and biochemical information (symptoms, CK level, triggers, etc.) should therefore be used to correctly identify the episodes of rhabdomyolysis. The severity of the event, then, should to be classified according to CK levels and complications.

CLINICAL FEATURES OF RHABDOMYOLYSIS

Rhabdomyolysis ranges in severity from asymptomatic elevation of CK levels in blood, to severe life-threatening episodes associated with very high CK levels, myoglobinuria and acute renal failure, multi-systemic failure. Clinically it manifests with a triad of symptoms: myalgia, weakness, and myoglobinuria, observed as the classically described tea-colored (or “coca-cola” or “porto”) urine. This rigid depiction of symptoms can be misleading as the triad is only observed in <10% of patients. Patients may also report tense and swollen muscles or muscle edema. Systemic manifestations may include tachycardia, general malaise, fever, and nausea and vomiting and as such are nonspecific. The clinical manifestations of acute kindey failure (AKI), disseminated intravascular coagulation, and multiorgan failure may subsequently appear.

COMPLICATIONS OF RHABDOMYOLYSIS AND TREATMENT

Major complications of rhabdomyolysis and their treatment are shown in Figure 1.

Myoglobinuria occurs only in the context of rhabdomyolysis. Acute kidney injury (AKI) associated with myoglobinuria is the most serious complication of both traumatic and nontraumatic rhabdomyolysis, and it may be life threatening. An estimated 10%-40% of patients with rhabdomyolysis develop AKI, and up to 15% of all cases of ARF can be attributed to rhabdomyolysis (Kasaoka et al 2010). The exact mechanisms by which rhabdomyolysis impairs the glomerular filtration rate are unclear. Experimental evidence suggests that intrarenal vasoconstriction, direct and ischemic tubule injury, and tubular obstruction all play a role (Bosch et al, 2009).

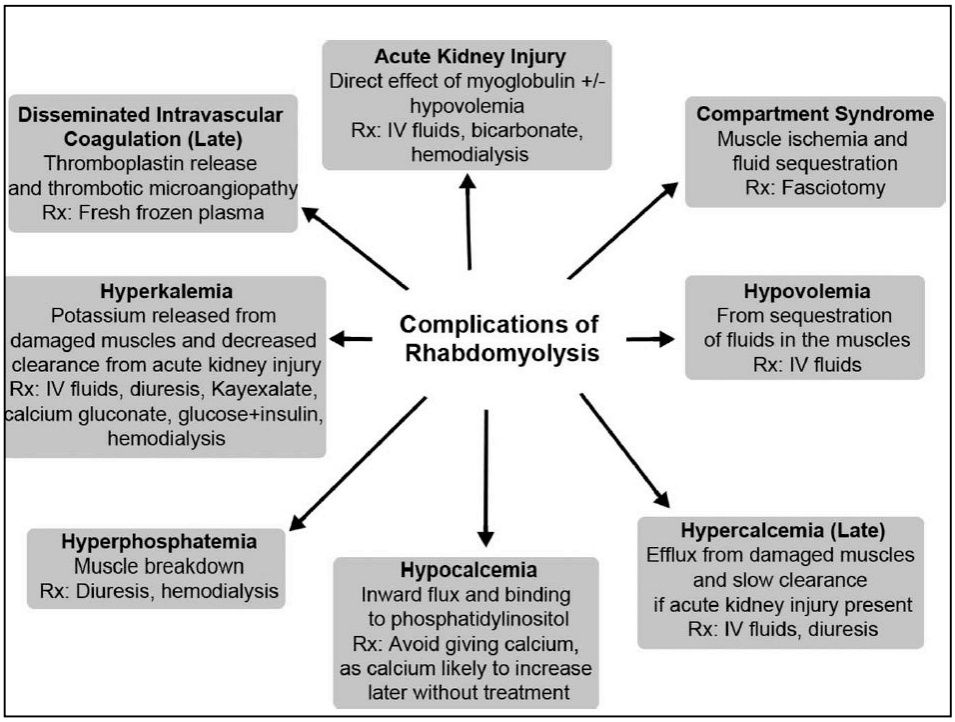


Fig. 1. Complications of rhabdomyolysis and treatments *(from Torres et al, 2015)*

PATHOMECHANISMS

Although the causes of rhabdomyolysis are very diverse, the pathogenesis appears to follow a final common pathway, ultimately leading to myocyte destruction and release of muscle components into the circulation. Irrespective of the initial insult, the final steps involve either direct myocyte injury or a failure of the energy supply within the muscle cells (Fig. 2).

In the normal myocyte, several sarcolemmal pumps accurately regulate cellular electrochemical gradients. The intercellular sodium concentration is normally maintained at 10 mEq/l by a sodium-potassium adenosine triphosphatase (Na/K-ATPase) pump located in the sarcolemma. The Na/K-ATPase pump actively transports sodium from the interior of the cell to the exterior. As a result, the interior of the cell is more negatively charged than the exterior because positive charges are transported across the membrane. The gradient pulls sodium to the interior of the cell in exchange for calcium by a separate ion exchange channel. Moreover, low intracellular calcium levels are also maintained by an active calcium exchanger (Ca2+ ATPase pump) that promotes calcium entry into the sarcoplasmic reticulum and mitochondria. The above processes depend on ATP as a source of energy. When muscle injury or ATP depletion occurs, the result is an excessive intracellular influx of Na^+ and Ca^{2+}. ATP depletion results in Na/K-ATPase and Ca^{2+} ATPase pump dysfunction, the

end result of which is an increased cellular permeability to sodium ions. Accumulation of sodium in the cytoplasm leads to an increase in intracellular calcium concentration, that leads to a sustained myofibrillar contraction that further depletes ATP. Futhermore, Ca^{2+} concentration determines the activation of intracellular proteolytic enzymes that degrade the muscle cell. Futhermore, the increase in intracellular Na^{+} draws water into the cell and disrupts the integrity of the intracellular space (Guis et al, 2005; Zutt et al, 2014; Torres et al, 2015; Olpin et al, 2015).

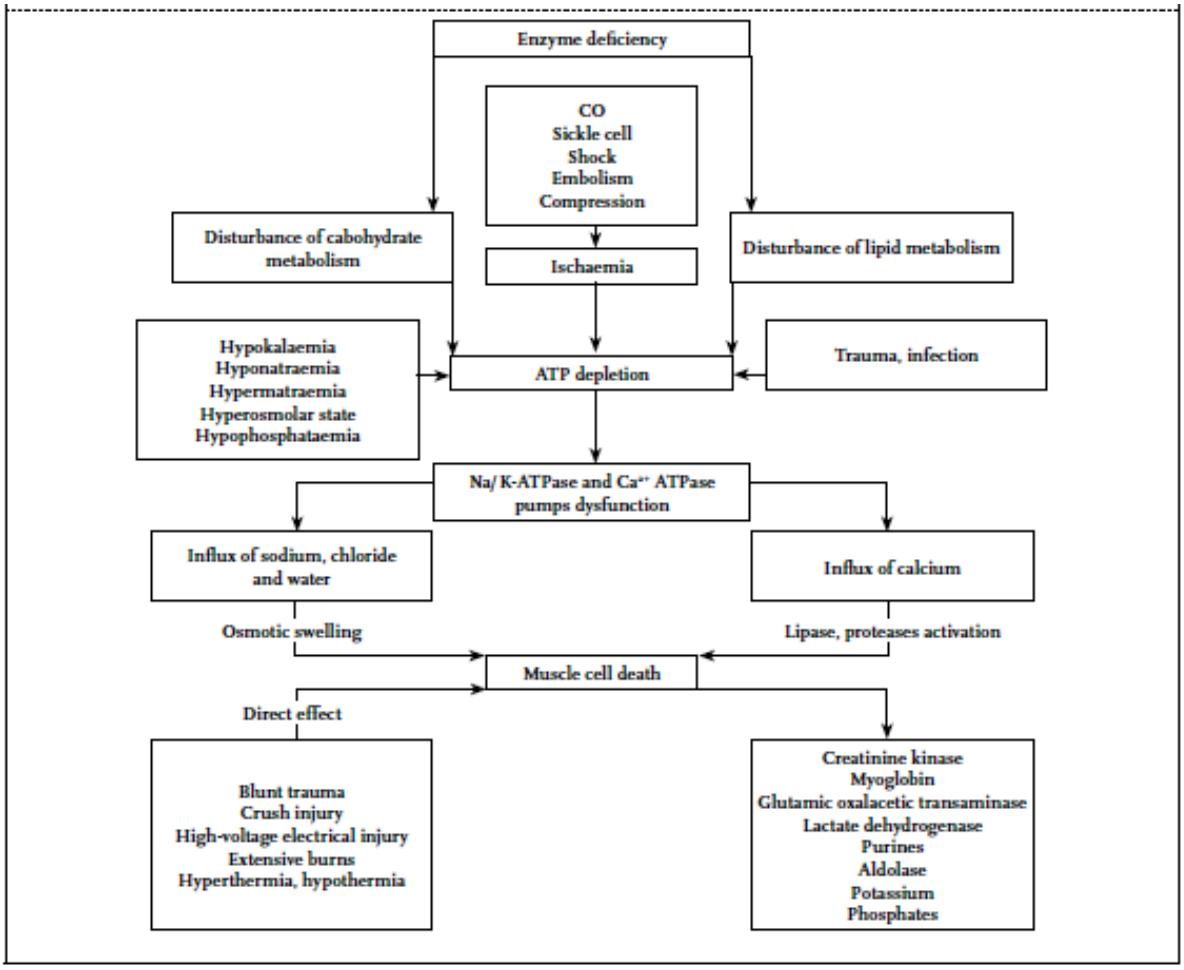


Fig. 2. Mechanisms of rhabdomyolysis *(From Kahn et al, 2009).*

As the myocyte degenerates, large quantities of potassium, aldolase,phosphate, myoglobin, CK, lactate dehydrogenase, aspartate transaminase and urate leak into the circulation. Under physiological conditions, the plasma concentration of myoglobin is very low (0 to 0.003 mg per dl). If more than 100 g of skeletal muscle is damaged, the circulating myoglobin levels exceed the

protein-binding capacity of the plasma and can precipitate in the glomerular filtrate. Excess myoglobin may thus cause renal tubular obstruction, direct nephrotoxicity, and acute renal failure.

CAUSES OF RHABDOMYOLYSIS

Several genetic and non-genetic (or acquired) causes of rhabdomyolysis have been identified (Fig. 3). It is to note that in individual cases both genetic and environmental factors may combine to trigger a rhabdomyolysis event.

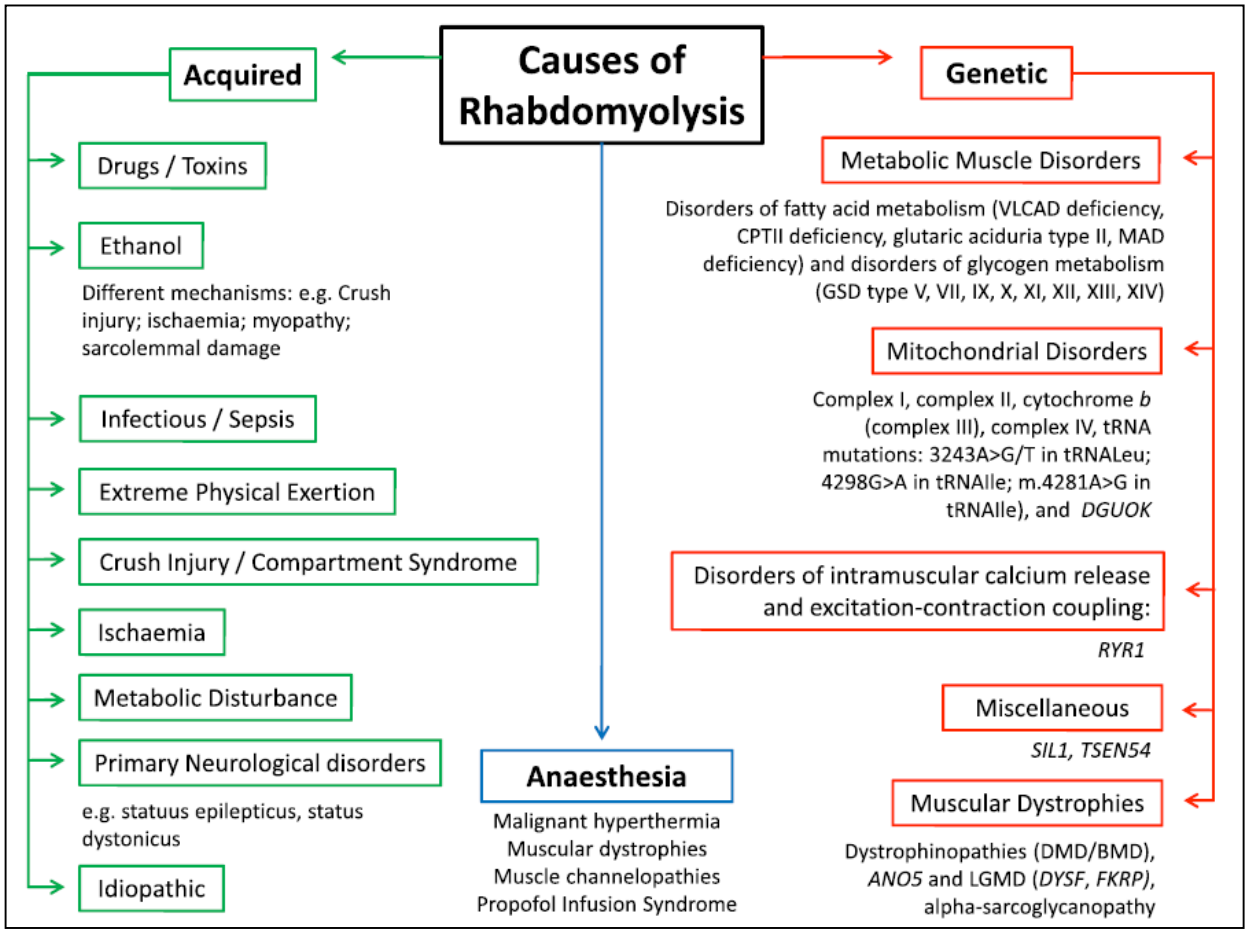


Fig. 3. Acquired and genetic conditions associated with Rhabdomyolysis..
(From Scalco et al, 2015)

Non-genetic (acquired) causes

The numerous non-genetic causes that have been demonstrated as cause of rhabdomyolysis are extensively shown in Table I. *"Crush syndrome"* is the term usually used to describe muscle destruction after direct trauma, injury, or compression. *Exercise/Exertion*: the occurrence of rhabdomyolysis has been especially studied in military personnel enrolled in basic military training; the incidence of exertional rhabdomyolysis was estimated 22.2 cases per 100,000 recruits per year (Alpers et al, 2010). Exertional rhabdomyolysis is usually characterized by low incidence in an active population, few complications relative to other causes in the acute setting, and infrequent long-term recurrence after return to prior levels of physical activity (Huynh et al, 2016; Lozowska et al, 2015). It is to note that serum CK levels will naturally rise after strenuous exercise in almost all normal humans, potentially up to 10 times the higher limit of normal, thus the limit between normal response to exercise and overt rhabdomyolysis is blurred (Kobayashi et al, 2005; Kenney et al, 2012). *Toxic:* the use of several drugs can determine rhabdomyolysis, via all the previously described pathomechanisms. The complete list is shown in Table I. Due to their large use in the global population, statins are the most frequently involved as cause of rhabdomyolysis. However, even if signs of myopathic toxicity are frequently reported (myalgias is observed in up to 10% of statin users), episodes of overt rhabdomyolysis remain a rare side effect (Joy and Hegele, 2009; Iwere et al, 2015; Collins et al, 2016) . *Infections:* Rhabdomyolysis has been described in all types of infections, ranging from localized muscle infections with erythema (bacterial pyomyositis) to patients with sepsis and no direct muscle infection. Proposed mechanisms for the development of rhabdomyolysis include tissue hypoxia secondary to sepsis or dehydration, toxin release, associated fever, direct bacterial invasion of muscle, or rigors/tremors. Classically, Legionella bacteria have been associated with bacterial rhabdomyolysis. Viral infections have also been implicated in rhabdomyolysis development, most commonly influenza A and B viruses (Tseytlin et al, 2016).

Table I. Non genetic causes of rhabdomyolysis *(modified from Warren et al, 2002)*

Exertion
Exercise; status epilepticus; delirium; psychosis; electric shock, electroconvulsive therapy; prolonged cardiopulmonary resuscitation and cardioversion; status asthmaticus; tetanus; prolonged myoclonus, dystonia or chorea; conga drumming; keyboard operation; raver's hematuria
Crush
External weight; prolonged immobility (including coma, Parkinson's disease); exaggerated lithotomy position and other surgical positions; "pseudo-crush" syndrome (torture victims, child abuse); pneumatic antishock garment
Ischemia
Arterial occlusion; compartment syndrome; cardiopulmonary bypass; vena cava ligation; sickle cell disease; air embolism; atrial myxoma; diabetes mellitus; increased capillary permeability syndrome
Metabolic
Hypokalemia; diabetic ketoacidosis; nonketotic hyperglycemic/hyperosmolar states; hyper/hyponatremia; hypophosphatemia;hypothyroidism; near drowning; renal tubular acidosis; pancreatitis
Extremes of body temperature
Fever; burns; hypothermia (exposure, hypothyroidism)
Drugs and toxins
Metabolic
statins; anticholinergics; antidepressants (all classes); antihistamines (diphenhydramine, doxylamine); arsenic; azathioprine; barbiturates;benzodiazepines; bezafibrate; carbon monoxide; clofibrate; cytotoxics; ethanol; ethylene glycol; fenfluramine; gemfibrozil; glutethamide; interferon- ; methanol; naltrexone; opiates; propofol; oxprenolol; labetolol; paracetamol; podophyllin; zidovudine; streptokinase; alteplase
Hypokalemia
Amphotericin; carbenoxolone; glycirrhizate (licorice); itraconazole; laxative abuse thiazides and other kaliuretics
Ischemia
-Aminocaproic acid; cocaine; vasopressin
Autoimmune
Cyclosporin; levodopa; nonsteroidals; penicillamine; phenylbutazone; phenytoin; trimethoprim–sulfamethoxazole
Membrane effect
Carbon tetrachloride; cimetidine; colchicine; didanosine; hydrocarbons; herbicides; iron dextran; metal fumes; quinidine; solvents; detergents; succinylcholine; toluene; vecuronium, pancuronium; snake/spider/hornet/bee/fugu/parrotfish venoms
Agitation
Hemlock (? quail eaters); ketamine; lithium; LSD; mercuric chloride; phencyclidine; salicylates; terbutaline
Neuroleptic malignant syndrome
Butyrophenones; levodopa and dopamine agonist withdrawal; lithium; pimozide; promethazine; thioxanthenes
Serotonergic syndrome
Amphetamines; Ecstasy; lithium; monoamine oxidase inhibitors; nefazodone; pethidine; tricyclic antidepressants; tryptophan; venlafaxine
Mechanism uncertain
Amiodarone; blowpipe dart poisoning; chromium picolinate; isoniazid; kidney beans; lamotrigine; nicotinic acid; peanut oil; pentamidine; valproate
Infections
Viral
Adenovirus; cytomegalovirus (CMV); Coxsackie; Enterovirus; Epstein–Barr (EBV); human immunodeficiency virus (HIV); herpes simplex (HSV); influenza A and B; measles; Varicella zoster
Bacterial
Bacillus spp.; Borrelia burgdorferi; Brucella; Campylobacter; Clostridia; Coxiella; Escherichia coli; Enterobacter; H. influenzae; Legionella; Leptospira; Listeria; Salmonella; Shigella; Staphylococcus; Streptococcus; tetanus; typhoid;
Other
Aspergillus; Candida; Mycoplasma; Plasmodium; Toxoplasma; Trichinella
Inflammatory and autoimmune muscle disease
Polymyositis; dermatomyositis; vasculitides; carcinoma (paraneoplastic necrotizing myopathy)

Genetic Causes

Genetic causes of myopathic rhabdomyolysis include metabolic myopathies (glycogen or lipid metabolism impairment; mitochondrial disease), calcium-related myopathies (*RYR1* mutation and other triadopathies), muscular dystrophies and other diseases.

- Metabolic myopathies

Metaoblic myopathies are caused by enzymatic defects of the biochemical pathways that produce ATP. It can result either from defects in substrate utilization (disorders of glycogen and lipid metabolism) or mitochondrial respiratory chain (mitochondrial myopathies). Typically they are divided into two main clinical presentations: dynamic symptoms (exercise intolerance / myalgia / cramps, with recurrent rhabdomyolysis) or static symptoms (progressive muscle weakness mimicking muscular dystrophies). Although this division based on clinical presentation is very helpful, it should be known that patients with dynamic symptoms may also present with some degree of muscle wasting occurring during the course of the disease, and conversely patients with permanent muscle weakness may also have exercise-related symptoms of fatigue and pain. The present work will be focused focused on those pathologies potentially manifesting with the "dynamic symptoms", including rhabdomyolysis.

A) Muscle glycogenosis

Deficiencies of virtually all enzymes that intervene in the synthesis or degradation of glycogen may cause glycogen storage disease (GSD) due to aberrant storage or utilization of glycogen. These disorders are inherited as autosomal recessive traits, with the exception of phosphoglycerate kinase and phosphorylase b kinase deficiencies, which are X-linked recessive (DiMauro *et al.,* 2004). Usually patients with glycogenosis and exercise intolerance develop symptoms early in exercise, but exercise intensity at which symptoms occur can vary according to the residual activity of the affected enzyme.

Glycogen Storage Disease Type V (GSDV) or McArdle disease. McArdle disease is the most frequent "dynamic" metabolic myopathy, caused by the genetic deficiency of the glycogenolytic enzyme myophosphorylase. The enzymatic defect causes the impossibility to mobilize glycogen deposits during anaerobic metabolism, the reduction of pyruvate production in the tricarboxylic acid

cycle, and the subsequent oxidative phosphorylation impairment bring to reduction of AcetylCoA production, with a cyclic worsening of function of tricarboxylic acid cycle. The disease is clinically characterized by childhood-onset of fatigue and exercise induced myalgia, exercise intolerance, frequently complicated by episodes of myoglobinuria with acute renal failure due to recurrent rhabdomyolysis. Patients usually present the pathognomonic 'second wind' phenomenon, that is the possibility of patients to continue exercise after some minutes rest, when the pain occurs. It is due to the shift to fatty acid oxidation and to the availability of glucose from other sources. A small proportion of patients develop a progressive weakness of axial and proximal muscles, especially of the upper limbs.

Diagnosis is based on: i) typical phenotype; ii) grip test: no increase of lactate and hyper-ammonia in respone to hand grip effort (Fig. 4); iii) muscle biopsy: increase in glycogen content, typical subsarcolemmal vacuolar blebs and no reactivity to myophosphorylase histochemical stain.

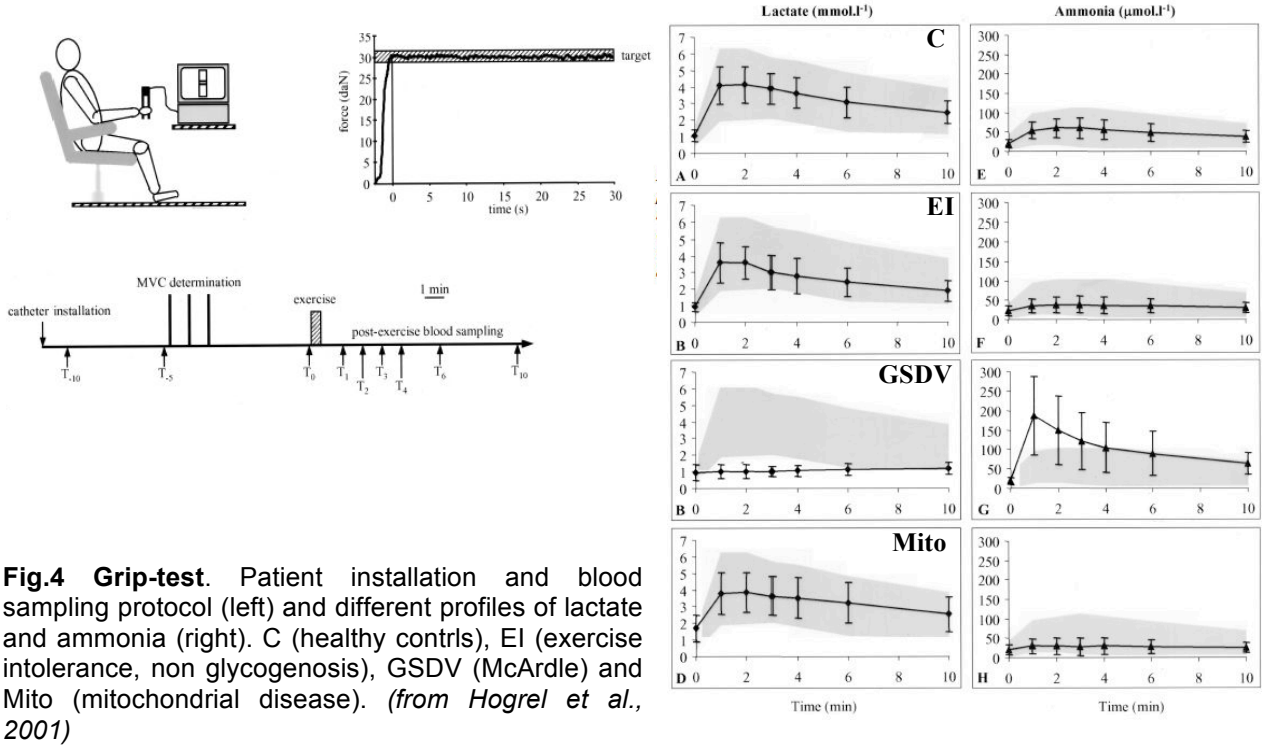


Fig.4 Grip-test. Patient installation and blood sampling protocol (left) and different profiles of lactate and ammonia (right). C (healthy contrls), EI (exercise intolerance, non glycogenosis), GSDV (McArdle) and Mito (mitochondrial disease). *(from Hogrel et al., 2001)*

Phosphorylase *b* Kinase (PHK) Deficiency (Glycogen Storage Disease Type IX): The degradation of glycogen is controlled both in liver and in muscle by a cascade of reactions resulting in the activation of phosphorylase, and phosphorylase b kinase (PHK) catalyses the conversion of myophosphorylase from the inactive (*b*) to the active (*a*) from. PHK is a decahexameric protein composed of four subunits, α, β, γ, and δ. The α and β subunits are regulatory, the γ subunit is catalytic, and the δ subunit is a calmodulin and confers calcium sensitivity to the enzyme. The hormonal activating signals for glycogenolysis, glucagon and adrenaline, activate the membrane-bound adenylate cyclase, which transforms ATP into cyclic AMP (cAMP) and interacts with the regulatory subunit of the cAMP-dependent protein kinase, resulting in phosphorylation of PHK. Ultimately, this activated PHK transforms glycogen phosphorylase into its active conformation, a process that is defective in GSD type IX.

According to the mode of inheritance and clinical presentation of PHK deficiency six different subtypes are distinguished: *(1)* X-linked liver glycogenosis (GSD IXa), by far the most frequent subtype; *(2)* combined liver and muscle PHK deficiency (GSD IXb); *(3)* autosomal liver PHK deficiency (GSD IXc); *(4)* X-linked muscle glycogenosis (GSD IXd); *(5)* autosomal muscle PHK deficiency (GSD IXe); and *(6)* heart PHK deficiency (GSD IXf), which, however is now recognised as being due to mutations in the γ2-subunit of AMP-activated protein kinase rather than to PHK deficiency. The myopathic variants present in a form that is clinically similar to McArdle disease with exercise intolerance, cramps, and rarely recurrent myoglobinuria in young adults (Abarbanel *et al*., 1986; Clemens *et al.,* 1990). Less frequent presentations have been described, including infantile weakness and respiratory insufficiency or late-onset weakness. A peculiarity in patients with muscular form of PHK deficiency has been the finding of normal lactate and ammonia responses on forearm exercise test, which has questioned whether PHK deficiency really is a metabolic myopathy. However, muscle morphology most often shows subsarcolemmal deposits of normal-looking glycogen. The myopathic form of PHK deficiency is caused by mutations in the gene on the X-chromosome for the α-subunit of PHK (*PHKA1*) (Wehner *et al.,* 1994; Burwinkel *et al.,* 2003).

Phosphofructokinase deficiency or GSD VII, first described by Tarui (Tarui *et al.,* 1965), is the most frequent glycolytic disorder. Phosphofructokinase is a tetrameric enzyme under the control of three genetic loci that code for muscle (M), liver (L) and platelet (P) subunits. Mature human muscle expresses only a M homotetramer (M4), whereas erythrocytes contain five isoenzymes combining the M and the L subunits (Toscano and Musumeci, 2007). There are two main myopathic variants, one manifesting as fixed weakness in adult life, although most patients recognise having suffered from exercise intolerance in their youth (Danon *et al.,* 1988; Argov *et al.,*1994); the other affecting infants or young children, who have both generalised weakness and symptoms of multisystem involvement (Danon *et al.,* 1981; Servidei *et al.,* 1986; Musumeci et al, 2012). In patients with typical PFK deficiency, mutations in *PFK-M* cause total lack of activity in muscle but only partial PFK deficiency in red blood cells, where the residual activity approximates 50% and is accounted for by the L4 isozyme (Nakajima *et al*., 2002).

Other glycolysis enzyme deficiencies are even rarer. **Phosphoglycerate mutase (PGAM)** deficiency has been described in about a dozen patients, and other GSDs in fewer or as single cases. Phosphoglycerate mutase (PGAM) is a dimeric enzyme with a muscle-specific (M) and a brain-specific (B) subunits. Skeletal and muscle tissues have a marked predominance of the MM isozyme, but symptoms only develop in skeletal muscle. The clinical picture is stereotypical: exercise intolerance and cramps after vigorous exercise, often followed by myoglobinuria. Molecular defects in the *PGAM* gene have been identified in some patients with GSD X (Vissing *et al*., 1999).

Phosphoglycerate kinase (PGK) is an ubiquitous enzyme, and clinical presentations of PGK deficiency depend on the isolated or associated involvement of three tissues, erythrocytes (hemolytic anemia), central nervous system (CNS) (seizures, mental retardation, strokes), and skeletal muscle (exercise intolerance, cramps, rhabdomyolysis). PGK is encoded by a X-linked gene (*PGK1*). Approximately ten patients with a myopathic form, with or without haemolytic anemia, have been reported (Spiegel *et al.,* 2009).

Phosphoglucomutase (PGM) catalyses the transformation of glucose-1-phosphate to glucose-6-

phosphate. Mutations have been detected in the *PGM1* gene, coding for the muscle-specific PGM1 isoform, in one patient with exercise-induced rhabdomyolysis episodes (Stojkovic *et al.,* 2009).

Deficiency in aldolase A (*ALDOA* gene) is a very rare disorder known to give hemolytic anemia (associated with moderate myopathy in few cases). Few patients suffering from isolated recurrent episodes of rhabdomyolysis since the age of 2 months, which were invariably triggered by febrile illnesses, have been described (Kreuder et al, 1996; Mamoune et al 2014; Hamel et al, 2015).

β-enolase (*ENO3* gene) deficiency is a very rare enzymatic defect of distal glycolysis, identified in few patients who complained of several episodes of rhabdomyolysis, with very aspecific laboratory findings (very mild rise in lactate during a forearm exercise test; minimal changes on muscle biopsy showed with no lipid or glycogen accumulation) (Comi et al, 2001; Musumeci et al, 2014).

Lactate dehydrogenase (*LDHM* gene) deficiency have also been identified in patients with exercise intolerance and rhabdomyolysis (Kanno *et al.,*1995).

Diagnostic process of muscle glycogenoses

When a disorder of glycolysis is suspected, routine laboratory results can give few clues guiding toward specific diagnoses. These include: 1) an increased bilirubin concentration and reticulocyte count, reflecting a compensated hemolysis which may occur in PFK and PGK deficiencies, and 2) an hyperuricemia which is commonly found in PFK deficiency and is attributed to excessive purine nucleotides degradation in the exercising muscles (Mineo *et al.,* 1985, 1987). Discrepancy between a high level of CK and low level of LDH are suggestive of LDH deficiency. The second step in the diagnostic process isforearm exercise test for measurement of lactate and ammonia levels: blunted lactate production and/or abnormal rise of ammonia levels, are characteristic features of several glycogenoses. Muscle biopsy analysis shows inconstant subsarcolemmal vacuoles and glycogen accumulation on PAS stain. This glycogen is normally digested by diastase, excepted in PFK deficiency which can also lead to accumulation of abnormally branched glycogen (polyglucosan) with a violine aspect on standard Hemateine-Eosine stain and resistance to diastase digestion.

Specific anomalies such as tubular aggregates may be observed in PGAM deficiency. A specific histochemical reaction is also available for PFK and may help to confirm the diagnosis of GSD VII. Conclusive evidence, however, comes from the biochemical analysis of enzyme deficiencies either on muscle biopsy for all enzymes, or in erythrocytes for PFK, PGK, and Aldolase A.

Table II. Main clinical and biological features of glycogen storage disorders with exercise intolerance (modified with permission from Dr. Laforet, 2012)

Type And defective enzyme	***Involved tissues***	***Main clinical symptoms***	***Forearm exercise test***	***Muscle biopsy***	***Gene (inheritance)***
V (McArdle) Myophosphorylase	Muscle	Exerc. intolerance, Rhabdomyolysis Second wind	No lactate increase Excessive rise of ammonia	Vacuolar myopathy; Increased PAS staining; Phosphorylase stain deficiency	*PYGM (AR)*
VII (Tarui) Phosphofructokinase	Muscle, Erythrocytes	Exerc. intolerance, Rhabdomyolysis, Muscle weakness *Hemolytic anemia*	No lactate increase Excessive rise of ammonia	Vacuolar myopathy Increased PAS staining Polyglucosans PFK stain deficiency	*PFK-M* (AR)
IX Phosphorylase b kinase	Muscle Liver	Exerc. intolerance, Rhabdomyolysis	Normal or blunted lactate increase	Inconstant vacuolar myopathy or increased PAS staining	*PHKA1* (XR)
Phosphoglycerate kinase	Muscle, Erythrocytes CNS Erythrocytes	Exerc. intolerance, Rhabdomyolysis, Muscle weakness Hemolytic anemia	No lactate increase Excessive rise of ammonia	Inconstant increased PAS staining	*PGK1* (XR)
X Phosphoglycerate mutase	Muscle	Exerc. intolerance, rhabdomyolysis	Blunted lactate production Excessive rise of ammonia	Increased PAS staining Tubular aggregates ((25 % of cases)	*PGAM* (AR)
XI Lactate dehydrogenase	Muscle	Exerc. intolerance, Rhabdomyolysis	Blunted lactate production	Normal PAS staining	*LDH-M* (AR)
XII Aldolase A	Muscle	Exerc. intolerance, Rhabdomyolysis	Not reported	Normal PAS staining	*ALDOA* (AR)
XIII β-enolase	Muscle	Exerc. intolerance,	No lactate increase	Normal PAS staining Negative muscle β-enolase staining	*ENO3* (AR)
XIV Phosphoglucomutase	Muscle	Exerc. intolerance, Rhabdomyolysis	Normal lactate increase Excessive rise of ammonia	Vacuolar myopathy Increased PAS staining	*PGM1 (*AR)

B) Disorders of lipid metabolism

Disorders of lipid metabolism affecting muscle may involve endocellular triglyceride degradation, carnitine uptake, long-chain fatty acids mitochondrial transport, or β-oxidation. The pathological hallmark of some of these diseases is an increased neutral lipid content, which may be observed on muscle biopsies specimen with the specific stainings of Sudan black or oil red O techniques by optic microscopy. The term of lipid storage myopathies is often used when the accumulation of lipid droplets in muscle fibers is uppermost, and associated with a vacuolated appearance on routine histological stains such as hematoxylin and eosin or Gomori Trichrome. Conversely, lipid metabolism disorders are inconstantly leading to a muscle lipidosis, and therefore awareness of their clinical features and main biological anomalies are essential for establishing accurate diagnosis.

Carnitine palmitoyl transferase II (CPT II) deficiency. Carnitine palmitoyl transferase II (CPT 2) deficiency is clinically characterized by muscle stiffness, myalgias, and exercise intolerance and episodes of rhabdomyolysis, usually triggered by prolonged exercise, cold, or fasting.

Very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency. Very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency most often occurs in childhood with cardiac or liver involvement, but rhabdomyolysis attacks have been increasingly reported in adults (Vianey-Saban *et al.,* 1998; Laforêt *et al.,* 2009). The juvenile or adult-onset myopathic form is very similar to CPT II deficiency, characterized by recurrent episodes of rhabdomyolysis triggered by prolonged exercise, fever, cold or fasting. However, in contrast to adult CPT II deficiency in which the clinical features are limited to skeletal muscle, early-onset extra-muscular symptoms may be observed in some patients with VLCAD deficiency. Pathologic findings in muscle biopsies are a moderate lipid storage in approximately one third of cases, predominating in type 1 fibres. The diagnosis of VLCAD deficiency relies on the measurement of plasma or blood spotted onto filter paper acylcarnitines by tandem mass spectrometry (MS/MS), allowing the detection of abnormal long-chain acylcarnitines with tetradecenoylcarnitine ($C_{14:1}$) as predominant species. Analysis of the

ACADVL gene shows a wide mutational spectrum, most of the mutations being private, with a clear correlation of genotype with disease phenotype (Andresen *et al.,* 1999).

Mitochondrial trifunctional protein (MTP) deficiency. Patients with MTP deficiency can be classified into two groups: long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and MTP (combined enzyme) deficiencies (Olpin *et al.,* 2005). The majority of MTP-deficient patients have an isolated deficiency of LCHAD activity due to mutations in LCHAD domain of the α-subunit (*HADHA* gene). Clinical manifestations are heterogeneous, but similar in LCHAD and MTP deficiencies. Severe encephalopathy and hepatopathy may occur in infancy often leading to death. A late-onset form may occur later in childhood, which predominant manifestations are rhabdomyolysis episodes, cardiomyopathy, pigmentary retinopathy and progressive sensorimotor axonal peripheral neuropathy (Spierkoetter *et al.,* 2004). Rhabdomyolysis episodes may be induced by exercise, illness or fasting, and are accompanied by life-threatening respiratory failure in 45% of patients. The sensorimotor neuropathy is a distinguishing feature which has not been reported in patients with other FAO defects, in combination with episodic rhabdomyolysis. Blood acylcarnitine profile may show an elevation of long-chain hydroxyacylcarnitines as well as 3-hydroxydicarboxylic aciduria. A prevalent missense mutation in the LCHAD domain of the α-subunit (c.1528G>C; p.Glu510Gln) has been identified in approximately 90% of mutant alleles in LCHAD deficiency. Unlike LCHAD deficiency, the molecular basis of MTP deficiency is heterogeneous with mutations identified in both *HADHA* and *HADHB,* the genes coding for the α- and β-subunits respectively.

Medium-chain acyl-CoA dehydrogenase deficiency (MCAD). MCAD deficiciency is the most common FAO in childhood (Di Donato and Taroni, 2004). Typical signs and symptoms are hyperammonemia,hypoketotic hypoglycaemia, and encephalopathy, of acute onset after a prolonged fast of intercurrent infection. Reye-like syndrome, coma, and sudden death may also occur in the second year of life. Skeletal muscle disease is very rare, but it seems that some patients may develop a mild myopathy with lipid excess in muscle, or rhabdomyolysis episodes after strenuous exercise or alcohol ingestion (Ruitenbeek *et al.,* 1995; Lang, 2009). In another

hand some MCAD-deficient individuals may remain asymptomatic throughout life. Increased octanoylcarnitine (C_8) and decenoylcarnitine ($C_{10:1}$) as well as their corresponding free acids (octanoic and cis-4-decenoic acids) are the most useful diagnostic markers in blood, and are also detectable in high amounts in urines. Plasma carnitine levels are usually lowered. Genetic studies showed that most symptomatic MCAD-deficient patients carry a homozygous c.985A>G (p.Lys304Glu) point mutation.

Multiple acyl-CoA dehydrogenase (MAD) deficiency. MAD deficiency, also known as glutaric aciduria type II (GAII), results in abnormal fatty acid, amino acid, and choline metabolism. The biochemical abnormalities are explained by deficiency of one of the two electron transfer flavoproteins which transfer electrons from acyl-CoA dehydrogenases to the respiratory chain: ETF (Electron Transfer Flavoprotein, coded by *ETFA* and *ETFB* genes) and ETF-QO (ETF-ubiQuinone Oxidoreductase coded by *ETFDH* gene). MAD deficiency has a wide range of clinical presentations, the most severely affected patients dying in the newborn period of congenital anomalies such as cystic renal dysplasia. Milder cases present later in childhood with hypoglycaemia, encephalopathy, muscle weakness or cardiomyopathy. Less severely affected patients might present with progressive muscle weakness or rhabdomyolysis episodes at adult age, and some of these patients show a dramatic response to riboflavin treatment. It has been demonstrated that riboflavin-responsive MAD deficiency is associated with mutations in *ETFDH* gene, at least in a large proportion of cases (Olsen *et al.,* 2007). Muscle histology always shows a muscle lipidosis. Urine organic acids are most often abnormal at the time of presentation (typically with C_5 to C_{10} dicarboxylic aciduria and acylglycine derivatives) and blood acylcarnitine analysis shows raised concentrations of all chain lengths acylcarnitines (C_4 to C_{16}). Plasma free carnitine levels are generally lowered. In addition, probably secondary impairments of respiratory chain enzymes deficiencies (RC) have been found in patients for whom such studies have been possible. *ETFDH* pathogenic mutations have also been identified in seven patients who carried the diagnosis of myopathic form of coenzyme Q10 deficiency (Gempel *et al*., 2007). All patients presented with fluctuating proximal myopathy, premature fatigue and high CK levels. Triggering factors could be infections, fasting, pregnancy or surgery. A dramatic clinical improvement was

noticed in all these patients after CoQ10 and riboflavin supplementation. Riboflavin treatment (100-400 mg/day) may induce within a few days, a dramatic improvement of muscle symptoms and encephalopathy in some patients with riboflavin-responsive MAD deficiency (Olsen *et al.,* 2007). A significant improvement of muscle weakness has also been observed after a few months of CoQ10 supplementation in patients with secondary coenzyme Q10 deficiency, but some of them improved even more with combined CoQ10 and riboflavin therapy (Gempel et al., 2007). Few patients have a deficiency that is similar to MAD deficiency but no mutations in *ETFA*, *ETFB*, or *ETFDH* identified. Recently, a defect in flavin adenine dinucleotide (FAD) biosynthesis or transportation was suspected in one of these MADD patients, which presented a dramatic improvement in the clinical and biologic abnormalities after treatment with riboflavine. Two mutations were identified in *SLC25A32* (solute carrier family 25, member 32) gene, that encodes the mitochondrial FAD transporter, an inner mitochondrial membrane carrier that imports FAD from the cytosol into the mitochondria (Schiff et al, 2016).

Phosphatidic acid phosphatase (LIPIN) deficiency. Mutations in *LPIN1* gene is an important and potentially fatal cause of recurrent episodes of acute myoglobinuria with onset in childhood, precipitated by febrile illnesses or anaesthesia (Zeharia *et al.,* 2008; Michot *et al.,* 2010). The *LPIN1* gene encodes the muscle-specific phosphatidic acid phosphatase (LIPIN), a key enzyme in triglyceride and membrane phospholipids biosynthesis, which catalyzes the conversion of phosphatidate to diacylglycerol in the triacylglycerol pathway. Age at first episode vary between 15 months and 7 years, with peak CK levels varying from 20,000 to 450,000 U/l. All the following tests give normal results: total and free carnitine, blood acylcarnitine profile, urinary organic acids, and FAO studies in fibroblasts or lymphocytes. Muscle biopsy findings usually shows normal lipid content or moderate lipid accumulation.

Table III. Lipid Myopathies: clinical and molecular features (modified with permission from Laforet et al, 2012)

Disorder	**Main neuromuscular symptoms**	Increase in muscle lipid droplets	Laboratory features	Gene
Carnitine palmitoyl transferase II (CPTII) deficiency	Rhabdomyolysis episodes	0 to +	Normal or moderately reduced plasma carnitine **Increased long-chain acylcarnitines (C_{16}, $C_{18:1}$, C_{18})**	*CPT2*
Very-long-chain acyl-CoA dehydrogenase (VLCAD) deficiency	Rhabdomyolysis episodes Cardiomyopathy	0 to +	Normal or moderately reduced plasma carnitine **Increased long-chain acylcarnitines ($C_{14:1}$ as main species)** Dicarboxylic aciduria	*ACADVL*
Mitochondrial trifunctional protein (MTP) deficiency	Rhabdomyolysis episodes Cardiomyopathy Axonal peripheral neuropathy	0 to +	**Increased long-chain 3-hydroxy-acylcarnitines** Dicarboxylic and 3-hydroxydicarboxylic aciduria	*HADHA* *HADHB*
Multiple acyl-CoA dehydrogenase (MAD) deficiency	Proximal and axial muscle weakness Rhabdomyolysis (rarely)	++ to +++	Low plasma carnitine **Increased acylcarnitines of all chain lengths** 2-hydroxyglutaric aciduria +/- acylglycines	*ETFDH*
Medium-chain acyl-CoA dehydrogenase (MCAD) deficiency	Muscle weakness Rhabdomyolysis episodes	0 to +	**Low plasma carnitine** Increased medium-chain acylcarnitines (C_6, C_8, C_{10}, $C_{10:1}$) Dicarboxylic aciduria + **acylglycines**	*ACADM*
Phosphatidic acid phosphatase deficiency	Rhabdomyolysis episodes	0 to +	Normal plasma carnitine Normal acylcarnitine profile Normal urinary organic acids	*LPIN1*

C) MITOCHONDRIAL MYOPATHIES

Mitochondrial myopathies due to respiratory chain anomalies rarely manifest as isolated rhabdomyolysis. Although some patients present with a clinical phenotype restricted to skeletal muscle, multivisceral and central nervous system involvement are generally the main clinical features of mitochondrial diseases often named “mitochondrial encephalomyopathies”.

Progressive external ophtalmoplegia (PEO) is the most frequent skeletal muscle symptom of mitochondrial myopathies. Patients with PEO often also complain of exercise intolerance, but without important rise of CK levels, nor rhabdomyolysis episodes. However, some mutations of

mitochondrial DNA may manifest by muscle weakness, exercise intolerance, and attacks of myoglobinuria (Andreu *et al.,* 1999; Kollberg *et al.,* 2009; Emmanuele *et al.,* 2011).

- Calcium-reltad myopathies

The triad (T-tubule and terminal sarcoplasmic reticulum) is the skeletal muscle substructure responsible for the regulation of excitation–contraction coupling and intracellular calcium homeostasis (Fig. 5). The number of skeletal myopathies caused by gene mutations in components of the triad (i.e. *RYR1, CASQ1*, *STIM1* and *ORAI1*), is rapidly growing, but the clinical and molecular features of these diseases are still largely unknown.

RYR1. Mutations in the skeletal muscle ryanodine receptor (RYR1) gene are a common cause of neuromuscular disease, ranging from various congenital myopathies to the malignant hyperthermia (MH) susceptibility trait without associated weakness. RYR1 mutations have recently been identified as cause for a substantial proportion of patients presenting with unexplained rhabdomyolysis and/or exertional myalgia (Sambuughin et al, 2009; Dlamini et al, 2013; Snoeck et al, 2015; Jungbluth et al, 2016; Voermans et al, 2016). This finding further suggest that exertional rhabdomyolysis and malignant hyperthermia are overlapping syndromes or slightly different manifestations of the same pathological process, recently proposed to be called human stress syndrome (Zhao et al, 2014; Snoek et al, 2016)

CASQ1. Calsequestrin (CASQ) is the main Ca2+ buffering protein in the terminal cisternae of sarcoplasmic reticulum (SR) and a regulator of ryanodine receptor (RYR1)-mediated Ca2+ release (Kawasaki T, Kasai M, 1994). Mutation in CASQ1 has recently been identified in association with a mild vacuolar myopathy in several Italian patients (Rossi et al, 2014; Di Blas et al, 2015). Its thight relationship with RYR1 suggests a potential involvement of mutations in *CASQ1* gene in patients presenting rhabdomyolysis (see aim 2 of this work).

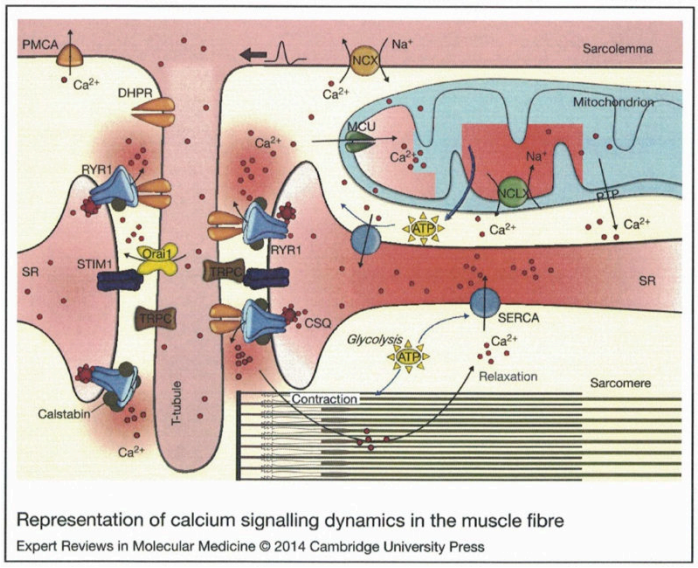


Fig. 5. **Representation of calcium signaling dynamics in the muscle fiber.** (*From Vallejo-Illaramendi et al, 2014)*

ORAI1 and STIM1. Signaling through the store-operated Ca(2+) release-activated Ca(2+) (CRAC) channel regulates critical cellular functions, including gene expression, cell growth and differentiation, and Ca(2+) homeostasis. Loss- and gain-of-function gene mutations in the genes encoding the main components of CRAC, *ORAI1* and *STIM1,* in human patients cause distinct disease syndromes. Loss-of-function mutations in *ORAI1* and *STIM1* that abolish CRAC channel function and SOCE are characterized by severe combined immunodeficiency (SCID)-like disease, autoimmunity, non progressive muscular hypotonia, and ectodermal dysplasia, with defects in dental enamel. By contrast, autosomal dominant gain-of-function mutations in these genes result in constitutive CRAC channel activation, SOCE, and increased intracellular Ca2+ levels that are associated with an overlapping spectrum of diseases, including non-syndromic tubular aggregate myopathy (TAM) and York platelet and Stormorken syndromes, two syndromes defined by thrombocytopenia, thrombopathy, and bleeding diathesis (Nesin et al, 2014; Endo et al, 2015; Lacruz and Feske, 2015).

DIAGNOSTIC PROCESS OF RHABDOMYOLYSIS

When facing an episode of rhabdomyolysis, the identification of the etiological cause can be extremely complex, long, and costly. Nevertheless, the correct identification of the underlying cause is of utmost importance for the correct information regarding prognosis (risk of recurrence) and for the family genetic counselling (Barca et al, 2015).

Several diagnostic work-up have been proposed to evaluate the etiological diagnosis of rhabdomyolysis (see Fig. 6, for children; other protocols are discussed in the Conclusions of the present work). However, when a non-genetic cause of rhabdomyolysis is not evident, despite the application of an accurate clinical evaluation, an expanded questionnaire regarding signs, symptoms, trigger, etc., and despite an extended diagnostic work up, frequently it is not easy to distinguish if the rhabdomyolysis episode is due to a genetic disorder or whether it is simply the result of an abnormal effort, of an infectious episode or effect of a toxic and thus to pose a complete and correct diagnosis.

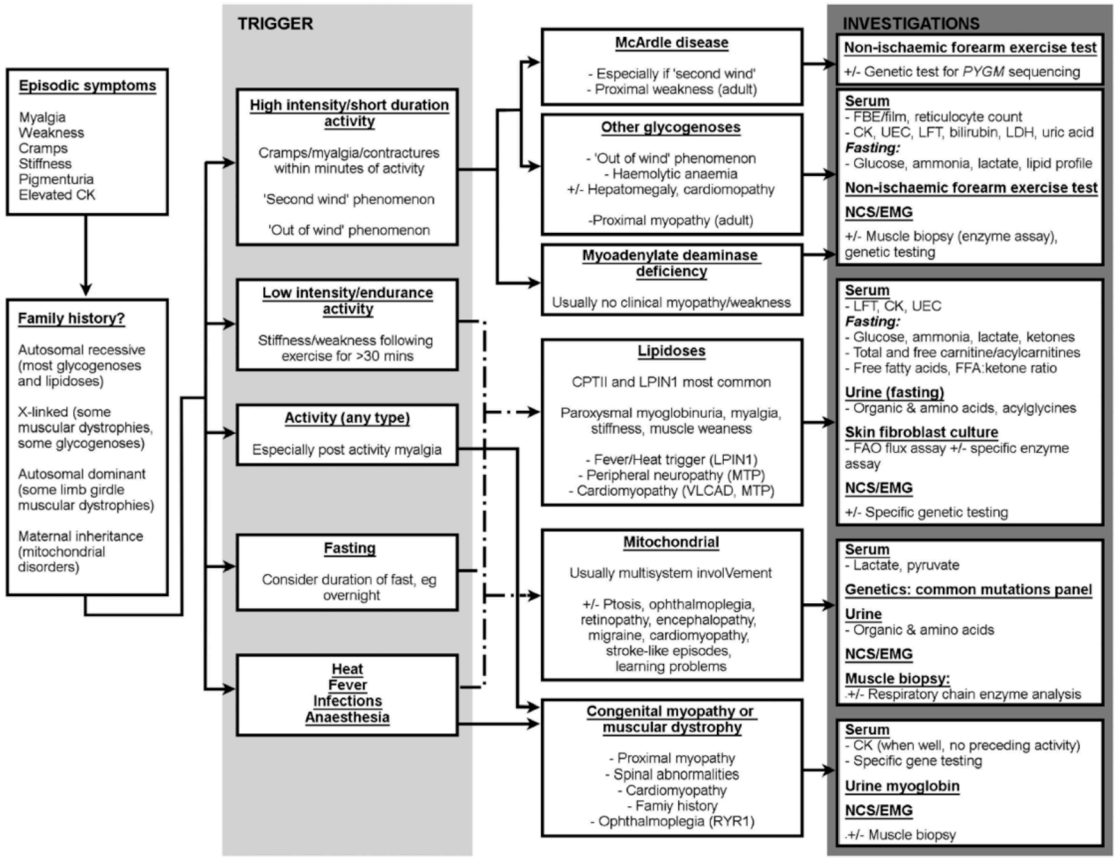


Fig. 6. Diagnostic work-up for rhabdomyolysis in children. Proposed by Chan et al (2015)

In recent years, the evolution of molecular techniques allowed to develop methods for the analysis of exomes ("whole exome sequencing") or of several genes involved in specific diseases ("gene panels"). These techniques allowed us to increase the detection rate, reducing time and cost analysis, and have identified new genes associated with myopathies. At the same time, it eased the identification of genetic variants whose pathogenicity is still doubtful, and it is still far from identifying all molecular mechanisms underlying the diseases (Savarese et al, 2014; Kuhn et al, 2016; Monies et al, 2016). A careful analysis of the clinical picture of the patient, the correct choice of diagnostic tools and their careful evaluation is mandatory for the proper selection and interpretation of molecular investigations.

The main objective of this study is, therefore, the evaluation of the diagnostic process of cases of rhabdomyolysis, to propose an updated, comprehensive, rational and cost effective protocol based on the latest information and techniques.

AIM 1

Identification and characterization of patients referring to Neuromuscular Centers for rhabdomyolysis (2000-2016): Clinical Features, Diagnostic work-up and final diagnoses

RATIONALE

When facing an episode of rhabdomyolysis, to determine the etiological cause can be extremely long, costly and complex. Nevertheless, the correct identification of the underlying cause is of utmost importance for correct information regarding prognostic purposes (risk of recurrence) and for the family genetic counselling.

Aims of this first part of the study were therefore: i) to describe clinical features of patients referring to tertiary neuromuscular center for rhabdomyolysis not related to clear external causes (such as trauma); ii) to evaluate the role of diagnostic tools used to determine molecular basis of rhabdomyolysis; iii) to determine the definite diagnostic rate of patients referring for rhabdomyolysis; iv) to identify peculiar features of specific diseases manifesting with rhabdomyolysis.

METHODS

A retrospective study was performed, including all patients that referred to the Paris Neuromuscular Referral Center for one or more episodes of rhabdomyolysis. The following criteria were selected:

- **Inclusion criteria**: patients referring from 01.01.2000 to 30.06.2016 for acute rhabdomyolysis, defined as: i) acute onset of suggestive signs and symptoms (myalgias /weakness/ dark urines) and/or ii) Increase of CK levels > 10 times of rest values.
- **Exclusion criteria**: i) diagnosis of muscle disease in the subject or in the family before the episode; ii) evidence of external cause of rhabdomyolysis (i.e. epilepsy, surgical intervention, trauma).

All clinical records and medical dossiers were collected and analyzed, and all the information were acquired regarding:

1. **Personal data**: age, sex, ethnic origins, familiarity for muscle disease; concomitant diseases.
2. **Clinical and laboratory features of the episode(s)**:
    a. Age at first episode; number of episodes; maximal CK level; type of trigger; time relation between trigger and symptoms onset.
    b. Clinical features of the episode: presence of myalgias, weakness, dark urines, muscle edema, hyperthermia, other symptoms.
    c. Consequences of the episode: hospitalization, renal insufficiency, dialysis, fasciotomy, other.
3. **Clinical and laboratory features during inter-critical periods**: CK and lactate levels at rest; presence of exercise intolerance, muscle cramps, myalgias, fixed weakness, muscle hyper- or hypotrophy, other clinical signs.
4. **Diagnostic tools that were performed**:
    a. Standard muscle biopsy: open muscle biopsy followed by histology and histochemistry (Haematoxylin and eosin, modified Gomori trichrome, Oil red O, periodic acid Schiff, adenosine triphosphatase, combined succinate dehydrogenase, cytochrome c oxidase, NADH dehydrogenase, myophosphorylase, phosphofructokinase,acid phosphatase, Congo red), Immunohistochemistry (usually Dystrophin, , α-, β-, γ– and δ-sarcoglycans, dysferlin, caveolin-3, MHC-1, a-dystroglycan) and/or Western blot (dystrophin, calpain-3, dysferlin, a-sarcogygan).
    b. EMG protocol: Nerve conduction studies (motor and sensory nerve conduction velocity of median, ulnar and peroneal nerves); Repetitive Nerve Stimulation (RNS) to test neuromuscular transmission (10 stimuli at 3Hz) in several nerve-muscle couples (axillary-trapezius, radial-anconeus, peroneal-tibialis anterior); Needle EMG to identify myopathic changes such as abnormal spontaneous activity; short, small and polyphasic Motor Unit Action Potential, etc. in proximal (trapezius, deltoid, vastus medialis) and distal (extensor digiti communis, tibialis anterior) muscles.
    c. EMG Long Exercise Test (LET): it consists in recording compound muscle action potential (CMAP) responses from abductor digiti minimi muscle before and after 5

minutes of maximal isometric contraction, to determine sarcolemmal excitability and membrane function after effort (see Aim 3 of the present study)

d. Handgrip test: measurement of blood concentrations of creatine kinase, lactate, and ammonia before and after isometric exercise at 70% of the maximal voluntary contraction during 30 seconds in non-ischemic conditions. The test was performed with standardized procedures in a single-center with specific tool (Hogrel et al, 2001 and 2015).

e. Muscle imaging (CT scan or MRI): either lower limbs CT or MRI scan; in selected cases whole body MRI.

f. Profile of acylcarnitines: analyzed by electrospray ionization tandem mass spectrometry from plasma or dried whole blood spots (DBS) on filter paper.

g. Dosage of total and free plasmatic carnitine

h. Chromatography of urinary organic acid: studied by gas chromatography-mass spectrometry

i. Dosage of CPT2 activity on lymphocytes: standard procedure of enzymatic dosage measurement comparing CPT2 activity to healthy controls and enzymatic reference (citrate synthase)

j. Dosage of beta-oxidation activity on lymphocytes or cultured fibroblasts: dosage of fatty acids labeled with 14C ((1-14C)butyrate , (1-14C)Octanoate, (1-14C)Palmitate) and Tritium (9,10-3H)Myristate, (9,10-3H)Palmitate)

k. Dosage of Mitochondrial respiratory chain activity on muscle biopsy: dosage of activity of single components of respiratory chain on muscle tissue.

l. Glyco(geno)lysis on muscle biopsy or blood cells: dosage of glucose-6-phosphate dehydrogenase, phosphoglycerate kinase, phoshofructokinase, triose phosphate isomerase, lactic dehydrogenase and aldolase on red blood cells or muscle tissue.

m. Genetic tests: specific candidate gene sequencing (by Sanger Sequencing) or gene panels performed in selected cases according to clinical phenotype or results of diagnostic tools performed: i) limb girdle muscular dystrophies gene panel (*ANO5, CAPN3, CAV3, DYSF, EMD, FKRP, SGCA, SGCB, SGCD, SGCG* and *TRIM32*) ; ii) Mitocapture panel (mitochondrial diseases gene panel, including 37 mitochondrial genes and 101 nuclear genes associated with mitochondrial functioning or disease, dr. Jardel, Paris).

To identify if different causes of rhabdomyolysis led to episodes with different characteristics, we classified the patients in three groups, according to their final diagnosis:

A) **Genetic diagnoses:** patients in whom the diagnosis of a genetically caused myopathy was posed by genetic, enzymatic or histopathological tests.

B) Non-genetic diagnoses: patients in whom a non-genetic diagnosis was confirmed or highly suspected, based on clinical and laboratory features. This includes:

a. **Pure exertional rhabdomyolysis and Compartment Syndrome (named together "exertional rhabdomyolysis").** Patients in which the cause of rhabdomyolysis was attributed to an extraordinary effort and in which muscle pathology was potentially excluded. It was defined if: i) episode(s) always related to effort intense / unusual / in predisposing conditions; ii) age at first episode > 12 years; iii) no major abnormalities on diagnostic tests, iv) no familiar cases.

b. **Drug induced =** likely related to statins or other toxic drugs

c. **Inflammatory muscle disease**

d. **Infectious disease**: likely related to muscular infectious disease

e. **Toxic =** likely related to alcohol or drug abuse

f. **Endocrine-related**

g. **Other:** i.e. rhabdomyolysis induced by Popliteal Artery Entrapment Syndrome

C) Undiagnosed: patients in which no diagnosis could be obtained.

In order to identify if specific diseases presented rhabdomyolysis with different characteristics, we then compared the features of patients affected by the most recurrent specific diagnosis: McArdle disease (Glycogenosis type 5, GSDV), Carnitine-Palmitoil-Transferase 2 deficiency (CPT2), RYR1-related myopathy, Beker Muscular Dystrophy (BMD), Phosphoglucomutase 1 Deficiency (PGM1), Very Long-Chain Acyl-Coenzyme A Dehydrogenase Deficiency (VLCAD), and pure exertional rhabdomyolysis or compartment syndrome ("exertional rhabdomyolysis").

We finally reviewed all the diagnostic tools that had been performed to reach the diagnosis in each patient, to identify the diagnostic power of each instrument routinely used in the diagnostic workup of the specific population of rhabdomyolysis.

Due to the retrospective nature of this study, despite a homogeneous style in the medical history collection, in the evaluation of patients and in the composition of discharge letters, not all information was available for all patients. The percentages are thus expressed referring to the total of available data and not to the total population.

RESULTS

Patient population

By the analysis of databases of Paris-Est Neuromuscular center we identified 208 patients that were referred during the period 2000-2016 for one or more episode of rhabdomyolysis, with a marked prevalence of males (2.7:1) and a mean age at first consultation of 34.2±12.9 years. Most patients had French origins, but we included patients coming from all over the world (Algeria, Antilles, Benin, Brazil, Cameroun, Ivory Coast, Gabon, Ghana, Greece, Guadeloupe, Guinee, Guyane, Ile de la Réunion, Iran, Italy, Kuwait, Mali, Morocco, Nigeria, Pakistan, Portugal, Senegal, Tahiti, Tunisia, Turkey, USA)

Clinical features of rhabdomyolysis

The main features of the episodes of rhabdomyolysis are shown in Figure 7 and Table IV. Almost all patients presented both a marked increase of CK levels and suggestive symptoms of rhabdomyolysis. Three patients were completely asymptomatic, and were included because of the incidental finding of marked increase of CK levels (up to 12.500, 18.000 and 28.900 U/L respectively, with an increase of 60, 150 and 25 times compared to their rest values). In 14 cases no maximum CK level was available because it was measured only days after onset or it was never measured. Almost half of the patients (n= 99, 47,8%) presented several episodes of rhabdomyolises, while 109 (52,2%) presented a single episode of rhabdomyolysis. In 80% of cases a trigger could be identified, and frequently it consisted in different types of effort (figure 7). Other triggers included fasting, infectious disease, drugs and cold temperatures.

The main symptom of rhabdomyolysis was muscle pain, occurring in 94,4% of our cases; dark colored urines were observed only in half of patients. Muscle edema (26%) was almost as frequent as muscular weakness (33,7%). Muscular weakness was noticed in 33% of patients; it is to note, however, that the presence of muscular weakness can be hidden by the prominent presence of muscle pain, that limits movements and interfere with clinical evaluation. The typical clinical triad muscle pain + dark colored urines + muscle weakness was noticed only in 18% of cases. Rarer but not unique symptoms included hyperthermia (defined as elevated body temperature occurring together with or after muscle symptoms) in 13 cases (6,3%), and loss of consciousness (in 5 cases) sometimes preceded by neuropsychiatric symptoms (confusion, behavior disturbance, in 3). Rhabdomyolyis episodes frequently had major complications, and required immediate and intensive medical care (see Table IV). 98 patients (47,4%) required hospitalization, sometimes only for rehydration and medical observation. Acute kideny insufficiency (AKI, defined by creatine levels) was noticed in 32 cases (15,7%), requiring dialysis in 12 (5,9%). Of note, patients presenting acute kidney injury had higher CK levels (mean 224,015 ± 261,133 U/L) than patients without AKI (73,939 ± 102,223) but no clear cut-off values could be observed: the range of maximum CK levels of patients affected by AKI and non affected by AKI had a great overlap (from 7.000 to 1.000.000 for AKI, and from 3.200 to 600.000 for patients without AKI). Other rare but severe complications included fasciotomy because of compartment syndromes, performed in 9 patients (anterior leg compartment in 8, linea alba in one) and the rare but severe Posterior Reversible Encephalopathy Syndrome (PRESS) following acute kidney injury in one case. No precise data could be obtained regarding the type of hospitalization (ICU, medical services, etc), the duration of the hospitalization or the type of medical interventions required except for dialysis.

Patient population	
Number of patients	208
Sex	152M; 56F (ratio 2.7)
Age at first visit	34,2±12,9 yrs
Pts w/ single episode	109 (52,2%)
Pts w/several episodes	99 (47,8%)
Features of acute episode	**n (%)**
Muscle pain	185 (94,4)
Myoglobinuria	102 (52,0)
Weakness	66 (33,5)
Muscle oedema	48 (25,7)
Hypertermia	13 (6,3)
Loss of Consciousness	5 (2,4)
Consequences of rhabdomyolysis	**n (%)**
Hospitalization	98 (47,4)
Acute kidney failure	32 (15,7)
Dialysis	12 (5,9)
Chronic Kidney disease	4 (1,9)
Fasciotomy	9 (5)
PRES	1 (0,5)

Table IV. Patient population and clinical features of rhabdomyolyses

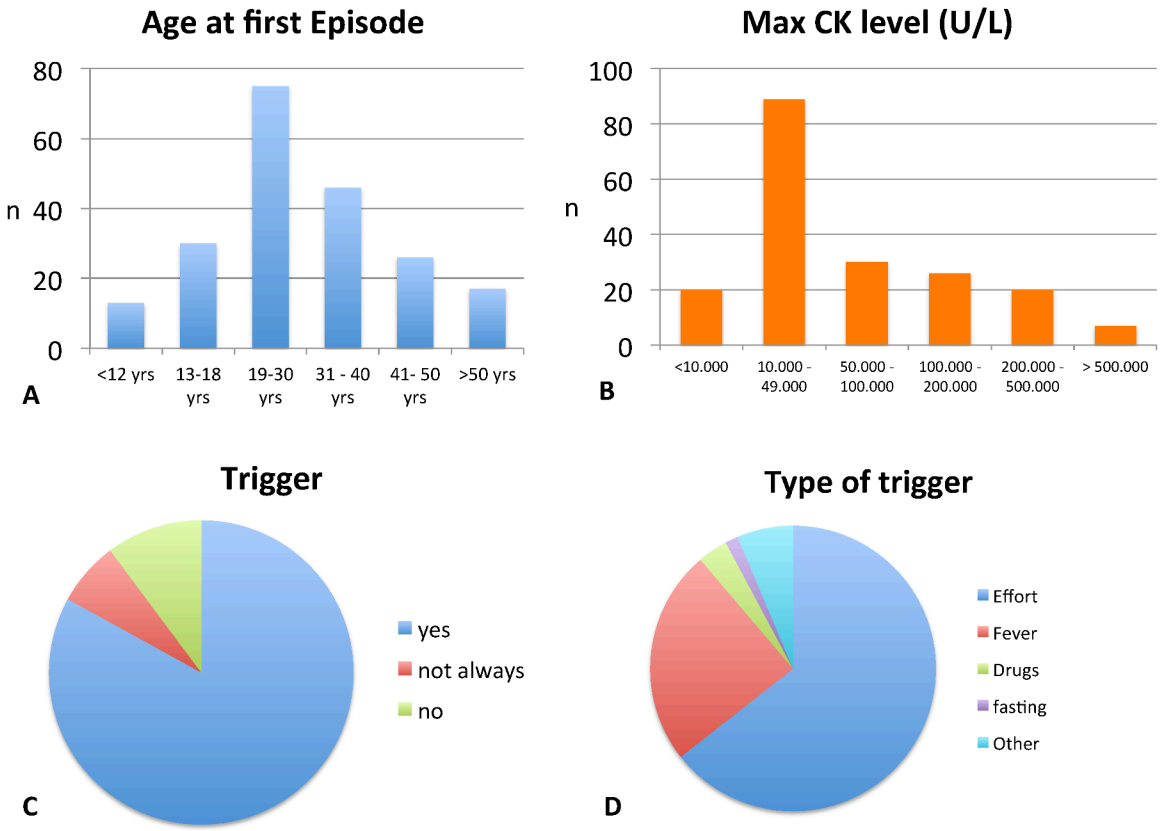


Fig. 7. Main features of rhabdomyolysis episodes: age at first episode (A), max CK level (B), presence of trigger (C); and type of trigger (D).

Final diagnosis

The final diagnoses obtained after the work-up are showed in Table V and Figure 8. A definitive diagnosis of genetic disease could be identified in 41 patients (19,7%); a non-genetic diagnosis was posed in 49 patients (23,6%) while no diagnosis could be achieved in more than half of our population (118; 56,7%). The most frequent genetic causes included GSDV (n = 11), CPT2 (n=11) and RYR1-related myopathies (n=8) in the genetic disease category, and pure exertional rhabdomyolysis (n = 37) in the non-genetic category.

Demographical data, features of episodes of rhabdomyolysis and inter-critical characteristics of patients grouped according to their final diagnoses are shown in Fig. 9 and Table VI and VII.

A) GENTIC DISEASES	*n = 41*
1.CPT2	11
2.McArdie disease	11
3.RYR1 myopathy	8
4.VLCAD	2
5.BMD	2
6.PGM1	2
7.Other (DM2, DM1, FKRP, LIPIN1, DNA2)	5
B) OTHER DIAGNOSES	***n = 49***
1.Exertional rhabdomyolyisis	36
2.Drug-induced rhabdo.	3
3.Infectious disease	3
4.Toxic rhabdomyolysis	2
5.Inflammatory	2
6.Compartment syndrome	1
7.Basedow	1
8.Popliteal Artery Entrapment Syndrome	1
C) UNDIAGNOSED.	***n = 118***

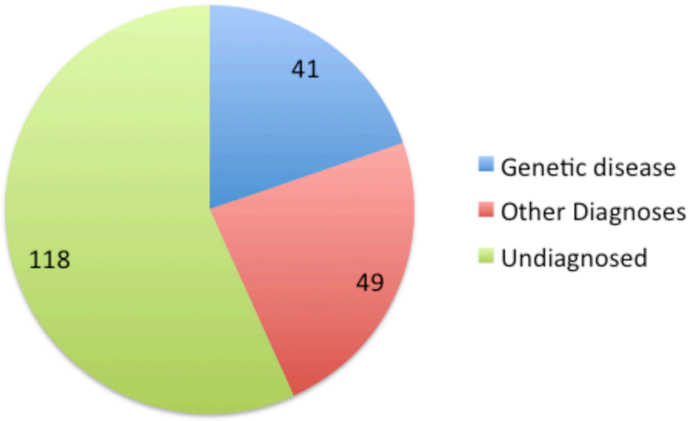


Table V and Fig. 8. Final diagnoses obtained in patients referred for rhabdomyolysis.

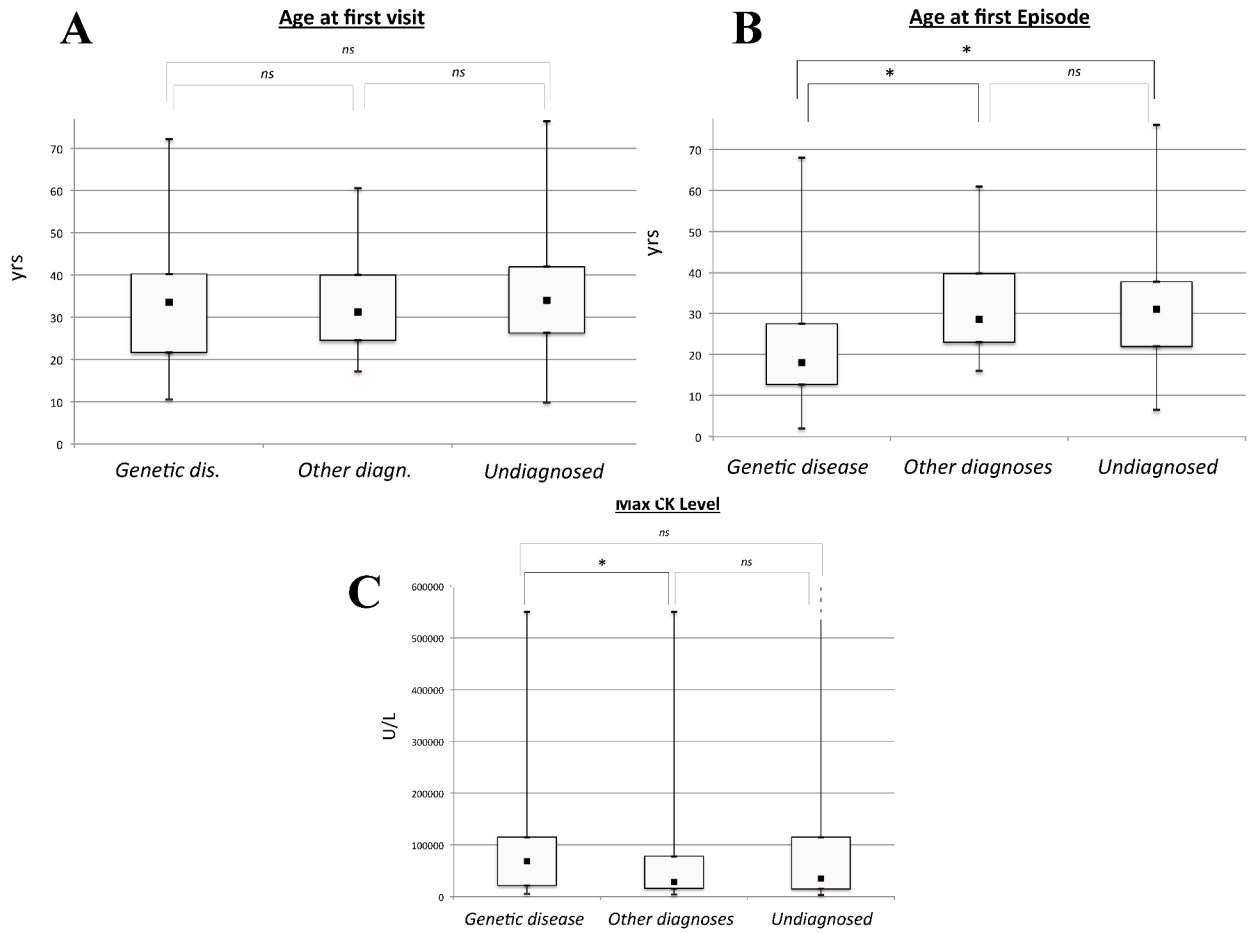


Fig. 9. Age at first consultation (A), age at first episode (B)and maximum CK level (C) of patients grouped according to their final diagnosis. Patients with genetic diseases present earlier episode of rhabdomyolysis and higher CK levels.

Table VI. Demographic data and characteristics of the episodes of rhabdomyolysis of patients grouped according to the final diagnosis

		Genetic Diseases		Non-genetic diseases		Undiagnosed pts	
	n	41		49		118	
	sex (ratio M:F)	27M, 14F (ratio 1,9)		40M, 9F (ratio 4,4)		85M, 33 F (2,6)	
	Age 1s consult (mean ±SD)	33,6 ± 14,8		33.8 ± 12.5		35,2 ± 12,1	
	Age 1st episode (mean ±SD)	22,5 ± 14.9		32,1 ± 12,3		31,4 ± 12,4	
		n	%	n	%	n	**%**
Age 1st episode	<12 ans	10	24,4	0	0,0	3	2,5
	13 - 18	12	29,3	3	6,1	15	12,7
	19 - 30	10	24,4	26	53,1	41	34,7
	31-40	4	9,8	8	16,3	34	28,8
	41-50	3	7,3	7	14,3	16	13,6
	>50	2	4,9	5	10,2	9	7,6
n. of rhabdomyolysis	Single Episode	14	34,1	36	73,5	59	50,0
	Two Episodes	6	14,6	11	22,4	28	23,7
	3-4 Episodes	3	7,3	2	4,1	23	19,5
	5 or more	18	43,9	0	0,0	8	6,8
Max CK level	<10.000	2	5,9	5	10,2	13	11,7
	10 - 49.999	11	32,4	28	57,1	50	45,0
	50 - 99.999	9	26,5	6	12,2	16	14,4
	100 - 199.999	5	14,7	8	16,3	13	11,7
	200 - 499.999	5	14,7	1	2,0	14	12,6
	> 500.000	2	5,9	1	2,0	5	4,5
Trigger	Yes	34	82,9	48	98	91	77,1
	Non always	3	7,3	0	0	11	9,3
	No	4	9,8	1	2	16	13,6
Type of triggers	Effort	31	64,6	41	80,4	60	56,6
	Fever	10	20,8	2	3,9	38	35,8
	Drugs	2	4,2	2	3,9	3	2,8
	Fasting	3	6,3	0	0	0	0
	Other	2	4,2	6	11,8	5	4,7
If effort: onset of symptos	During effort	11	42,3	16	41,0	10	18,2
	Hours after effort	14	53,8	17	43,6	33	60,0
	Days after effor	1	3,8	6	15,4	12	21,8
Clincal Features of episodes	Weakness (A)	14	35,9	10	20,8	42	37,8
	Myalgias (B)	36	94,7	44	91,7	108	**97,3**
	Dark colored urines (C)	29	74,4	20	42,6	54	52,3
	Muscle edema	4	10,8	17	38,6	27	26,2
	Hypertermia	0	0,0	3	6,1	10	8,5
Clinical Triad (#)	3 sypmtoms	10	28,5	3	6,5	24	22,6
	2 symptoms	17	48,6	20	43,5	43	40,6
	1 symptom	7	20,0	22	47,8	37	34,9
	no symptoms	1	2,9	1	2,2	2	1,9
Consequences	Hospitalization	18	43,9	23	46,9	57	48,7
	Acute Kideny Injury	6	15,0	7	14,3	19	16,4
	Dylaisis	2	4,9	2	4,1	8	6,9
	Chronic Kideny Injury	1	2,4	0	0,0	3	2,6
	Fasciotomy	1	2,4	3	6,3	6	5,3

Table VII. Inter-critical features of patients grouped according to the final diagnosis

		Genetic Diseases		Non-genetic diseases		Undiagnosed pts	
		n	%	n	%	n	%
Rest CK	normal (<250)	20	48,8	40	85,1	86	75,4
	slightly elevated (251 -500)	12	29,2	6	12,8	19	16,7
	elevated (501 -1000)	5	12,2	0	-	8	7
	very high(> 1000)	4	9,8	1	2,1	1	0,9
Inter Critical Features	Exerc. Intol	24	58,5	6	12,5	38	33
	Cramps	12	31,6	5	10,4	21	18,3
	myalgias	29	74,4	13	27,1	50	43,1
	weakness	8	19,5	1	2,1	13	11,2
	Hypo- or hypertrophy	3	7,5	2	4,2	9	7,9
	other signs	7	18,9	4	9,1	21	19,1

Patients that were finally diagnosed with a genetic disease presented an earlier first episode of rhabdomyolysis, and frequently recurrent episodes. The age at first consultation was not a discriminant between the two groups.

In the “genetic diseases” subset, patients presented episodes with a higher maximum CK level compared to patients with rhabdomyolysis due to other causes (in 70% of which CK levels were <100,000 U/L). In each category, the effort was the most frequent trigger. Fasting, as trigger, was always associated with genetic diseases, while the use of potentially toxic drugs could be the trigger of rhabdomyolysis also in genetic diseases. The clinical features of the episodes were similar in all categories. However, it is to note that the complete triad muscle weakness + myalgias + dark-colored urines was observed only in 6.5% of patients with non-genetic diseases vs 28% of genetic disease. In the inter-critical period, patients that received a genetic diagnosis presented more frequently, but not always, signs and symptoms of a subjacent myopathy (high CK levels, exercise intolerance, myalgias, fixed weakness, etc.).

However, it is important to remind that the vast majority of patients did not obtain a definitive diagnosis. The “undiagnosed” group, potentially extremely heterogeneous including both genetic and non-genetic diseases, presented the characteristics and clinical features of the other two groups.

The different characteristics of patients affected by the most recurrent specific diagnosis are shown in the following figures and tables.

Table VIII. Demographic data and characteristics of the episodes of rhabdomyolysis of patients grouped according to their diagnosis

		CPT2	GSDV	RYR1	BMD	PGM1	VLCAD	Ex. Rhabdomyolysis
	n	11	11	8	2	2	2	37
	Sex	6M; 5F	8M; 3F	7M; 1 F	2M	1 M; 1F	2F	32M; 5F
	Ratio M:F	1,2	2,67	7		1		6,4
	Age 1s consult (mean ±SD)	32,5±9,7	41,3±18,8	33,2±10,1	16,65	28	n.a.	32,2±12,1
	Range	16,7 -45,9	18,1 -72,2	20,6 -40,6	17 ; 16,3	20,2; 35,8	NA, 25,7	17,2 - 60,5
		%	%	%	%	%	%	%
Age 1st episode	<12 ans	63,6	0,0	0,0		50,0	50,0	0,0
	13 - 18	27,3	27,3	25,0	100,0	50,0	50,0	2,8
	19 - 30	9,1	36,4	37,5				63,9
	31-40	0,0	18,2	12,5				13,9
	41-50	0,0	9,1	25,0				11,1
	>50	0,0	9,1	0,0				8,3
n. of rhabdomyolysis	Single Episode	9,1	36,4	50,0	100,0			75,7
	Two Episodes	9,1	18,2	25,0		50,0		21,6
	3-4 Episodes	27,3	0,0	0,0				2,7
	5 or more	54,5	45,5	25,0		50,0	100	0,0
Max CK level	<10.000	0,0	14,3	0,0	50,0		50,0	8,1
	10 - 49.999	0,0	28,6	37,5	50,0	100	50,0	56,8
	50 - 99.999	33,3	14,3	25,0				16,2
	100 - 199.999	22,2	28,6	12,5				16,2
	200 - 499.999	33,3	14,3	12,5				2,7
	> 500.000	11,1	0,0	12,5				0,0
		CPT2	GSDV	RYR1	BMD	PGM1	VLCAD	Ex. Rhabdomyolysis
Clincal Features and consequences of episodes	Weakness	45,5	22,2	25,0		50,0	50,0	13,9
	Myalgias	100	100,0	75,0	100,0	100,0	100,0	88,9
	Dark Urines	90,0	90,0	37,5	50,0	50,0	50,0	45,7
	Muscle Oedema	0,0	22,2	12,5		50,0		40,6
	Hyperthermy	0,0	0,0	0,0				8,1
	Hospitalization	45,5	45,5	75,0		50,0		48,6
	Acute Kidey Injyr	18,2	27,3	12,5				13,5
	Dyalysis	9,1	9,1	0,0				2,7
	Chronic renal disease	0,0	0,0	0,0				0,0
	Fasciotomy	0,0	0,0	12,5				5,6

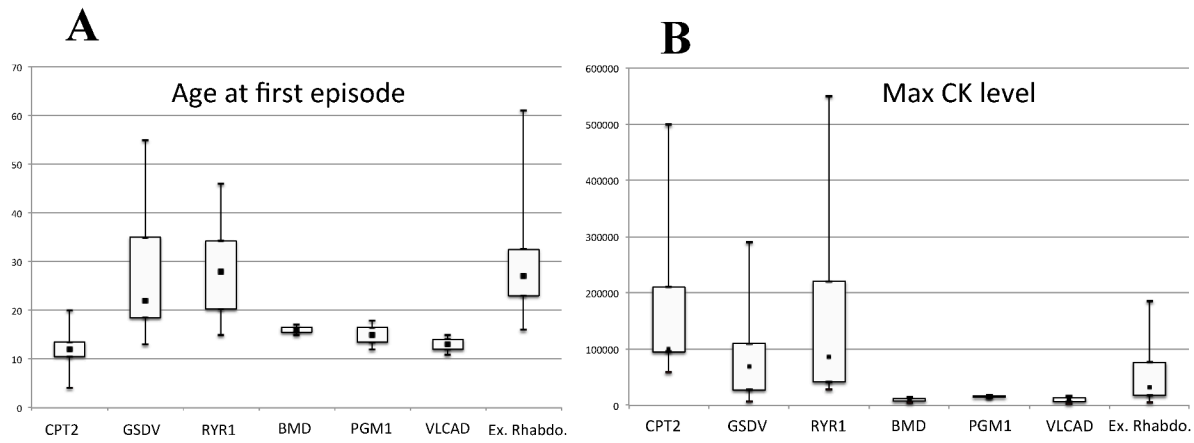


Fig. 10. Age at first episode (A) and Max CK (B) levels of patients grouped according to their final diagnosis. Patients with CPT2, PGM1 and VLCAD deficiency invariably present first episode <20 years of age (A). BMD, PGM1 and VLCAD reach lower levels of max CK, indicating milder episodes of rhabdomyolysis. RYR1 and CPT2 can reach the higher CK values during episodes (B).

Table IX. Type of trigger of rhabdomyolysis in patients grouped according to their final diagnosis. Effort is the most frequent trigger in all patients but VLCAD and CPT2 patients, in which rhabdomyolysis can be triggered also by fever, fasting or other. The relationship between effort and symptoms onset cannot guide toward specific diagnoses.

		CPT2	GSDV	RYR1	BMD	PGM1	VLCAD	Ex. Rhabdomyolysis
		%	%	%	%	%	%	%
Trigger	yes	81,8	100	87,5	50,0	50,0	50,0	100,0
	not always	9,1		12,5			50,0	
	no	9,1			50,0	50,0		
Type of triggers	effort	50,0	91,7	75,0	100,0	100,0	25,0	100,0
	fever	31,3		25,0			50,0	
	drug	0,0	8,3					
	fasting	12,5					25,0	
	other	6,3						
If effort: onset of symptos	during effort	37,5	50,0	40,0	100,0			40,0
	hours after effort	62,5	50,0	80,0				45,7
	days after effor	0,0		20,0				14,3

Table X. Inter-critical features of patients grouped according to their final diagnosis. Rest CK levels distinguish GSDV patients (invariably elevated) from the other groups, presenting normal or slightly increase of CK levels.

		CPT2	GSDV	RYR1	BMD	PGM1	VLCAD	Ex. Rhabdomyolysis
		%	%	%	%	%	%	%
Rest CK values	normal: <250	81,8	0	62,5		100	100,0	85,7
	slightly elev (251 -500)	18,2	36,4	37,5	100,0			14,3
	high (501 -1000)	0,0	36,4					
	very high (> 1000)	0,0	27,3					
Inter-critical features	exercise intolerance	36,4	90,9	37,5	50,0	100	100,0	8,1
	cramps	20,0	36,4	25,0		50,0	100,0	10,8
	myalgias	80,0	90,9	50,0	50,0	100	100,0	24,3
	weakness	0,0	36,4	0,0		50,0	50,0	0,0
	hypo or hypertrophy	0,0	9,1	0,0				5,4
	other signs	0,0	16,7	25,0		50,0		0,0

GSDV patients presented more homogeneous and identifiable clinical and laboratory features. In inter-critical period (usually since childhood) they presented the typical exercise intolerance, associated with myalgias and in 16,7% of cases the pathognomonic “second wind” phenomenon”. Rest CK level was invariably above normal limits (even >1000 U/L). First rhabdomyolysis occurred more frequently in the 2nd-3rd decades, and was frequently recurrent. The trigger was always exercise, except in one case that was triggered by concomitant statin use. Typical feature is muscle oedema, occurring more frequently than in other disases.

CPT2 patients presented the earliest episodes of rhabdomyolysis (always 1st – 2nd decade), usually triggered by effort or infectious diseases or fasting, recurrent, and characterized by very high CK levels (>50,000 U/L). During inter-critical periods no weakness was observed, and generally muscle symptoms were rare except for muscle pain. CK levels were normal to slightly elevated. RYR1 mutated patients frequently did not present myopathic signs in inter-critical periods (no muscle signs or symptoms, normal CK level). Rhabdomyolysis occurred in 2^{nd} -5^{th} decade, usually triggered by effort or fever, and was characterized by very high CK levels. In 50% of patients a single episode of rhabdomyolysis was reported. All these features make the diagnosis between RYR1-mutated patients and “pure exertional rhabdomyolysis” patients impossible, based only on clinical features.

Diagnostic tools

The retrospective analysis of diagnostic tools' results performed in patients affected by different disease are shown in Figure 11.

The EMG is frequently the first exam performed in the diagnostic work-up of suspected myopathies. However, in patients with rhabdomyolysis, the exam results frequently normal (85/109, 78%) and rarely shows non specific myopathic signs (19/109, 17,4%), irrespective of the final diagnosis. The relevance of this exam can be increased with provocative tests, as the Long Exercise test, that in most GSDV and in one PGM1 patient shows a peculiar pattern of immediate decrease of CMAP (Compund Muscle Action Potential) after effort, indicating a post-exercise muscle cells unexcitability (see aim 3 of this thesis for further information). The handgrip test shows a peculiar and pathognomonic result in GSDV patients (no lactate increase and hyperammonia after effort, 100%, n =6)), and isolated post-exercise hyperammonia in PGM1 patients (100%, n =2). The profile of acylcarnitines is useful in the diagnosis of lipid disorders, showing recurrent patterns in CPT2 patients (increase in C16, C16:1 and C18:1 with reduction of the final product of beta-oxidation C2: found in 3/7 CPT2 patients, 43%) and VLCAD (increase in C14 and C14:1, found in 2/2 patients); the isolated increase of long chains is more unspecific (secondary beta-oxidation dysfunction?), but do not exclude the diagnosis of CPT2 (observed in 4/7 CPT2 patients, 58%).

The muscle biopsy shows several and different types of abnormalities in every groups. In GSDV (9/9 biopsies) muscle biopsy showed typical vacuolar blebs, increase in glycogen content and absence of myophosphorylase, in PGM1 (2/2) increase of glycogen content and in BMD (2/2) mild dystrophic changes, with abnormal IHC/WB of dystrophin protein. CPT2 and RYR1 muscle biopsies show more variable abnormalities, ranging from normal to lipid accumulation, mild unspecific myopathic changes, glycogen accumulation, or consequences of rhabdomyolysis. Finally exertional rhabdomyolysis (ER) muscle biopsy showed subtle histopathological abnormalities.

Other diagnostic tools performed include: CPT2 dosage on white blood cells (abnormally reduced in 8 cases/87 studied); beta oxidation activity on lymphocytes or cultured fibroblasts (abnormal but

non pathognomonic in 2/11 patients); dosage of mitochondrial respiratory chain activity on muscle biopsy (isolated complex defect in 5 and global reduction of activity in 2 undiagnosed patient, normal activity in 12); study of glyco(geno)lysis enzyme on muscle biopsy or blood cells (isolated enzymatic defect in 2 and global reduction of activity in 2 undiagnosed patient; 2 myophosphorylase deficiency; 14 normal tests); halothane and caffeine in vitro muscle contracture tests (normal in 2 ER and 1 undiagnosed patients, abnormal in 1 undiagnosed patient – not studied for *RYR1* mutations).

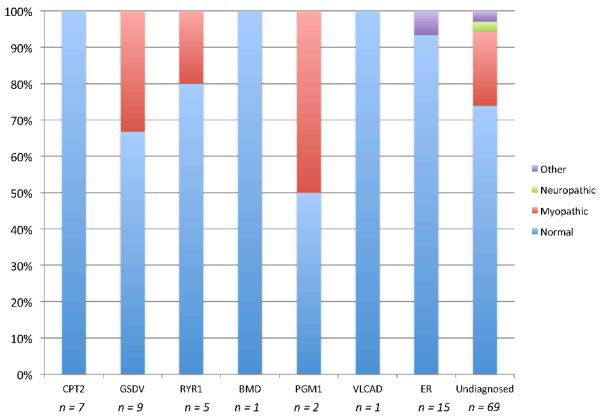
The genetic tests performed are shown in Table XI. A total of 102 test were performed, 37 of them resulting positive (for 4 patients with confirmed genetic no genetic data were available, and the diagnosis was based on biochemical analysis). More specifically, the most frequent test performed was *CPT2* gene, which resulted positive only in 6/27 patients (22,2%). *PYGM* gene was studied in patients highly suggestive of GSDV, based on clinical features or diagnostic tools results, thus gene mutations were always identified (10/10 patients). *RYR1* was routinely studied only in recent years, and resulted positive in a high percentage of patients (33%, 8/24). Genetic panels for limb girdle muscular dystrophies (LGMD, Dr F. Leturcq) and mitochondrial myopathies (Mitocapture, dr C. Jardel) were developed in the last few years, and were used in selected cases. Only the Mitocapture identified a new pathogenic mutation in *DNA2* gene in one case.

	n of test	positive	%positive
CPT2	27	6	22,2
RYR1	24	8	33,3
PYGM	10	10	100,0
MELAS/MERRF	8	0	0,0
DMD	6	2	33,3
LPIN1	4	1	25,0
PGM1	3	2	66,7
ACAVDL	2	2	100,0
DMPK	2	1	50,0
FSHD	2	0	0,0
ANO5	1	0	0,0
CACNA1S	1	0	0,0
CNBP	1	1	100,0
FKRP	1	1	100,0
Gene Panel Mito	3	1	33,3
Gene Panel LGMD	1	0	0,0

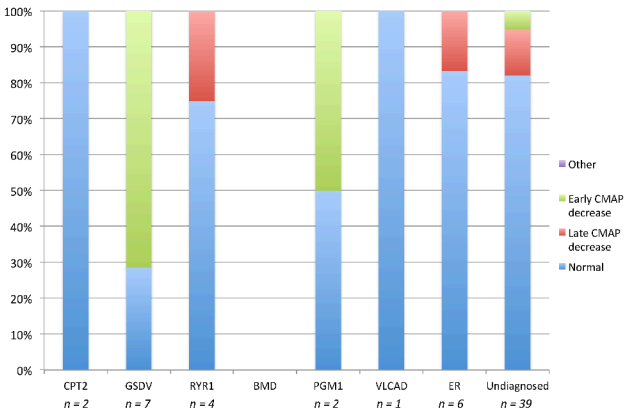
Table XI. Genetic test performed and % of positive results. CPT2 and RYR1 were the most frequently studied genes; PYGM and ACAVDL genes, when performed, always resulted mutated.

Fig. 11 (In the next page). Diagnostic tools results in patients classified according their final diagnosis (ER = exertional rhabdomyolysis): Standard EMG/ENG (A), EMG-Long Exercise Test (B), Handgrip Test (C), profile of acylcarnitines (D) and Muscle Biopsy (E). EMG is normal in majority of patients, otherwise presenting mild non specific myopathic signs (A). EMG with long exercise test detected an early decrease in post-exercise CMAP in >70% of GSDV patients and one PGM1 (B, see aim 3 of the present work). The handgrip test shows a typical profile in 100% of GSDV patients (no lactate increase + hyperammonia); all PGM1 patients presented the more aspecific reduced increase in post-exercise ammonia (C). The profile of acylcarnitines was useful in diagnosis of CPT2 and VLCAD (D). Muscle biopsy showed the typical GSDV profile in all GSDV patients, a mild dystrophic pattern in all BMD patients; in other diseases, it was more variable and less useful in guiding diagnosis.
Abbr.: ER = Exertional Rhabdomyolysis

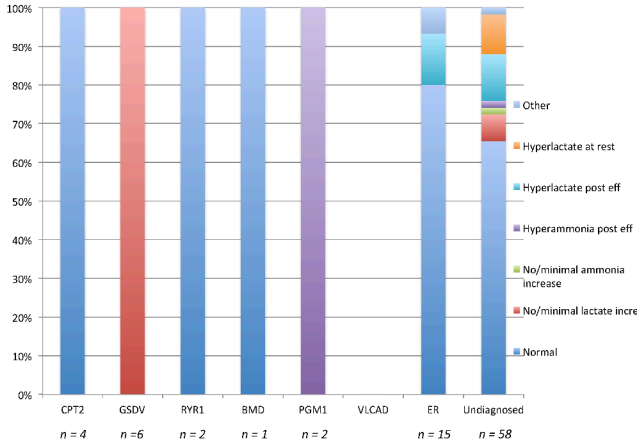
A


Standard EMG / ENG
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CPT2 n = 7
GSDV n = 9
RYR1 n = 5
BMD n = 1
PGM1 n = 2
VLCAD n = 1
ER n = 15
Undiagnosed n = 69
Other
Neuropathic
Myopathic
Normal

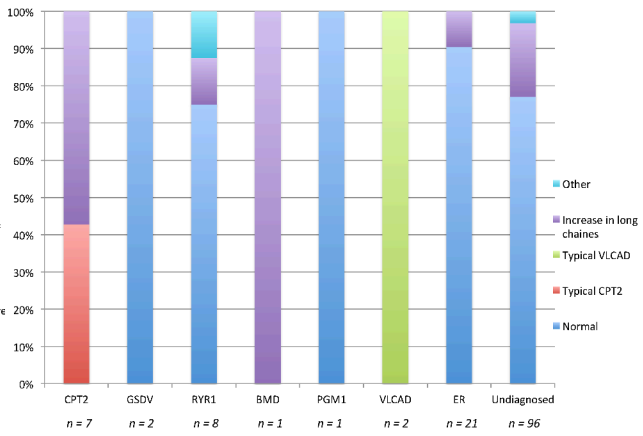
B


EMG Long Exercise Test
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CPT2 n = 2
GSDV n = 7
RYR1 n = 4
BMD
PGM1 n = 2
VLCAD n = 1
ER n = 6
Undiagnosed n = 39
Other
Early CMAP decrease
Late CMAP decrease
Normal

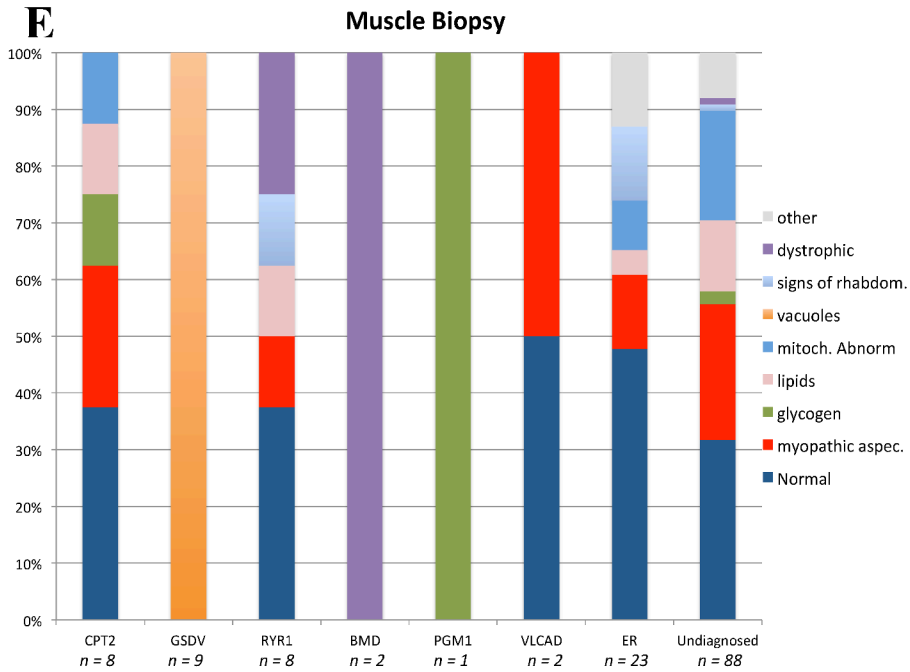
C


Grip Test
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CPT2 n = 4
GSDV n =6
RYR1 n = 2
BMD n = 1
PGM1 n = 2
VLCAD
ER n = 15
Undiagnosed n = 58
Other
Hyperlactate at rest
Hyperlactate post eff
Hyperammonia post eff
No/minimal ammonia increase
No/minimal lactate incre
Normal

D


Profile of Acylcarnitines
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CPT2 n = 7
GSDV n = 2
RYR1 n = 8
BMD n = 1
PGM1 n = 1
VLCAD n = 2
ER n = 21
Undiagnosed n = 96
Other
Increase in long chaines
Typical VLCAD
Typical CPT2
Normal

E


Muscle Biopsy
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
CPT2 n = 8
GSDV n = 9
RYR1 n = 8
BMD n = 2
PGM1 n = 1
VLCAD n = 2
ER n = 23
Undiagnosed n = 88
other
dystrophic
signs of rhabdom.
vacuoles
mitoch. Abnorm
lipids
glycogen
myopathic aspec.
Normal

Relevant Cases

Few specific cases of this cohort need to be discussed individually for their clinical features or diagnosis.

S.D (F, 53 years) presented with two episodes of rhabdomyolysis (max CK 15,000 and 37,000 U/L) over 2 years, the first triggered by an infectious disease and the second by an effort (1h tennis). CK levels never lowered to normal values (rest CK 1,000 – 2,000), and the patient reported exercise intolerance and muscle pain. The diagnosis of myeloma stage 1 (IgG k) was posed after the identification of monoclonal peak, soon after the first episode. In the following months the patient was treated for the hematologic disease, but she reported persistent exercise intolerance, myalgias and high CK levels. The most frequent metabolic myopathies and dystrophies were excluded. The identification of immunoglobulin deposition on the muscle membrane was confirmed on the first biopsy (otherwise showing only small foci of necrosis-regeneration). This suggested a direct correlation between myopathy and myeloma, as previously reported in few sporadic cases (Rodolico et al, 2006).

L.S. (F, 35 years) presented several episodes of rhabdomyolysis with max CK levels 19,000 U/L, without any evident trigger. Out of the episode, she reported only frequent and persistent muscle pain, and CK levels at rest were slightly elevated (400 U/L). All diagnostic tests were normal (muscle biopsy, several EMG, acylcarnitines, GAA activity); however, on one EMG the neurologist reported one or two doubtful myotonic discharges. For this reason, genetic test for myotonic dystrophy type 1 and 2 were performed, and the CCTG amplification on ZNF9 gene was identified. To our knowledge, this is the first report of rhabdomyolysis in myotonic dystrophy type 2.

B.D, (M, 23 years) first consulted a neurologist after a rhabdomyolysis with CK >58,000 U/L after a body building training. Clinical evaluation showed distal weakness on upper limbs and distal hypotrophy on lower limbs, associated with hand myotonia. Genetic analysis confirmed the diagnosis of DM1 (230 CTG repeats on *DMPK* gene).

D.J. (M, 46 years) first consulted because the acute onset of myalgias, weakness and dark urines after a moderate effort; CK level was 11,400 U/L; no AKI sings were noted. Clinical features (proximal upper limbs muscular weakness, skin rush) and histopathological features led to the

diagnosis of dermatomyosytis. Dermatomyosytis manifesting with rhabdomyolysis is rarely reported, and need to be carefully recognized, monitored and treated (i.e. steroids) because of its frequent and severe complications (AKI).

Q.G. (M, 33 years), affected by sickle cell trait, presented several episodes of bilateral pain and muscle edema, both localized in anterior and lateral compartment of legs, appearing invariably during prolonged walking; in several occasion (the most intense) high CK levels were detected (> 12,000 U/L). Muscle biopsy, EMG and muscle MRI were not diriment. Lower limbs arteriography with plantar flexion showed a narrowing of the left popliteal artery, thus suggesting a popliteal artery entrapment syndrome associated with the known risk factor for rhabdomyolysis, the sickle cell trait (Connes et al, 2015; Nelson et al, 2016).

B.S (F, 41 years), reported since childhood several episodes suggesting rhabdomyolysis (muscle pain, dark urines), usually triggered by exercise or fasting. She was hospitalized for the first time at the age of 40, because of intense and diffuse pain and functional impairment after a prolonged walk. CK levels were 21,000 U/L. Few days later, despite accurate treatment (rehydration, hemodialysis because of anuria), she presented tonic-clonic seizures associated with muscle pain and stiffness, posterior headache, visual disturbances, difficulty in reading and low-grade fever. Cerebral MRI showed signs compatible with PRES (Posterior Reversible Encephalopathy Syndrome) related to AKI. Clinical and radiological findings normalized few weeks later and the patients did not present any consequences. In the same days she received the diagnosis of CPT2 deficiency.

R.A (M, 69 years) consulted for an episode of rhabdomyolysis triggered by an infectious disease (CK 91,000). Inter-critical CK level was normal. He presented high blood lactate (4,1 Mmol/L), deafness, bilateral ptosis, without any other myopatic sign/symptom. Muscle biopsy was unspecific. Mitochondrial DNA analysis showed the presence of multiple deletions, MELAS/MERRF mutations were absent, and the gene panel for mitochondrial diseases (Mitocapture) identified one pathogenic mutation in *DNA2* gene.

In four patients the diagnostic workup after the episode of rhabomyolysis let to the identification of asymptomatic cardiomyopathy (2 dilated cardiomyopathy and 2 left ventricular dysfunction);

muscle biopsies showed mild myopathic signs in 1, glycogen accumulation in 1, and were normal in 2. Despite the diagnostic workup none of them received a final genetic diagnosis. Cardiological investigations are thus indicated in all cases of rhabdomyolysis.

COMMENT

Several previous studies reviewed the clinical features of rhabdomyolysis, its pathomechanisms, causes and treatments (Warren et al, 2002; Khan, 2009; Torres et al, 2015; Chavez et al, 2016). Most of the works were performed in the setting of Internal Medecine Services or Emergency Rooms, and thus included mainly secondary causes of rhabdomyolysis, such as trauma, surgery, endocrine pathologies or drugs. Other studies were focused on exertional rhabdomyolysis in healthy people, such as military trainees or sporty subjects (Nelson et al, 2016). A recent study tried to describe the characteristics of potential myoapthic patients among subjects presenting rhabdomyolsys (Scalco et al, 2016).

The aim of this part of the study was to evaluate the clinical features, diagnostic work-up and diagnostic yield in a large series of non-selected patients referred for rhabdomyolysis to a Neurmouscular Center in the last 16 years. In this retrospective work we collected a large population of patients referred for rhabdomyolysis to a Referral Center for Neuromuscular disorders, thus including all the potentially myopathic patients that manifested with this dramatic event. Due to the setting of the study (Neuromuscular Center) and the inclusion criteria, we excluded all the evident secondary causes of rhabdomyolysis. The main limitation of this study is its retrospective nature, and its results reflect the clinical variability in diagnostic process and not a predefined common protocol. However, to minimize this hurdle the study was conducted in a single center, in which few experts in neuromuscular diseases work and collaborate in a rather homogenous way. This smoothen the potential variability of the diagnostic processes.

During the study period, new information was obtained in the evolving world of metabolic myopathies. For example, a great number of new genes were discovered, or were identified as associated with rhabdomyolysis (i.e. *RYR1,* in the last few years); new diagnostic techniques were developed or improved (i.e. handgrip test, EMG provocative test, gene panels); or abandoned (i.e.

halothane and caffeine in vitro muscle contracture tests for hyperthermia). As such, this study includes patients that underwent different diagnostic processes that were always the most up-dated for the time of study.

In the present study we confirm that rhabdomyolysis can be a dramatic event, that frequently requires a rapid and intensive medical care (almost 50% of patients required hospitalization), it is frequently recurrent, and can be complicated by acute kidney injury, compartment syndrome requiring fasciotomy (9 cases) or even Posterior Reversible Encephalopathy Syndrome due to AKI. Rhabdomyolysis has a major impact on quality of daily life of patients (risk of recurrence, fear of efforts or fever, etc) and on families (is there a genetic disease underlying this event?).

In the present study we demonstrate that, in case of, rhabdomyolysis the etiological diagnosis is still very challenging, when an external cause is not evident. Only 20% of the included patients received a final diagnosis of genetic disease, while 23% received a diagnosis of non-genetic cause of rhabdomyolysis. However, it has to be noted that the last category included mostly the so called "pure exertional rhabdomyolysis" (ER); this entity is a diagnosis *per exclusionem*, posed when a peculiar effort alone is thought to be the cause of rhabdomyolysis. However, here we demonstrate that RYR1-related rhabdomyolysis patients were clinically not different from ER patients. They were normal in the inter-critical periods, presented frequently isolated episodes of rhabdomyolysis, triggered by effort. *RYR1* was found mutated in one third of the examined patients, thus it can be considered as a frequent cause of rhabdomyolysis and need to be studied in all patients. *RYR1*-related rhabdomyolysisdiagnosis has several implications for prognosis (risk of recurrence, risk of general anesthesia, etc) and for genetic counseling (autosomal dominant transmission).

The clinical features of the episodes or the inter-critical clinical and laboratory features could guide toward specific molecular diagnoses only in few cases. GSDV is the easiest diagnosis, because of the typical clinical features and the unique results of diagnostic work up. CPT2 need to be suspected when the trigger is a long effort, or fasting, or fever. VLCAD is suspected when the profile of acylcarnitines is supportive of the diagnosis, if normal it should be repeated during episodes or after prolonged fasting (8 hours). Overall, the vast majority of patients presenting to neuromuscular specialists for rhabdomyolysis do not receive a final diagnosis (118, around 50%)

despite an extensive work up. This work-up is expensive and time consuming for patients and doctors, and it is still insufficient for the diagnosis of rhabdomyolysis.

The Next Generation Sequencing (NGS) techniques applied to the whole exome, or to gene panels can study several genes implicated in specific diseases in a single procedure. In France several panels were set up, including one for limb-gridle muscular dystrophies (LGMD), and one for mitochondrial disease (Mitocapture). These panels are used in standard diagnosis of genetic diseases, and were used also in specific cases of rhabdomyolysis, when indicated. A gene panel specific for metabolic myopathies with rhabdomyolysis is actually under development and it will be discussed in aim 4 of this thesis. The NGS techniques are very promising for increasing the diagnostic rate of patients presenting with rare diseases, however can not be blindly used as a first step in a diagnostic work-up, but need to be implemented after a careful selection of candidates and after the exclusion of: i) genetic diseases specifically suggested by first-step exams; ii) non-genetic diseases that can be excluded by first-step exams.

AIM2:

Identification and characterization of a new cause of rhabdomyolysis: *CASQ1*-related myopathy

RATIONALE

Ca^{2+} transients are crucial second messengers during excitation-contraction (EC) coupling in the skeletal muscle. Synchronous Ca^{2+} release from the sarcoplasmic reticulum (SR) terminal cisternae (TC) into the muscle fibre cytoplasm, and its subsequent re-uptake into the SR store, is granted by a structural and functional unit of Ca^{2+} sensors, release channels, buffering proteins and ATP-dependent Ca^{2+} pumps, usually referred to as triad (Rossi AE and Dirksen RT, 2006), spanning from the lumen of SR, across the cytoplasm and into the extracellular matrix. A number of Ca^{2+} handling diseases involving components of triad junction have been recently described (Dowling et al, 2014). They underline the importance of its single constituents in maintaining a correct Ca^{2+} homeostasis and release/uptake dynamics for a proper function of muscle tissues. Calsequestrin (CASQ) is the main Ca^{2+} buffering protein in the terminal cisternae of sarcoplasmic reticulum (SR) and a regulator of ryanodine receptor (RYR1)-mediated Ca^{2+} release (Kawasaki T and Kasai M, 1994). Its two isoforms, CASQ1 and CASQ2, are preferentially expressed in skeletal and cardiac/smooth muscle tissues, respectively, with CASQ1 being largely predominant isoform in fast twitch (type II) fibres (Lamboley et al, 2013). CASQ1 buffers the Ca^{2+} present in the SR terminal cisternae and prevents it from precipitating, thus maintaining a large-capacity buffering gradient that can rapidly release large amounts of Ca^{2+} upon stimulus. CASQ1 is physically anchored to RYR1 through the interaction with triadin and junction (Zhang et al, 1997), and proposed to act as an SR Ca^{2+} sensor and regulator of Ca^{2+}-release kinetics by RYR1. CASQ1 binds Ca^{2+} ions with high capacity both through high- and low-affinity binding sites. Occupancy of high- and low-affinity sites promotes step-wise polymerization of CASQ1 proteins in long chains, which are further stabilized by coordinate binding of additional Ca^{2+} ions between its adjacent chains. High dissociation rate of CASQ1 low-affinity Ca^{2+} binding sites assures that release of non-bound, free TC Ca^{2+} easily destabilizes the CASQ1 polymeric network, resulting in a precise

kinetics of Ca^{2+} release (Sanchez et al, 2012). Thus, both Ca^{2+} binding residues and those involved in CASQ1 polymerization have an important role in regulating the kinetics of Ca^{2+} buffering and release.

The physiological importance of CASQ1 is underlined by the findings from CASQ1 null mice, which undergo spontaneous lethal episodes, similar to human malignant hyperthermia (MH), when exposed to stressful conditions (e.g. mating, heat shock, anaesthetics) (Dainese et al, 2007). iIn these mice, fast-twitch muscle (e.g. EDL) of male display SR Ca^{2+} depletion and signs of Ca^{2+} leakage at increasing temperatures (Protasi et al, 2011), which might be the basis of spontaneous contractures seen in the lethal episodes of CASQ1 KO mice.

Despite this crucial role, the first human myopathy associated with *CASQ1* mutations was only recently reported by two Italian groups (Rossi et al, 2014; Di Blasi et al, 2015). The authorsdescribed a common *CASQ1* mutation, p.Asp244Gly, in patients presentinga non-debilitating vacuolar myopathy, first described at the histopathological level by Tomelleri et al. (2006). The p.Asp244Gly mutation identified was shown to impair CASQ1 polymerization dynamics and, consequently, the Ca^{2+} release kinetics (Rossi et al, 2014; Di Blasi et al, 2015, D'Adamo et al 2016). The Asp244 amino-acid is located in a a conserved high-affinity Ca^{2+}-binding site of CASQ1. Another group reported a positive correlation of *CASQ1* single nucleotide polymorphism (SNP) 175A>G with the onset of heat stroke in a cohort of 150 patients (Li et al, 2014)

In this study we report the clinical and molecular characterization of the largest cohort of CASQ1 mutated patients, including 16 patients carrying the common p.Asp244Gly mutation (two already described [9]) and one patient carrying a novel *CASQ1* mutation (p.Gly103Asp), identified by genetic screening of unresolved vacuolar myopathies. We expanded the clinical spectrum of the disease, identify a peculiar muscle MRI pattern, and further deepened molecular, immunohistochemical and ultrastructural characterization of muscle biopsies, thus providing new details on the pathogenetic aspects of this myopathy.

METHODS

Informed consent and ethics

Every patient signed an informed consent to collect and study biological material and clinical data. The study was carried out in accordance with the ethical rules and guidelines issued by the Ethical committee of Azienda Ospedaliera di Padova and by Helsinki declaration. The collection of muscle biopsies was conducted as part of standard diagnostic procedures.

Participants

All patients with genetically unresolved vacuolar myopathy with histopathologcal features similar to those described in the report of Rossi and colleagues (2014) were selected from the database of patients referring to the Centre for Muscle Diseases at University Hospital of Padova and underwent the genetic screening for *CASQ1* gene mutation. Histopathological selection criteria included: i) presence of optically empty vacuoles and/or amorphous material debris; ii) absence of rimmed vacuoles; iii) no other causes of myopathy (i.e. normal muscular protein levels and acid maltase level; no exposure to chloroquine or colchicine). Forty five unrelated patients (38 males and 7 females) were included in the study.

Genetic Analysis

Genomic DNA was extracted from peripheral blood leucocytes by standard salting out procedure. Tetraprimer ARMS (Amplification Refractory Mutation System) were designed to verify the presence of c.731A>G variant and positive results were confirmed by Sanger sequencing (primers and PCR conditions available on request).

Total RNA was isolated from patients' skeletal muscle biopsies following the TRIzol (Life Technology) standard protocol, and RNA was reverse-transcribed using SuperScript™III Reverse Transcriptase (Life Technology). *CASQ1* cDNA (NM_001231) was amplified and coding sequence analyzed by Sanger sequencing. Any variation was checked against public databases (dbSNP, Exome Variant Server and 1000Genomes) to take note of common SNPs. Potential functional effects of the novel variants were evaluated using *in silico* prediction tools: MutationTaster (http://www.mutationtaster.org/), SIFT (http://sift.jcvi.org/), and PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/). Haplotype analysis was based on genotyping of four

dinucleotide polymorphic repeats in a region of about 400 kb around CASQ1 gene (see Supplementary material for haplotype analysis procedure; and Supplementary Table I for primer sequences).

Clinical Evaluation

The clinical features and progression of *CASQ1*-mutated patients were evaluated by revision of medical records, structured questionnaire and direct specific clinical evaluation. Patients were interviewed about family history, clinical course, type and age of disease onset and rate of progression. The clinical features were classified by the presence of clinical signs and symptoms, like tendon contraction, muscular hypo- or hypertrophy, scoliosis, scapular winging, dysphagia. Muscle weakness was evaluated by manual muscle testing and graded according to Medical Research Council score from 0 (absence of any contraction) to 5 (normal strength).

Patients underwent a cardiac evaluation that included physical examination, electrocardiography (ECG) and echocardiography including M-mode, two dimensional, and Doppler echocardiography to detect the presence of abnormal wall motion and regional structural abnormalities.

A complete respiratory evaluation including physical examination, arterial blood gas analysis and spirometry in upper position was performed.

Muscle Imaging

Patients underwent muscle MRI assessment (1.5 T, Avanto, Siemens, Erlangen, Germany) with T1-weighted axial scans and Short-Tau Inversion Recovery (STIR) sequences. The examination included pelvic, lower and upper limbs muscles. The degree of degeneration and fatty infiltration was graded in T1-weighted axial scans by experienced radiologists according to Mercuri's classification. A final score ranging from 1 (normal) to 4 (severe involvement) was assigned to each muscle. Hyperintense signals in STIR sequences were used to assess the degree of oedema, and graded into 3 levels (absent, mild or moderate).

Histology and fluorescence microscopy

At the time of diagnosis vastus lateralis biopsies were obtained from 10 patients. Serial cryosections of muscle biopsies were stained with hematoxylin–eosin (H&E), ATPase, NADH and antibodies against the components of calcium homeostasis machinery (CASQ1, RYR1, SERCA1,

STIM1, ORAI1 and SERCA2) (see supplementary material for details about antibodies and procedures). Single fibers were followed throughout serial sections, counted and distinguished for the fiber type, presence of single or multiple vacuoles and immunoreactivity of the vacuoles for each indicated antibody.

Electron Microscopy

Ultrathin section of muscle specimens was fixed in glutaraldehyde and osmium tetroxide and contrasted with 4% uranyl acetate and lead citrate and examined with a transmission electronic microscope (TEM Philips 410 T) (See supplementary material for details).

RESULTS

CASQ1 gene mutation analyses

Forty five unrelated patients were tested for the presence of c.731A>G (p.Asp244Gly) variant, and the mutation was identified in 10 subjects in heterozygous state. The collection of more detailed information about the pedigrees identified others 6 affected family members that were recruited in the study. Overall we identified the common c.731A>G (p.Asp244Gly) mutation in 16 patients belonging to 9 unrelated pedigrees.

The hypothesis of a common ancestor origin for p.Asp244Gly mutation was confirmed by reconstructing the haplotype using four polymorphic dinucleotide repeats mapping on chromosome 1q23.2 near *CASQ1* gene (400Kb). The specific haplotype was shared by all analyzed family members and segregated with p.Asp244Gly mutation (Figure 12), confirming the hypothesis of a common ancestor of all Italian patients and consistently with the previously published studies.

Screening of the entire *CASQ1* gene sequence was performed in patients resulting negative for the p.Asp244Gly variation. This technique revealed a new heterozygous variant, c.308G>A, in one patient. This variation has never been reported previously in public genetic databases of genomic variants (dbSNP, 100Genomes, EVS), and determines an amino acid change from highly conserved glycin to aspartic acid at codon 103 of the protein (p.Gly103Asp).

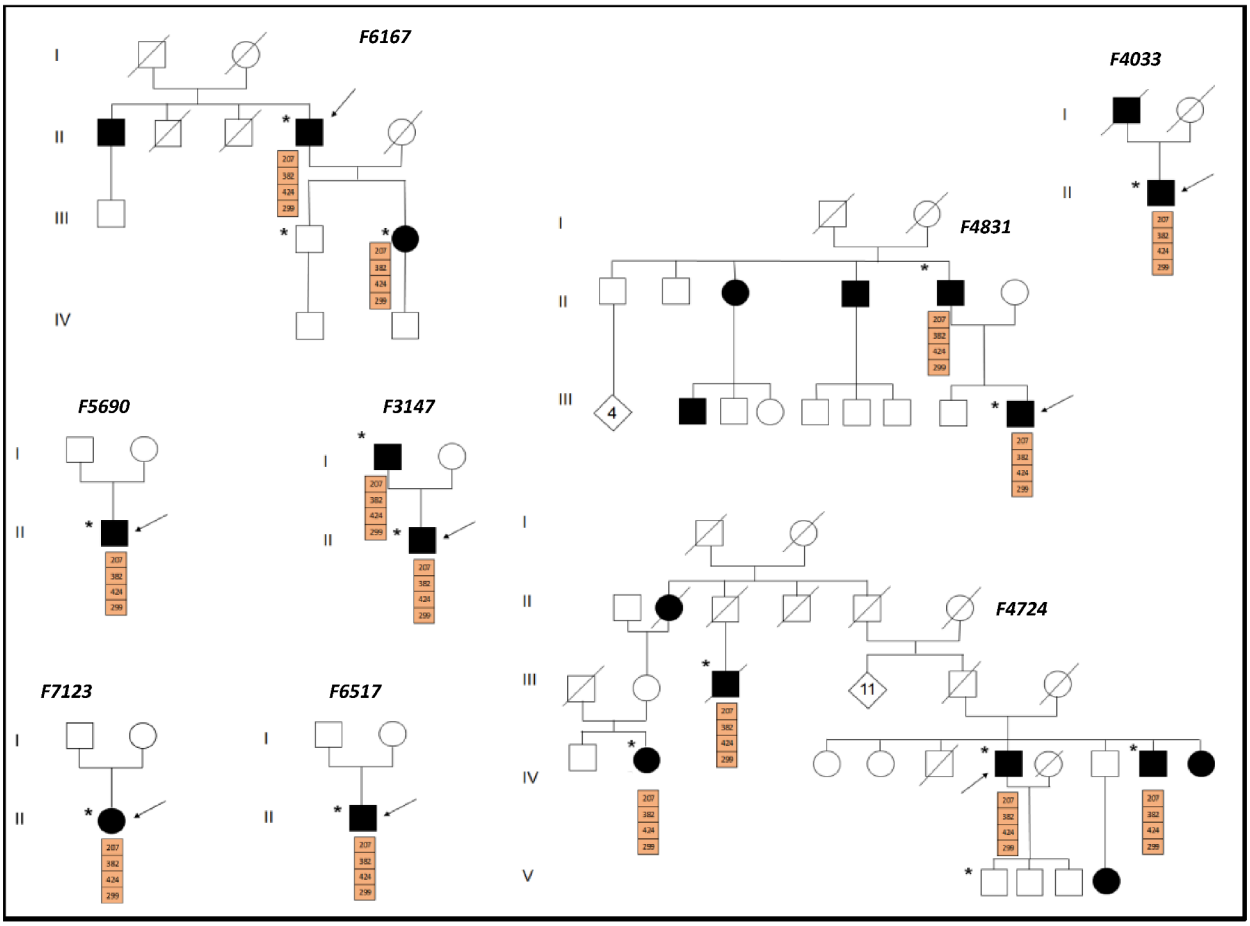


Figure 12. Family trees of eight *CASQ1* p.Asp244Gly mutated families with haplotype reconstruction.
Black arrows: probands; asterisk: subjects available for genetic analysis.

Clinical evaluation

Seventeen CASQ1-mutated patients were included in the study (14 males, 3 females) (Table XII). Age at last evaluation ranged from 16 to 83 years (mean 54±18). Seven patients aged 33 to 71 years were still asymptomatic, and the diagnosis was posed because of asymptomatic hyperCKemia (7 patients) or because relatives of symptomatic patients. In the remaining patients, first symptoms occurred at an age of 47 ± 18 years (range 15 -70). First reported symptoms included exercise intolerance (4 cases), muscle pain (4), proximal weakness in lower limbs (3) and frequent falls (2). Of note, three patients reported minimal symptoms since childhood (running and sport difficulties, scoliosis), even if overt disease manifested in the third or fourth decade. CK levels at disease onset or before first symptoms were elevated in all patients at rest, ranging from 1.5 to 11 times above normal values (mean x7,5). Rhabdomyolysis episodes were reported in 3 cases after efforts (myalgias and increase of CK levels up to x21 normal value). All patients were ambulant without aids at last evaluation.

Neurological evaluation showed normal muscle strength in 9/15 patients in upper limbs muscles and in 10/15 patients in lower limb muscles. In the remaining patients muscle weakness was always mild to moderate (3+ to 4+/5 MRC scale) and was observed more frequently in triceps brachii (5 cases), scapular muscles (4), biceps brachii and deltoid (1) in upper limbs, and hip flexors (5 cases), knee flexors (3), knee extensors and ankle dorsiflexors (1) in lower limbs. Two patients presented mild neck flexor weakness. Muscle trophism was normal in 6 patients, while the others presented scapular winging (6 cases), quadriceps hypotrophy (4 cases) or pectoral hypotrophy associated with distal lower limbs hypotrophy (3 patients). Knee deep tendon reflexes were brisk in 7 patients. Two patients presented mild joint contractures (knee and elbow).

Pneumological evaluation revealed a mild decrease of respiratory muscle strength in two patients (mild reduction of maximal inspiratory and expiratory pressure). Cardiac evaluations showed a non-physiological left ventricular concentric remodeling in patient n. 17 (59 years, no risk factors, no neuromuscular signs except for high CK levels) and hypertensive heart disease in patient n. 9 (79 years). No cardiac abnormalities were observed elsewhere. No cases of malignant hyperthermia were reported.

Muscle imaging

Ten patients (median age 48, range 16 – 79) underwent a muscle MRI of upper and lower limbs (Figure 13 and 14). The four youngest patients (aged between 16 and 37) showed normal signal in T1-weighted sequences. In the remaining patients, a peculiar pattern of fibro-fatty substitution was observed, with severity of degeneration correlated with the age. First alterations appeared in the distal portion of vastus lateralis, in semitendinosus muscle and in gluteus maximus. With increasing patients' age, the fibro-fatty substitution spread to all anterior thigh muscles, with relative sparing of vastus intermedius, to posterior thigh muscles with relative sparing of sartorius and proximal portion of biceps femoris, and to posterior leg, more severe in gastrocnemius. Of note, a remarkable asymmetry was observed in all patients. In upper limbs, only the two oldest patients presented mild involvement of triceps brachii (2/2) and deltoid and subscapularis (1/2). STIR sequences revealed mild intrafascicular oedema in all patients with a variable distribution: gastrocnemius in three patient, posterior thigh muscles in two patients, anterior thigh muscles (vastus lateralis and rectus femoris) in one patient and triceps brachii in one patient.

For two patients a radiological follow-up was available. Patient n. 1 showed a mild evolution (Mercuri score changed from 1 to 2a) of fibro-fatty involution during a four-year observational period (from 48 to 52 years). One patient (n. 9) underwent to MRI at age of 77 and 78. The severe fibro-fatty substitution at baseline was unchanged after one year.

Table XII: Patient Population. Clinical features of CASQ1 patients included in the study

Pt n., sex	Family n.	Age at first symptoms	First symptoms	CK at onset (x n.v.)	Age at last evaluation	Upper limbs muscular weakness	Lower limbs muscular weakness	Axial muscles weakness	Muscle trophism	Deep reflexes	Associated pathologies
1, F	1a	34	muscle pain, frequent falls	x1,5	52	triceps brachii; scapular muscles	knee flexors, hip flexors	Neck flexor	scapular winging	very brisks	Hemicrania
2, M	1b	65	muscle pain	x6 (x10 after effort)	67	triceps brachii; scapular muscles	hip flexors, ankle dorsiflexion	no	quadriceps hypotrophy	absent	Hypertension, Dupuytren sdr
3, M	1b	70	fatigue, difficulty in climbing stairs	x4	83	n.a.	n.a.	n.a.	normal	brisk	Hypertension, sensorineural hearing loss
4, M	1b	45	exercise intolerance	x7	55	no	no	no	quadriceps hypotrophy	brisk	Sensorineural hearing loss
5, M	2	47	exercise intolerance, muscle pain	x7	60	triceps brachii	knee flexors, hip flexors	no	quadriceps hypotrophy, scapular winging	brisk	Hypertension
6, F	2	35		x11	37	no	no	no	mild scapular winging	brisk	
7, M	3	asymptomatic (37)	hyperCKemia	x11 (x27 after effort)	37	no	no	no	normal	brisk	
8, M	4	43	exercise intolerance	x5	61	no	no	no	normal	normal	
9, M	5	65	frequent falls, mild lower limb proxymal weakness	x4	79	biceps brachii, triceps brachii	knee flexors, hip flexors, knee extensor	no	scapular winging, muscle hypotrophy of quadriceps, pectoral and distal leg	reduced	Chronic Obstructive Pulmonary Disease
10, M	5	asymptomatic (35)	hyperCKemia	x2 (x21 after effort)	35	no	no	no	normal	very brisk	Multiple sclerosis
11, M	6	asymptomatic (69)		n.a.	69	deltoid, scapular muscles, triceps brachii	no	no	scapular winging	normal	Ulcerative colitis
12, M	6	asymptomatic (33)	hyperCKemia	x12	33	no	no	no	scapular winging	normal	
13, F	7	asymptomatic (71)	hyperCKemia	x5	71	scapular muscles	no	Neck flexor	normal	normal	Hypertension
14, M	8	n.a.	exercise intolerance, muscle pain	n.a.	52	n.a.	n.a.	n.a.	n.a.	n.a.	
15, M	9	15	mild proxymal weakness	x11	16	no	hip flexors	no	pectoral and distal leg muscles hypotrophy	normal	
16, M	9	asymptomatic (59)	hyperCKemia	x4	59	no	no	no	pectoral and distal lower limb muscle hypotrophy	normal	
17, M	10	asymptomatic (59)	hyperCKemia	x3	59	no	no	no	normal	normal	

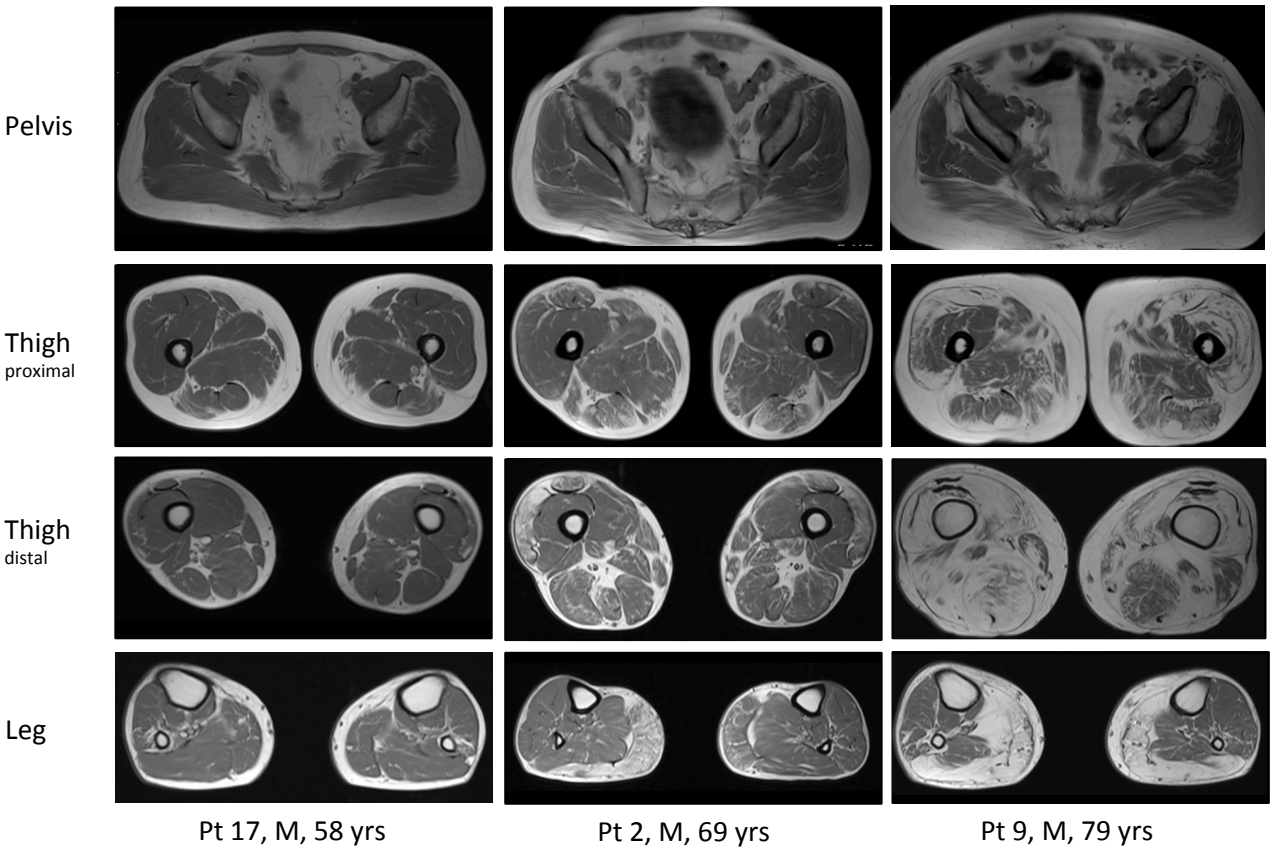


Fig. 13 Lower Limb Muscle MRI, T1W sequences in three patients of different age. Age-dependent and asymetric degree of fibro-fatty substitution can be observed initially in the distal portion of vastus lateralis, semitendinosus muscle and in gluteus maximus, the spreading to all anterior thigh muscles (with relative sparing of vastus intermedius), to posterior thigh muscles (with relative sparing of sartorius and proximal portion of biceps femoris), and to posterior leg (more severe in gastrocnemius).

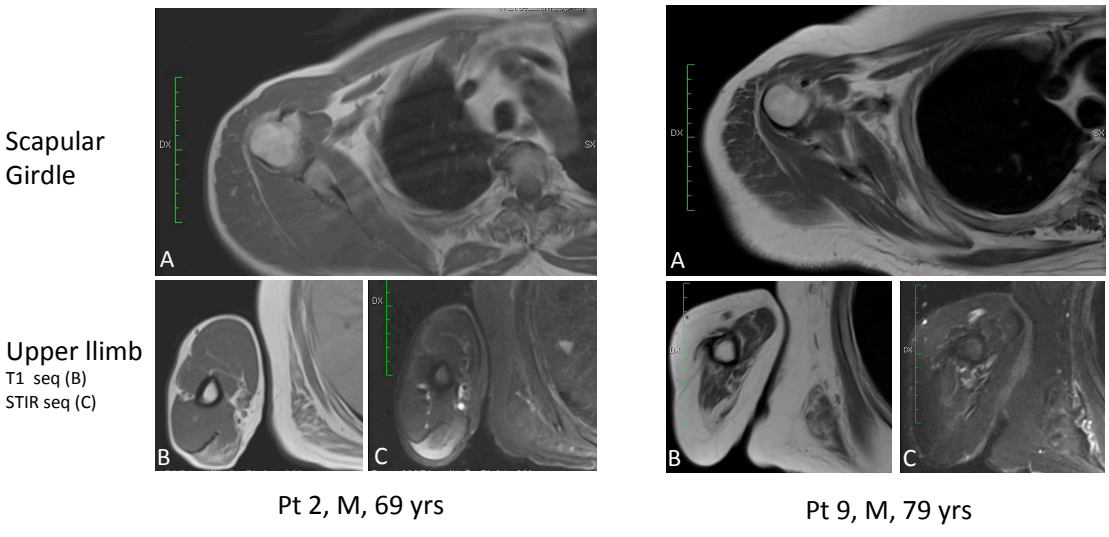


Fig. 14. Upper Limb Muscle MRI, T1W and STIR sequences in the two oldest patients.

Histopathology and Immunofluorescence

Serial cryosections of nine patients carrying the p.Asp244Gly and one patient with p.Gly103Asp mutation in CASQ1 gene were analyzed for histological properties and distribution of the proteins involved in maintaining Ca^{2+} homeostasis in muscle fibers. On average, 116±59 of fibers were analyzed per patient. As already described by Rossi et al (2014), p.Asp244Gly mutation in *CASQ1* resulted in the appearance of optically empty vacuoles in H&E staining as well as in acidic ATPase (pH 4.35). We also observed increase in fiber size variations (6-86 μm), rare necrotic fibers, central nuclei, splitting and activation of acid phosphatase. Immunofluorescence analysis of sarcoplasmic Ca^{2+} influx/efflux machinery proteins showed that the optically empty vacuoles did not show any reactivity within the vacuoles, but rather displayed enrichment in fluorescence signal on the vacuole margins (RYR1, ORAI1, SERCA1, SERCA2 and STIM1) or in the sarcoplasmic tubular network surrounding the vacuoles (CASQ1). Vacuoles were observed almost exclusively in fast twitch type II fibers (204/488 type II fibers with vacuoles, versus 4/557 type I fibers with vacuoles) and most of the fibers contained multiple vacuoles (82±14% of all vacuolated fibers). CASQ1 and SERCA1 displayed the highest levels of enrichment (respectively in 86±16% and 74±20% of type II vacuolated fibers), while lower levels of enrichment on vacuole margins were observed for ORAI1 (72±30%), RYR1 (60±23%) and STIM1 (57±33%). The weakest increase in signal and the smallest frequency of enrichment of vacuoles was seen for SERCA2, normally expressed at low levels in fast twitch fibers (38±18% of vacuolated fibers). (Fig 15).

The novel missense mutation p.Gly103Asp resulted in a different muscle morphology, showing either vacuoles fulfilled of amorphous material (in 15/62 fibers) or NADH-positive debris (in 10/62), without any optically empty vacuole. As for the p.Asp244Gly biopsies, abnormalities were observed in type II fibers only. The intra-fiber vacuoles were strongly immunoreactive for CASQ1, ORAI1, RYR1, SERCA1 and STIM1 and weakly reacting with SERCA2.

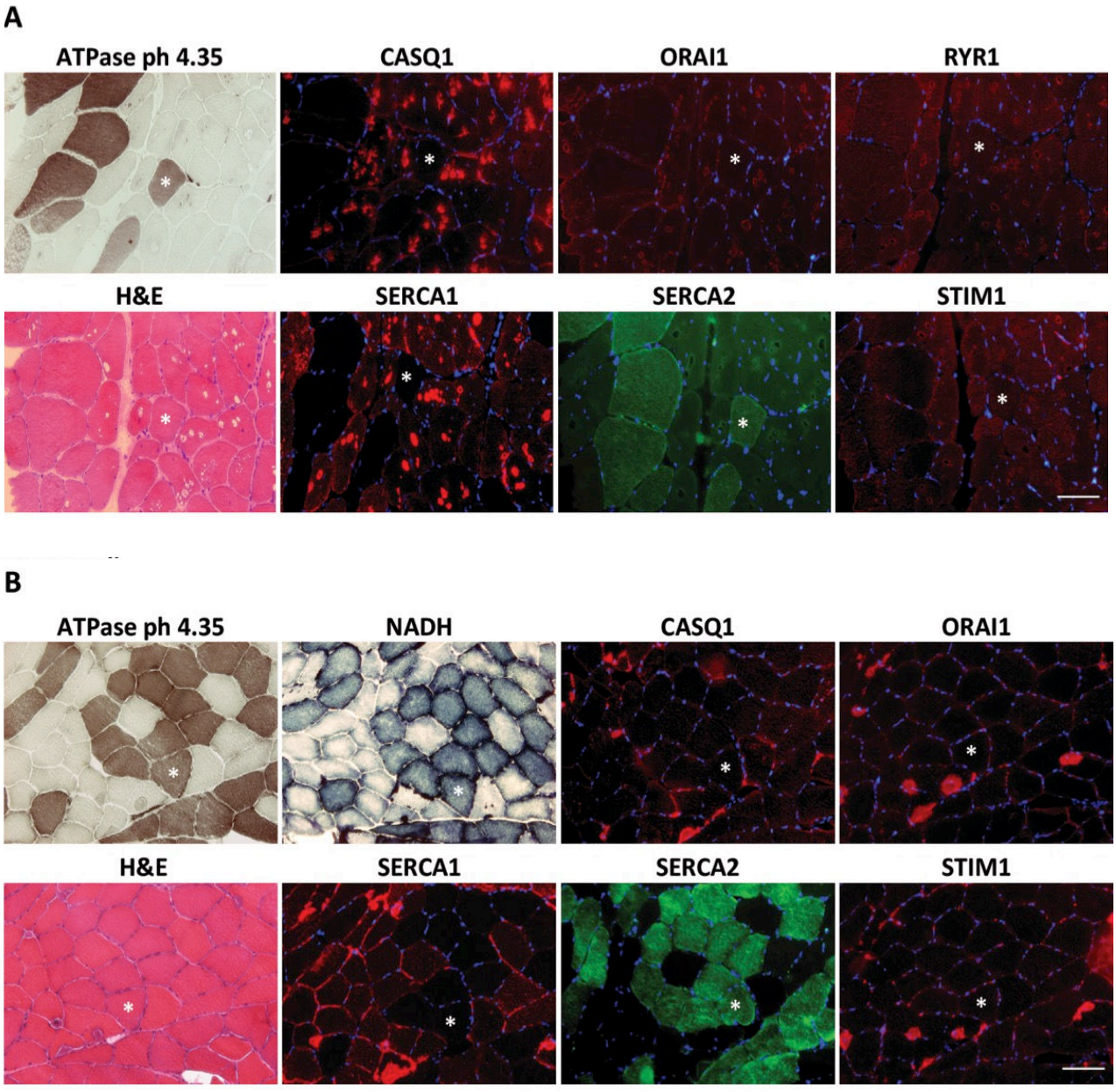


Fig. 15 Histopathology and immunofluorescence. Serial cryosections of one patient carrying the p.Asp244Gly mutation (A) and one patient with p.Gly103Asp (B).

COMMENT

Rossi et al. (2014) and Di Blasi et al. (2015) described the first cohorts of patients affected by *CASQ1*-related myopathy, clinically characterized by high CK levels, mild proximal weakness and myalgias, thus confirming the important role of CASQ1 in muscle calcium homeostasis. The disease was associated with a typical histopathological picture with at the EM level inclusions of different size and shape. In the original reports all patientscarried the same mutation c.731A>G (p.Asp244Gly) suggesting a founder effect in northern Italy. In our study we expand the clinical and molecular characteristic of the disease associated with the common p.Asp244Gly mutation and confirm the slow progression of the disease and the paucity of symptoms at onset that can be complicated by an increased disability due to the progressive course of muscle weakness in later age. Furthermore, we identified a peculiar muscle MRI pattern and a novel *CASQ1* gene mutation leading to a different histopathological picture.

The *CASQ1* genetic screening in unresolved vacuolar myopathies referred to our Unit confirmed the high prevalence of CASQ1 mutations (11/45; 24%). The identification of the recurrent p.Asp244Gly in 16 patients and the haplotype study confirm the presence of a founder effect in Italian patients, and in particular in patients coming from the north-eastern part of our region (Veneto). The haplotype reconstruction identified unknown family linkages in our patients cohort. We also identified a novel *CASQ1* mutation, p.Gly103Asp, in a patient coming from a region (Tuscany) of central Italy. The pathogenicity of the common mutation was previously demonstrated by several genetic and *in vivo* approaches (Rossi, DI Blasi, D'Adamo), but the pathogenicity of the newly identified mutation can only be inferred because no patient's cultured muscle tissue was available for functional studies. We think this mutation may be pathogenetic because a) it is has never been reported in genetic databases (dbSNP, 1000Genomes, EVS) including the newly launched Genome Aggregation Database of the Broad Institute including 126,216 exome sequences and 15,136 whole-genome sequences from unrelated individuals sequenced as part of various disease-specific and population genetic studies (http://gnomad.broadinstitute.org/); b) it is located in a highly conserved residue. Two *in silico* prediction tools suggest a pathogenic role (MutationTaster indicate as highly probable the damaging effect, p=0.999; same results with *SIFT)*

and only one tool (*Polyphen2*) indicates a possible benign effect; c) the glycine in position 103 is located in an important functional domain of the protein, a [Ca2+] independent high affinity Ca2+-binding site, suggesting that a mutation in this region is probably damaging; and d)the clinical features of the patient (myalgias, exercise intolerance, hyperCKemia) are consistent with the other CASQ1-mutated patients.

In our case series the clinical onset typically occurs on the 3rd-4th decade with relatively mild symptoms, such as myalgias, exercise intolerance or mild proximal weakness, and the clinical evolution is usually slow. HyperCKemia is common and sometimes the unique clinical sign, confirming that the disease can be frequently undiagnosed and that the condition could be more frequent than supposed. However, our study suggests that caution should be put in deriving long term prognosis in these patients since in the oldest patients the disease evolves in a slowly progressive proximal and axial myopathy. Indeed in our case series we identified some patients presenting with unrecognized clinical symptoms since the very young age, indicating a very early subtle onset, and older patients presenting with severe muscular weakness significantly affecting motor function. Muscle MRI studies further confirm the progression of the disease toward severe fibro-fatty substitution. The recurrence of rhabdomyolysis in three case (marked increase in CK levels and myalgias after effort) and the exercise intolerance indicate that CASQ1 myopathy should be classified also as a metabolic myopathy with dynamic symptoms, sometimes without fixed weakness.

The clinical features in our case series were quite homogeneous. Muscular weakness was relatively mild and age-dependent, and mostly involved triceps brachii and scapular muscles in upper limbs, hip and knee flexors in lower limbs. We frequently observed muscle hypotrophy involving quadriceps, scapular muscles or pectoral muscles. This is different from the cohort described by Di Blasi et al (2016), in which two patients, aged 29 and 81 years, presented with mild muscle hypertrophy. Interestingly, an accurate neurological examination showed brisk knee reflexes in 7/15 patients, a sign unfrequently observed in myopathic patients with quadriceps involvement. This feature could indicate a possible role of CASQ1 in central nervous system. The clinical “red flags” that have to be taken into account for diagnosing CASQ1-related myopathy are

proximal and axial muscular weakness, with scapular winging and quadriceps hypotrophy with relative sparing of rectus anterior, brisk patellar reflexes, mild contractures (ankle, but also knee and wrist), and very high CK levels. The diagnosis of CASQ1 myopathy can also be suspected on the basis of the peculiar MRI pattern. The asymmetric age-dependent fibro-fatty substitution of distal portion of vastus lateralis, semitendinosus, gluteus maximus and gastrocnemius medialis, with relative sparing of vastus intermedius, sartorius and proximal portion of biceps femoris is recurrent in our case series.

In one patient left ventricle concentric remodeling was observed in the absence of other known causes. This patient did not show any neuromuscular sign / symptom except for high CK levels. The association between heart disease and *CASQ1* to date has never been described, and *CASQ1* is not highly expressed in heart. A single case observation cannot lead to definitive conclusions, but, if confirmed, could help to extend the phenotypic spectrum of disease. Furthermore, this finding indicates the necessity for careful monitoring of cardiac function in all CASQ1-mutated patients.

The most important clue for the diagnosis of CASQ1-related myopathy is the histopathological picture. In p.Arg244Gly mutated patients it is always characterized by myopathic changes and vacuolar inclusions, optically empty in standard colorations. These vacuoles strongly react with antibodies against SR calcium-machinery proteins. This finding suggests that abnormal polymerization and aggregation of mutated *CASQ1* lead not only to a decreases the release of Ca2+, as previously reported (D'Adamo et al, 2016) but also to accumulation of all the other interacting proteins.

The slow evolution of the CASQ1-myopathy could be due to redundance of the complex molecular mechanisms regulating calcium homeostasis that can supply the abnormal functioning of calsequestrin, and that still need to be further investigated. The triadopathies are muscle pathologies that include an emerging group of diseases due to genetic defects in one of the components of the triades (RYR1, ORAI1, STIM1, CASQ1). These pathologies frequently present with overlapping phenotypic and molecular and histopathological characteristics, that are being clarified in the recent few years. Each gene may be associated with different clinical pictures, but

similar histopathological changes can be caused by mutations in different genes. For example, myalgias or recurrent rhabdomyolysis can be observed in *CASQ1* but also in *RYR1* and *ORAI1*-related myopathies (Dlamini et al, 2013, Endo et al, 2014). In these myopathies histopathological features are different, being characterized either by core-like structures on oxidative stains (*RYR1*) or by the presence of tubular aggregates (*ORAI1*) or vacuolar inclusions (*CASQ1*). Our patient presenting the p.Gly103Asp CASQ1 mutation showed NADH-positive inclusion on muscle biopsy, thus further blurring the boundaries of our actual knowledge.

Supplementary material

- Haplotype study

Supplementary Table 1: Primer sequences

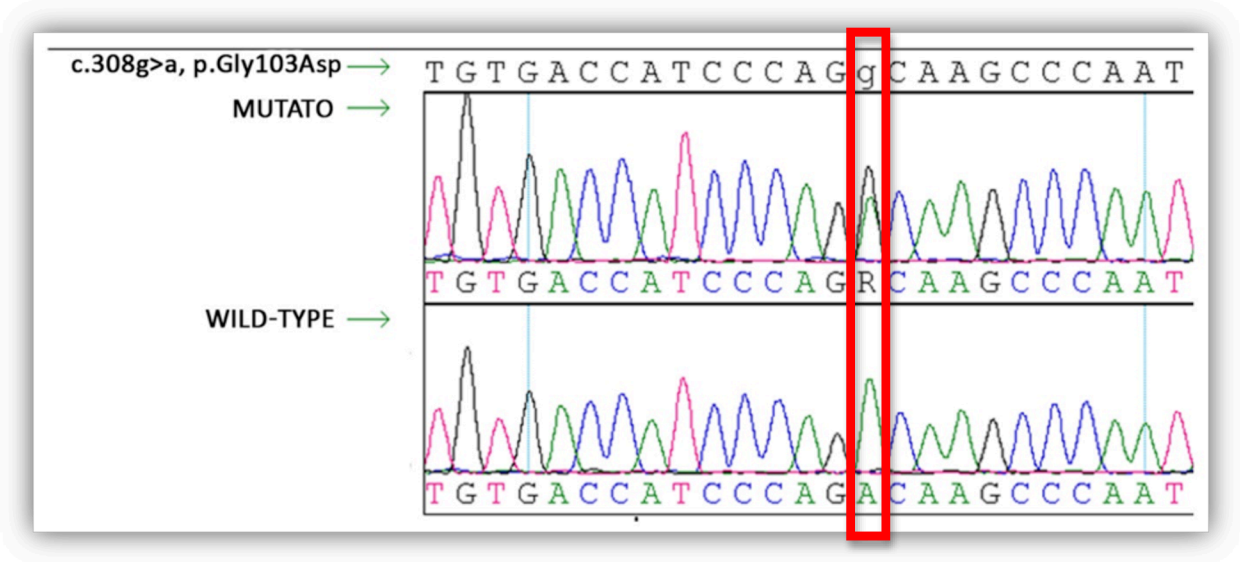
PRIMER NAME	SEQUENCE (5'-3')
chr1_160402793F	TGTAAAACGACGGCCAGTTATGAGACTAGGGCCCTCGT
chr1_160402793R	GGCAAGCATGGTTAGGATCTG
chr1_160082463F	TGTAAAACGACGGCCAGTAGTGTTGTCCTCTCCCCTGA
chr1_160082463R	AAAGCAGGAGGATGGAAGGC
chr1_160019166F	TGTAAAACGACGGCCAGTTGGCACCGCTCTTAGTACAT
chr1_160019166R	CCTGGCCCTATGATGATGGA
chr1_160008864F	TGTAAAACGACGGCCAGTTAGACAGCAGGGGAATGGGA
chr1_160008864R	GACCCATGATCACCACCAACT

Procedure: starting position of the 4 dinucleotide polymorphic repeats: 160402793, 160082463, 160019166, 160008864 on chromosome 1. Each forward primer is tailed with an M13 sequence and a third primer (M13F with fluorescent dye) is used in the PCR reaction to obtain fluorescent PCR products as previously described (Schuelke M. , 2000). PCR products is then pooled and separated on an ABI PRISM 3700 DNA Sequencer (Applied Biosystems, Foster City, CA). The physical positions of each microsatellite is based on the UCSC Human Genome Database (http://genome.ucsc.edu, hg19).

Immunofluorescene studies

Antibodies: mouse monoclonal calsequestrin (Thermo Fisher, Waltham, MA, USA, clone VIIID12); ryanodine receptor, RYR1 (Abcam, Cambridge, UK, clone 34C), SERCA1 (Santa Cruz, CA, USA, clone VE121G9); STIM1 (BD, San Jose, CA, USA, clone 44/GOK), rabbit polyclonal ORAI1 (Sigma-Aldrich Srl, Milan, Italy); goat polyclonal SERCA2 (Santa Cruz, N19).

Procedure: muscle cryosections were collected on Superfrost slides, fixed with 4% paraformaldehyde (PFA) for 10 minutes, permeabilized with 0.3% Triton X-100, incubated in blocking solution (0.5% BSA, 10% horse serum in PBS) for 30 min and incubated with antibodies at 4°C overnight (0.5% BSA, 2% horse serum in PBS). Anti-goat Cy2-coniugated (Jackson Laboratory, Sacramento, CA, USA), anti-mouse Cy3-coniugated or anti-rabbit Cy3-coniugated (Invitrogen, Carlsbad, CA, USA) secondary antibodies were incubated (0.5% BSA, 2% horse serum in PBS) at RT for 1h. Slides were mounted using Vectashield medium with DAPI stain (Vector, Burlingame, CA, USA) and examined on upright microscope (Olympus BX60, Tokyo, Japan) equipped with a CCD camera (DP70, Olympus). Images were post-processed using ImageJ suite.



Supplementary Fig. 1 Chormatogram of the c.398G>A mutation, Sanger sequencing

AIM 3

Clinical and genetic spectrum in limb-girdle muscular dystrophy type 2E

RATIONALE

Sarcoglycanopathies are four recessive limb girdle muscular dystrophies (LGMD) that represent 20-25% of all Limb-Girdle Muscular Dystrophies (LGMDs) and 40-65% of LGMD cases with infantile onset (Vainzof et al, 1999; Fanin et al, 2009). Sarcoglycanopathies are caused by mutations in genes encoding four transmembrane glycoproteins, α-, β-, γ-, and δ-sarcoglycan, that form a tetrameric complex at the cell membrane of skeletal and cardiac muscle. Sarcoglycans play an important role in stabilizing the dystrophin-associated glycoprotein (DAG) complex localized in the sarcolemma of muscle fibres (Ervasti et al, 1990; Chan et al , 1998; Hack et al, 2000; Sandonà et al, 2009).

Mutations in individual sarcoglycans are responsible for LGMD2C (γ-sarcoglycan, SGCC gene at 13q12) (Noguchi et al, 1995), LGMD2D (α-sarcoglycan,SGCA at 17q219) (Roberds et al, 1994), LGMD2E (β-sarcoglycan, SGCB at 4q12) (Bonnemann et al, 1995; Lim et al, 1995) and LGMD2F (δ-sarcoglycan, SGCD at 5q33) (Nigro et al, 1996). LGMD2C and 2D are the most frequent and extensively studied of the four sarcoglycanopathies, with a clinical phenotype characterized by progressive skeletal muscle weakness ranging from a severe Duchenne-like dystrophy to a relatively mild LGMD (Eymard et al, 1997; Angelini et al, 1995; Ozawa et al, 2005; Nalini et al, 2010; Ferreira et al, 2011). LGMD2E is a rare cause of sarcoglycanopathy, with an estimated prevalence of 0.86 x10-6 cases (Fanin et al, 1997), and has generally been believed to be associated with a severe skeletal muscle phenotype, and cardiomyopathy (Melacini et al, 1999; Barresi etal, 2000; Politano et al, 2001; Fanin et al, 2003). One case of exercise-induced myoglobinuria as presenting sign of primary beta-sarcoglycanopathy has been reported (Cagliani et al, 2001). Rhabdomyolysis in other sarcoglycanopathyies (LGMD2D and LGMD2C) has been

described, thus indicating that sarcoglycan genes should be added to the differential diagnosis in the work up of rhabdomyolisis, exercise intolerance, and hyperCKemia, even in the absence of overt muscle weakness or with normal α-sarcoglycan immunohistochemistry in muscle biopsy (Mongini et al, 2002; Pena et al, 2010; Ceravolo et al, 2014; Tarnopolsky et al, 2015). However, knowledge about the specific features of LGMD2E is scarce, because no large cohort has been reported.

The aim of this part of the study was to detail the specific clinical pattern of LGMD2E (age of onset; clinical course; rate of progression; skeletal muscle involvement; rate of rhabdomyolysis; and heart and respiratory function) from a large population of patients affected by LGMD2E. We also evaluated biochemical and molecular data, to identify possible correlations between phenotype, genotype, and protein expression levels and to recognize prognostic factors.

MATERIAL AND METHODS

Study design and patient cohort

The study was designed as an observational, cross-sectional study of clinical features and natural course of LGMD2E. All patients diagnosed with LGMD2E underwent an extensive clinical evaluation and an accurate revision of medical records was performed.

The clinical characteristics at time of diagnosis and the progression of the disease were evaluated by the revision of medical records and by the administration of a specific questionnaire. Patients were divided in three subgroups according to phenotype, defined as “severe” or “mild” if loss of ambulation occurred before or after 18 years of age. The phenotype of patients younger than 18 years and still ambulant was defined as “unknown”.

Clinical evaluation of muscle function

Family history, consanguinity and medical history were recorded for each patient. Patients were asked about the first symptoms of the disease, and age of onset. The progression of muscle

weakness was described by noting the age of loss of specific functions: running, climbing stairs, rising from a chair, and walking without support. The presence and the age of onset of clinical signs and symptoms were recorded (weakness, muscle pain, etc). All the CK measurements available were also reported. Muscle weakness was evaluated by manual muscle testing and graded according to Medical Research Council score from 0 (absence of any contraction) to 5 (normal strength). Fifteen muscle groups of proximal and distal limbs and axial muscles were selected for statistical analysis.

Evaluation of cardiac function

Symptoms and signs of cardiac involvement were noted. Patients underwent cardiac evaluation, including physical examination, electrocardiography (ECG) and echocardiography. All rhythm abnormalities were noted. Echocardiographic examination included M-mode, two dimensional, and Doppler echocardiography to detect the presence of abnormal wall motion and regional structural abnormalities in both ventricles and to measure cardiac volumes and ejection fraction.

Evaluation of respiratory function

The respiratory section of the questionnaire investigated signs and symptoms of chronic respiratory insufficiency, such as rest or exercise dyspnoea, morning headache and recurrent pneumonia and ineffective cough. The number of hospitalization for acute respiratory insufficiency was recorded. Use of a mechanical respiratory support, either invasive or non-invasive, was noted, as well as the number of hours of ventilation per day. The respiratory evaluation included assessment of sitting vital capacity and arterial blood gas analysis.

Sarcoglycan proteins studies

At the time of diagnosis, an open skeletal muscle biopsy was obtained after informed consent from patients or their parents. Muscle biopsies were performed in 23 patients, and immunohistochemistry and/or western blot studies of muscle proteins were available for 20 cases. The level of sarcoglycans was assessed by visual inspection using a panel of monoclonal antibodies (Novocastra, Newcastle- upon-Tyne, UK) and scored as “normal”, “slightly reduced”,

"markedly reduced" or "absent". The skeletal muscle samples used as controls were obtained from patients free of any neuromuscular disorder.

SGCB gene analyses

All patients and their parents gave informed consent for the molecular analysis of SGCB gene. The identification of SGCB gene mutations was obtained by single-strand conformation polymorphism (SSCP)/direct sequencing or direct sequencing of genomic DNA extracted either from muscle or blood leucocytes.

RESULTS

Patient cohort

Thirty-two patients from 25 families (16 males and 16 females) were included (19 from Italy, 7 from Denmark, and 6 from France). According to phenotype classification, 15 patients were scored as severe, 12 as mild, and 5 as "unknown", because younger than 18 and still ambulant. The age at last evaluation ranged from 7 to 67 years. Most patients were of Caucasian descent (15 from Italy, 7 from Denmark, 3 from France, 1 from Albania and 1 from Greece); three unrelated patients originated from the Tunisian Sephardic Jewish community, two siblings were Jordanian and one patient came from the Ivory Coast. Consanguinity was reported in seven families. Two patients were deceased: a severe patient at the age of 14 due to cardiac failure, and a mild patient at 60 from pneumonia.

Muscle function

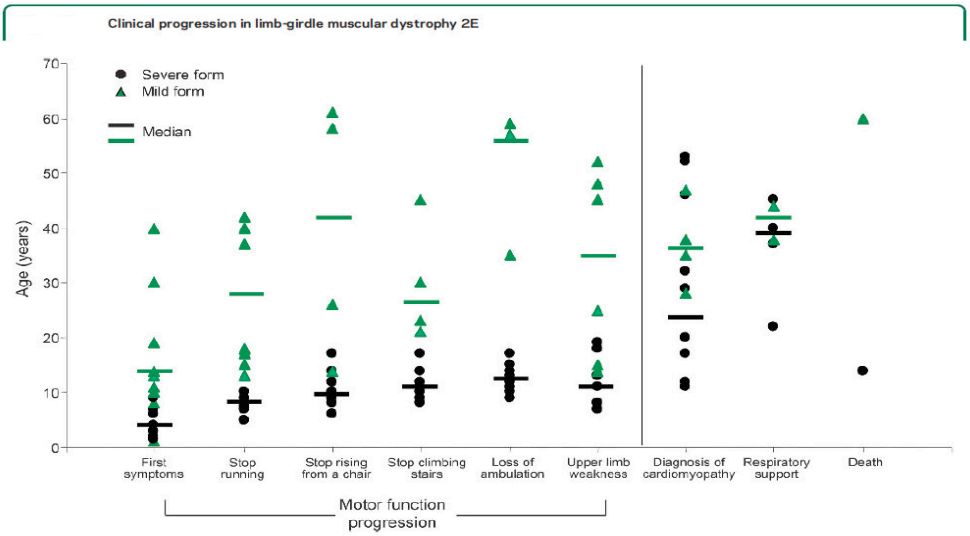
The main clinical symptoms are listed in Table XIII. The progression of motor impairment is represented in Figure 16. The age at onset ranged from 1.5 to 40 years. The first symptoms occurred before age 10 in all severe patients. At onset symptoms were predominately in pelvic girdle muscles, with difficulty in running (41%), gait abnormalities (34%), and difficulties in rising from the floor (22%). Three patients had been diagnosed at a pre-symptomatic stage due to

Table XIII. Patient population and clinical features

	Total	Severe phenotype	Mild phenotype	Unknown phenotype
No. of patients	32	15	12	5
Sex, M/F	16/16	7/8	7/5	2/3
Age at onset, y, average ± SD (range)	9.3 ± 8.9 (1.5-40)	4.8 ± 2.4 (1.5-9)	16.4 ± 11.2 (1.5-40)	5.8 ± 2.4 (3-9)
Age at last evaluation, y, average ± SD (range)	32 ± 19.6 (7-67)	28 ± 17.9 (13-63)	45 ± 15.6 (19-67)	10.4 ± 3.4 (7-16)
Deceased patients, n (%)	2 (6)	1 (7)	1 (8)	0
Motor function, n (%)				
Signs and symptoms				
Loss of independent ambulation	20 (63)	15 (100)	5 (42)	0
Calf hypertrophy	25 (78)	11 (73)	10 (83)	4 (80)
Tendon contractures	22 (69)	13 (87)	6 (50)	3 (60)
Scoliosis	19 (59)	10 (67)	7 (58)	2 (40)
Scapular winging	19 (59)	8 (53)	9 (75)	2 (40)
Tiptoe gait pattern	18 (56)	12 (80)	6 (50)	2 (40)
Muscle pain	10 (31)	0	9 (75)	2 (40)
Macroglossia	10 (31)	6 (40)	3 (25)	1 (20)
Dysphagia	1 (3)	0	1 (8)	0
Osteoporosis	7 (22)	5 (33)	1 (8)	1 (20)
Muscle function treatment				
Physical therapy	17 (63)	9 (60)	8 (67)	3 (60)
Drug therapy: glucocorticoids	7 (22)	4 (27)	2 (17)	1 (20)
Creatine and vitamins	3 (9)	3 (20)	0	0
Cardiac function, n (%)				
Cardiac involvement	20 (63)	11 (73)	7 (58)	2 (40)
Dilated cardiomyopathy	6 (19)	5 (33)	1 (8)	0
Hypertrophic cardiomyopathy	2 (6)	1 (7)	1 (8)	0
Wall motion abnormalities	8 (25)	4 (27)	4 (33)	0
Other[a]	3 (9)	0	1 (8)	2 (40)
Heart rhythm abnormalities[b]	9 (28)	5 (33)	4 (33)	0
Signs and symptoms				
Palpitations	10 (31)	6 (40)	4 (33)	0
Edemas	6 (19)	6 (40)	0	0
Other[c]	8 (25)	3 (20)	5 (42)	0
Drug treatment for cardiac function (angiotensin-converting-enzyme inhibitor; β-blocker; digoxin; diuretic)				
One drug	10 (31)	2 (13)	6 (50)	2 (40)
Two drugs	3 (9)	2 (13)	1 (8)	0
Three or more drugs	3 (9)	3 (19)	0	0
Respiratory function, n (%)				
Respiratory support	6 (19)	4 (27)	2 (17)	0
Invasive	4 (13)	3 (19)	1 (8)	0
Noninvasive	2 (6)	1 (7)	1 (8)	0
Signs and symptoms				
Dyspnea at rest	5 (16)	4 (27)	1 (8)	0
Effort dyspnea	5 (16)	1 (7)	4 (33)	0

[a] Other: ischemic cardiopathy; asymptomatic pseudonecrosis signs at ECG.
[b] Heart rhythm abnormalities: ventricular ectopic beats, left/right branch block; third degree atrioventricular block; extrasystolic arrhythmia; episodic ventricular tachycardia.
[c] Other: syncope; chest pain; hypotension; cardiac dyspnea.

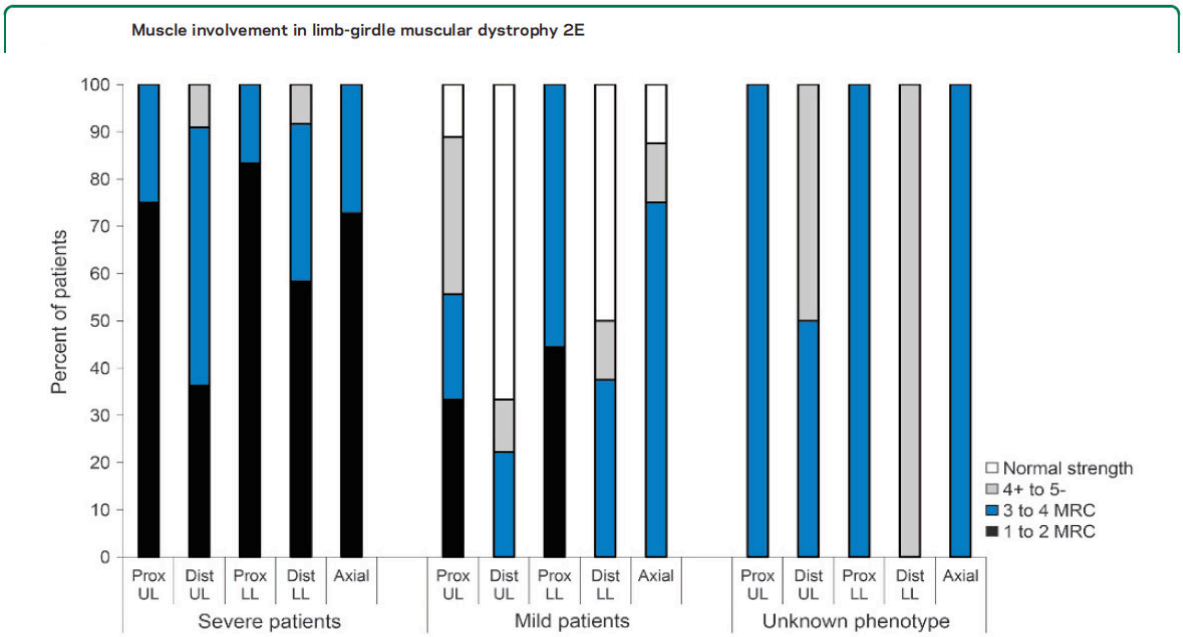
incidental finding of increase of CK levels. All severe patients were wheelchair bound, in accordance with the definition of severe phenotype, at a mean age of 13 years (range 9-17). Age at loss of ambulation was between 23 and 59 years in the five mild patients (mean 40 years). The decline of muscle function was homogeneous and rapid in the severe patients, mimicking the course of Duchenne muscular dystrophy. Mild patients showed a more variable course of the disease (Figure 16). Frequent clinical signs included calf hypertrophy (78% of patients), tiptoe gait pattern (56%), tendon contracture (69%), scapular winging (59%), and scoliosis (59%). No patients reported dark colored urines, myalgias, transient weakness associated with increase of CK level triggered by exercise, fever or other and thus no episode of rhabdomyolysis could be clearly identified. However,muscle pain was reported in 75% of mild, 40% of unknown phenotype, and never in severe cases. Macroglossia was found in 31% of patients, with almost double the prevalence in severe vs. mild cases. Neurological examination showed proximal muscle weakness in all patients, predominantly in lower limb muscles (Figure 17). Muscle weakness was more diffuse in severe patients, involving both proximal and distal muscles of lower and upper limbs. Distal muscles were severely affected only in patients with severe or unknown phenotype. Axial muscle weakness was found in 22 of 25 examined patients (88%).



Age at loss of different clinical abilities and onset of cardiomyopathy or respiratory insufficiency in patients with severe and mild limb-girdle muscular dystrophy 2E. Bars represent median for severe and mild forms. A more rapid motor function deterioration and an earlier onset of cardiomyopathy or respiratory insufficiency in patients with severe disease compared to mild is observed, with a wider variability in the milder patients.

Fig. 16. Clinical Progressoin in LGMD2E, evaluated by *ad hoc* questionnaires

Fig. 17. Muscle involvement in LGMD2E: muscle strength measured with MRC score.



Medical Research Council (MRC) score was used to measure muscle strength in 15 muscle groups in the upper and lower limbs and in axial muscles. Bars represent the percent of patients with specific MRC score. All patients with limb-girdle muscular dystrophy 2E presented a significant weakness involving axial, pelvic, and shoulder girdle muscles. Distal muscles were significantly affected in the patients with severe disease. Dist = distal; LL = lower limbs; Prox = proximal; UL = upper limbs.

Cardiac function

Cardiac involvement was found in 20/32 patients (11/15 severe, 7/10 mild and 3/5 unknown). Cardiac involvement was independent of skeletal muscle phenotype. Dilated cardiomyopathy was detected in six cases, localized wall motion abnormalities in eight, and hypertrophic cardiomyopathy in two. Conduction or heart rhythm abnormalities were reported in 9 patients, including right or left bundle branch block, third degree AV block, supraventricular arrhythmia, ventricular tachycardia, and ventricular ectopic beats (Table XIII). Frequent signs and symptoms included palpitations (9 cases) and lower limb oedema (5). One patient required pacemaker implantation. One patient, affected by dilated cardiomyopathy, had experienced cardiac arrest at 54 years and was resuscitated. One patient presented symptomatic ventricular ectopic beats at the age of 16, without any further cardiologic alteration until the latest echocardiography evaluation at age of 34. Sixteen patients (7 severe, 7 mild and 2 unknown phenotype) received cardiac treatments (ACE inhibitors, beta-blockers, diuretics), most often with a combination of two or more drugs (Table XIII).

Respiratory function

Details on respiratory symptoms are reported in Table XIII. Six patients (19%) presented respiratory insufficiency requiring invasive mechanical ventilation (3 severe, 1 mild) or non-invasive ventilation in 2 cases (1 severe and 1 mild). Age at mechanical ventilation ranged between 22 and 45 years (median: 39) for severe patients and 38 and 44 years for mild patients (Figure 16). Spirometry data were available for 22 patients (9 severe, aged 18.9±7.7; 10 mild, aged 40,7±15.3; 3 unknown,11.2±4.4). Forced vital capacity did not differ significantly among the different phenotypes (60 ± 26 % of predicted value in severe patients, 71 ± 33 % in mild patients and 67 ± 6 % in unknown phenotype patients).

Sarcoglycan proteins studies

Sarcoglycan imunofluorescence and immunoblotting analyses were available in twenty patients (Table XIV). Deficiency of one or more sarcoglycans was obvious in the muscle biopsy of all patients. In eight patients (seven severe, one unkown) the deficiency of α, β-, γ-, and δ-sarcoglycan was complete. Two other severe patients had complete deficiency of one or more sarcoglycans. No patients presenting with a mild phenotype had a complete deficiency of sarcoglycans. Among the milder patients, a variable reduction of sarcoglycans was observed.

In order to test the hypothesis that a complete vs partial reduction of β -sarcoglycan level be associated with a more or less severe phenotype, a time-to-event analysis of loss of ambulation (using age as a time variable) was carried out comparing patients with absence or partial expression of b-sarcoglycan protein. The Kaplan-Meyer survival curve for loss of ambulation comparing patients with complete (n=9) or partial (n=9) defect of β -sarcoglycan is shown in Figure 18. Median ages at loss of ambulation were compared between the two groups by log-rank test. The median age at loss of ambulation was significantly lower in patients with observed absence of protein (13 years) compared to residual protein expression (50 years) ($p<10\text{-}4$).

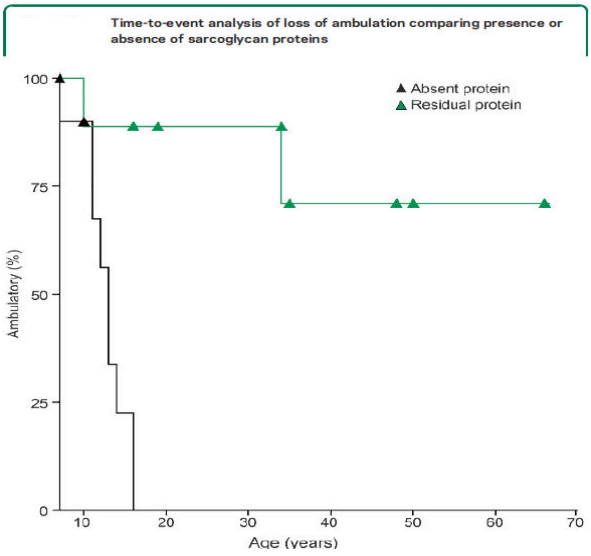


Fig. 18. Time-to-event analysis of loss of ambulation comparing presence or absence of sarcoglycan protein on muscle tissue . In case of absence of the protein, median age of loss of ambulation is predicted to occur at a median age of 13 years, while if a residual level of protein is present the loss of ambulation is predicted to occur at a median age of 50 ($p<10-4$).

SGCB gene analyses

Both SGCB mutated alleles were identified in all patients. Seventeen different mutations were found: seven missense, two nonsense, one splice site and seven frame-shift insertion/duplication or deletion (Table XIV). Five mutations were novel:c.518_519insC, c.543C>G, c.754_957del, c.36_37delinsG, c.1A>G. Three mutations were recurrent. The 32 bp duplication in exon 1 (c.-22_10dup; p.Ala8Glyfs*22), corresponding to 16% of the mutated alleles, predicted a mild phenotype being found only in mild patients (3 from Tunisia, one from Italy and one from Ivory Coast). The 8 bp duplication in exon 3 (c.377_384dup; p.Gly129Glnfs*2; 20% of mutated alleles) was detected only in severe patients (5 homozygous and 3 compound heterozygous with other null mutations). The c.341C>T (p.Ser114Phe) was observed in 6 cases (14% of mutated alleles; 3 homozygous and 3 compound heterozygous) that presented with a clinical phenotype classified either as severe or mild.

Table XIV. Correlations between genotype, protein expression and phenotype.

SGCB gene mutation and sarcoglycan protein expression studies in limb-girdle muscular dystrophy 2E

Mutation	Exon	Protein change	Type of mutation	Ethnicity	No. of mutated alleles	Sarcoglycan expression	Phenotype
c.-22_10dup	1	p.Ala8Glyfs*22	Frameshift	Tunisia, Ivory Coast, Italy	10: 5 homozygous	↓ or ↓↓	Mild ± CM
c.1A>G[a]	1	p.Met1 ?	Nonsense	Jordan	4: 2 homozygous	0	Unknown + CM
c.33+1 G>C	Intron 1	p.(?)	Splicing	Italy	2: 1 homozygous	NA[b]	Unknown
c.36_37delinsG[a]	2	p.Ser13Valfs*6	Frameshift	Italy	4: 2 homozygous	NA[b]	Mild + CM
c.85A>T	2	p.Arg29*	Nonsense	Italy	1: 1 heterozygous with c.377_384dup	NA[b,26]	Severe
c.212T>G	3	p.Leu71Arg	Missense	France	1: 1 heterozygous with c.334_337del	↓	Mild
c.275T>C	3	p.Ile92Thr	Missense	Greece	2: 1 homozygous	NA[b,27]	Severe + CM
c.341C>T	3	p.Ser114Phe	Missense	Denmark, France	9: 3 homozygous	↓ or ↓↓	Severe or mild ± CM
					2 heterozygous with c.543C>G	Normal or ↓	Mild, no CM
					1 heterozygous with c.499 G>A	0	Severe + CM
c.334_337del	3	p.Gln112Tyrfs*9	Frameshift	France	1: 1 heterozygous with c.212T>G	↓	Mild
c.377_384dup	3	p.Gly129Glnfs*2	Frameshift	Italy	13: 5 homozygous	0	Severe + CM
					1 heterozygous with c.699_702del	0	Severe + CM
					1 heterozygous with c.552T>G	0	Severe + CM
					1 heterozygous with c.85A>T	0	Severe
c.499 G>A	4	p.Gly167Ser	Missense	Denmark	1: 1 heterozygous with c.341C>T	0	Severe + CM
c.552T>G	4	p.Tyr184*	Nonsense	Italy	3: 1 homozygous	0	Unknown
					1 heterozygous with c.377_384dup	0	Severe + CM
c.518_519insC[a]	4	p.Pro173Profs*12	Frameshift	Albania	2: 1 homozygous	0	Severe + CM
c.543C>G[a]	4	p.Ser181Arg	Missense	Denmark	2: 2 heterozygous with c.341C>T	Normal or ↓	Mild, no CM
c.544A>G	4	p.Thr182Ala	Missense	Italy	2: 1 homozygous	NA[c,24]	Severe + CM
c.699_702del	5	p.Phe233Leufs*16	Frameshift	Denmark, Italy	5: 2 homozygous	0	Severe + CM
					1 heterozygous with c.377_384dup	0	Severe + CM
c.754_957del[a]	6	p.Glu252*	Nonsense	Italy	2: 1 homozygous	0	Severe

Abbreviations: ↓ = Slightly reduced; ↓↓ = markedly reduced; 0 = absent; CM = cardiomyopathy; NA = not available.
[a] Novel mutation.
[b] Predicted absent.
[c] Predicted reduced.

COMMENT

For rare diseases, small patient populations limit the ability to collect valid data, which inhibits progress in research and care. This limitation can be effectively overcome by collaborative efforts among specialized centres sharing experience in clinical evaluation and management of such patients. The aim of this study was to collect accurate clinical, genetic and protein expression data in a large number of LGMD2E patients and thereby learn the clinical features of the disease, and in particular the occurrence of rhabdomyolysis. We also evaluated whether there exists any genotype/phenotype correlation, and whether prognostic factors can be identified. Four tertiary referral centres contributed to the data collection, and report the largest series of LGMD2E patients to date.

Muscle weakness in LGMD2E, as expected, predominates in proximal muscles, with a more sever affection of lower vs. upper limbs. However, a distal involvement is frequent and prominent in severe form, even at the early stages of the disease (ankle dorsiflexion and wrist extension), as well as axial weakness. This peculiar pattern of muscle involvement could help in differentiation between a severe or mild phenotype since early stages of the disease. Calf and tongue hypertrophy is frequent. LGMD2E patients often present tiptoe walking due to Achilles tendon contractures appearing early in the disease, and subsequently develop contractures in elbows and knees.

In the present cohort we never detected episodes of rhabdomyolysis, clinically or biochemically defined. However, muscle pain was frequently reported in “active patients”, and in particular in 75% of mild patients and 40% of unknown phenotype, while it was never reported in severe cases (non ambulant). The presence of this symptom only in active patients suggests that the defective membrane could be challenged only in mobile patients and therefore in this category of patient if the stress is sufficient may potentially result in rhabdomyolysis

In the present study we also observed that cardiac involvement is frequent in LGMD2E, affecting more than 50% of patients as reported in previous studies (Barresi et al, 2000; Politano et al, 2001; Fanin et al, 2003). Cardiac involvement was independent of skeletal muscle phenotype and occurred at all stages of the disease, even before skeletal muscles were affected. This discrepancy is surprising, but has been reported for related muscular dystrophies with cardiac involvement (Melacini et al, 1996). It is usually characterized by a dilated cardiomyopathy preceded by localized wall motion abnormalities or fibrosis signs on ECG studies. In our cohort, two patients were affected by hypertrophic cardiomyopathy, but the correlation with β-sarcoglycanopathy is doubtful because of concomitant hypertension in one case and lack of follow-up in the other. Heart rhythm and conduction abnormalities were also frequent in LGMD2E (28% of patients). Heart conduction disturbance in LGMD2E has been reported before, and was usually observed after diffuse myocardial involvement (Melacini et al, 1999; Fanin et al, 2003). In our series, at least two patients presented either symptomatic ventricular ectopic beats or right bundle branch block before the onset of other overt cardiac involvement, suggesting the need for close monitoring of cardiac function in LGMD2E.

A severe respiratory involvement requiring mechanical ventilation is less frequent, occurring in the later stages of the disease, and only in wheelchair bound patients. In our series, respiratory muscle involvement never preceded limb muscle involvement as in many other LGMDs except LGMD2I, that can be characterized by early and prominent respiratory involvement (Poppe et al, 2003; Boito et al, 2005). The age at start of assisted ventilation was independent of phenotype and disease duration (median 39 years of age for severe and 41 for mild patients).

Seventeen different mutations were found distributed over the entire SGCB gene. Three mutations were recurrent. The frame-shift mutation c.377_384dup (exon 3) was found in 8 patients coming from Northern Italy. Interestingly, the c.377_384dup was previously found to be in linkage disequilibrium with neighboring polymorphisms in the population of Northern Italy, suggesting a founder effect combined with a relative genetic isolation (Fanin et al, 2000), and our new cases add to this conclusion. All the patients carrying the c.377_384dup presented with a severe

phenotype that included cardiomyopathy in 3 cases, and all showed a complete absence of the four sarcoglycan proteins in skeletal muscle. The c.377_384dup is a truncating mutation, and it has been shown that truncated β -SG, missing the extracellular domain, accumulate around the nucleus and are not properly localized at the plasma membrane, thus disrupting the sarcoglycan complex assembly (Holt et al, 1998). Moreover, β -SG is essential for the formation of the β /d-SG core structure, and disruption of this core affects targeting of the sarcoglycan complex to the plasma membrane, which explains the complete lack of the complex in muscle biopsies Shi et al, 2004).

The c.-22_10dup homozygous mutation was found in five patients presenting a mild phenotype and cardiac abnormalities. All patients presented a variable residual level of sarcoglycans,. This mutation encompasses the first ATG codon (start codon) of SGCB gene and it is predicted to be frame-shifting with a premature stop codon 22 amino acid downstream. To reconcile the discrepancy among a frame-shift mutation, the biochemical data of residual sarcoglycan expression in skeletal muscle and the mild phenotype of patients carrying this mutation, we hypothesize that the c.-22_10dup alters the Kozak consensus sequence, diminishing the amount of protein translated from the first AUG codon and allowing the recognition of the second AUG that is predicted to drive the translation of a full length protein. To test this hypothesis, we used the DNA TIS Miner and the ATGpr algorithms (http://www.hri.co.jp/atgpr/ATGpr_sim.html) (Nishikawa et al, 2000). Both algorithms predicted that, in the mutated sequence, translation would start from the second ATG upstream of the annotated SGCB ORF, but with less efficiency compared with the reference wild type ATG. Finally, the c.341C>T missense mutation was found in 6 patients. This mutation is located few amino acids downstream of the transmembrane domain in the C-terminal extracellular domain. The N-terminal half extracellular domain is important for sarcoglycan interaction and disruption of the b/d-SG interaction will affect membrane localization (Chen et al, 2006). Immunofluorescence analyses in our patients further confirm these findings. Patients carrying this mutation present different phenotypes, severe or mild, with or without cardiomyopathy. To explain this phenotype variability, we hypothesize that other, unrecognized

polymorphisms in the sarcoglycans or other related proteins may further modify the stability of the sarcoglycan complex or other still unknown mechanisms.

As previously described in smaller cohorts of sarcoglycanopathies, the level of protein expression in skeletal muscle predicts disease severity (Guglieri et al, 2008). In our cohort a complete absence of sarcoglycan complex is common in severe patients, and it was never detected in milder patients in whom a variable reduction in one or more sarcoglycans was noted. Loss of ambulation occurred significantly earlier in in patients with observed absence of protein compared to residual protein expression. As expected, there was a correlation between the predicted effect of mutations on protein sequence, and immunofluorescence/immunoblot results. All mutations predicted to cause a frameshift or a premature termination codon resulted in absent labeling for sarcoglycans, and missense mutations showed variable sarcoglycan labeling, from absent to normal, reflecting different impact of individual aminoacid changes on sarcoglycan assembly. Sarcoglycan expression in muscle is a strong predictor of age at loss of ambulation and may be used as prognostic factor in patients stratification for future clinical studies.

This study demonstrates in a large multicentric cohort that LGMD2E may present a highly variable phenotype and that clinical course may vary. We could not identify episodes of rhabdomyolysis in LGMD2E patients, however we could observe the presence of frequent muscle pain in active patients (75%). This can suggest an increased membrane fragility that in specific cases can lead to rhabdomyolysis. We also confirm the high prevalence of cardiac involvement in LGMD2E, characterized by structural and rhythm abnormalities that can appear at any stage of the disease, indicating that cardiac function should be carefully monitored since early stages of the disease. A severe respiratory involvement is less frequent and late-appearing. The knowledge of SGCB gene mutations and sarcoglycan protein expression offer a prediction of the severity of the phenotype.

AIM4:

The role of EMG with long exercise test in the diagnosis of McArdle disease

RATIONALE

McArdle disease (Glycogenosis type V, GSDV) is the most frequent metabolic myopathy caused by deficiency, usually complete absence, of the glycogenolytic enzyme myophosphorylase, due to mutations in the *PYGM* gene, encoding glycogen phosphorylase, muscle form (McArdle, 1951; Bruno et al, 2006; Nogales-Gadea et al, 2015).

GSDV patients present a typical phenotype characterized by juvenile onset of exercise intolerance with muscle pain and early fatigue. Patients generally present the pathognomonic phenomenon of “second wind”, characterized by enhanced tolerance to aerobic exercise occurring within 10 minutes after starting the effort. Beyond the typical exercise intolerance, patients often describe severe symptoms during resting periods after abnormal efforts. These include profound fatigue and intense muscle pain, persistent muscular contractures, and myoglobinuria with possibly acute renal failure due to rhabdomyolysis. A small proportion of patients develop a progressive weakness of axial and proximal upper limb muscles in adult age (after the age of 50) (Nadaj-Pakleza et al, 2009; Vieitez et al, 2011; Lucia et al, 2012). Despite a homogeneous enzymatic background, patients can present phenotypes of variable severity (Aquaron et al, 2007).

The diagnosis of GSDV, which should be raised in patients with short exercise intolerance and high CK levels at rest, relies on the following analysis: non-ischemic forearm exercise test, cycling tests, muscle biopsy and gene testing. The non-ischemic forearm exercise test (also known as the Grip Test) typically shows no increase in plasma lactate concentration and hyperammoniemia (Hogrel et al 2001 and 2015; Kazemi-Esfarjani et al, 2002). The cycling test is also a specific, sensitive, and simple test that is based on the pathognomonic heart rate response observed in the "second wind" phenomenon (Vissing and Haller, 2003). The muscle biopsy is

commonly no longer needed when *in vivo* metabolic studies are suggestive of McArdle disease as *PYGM* mutations are easily detected by specialized genetic laboratories.

The pathogenic mechanisms that underlie the disease are complex and go beyond the only glycolytic pathway blockade. The incorrect utilization of glycogen secondarily determines absence of lactate production, defective acidification of muscle cells, and limited production of ATP. This leads to impairment of membrane pumps function, impaired excitation–contraction coupling and post-exercise sarcolemmal inexcitability (Vissing and Haller, 2012).

The functional consequences of ion channel abnormalities on muscle membrane excitability could be studied by electromyography (EMG) with exercise tests in patients with muscle channelopathies (Fournier et al, 2004). Thus, the aim of this study was to determine the role of EMG associated with long exercise test (LET) in the diagnosis of GSDV and in the evaluation of the secondary biochemical and ionic pump defects of the disease.

METHODS

Patient selection

Twenty-five consecutive patients with diagnosis of GSDV (18M, 7F, age 39,2 ±16,3 years) were included in the study. The diagnosis was confirmed by molecular analysis of *PYGM* gene and/or assessment of myophosphorylase activity on muscle biopsy. Severity of the disease was graded according to the currently used scale (Martinuzzi et al, 2003): 0 = asymptomatic or minimally symptomatic (mild exercise intolerance, but no functional limitation in any daily life activity); 1 = exercise intolerance, contractures, myalgia, and limitation of acute strenuous exercise, and occasionally in daily life activities, without myoglobinuria or muscle wasting and weakness; 2 = same as 1, plus recurrent exertional myoglobinuria, moderate restriction in exercise, and limitation in daily life activities; 3 = same as 2, with fixed muscle weakness and severe limitations on exercise and most daily life activities.

Two patient groups were selected as controls: a group of 14 healthy subjects and a group including 25 consecutive patients (13M, 12F, age 35.9 ±10.5 years) referring to the Neuromuscular Center of Paris for metabolic acute rhabdomyolysis (confirmed by CK levels > 5.000 U/L and/or

increase >20 times of rest CK levels) in which subsequent diagnostic tests (i.e. grip test, muscle biopsy, genetic analyses, etc) excluded the diagnosis of GSDV. Rhabdomyolysis could be relied to a molecular diagnosis in only 6 patients: 2 with phosphoglucomutase deficiency (2 mutations in *PGM1* gene*)*, 3 with very long chain acyl-CoA dehydrogenase (VLCAD) deficiency (*ACADVL* gene) and 1 with long chain acyl-CoA dehydrogenase (LCHAD) deficiency (*HADHA* gene).
The study was conducted after obtaining written informed consent from each patient, according to the European Union and French bioethics laws, as well as the Convention of Helsinki.

EMG Protocol

Patients and controls were examined using the standardized EMG protocol previously described by our group for muscle channelopathies[13]. The overall duration of the protocol was about 40 minutes.

1. Long Exercise Test (LET). The analysis of the compound muscle action potential (CMAP) amplitude before and after exercise provides information on changes in the number of active fibers and on their ability to depolarize and repolarize. CMAPs were evoked by supramaximal ulnar nerve stimulation at the wrist using a bipolar bar electrode, and recorded from right abductor digiti minimi (ADM) muscle using skin electrodes. CMAPs were first monitored before exercise every 1–2 minutes to allow baseline stabilization. The patient was then asked to contract the ADM as strongly as possible in isometric conditions for 5 minutes. A bandage around the tested hand prevented articulation displacements and facilitated isometric effort during the exercise test. After completion of the exercise, the patient was instructed to completely relax while CMAPs were measured at regular time intervals after the end of exercise: 2 seconds immediately after cessation of exercise, then every minute for 5 minutes, and finally every 5 minutes until 30 minutes.
Technical precautions include: careful positioning of recording electrodes to ensure maximal CMAP amplitude; careful positioning of stimulator electrode in the best position to obtain supramaximal activation of the nerve at each time point; brief (2-3 seconds) resting periods during

effort, every 45-60 seconds, to prevent ischemia; skin temperature monitoring between 32-34°C; frequent absorption of sweat film that can alter the CMAP response.

2. Nerve conduction studies: motor and sensory nerve conduction velocity of median, ulnar and peroneal nerves.

3. Repetitive Nerve Stimulation (RNS): to tsest neuromuscular transmission (10 stimuli at 3Hz) in several nerve-muscle couples (axillary-*trapezius*, radial-*anconeus*, peroneal-*tibialis anterior*).

4. Needle EMG: to identify myopathic changes such as abnormal spontaneous activity; short, small and polyphasic Motor Unit Action Potential, etc. Proximal (*trapezius*, *deltoid*, *vastus medialis*) and distal (*extensor digiti communis*, *tibialis anterior*) muscles were studied.

Statistical Analysis

Descriptive statistics (mean, range, standard deviation, standard error of the mean) were used to describe patient's demographic and clinical characteristics for continuous variables. The Shapiro–Wilk test was used to check for normality of the data. For normally distributed variables, repeated measures analysis of variance was performed. ROC curves were calculated with specific tool (Eng, 2014; available at http://www.jrocfit.org).

RESULTS

Patient population

The characteristics, clinical features and results of diagnostics tests of the twenty-five GSDV patients included in the study are shown in Table XV. All patients were symptomatic, with different degrees of severity: 3 patients were classified in class 1 (exercise intolerance), 14 in class 2 (+ rhabdomyolysis) and 8 in class 3 (+ fixed weakness). CK levels were lower than 2x normal values in 6/25 patients in at least one evaluation. Grip test showed a typical GSDV pattern with absence of lactate production and hyperammoniemia post-exercise in 13/13; two siblings (pt. 24 and 25) presented also elevated lactate levels at rest. Muscle biopsy (n = 14) showed in all patients the peculiar features, such as glycogen vacuoles and no expression of myophopshorylase.

Table XV (next page) GSDV patients: demography, clinical features and diagnostic tools.

Pt n, Sex	Age first symptoms (decade)	Age at EMG	Class of severity	Lab features	Grip Test	Muscle Biopsy	EMG		PYGM gene analysis	
				CK levels (min-max, U/L)	Profile	Histopathologycal features	LET : min CMAP amplitude (%; time point)	Needle EMG	Allele 1	Allele 2
1, F	1st - 2nd	30	3	**230** - 2.200	Typical profile	n.p.	0,43 (30')	normal	c.148C>T, p.(Arg50*)	c.2392T>C, p.(Trp798Arg)
2, M	1st	47	3	1.789	n.p.	Typical histopathology	0,61 (30')	**Minimal myopathic changes (deltoid)**	c.148C>T, p.(Arg50*)	**not found**
3, M	1st - 2nd	57	2	409 - 2169	n.p.	n.p.	0,65 (1')	normal	c.148C>T, p.(Arg50*)	c.1190T>C, p.(Leu397Pro)
4, M	1st	60	2	2.200	n.p.	Typical histopathology	0,63 (2")	normal	**n.a.**	**n.a.**
5, M	1st	37	1	613 - 10.000	n.p.	n.p.	0,75 (5')	normal	c.148C>T, p.(Arg50*)	c.613G>A, p.(Gly205Ser)
6, M	1 st	35	2	1.667 - 8.300	Typical profile	n.p.	0,70 (2")	normal	c.148C>T, p.(Arg50*)	c.148C>T, p.(Arg50*)
7, F	1st - 2nd	33	2	648 - 3.200	Typical profile	n.p.	0,73 (30')	normal	c.148C>T, p.(Arg50*)	c.808C>T; p.(Arg270*)
8, M	1st - 2nd	26	2	3.800 - 90.000	Typical profile	Typical histopathology	0,59 (1')	normal	c.148C>T, p.(Arg50*)	c.148C>T, p.(Arg50*)
9, F	4th	39	2	**380** - 6.000	n.p.	Typical histopathology. Enzymatic activity: 1 U/g	**0,93 (20')**	normal	c.148C>T, p.(Arg50*)	**not found**
10, M	1st - 2nd	84	3	330	n.p.	n.p.	0,77 (30')	**Myopathic changes**	c.148C>T, p.(Arg50*)	c.2353C>T; p.(Gln785*)
11, M	1st - 2nd	42	2	726 - 10.000	n.p.	Typical histopathology	0,67 (5')	**Minimal myopathic changes (deltoid)**	c.148C>T, p.(Arg50*)	c.148C>T, p.(Arg50*)
12, M	1st - 2nd	43	3	n.a.	n.p.	Typical histopathology	0,59 (5')	normal	c.148C>T, p.(Arg50*)	c.148C>T, p.(Arg50*)
13, F	1st	17	2	512 - 3.700	Typical profile	n.p.	0,60 (5')	**Minimal myopathic changes (deltoid)**	c.2262delA, p.(Lys754Asnfs*49)	c.2262delA, p.(Lys754Asnfs*49)
14, M	1st	20	1	613 - 290.000	n.p.	Typical histopathology	0,77 (2")	normal	c.1A>G, p.(0?)	c.2262delA, p.(Lys754Asnfs*49)
15, M	3rd	49	3	528 - 9099	Typical profile	Typical histopathology	**0,95 (20')**	normal	c.148C>T, p.(Arg50*)	c.1804C>T, p.(Arg602Trp)
16, M @	2nd	18	1	1019	Typical profile	Typical histopathology	0,45 (10')	normal	c.148C>T, p.(Arg50*)	c.699delCCinsAAA (#)
17, M	1st - 2nd	72	3	**286** - 8.600	Typical profile	Typical histopathology	0,69 (5')	**Minimal myopathic changes (deltoid)**	c.148C>T, p.(Arg50*)	c.2262delA, p.(Lys754Asnfs*49)
18, M	2nd	47	3	2000 - 100.000	Typical profile	Typical histopathology	0,65 (30')	normal	c.613G>A, p.(Gly205Ser)	**not found**
19, M	2nd	39	2	4.105 - 8.772	n.p.	Typical histopathology	0,36 (20')	normal	c.1098G>A, p.(Trp366*)	c.1098G>A, p.(Trp366*)
20, F @	2nd	26	1	2.000 - 4.795	n.p.	n.p.	0,83 (1')	normal	c.148C>T, p.(Arg50*)	c.699delCCinsAAA (#)
21, M	2nd	48	2	560 - 2.800	Typical profile	Typical histopathology	0,69 (10')	normal	c.1475G>A, p.(Trp492*)	c.613G>A, p.(Gly205Ser)
22, M	1st - 2nd	38	2	5.000	Typical profile	n.p.	0,48 (20')	normal	c.148C>T, p.(Arg50*)	c.1768+1G>A; p.(0?)
23, F	2nd	25	2	**350** - 3.500	Typical profile	n.p.	0,53 (30')	normal	c.148C>T, p.(Arg50*)	c.2262delA, p.(Lys754Asnfs*49)
24, M §	1st - 2nd	18	2	653 - 42.000	**Hyperlactatemia at rest; no lactate increase, post-effort hyperamoniémia**	Typical histopathology	0,51 (10')	normal	c.148C>T, p.(Arg50*)	c.2056G>A, p.(Gly686Arg)
25, F §	1st	29	2	**100** - 3.418	**Hyperlactatemia at rest; no lactate increase, post-effort hyperamoniémia**	n.p.	0,82 (2")	normal	c.148C>T, p.(Arg50*)	c.2056G>A, p.(Gly686Arg)

@, § = siblings

***normal values**: <190 U/L*

***typical profile=** normal baseline values, no lactate increase and post-effort hyperammoniemia*

***Typical histopathology =** vacuolar blebs, increase in glycogen content, no myophoshorylase.*
***Enz. activity** n.v. = 45 - 81 U/g*

LET = Long Exercise Test; CMAP = Compound Muscle Action Potential

= novel mutation n.a = not available

EMG Protocol

The standard needle EMG showed non-specific myopathic changes in 5/25 GSDV patients and 6/25 with rhabdomyolysis. No abnormalities were found in neuromuscular transmission with RNS or in short exercise test.

Changes in CMAP amplitudes along the LET of the three cohorts are shown in Fig. 19. All patients and controls had CMAP values within normal values at baseline. The results are thus expressed as a percentage of the reference value measured before exercise (CMAP amplitude before exercise = 100%). Healthy controls presented a mild drop (-10%) in amplitude immediately after effort, rapidly normalized within the first minute. In GSDV patients LET disclosed an abnormal post-exercise drop in CMAP amplitude in 23/25 GSDV patients (92%). The decrement usually appeared immediately after exercise (average -19% ±15) and reached its maximum (up to -27%±14) at 20 min, after a transient plateau phase (lasting 5 to 10 min). No correlations could be found between CMAP decrease in LET and age or sex.

Two GSDV patients (Pt 9 and 15) had normal LET (decrease of CMAP amplitudes <10%). Pt.15 (M, 58 yrs) was very sportive in his youth (cycling), and was diagnosed at age 55 yrs because of high CK levels. He presented mild exercise intolerance, without rhabdomyolysis episodes and *PYGM* molecular analysis identified a missense mutation (c.1804C>T, p.Arg602Trp) in association with the recurrent mutation (c.148C>T, p.Arg50*). Pt 9 (F, 40 yrs) presented a typical but GSDV phenotype with late onset and absence of rhabdomyolysis episodes. Muscle biopsy morphological analysis showed typical features, but a low residual myophosphorylase activity was detected on muscle biopsy (1 U/G, vn 45 - 81), and only one mutation (p.Arg50*) was identified.

The heterogeneous population of rhabdomyolysis presented minimal changes during LET. In detail, 16/25 patients (64%) had normal LET, 5 (24%) a late (>20 min) decrease in CMAP amplitudes (up to -29%), 3/25 patients (8%) an immediate and persistent drop (up to -28%) of amplitudes and 1/25 an immediate and persistent (+56%) increase of CMAP amplitudes. No peculiar patterns were observed in patients with a confirmed molecular diagnosis (LCHAD, VLACD, PGM1).

Overall, the pattern of LET in the 25 GSDV patients was significantly different from healthy controls and from rhabdomyolysis ($p<0.001$). In order to identify GSDV *vs* rhabdomyolysis patients, the test showed a sensibility of 92%, a specificity of 88%. ROC curves of sensibility and specificity of CMAP amplitude, comparing GSDV patients only *vs* "rhabdomyolysis" (Fig. 20), confirm the accuracy of the test at all the different time points in these populations, with an Area Under Curve > 0.85 in all time points after 1 minute.

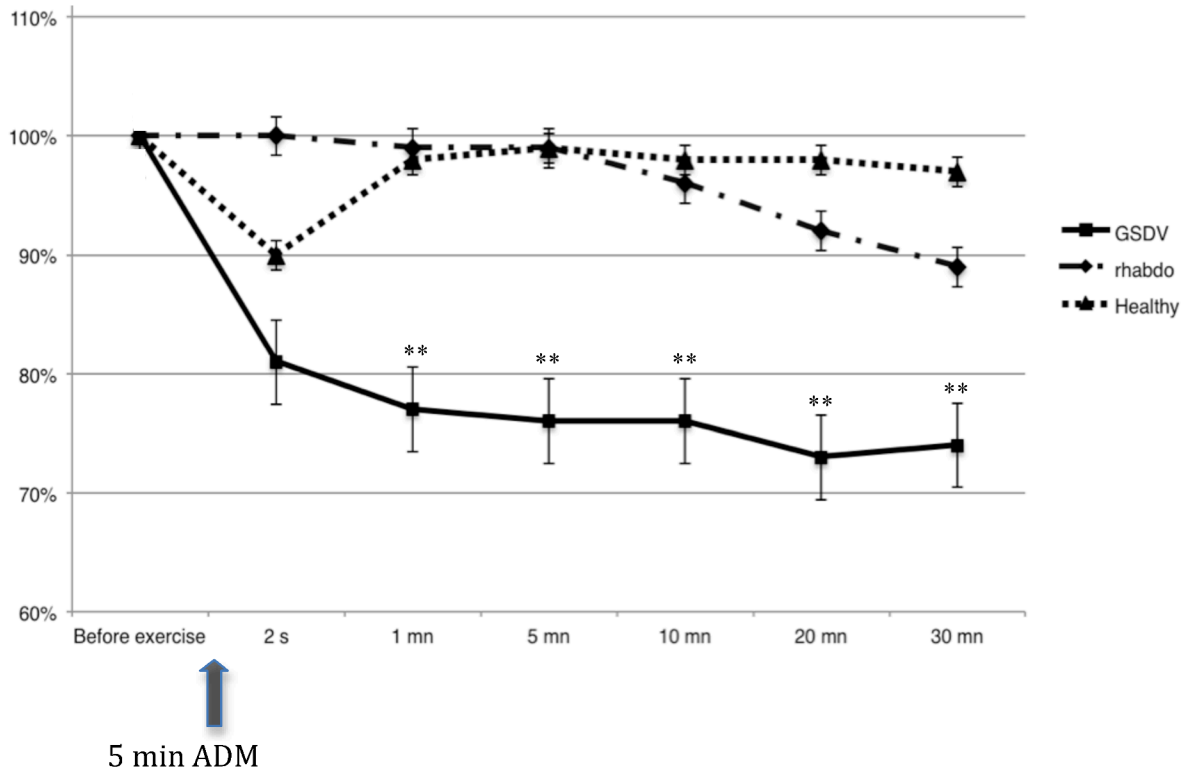


Fig. 19. CMAP amplitude at baseline (before effort) and at different time points after 5 min of isometric contraction of ADM. Values are expressed as percentage of the reference value measured before exercise (Mean ± Standard Error). Asterisks indicate *p* values compared to healthy controls. * = $p<0,05$; ** = $p<0,001$

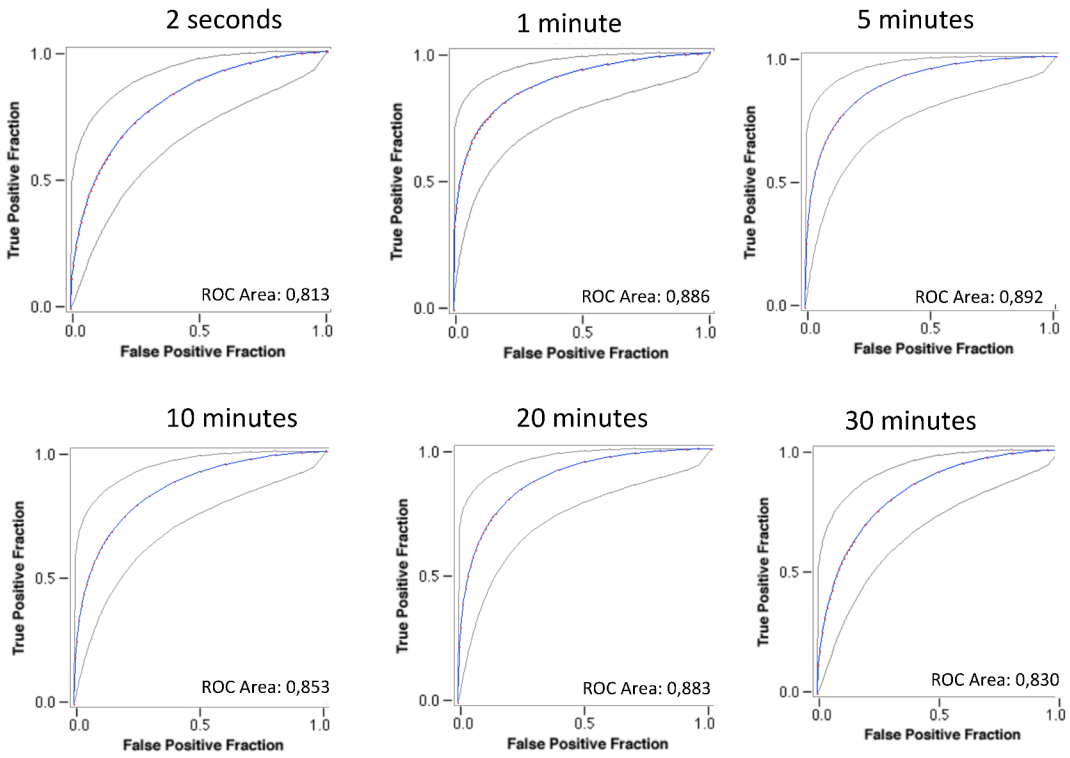


Fig. 20. ROC curves of CMAP amplitudes comparing GSDV *vs* "rhabdomyolysis" patients at different time points after the effort.

COMMENT

The clinical and biological features of McArdle disease are clear, characterized by childhood-onset of short-term exercise intolerance with second wind phenomenon and resting hyperCKemia. However, the diagnosis of McArdle disease is often made in adulthood (from 2nd to 5th decade) despite the very early onset of symptoms, thus indicating that the diagnostic process could be improved with non-invasive test avoiding muscle biopsy (Lucia et al, 2012). In the current study we demonstrated the role of provocative neurophysiological tests, and in particular the LET, in a large series of GSDV patients and compared it with the diagnostic tools actually in use.

Only few studies evaluated the role of EMG in McArdle disease, and its provocative tests. Dyken et al. proposed the first specific neurophysiological test for McArdle disease in 1967 (Dyken et al). They observed the consequences of repetitive stimulation of the ulnar nerve with

supramaximal stimuli at a rate of 18/sec, recording from the *abductor digiti minimi* muscle by a concentric needle electrode. This test produced a considerable (>75%) drop in the amplitude of the evoked response over a 100-sec period in affected individuals and could also produce an electrically silent 'contracture'. However, subsequent studies did not confirm the specificity of this test (Cochrane et al, 1973). This test is no longer performed being unacceptably painful for patients and unreliable.

Other EMG protocols evaluated different characteristics of the effort in McArdle patients. In placebo-controlled trials testing creatine in McArdle disease, EMG was used to evaluate muscle function during exercise protocols (Vorgerd et al, 2000 and 2002). To this aim, amplitude and frequency of EMG signal were recorded from gastrocnemius during 2 minutes of isometric foot plantar flexion (30% of maximal voluntary contraction). The EMG study was also studied during two cycling tests (an incremental test and a constant workload test) as indicators of motor unit recruitment to evaluate the possible contribution of an altered skeletal muscle recruitment strategy to the abnormal exercise tolerance of individuals with McArdle disease (Rae et al, 2010). The main finding of this study was that McArdle patients required an excessive muscle mass recruitment for a given power output and that the state of contractility of their muscles is reduced compared with healthy people. However, all these protocols were confined to the research field, did not include larger controlled case series and were not applied to diagnostic work-up of myopathic patients.

1. LET is useful in the diagnosis of GSDV

Our study indicates that Long Exercise Test (LET) can be usefully and easily added in the diagnostic process of suspected McArdle disease. GSDV was chosen to study the role of EMG with long exercise test for several reasons. GSDV is the most frequent muscle glycogenosis. The vast majority of GSDV patients are biochemically homogeneous, presenting a complete absence of enzymatic activity; this determines a complete absence of lactate production that limits the variability of the response to LET. GSDV patients' symptoms are especially evident during exercise tasks involving both aerobic and anaerobic glycolysis for muscle ATP production. Two types of effort are more likely to cause symptoms: brief intense isometric exercise, such as lifting heavy

weights, or less intense but sustained dynamic exercise, such as running or climbing a hill (Di Mauro, 2007). In EMG with LET the patients are asked to perform an intense isometric contraction of a little muscle (ADM), thus mimicking the first type of effort.

The LET pattern of GSDV patients was significantly different from controls, characterized by a marked drop in CMAP amplitude (-19% on average) appearing immediately after the effort (usually within the first minute) and lasting until the end of the 30-min observational period (Fig. 19). This pattern was observed only in 3 patients referred for rhabdomyolysis (one with phosphoglucomutase deficiency and two undiagnosed) and in no healthy controls. Patients referring for rhabdomyolysis were chosen as control group because one the main aim of this study was to evaluate the role of EMG in the diagnostic process of GSDV, and frequently the first cause of medical consultation of GSDV patients is an episode of rhabdomyolysis. This serious event can be observed in several genetic and non-genetic diseases, but a definitive molecular diagnosis is not frequently achieved despite several diagnostic tests (Scalco et al, 2015).

In this study, we demonstrated that the LET could differentiate the patients affected by GSDV from patients presenting rhabdomyolysis due to other causes. The late-onset decrease of CMAP amplitude in our population of patients referred for rhabdomyolysis ($p<0.05$ compared to healthy controls) cannot be deeply understood, because the diseases underlying this event in our case series are very heterogeneous and often undetermined. The LET pattern observed in patients with GSDV is also different from all the previously published LET patterns for channelopathies; these diseases do not enter in the differential diagnosis of GSDV and thus were not included in this study.

Our findings confirm the first report of an EMG exercise test in one patient suffering from McArdle disease (Lorenzoni et al, 2005). In this reported patient, a decrease in CMAP amplitude was observed after short effort (60-90 sec) in several nerve-muscle couples. However, these findings were never confirmed in larger series. Furthermore, the long effort proposed in our study is more prone to reproduce clinical symptoms, and the longer observation after the effort let us to observe the persistence of the abnormalities and to increase the sensibility of the test.

There are actually in use some diagnostic tools that can easily and specifically guide toward the diagnosis of GSDV. The grip test is actually the most used and accurate tool in the diagnosis of McArdle disease: when performed with standardized procedures it has a sensitivity up to 100%, a specificity of 99.7% and a positive predictive value of more than 90% (Hogrel et al, 2015). Muscle biopsy easily detects specific features (i.e. increase in glycogen content, typical subsarcolemmal vacuolar blebs) and can be used to perform specific biochemical and histoenzymatic (no reactivity to myophosphorylase histochemical stain).

The standard EMG is routinely performed in patients with suspected myopathy or hyperCKemia, but has a limited role in the etiologic diagnostic process of a suspected GSDV, being able to identify only non-specific myopathic changes in a small proportion of cases (20% in our series). In this study, we demonstrated that the LET increases the diagnostic power of EMG in the diagnosis of GSDV compared to patients referring for rhabdomyolysis (ROC curves > 0.85 at all time points from 1 to 20 min) and normal subjects. This test is less sensitive and specific than other diagnostic tools. However, it has favorable characteristics: it is easily performed during a standard diagnostic exam and is well tolerated by patients; it is safer than cycling tests, which may – rarely – determine rhabdomyolysis; and it is non-invasive, in contrast with muscle biopsy. The LET thus nicely complement the grip test, for which the diagnostic power for McArdle has been proved to be excellent (Kazemi-Esfarjani et al, 2002; Hogrel et al, 2015). Furthermore, its correlation with clinical phenotype and its repeatability indicate LET as a potential outcome measure in McArdle disease.

2. LET correlates with clinical phenotype.

Two patients among our cohort of GSDV patients had normal LET. They both presented a mild phenotype, and a residual myophosphorylase enzymatic activity was demonstrated in one. It has been previously demonstrated that a residual amount of myophosphorylase activity is sufficient for allowing a minimal lactate production, and thus to mitigate biochemical secondary dysfunction, attenuate muscle symptoms and increase effort abilities (Vissing et al, 2009). The LET results that we observed in these two patients further confirm these observations.

3. LET as potential outcome measure in McArdle disease.

No curative treatment is currently available for McArdle disease. Some dietary treatments (i.e. sucrose before exercise, carbohydrate-rich diet) and training protocols were demonstrated to be effective in alleviating exercise intolerance symptoms (Vissing and Haller, 2003; Ollivier et al, 2005; Andersen and Vissing, 2008; Andersen et al, 2008, Santalla et al, 2014). However, no large controlled studies are available, and outcome measures to evaluate effectiveness are still scarce. The 31P Magnetic Resonance Spectroscopy can be used in McArdle disease to measure intracellular pH and concentrations of the energy-rich compounds phospho-creatine and ATP (Vorgerd et al, 2000). However, this technique requires specialized centers, is time-consuming and expensive, and is therefore not an ideal tool neither in the diagnosis of GSDV nor as outcome measure in multi-center treatment trials. The outcome measures actually used for McArdle disease include exercise cycling test, 12-minute walk test, quality of life and pain or fatigue scales (Quinlivan and Vissing, 2007; Quinlivan et al, 2015; Ricci et al, 2015). The cycling test accurately measures effort abilities, but it doesn't take into account all the post-exercise effects of the effort, such as pain and fatigue, that prominently affect patients' daily life (Vissing and Haller, 2003). Few quality of life and pain scales study these aspects, but in a qualitative more than quantitative manner and from a subjective more than objective perspective (Ollivier et al, 2005). The LET that we evaluated in this study can be thus usefully used as potential outcome measure to evaluate post-effort dysfunction of GSDV muscles.

4. Mechanisms of post-exercise symptoms and LET Abnormalities in McArdle disease.

The mechanisms underlying the post-exercise abnormalities observed in LET are complex. The absence of myophosphorylase completely blocks anaerobic glycolysis, which is fundamental in normal muscle function during high-intensity exercise. However, beyond the simple failure of energy production for muscle contraction, several pathophysiological mechanisms lead to sarcolemmal dysfunction in McArdle disease.

The failure of lactate production reduces the normal fall of muscle pH. The abnormally high pH decreases the creatine kinase reaction (PCr hydrolysis + ADP phosphorylation) that is normally stimulated by [H+] production via glycolysis. Consequences of this include the presence of

elevated levels of ADP during exercise and inhibition of ATPases, including Na–K ATPase and Ca–ATPase. Secondarily, AMP levels are increased (via the adenylate kinase reactions), thus accelerating AMP deamination (via myoadenylate deaminase) and increasing ammonia production (that is systematically observed during the grip test). Furthermore, several studies in GSDV patients demonstrated loss of osmotic effect related to lactate accumulation. The absence of increase of water content in exercised muscle is cause of the exaggerated rise in blood and presumably extracellular potassium, that contributes to sarcolemmal inexcitability and impaired Na–K pump function. Finally, a deficient substrate-level phosphorylation of ADP such as blocked ATP production in anaerobic glycolysis was observed, that preferentially supply ATP for the membrane sodium–potassium ATPase. This further contributes to impairment of membrane pump function and finally to the abnormal post-effort fatigue and sarcolemmal failure (Gruener et al, 1968; Brandt et al, 1977; Haller et al, 1998; Fauler et al, 2012; Vissing and Haller, 2012; Ørngreen et al, 2015; Nogales-Gadea et al, 2016). All these secondary cellular events in GSDV are strongly related to the post-effort symptoms, such as intense and prolonged fatigue, persistent muscular pain and increase in muscle volumes leading to acute rhabdomyolysis. These symptoms are often underestimated by physicians and researchers, actively studying exercise capacity of GSDV patients, but have a major impact on quality of daily life of GSDV patients. The post-exercise sarcolemmal inexcitability observed on LET in GSDV explore and confirm these complex biochemical mechanisms determined by the absence of myophosphorylase activity, beyond the simple glycolytic blockade.

In conclusion, in our study we demonstrated that LET can guide clinicians toward the diagnosis of GSDV, being a highly sensitive and specific test, that can be easily performed in an outpatient setting and well tolerated by patients. It correlates well with GSDV phenotype, and with the very disabling post-effort symptoms, thus can be studied as a potential outcome measure. Thus, LET is an important *in vivo* study of muscle functioning not only in “primary” muscle channelopathies but also in metabolic myopathies that secondarily affect ionic pumps.

Aim 5

Construction of a Gene Panel for Rhabdomyolysis

In recent years, the evolution of molecular techniques allowed to develop new more efficient methods (NGS, Next Generation Sequencing) for the analysis of exomes ("whole exome sequencing") or of multiple genes involved in specific diseases ("gene panels"). These promising techniques are more and more used especially in rare genetic diseases, for example in LGMDs and in mitochondrial diseases. More recently a specific panel for rhabdomyolysis has been proposed by Olpin et al (2015) and is currently under evaluation in England.

Generally, NGS allows an increased mutation detection rate, a reduction of time and cost, and identification of mutations in several genes not previously associated with muscle disorders. On the other hand, NGS still has several limitations: the analysis of the large generated amount of data (output) requires specialized centres and it is time consuming; the pathogenicity of several genetic variants is doubtful; NGS output are difficult to be analysed in single patients (and this is frequently the case in rhabdomyolysis). The good selection of candidate genes to be studied in a panel and the correct identification and characterization of good patients candidate for “gene panels” are mandatory for best results.

Medline search including “rhabdomyolysis”, “myoglobinuria”, “metabolic myopathy” was done and 14,686 articles were identified (8,329 rhabdomyolysis, 2,108 myoglobinuria and 4, 249 metabolic myopathy). The articles were screened and all genes reported to be associated with rhabomyolysis were selected.

The screening led us to propose a “rhabdomyolysis panel” that includes 32 genes that are mutated in diseases known to manifest with rhabdomyolysis and genes that participate in the mechanisms known to be disrupted in rhabdomyolysis (See Table XVI). These genes are involved in: i) glycogen metabolism; ii) lipid metabolism; iii) calcium homeostasis; iv) others (lipin, *ISCU*). Beside *ISCU* no other genes involved in mitochondrial myopathies or genes involved in LGMDs were included since specific panels are already available and in use (Mitocapture and LGMDs).

Table XVI. Proposal of a large gene panel for metabolic myopathies and rhabdomyolysis.

Human Gene nomenclaure (Symbol)	Gene/Locus MIM number	Associated disease	Phenotye MIM number
Disorders of fatty acid oxidation			
Acyl-CoA dehydrogenase, very long chain (ACAVDL)	60957	Very long chain deficiency (VLCAD)	201475
Carnitine Palitoyltransferase 2 (CPT2)	600650	CPTII deficiency (CPT2)	608836
Electron-transfer-flavoprotein αpolypeptide (ETFA)	608053	Multiple acyl-CoA dehydrogenase deficiency (MADD)	231680
Electron-transfer-flavoprotein βpolypeptide (ETFB)	130410	MADD	231680
Electron-transfer-flavoprotein dehydrogenase (ETFDH)	231675	MADD	231680
Trifunctinoal protein, α-subunit (HADHA)	600890	Long chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiency	609016
Trifunctinoal protein β-subunit (HADHB)	143450	Trifunctional protein deficiency	609015
Solute carrier family 22, member 5 (SLC22A5)	603377	Primary carnitine deficiency	212140
Solute carrier family 25, member 32 (SLC25A32)	610815	Exercise intolerance, riboflavin-responsive	616839
Flavin Adenin Dinucleotide Synthase (FLAD1)	610595	Lipid storage myopathy due to flavin adenine dinucleotide synthetase deficiency	255100
Calcium-related disorders			
Ryanodine Receptor 1 (RYR1)	180901	Central core disease; Malignant hyperthermia susceptibility	117000; 145600
Calsequestrin 1 (CASQ1)	114250	Myopathy, vacuolar, with CASQ1aggregates	616231
ORAI Calcium Release-Activated Calcium Modulator 1 (ORAI1)	610277	Myopathy with tubular aggregates; Immunodeficiency	615883; 612782
Stromal Interaction Molecule 1 (STIM1)	605921	Myopathy with ubular aggregates, Immunodeficiency Stormorken syndrome	160565; 612783; 185070;
ATPase Sarcoplasmic/Endoplasmic Reticulum Ca2+ Transporting 1 (SERCA1)	108730	Brody myopathy	601003
Calcium channel, voltage-dependent, L type, alpha 1S subunit, (CACNA1S)	114208	Malignant hyperthermia susceptibility; Hypokalemic periodic paralysis type 1	601887; 170400

Human Gene nomenclaure (Symbol)	Gene/Locus MIM number	Associated disease	Phenotye MIM num
Disorders of glycogen metabolism			
Glycogen synthase 1 (GYS1)	138570	Glycogen storage disease 0 (GSD0)	611556
Glucosidase, αacid (GAA)	606800	Glycogen storage disease II (GSD2)	232300
Amylo-α1,6-glucosidase, 4-α-glucanotransferase (AGL)	610860	Glycogen storage disease III (GSD3)	232400
Glucan (1,4-α) branching enzyme (GBE1)	607839	Glycogen storage disease IV (GSD4)	232500
Phosphorylase, glycogen, muscle (PYGM)	608455	Glycogen storage disease V (GSD5)	232600
Phosphofructokinase, muscle (PFKM)	610681	Glycogen storage disease VII (GSD7)	232800
Phosphorylase kinase, α1 (PHKA1)	311870	Glycogen storage disease Ixd (GSD9D)	300559
Phosphoglycerate mutase 2 (PGAM2)	612931	Glycogen storage disease X (GSD10)	261670
Lactate dehydrogenase A (LDHA)	150000	Glycogen storage disease XI (GSD11)	612933
Aldolase A, fructose biphosphate (ALDOA)	103850	Glycogen storage disease XII (GSD12)	611881
βenolase 3 (muscle) (ENO3)	131370	Glycogen storage disease XIII (GSD13)	612932
Phosphoglucomutase 1 (PGM1)	171900	Glycogen storage disease type XIV (GSD14)	612934
Glycogenin 1 (GYG1)	603942	Glycogen storage disease XV (GSD15)	613507
Phosphoglycerate Kinase 1 (PGK1)	311800	Phosphoglycerate kinase 1 (PGK1) deficiency	300653
Disorders of phospholipid metabolism			
Lipin-1 (LPIN1)	605518	Myoglobinuria, acute recurrent	268200
Other disorders			
Iron-sulfur cluster assemby enzyme (ISCU)	611911	Myopathy with lactic acidosis	255125

Due to the rapid evolution of knowledge in these rare diseases, and the increasing number of new genes that are being discovered, a cost - benefit analysis suggests that selected patients should be studied with the sequencing of all the exome (WES), followed by subsequent analysis only of the genes pre-selected in the panel. In fact, the slightly higher cost of WES compared to the sequencing of panel genes would be offset by the possibility of studying other genes without further sequencing analysis, if the first-step analysis resulted negative. On the other hand, it is not feasible to propose a complete WES (sequencing and analysis of all exomes) for all patients with rhabdomyolysis, because it is time consuming and expensive, it would be difficult to distinguish pathogenic mutations from the numerous variants identified (especially if parents are not included in the WES), and it would increase the ethical issues of the analysis (i.e. for accidental discovery of pathogenic mutations non-related to rhabdomyolysis).

CONCLUSIONS

The main objective of this study was the amelioration of the diagnostic process for patients with suspected myopathy manifesting with rhabdomyolysis.

The first step was the identification of the current diagnostic work up in rhabdomyolysis and estimate of the diagnostic yield.

We analyzed the clinical features and diagnostic procedures performed in patients with rhabdomyolysis (aim1) in a retrospective study including all consecutive patients (n = 208) referred for rhabdomyolysis to a Reference Centre for Neuromuscular Diseases. We verified that the final diagnosis could not be identified in 118 patients (57%), despite an extensive work up. A genetic disease was observed in 19,7% and a non-genetic cause was found in 23,6%. Some diagnostic tests were confirmed to have high specificity and/or sensitivity for specific diseases, such as: grip tests in the diagnosis of McArdle's disease and in phosphoglucomutase 1 deficit; profile of acylcarnitines in VLCAD and CPT2 deficiency; muscle biopsy in McArdle's disease and in some muscular dystrophies. Overall, however, the diagnostic tools used in the last 16 years were insufficient in guiding the diagnostic process to a defined etiologic diagnosis. We also identified few cases presenting rhabdomyolyses due to rare diseases (i.e. *DNA2* mutations), or presenting rare causes of rhabdomyolysis rare (i.e. myeloma; hyperthyroidism; dermatomyosytis) or previously unknown (i.e. Myotonic Dystrophy Type 2). The clinical features of the episode of rhabdomyolysis (i.e. trigger, max CK, symptoms, etc.) could not guide toward a specific diagnosis, and most of the cases remained unsolved despite the long and costly workup.

In the second aim of the study (aim 2) we identified and characterized a new genetic cause of rhabdomyolysis, the CASQ1 – related myopathy. This disease has been recently discovered in Italian patients affected by mild myopathy, all carrying the same mutation (p.Asp244Gly) because of a founder effect. In the present study, we collected data concerning 17 patients, and we widened the clinical spectrum, including rhabdomyolysis and severe course in older patients, expanded the *CASQ1* mutational spectrum by identifying a novel mutation. We provided information about the clinical features, muscle imaging and histopathology that can orient towards the diagnosis of CASQ1-related myopathy.

In the third aim we investigated the prevalence of rhabdomyolyses in a large population of beta-sarcoglycanopathy patients. In a multicentric International study we collected natural history data of 32 LGMD2E patients with different disease severity. We did not detect any case of overt rhabdomyolysis; however, we confirmed the high prevalence of muscle pain in active patients, suggesting the presence of membrane fragility exacerbated by muscle activity. We described disease evolution, and correlations between genotype, protein expression, and phenotype.

The fourth objective (aim 4) focused on diagnostic tools to be used in cases of rhabdomyolysis, and in particular on the role of EMG with provocative test: the Long Exercise Test (LET) for the diagnosis of McArdle disease (GSDV). LET differentiate GSDV patients from patients referred for rhabdomyolysis from other causes and healthy subjects. LET showed a marked and immediate reduction of compound action potential (CMAP) amplitude after effort, correlated to membrane inexcitability that occurs after exertion in GSDV patients. We also demonstrated that LET can also be used as an outcome measure, because it is related to the severity of symptoms and is consistent with post-exercise symptoms reported by patients.

Finally, we proposed a rhabdomyolysis gene panel to be used in undiagnosed patients. This panel includes genes involved in glycogen, lipid metabolism and in calcium homeostasis.

Proposal of a diagnostic work-up for rhabdomyolysis

Few diagnostic flow charts for rhabdomyolysis have been published in recent years: these are certainly helpful, but they are based more on theoretical basis and expertise of neuromuscular specialists than on clinical data obtained from large case series. In fact, no population studies have been performed so far in myopathies manifesting with rhabdomyolysis.

The flow chart proposed by Zutt et al (2014) is shown in Figure 21. This elegant and complete protocol has some limitations that need to be underlined. First, the authors propose to stop investigations after blood tests in patients presenting a single episode of rhabdomyolysis, if no positive family history or myalgias/cramps are present. This should be avoided, because of the unknown risk of recurrence, the high impact of rhabdomyolysis on quality of daily life of patients, and the potential costs of the intensive medical care needed in acute episodes. In the present

study, we report several patients without any past medical history or family history that after a single episode were diagnosed with a genetic disease (frequently *RYR1* mutations) and, more importantly, with potentially treatable disorders disclosed by rhabdomyolysis (i.e. myeloma, dermatomyositis, etc.). Few additional exams are therefore needed even after a single episode of rhabdomyolysis. Muscle biopsy is correctly proposed as a second line exam after non-ischemic grip test and acylcarnitines studies, and we agree that it remains an important tool to be performed in case of rhabdomyolysis. However, in our study we showed that only few histopathological features could certainly guide toward specific diagnoses, because more often muscle biopsy shows unspecific myopathic features. Furthermore, biochemical assays on muscle tissue or blood cells seem less useful in guiding toward specific diagnosis. The high cost (in money and time), the low number of specialized laboratories available, the numerous technical precautions and limitations (i.e. difficulties in sample collection, transportation and conservation, etc.) and their low sensitivity limit their use for diagnostic purposes. They remain very useful, for example, for the confirmation of doubtful genetic defects.

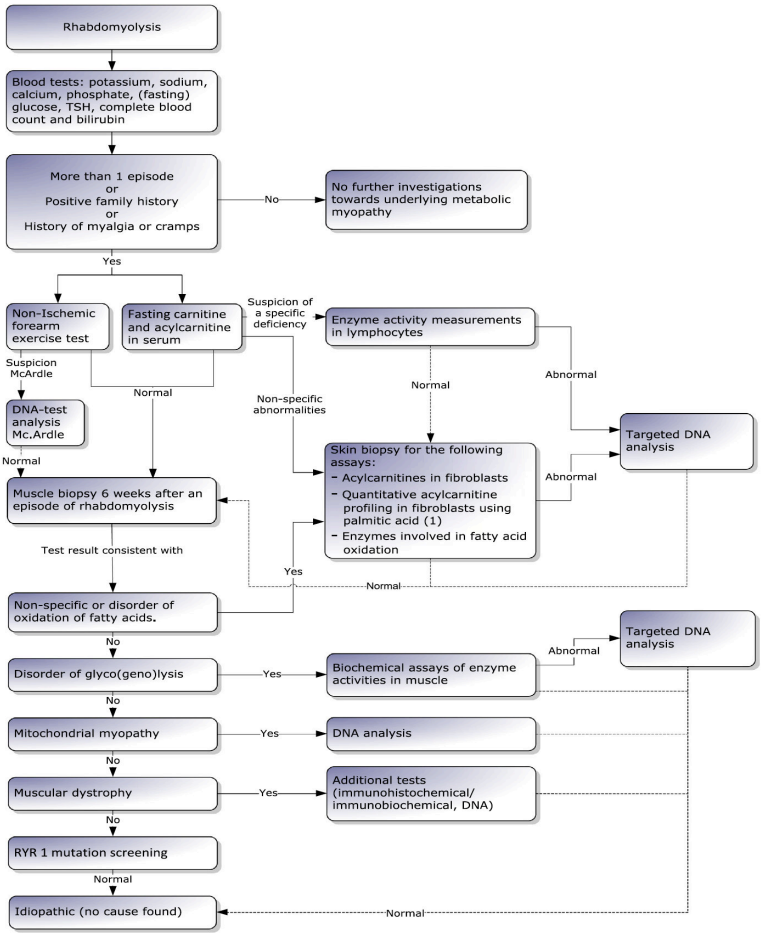


Fig. 21. Diagnostic flow-chart proposed by Zutt et al, 2014.

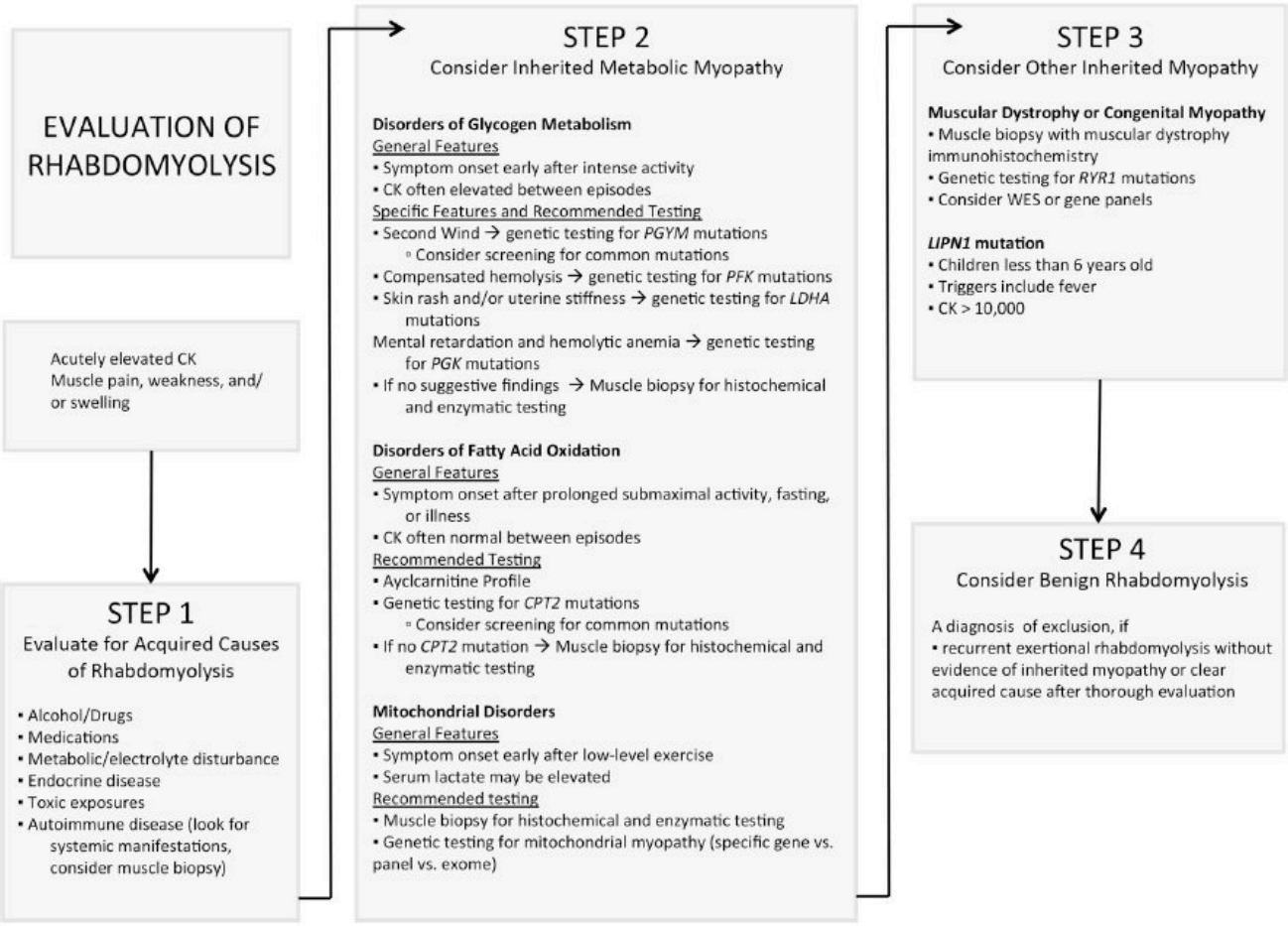


Fig. 22. Diagnostic flow-chart proposed by Nance and Mammen (2015).

The protocol proposed by Nance and Mammen (2015) is shown in Fig. 22. This very elegant protocol underlines the importance of excluding acquired causes of rhabdomyolysis as a first step, and the importance of an accurate evaluation of general clinical features (step 2). Unfortunately, in our study we demonstrated that rarely the features of the episodes, the relationship between effort and symptoms onset and the concomitant clinical clues are helpful to direct genetic tests. The authors include the most recent scientific and technical advances such as NGS (step 3), and in step 4 suggest that the diagnosis of “Benign Rhabdomyolysis” is a diagnosis of exclusion, that can be posed in exertional rhabdomyolysis only after that several diagnostic tools have been performed and resulted negative. It is to note, however, that due to the potential severity of the episode of exertional rhabdomyolyses and the unknown risk of recurrence, the term “benign” seems misleading.

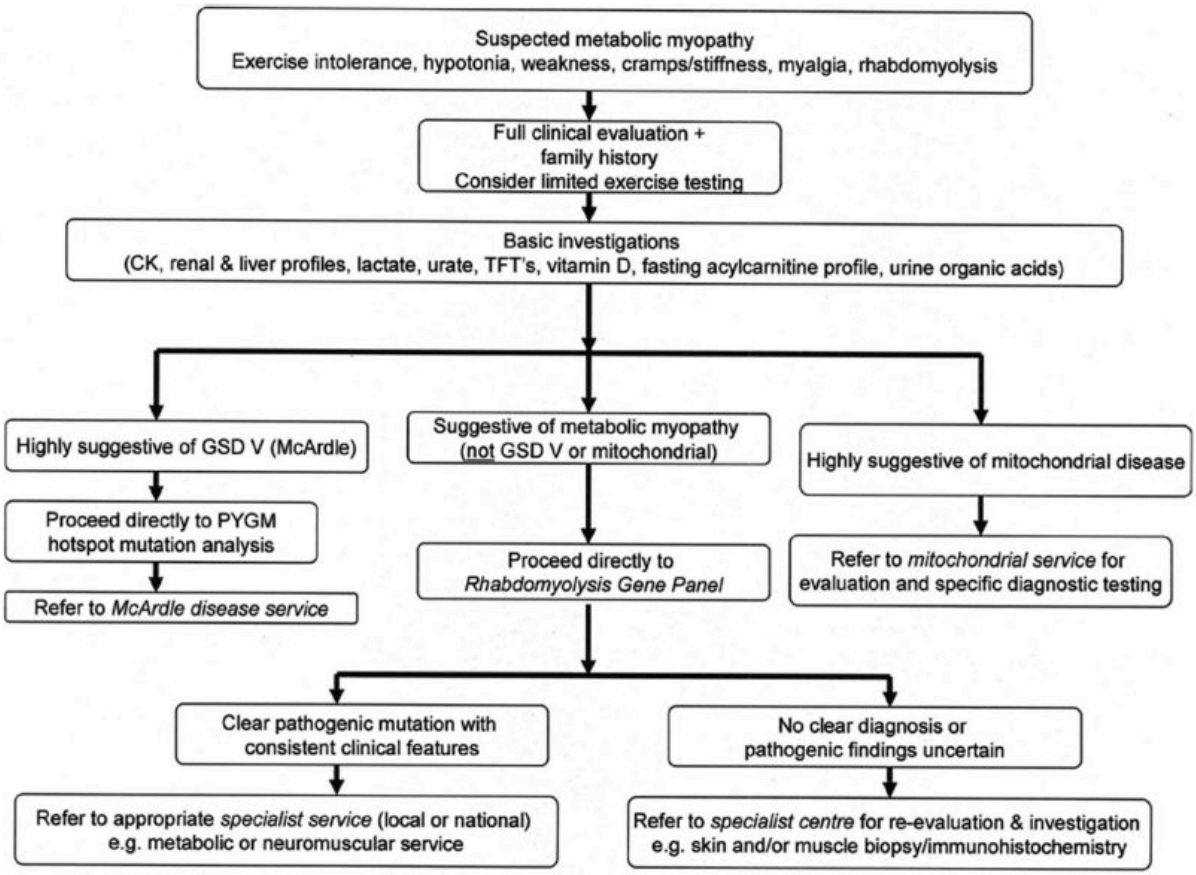


Fig. 23. Diagnostic flow-chart proposed by Olpin et al (2015).

The flow chart proposed by Olpin et al (2015) (Fig. 23) is centered on the importance of a specific gene panel for rhabdomyolysis. The authors suggest to proceede directly to the Gene Panel after few basic investigations (blood tests and possibly limited exercise testing) and the exclusion of GSDV or mitochondrial disease by clinical evaluation. Neuromuscular specialists and deeper investigations are envisaged only if no clear diagnosis is acquired from NGS. With this approach, several non-genetic rhabdomyolyses and rare diseases would be studied with NGS, with a high cost and scarce results.

Here we suggest a new diagnostic algorithm, shown in Fig. 24, that integrates the actual knowledge on metabolic myopathies and rhabdomyolysis with the results of the present study. The first step includes an accurate medical history, clinical evaluation, and family history to delineate the scenario in which rhabdomyolysis occurred and, rarely, suggest specific diagnoses. If an evident external cause is excluded (i.e. trauma, epilepsy, etc.), few selected first line exams, that include blood exams, acylcarnitines, grip test and EMG, need to be performed:

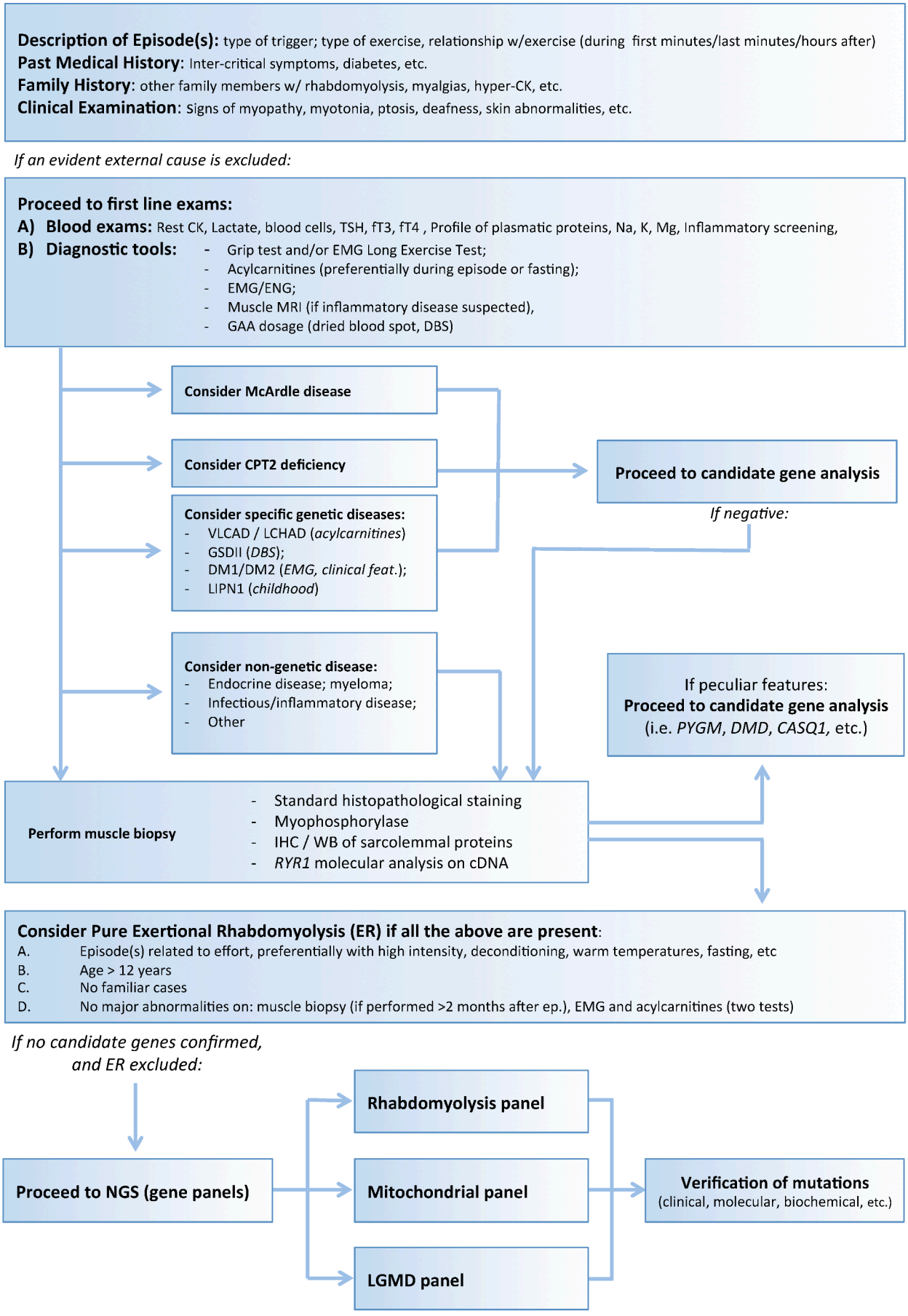
i) To guide toward the diagnosis of the most frequent or peculiar causes of rhabdomyolysis: GSDV diagnosis with Grip Test (eventually EMG with Long Exercise Test); CPT2 and LCHAD with the typical profile of acylcarnitines; etc.
ii) To disclose some of the non-genetic causes of rhabdomyolysis that can be potentially treatable and cannot be distinguished from genetic diseases: endocrine disease (thyroid), myeloma (profile of blood proteins); inflammatory myopathies; etc.
iii) To screen for treatable muscle disorders with low-costs tests: GSDII with enzymatic dosage in Dried Blood Spot.
iv) To identify signs of diseases in which gene panels are scarcely sensitive (i.e. myotonic discharges on EMG in DM1 and DM2)
v) To identify signs that can suggest specific diagnosis: hemolytic anemia in PGK; peripheral neuropathy in LCHAD; etc.

The muscle biopsy should be performed as a second step in all non-diagnosed patients. Muscle tissue analysis should include at least:

i) Standard histopathological stains (to evaluate muscle structure, glycogen and lipid content, mitochondrial features, inflammatory elements, etc.);
ii) Myophosphorylase stain;
iii) Western-blot (WB) and/or immunohistochemistry (IHC) for sarcolemmal proteins.

If no peculiar patterns can be evident on muscle tissue, the analysis of cDNA for *RYR1* gene should be performed. This analysis is preferred to the DNA analysis extracted from blood cells, because it is easier, quicker and more reliable for this large gene in which several variations are usually identified.

Fig. 24. Proposition of a diagnostic work-up for patient referred for rhabdomyolysis.



The “pure exertional rhabdomyolysis" remains a diagnosis of exclusion that can be carefully posed in non-myopathic patients when: i) the episode is triggered by effort in favoring conditions, ii) no major abnormalities are observed on diagnostic tools, iii) family history is negative for rhabdomyolysis, high CK or intense myalgias; iv) no *RYR1* variations are detected. It is to note, however, that in patients with “pure exertional rhabdomyolysis” it is not possible to exclude rare diseases, milder mutations in pathogenic genes or genetic variations that affect the physiological response to exercise. Therefore, the risk of recurrence and complications should not be completely excluded.

For the non-diagnosed patients, the combination of medical history, laboratory findings and histopathological features should guide toward NGS, without any further biochemical or functional test. We propose three main gene panels that are under study (“rhabdomyolysis panel”, see Aim 5) or already available for diagnostic purposes (“mitochondrial disorders” and “Limb-Girdle Muscular Dystrophies”).

Patients presenting clinical features suggestive for mitochondrial disorders (i.e. short stature, ptosis, deafness, etc.), increased levels of lactate, peripheral neuropathy on EMG and/or mitochondrial abnormalities on muscle biopsy should be preliminary studied with a specific panel for mitochondrial diseases. The one in use in France, for example, includes 101 nuclear genes and all the mitochondrial DNA (37 genes) associated with diseases or involved in mitochondrial functioning (Mitocapture). Patients presenting very high CK levels, myopathic changes on EMG, and most importantly histopathological features suggesting muscular dystrophy and normal sarcolemmal proteins on IHC/WB, should be first studied with the specific panel for LGMDs (patients presenting abnormal IHC/WB for sarcolemmal proteins will be first screened by a candidate gene approach). All other patients should be studied with the “rhabdomyolysis panel”.

In conclusion, the diagnostic algorithm that we propose will allow cost reduction and time optimization, and hopefully will increase the rate of etiological diagnosis of rhabdomyolysis, a serious event with major impact on patients' lives that, to date, remains poorly diagnosed.

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