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New insights into McArdle disease: Characterization of the murine model and development of new diagnosis and therapeutic approaches

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Memòria presentada per Astrid Brull Cañagueral per optar al grau de Doctora en

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A la iaia Carmeta

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List of Abbreviations

AAV: adeno-associated virus

AAV8: AAV serotype 8

ACE: angiotensin-converting enzyme

ADP: adenosine diphosphate

Akt: protein kinase B

AMPD1: adenylate daminase gene

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

ATP: adenosine triphosphate

bp: base pair

Cas: CRISPR-associated protein

CK: creatine kinase

CMV: citomegalovirus

CRISPR: clustered regularly interspaced short palindromic repeat

crRNA: CRISPR RNA

DHAP: dihydroxyacetone phosphate

DMD: Duchenne muscular dystrophy

DMEM: Dulbecco's modified Eagle's medium

DNA: deoxyribonucleic acid

DSB: DNA double-strand break

EDL: extensor digitorum longus

FBS: fetal bovine calf serum

FF: fast fatigable

FFA: free-fatty acid

FR: fatigue-resistant

GAPDH: glyceraldehyde-3-phosphate dehydrogenase protein

GBE: glycogen branching enzyme

gc: genome copies

GDE: glycogen debranching enzyme

GFP: green fluorescent protein

GLUT4: glucose transporter type 4

GN: glycogenin

GP: glycogen phosphorylase

GP-BB: brain isoform of glycogen phosphorylase protein

GP-LL: liver isoform of glycogen phosphorylase protein

GP-MM: skeletal-muscle isoform of glycogen phosphorylase protein

GS: glycogen synthase

GSD: glycogen storage disease

GSK3α/ß: glycogen synthase kinase 3 α/ß protein

gRNA: guide RNA

HDR: homology-directed repair

HPLC: high-performance liquid chromatography

hPYGM: human *PYGM* cDNA

HR: homologous recombination

IMP: inosine monophosphate

IRS: insulin receptor substrate

LDH: lactate dehydrogenase

MAF: minor allele frequency

MyHC: myosin heavy chain

NAD: oxidized nicotinamide adenine dinucleotide

NADH: reduced nicotinamide adenine dinucleotide

NHEJ: error-prone nonhomologous end-joining

NMD: nonsense-mediated mRNA decay

pAkt: phospho-protein kinase B

PAM: protospacer adjacent motif

pAMPKα: phospho-AMP-activated protein kinase subunit alpha

PAS: periodic acid-Schiff

PCr: phosphocreatine

PCR: polymerase chain reaction

PCR-RFLP: PCR-restriction fragment length polymorphism

PDH: pyruvate dehydrogenase

PDK1: phosphoinositide-dependent kinase 1

PGM: phosphoglucomutase

pGS: phospho-glycogen synthase

pGSK3α/ß: phospho-glycogen synthase kinase 3 α/ß protein

PhB: phenylbutyrate

PHK: phosphorylase kinase

P_i: inorganic phosphate

PI3K: phosphatidylinositol 3-kinase

^{31}P-MRS: magnetic resonance spectroscopy

PP1: phosphorylase phosphatase 1

PSF: penicillin/streptomycin/fungizone

PTC: premature termination codon

PYGB: human brain isoform glycogen phosphorylase-encoding gene

Pygb: mouse brain isoform glycogen phosphorylase-encoding gene

PYGL: human liver isoform glycogen phosphorylase-encoding gene

Pygl: mouse liver isoform glycogen phosphorylase-encoding gene

PYGM: human skeletal-muscle isoform glycogen phosphorylase-encoding gene

Pygm: mouse skeletal-muscle isoform glycogen phosphorylase-encoding gene

Pygm$_{oa}$: sheep skeletal-muscle isoform glycogen phosphorylase-encoding gene

qPCR: quantitative PCR

RFLP: restriction fragment length polymorphism

RFP: red fluorescent protein

RNA: ribonucleic acid

SD: standard desviation

SERCA: SR Ca^{2+} ATPase

SR: sarcoplasmic reticulum

ssDNA: single-standed DNA

ssODN: single-stranded oligodeoxynucleotide

TA: tibialis anterior

TALEN: transcription activator–like effector nucleases

tracrRNA: trans-activating crRNA

TSA: trichostatin A

UDP: uridine diphosphate

UTP: uridine triphosphate

UTR: untranslated region

VPA: sodium valproate

WBC: white blood cell

WT: wild-type

ZFN: zinc-finger nucleases

Abstract

McArdle disease (glycogenosis type V), the most common muscle glycogenosis, is caused by inherited deficiency of the muscle isoform of glycogen phosphorylase. Patients are unable to obtain energy from their muscle glycogen, thus typically experience exercise intolerance with premature fatigue and contractures, sometimes accompanied by rhabdomyolysis and myoglobinuria. Currently, there is no therapy to restore the enzyme activity in patients, but the recently developed *p.R50X/p.R50X knock-in* McArdle mice opens the door to the study of the phenotypic consequences of muscle glycogen unavailability, as well as, to the evaluation and testing of new therapeutic approaches for this disorder.

The main goal of this PhD thesis was to deepen into the phenotypic characterization of the murine model of McArdle disease, focusing our efforts on the study of the regulation of glycogen metabolism from a glycogen phosphorylase deficiency and the subsequent high glycogen content in the skeletal muscle cell, as well as, the development of an *in vitro* model of the disease that mimics the absence of the muscular isoform of glycogen phosphorylase, and subsequent glycogen accumulation observed in skeletal muscle from McArdle patients. Using the *in vitro* model, we demonstrated the efficacy of sodium valproate, an inhibitor of histone deacetylase, as a potential therapy for McArdle disease, thus we observed the re-expression of the brain isoform of glycogen phosphorylase and a dose-dependent reduction in glycogen accumulation. In addition, we developed a non-invasive, functional and complementary test for the diagnosis of McArdle disease by flow cytometry, which allows the detection of muscle isoform of glycogen phosphorylase expression in T lymphocytes, without the need to perform a muscular biopsy.

Resum

La malaltia de McArdle (glicogenosis tipus V), és la glicogenosis muscular més freqüent i està causada per la manca de la isoforma muscular de la glicogen fosforilasa. Els pacients són incapaços d'obtenir l'energia de les seves reserves de glicogen al múscul, i com a conseqüència, presenten intolerància a l'exercici amb fatiga prematura i contractures, a vegades acompanyada per rabdomiolisis i mioglobinuria. Actualment, no hi ha cap teràpia capaç de restaurar l'activitat de l'enzim en pacients, però el recent desenvolupat model murí de la malaltia de McArdle obre les portes a l'estudi de les conseqüències fenotípiques de la indisponibilitat del glicogen muscular, així com, a l'estudi de noves aproximacions terapèutiques per a aquest trastorn.

Aquesta tesi doctoral s'ha basat en la caracterització fenotípica del model murí de la malaltia de McArdle, centrant tots els esforços en l'estudi de la regulació del metabolisme del glicogen pel que fa a la manca de la glicogen fosforilasa i la subsequent acumulació de glicogen, així com en el desenvolupament d'un model *in vitro* de la malaltia que imita l'abscència de la isoforma muscular de la glicogen fosforilasa i l'acumulacio de glicogen observada en el múscul esquelètic dels pacients amb la malaltia de McArdle. Utilitzant aquest model, hem demostrat l'eficiència del valproat de sodi, un inhibidor de les histones desacetilases, com a possible teràpia per a la malatia de McArdle, ja que hem observat la reexpressió de la isoforma cerebral de la glicogen fosforilasa i una reducció dosi-depenent en l'acumulació de glicogen després del tractament amb aquest fàrmac. A més, hem desenvolupat un test no invasiu, funcional i complementari per al diagnòstic de la malaltia de McArdle mitjançant la citometria de flux, que permet la detecció de l'expressió de la isoforma muscular de la glicogen fosforilasa en limfòcits T, sense necessitat de dur a terme una biòpsia muscular.

1. Introduction

1. Introduction

1.1 Skeletal muscle: the role of muscle fibers

In humans about 40% of body weight is skeletal muscle, and about another 10% is smooth and cardiac muscle showing the importance of the skeletal muscle in the mammals. Skeletal muscle is the tissue responsible for a large range of activities, from supporting the body weight during long periods of upright standing, to performing explosive movements in response to an unexpected threat. To carry out these different activities our muscles are composed of muscle cells (also known as myocites or muscle fibers) which present large differences in metabolic profile, contractile speed, and cellular Ca^{2+} handling [1]. In mammals, there are four major different fiber types (I, IIA, IIX/D and IIB) each one composed by a different myosin heavy chain (MyHC) isoform [2-5].

These different MyHC isoforms are encoded by a multigene family (*MYH* family), the members of which are expressed in a tissue-specific and developmentally regulated manner. Additionally, in mammals, the four major fiber types are variously distributed in body muscles including limb, trunk and head muscles. However, the relative proportion of any fiber type may vary between species. In this regard, it is important to mention that in humans the fast-twitch IIB fibers are not present at all [6, 7]. In many species type II fibers are more numerous in forelimbs than in hindlimbs, and accordingly, in humans, upper limb muscles are faster than lower limb muscles [7]. In addition to the four major fiber types, specific MyHC isoforms such as embryonic and neonatal/perinatal/fetal isoforms are also present in developing muscle, thus providing a useful marker for regenerating fibers [7-9].

The main feature of skeletal muscle is its heterogeneity and plasticity allowing the same muscle to be used for various tasks adjusting their phenotypic properties to altered functional demands. Thus, muscle fibers adapt their phenotypic properties such as their size or thickness, their extracellular surroundings (e.g. capillary density), contractile properties, sarcoplasmic reticulum (SR) development, resistance to fatigue or mitochondrial content to better cope with their metabolic activities (Table 1) [1, 7, 9]. By means of a powerful and accurate nervous system control, skeletal muscle can adjust the performance of a muscle adapting its power output to the motor task on a very short time base; or can adapt their contractile properties by changing their structure on a long-term basis [10]. In this regard, muscle fibers can change their phenotype under certain conditions such as neuromuscular activity, mechanical loading or unloading, altered hormonal profiles (especially of the thyroid hormones), and aging. Increased neuromuscular activity, mechanical loading and hypothyroidism induce fast-to-slow transitions, whereas reduced neuromuscular activity, mechanical unloading and hyperthyroidism elicit slow-to-fast transitions [9]. Additionally, it has been suggested that aging causes fast-to-slow transitions [11].

Gene	MYH7	MYH2	MYH1	MYH4
Protein	MyHCIß	MyHCIIa	MyHCIId	MyHCIIb
Expression	I fibers (and heart)	IIA fibers	IIX/D fibers	IIB fibers
Contraction Speed (fast/slow)	Slow	Fast	Fast	Fast
Metabolism profile (oxidative/glycolytic)	Oxidative	Oxidative/Glycolytic	Glycolytic	Glycolytic
Sarcoplasmic reticulum development	Poorly developed	Richly developed	Richly developed	Richly developed
Resistance to fatigue	FR	FR	Intermediate	FF
Mitochondrial content	Rich	Rich	Rich	Poor
Thickness of the Z line	Thick	Thin	Thin	Thin
SDH staining	Strong	Strong	Moderate to Strong	Weak

Table 1: Summary of the phenotypic properties of the major fiber types: type I, type IIA, type IIX/D and type IIB. FF, fast fatigable; FR, fatigue-resistant. [1, 7, 9, 12]

1.2 Properties of the different muscle fiber types

a) *Contractile properties*: Every muscle of the body is composed by a mixture of so-called fast-twitch (type II) and slow-twitch (type I) muscle fibers, with still other fibers gradated between these two extremes. The muscles that are adapted for rapid and powerful contractions are composed mainly of fast-twitch fibers, whereas the muscles that respond slowly but with prolonged, continued muscle activity are composed mainly of slow-twitch fibers [12].

b) *Fiber size*: In mammals, fiber thickness is in general inversely related to the aerobic oxidative activity or to mitochondrial density, being the oxidative slow (type I) and fast (type IIA)-twitch fibers thinner than the glycolytic type IIX/D and II B fibers [7].

c) *Capillary density*: Blood flow in muscles increases several times during exercise. Nevertheless, the number of capillary vessels surrounding a muscle fiber is higher for slow than for fast-twitch fibers. As indicated by the predominance of oxidative metabolism in slow fibers, an increase in the number of capillary vessels means a better oxygen supply, thereby promoting oxidative phosphorylation [7].

d) *Mitochondrial content*: Mitochondrial density and structure varies in relation to fiber type. Slow type I fibers have more mithochondrial volume and exhibit more densely packed cristae than fast type II fibers and among fast type II, type IIA fibers have more mitochondrial content than type IIX/D fibers [7].

Moreover, two distinct mitochondrial subpopulations exist in slow and fast-twitch fibres with different subcellular localization, morphology, and biochemical properties [7, 13]. Subsarcolemmal mitochondria show a large, lamellar shape, whereas intermyofibrillar mitochondria are smaller and more compact and located

between the myofibrils in close proximity to the triads where calcium is released from SR. Exist some differences in mitochondrial subpopulations content between fibers types in rat muscles, being subsarcolemmal mitochondria more abundant in oxidative fibers than in fast-twitch glycolytic fibers [7, 14].

e) *SR development*: Calcium triggers contraction by reaction with troponin-tropomyosin protein complex that in the absence of calcium prevent interaction of actin and myosin avoiding the muscular contraction, so calcium kinetics have a direct impact on the dynamic properties of the muscle fibers.

SR is responsible for controlling the concentration of intracellular cytosolic free calcium and this task is accomplished by the SR through calcium release, via the SR terminal cisternae, and calcium uptake, via the SR longitudinal tubules, with important contributions of cytoplasmic calcium buffers, mitochondria and sarcolemma [7]. Since a richer developed SR is observed in fast-twitch muscle fibers compared with slow-twitch muscle fibers, a correlation between SR development and speed of contraction has been observed [7].

f) *Resistance to fatigue*: After an intense activation of skeletal muscles, a decrease in the contractile function and a decline in performance is observed. Fatigue is highly dependent on the capacity of the aerobic metabolic system, thus slow-twitch muscle fibers are more fatigue resistant than fast-twitch fibers under normal conditions [1] because of their greater adaptation to an oxidative metabolism as indicated by their high mitochondrial content compared with fast-twitch glycolytic fibers.

1.3 Energy requirements during muscle contraction

The immediate energy source during muscle contraction is provided by ATP hydrolysis to ADP and P_i but the intracellular store of ATP is small (5-6 mM in humans) [15]. Even though energy expenditure in muscle at rest is low, skeletal muscle fibers can undergo extremely large and sudden changes in energy consumption when switching from rest to contractile activity; thus when the muscle is fully activated, the store is depleted in a few seconds and fast and efficient mechanisms of ATP turnover are needed to avoid ATP depletion [1, 7].

Energy consumption during contraction has two main components: ATP consumed by myofibrillar ATPase (~70%) and ATP consumed by ionic transport (~30%), mainly calcium [16]. MyHC isoforms with a highest rate of cross-bridges cycling consume ATP more rapidly than MyHC isoforms with a lowest rate. The cross-bridge cycle refers to the cycle of force-generating attachment/detachment interactions between myosin head-groups and actin, powered by the hydrolysis of ATP [12], therefore, during contraction fast-twitch MyHC isoforms such as type IIA, IIX/D and IIB, consume ATP more rapidly than the slow-twitch isoform (type I) [1, 9]. However, there are other ATP consuming proteins in skeletal muscles. For instance, exist two SR Ca^{2+} ATPase (SERCA) pump isoforms, SERCA1 in fast-twitch type II fibers and SERCA 2 in slow-twitch type I fibers. This is another major ATP consuming protein and the density of pumps is much higher in fast than in slow-twitch fibers [1].

So the mechanisms of ATP resynthesis can be divided into anaerobic and aerobic metabolism, of which the anaerobic pathways are faster and therefore dominate during high-intensity physical activity of short duration (fast twitch type II fibers), whereas the aerobic pathways predominate during prolonged submaximal exercise (slow-twitch type I fibers) (Figure 1) [1].

1.3.1 Anaerobic metabolism

Two main anaerobic pathways provide ATP regeneration in muscle fibers: degradation of phosphocreatine (PCr) and breakdown of muscle glycogen to lactate.

During the transition from rest to activity, PCr is the main source of ATP supply in mammalian skeletal muscles and is rapidly degraded by creatine kinase (CK), which catalyzes the reaction: PCr + ADP $\longleftrightarrow$ Cr + ATP in the attempt to keep constant ATP levels [1].

The PCr-CK system is localized close to the sites of ATP consumption (myofibrils and SR) and provides the fastest and most effective way of ATP regeneration during brief bouts of contractile activity.

Significant accumulation of by-products occurs during short-duration intense exercise, increasing P_i and ADP concentration. But such increase in ADP concentration can also support directly ATP regeneration, since a minor amount of ATP is resynthesized via adenylate kinase, generating one ATP and one adenosine monophosphate (AMP) from two ADP molecules. When AMP concentration increases is deaminated to inosine monophosphate (IMP) and NH_4^+ [17, 18]. Moreover, the AMP increase activates AMP-activated protein kinase (AMPK), an energy sensing/signaling protein that increases ATP production by activating glucose uptake, promoting glucose transporter type 4 (GLUT4) translocation to the membrane and free-fatty acids (FFA) oxidation (via inhibition of acetyl-CoA carboxylase, the rate-limiting enzyme for FFA synthesis) while at the same time switching off glycogen synthesis and protein synthesis [19-21].

However, the PCr-CK system is quickly overwhelmed during sustained exercise, the muscle cell must then rely on the glycolytic pathway to provide ATP for

continued contraction. The increase of AMP and P_i concentrations following the start of muscular contraction activate glycogen breakdown and glucose is released from glycogen stores by glycogen phosphorylase (GP) [7]. The glucose residues enter glycolysis and are ultimately converted to pyruvate, which can be converted to lactate by lactate dehydrogenase (LDH) with reoxidation of NADH. In principle, the rate at which lactate formation occurs depends on the availability of oxygen relative to the energy demand. Thus, lactate accumulation occurs mainly during heavy exercise when the rate of ATP consumption is high [1].
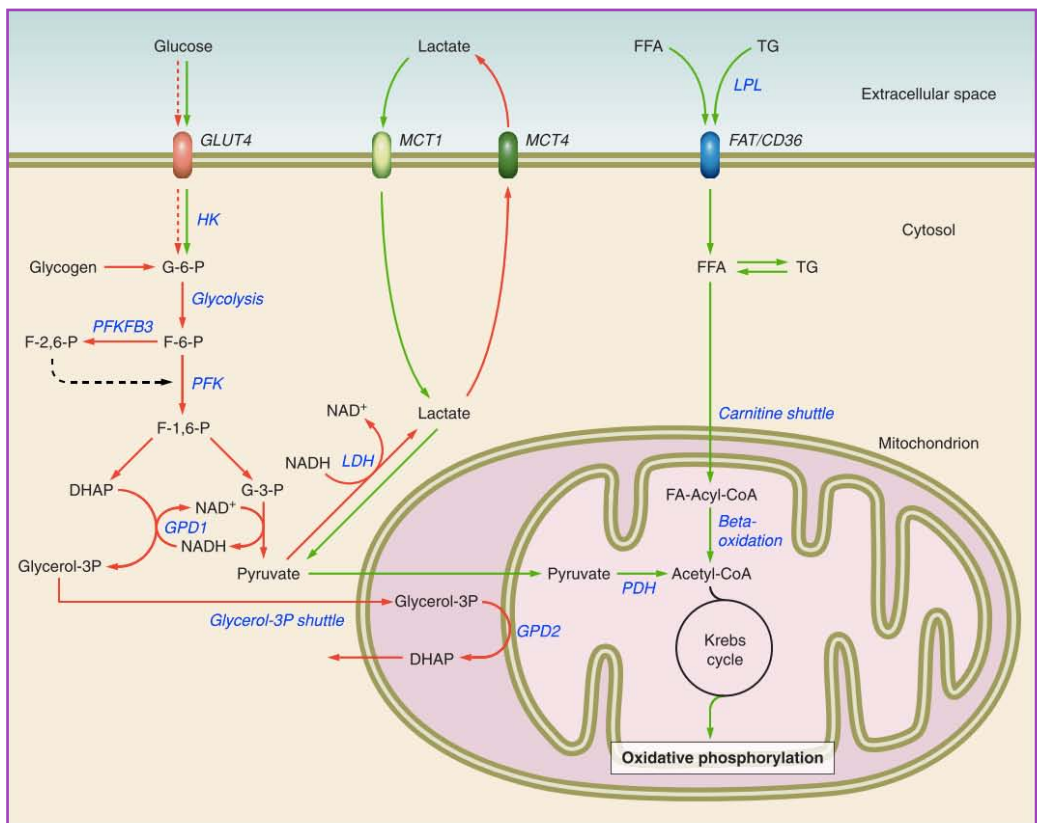
Figure 1: **Schematic representation of the differences in glucose, lactate, and fatty acid metabolism between fast and slow muscle fibers**. Pathways prevalent in fast or slow muscle fibers are shown as red or green arrows, respectively. DHAP, dihydroxyacetone phosphate; GLUT4, glucose transporter 4; F-6-P, fructose 6-phosphate; FAT/CD36, fatty acid translocase; FFA, free fatty acids; F-1,6-P, fructose 1,6-bisphosphate; F-2,6-P, fructose 2,6-bisphosphate; G-3-P, glyceraldehyde 3-phosphate; G-6-P, glucose 6-phosphate; GPD1, glycerolphosphate dehydrogenase 1 (cytoplasmic); GPD2, glycerolphosphate dehydrogenase 2 (mitochondrial); HK, hexokinase; LDH, lactate dehydrogenase; MCT1, monocarboxilic acid transporter 1; MCT4, monocarboxilic acid transporter 4; PDH, pyruvate dehydrogenase; PFK, phosphofructokinsase; PFKFB3, phosphofructokinasefructose bisphosphatase 3; TG, triglycerides [7].

Alternatively to anaerobic metabolism, pyruvate can enter the Krebs cycle via the pyruvate dehydrogenase (PDH) complex, which in skeletal muscle controls the glucose oxidation pathway by catalyzing the irreversible decarboxylation of pyruvate to acetyl-CoA [7].

The onset of contraction also results in the release of calcium stores from the SR, causing the activation of phosphorylase kinase (PHK) and subsequent phosphorylation and activation of GP [22].

1.3.2 Aerobic metabolism

But during prolonged, continued muscle activity, glycolytic metabolism do not produce enough ATP per glucosyl to meet the energy requirements for muscle contraction, thus oxidative metabolism of carbohydrates and lipids are the dominating ATP-producing systems [23].

ATP resynthesis via mitochondrial oxidative phosphorylation utilizes acetyl-CoA derived either from pyruvate generated by the activity of PDH or from FFA via ß-oxidation [7]. Acetyl-CoA is oxidated to CO_2 by the Krebs cycle, with most of the energy of oxidation temporarily held in the electron carriers $FADH_2$ and NADH. During aerobic metabolism, these electrons are transferred to O_2 via the mitochondrial respiratory chain, and the energy of electron flow is trapped as ATP by oxidative phosphorylation [24].

1.4 Glycogen metabolism

1.4.1 Glycogen

In a wide range of organisms, excess glucose is converted to polymeric forms for storage — glycogen in vertebrates and many microorganisms, starch in plants.

Glycogen is a branched polymer consisting of linear chains (α-1,4-glycosidic linkages) of glucose residues with further chains branching off (α-1,6-glycosidic linkages) every 8 to 12 glucoses and serves as an osmotically neutral means to store glucose in cells in times of nutritional plenty for utilization in times of need (Figure 2) [24, 25].

Although predominantly composed of glucose residues, glycogen contains other traces constituents, notably glucosamine and phosphate. Additionally, it forms aggregates with the enzymes responsible for its synthesis and degradation (such as glycogenin (GN), glycogen synthase (GS), GP, as well as several regulatory proteins including PHK and members of the protein phosphatase 1G family) [25].
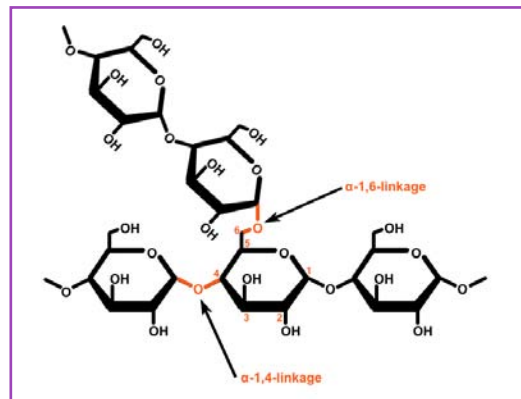
Figure 2: Schematic representation of the branch points. [25]

In vertebrates, glycogen is found primarily in the liver and skeletal muscle and may represent up to 10% of the weight of liver and 1% to 2% of the weight of muscle [24, 25].

In muscle, glycogen provides a quick source of energy for either aerobic or anaerobic metabolism and can be exhausted in less than an hour during vigorous activity [24]. On the contrary, liver glycogen serves as a reservoir of glucose for other tissues when dietary glucose is not available.

The general mechanisms for storing and mobilizing glycogen are the same in muscle and liver, but the enzymes differ in subtle yet important ways that reflect the different roles of glycogen in the two tissues.

1.4.2 Glycogen breakdown

In muscle, glycogen is degraded to retrieve glucose to fuel contraction and in liver to provide glucose for export to the bloodstream. Skeletal muscles are unable to release glucose (as muscles lack the glucose 6-phosphatase enzyme) and muscle glycogen is mainly a local energy substrate for exercise. In both skeletal muscle and liver, the glucose units of the outer branches of glycogen enter to the glycolytic pathway through the action of three enzymes: GP, glycogen debranching enzyme (GDE) and phosphoglucomutase (PGM). GP catalyzes the phosphorolytic cleavage of α-1,4-glycosidic linkage between two glucose residues at a nonreducing end of glycogen, releasing the terminal glucose residue as α-D-glucose 1-phosphate (Figure 3) [24, 26].

GP acts repetitively on the nonreducing ends of glycogen branches until it reaches the last four glucose residues before an α-1,6-branch point, where its action stops. Further degradation by GP can occur only through the action of the GDE, formally known as oligo (α1,6) to (α1,4)-glucantransferase, which catalyzes two successive reactions that transfer branches. First, the transferase activity of the enzyme shifts a block of three glucose residues from the branch to a
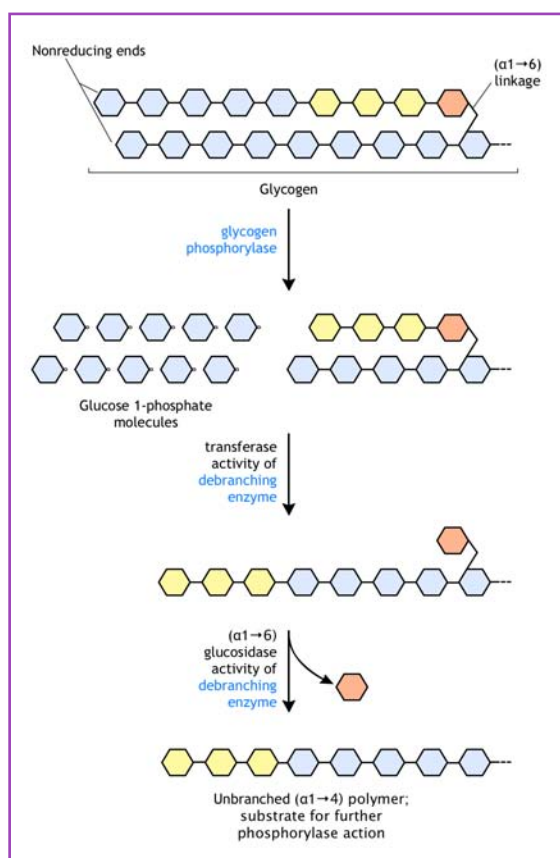
Figure 3: Glycogen breakdown near an (α1→6) branch point. [24]

nearby nonreducing end by an α-1,4-glycosidic linkage. Then, the remaing glucose residue is released as free glucose by the glucosidase activity of the GDE [24]. Once these branches are transferred and the glycosyl residue at C-6 is hydrolyzed, GP activity can continue (Figure 3).

Glucose 1-phosphate formed in the GP reaction is converted to glucose 6-phosphate by PGM. The glucose 6-phosphate formed from glycogen in skeletal muscle can enter glycolysis and serve as an energy source to support muscle contraction [24].

A primary stimulus for hepatic glycogenolysis is nutritional deprivation, with corresponding elevation of counterregulatory hormones such as glucagon. Breakdown of muscle glycogen accompanies exercise, under conditions of increased AMP and Ca^{2+} [7].

1.4.3 Glycogen synthesis

Glycogen synthesis follows a simple but strictly ordered process, resulting in a complex structure. The starting point for synthesis of glycogen is GN. GN self-glucosylates to form an oligosaccharide primer chain. Via its extreme C-terminus, GN can interact directly with GS which is responsible for the formation of the large majority of the α-1,4-glycosidic linkages of glycogen, utilizing UDP-glucose as the glucose donor [25, 27]. To initiate this step, glucose 6-phosphate, derived from either hexokinase I and hexokinase II in muscle and hexokinase IV (glucokinase) in liver, is converted to glucose 1-phosphate by the PGM. Then, glucose 1-phosphate is converted to UDP-glucose by the action of UDP-glucose pyrophosphorylase at the expense of converting UTP into UDP [24, 25].

UDP-glucose is the immediate donor of glucose residues in the reaction

catalyzed by GS, which promotes the transfer of the glucose residue from UDP-glucose to a nonreducing end of a branched glycogen molecule.

GS cannot make the α-1,6-bonds found at the branch points of glycogen; these are formed by the glycogen branching enzyme (GBE), also called glycosyl-(4,6)-transferase (Figure 4). The GBE catalyzes the transfer of a terminal fragment of 6 or 7 glucose residues from the nonreducing end of a glycogen branch to the C-6 hydroxyl group of a glucose residue at a more interior position of the same or another glycogen chain, creating a new branch. Further glucose residues may be added to the new branch by GS. The biological effect of branching is to make the glycogen molecule more soluble and to increase the number of nonreducing ends. This increases the number of sites accessible to GP and GS, both of which act only at nonreducing ends [24].
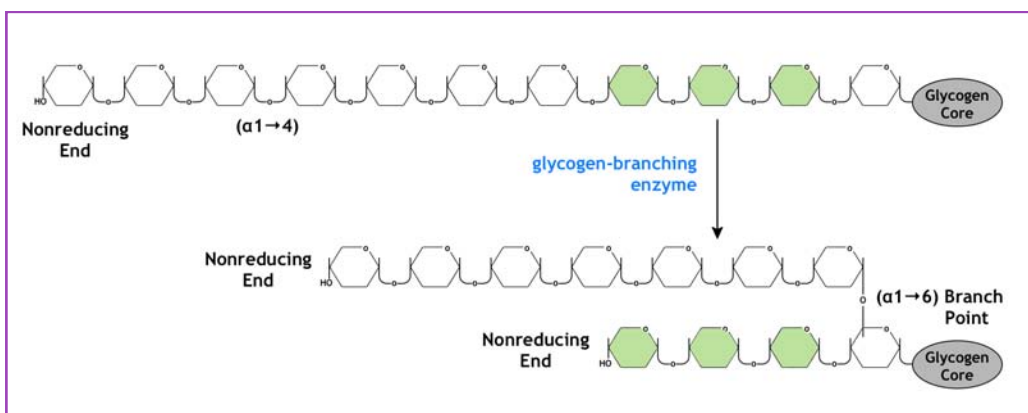
Figure 4: Branch synthesis in glycogen. [24]

1.4.4 Glycogen phosphorylase (GP)

GP (1,4 α-D-glucan: orthophosphate α-D-glucosyltransferase, EC 2.4.1.1) catalyzes the first step in the glycogen breakdown being responsible of the intracellular degradation of glycogen into glucose 1-phosphate [26], thus plays a

crucial role for maintaining the cellular and organismal glucose homeostasis. GP was the first allosteric enzyme to be discovered [28, 29] and the first protein reported to be regulated by reversible protein phosphorylation [30].

1.4.4.1 Isoforms

In mammals exist three different isoforms of the GP: muscle (GP-MM), brain (GP-BB, OMIM database number 38550), and liver (GP-LL, OMIM database number 613741) according to the tissue in which they are preferentially expressed in adult mammals (Figure 5) [31]. Rabbit, rat and human GP-MM sequences are 97% identical, whereas human brain, muscle and liver phosphorylases are 80% identical in sequence comparisons [32], suggesting a common origin by gene duplication [33]. All of these isoforms are interconverted between *b* and *a* forms by covalent phosphorylation/ dephosphorylation but they differ in their responsiveness to allosteric ligands. The physiological role of each isoform varies greatly such as is reflected in their complex regulatory mechanisms, allowing tissue and cell-type specific glycogen metabolism.

1.4.4.1.1 Muscle glycogen phosphorylase (GP-MM)

GP-MM is codified by *PYGM* gene (HGNC: 9726), which is located in chromosome 11, and its physiological role is to provide the fuel for the energy production required for muscular contraction. Although GP-MM is mainly found in the skeletal muscle and in a minor proportion, in the heart muscle, it can be also expressed in the brain [34, 35]. Mutations in this gene cause glycogenoses type V, also known as McArdle disease (Table 2 and 3, Figure 8).

GP-MM can be highly activated by phosphorylation and exhibits full activity in the presence of AMP, which it binds cooperatively [31].

GP-MM *b* is the form found in resting muscle and may be activated by AMP or IMP and is inhibited by glucose, glucose 6-phosphate, ATP as well as purine analogues such as caffeine and adenosine [36, 37]. In response to nervous or hormonal stimulation, GP-MM *b* is phosphorylated at Ser-15 by PHK, thus becoming a more active enzyme, GP-MM *a*. GP-MM *a* is not dependent on AMP [38], although its activity can be increased by AMP about 5-10% [39]. GP-MM is composed of two identical monomers and upon Ser-15 phosphorylation, the N-terminal residues change their conformation from disordered to ordered and their contacts from intrasubunit to intersubunit [37]. GP-MM *a* can be reconverted to GP-MM *b* by the action of phosphorylase phosphatase 1 (PP1).

In muscle, superimposed on the regulation of GP-MM by covalent modification are two allosteric control mechanisms. a) Ca^{2+}, the signal for muscle contraction, binds to and activates PHK, promoting conversion of GP-MM *b* to the active *a* form. b) AMP, accumulated during muscle contraction as a result of ATP breakdown, binds to and activates GP-MM. When ATP levels are restored, ATP blocks the allosteric site to which AMP binds, inactivating phosphorylase [24].

1.4.4.1.2 Liver glycogen phosphorylase (GP-LL)

GP-LL is codified by *PYGL* gene (HGNC: 9725), which is located in chromosome 14. The physiological role of GP-LL isoform is to ensure a constant supply of glucose for extrahepatic tissues, especially the central nervous system, which relies on glucose as its major source of fuel [40]. Mutations in *PYGL* gene cause glycogenoses type VI, also known as Hers disease (Table 2).

The liver and muscle isoforms fulfill different physiological requirements and accordingly respond differently to allosteric effectors. The liver isoform is less sensitive to intracellular ligand and consequently its activation by AMP and other

nucleotides is very weak and it is completely non-cooperative [41]. The GP-LL *b* is inactive, only shows a 10-20% of activity in the presence of AMP and is insensitive to inhibition by ATP or glucose 6-phosphate [42-44]. It depends strongly on phosphorylation to achieve activity and its phosphorylation state is subject to hormonal control, thus GP-LL isoform activity is regulated primarily extracellularly in keeping with the function of this isoform in providing glucose outside of the cell [45].

1.4.4.1.3 Brain glycogen phosphorylase (GP-BB)

GP-BB is codified by *PYGB* gene (HGNC: 9723), which is located in chromosome 20. It is associated primarily with provision of an emergency glucose supply during short anoxic or hypoglycemic episodes [33], and in brain might also be associated with the supply of energy to glial cells for rapid neurotransmitter clearance [46]. This isoform predominates in human heart and brain, although the muscle isoform is also detectable in these tissues, together with a third isoform, which is a hybrid of the brain and muscle isoforms [34]. Moreover, GP-MM is the major isoform found in all human and rat foetal tissues, including in the muscle, although its postnatal expression is dramatically reduced in the vast majority of tissues with the exception of the brain and heart. Together with GP-LL, it is also expressed in regenerating mature fibers, but not in adult non-regenerating mature fibers, where only the muscle isoform is expressed. Unlike muscle isoform, the brain isoform is poorly activated by phosphorylation and its phosphorylated form (*a*) is more potentially activated by AMP which it binds non-cooperatively [47].

In comparison to GP-MM and GP-LL isoforms, there have been no diseases associated with a defect in GP-BB.
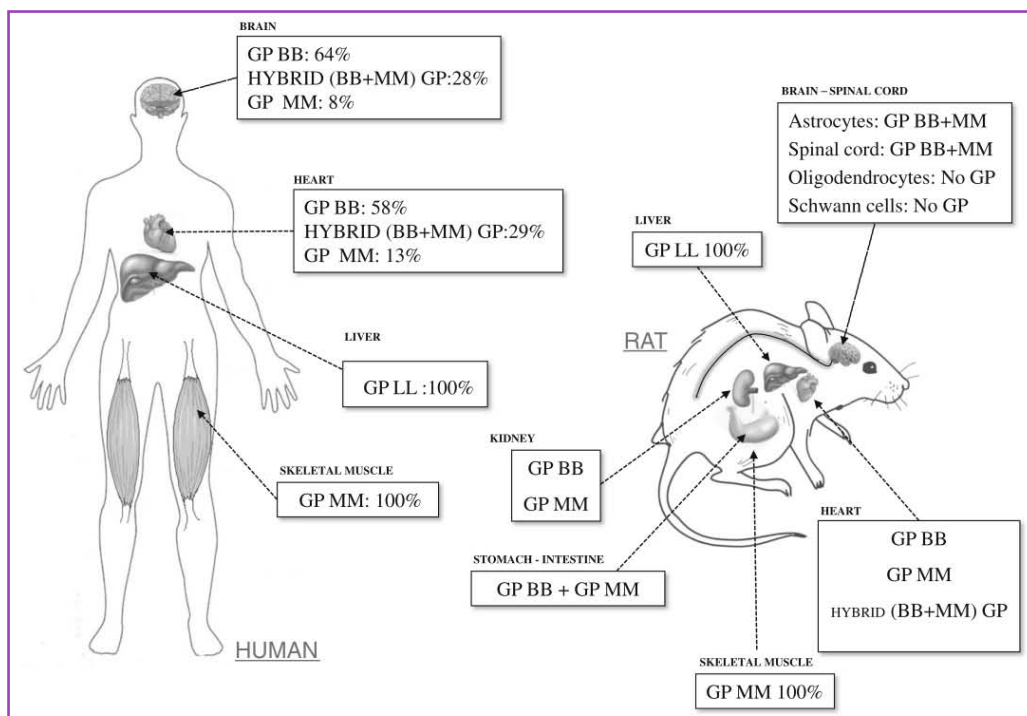
Figure 5: Summary of the expression pattern of the different glycogen phosphorylase isoforms in human and rat tissues. GP-MM, skeletal-muscle isoform of glycogen phosphorylase; GP-BB, brain isoform of glycogen phosphorylase; GP-LL, liver isoform of glycogen phosphorylase [48].

1.4.4.2 Muscle Glycogen Phosphorylase: Structure and domains

GP-MM is composed of two identical monomers of 842 amino acids with a molecular weight of 97,434 Da [32] and an essential cofactor, pyridoxal 5'-phosphate, linked via a Schiff base to Lys 681 in each subunit [37].

Each monomer is composed by two different α/ß domains, an N-terminal and a C-terminal domain (Figure 6). The N-terminal domain comprises the residues 1-484 and is composed by fifteen α-helices and nine-stranded, mostly parallel ß-sheet core [32, 49]. This domain contains: the site of Ser-15 phosphorylation; the phosphorylated Ser-15 contacts; the AMP allosteric effector and glycogen binding sites; and part of the catalytic domain. Whereas the C-terminal domain comprises

residues 485-842 and consists of five α -helices and six ß-strands. This domain includes the major part of the catalytic domain, as well as the pyridoxal 5'-phosphate binding and nucleoside inhibitor sites [32, 50].
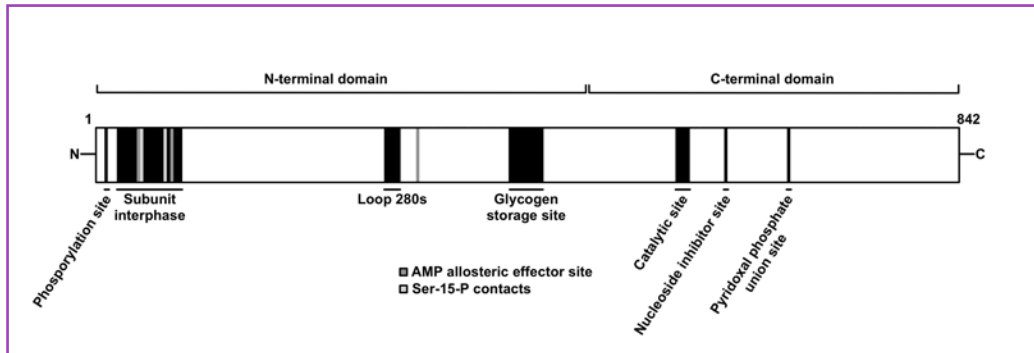
Figure 6: Graphic representation of the primary structure of the muscle glycogen phosphorylase monomer. [49]

GP-MM is regulated by reversible phosphorylation and allosteric effectors and can exist in two different structural conformations, depending on the phosphorylation state of the enzyme and the presence or absence of various ligands (Figure 7): the dephosphorylated low-activity and low substrate affinity GP-MM *b* and the phosphorylated high-activity and high substrate affinity GP-MM *a*. In both forms, allosteric effectors can promote equilibrium between a less active T state and a more active R state (Figure 7).

The *a* and *b* forms differ in their secondary, tertiary and quaternary structures; the active site undergoes changes in structure and, consequently, changes in catalytic activity as the two forms are interconverted.

GP-MM exhibits control by ligands at five binding sites:

a) *Phosphorylated Ser-15 site*: In the inactive T state of GP-MM *b*, the N-terminal residues 10-20 are poorly ordered. They are mainly made up of basic amino acids and generate intrasubunit contacts at an acidic region [37]. In this state, Ser-15

is turned in toward Glu-502 and Arg-17 interacts with either Glu-106 or Asp-110. When Ser-15 is phosphorylated, the N-terminal residues 10-23 swing through ~120º with respect to their initial position [36] and change their contacts from intrasubuint to intersubunit contacts, making ionic contacts with Arg-70 from its own subunit and with Arg-44 from the other subunit [37].

The tertiary and quaternary structural changes promoted by phosphorylation affect the subunit interface that is composed mainly of the interactions between residues 20-80 and residues 36-45 of the other subunit [37].

In the GP-MM *a* form, the structural changes prompted by phosphorylation of Ser-15 cause the ordering of the N-terminal tail and the disordering of the C-terminal tail. The contact between the two arginines and the phosphorylated Ser-15 prompt the creation of a high-affinity AMP site.

b) *AMP allosteric site*: GP-MM is also regulated by AMP. AMP allosteric effector site is found in the N-terminal domain and is composed by a cleft at the interface of the two subunits [51].

In the T state of GP-MM *b*, the AMP site is open and AMP can be bound, but the contacts are almost entirely between the AMP phosphate and Arg-310 and Arg-311 [36]. The structural changes prompted by phosphorylation cause an increase in the interactions between the enzyme and AMP in the R state [51-53], increasing the AMP affinity. However, in contrast with phosphorylation-mediated activation, the activation of GP-MM *b* by AMP does not generate structural changes in the N-terminal residues [54].

c) *Catalytic site*: The catalytic site is located below the molecular surface at the base of a narrow tunnel formed between the interface of the two domains, and

close to the essential cofactor pyridoxal 5'-phosphate bound covalently at the Lys-681 [36].

Figure 7: Schematic representation of the allosteric and covalent activation mechanism for glycogen phosphorylase. T and R subunits are shown as squares and circles, respectively [36].
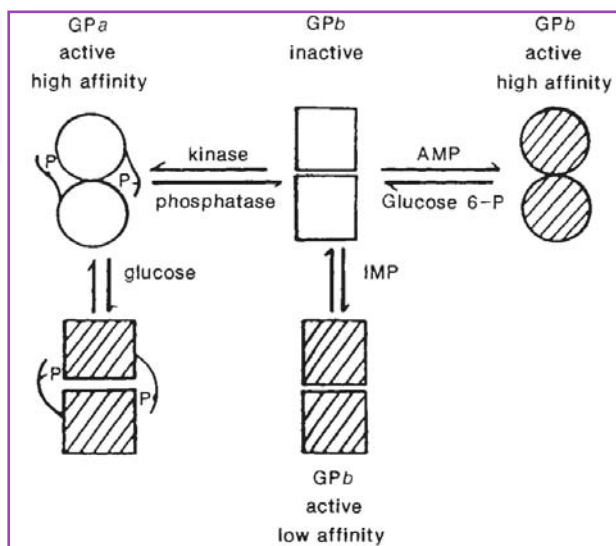
In the T state, access to the catalytic site is blocked by the loop of chain residues 281-289 (280's loop) and the side chain of Asp-284 is directed toward the catalytic site and is linked via two water molecules to the 5'-phosphate of the pyridoxal phosphate. On transition to the R state, conformational changes at the catalytic site occur to convert it into a form capable of binding phosphate. The 280's loop is disordered and displaced from the catalytic site and Arg-570 occupies the position close to that previously occupied by Asp-284 in the T state and helps to create a higher affinity recognition site for the phosphate substrate [37].

d) *Glycogen storage site*: GP-MM also presents a glycogen binding site which exerts regulatory properties. It is located at the surface of the enzyme, away from the catalytic and allosteric sites and is mainly formed by residues 398-438 [37].

Activation of phosphorylase by either phosphorylation or AMP leads to the association of the subunits from dimers to tetramers. Tetramers exhibit less activity than dimers but the glycogen binding enhances activity and promotes dissociation of

less active tetramers to active dimers through competition for binding at the storage site [37].

e) *Nucleoside inhibitor site*: The nucleoside inhibitor site is situated at the entrance of the catalytic site and binds purine analogs or related heterocyclic ring compounds such as adenosine, caffeine, flavin mononucleotide, NADH and AMP at high concentrations (>2 mM). The fused ring compounds intercalate two aromatic rings of Phe-286 and Tyr-614 and stabilize the location of the 280's loop in the inactive state of the protein [55].

1.5 Glycogenoses

Glycogenoses, also known as glycogen storage diseases (GSD), are inborn errors of glycogen or glucose metabolism. There are fifteen types of GSD [56] principally affecting the liver and muscle, owing to the significance of glycogen and glucose metabolism in these organs. Nevertheless, GSD have a broad clinical spectrum and many tissues may also be affected. They are caused by defects in enzymes or transporters involved in the synthesis or degradation of glycogen. These defects can be inherited in an autosomal or X-linked manner and depending on which enzyme is affected will lead to a type of GSD (Table 2 and Figure 8).

In the GSD where skeletal muscle is affected (GSD types 0b, II, IIb, IIIa, IIId, IV, V, VII, IXb, IXd and X to XV), the blockage in this tissue of either glycogen breakdown (glycogenolysis), or of the anaerobic catabolism of glucose 6-phosphate (glycolysis), results in an impairment of muscle performance, owing to i) and increase in glycogen storage that disrupts contractile function and/or ii) a reduced substrate turnover below the block, which inhibits skeletal muscle ATP production [57]. This blockage causes two major clinical presentations: i) recurrent and reversible exercise-related muscle pain, cramps, often culminating in muscle

breakdown (rhabdomyolysis) with myoglobinuria; and ii) slowly progressive muscle weakness [58].

Type (name)	Enzyme affected	Inheritance	Primary organ affected	Symptoms	References
0a	Glycogen synthase-2	Autosomal Recessive	Liver	-Presents in infancy and childhood -Neonatal hypoglycemia -Fasting hypoglycemia -Fasting hyperketonemia -Hyperglycemia and hyperlactatemia with feeding	[56, 59-61]
0b	Glycogen synthase-1	Autosomal Recessive	Muscle	-Cardiomyopathy and exercise intolerance associated with complete absence of muscle glycogen -Seizures, tonic-clonic (rare) -Risk of sudden death in childhood due to cardiac arrest	[56, 62, 63]
Ia (von Gierke's)	Glucose 6-phosphatase	Autosomal Recessive	Liver and kidneys	-Typically manifests during the first year of life with severe hypoglycemia and hepatomegaly caused by the accumulation of glycogen -Affected individuals exhibit growth retardation, delayed puberty, lactic acidemia, hyperlipidemia, hyperuricemia, and in adults a high incidence of hepatic adenomas	[56, 64]
Ib	Glucose 6-phosphatase transporter T1	Autosomal Recessive	Liver and kidneys	-Differ from Ia in that patients are prone to bacterial and fungal infections due to impaired leukocyte function -Neutropenia -They are also prone to chronic pancreatitis, chronic inflammatory bowel disease and Crohn's disease	[56, 65, 66]
Ic	Glucose 6-phosphatase transporter T2	Autosomal Recessive	Liver and kidneys	-Differ from Ib in that patients don't present leukocyte dysfunction	[56, 67, 68]
Id	Glucose 6-phosphatase transporter T3	Autosomal Recessive	Liver and kidneys	-Caused by a defect in a third type of glucose 6-phosphatase transporter, responsible for transport of glucose out of the endoplasmic reticulum	[56]
II (Pompe's)	α-1,4-glucosidase (acid maltase)	Autosomal Recessive	Skeletal and cardiac muscle	-Lysosomal glycogen storage disease -In the classic infantile form (Pompe disease), cardiomyopathy and muscular hypotonia are the cardinal features; in the juvenile and adult forms, involvement of skeletal muscles dominates the clinical picture -Infantile onset is characterized by muscle weakness and heart involvement (cardiomegaly and congestive heart failure) -Adult onset is characterized by fatigue and proximal weakness and respiratory failure -Better prognosis is associated with later onset disease	[56, 69]
IIb (Danon's)	Lysosome associated membrane protein-2	X-linked dominant	Skeletal and cardiac muscle	-Lysosomal glycogen storage disease with normal acid maltase -Multifactorial disease which predominately affects cardiac and skeletal muscle -Clinical features include cardiomyopathy, myopathy and variable degrees of mental retardation	[56,70]
IIIa (Cori's or Forbes's	Glycogen Debranching Enzyme	Autosomal Recessive	Liver, skeletal and cardiac muscle	-Disorder associated with an accumulation of abnormal glycogen with short outer chains. Most patients are enzyme-deficient in both liver and muscle -Lack of both glycosidase and transferase activity in liver and muscle -Clinically, patients with GSD III present in infancy or early childhood with hepatomegaly, hypoglycemia, and growth retardation. Muscle weakness in those with IIIa is minimal in childhood but can become more severe in adults; some patients develop cardiomyopathy	[56, 71]

Type (name)	Enzyme affected	Inheritance	Primary organ affected	Symptoms	References
IIIb	Glycogen Debranching Enzyme	Autosomal Recessive	Liver	-Lack of both activities in liver only (about 15%)	[56, 71]
IIIc	Glycogen Debranching Enzyme	Autosomal Recessive	Liver, skeletal and cardiac muscle	-Selective loss of glycosidase activity	[56, 71]
IIId	Glycogen Debranching Enzyme	Autosomal Recessive	Liver, skeletal and cardiac muscle	-Selective loss of translocate activity	[56, 71]
IV (Andersen's)	Glycogen Branching Enzyme	Autosomal Recessive	Liver, skeletal and cardiac muscle	-Tissue accumulation of abnormal glycogen with fewer branching points and longer outer branches, resembling an amylopectin-like structure, also known as polyglucosan -Clinically heterogeneous disorder -"Classic" hepatic presentation is liver disease of childhood, progressing to lethal cirrhosis -The neuromuscular presentation is divided in 4 groups depending of age at onset: perinatal, presenting as fetal akinesia deformation sequence (FADS) and perinatal death; congenital, with hypotonia, neuronal involvement, and death in early infancy; childhood, with myopathy or cardiomyopathy; and adult, with isolated myopathy or adult polyglucosan body disease	[56, 72, 73]
V (McArdle's)	Muscle Glycogen phosphorylase	Autosomal Recessive	Skeletal muscle	-Onset of exercise intolerance and muscle cramps in childhood or adolescence -Transient myoglobinuria may occur after exercise, due to rhabdomyolysis -Severe myoglobinuria may lead to acute renal failure -Patients may report muscle weakness, myalgia, and lack of endurance since childhood or adolescence -In adult life, there is persistent and progressive muscle weakness and atrophy with fatty replacement -Second wind phenomenon	[56, 74, 75]
VI (Hers's)	Liver Glycogen phosphorylase	Autosomal Recessive	Liver	-Presentation in early childhood -Clinical presentation: is one of mild to moderate hypoglycemia, mild ketosis, growth retardation, and prominent hepatomegaly -Heart and skeletal muscle are not affected	[56, 76]
VII (Tarui's)	Muscle phosphofructokinase	Autosomal Recessive	Skeletal muscle	-Characterized clinically by exercise intolerance, muscle cramping, exertional myopathy, and compensated hemolysis -Myoglobinuria may also occur -Total and partial loss of muscle and red cell phosphofructokinase activity	[56, 77]
IXa1 (VIII, formerly)	α-2 subunit of hepatic phosphorylase kinase	X-linked recessive	Liver and erythrocytes	-No phosphorylase activity in liver or erythrocytes -Glycogen storage disease IXa is one of the mildest of the glycogenoses of man -Clinical symptoms include hepatomegaly, growth retardation, elevation of glutamate-pyruvate transaminase and glutamate-oxaloacetate transaminase, hypercholesterolemia, hypertriglyceridemia, and fasting hyperketosis -These clinical and biochemical abnormalities gradually disappear with age, and most adult patients are asymptomatic	[56, 78-80]
IXa2	α-2 subunit of hepatic phosphorylase kinase	X-linked recessive	Liver	-No phosphorylase activity in liver -The same clinical presentation as IXa1	[56, 78-80]
IXb	ß subunit of hepatic phosphorylase kinase	Autosomal Recessive	Liver and skeletal muscle	-Hepatomegaly and marked accumulation of glycogen in both liver and muscle -Diarrhea -Hypotonia and mild weakness	[56, 80, 81]

Type (name)	Enzyme affected	Inheritance	Primary organ affected	Symptoms	References
IXc	ɣ subunit of phosphorylase kinase	Autosomal Recessive	Liver	-Onset in childhood -Hepatomegaly, hypotonia, growth retardation in childhood, and liver dysfunction -Symptoms improve with age in most cases; however, some patients may develop hepatic fibrosis or cirrhosis	[56, 80, 82]
IXd	α-1 subunit of hepatic phosphorylase kinase	X-linked recessive	Skeletal muscle	-Relatively mild metabolic disorder characterized by variable exercise-induced muscle weakness or stiffness -Most patients have adult-onset of symptoms, and some can remain asymptomatic even in late adulthood -The phenotype is usually only apparent with intense exercise	[56, 83]
X	Muscle phosphoglycerate mutase	Autosomal Recessive	Skeletal muscle	-Onset in adolescence of exercise-induced cramps, occasional myoglobinuria, and intolerance for strenuous exercise -Pigmenturia	[56, 84]
XI	Muscle lactate dehydrogenase	Autosomal Recessive	Skeletal muscle	-Exercise intolerance and recurrent myoglobinuria -Erythematous squamous skin lesions	[56, 85]
XII	Aldolase A	Autosomal Recessive	Skeletal muscle and red cells	-Hemolytic anemia -Mental retardation and myopathy (in some patients)	[56, 86]
XIII	ß-enolase	Autosomal Recessive	Skeletal muscle	-Exercise intolerance, myalgias, and increased serum creatine kinase	[56, 87]
XIV	phosphoglucomutase 1	Autosomal Recessive	Liver, skeletal and cardiac muscle	-Characterized by a wide range of clinical manifestations and severity -The most common features include cleft lip and bifid uvula, apparent at birth, followed by hepatopathy, intermittent hypoglycemia, short stature, and exercise intolerance with episodes of rhabdomyolysis, often accompanied by increased serum creatine kinase, normal elevation of lactate, and hyperammonemia on a forearm-exercise test -Less common features include dilated cardiomyopathy, and hypogonadotropic hypogonadism	[56, 88, 89]
XV	Glycogenin 1	Autosomal Recessive	Skeletal and cardiac muscle	-Muscle weakness associated with the depletion of glycogen in skeletal muscle and cardiac arrhythmias associated with the accumulation of abnormal storage material in the heart	[56, 90]

Table 2: Summary of 15 types of Glycogen Storage Disease (GSD).
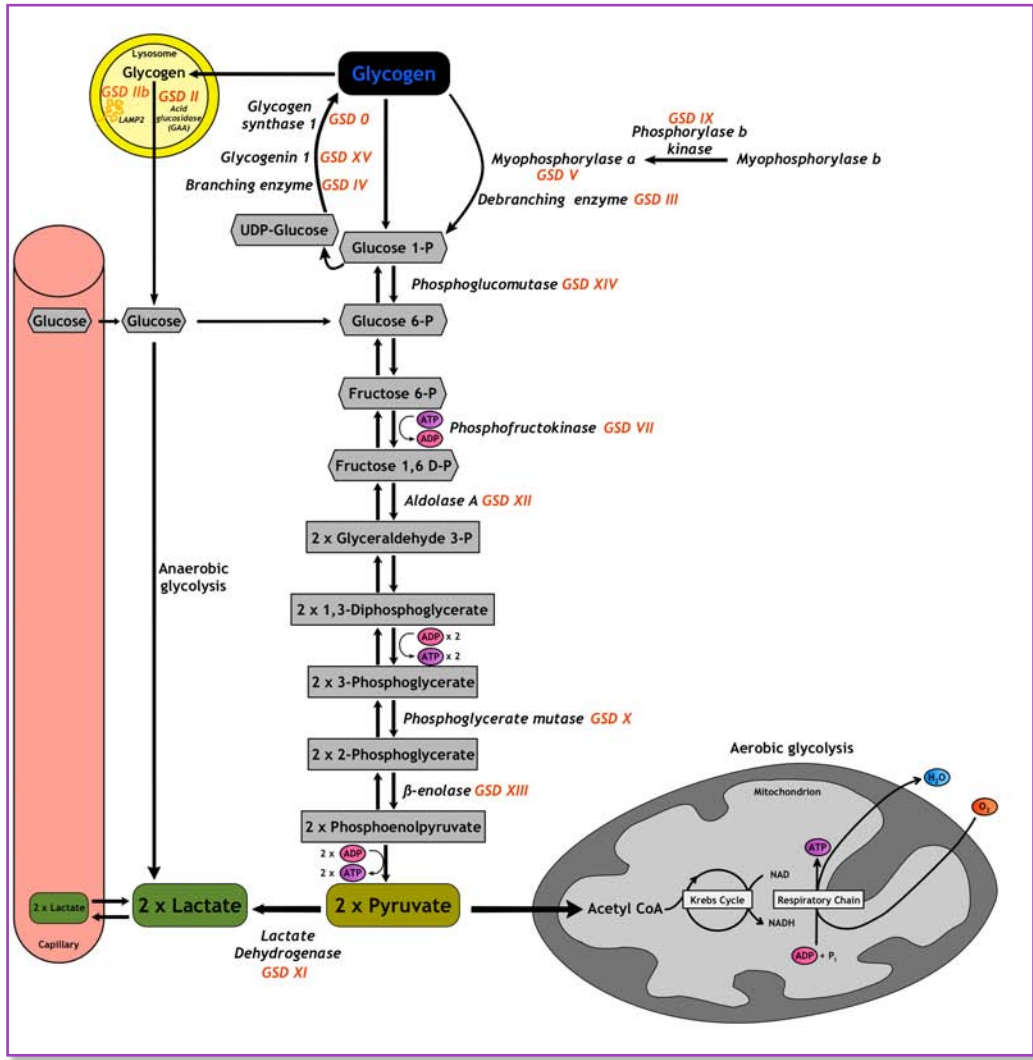
Figure 8: **Schematic representation of skeletal muscle glycogen metabolism.** Glycogen and glucose are metabolized anaerobically (with ATP generated by substrate-level phosphorylation and pyruvate reduced to lactate) as well as aerobically (with pyruvate metabolized to acetyl CoA with ATP produced via oxidative phosphorylation). The figure also indicates the steps in the pathway that are affected in various glycogen storage diseases that cause defects in skeletal muscle function. *GSD II*, Pompe disease; *GSD IIb*, Danon disease; *GSD III*, Cori or Forbes disease; *GSD IV*, Andersen disease; *GSD V*, McArdle disease; *GSD VII*, Tarui disease. GSD, glycogen storage disease; UDP, uridine diphosphate; LAMP2, lysosome associated membrane protein-2; ATP, adenosine triphosphate; ADP, adenosine diphosphate; P_i, inorganic phosphate; NAD, oxidized nicotinamide adenine dinucleotide; NADH, reduced nicotinamide adenine dinucleotide [24, 56-58, 75, 91].

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1.6 McArdle disease

1.6.1 Definition

McArdle disease, also termed GSD type V or glycogenoses type V (OMIM® number 232600) is a pure myopathy caused by inherited deficiency of GP-MM, also known as myophosphorylase. It is an autosomal recessive disorder caused by pathogenic mutations in both alleles of human GP-MM-encoding gene (*PYGM*), which is located in chromosome 11q12-11q13 [75]. It was first described by Brian McArdle in 1951 [74] but it was not until 1959 that the enzyme defect was identified [92]. It is one of the most frequent genetic myopathies, e.g., with a prevalence of ~1/167,000 in Spain, with both sexes similarly affected [93].

1.6.2 Clinical presentation

There is phenotype heterogeneity among patients, and clinical severity can be classified into four categories, according to the Martinuzzi scale [94]:

- 0: asymptomatic or virtually asymptomatic (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; no record of myoglobinuria, no muscle wasting or weakness
- 2: same as 1, plus recurrent exertional myoglobinuria, moderate restriction in exercise, and limitation in daily life activities
- 3: same as 2, plus fixed muscle weakness, with or without wasting, and severe limitations on exercise and most daily life activities
  Renal failure can occasionally occur but it is usually reversible [93, 95].
  Additionally, the time of onset of the disease symptoms can vary between

patients. In this regard, the majority of patients (86%) from the Spanish McArdle disease registry reported that symptoms started within the first two decades of life; indeed, in 58% of patients, symptoms started in the first decade, typically in school sport activities [93]. Yet in many cases the disease is not correctly diagnosed until years later. One reason for clinical heterogeneity might lie in different dietary regimens (e.g. low vs high carbohydrate content) and lifestyles, but also genetics must play a role. Genetic variants of several candidate genes that modulate human responses to exercise can also have a role in determining the severity of the McArdle disease phenotype. For example, it has been described that the coexistence of *PYGM* mutations along with specific genetic variants for the adenylate deaminase (*AMPD1*) may account for more severe phenotypes of the disease. Additionally, the insertion/ deletion polymorphism of the angiotensin-converting enzyme (*ACE*) gene also modulates the McArdle disease phenotype, with the insertion allele improving cardiovascular function and higher uptake of blood glucose into skeletal muscle fibers and favors a less-severe clinical presentation of the disease [75, 96].

1.6.2.1 Exercise intolerance

McArdle patients are unable to obtain energy from their muscle glycogen stores and, as a result, commonly experience exercise intolerance, mainly in the form of acute crises of early fatigue and muscle stiffness and contractures, especially at the start of exercise, which usually disappear if exercise is stopped or intensity is reduced. They are sometimes accompanied by marked muscle damage or rhabdomyolysis as indicated by the efflux of intra-muscle proteins to the bloodstream, e.g. CK and myoglobin [75, 97]. Thus, high serum CK activity (typically >1,000 U/L) after exercise is a common finding that can be accompanied by myoglobinuria (or 'dark urine'). The main danger of exertional rhabdomyolysis is acute renal failure as well as hyperkalemia, with the former eventually leading to

chronic renal failure, although reported cases are rare [93, 95].

Patients' exercise intolerance is specially evident during exercise tasks involving aerobic and anaerobic glycolysis for muscle ATP production [75]. Thus, muscle crises are typically triggered by static or isometric muscle contractions (e.g. lifting a heavy weight, handgrip exercises) where high mechanical demands are relying on a relatively small muscle mass and the sustained muscular contraction dramatically increases the pressure inside the muscle, causing the supply of oxygenated blood to be transiently cut off or at least considerably decreased [75, 93, 96]. In this situation, muscles rely on an anaerobic energy supply from intracellular glycogen stores. This can be aggravated by the fact that excessive glycogen accumulation (~2-3 times higher than in healthy people) may mechanically disrupt the contractile apparatus [96]. In McArdle patients, muscle crises also can be triggered by dynamic "aerobic" exercises, involving large muscle mass and smaller mechanical loads (e.g. stair-climbing, running or brisk walking). Indeed, their muscle oxidative capacity is impaired; owing to their inability to produce pyruvate because of blocked glycogenolysis [75]. The reduced rate of oxidative phosphorylation in these patients is reflected on phosphorus magnetic resonance spectroscopy (31P-MRS) by significantly greater PCr consumption and lower ATP concentrations than in healthy controls after submaximal isometric calf contractions [96] or higher intracellular ADP concentrations at the beginning of recovery from ischemic exercise [97]. The resultant marked decrease in skeletal muscle phosphorylation capacity ($[ATP]/[ADP][Pi]$), leads to the accumulation of P_i, and probably also ADP, in patient's muscles, thereby potentially inhibiting (i) myofibrillar ATPase, (ii) SERCA pumps, and (iii) Na^+-K^+ ATPase pump reactions, leading to premature muscle fatigue and contractures [75, 97].

A deficiency in the glycogen-dependent ATP supply to Na^+-K^+ ATPase pump in

skeletal muscle fibers might result in downregulation of these pumps in the cells, leading to exercise-induced hyperkalemia and accelerated loss of membrane excitability [75]. Moreover, the accumulation of potassium, P_i and ADP in working muscles, due to the reduced muscle phosphorylation potential leads to an excessive release of these substances into the blood, thereby stimulating vascular smooth muscle and metabolically sensitive nerve afferents in skeletal muscle. This might explain, al least partly, the hyperkinetic cardiovascular response exhibited by patients with McArdle disease during dynamic exercise (i.e. increased cardiac output: oxygen uptake ratio) [98, 99].

1.6.2.2 The Second Wind phenomenon

One of the characteristic features of the McArdle disease is the so-called second wind phenomenon [100], what denotes a sudden, marked improvement in the tolerance to 'aerobic', dynamic exercise (walking or cycling) after ~8–10 min of exercise accompanied by a decrease of undue exertional fatigue, breathlessness and heart rate. Most patients refer to it as the ability to resume exercise if they take a brief rest once appearing premature fatigue early in exercise [96]. The majority of patients (86%) repeatedly experience the second wind phenomenon over life [93]. It is easily detectable by the diagnostic cycle ergometer test, where all adult patients show a decrease in early external tachycardia (from ~140-150 to ~120 beats/min) starting after around 7-8 min of a constant load (~40 W for most adults), cycle exercise eliciting a heart rate value of 60-70% of the predicted maximum heart rate (i.e., 220 beats/min minus age in years), and most reporting a decrease in local leg-muscle pain [93, 97, 101]. The second wind phenomenon is also detectable in children although the magnitude of the phenomenon is less marked than in adults - that is, lower decrease in exertional tachycardia after around 7 min [102].

The second wind phenomenon could be attributed to increased uptake of glucose and combustion of free-fatty acids, as during the beginning of submaximal exercise, muscle glycogen is the most important fuel [103]. The first few minutes of exercise act as a warm-up (e.g. inducing muscle vasodilatation), where but later, blood-borne glucose and free-fatty acids (FFA) are the major fuels. During the beginning of exercise, McArdle's disease patients thus experience trouble, as they are unable to use muscle glycogen. As exercise continues, they can use blood-borne glucose and FFA and no problems are to be expected (second wind phase), because of more circulating glucose and FFA are available to working muscle fibers that can oxidize these substrates [100, 104]. In fact, the second wind phenomenon is abolished by glucose infusion [104] or sucrose ingestion before exercise [105].

1.6.2.3 Muscle fixed weakness

Around 25% of patients from the Spanish registry also develop fixed muscle weakness and wasting (affecting more proximal than distal muscles) which is aggravated with aging [93, 106]. This phenomenon might be attributed, at least partly, to the cumulative effect of repeated episodes of rhabdomyolysis [107].

1.6.3. Mutations and polymorphisms

To date 148 pathogenic mutations and 39 polymorphisms (with a minor allele frequency (MAF) ≥ 1%) have been reported (Table 3 and 4) [49, 108]. The majority of mutations (91%) are located in exonic regions and only a 9% in intronic regions. McArdle disease has a high genetic heterogeneity, which include missense and nonsense mutations, splice junction, insertions, inversions and deletions along the twenty exons of *PYGM*. Missense mutations are the main type and account for 50% of all described mutations (75/148). The second most common type of mutation in *PYGM* are deletions (18%; 26/148), followed by nonsense mutations (13%; 19/148)

and mutations that affect RNA splicing (11%; 16/148). Few mutations are duplications (7/148), deletions/insertions (3/148), or missing of the starting amino acid (2/147) [49].

Mutations can be found in all the exons, but there are some exons that contain more mutations than others. For example, exon 1 is the densest in number of mutations (*N*=17) and includes the most common mutation (*p.R50X*) in the Caucasian population [49]. Exon 17 is the second densest in mutations (*N=14*) and harbours the most frequent mutation in Japanese population, *p.F710del* [109]. Other exons such as exons 10, 11, 12, 14, and 20 are also highly mutated and include 32% of *PYGM* mutations. Whereas exon 7 is very low in mutations with only one mutation described [110].

Among the described mutations, there is one mutation that predominates beyond the others in the studied Caucasians populations [93, 111-114]. This is *p.R50X* and is by far the most frequently found: ~55% of the mutated alleles in the Spanish registry of patients (*N=239*) [93], 68%-72% (France) [112, 113], 58% (Germany) [115], 43% (Italy) [111], 31% (Netherlands) [116], 77%-81% (UK) [95, 117], 63%-60% (USA) [118, 119], and 50% (Brazil) [114]. However, among patients of Asian descend, the *p.R50X* mutation has only been described in a Korean patient [120] and has yet not been detected in the Japanese population [109].

Depending on the population, *p.G205S* is the second or third most frequent mutation, accounting for 3%-10% of the mutated alleles in some cohorts [93, 113, 119, 121], but also being hardly detectable or not detectable at all in other cohorts [109, 111, 113, 122]. In Spanish population, *p.W798R* mutation is the second most frequent mutation [93] and *p.F710del* is the most frequent mutation in Japanese population [109].

Transcriptomic studies have revealed the pathogenic effect of some mutation such as intronic mutations, large deletions and silent mutations [123, 124]. It has been described that mutations in the donor or acceptor splicing site such as *c. 1768+1G>A* or *c.528-8G>A* can cause deletions and alterations in the open-reading frame, leading to a premature termination codon (PTC) [124, 125]. Other studies have also revealed the deletion of the full exon 17 in the transcript sequence of one patient [124] and have also been observed that some silent mutations (*p.K215K*, *p.K609K* and *p.G810G*) do not alter the amino acid sequence but affect splicing [123, 124].

The generation of a PTC directly by inherited mutations such as nonsense mutations, or indirectly by mutations that alter the open-reading frame can lead to the "nonsense-mediated mRNA decay" (NMD) mechanism. NMD is a homeostatic mechanism that regulates the quality of the transcripts inside each cell by degrading those that contain PTCs [120, 124, 126]. A study of 28 Spanish McArdle patients found that NMD was acting in 92% of them [126], therefore therapies trying to control this mechanism are being considered as a potential treatment strategies in McArdle patients [127, 128].

Exon	Codon	Nucleotide change	Amino acid sequence change	Previous nomenclature	Type of mutation	First description
1	1	c.1A>C	p.M1?	M0L; p.M1L	Missing starting codon	[129]
1	1	c.1A>G	p.M1?	M0V; p.M1V	Missing starting codon	[130]
1	5	c.13_14delCT	p.L5VfsX22		Deletion	[131]
1	5	c.14delT	p.L5RfsX21	L5RfsX20	Deletion	[113]
1	10	c.21_28dup8	p.K10TfsX19		Duplication	[132]
1	16	c.46delGinsTT	p.V16FfsX12		Deletion/ insertion	[133]
1	26/27	c.78_79delTG	p.E27AfsX50	T25fs	Deletion	[134]
1	36	c.107T>G	p.L36R		Missense	[113]
1	36	c.107T>C	p.L36P		Missense	[135]
1	37	c.111_133del	p.H37QfsX33	c.108_130del	Deletion	[113]
1	50	c.148C>T	p.R50X	R49X	Nonsense	[119]
1	51	c.152 A>G	p.D51G		Missense	[136]
1	53	c.158_160delACT	p.Y53del		Deletion	[136]
1	53	c.159C>G	p.Y53X	Y52X	Nonsense	[137]
1	55	c.164_168delCTCTG	p.A55GfsX21	c.163_167delGCTCT	Deletion	[113]
1	73	c.212_218dupCGCAGCA	p.Q73HfsX7		Duplication	[121]
1	75	c.225 C>A	Y75X		Nonsense	[136]
2	83	c.247 A>T	p.I83F		Missense	[121]
2	85	c.255C>A	p.Y85X	Y84X	Nonsense	[122]
2	94	c.280C>T	p.R94W	R93W	Missense	[122]
2	102	c.305delA	p.N102TfsX4	p.N102DfsX4	Deletion	[111]
2	114	c.341delA	p.Y114SfsX181		Deletion	[136]
3	116	c.347T>C	p.L116P	L115P	Missense	[138]
3	125	c.373G>T	p.E125X	E124X	Nonsense	[139]
3	134	c.402delC	p.N134KfsX161		Deletion	[121]
3	135	c.403G>A	p.G135R		Missense	[135]
3	136	c.407G>A	p.G136D		Missense	[112]
3	136	c.407delG	p.G136AfsX159		Deletion	[93]
3	139	c.415C>T	p.R139W	R138W	Missense	[116]
4	153	c.458T>C	p.L153P		Missense	[113]
4	153	c.458T>G	p.L153R		Missense	[113]
4	157	c.470G>T	p.G157V		Missense	[115]
4	159	c.475G>A	p.G159R		Missense	[111]
4	161	c.481C>T	p.R161C		Missense	[115]
4	170	c.509_511delAGA	p.K170del		Deletion	[112]

Exon	Codon	Nucleotide change	Amino acid sequence change	Previous nomenclature	Type of mutation	First description
4	174	c.521G>A	p.G174D		Missense	[121]
5	194	c.580C>T	p.R194W	R193W	Missense	[140]
5	205	c.613G>A	p.G205S	G204S	Missense	[119]
5	205	c.614G>A	p.G205D		Missense	[113]
5	211	c.632delG	p.S211TfsX84		Deletion	[141]
5	215	c.645G>A	p.K215K		Silent/splicing	[124]
6	230	c.689C>G	p.P230R		Missense	[111]
6	239	c.715_717delGTC	p.V239del		Deletion	[111]
6	246	c.736T>C	p.S246P		Missense	[113]
7	270	c.808C>T	p.R270X	R269X	Nonsense	[110]
8	292	c.875T>C	p.L292P	L291P	Missense	[125]
8	298	c.892dupT	p.Y298LfsX24	Y298LfsX35	Duplication	[113]
8	324	c.970C>G	p.R324G		Missense	[93]
9	337	c.1010A>G	p.Q337R		Missense	[115]
9	349	c.1045G>A	p.E349K	E348K	Missense	[139]
9	354	c.1061T>C	p.L354P		Missense	[142]
9	362	c.1086G>A	p.W362X	W361X	Nonsense	[143]
10	365	c.1094C>T	p.A365V	p.A365E	Missense	[111]
10	366	c.1098G>A	p.W366X		Nonsense	[113]
10	377	c.1129A>T	p.N377Y		Missense	[135]
10	379	c.1136C>T	p.T379M		Missense	[112]
10	383	c.1147G>A	p.E383K		Missense	[115]
10	384	c.1151C>A	p.A384D		Missense	[111]
10	384	c.1151C>T	p.A384V		Missense	[108]
10	385/386	c.1155_1156delGG	p.E386Afsx88		Deletion	[115]
10	388/390	c.1162_1169delTGGCCGGTinsA	p.W388SfsX34	insA/del 8bp in exon 10	Insertion/Deletion	[144]
10	397	c.1190T>C	p.L397P	L396P	Missense	[145]
10	398	c.1193C>T	p.P398L		Missense	[136]
11	428	c.1282C>T	p.R428C		Missense	[111]
11	442	c.1325T>A	p.M442K		Missense	[93]
11	449	c.1345G>C	p.G449R		Missense	[112]
11	450	c.1349C>T	p.S450L		Missense	[115]
11	452	c.1354dupC	p.A452GfsX23		Duplication	[115]
11	454	c.1362_1394delCGGCGTGGCGCGCATCCACTCCGAGATCCTCAA	p.N454_L464	c.1361_1393del	Deletion	[146]
11	455	c.1363G>C	p.G455R		Missense	[147]
11	456	c.1366G>A	p.V456M		Missense	[148]

Exon	Codon	Nucleotide change	Amino acid sequence change	Previous nomenclature	Type of mutation	First description
12	486	c.1457G>A	p.G486D		Missense	[115]
12	488	c.1463C>A	p.T488N	T487N	Missense	[149]
12	488	c.1463C>T	p.T488I		Missense	[112]
12	489	c.1466C>G	p.P489R	Pro488Arg	Missense	[150]
12	490	c.1468C>T	p.R490W		Missense	[121]
12	490	c.1469G>A	p.R490Q		Missense	[112]
12	491	c.1470dupG	p.R491AfsX7		Duplication	[121]
12	491	c.1471C>T	p.R491C		Missense	[111]
12	492	c.1475G>A	p.W492X	p.R492X	Nonsense	[111]
12	494	c.1480delG	p.V494FfsX45		Deletion	[151]
13	511	c.1531delG	p.D511TfsX28	p.D510fs	Deletion	[152]
13	513	c.1537A>G	p.I513V		Missense	[114]
13	534	c.1601delA	p.D534VfsX5		Deletion	[139]
14	541	c.1621G>T	p.E541X	E540X	Nonsense	[153]
14	543	c.1627A>T	p.K543X		Nonsense	[111]
14	543	c.1628A>C	p.K543T	L542T	Missense	[119]
14	570	c.1709G>A	p.R570Q		Missense	[112]
14	570	c.1708C>T	p.R570W		Missense	[115]
14	574	c.1722T>G	p.Y574X		Nonsense	[154]
14	575	c.1723A>G	p.K575E		Missense	[115]
14	576	c.1726C>T	p.R576X	R575X	Nonsense	[130]
14	577	c.1730A>G	p.Q577R		Missense	[121]
14	587	c.1760T>C	p.L587P		Missense	[155]
15	590	c.1769G>A	p.R590H		Missense	[112]
15	599	c.1797delT	p.F599LfsX6		Deletion	[116]
15	602	c.1804C>T	p.R602W	R601W	Missense	[139]
15	602	c.1805G>A	p.R602Q		Missense	[156]
15	609	c.1827G>A	p.K609K		Silent/splicing	[123]
16	648	c.1942A>T	p.N648Y		Missense	[136]
16	650	c.1948C>T	p.R650X		Nonsense	[112]
16	650	c.1948delC	p.R650EfsX8		Deletion	[113]
16	655	c.1963G>A	p.E655K		Missense	[145]
17	657/726	c.1970_2177del	p.V657GfsX21	p.G657_G726	Deletion	[111]
17	660	c.1979C>A	p.A660D	A659D	Missense	[157]
17	662	c.1985A>C	p.D662A	661 Asp-Ala	Missense	[158]
17	666	c.1996C>G	p.Q666E	E665Q	Missense	[130]
17	670	c.2009C>T	p.A670V		Missense	[159]

Exon	Codon	Nucleotide change	Amino acid sequence change	Previous nomenclature	Type of mutation	First description
17	685	c.2053A>T	p.N685Y	N684Y	Missense	[160]
17	686	c.2056G>A	p.G686R	G685R	Missense	[130]
17	687	c.2059G>C	p.A687P	A686P	Missense	[161]
17	692	c.2075_2076delCCinsAAA	p.T692KfsX30	691delCC/insAAA	Insertion/ Deletion	[162]
17	695	c.2083G>A	p.G695R		Missense	[163]
17	704	c.2111C>T	p.A704V	A703V	Missense	[139]
17	705	c.2113_2114delGG	p.G705RfsX16		Deletion	[111]
17	710	c.2128_2130delTTC	p.F710del		Deletion	[125]
17	715	c.2143C>T	p.R715W		Missense	[112]
18	733	c.2199C>G	p. Y733X		Nonsense	[113]
18	754	c.2262delA	p.K754NfsX49	753 delA	Deletion	[164]
18	755	c.2263C>T	p.Q755X	Q754X	Nonsense	[139]
18	771	c.2310_2311dupCC	p.R771PfsX33		Duplication	[165]
18	771	c.2312G>A	p.R771Q		Missense	[113]
19	779	c.2337_2339delAGA	p.E779del		Deletion	[120]
19	784	c.2352C>A	p.C784X		Nonsense	[121]
19	785	c.2353C>T	p.Q785X		Nonsense	[113]
20	795/796	c.2385_2386delAA	p.E797VfsX18	794/795 delAA	Deletion	[140]
20	798	c.2392T>C	p.W798R	W797R	Missense	[166]
20	808	c.2422A>C	p.T808P		Missense	[113]
20	809	c.2425dupT	p.S809FfsX7	S809FfsX6	Duplication	[113]
20	810	c.2430C>T	p.G810G		Silent/ splicing	[135]
20	814	c.2441G>A	p.S814N		Missense	[167]
20	815	c.2444A>C	p.D815A		Missense	[111]
20	822	c.2465C>A	p.A822D		Missense	[135]
20	826	c.2477G>C	p.W826S		Missense	[111]
Intron 3	-	c.425-26A>G			Splicing	[168]
Intron 4	176	c.528-8G>A	p.Q176_M177insVQ		Splicing	[124]
Intron 5	-	c.856-601G>A		IVS5-601G>A	Splicing	[163]
Intron 6	-	c.772-2A>T		IVS6-2A>T	Splicing	[115]
Intron 7	-	c.855+1G>C			Splicing	[111]
Intron 7	-	c.855+5G>A			Splicing	[93]
Intron 8	-	c.999+20C>T			Splicing	[135]
Intron 9	-	c.1092+1G>A			Splicing	[111]
Intron 9	-	c.1092-1G>T		c.1093-1G>T	Splicing	[111]
Intron 10	-	c.1239+1G>A		IVS10+1G>A	Splicing	[111]

Exon	Codon	Nucleotide change	Amino acid sequence change	Previous nomenclature	Type of mutation	First description
Intron 14	-	c.1768+1G>A		*(1844+1GA); IVS14+1G>A; p.V568AfsX16*	Splicing	[125]
Intron 16-17		c.(1969+214)_(2177+369)del			Deletion	[124]
Intron 18	-	c.2312+3G>C		*IVS18+3G>C*	Splicing	[112]
Intron 19	-	c.2379-1G>A		*c.2380-1G>A*	Splicing	[111]

Table 3: Described mutations in the *PYGM* gene [49].
Nucleotide numbering uses +1 as the A of the ATG translation initiation codon in the reference sequence, with the initiation codon as codon 1.
GeneBank reference sequences are: NM_005600.1 and AH002957.1
All the mutations described in this table can be found at http://databases.lovd.nl/shared/variants/PYGM.

As reviewed in [49], up to 39 polymorphisms, with a MAF ≥ 1% have been described in *PYGM* (Table 4). Among them, 82% (32 out of 39) are found in intronic regions, whereas only 18% (7 out of 39) are exonic. The majority of intronic polymorphisms (59%; 19 out of 39) are found in introns 5, 16, and 17, whereas the rest are equally distributed throughout the remaining introns (with the exception of introns 2, 6, 7, and 19, where no polymorphisms have been described).

With regard to their distribution, none of the polymorphisms are found in functionally relevant intronic regions such as 5' and 3' splice sites, the polypyrimidine tract, or the branch point. As for exonic polymorphisms, only one of them is found in the 5'UTR of the exon 1 sequence (rs483962), whereas the remaining six (rs71581787, rs11231866, rs11231865, rs114138772, rs61736659, and rs2230309) are found in the coding region of the gene (exons 10, 11, 12, 13, 16 and 17, respectively). Four of these polymorphisms cause a change in the encoded amino acid (rs71581787, rs11231866, rs61736659, and rs2230309), whereas the other two (rs11231865 and rs114138772) do not change the amino acid sequence (synonymous change).

Apart from the 39 polymorphisms found in *PYGM* with a MAF ≥ 1%, an additional list of +200 polymorphisms can be found in the UCSC genome browser

(GRCh37/h19 assembly, chr11:64513861-64528187, dbSNP141, "All SNPs [141]") for the *PYGM* gene.

Polymorphism	Position (dbSNP build 141)	Strand	DNA sequence change	Exon	Codon change	Allele frequency	HapMap populations	References
rs483962	chr11:64527751	Negative	C>T	1	5'UTR	C:28.4%; T:71.6%	MAF >5% in all 11 populations	N.A.
rs477549	chr11:64527080	Negative	c.243+48A>G	Intron 1	N.A.	A:74.8%; G:25.1%	MAF >5% in all 11 populations	N.A.
rs118038492	chr11:64526352	Positive	c.243+775C>T	Intron 1	N.A.	C:98.7%; T:1.2%	Not. Det.	N.A.
rs61884454	chr11:64525843	Positive	c.424.66A>G	Intron 3	N.A.	A:2.3%; G:97.6%	Not. Det.	N.A.
rs489192	chr11:64525644	Negative	c.660.+74A>C	Intron 4	N.A.	A:71.8%; C:28.1%	MAF >5% in all 11 populations	N.A.
rs490980	chr11:64525464	Positive	c.660+254C>T	Intron 4	N.A.	C:35.8%; T:64.1%	MAF >5% in CEU,CHB,JPT, YRI	N.A.
rs589691	chr11:64525216	Positive	c.855+35C>T	Intron 5	N.A.	C:34.0%; T:66.0%	MAF >5% in all 11 populations	N.A.
rs630966	chr11:64524911	Positive	c.855+340C>G	Intron 5	N.A.	C:28.0%; G:72.0%	Not. Det.	N.A.
rs7938455	chr11:64524781	Positive	c.855+470C>T	Intron 5	N.A.	C:80.0%; T:19.4%	Not. Det.	N.A.
rs73494206	chr11:64524752	Positive	c.855+499A>G	Intron 5	N.A.	A:95.0%; G:5.0%	Not. Det.	N.A.
rs547066	chr11:64523494	Positive	c.855+1756A>C	Intron 5	N.A.	A:22.5%; C:77.5%	MAF >5% in 9 populations (A<5% in ASW and YRI)	N.A.
rs75633423	chr11:64523035	Positive	c.855+2215C>G	Intron 5	N.A.	C:1.4%; G:98.6%	Not. Det.	N.A.
rs625172	chr11:64522066	Positive	c.999+99A>G	Intron 8	N.A.	A:30.3%; G:69.6%	MAF >5% in all 11 populations	N.A.
rs71581787	chr11:64521406	Positive	c.1184A>G	10	p.395T>M	A:2.1%; G:97.9%	Not. Det.	N.A.
rs192139668	chr11:64521279	Positive	c.1239+72C>T	Intron 10	N.A.	C:98.8%; T:1.2%	Not. Det.	N.A.
rs11231866	chr11:64521154	Positive	c.1240C>G	11	p.414R>G	C:9.0%; G:91%	Not. Det.	N.A.
rs2959652	chr11:64520942	Positive	c1240+49G>T	Intron 11	N.A.	G:35.9%; T:64.1%	MAF >5% in all 11 populations	N.A.
rs11231865	chr11:64520569	Positive	c.1494G>A	12	syn	A:3.7%; G:96,3%	MAF >5% in ASW, LWK, YRI (A<5% in CEU, MEX, MKK, TSI)	N.A.
rs565688	chr11:64520374	Positive	c.1518+171A>T	Intron 12	N.A.	A:89.0%; T:11.0%	A:100.0% in CEU, CHB, JPT, YRI	N.A.
rs7126110	chr11:64520255	Positive	c.1518+290C>G	Intron 12	N.A.	C:93.4%; G:6.5%	C:100.0% in JPT, YRI (G<5% in CEU, CHB)	N.A.

Polymorphism	Position (dbSNP build 141)	Strand	DNA sequence change	Exon	Codon change	Allele frequency	HapMap populations	References
rs114138772	chr11:64519926	Positive	*c.1569C>G*	13	syn	C:1.2%; G:98.8%	Not. Det.	[135]
rs686171	chr11:64519345	Positive	*c.1768+51G>A*	Intron 14	N.A.	A:17.5%; G:82.5%	Not. Det.	N.A.
rs532747	chr11:64519062	Positive	*c.1827+7C>T*	Intron 15	N.A.	C:16.7%; T:83.3%	MAF >5% in 10 populations (C<5% in YRI)	N.A.
rs61736659	chr11:64518809	Positive	*c.1957C>G*	16	p.653L>V	C:1.0%; G:99.0%	Not. Det.	N.A.
rs111543138	chr11:64518530-31	Positive	*c.1969+267insTG*	Intron 16	N.A.	(-):95.4%; TG:4.6%	Not. Det.	N.A.
rs592521	chr11:64518525	Negative	*c.1969+272C>T*	Intron 16	N.A.	C:14.7%; T:85.3%	Not. Det.	N.A.
rs2071320	chr11:64518520	Negative	*c.1969+277A>G*	Intron 16	N.A.	A:14.5%; G:85.5%	Not. Det.	N.A.
rs592532	chr11:64518517	Negative	*c.1969+280A>G*	Intron 16	N.A.	A:85.6%; G:14.4%	Not. Det.	N.A.
rs592546	chr11:64518511	Positive	*c.1969+286C>G*	Intron 16	N.A.	C:85.5%; G:14.5%	Not. Det.	N.A.
rs506354	chr11:64518504	Positive	*c.1969+293C>T*	Intron 16	N.A.	C:15.7%; T:84.3%	Not. Det.	N.A.
rs2230309	chr11:64517999	Negative	*c.2026A>G*	17	p.G676S	A:1.8%; G:98.2%	Not. Det.	N.A.
rs474006	chr11:64517326	Positive	*c.2177+522A>G*	Intron 17	N.A.	A:71.0%; G:29.0%	Not. Det.	N.A.
rs608261	chr11:64517317	Negative	*c.2177+531A>G*	Intron 17	N.A.	A:1.6%; G:98.4%	Not. Det.	N.A.
rs1207113	chr11:64517047	Positive	*c.2177+801A>G*	Intron 17	N.A.	A:69.8%; G:30.2%	Not. Det.	N.A.
rs9704315	chr11:64517039	Positive	*c.2177+809A>G*	Intron 17	N.A.	A:4.8%; G:95.2%	Not. Det.	N.A.
rs555974	chr11:64516477	Positive	*c.2177+1371G>T*	Intron 17	N.A.	G:72.1%; T:27.9%	Not. Det.	N.A.
rs28398896	chr11:64516308	Positive	*c.2177+1540A>G*	Intron 17	N.A.	A:5.7%; G:94.3%	Not. Det.	N.A.
rs566653	chr11:64515622	Positive	*c.2177+2226A>G*	Intron 17	N.A.	A:2.0%; G:98.0%	A<5% in CEU, YRI	N.A.
rs569602	chr11:64514506	Positive	*c.2312+190A>G*	Intron 18	N.A.	A:80.0%; G:20.0%	MAF >5% in all 11 populations	N.A.

Table 4: Described polymorphisms in the *PYGM* gene [49].
GeneBank reference: NG_013018.1
5'UTR, 5' untranslated region; N.A., nonapplicable; A, adenosine; G, guanosine; C, cytidine; T, thymidine; (-), lack of insertion TG; MAF, minor allele frequency; CEU, Utah residents with ancestry from northern and western Europe; CHB, Han Chinese in Beijing, China; JPT, Japanese in Tokyo, Japan; YRI, Yoruba in Iabadan, Nigeria; ASW, African ancestry in South Western United States; LWK, Luhya in Webuye, Kenya; MEX, Mexican ancestry in Los Angeles, CA; MKK, Masai in Kinyawq, Kenya; TSI, Toscani in Italy; Not. Det., not determined.
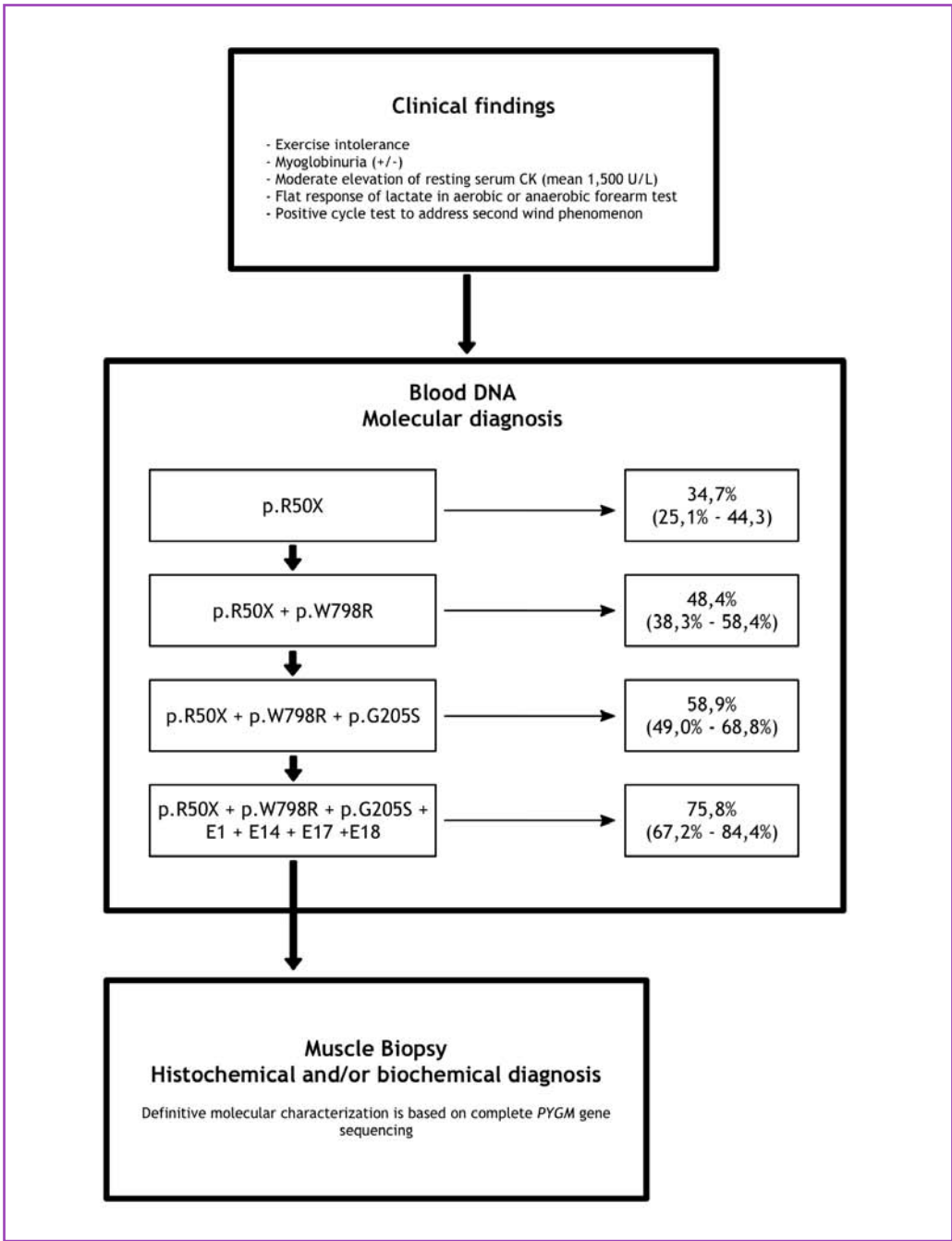
1.6.4 Diagnosis

As mentioned above, there is phenotype heterogeneity among McArdle patients and in addition, different GSD can have similar symptoms as McArdle disease, so sometimes carrying out a good diagnostic can be difficult. For this reason it is necessary to have an established standard diagnostic protocol to provide an easy and speedy way to diagnose all patients with a correct diagnosis.

The main tools traditionally used for diagnosing McArdle disease include clinical features; exercise intolerance with or without fixed muscle weakness, and the so-called second wind phenomenon; high serum levels of total CK activity; and absence of GP-MM activity in muscle biopsies.

Because McArdle disease is an autosomal recessive disorder, all patients have a pathogenic mutation in both copies of *PYGM*, being either homozygous or compound heterozygotes. Currently, the diagnosis of McArdle disease is based on the molecular analysis of DNA obtained from any accessible source as blood, hair follicle, buccal scraping samples, among others. However, DNA analysis of *PYGM* mutations can be tedious, since the gene contains 20 exons, but the fact that some mutations are clearly more prevalent in most populations (e.g, *p.R50X*, with an allele frequency 50% in Caucasian patients) and some exons are highly dense in mutations can facilitate genetic diagnosis. For instance, an algorithm was developed for Spanish patients' diagnosis [121] (Figure 9). Thus, Rubio and co-workers recommended to start searching for the commonest *p.R50X* mutation, followed by the relatively frequent *p.W798R* and *p.G205S* mutations, and thereafter by the complete sequencing of exons 1, 14, 17 and 18. This approach allowed the identification of the causative mutation in 75,8% of patients in a relatively cheap and simple manner [121].

Figure 9: **Diagnostic flow chart for diagnosis of McArdle disease**. (+/-) indicates presence or absence of the particular clinical feature. In the Blood DNA molecular diagnosis square, left boxes indicate the mutations and exons screened, left flow chart (thick arrow-lines) shows each of the consecutive steps of molecular screening, and right boxes represent the percentage (95% confidence interval) of patients in whom the two mutant alleles were identified by using the corresponding consecutive step of molecular screening (horizontal thin arrow-lines). E, exon. [121].

Specific PCR-RFLP designs have been developed for identification of common *PYGM* pathogenic mutations such as *p.R50X*, *p.G205S* or *p.W798R*, whose presence can be evidenced by the digestion with the enzymes NlaIII [119], HaeIII [169] and BsrBI [169], respectively. As not all mutations can be detected with above-mentioned approach, gene sequencing after PCR amplification is the most frequently utilized technique for screening the different *PYGM* mutations [111, 113-115]. However, this method is likely to be gradually replaced by whole gene next generation approaches.

In undiagnosed patients or in patients in whom only one mutation has been found, the RNA analysis of muscle biopsies samples might help to identify mutations that cannot be detected in DNA analysis and to unveil their effect at the transcriptional level [124]. The described methods for this approach include cDNA amplification in two overlapping fragments and sequencing reactions with eight internal fragments [124]. In addition, quantification of *PYGM* transcripts can be performed with specific primers and probes for quantitative PCR (qPCR) [126].

Two newer and less frequently used techniques have also been described. The first technique is high resolution melting [136, 170], which has been adapted for the study of the 20 exons of *PYGM* [171]. With this method the entire coding region and intronic flanking sequences are amplified in 18 different PCR fragments. If abnormalities in the melting profile of the PCR product are found, the fragment is sequenced. In this way, the sequencing costs in the screening can be reduced by 85%. The second technique is massive parallel sequencing, which has been applied for the diagnosis of patients with different GSDs [132]. This technique was used for studying 16 genes causative of muscle or liver GSDs, resulting in the correct diagnosis in 11 out of 17 patients, some of them harboring large deletions affecting several exons.

1.6.5 Therapy

There is no treatment to restore GP-MM activity in McArdle patients for the moment, but several types of treatment have been studied to reduce the symptoms in these patients, with different controversial results.

(a) *Nutritional supplements*: No significant beneficial effects have been reported in McArdle patients receiving nutritional supplements such as branched chain amino acids [172], depot glucagon [173], dantrolene sodium [174], verapamil [175], vitamin B6 [176] (except in one recent case report [177]), high-dose oral ribose [178]. More controversial are the results for creatine supplementation: low-dose supplementation (60 mg/kg per day for 4 weeks) attenuated muscle complaints in five of nine McArdle patients, but higher doses (150 mg/kg per day) exacerbated exercise-induced myalgia for unknown reasons [179].

(b) *"Read through" compounds*: Because almost 50% of Caucasians with McArdle disease present the nonsense *p.R50X* mutation, treatment with compounds that could potentially induce "read through" of the generated PTC could help to re-express GP-MM activity; however, in a short-term (10 days) gentamicin treatment failed to normalize ^{31}P-MRS indicators of GP-MM deficiency in the muscle of McArdle patients [127].

(c) *Induction of the expression of brain and liver glycogen phosphorylase isoforms in the muscle*: As mentioned above, GP-BB and GP-LL are only expressed in muscle tissue during its development and in regenerating mature fibers. Thus, any pharmacological treatment able to upregulate the expression of GP-BB and GP-LL in the skeletal muscle of McArdle patients could theoretically alleviate the symptoms of the disease by partially restoring muscle glycogen breakdown. In fact, a recent study with sodium valproate (VPA) treatment, an inhibitor of histone deacetylase, showed

an increase in the expression of phosphorylase in muscle fibers of treated McArdle sheep [180].

(d) *Gene therapy*: Gene therapy strategies have also been evaluated either *in vitro*, i.e. in human and sheep myoblast cultures deficient for GP-MM [181], or *in vivo*, i.e. in the ovine model of McArdle disease [182]. Recombinant adenoviral vector expressing the wild-type (WT) human GP-MM cDNA under the control of the Rous sarcoma virus promoter have been used to restore the human GP-MM in human and sheep myoblast cultures from McArdle patients and the ovine model of McArdle disease, respectively [181]. In contrast, after the treatment of the ovine model of McArdle disease via intramuscular injection into semitendinosus muscle with a modified adenovirus 5 (AdV5) and adeno-associated virus 2 (AAV2) vectors containing GP-MM expression cassettes, functional GP-MM expression was only observed in the regenerating fibres surrounding the injection site and some expression of the other two isoforms of glycogen phosphorylase (brain and liver isoforms) was also observed [182].

(e) *Nutritional interventions before exercise*: To ensure that sufficient blood glucose is constantly made available to the working muscle during daytime and thus protecting, the muscle from rhabdomyolysis risk, some interventions can be made:

- adopting a diet with a high proportion (65%) of complex carbohydrates (such as found in vegetables, fruits, cereals, bread, pasta, and rice) and a low proportion (20%) of fat [183].

- ingesting simple carbohydrates: in adults, 30-40 g of glucose, fructose, or sucrose approximately 5 minutes before engaging in strenuous exercise such as a brisk walking or hiking [184, 185], which translates to 400-500 ml of most commercially available sport drinks, or higher doses, 75 g of sucrose 30-40 min pre-

exercise [105]; and in children, 20 g of glucose before any vigorous exercise bout, for instance, physical education classes [186].
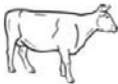
(f) _Exercise_: Some studies have shown that supervised light-moderate intensity aerobic [48, 93, 97, 184, 186, 187] or resistance exercise training can improve the functional capacity and/or clinical course of the disease [188, 189].

1.6.6 Animal models

Two naturally occurring animal models of McArdle disease have been reported in the literature (Table 5). The first described animal was a bovine model that was identified in a Charolais cattle of New Zeland [190]. It presented with continued recumbency, severe rhabdomyolysis and electrolyte imbalance after forced exercise. The disease phenotype is caused by a C-to-T change at codon 489, which generates an arginine-to-tryptophan substitution [191].

The second model was identified in a Merino sheep flock of Western Australia [192]. It showed intolerance exercise, lack of GP-MM and excess of muscle glycogen. The disease is caused by a single base change at the 3' splice site of intron 19 in _Pygm_$_{oa}$, which activates a cryptic splice site within exon 20. This results in the disruption of the reading frame and in a PTC that truncates the last 31 amino acids of the protein.

Both animal models mimics the human disease phenotype but they present obvious difficulties in their manipulation and reproduction, and they are difficult to share among different research groups (advantages and disadvantages of the different animal models are summarized in Table 5). These limitations prompted our laboratory to develop a knock-in mouse homozygous for the most common pathogenic mutation among Caucasian population, _p.R50X_ [193]. Biochemical and

Animal Model	Phenotype	Advantages	Disadvantages	Therapies evaluated
Bovine (Mutation: *p.W489R*)	Exercise intolerance Rhabdomyolysis Blood analysis abnormalities: -↑ K⁺ -↑ Phosphate -↓ Ca²⁺ -↑ CK Histopathology: -No staining for GP-MM activity -Subsarcolemmal glycogen vacuoles -Some central nuclei -PAS staining *within normal limits*	Spontaneous animal model Muscle fiber type composition (I and IIA fibers) similar to humans Mitochondria density volume per fiber volume (2-5%) similar to humans	Difficult to obtain animals for experimentation owing to: -Long gestation period (270-295 days) -Only 1 calf per birth. -Difficult to manipulate (average cow weight ~450 kg) -Difficult to share with other research groups -High maintenance costs	None
Ovine (Mutation: truncation of the last 31 amino acid of the protein)	Exercise intolerance Histopathology: -No staining for GP-MM activity -PAS staining with high glycogen content	Spontaneous animal model Animal size and muscle mass comparable to humans Musle fiber type composition (I and IIA fibers) similar to humans Mitochondria density volume per fiber volume (2-5%) similar to humans	Difficult to obtain animals for experimentation owing to: -Long gestation period (147 days) -1-3 lambs per birth -Difficult to manipulate (sheep weight 45-100 kg) -Difficult to share with other research groups -High maintenance costs	Gene therapy: AAV2/AdV5-*Pygm* vectors Pharmacologic: Valproic acid Notexin
Murine (Mutation: *p.R50X*)	Exercise intolerance ↑ blood CK Myoglobinuria Histopathology: -No staining for GP-MM activity -PAS staining with high glycogen content Molecular studies: -Lack of GP-MM protein -Lack of GP-MM activity -Very low *Pygm* mRNA (10% of normal levels)	Presents with a complete McArdle disease phenotype Presents the most common mutation in Caucasian McArdle patients (p.R50X) and allows to evaluate different therapies (*e.g.* read through compounds) Easy to manipulate (mouse weight 20-45 g) Easy to share with other research groups Low maintenance costs Easy to obtain animals for experimentation: -Short gestation period (19-21 days) -High litter size (7-12 pups per litter)	Genetically modified animal model: 34 bp LoxP sequence is present in intron 1 Prenatal mortality in homozygous mice 25% mortality in homozygous mice after weaning and before 3 months of age Higher glycogen accumulation in homozygous mice muscles than in human patients Different muscle fiber type composition (predominantly IIX and IIB fibers) compared to humans (predominantly I and IIA fibers) Different mitochondria density volume per fiber volume (30%) compared to humans (2-5%)	None

Table 5: Main features of the different animal models of McArdle disease. CK,creatine kinase; GP-MM, muscle glycogen phosphorylase isoform; PAS, Periodic acid-Schiff [48].

molecular analyses revealed that mice homozygous for this mutation have the same muscle phenotype as McArdle patients, that is, complete absence of GP-MM and activity, very low transcript levels of *Pygm* (~90% lower compared with WT mice), as well as, massive glycogen accumulation. Further characterization also showed mice exercise intolerance as evidenced by their very poor performance in wire grip and treadmill tests, elevated serum CK-activity levels and myoglobinuria.

Thus, this knock-in mouse model might represent a useful tool in helping understanding the pathophysiology of this disorder, as well as, being an adequate model for evaluating potential therapies.

2. Aims

Aims

This PhD thesis will focus on the characterization of the murine model of McArdle disease and to achieve a better understanding of the pathophysiologic pathways associated to GP-MM deficiency, as well as, seeking and assessing new therapeutic approaches.

Specifically, the objectives of this PhD thesis can be defined as:

1.- Phenotypic characterization of the murine model of McArdle disease, including:

a.- To analyze whether the genes coding the main regulatory enzymes associated to glycogen metabolism are differently expressed in McArdle mice and whether their expression vary among metabolically different muscle types

b.- To analyze whether glycogen synthesis is down-regulated in the muscles of McArdle mice to prevent deleterious glycogen accumulation and muscle glycogen unavailability

c.- To analyze whether complete or even partial glycogen phosphorylase deficiency has important muscle phenotype consequences in mice, including a marked impairment in maximal endurance capacity

d.- To analyze whether insulin-stimulated glycogen pathway is down-regulated in the muscles of McArdle mice

2.- Seeking and assessing new therapeutic approaches for McArdle disease, including:

a.- To test whether VPA is able to up-regulate non-GP-MM isoforms in an *in vitro* model of McArde disease

b.- To test whether an adeno-associated virus 2/8 (AAV2/8) containing human muscle glycogen phosphorylase expression cassette under the muscle creatine kinase (MCK) promoter (CK7) is able to restore muscle glycogen phosphorylase isoform in the murine model of McArdle disease

3.- To evaluate whether GP-MM expression analysis by flux cytometry in white blood cells (WBC) can be used as a new and less invasive diagnostic method for McArdle disease

3. Results

3. Results

This is a PhD thesis written by publications. It is composed of five articles, and the results are:

3.1 Article 1:

Brull A*, de Luna N*, Blanco-Grau A, Lucia A, Martin MA, Arenas J, Martí R, Andreu AL, Pinós T. *Phenotype consequences of myophosphorylase dysfunction: Insights from the McArdle mouse model*. J Physiol (2015) 593(12);2693–2706

3.2 Article 2:

De Luna N*, Brull A* , Guiu JM, Lucia A, Martin MA, Arenas J, Martí R, Andreu AL, Pinós T. *Sodium valproate increases glycogen phosphorylase brain isoform: looking for a compensation mechanism in McArdle disease using a mouse primary skeletal-muscle culture in vitro*. Dis Model Mech (2015) 8(5):467-72

3.3 Article 3:

Nogales-Gadea G*, Brull A*, Santalla A, Andreu AL, Arenas J, Martín MA, Lucia A, de Luna N, Pinós T. *McArdle Disease: Update of reported mutations and polymorphisms in the Pygm gene*. Human Mutation, 2015. 36(7): p. 669-78.

3.4 Article 4:

Nogales-Gadea G, Santalla A, Brull A, de Luna N, Lucía A, Pinós T. *The pathogenomics of McArdle disease-genes, enzymes, models, and therapeutic implications*. Journal of inherited metabolic disease, 2014. 38(2): p. 221-30.

3.5 Article 5:

Santalla A, Nogales-Gadea G, Ortenblad N, Brull A, de Luna N, Pinós T, Lucia A. *McArdle Disease: A unique study model in Sports Medicine*. Sports Medicine, 2014. 44(11): p. 1531-44.

3.6 Article 6:

Pinós T, Lucia A, Arenas J, Brull A, Andreu AL, Martin MA, Nogales-Gadea G. *Minimal symptoms in McArdle disease: A real PYGM genotype effect?*. Muscle Nerve, 2015. Epub ahead of print.

*Both authors contributed equally to this work

3.1 Article 1

Results

Phenotype consequences of myophosphorylase dysfunction: insights from the McArdle mouse model

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Key points

- This is the first study to analyse the effect of muscle glycogen phosphorylase depletion in metabolically different muscle types.
- In McArdle mice, muscle glycogen phosphorylase is absent in both oxidative and glycolytic muscles.
- In McArdle mice, the glycogen debranching enzyme (catabolic) is increased in oxidative muscles, whereas the glycogen branching enzyme (anabolic) is increased in glycolytic muscles.
- In McArdle mice, total glycogen synthase is decreased in both oxidative and glycolytic muscles, whereas the phosphorylated inactive form of the enzyme is increased in both oxidative and glycolytic enzymes.
- In McArdle mice, glycogen content is higher in glycolytic muscles than in oxidative muscles. Additionally, in all muscles analysed, the glycogen content is higher in males than in females.
- The maximal endurance capacity of the McArdle mice is significantly lower compared to heterozygous and wild-type mice.

Abstract   McArdle disease, caused by inherited deficiency of the enzyme muscle glycogen phosphorylase (GP-MM), is arguably the paradigm of exercise intolerance. The recent knock-in (p.*R50X*/p.*R50X*) mouse disease model allows an investigation of the phenotypic consequences of muscle glycogen unavailability and the physiopathology of exercise intolerance. We analysed, in 2-month-old mice [wild-type (*wt/wt*), heterozygous (*p.R50X/wt*) and *p.R50X/p.R50X*)], maximal endurance exercise capacity and the molecular consequences of an absence of GP-MM in the main glycogen metabolism regulatory enzymes: glycogen synthase, glycogen branching enzyme and glycogen debranching enzyme, as well as glycogen content in slow-twitch (*soleus*), intermediate (*gastrocnemius*) and glycolytic/fast-twitch (*extensor digitorum longus*; *EDL*) muscles. Compared with *wt/wt*, exercise capacity (measured in a treadmill test) was impaired in *p.R50X/p.R50X* (~48%) and *p.R50X/wt* mice (~18%). *p.R50X/p.R50X* mice showed an absence of GP-MM in the three muscles. GP-MM was reduced in *p.R50X/wt* mice, especially in the *soleus*, suggesting that the function of 'slow-twitch' muscles is less dependent on glycogen catabolism. *p.R50X/p.R50X* mice showed increased glycogen debranching enzyme in the *soleus*, increased glycogen branching enzyme in the *gastrocnemius* and *EDL*, as well as reduced levels of mucle glycogen synthase protein in the three muscles (mean ~70%), reflecting a protective mechanism for preventing deleterious glycogen accumulation. Additionally, glycogen content was highest in the *EDL* of *p.R50X/p.R50X*

A. Brull and N. de Luna have contributed equally to this work.

Results

mice. Amongst other findings, the present study shows that the expression of the main muscle glycogen regulatory enzymes differs depending on the muscle phenotype (slow- *vs.* fast-twitch) and that even partial GP-MM deficiency affects maximal endurance capacity. Our knock-in model might help to provide insights into the importance of glycogen on muscle function.

Abbreviations   CK, creatine kinase; *EDL, extensor digitorum longus*; GBE, glycogen branching enzyme; GDE, glycogen debranching enzyme; GP-MM, muscle glycogen phosphorylase; GS, glycogen synthase; GSD V, glycogen storage disease type V; GS-M, muscle glycogen synthase; PAS, periodic acid–Schiff; pGS-M, muscle phospho$_{SER640}$ glycogen synthase; *Pygb*, brain glycogen phosphorylase gene; *Pygl*, liver glycogen phosphorylase gene; *Pygm*, muscle glycogen phosphorylase gene.

Introduction

McArdle disease or glycogenosis type V [glycogen storage disease type V (GSD V) myophosphorylase deficiency; OMIM® database number 232600; http://www.omim.org] is an inborn disorder of skeletal-muscle carbohydrate metabolism (McArdle, 1951) caused by pathogenic mutations in both alleles of the *PYGM* gene, encoding the muscle isoform of *glycogen phosphorylase* (GP-MM; also known as *myophosphorylase*) (Lucia *et al.* 2008). Because GP-MM catalyses and regulates the breakdown of glycogen into glucose 1-phosphate in muscle fibres, patients are unable to obtain energy from their muscle glycogen stores (Dimauro *et al.* 2002). Yet endogenous muscle glycogen is a primary fuel source during exercise, with low glycogen availability impairing muscle function and basic cellular events (Ortenblad *et al.* 2013). Thus, McArdle disease provides a efficient model for studying the phenotypic consequences of muscle glycogen unavailability (Santalla *et al.* 2014). In terms of exercise capacity, patients typically present with 'exercise intolerance', in the form of acute crises of early fatigue and muscle stiffness and contractures, sometimes accompanied by marked muscle damage or rhabdomyolysis, as indicated by the efflux of intramuscle proteins to the bloodstream, such as creatine kinase (CK) (Lucia *et al.* 2008).

By contrast to clinical research, naturally occurring or laboratory-generated animal disease models allow more mechanistic studies to be performed that provide insights into the pathophysiology of a disorder. The first reported naturally occurring animal model of McArdle disease was a Charolais calf, showing continued recumbency, severe rhabdomyolysis and electrolyte imbalance after forced exercise (Angelos *et al.* 1995; Tsujino *et al.* 1996). The second model was identified in the Merino sheep flock of Western Australia (Tan *et al.* 1997); these animals were devoid of GP-MM and exhibited exercise intolerance and an excess of muscle glycogen (Tan *et al.* 1997). Although the discovery of the ovine and bovine animal models

represented a step forward to clarying the molecular characteristics of the disease (Tan *et al.* 1997), both models presented obvious difficulties in their manipulation and were difficult to share among different research groups.

Such limitations prompted us to develop a 'knock-in' mouse homozygous for the most common pathogenic mutation causing McArdle disease among Caucasians (i.e. the *PYGM p.R50X* mutation) (Nogales-Gadea *et al.* 2012*b*). Biochemical and molecular analyses of the *gastrocnemius* muscle extracts from the 2-month-old *Pygm p.R50X/p.R50X* mice revealed a complete absence of GP-MM protein and a clear McArdle-like phenotype, including basal 'hyperCKaemia' (Nogales-Gadea *et al.* 2012*b*). Further analyses were missing that might help to unravel the molecular and functional consequences of GP-MM deficiency and subsequent glycogen unavailability, as well as the physiopathology of exercise intolerance. Accordingly, in the present study, we analysed exercise capacity in young adult (2-month-old) healthy mice [wild type (*wt/wt*)), heterozygous (*pygm p.R50X/wt*) and homozygous (*pygm p.R50X/p.R50X*)], as well as the molecular consequences of an absence of GP-MM (particularly with respect to the main regulatory enzymes of glycogen metabolism) in three different types of skeletal muscles in terms of predominant metabolic phenotype: more oxidative/slow-twitch type (*soleus*), intermediate (*gastrocnemius*) and more glycolytic/fast-twitch (*extensor digitorum longus*; *EDL*). We also analysed the possible role of the sex of the mice in the outcomes. Our main hypotheses were: (i) the main regulatory enzymes are expressed differently depending on the muscle (slow- *vs.* fast-twitch) phenotype; (ii) glycogen synthesis is down-regulated in the muscles of McArdle mice to prevent deleterious glycogen accumulation and muscle glycogen unavailability; and (iii) complete (*p.R50X/p.R50X*) or only partial (*p.R50X/WT*) GP-MM deficiency has important muscle phenotype consequences in mammals, including a marked impairment in maximal endurance capacity.

Methods

Ethical approval

All experimental procedures were approved by the Vall d'Hebron Institutional Review Board (protocol number 13/04 CEEA; 35/04/08) and were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 1 2 3) and Spanish laws (32/2007 and R.D. 1201/2005).

Animals

Previously developed *p.R50X/p.R50X* knock-in McArdle mice presented a mixed 129/sv and C57Bl/6J genetic background as a result of the implantation of embryonic stem cells (with a 129/sv nuclear background) carrying the *p.R50X* mutation into blastocysts (with a C57Bl/6J nuclear background) (Nogales-Gadea *et al.* 2012*b*). To reduce the number of 129/sv derived genes, heterozygous (*p.R50X/wt*) mice were backcrossed during 10 generations with normal (*wt/wt*) C57Bl/6J mice. All mice were killed by cervical dislocation immediately before muscle removal.

Exercise capacity

Mice ($n = 35$; 18 males) were exercised on an enclosed treadmill (Harvard Apparatus, Panlab, Barcelona, Spain) supplied with an electrified grid at the rear of the belt to provide motivation (shocks of 0.2 mA; 1 Hz, 200 ms). After a warm-up period (5 min at 5 cm s^{-1}), the treadmill speed was increased to 15 cm s^{-1} for 5 min and, subsequently, by 5 cm s^{-1} every 5 min until exhaustion. This protocol was identical to that reported previously (Nogales-Gadea *et al.* 2012*b*), with the exception of treadmill inclination, which was 0% in the present study (compared to 25% in the previous study). Mice were defined as exhausted when they spent more than 5 s (continuous) on the electric grid and were unable to continue running at the next speed increase (Fiuza-Luces *et al.* 2013). We determined the maximum distance completed by each mouse as an index of maximum endurance capacity (Hoydal *et al.* 2007).

Blood variables

Prior to blood extraction, mice were immobilized with a Harvard Apparatus Rodent Restrainer (Harvard Apparatus, Holliston, MA, USA). Subsequently, blood samples were collected from the saphenous vein and diluted one-third with phosphate-buffered saline. The diluted blood was centrifuged at 3000 *g* for 5 min at 4°C and the supernatant (plasma) was collected to determine CK activity and glucose, ammonia and lactate concentration with a COBAS 6000 analyser (Roche Diagnostics, Mannheim, Germany), as well as plasma free fatty acid (FFA) levels with a specific assay kit (Biovision, Inc., Milpitas, CA, USA) in accordance with the manufacturer's instructions.

mRNA analysis

RNA samples were obtained from *gastrocnemius*, *soleus* and *EDL* muscles as described previously (Nogales-Gadea *et al.* 2012*b*) using Trizol in accordance with the manufacturer's instructions (Invitrogen, Carlsbad, USA). To eliminate any traces of DNA, RNA was treated with the *DNAse I*, amplification grade (Invitrogen). cDNA was synthesized from 500 ng of muscle total RNA using the high-capacity cDNA archive kit (Applied Biosystems, Foster City, CA, USA). Real-time PCR (quantitative PCR) was performed using a 7500 real-time PCR System (Applied Biosystems), with the TaqMan fluorogenic probes: (i) glycogen phosphorylase, muscle isoform (*Pygm*) gene (Mm00478582_m1); (ii) glycogen phosphorylase, brain isoform (*Pygb*) gene (Mm00464080_m1); and (iii) glycogen phosphorylase, liver isoform (*Pygl*) gene (Mm00500078_m1). The results were normalized to peptidylprolyl isomerase A (cyclophilin A, *Ppia*) gene mRNA levels (probe Mm02342430_g1).

Western blot analysis

Samples from *gastrocnemius*, *soleus* and *EDL* muscles were homogenized in 20 volumes (1 ml per 50 mg of tissue) of cold homogenization buffer (40 mM β-glycerophosphate, 40 mM NaF, 10 mM EDTA and 20 mM ß-mercaptoethanol, pH 6.8) and centrifuged at 10,000 *g* for 10 min at 4°C. Proteins (20 μg) were resolved on 8% SDS polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Immun-Blot® PVDF membrane; Bio-Rad, Hercules, CA, USA). Primary antibodies against GP-MM (generated in the laboratory of Professor Martinuzzi, K.S, Lausanne, Switzerland), muscle phospho$_{SER15}$ GP-MM (pGP-MM) (kindly provided by Dr K. Sakamoto, A.M, Conegliano, Italy), muscle glycogen synthase (GS-M) (ref. #3893; Cell Signaling Technology, Inc., Danvers, MA, USA), muscle phospho$_{SER640}$ glycogen synthase (pGS-M) (ref. GTX22479; GeneTex, Inc., Irvine, CA, USA), glycogen branching enzyme (GBE) (ref. ab103133; Abcam, Cambridge, UK) and glycogen debranching enzyme (GDE) (ref. TA310177; OriGene, Rockville, MD, USA) were used. Primary antibody against glyceraldehyde-3-phosphate dehydrogenase protein (Ambion, Austin, TX, USA) was used to normalize protein levels. The horseradish peroxidase-conjugated secondary antibodies included rabbit anti-mouse (Dako, Glostrup, Denmark), goat anti-rabbit (Jackson Laboratories, Baltimore Pike, PA, USA) and donkey

Results

anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Membranes were developed with Immobilon Western Chemiluminiscent HRP Substrate (EMD Millipore, Billerica, MA, USA). Images were obtained with Fujifilm LAS 3000 imager (R&D Systems, Minneapolis, MN, USA) and quantified with Image J, version 1.37 (NIH, Bethesda, MD, USA).

Measurement of glycogen content

The glycogen content in the muscles of *wt/wt* and *p.R50Xp.R50X* mice was measured as described previously (Lo *et al.* 1970). Of note, glycogen was not measured in *p.R50X/wt* mice because preliminary data from our laboratory from the *quadriceps*, *biceps* and *gastrocnemius* muscles of six 2-month-old mice (three of each sex) showed almost identical glycogen content in *p.R50X/wt* and *wt/wt* mice, which is also consistent with our previous study (Nogales-Gadea *et al.* 2012*b*). Briefly, ∼150 mg of tissue was boiled for 30 min with 30% KOH and, subsequently, 1.2 volumes of 95% ethanol were added to precipitate glycogen. After a centrifugation step (25 min at 840 *g*), the glycogen pellet was resuspended in 0.3 ml of water. Next, 0.1 ml of 5% phenol was added to 0.1 ml of sample and treated with 0.5 ml of H_2SO_4 (to hydrolyse glycogen to glucose). The mixture was allowed to stand for 30 min at room temperature and the glucose released was measured spectrophotometrically at 490 nm. A standard curve made with glycogen purified from rabbit liver (Sigma-Aldrich, St Louis, MO, USA), ranging from 0.1 to 0.8 mg mL^{-1}, was processed in parallel. The results were expressed as mg glycogen (g tissue)$^{-1}$.

Histochemical analysis

Gastrocnemius, *soleus* and *EDL* muscles were fixed in cold methyl butane for 30 s and samples were maintained in liquid nitrogen until analysis. To determine muscle morphology, 8 μm sections were stained with haematoxylin & eosin and slides were first incubated 5 min in haematoxylin (Merck-Millipore, Billerica, MA, USA); subsequently, after two washes with 1% hydrochloric acid and ammonia water (ammonium hydroxide), slides were incubated with 2% eosin. We also performed Gomory's trichrome staining to investigate the possible increment of connective tissue or mitochondrial accumulation among other histological defects. Glycogen content was analysed with periodic acid–Schiff (PAS) staining by sequentially incubating the sections with: periodic acid (0.5%) for 5 min, water wash, Schiff's solution for 1 min, water for 1 min, haematoxylin for 1 min, a water wash, alcohol-xylol dehydration and DPX mounting. Succinate dehydrogenase staining was performed to measure oxidative muscle fibres, which

stained darker using this stain. Briefly, muscle sections were incubated in 2.7% sodium succinate and 0.2 M phosphate buffer (pH 7.6) at 37°C for 30 min; after water washes, sections were mounted in an hydrophilic mount medium (Aquatex; Merck-Millipore). For GP-MM activity staining, skeletal muscle sections were incubated for 45 min with a solution containing 1% glucose 1-phosphate, 0.2% AMP and 0.02% glycogen in 0.1 M sodium acetate buffer (pH 5.6). Sections were washed with water, Lugol's iodine was applied for 3 min and samples were mounted with Aquatex. Stained sections were analysed and images were obtained with an inverted microscope (IX 71 Inverted Microscope; Olympus Corp., Tokyo, Japan).

Statistical analysis

All statistical analyses were performed using the IBMS SPSS, version 20.0 (IBM Corp., Armonk, NY, USA) with α set at 0.05 and data are reported as the mean ± SD. We used two-factor [*Pygm* genotype (*p.R50X/p.R50X*, *p.R50X/wt*, *wt/wt*), sex (male, female)] ANOVA for body mass and exercise capacity, and a three-factor [(*Pygm* genotype, sex, muscle type (*soleus*, *gastrocnemius*, *EDL*)] ANOVA was applied for the different muscle biochemical variables. The Bonferroni test was applied *post hoc*. For statistical purposes, undetectable values were considered as zero.

Results

Body mass

No differences existed in body mass across *Pygm* genotypes ($P = 0.517$), except for an obvious sex effect ($P < 0.001$) with female mice showing lower body mass than their age-matched male referents (data not shown) (Table 1).

Exercise capacity

The maximal endurance capacity (expressed as total distance run) of the homozygous (*p.R50X/p.R50X*) mice was significantly lower compared to heterozygous (p.*R50X/wt*) and normal (*wt/wt*) mice (∼48% and ∼37%, respectively, $P < 0.001$) and the capacity of *p.R50X/wt* mice was also lower compared to the *wt/wt* mice (∼18%, $P = 0.025$). No sex effect was found ($P = 0.365$) (Table 1).

Blood variables

Overall, there was a significant *Pygm* genotype effect for the blood variables investigated but such an effect was not noted for sex (for a detailed presentation of the results and of all *P* values, see Table 1). Briefly, the homozygous mice showed overall lower reliance on glycolytic

Results

Table 1. Body mass, exercise capacity and basal blood variables (mean ± SD)

	Pygm genotype			Post hoc comparisons			Main effects		
	p.R50X/p.R50X (n = 9) (five males)	p.R50X/wt (n = 15) (seven males)	wt/wt (n = 11) (six males)	p.R50X/p.R50X vs. p.R50X/wt	p.R50X/R50X vs. wt/wt	p.R50X/wt vs. wt/wt	Sex	Pygm genotype	Interaction sex × genotype
Body mass (g)	24.2 ± 4.4	24.3 ± 3.5	25.0 ± 3.1	$P = 0.999$	$P = 0.901$	$P = 0.907$	**$P < 0.001$**	$P = 0.517$	$P = 0.717$
Exercise capacity (m)	376.3 ± 81.3	600.1 ± 107.1	729.6 ± 120.5	**$P < 0.001$**	**$P < 0.001$**	**$P = 0.025$**	$P = 0.365$	**$P < 0.001$**	$P = 0.479$
[Glucose] (mg dl^{-1})	110.0 ± 22.2	153.7 ± 23.8	147.9 ± 30.1	**$P = 0.004$**	**$P = 0.013$**	$P = 0.871$	$P = 0.698$	**$P < 0.001$**	$P = 0.932$
[Lactate] (mM l^{-1})	1.9 ± 0.2	4.2 ± 0.3	4.3 ± 0.6	**$P < 0.001$**	**$P < 0.001$**	$P = 1.000$	$P = 0.877$	**$P = 0.001$**	$P = 0.395$
[Ammonia] (μM l^{-1})	497.7 ± 136.1	235.0 ± 90.7	188.4 ± 78.0	**$P < 0.001$**	**$P < 0.001$**	$P = 0.944$	$P = 0.474$	**$P = 0.001$**	**$P = 0.028$**
[FFA] (mM l^{-1})	0.77 ± 0.14	0.56 ± 0.18	0.68 ± 0.19	$P = 0.101$	$P = 0.766$	$P = 0.190$	$P = 0.696$	$P = 0.073$	$P = 0.213$
Serum CK activity (U L^{-1})	1179.6 ± 660.4	818.3 ± 1068.0	108.0 ± 57.4	$P = 0.701$	$P = 0.090$	$P = 0.241$	$P = 0.178$	$P = 0.093$	$P = 0.142$

CK, creatine kinase; FFA, free fatty acids. Significant P values are shown in bold.

metabolism (i.e. lower lactate levels) and a trend towards an increased use of FFAs, together with higher ammonia levels, compared to both *p.R50X/wt* and *wt/wt* mice, although essentially no differences were observed between the latter two groups.

Muscle phenotype (enzymes of glycogen metabolism) of healthy (*wt/wt*) mice

We first studied the protein levels of glycogen catabolic (GP-MM and GDE) and anabolic enzymes (GS-M and GBE) in muscles with a predominantly slow-twitch (*soleus*), intermediate (*gastrocnemius*) and fast-twitch phenotype (*EDL*) in healthy (*wt/wt*) mice of both sexes (Fig. 1). For glycogen catabolic enzymes (along with GP-MM, GDE protein is also involved in glycogen breakdown transferring the last four glucose units from a glycogen branch to a nearby branch), we only found a significant muscle effect for GP-MM ($P = 0.001$), with the muscle with a more fast-twitch, glycolytic phenotype (i.e. *EDL*) showing the highest enzyme levels (Fig. 1*B*). We also found a significant sex effect for GP-MM only ($P = 0.006$; GDE: $P = 0.597$) and no muscle × sex effect (GP-MM: $P = 0.227$; GDE: $P = 0.266$) or no significant *post hoc* differences between sexes for any of the two enzymes.

Concerning glycogen anabolic enzymes (GS-M and GBE), we also found a significant muscle effect ($P < 0.001$ for both enzymes), with a pattern opposite to that shown by glycogen catabolic enzymes, in that the highest protein levels were shown in the most oxidative muscle (*soleus*) (Fig. 1*C*). A significant sex effect was found for the two enzymes (GS-M: $P < 0.001$; GBE: $P = 0.030$) and a muscle × sex effect was found for GBE ($P = 0.043$) but not for GS-M ($P = 0.285$).

GP-MM (protein and transcript) levels in muscle: effect of *Pygm* genotype, muscle type and sex

We found a significant *Pygm* genotype and muscle effect (both $P < 0.001$) but no sex effect ($P = 0.813$) for GP-MM (Fig. 2). Thus, *p.R50X/p.R50X* mice showed a total absence of GP-MM protein in the three muscles analysed (*soleus*, *gastrocnemius*, *EDL*) and the levels of this enzyme were also significantly lower in heterozygous mice compared to *wt/wt* mice in the three muscles (*soleus*, ~60%; *gastrocnemius* and *EDL* muscles, both ~35%) (Fig. 2*B*). *Post hoc* analysis for the *p.R50X/wt* genotype showed that, in heterozygous mice, GP-MM levels were lowest in the most slow-twitch oxidative muscle (*soleus*).

Similar results were found for *Pygm* transcript levels (i.e. $P < 0.001$ for both the genotype and muscle effect and $P = 0.469$ for the sex effect) (Fig. 2*C*). The mean values of *p.R50X/p.R50X* mice were ~90% lower compared to those found in *wt/wt* mice in the three muscles, and the *Pygm*

mRNA levels were also lower in *p.R50X/wt* mice compared to the *wt/wt* controls in the three muscles (*soleus*, ~55%; *EDL*, ~44%; *gastrocnemius*, ~35%). *Pygb* and *Pygl* mRNA levels were also determined in the three muscles and were negligible (all individual values <5% of normal values) irrespective of *Pygm* genotype (data not shown).

Effect of *Pygm* genotype on muscle phenotypes (enzymes of glycogen metabolism)

We also investigated whether the expression of other enzymes directly involved in glycogen degradation (GDE) or synthesis (GBE and GS-MM) was altered in homozygous mice compared to the rest of the mice. We found a significant genotype, muscle, sex, genotype × muscle and genotype × sex effect (all $P < 0.001$) for GDE (Fig. 3). In *post hoc* analysis, GDE levels were highest in homozygous mice in the *soleus* but not in the other two muscles, where no significant differences were found between genotypes (Fig. 3*B*). No differences were found between sexes for any of the *Pygm* genotypes. By contrast, we found a significant genotype, muscle and genotype × muscle effect (all $P < 0.001$) for GBE but no sex effect ($P = 0.630$) (Fig. 3*C*). In *post hoc* analyses, significant differences between genotypes were found for both the *gastrocnemius* and *EDL* muscles, where enzyme levels were higher in homozygous mice compared to the other two groups ($P < 0.001$).
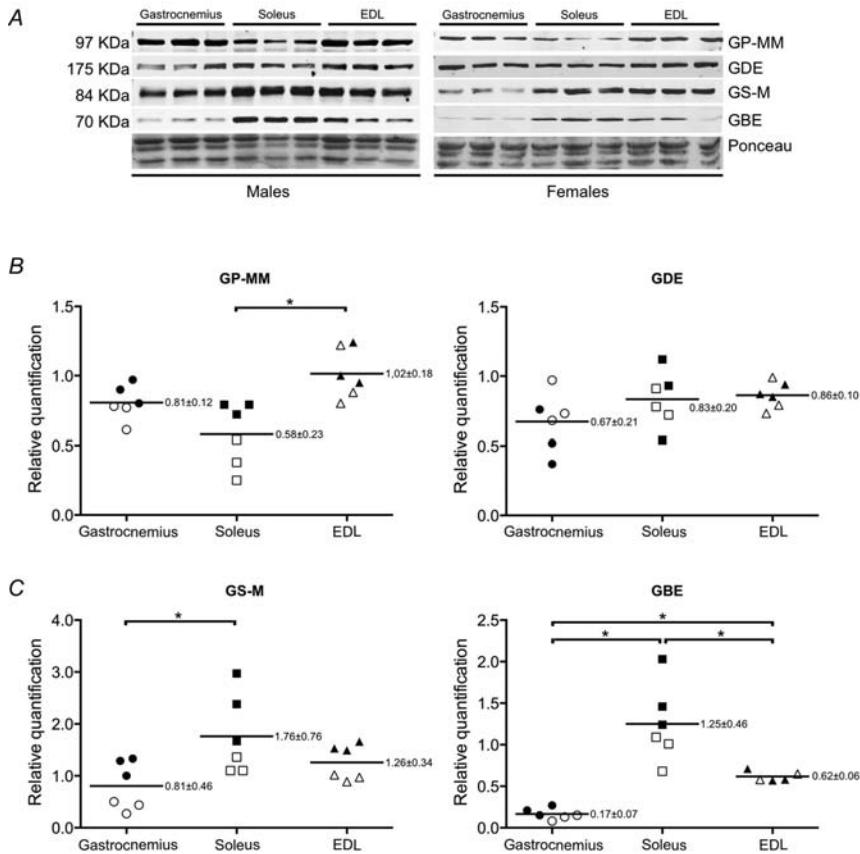
Figure 1. Levels of enzymes of glycogen metabolism enzymes in the muscles of healthy mice
A, western blot analyses. *B* and *C*, levels of the two catabolic (GP-MM and GDE) and anabolic enzymes (GS-M and GDE), respectively. Data are shown as the mean ± SD and individual values, with males indicated by black coloured squares, dots or triangles. Significant *post hoc* differences are indicated in parenthesis (*$P < 0.01$).

We found a significant genotype, muscle and genotype × muscle effect (all $P < 0.001$) for GS-M but no sex effect ($P = 0.249$) (Fig. 4). In *post hoc* analyses, significant differences between genotypes were found for the three muscles, with GS-M levels being lower in homozygous mice compared to the other two groups ($P < 0.001$) (Fig. 4*B*). We found similar results for the inactive, phosphorylated form of the abovementioned enzyme (pGS-M), in that a significant genotype and muscle effect (both $P < 0.001$) existed, with no genotype × muscle effect ($P = 0.532$) or sex effect ($P = 0.271$); in *post hoc* analyses, significant differences between genotypes were found for the three muscles, with enzyme levels being higher in homozygous mice compared to the other two groups ($P < 0.001$) (Fig. 4*C*).

Glycogen content

We found a significant genotype, muscle, sex, genotype × muscle and genotype × sex effect (all $P < 0.01$)

for muscle glycogen content (Fig. 5). Compared with *wt/wt* mice, the content of glycogen in *p.R50X/p.R50X* mice was much higher: ∼86-fold higher in the *EDL*, ∼46-fold higher in the *gastrocnemius* and ∼29-fold higher in the *soleus*. In *p.R50X/p.R50X* mice, the glycogen content in the *EDL* was significantly higher than in the other two muscles ($P < 0.05$). This was in contrast to normal mice, which showed the highest glycogen content in *gastrocnemius* muscle. In these mice, significant differences were found between the *soleus* and the other two muscles ($P < 0.05$ *vs. EDL* and $P < 0.01$ *vs. gastrocnemius*). Finally, glycogen content was higher in *p.R50X/p.R50X* males ($P < 0.05$) compared to *wt/wt* females, although no sex differences were found in the *wt/wt* group.

Histochemical analysis in *p.R50X/p.R50X* mice muscles

In all of the stained sections, we observed a high number of vacuoles in muscle fibres (Figs 6*A*–*D*). Vacuoles were positive for PAS staining (Fig. 6*C*). Notably, the
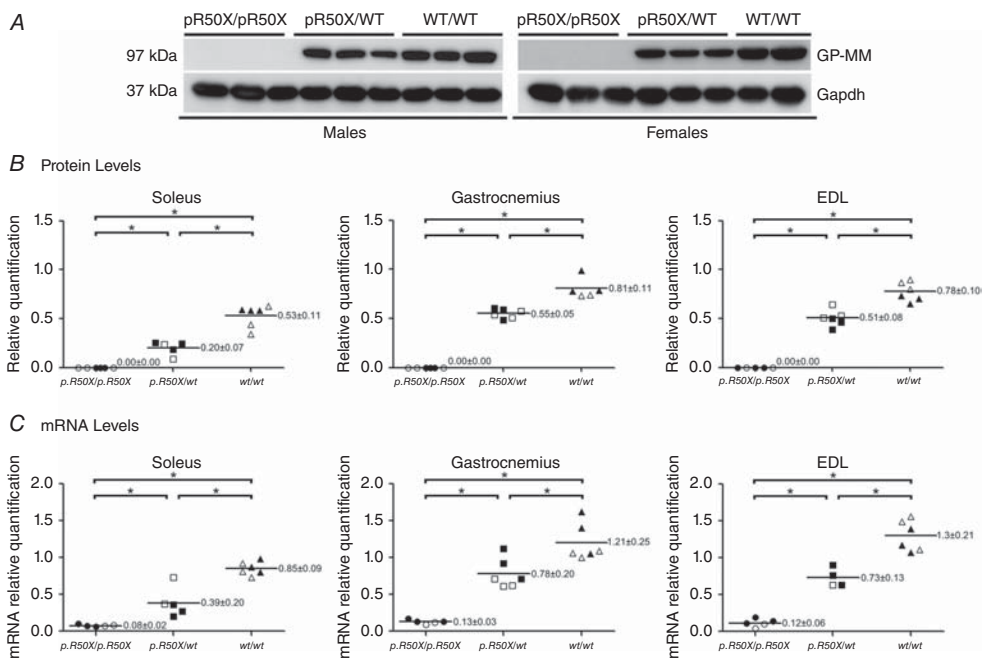
Figure 2. Comparison of protein and transcript levels of GP-MM according to muscle type and *Pygm* genotype
A, western blot analyses in *gastrocnemius* muscle. *B* and *C*, protein and transcript levels, respectively, in muscles. Data are shown as the mean ± SD and individual values, with males indicated by black coloured squares, dots or triangles. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Significant *post hoc* differences are indicated in parenthesis (*$P < 0.01$). In heterozygous mice, GP-MM levels were significantly lower in the *soleus* muscle compared to the *gastrocnemius* and *EDL* (both $P < 0.001$).

polysaccharide positive vacuoles were preferentially located in the oxidative muscle fibres (the latter correspond to the darker fibres in Fig. 6*D*). No histological differences were observed between *soleus*, *gastrocnemius* and *EDL* because all three muscles presented glycogen vacuoles and an increment of PAS positive fibres. We found no differences between sexes.

Discussion

We recently reported the generation and characterization of a McArdle knock-in mouse model carrying the *p.R50X* mutation in both *Pygm* gene copies that faithfully reproduced the McArdle disease phenotype observed in patients (Nogales-Gadea *et al.* 2012*b*). However, only the *gastrocnemius* muscle was sampled and the question of whether muscles with different contractile/metabolic profiles are distinctly affected by the absence of GP-MM

has not yet been evaluated. Indeed, previous human research on the disease (as well as diagnostic biopsies) has typically focused on the analysis of a single muscle type (normally *biceps brachii* or *quadriceps*) (Kohn TA, 2014). Yet the mammalian skeletal muscle is heterogeneous in nature, comprising four major fibre types: I, IIA, IID and IIB (Schiaffino & Reggiani, 2011). Type I fibres are more predominant in 'slow-twitch' muscles (such as typically the *soleus* muscle), whereas IIA, IID and especially IIB fibres are predominant in 'fast-twitch' muscles (such as *EDL*) (Schiaffino & Reggiani, 2011). Additionally, these four fibre types also diverge in their amount of glycolytic enzymes: although slow-twitch-fibres are typically oxidative, fast-twitch fibres are either oxidative (IIA) or glycolytic (IID and IIB) (Schiaffino & Reggiani, 2011; Murphy *et al.* 2012). One advantage of the McArdle mouse model compared to studies with McArdle patients is that the former permits a thorough molecular and biochemical characterization of the disease in several
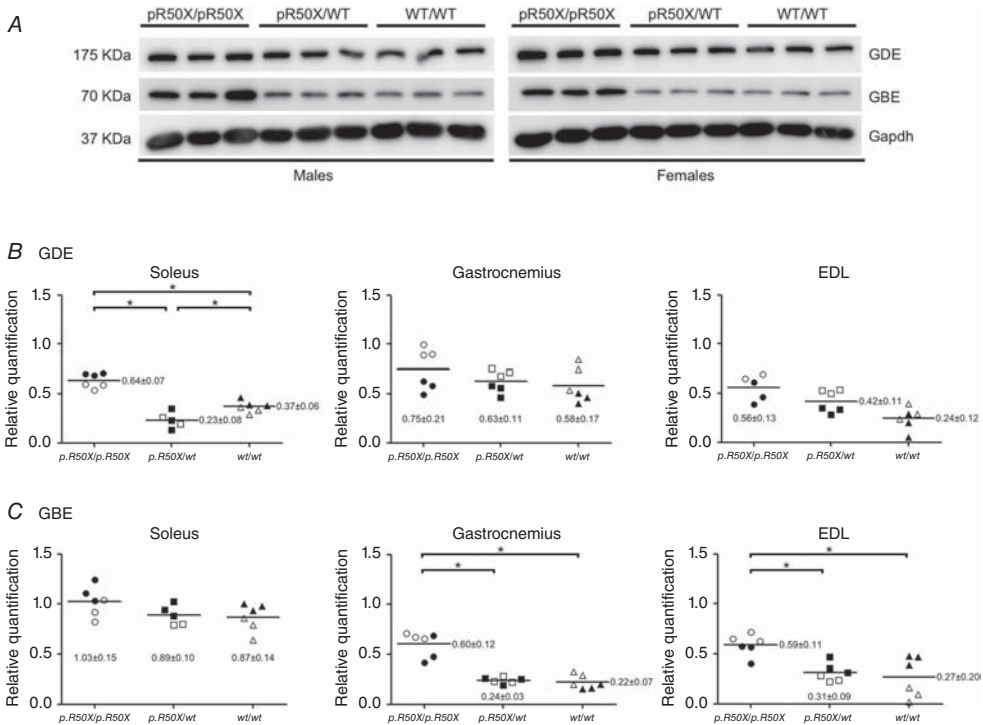
Figure 3. Effect of *Pygm* genotype and muscle type on GBE and GDE
A, western blot analyses in the *gastrocnemius* muscle. *B*, effect of *Pygm* genotype and muscle type on GBE. *C*, effect of *Pygm* genotype and muscle type on GDE. Data are shown as the mean ± SD and individual values, with males indicated by black coloured squares, dots or triangles. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Significant *post hoc* differences are indicated in parenthesis (*$P < 0.01$; **$P < 0.05$).

different muscle types. Thus, additional novelties of the present study were the determination of several enzymes involved in glycogen metabolism across the three possible *Pygm* genotypes, together with the assessment of potential differences between sexes. In the present study, we first corroborated that the *p.R50X/p.R50X* mice exhibited a McArdle-like phenotype, as shown by their very poor performance in the treadmill tests and their hyper-CKaemia. This is consistent with the findings of previous studies (Nogales-Gadea *et al.* 2012*b*) and an additional novelty of the present study is that treadmill inclination was set at 0%. This allowed us to determine that glycogen unavailability also considerably impairs maximal capacity in longer bouts of exertion (i.e. during a test with in which normal mice ran >700 m) (compared to ∼500 m in our previous study) (Nogales-Gadea *et al.* 2012*b*).

We analysed the molecular consequences of McArdle disease on glycogen metabolism in three different muscles: *soleus*, *gastrocnemius* and *EDL*. First, we analysed, within healthy mice, the expression of the four proteins more directly involved in glycogen metabolism. On the one

hand, we observed that, in *wt/wt* mice, there was a higher content of glycogen anabolic enzymes (GS-M and GBE) in the most slow-twitch muscle (i.e. *soleus*). This is in agreement with findings previously reported in wild-type rats, where four-fold higher GBE protein levels were reported in the *soleus* compared to the *EDL* muscle (Murphy *et al.* 2012) (compared to five-fold higher levels in our *wt/wt* mice). The data on GBE are also in support of previous research suggesting that glycogen granules might present more ramifications and be more densely packed in slow-twitch compared to fast-twitch fibres (Murphy *et al.* 2012). More research is needed to determine why the ability for glycogen ramification and storage is highest in the muscles that are less reliant on glycogen metabolism. By contrast, in *wt/wt* mice, the content of glycogen catabolic enzymes (GP-MM, GDE) was highest in the muscle with a more characteristic fast-twitch phenotype (i.e. *EDL*). This is also consistent with previous research conducted in healthy rats, where GP-MM and GDE protein levels were higher in *EDL* compared to *soleus* muscle (Murphy *et al.* 2012). In humans,

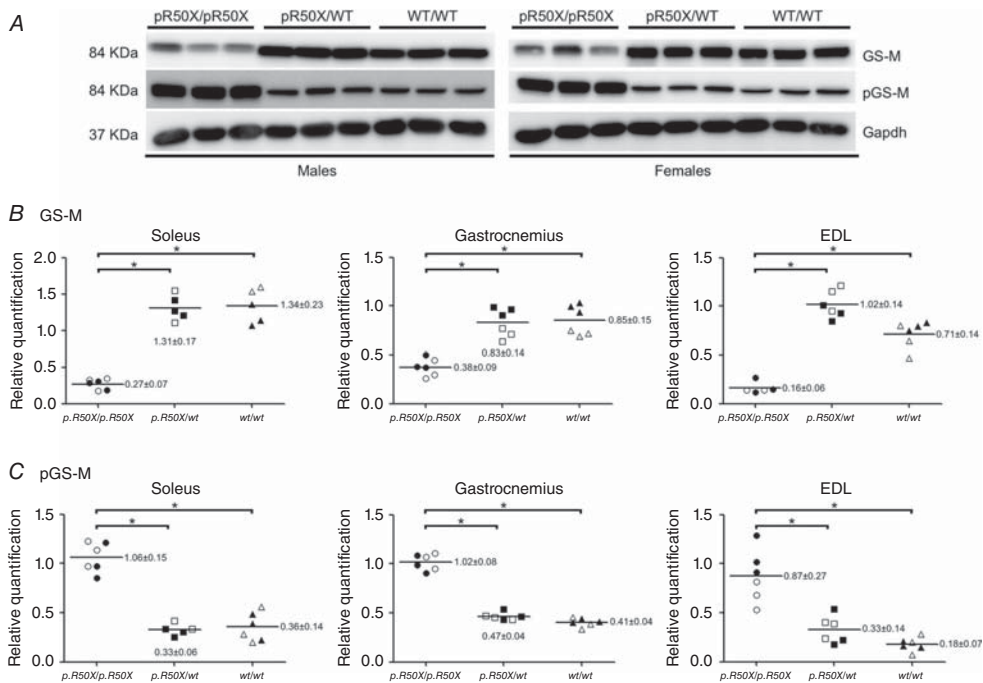**Figure 4. Effect of *Pygm* genotype and muscle type on GS-M and pGS-M**
A, western blot analyses in the *gastrocnemius* muscle. *B*, effect of *Pygm* genotype and muscle type on GS-M. *C*, effect of *Pygm* genotype and muscle type on pGS-M. Data are shown as the mean ± SD and individual values, with males indicated by black coloured squares, dots or triangles. GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Significant *post hoc* differences are indicated in parenthesis (*$P < 0.01$).

slow-twitch oxidative (type I) fibres were reported to have lower GP-MM content in the *triceps brachii*, although no differences were found for the *vastus lateralis muscle* (Daugaard & Richter, 2004), and type I fibres would exhibit lower rates of glycogenolysis during maximal contractions

compared to type II fibres (Vollestad *et al.* 1992; Greenhaff *et al.* 1993).

In McArdle mice, complete depletion of GP-MM protein was observed in the three muscles (*soleus*, *gastrocnemius* and *EDL*), which was accompanied by a 90% reduction in *Pygm* mRNA levels, as a result of the so-called 'non-sense mediated decay mechanism' (i.e. a protective cellular mechanism that eliminates aberrant transcripts), as previously reported in McArdle patients (Nogales-Gadea *et al.* 2008). These results suggest that GP-MM protein and mRNA levels might be equally depleted in all the muscles of a patient, regardless of their fibre type composition. However, in *p.R50X/wt* mice, the decrease in both GP-MM mRNA and protein was more pronounced in the slow-twitch *soleus* muscle than in the more fast-twitch *gastrocnemius* and *EDL* muscles. These results, together with the abovementioned observation indicating that both GP-MM and GDE proteins were less expressed in the *soleus* of healthy mice (and that this muscle showed the lowest glycogen content) (Fig. 5), might indicate that slow-twitch muscles are not as dependent on glycogen catabolism for their proper function as fast-twitch muscles and might be less affected by GP-MM depletion. Of note, although each of the three muscles studied has a clearly predominant metabolic profile, one limitation of the present study is that we did not study molecular phenotypes within the different fibre
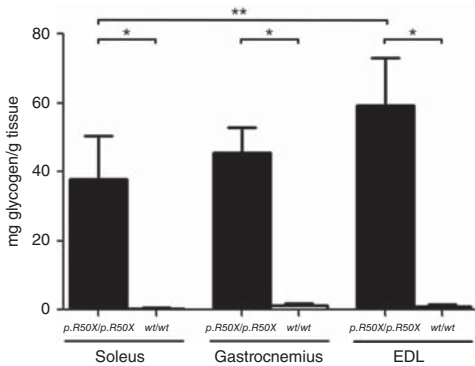
Figure 5. Effect of *Pygm* genotype and muscle type on muscle glycogen content
Data are shown as the mean ± SD. Significant *post hoc* differences are indicated in parenthesis (*$P < 0.01$; **$P < 0.05$).
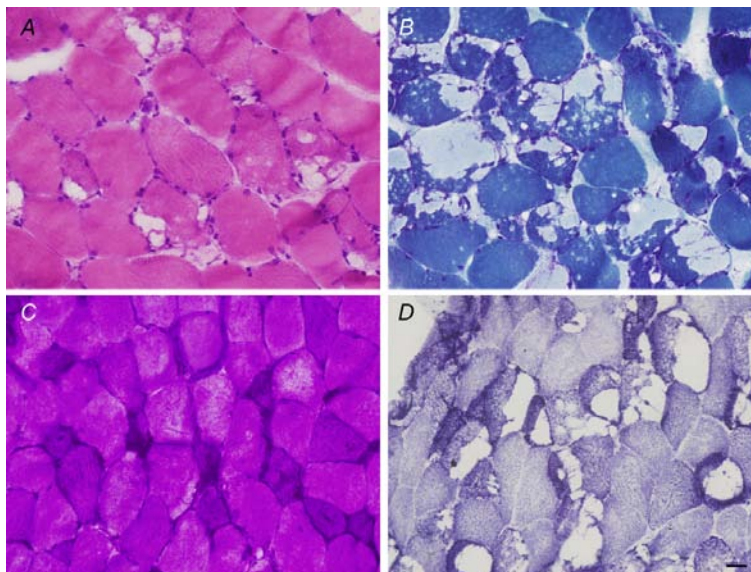
Figure 6. Histochemical analysis of a *gastrocnemius* muscle from a McArdle mouse
Haematoxylin and eosin (*A*), Gomory's trichrome (*B*), PAS (*C*) and succinate dehydrogenase (*D*) staining are shown. Scale bar = 50 μm.

subtypes of the three muscles assessed. Furthermore, we cannot discard a certain 'training-effect' influencing the results with respect to the *soleus* muscle compared to the other two muscles, with the former having an important postural role, and thus a more chronic type of activity.

With regard to glycogen synthesis, it has been previously reported that muscle biopsies from McArdle patients present lower levels of mRNA, protein and activity of GS-M compared to healthy controls (Nielsen *et al.* 2002; Nogales-Gadea *et al.* 2012*a*); however, higher levels of the phosphorylated (i.e. less active) form of the enzyme (pGS-M) were also observed in these patients (Nielsen *et al.* 2002; Nogales-Gadea *et al.* 2012*a*). Similarly, in the present study, we observed much lower levels of total GS-M protein (∼70% on average) but higher levels of its phosphorylated form in all three muscles from McArdle mice compared to normal mice (∼70% and ∼68%, respectively). This result might reflect a cellular mechanism aimed at preventing excessive, deleterious glycogen accumulation as a result of the lack of glycogen catabolism in McArdle mice. It was previously reported that high glycogen in skeletal muscles decreases insulin-stimulated glycogen synthesis, as well as GS activation (Jensen *et al.* 2006). Therefore, the high glycogen accumulation in the muscles of McArdle mice might be one of the contributors to GS inactivation. Indeed, GP-MM forms two complexes in which GS is also present. The presence of muscle GP-MM/GS complexes suggests that their common allosteric regulators and covalent modifiers function better if both proteins are in physical vicinity (Nogales-Gadea *et al.* 2012*a*). Along with GS-M, GBE also participates in glycogen synthesis by creating new branches and generating a molecule with a helical structure of 12 concentric tiers (Gibson *et al.* 1971; Caudwell & Cohen, 1980; Melendez *et al.* 1999). Interestingly, in the present study, we observed that, compared to normal and heterozygous mice, GBE was up-regulated in the homozygous mice muscles with an 'intermediate' (*gastrocnemius*) or 'glycolytic' phenotype (*EDL*) but not in the *soleus* muscle. These results suggest that, in homozygous mice, GBE is up-regulated in those muscles (*gastrocnemius* and *EDL*) that present the lowest GBE basal levels in healthy conditions (Fig. 1*C*), probably as a compensatory mechanism (i.e. to accommodate higher amounts of glycogen in more tightly packed granules).

Regarding glycogen content in healthy mice, the *soleus* presented the lowest levels. Similar findings were found in the homozygous mice. These differences in glycogen content between muscles can be explained by their different fibre type composition. We previously reported higher glycogen accumulation in the type II fibres from the *gastrocnemius* muscle of homozygous mice compared to type I fibres (Nogales-Gadea *et al.* 2012*b*). Taken together, all of these results suggest that muscles with a larger proportion of type II fibres might present a higher

glycogen content than those with a higher proportion of type I fibres. However, our histochemistry results in the homozygous mice add an additional layer of complexity: within each muscle (*soleus*, *gastrocnemius* and *EDL*), and regardless of their predominant muscle fibre type, the largest vacuoles of glycogen content were found in the more oxidative fibres. Further studies are needed to specifically determine which fibre type accumulates more glycogen and whether there is a shift in muscle fibre type composition between healthy and McArdle mice.

When analysing blood metabolites, we observed significantly lower glucose and lactate but higher ammonium levels in *p.R50X/p.R50X* mice compared to their wild-type counterparts. Regarding the latter, as a result of reduced glycolytic flux, there is frequently a mismatch between ATP consumption and production in the muscles of these patients (Santalla *et al.* 2014). Thus, two ADP molecules can combine to regenerate ATP by the myokinase pathway in an attempt to keep up with ATP demand. In this reaction, AMP is produced and removed by AMP deaminase 1, resulting in the production of NH_4^+ and inosine monophosphate. Indeed, there is evidence of higher levels of muscle ADP (Mineo *et al.* 1985) and plasma NH_4^+ in McArdle disease patients compared to non-patients (Brooke *et al.* 1983; Mineo *et al.* 1985) (as well as in *p.R50X/p.R50X* mice compared to their *wt/wt* controls in the present study), resulting in high oxidative stress in muscle fibres (Kitaoka *et al.* 2013), which, in turn, could lead to the basal muscle damage commonly observed in patients (Kohn TA, 2014) and, in the present study, in mice.

Heterozygosity for the *p.R50X* mutation resulted in lower GP-MM levels (*soleus*, ∼60%; *gastrocnemius* and *EDL* muscles, both ∼35%) and reduced exercise capacity (∼18%) compared to healthy mice. The latter finding apparently contrasts with the autosomal recessive nature of the disease in humans, where heterozygous individuals are traditionally considered to be asymptomatic, at least during normal activities of daily living (Andersen *et al.* 2006). On the other hand, the reduction of GP-MM levels in heterozygous compared to healthy mice does not result in glycogen accumulation leading to fragile fibres in the former, whereas the latter phenomenon is clearly observed in p.*R50X/p.R50X* mice (Nogales-Gadea *et al.* 2012*b*). This might suggest that the partially compromised exercise performance of heterozygous mice is more related to some degree of metabolic limitation (i.e. possibly lower maximal glycolytic flux) rather than to the myopathy-induced structural alterations known to occur in McArdle patients and mice, as reflected by their hyperCKaemia and myoglobinuria, but not reported in heterozygous individuals (Andersen *et al.* 2006; Nogales-Gadea *et al.* 2012*b*; Santalla *et al.* 2014). Irrespective of the fact that they are usually asymptomatic in daily activities, further research is needed to determine whether the maximal endurance capacity of

Results

individuals who are carriers of a pathogenic mutation in only one of the copies of *PYGM* gene is also impaired compared to non-carriers.

The bulk of animal research in exercise/muscle physiology comprises studies performed in rats. Yet the generation of transgenic mouse models of human cardiovascular or neuromuscular disease has led to a progressive shift from rat to mouse studies and, consequently, to a growing interest in understanding the physiological responses to exercise in the latter species (Bogue, 2003). Thus, our McArdle model might help our understanding of the regulation of glycogen metabolism, as well as confirm the importance of glycogen on muscle function and the phenotypic consequences of the unavailability (whether complete or not) of this substrate. In this regard, we showed no overall differences between sexes in most of the muscle variables that we studied. To the best of our knowledge, no study has yet specifically assessed possible between-sex differences in humans in the muscle enzymes investigated in the present study. On the other hand, although there appears to be no inherent between-sex difference in basal levels of muscle glycogen, some sex-related differences have been reported in the breakdown and metabolism of carbohydrates, with women tending to oxidize less total carbohydrate than men (although the mechanism behind this phenomenon remains unclear) (Tarnopolsky, 2008). Finally, the present study is not without limitations. First, although we measured the maximal endurance capacity of the mice and were able to document the marked exercise capacity limitation of the McArdle mice, we did not identify a unique feature of the disease in patients: the so-called 'second wind phenomenon' (Braakhekke *et al.* 1986; Vissing & Haller, 2003), which is considered as the ability to resume dynamic exercise (e.g. brisk walking) if patients take a brief rest upon the appearance of premature fatigue (Di Mauro, 2007). The second wind can be objectively detected during a constant-load cycle-ergometer test, with patients showing a clear decrease in early exertional tachycardia after ∼7–8 min, and most reporting a decrease in local leg muscle pain (Braakhekke *et al.* 1986; Vissing & Haller, 2003). Indeed, the first few minutes of exercise would act as a warm-up (inducing muscle vasodilatation), after which more bloodborne fuels (FFAs and glucose) are available to be oxidized in working muscle fibres, resulting in the attenuation of exercise intolerance (Haller & Vissing, 2002). More work is underway by our group aiming to determine whether this phenomenon also occurs in the mouse model during constant-load treadmill exercise. However, recent research has indicated that the cardiovascular response to treadmill exercise in mice is masked by a stress-associated heart rate increase, which makes it difficult to obtain reliable measurements of this variable during exercise (Andreev-Andrievskiy *et al.* 2014). (Of note, the second wind is the only feature of the disease that has yet not been reported in the McArdle mouse). Further research is also needed to determine whether spontaneous locomotor activity or gas exchange parameters indicative of substrate utilization (such as respiratory exchange ratio) are affected in the McArdle mouse model.

Our main findings can be summarized. Both complete and partial deficiency of GP-MM resulted in impaired maximal endurance capacity, thereby reflecting the key role that GP-MM plays in muscle function in mammals. Expression of the main muscle glycogen regulatory enzymes (and thus muscle glycogen content) differed depending on the muscle predominant phenotype (slow- *vs.* fast-twitch). On the other hand, the glycogen synthesis machinery was down-regulated in *p.R50X/p.R50X* mice, probably reflecting a protective mechanism to prevent deleterious glycogen accumulation. Finally, except for glycogen content in normal mice, the variables under investigation were not influenced by sex. Our knock-in model, which closely mimics the phenotype manifestations of the paradigm of exercise intolerance in humans (i.e. McArdle disease), might help to provide insights into the importance of glycogen on muscle function. Further research might also determine how these mice adapt to a training programme in the face of their marked metabolic limitation.

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Additional information
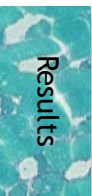
Competing interests

Author contributions

AB, NdL, ALA and TP designed and performed the experiments. AB, ALA, AL and TP collected and interpreted the data. RM, ABG, MAM, JA and AL revised the manuscript and provided critical intellectual suggestions. AL, ALA and TP wrote the manuscript.

Results

Results

Funding

3.2 Article 2

The Company of **Biologists**

RESEARCH ARTICLE

Sodium valproate increases the brain isoform of glycogen phosphorylase: looking for a compensation mechanism in McArdle disease using a mouse primary skeletal-muscle culture *in vitro*

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ABSTRACT

McArdle disease, also termed 'glycogen storage disease type V', is a disorder of skeletal muscle carbohydrate metabolism caused by inherited deficiency of the muscle-specific isoform of glycogen phosphorylase (GP-MM). It is an autosomic recessive disorder that is caused by mutations in the *PYGM* gene and typically presents with exercise intolerance, i.e. episodes of early exertional fatigue frequently accompanied by rhabdomyolysis and myoglobinuria. Muscle biopsies from affected individuals contain subsarcolemmal deposits of glycogen. Besides GP-MM, two other GP isoforms have been described: the liver (GP-LL) and brain (GP-BB) isoforms, which are encoded by the *PYGL* and *PYGB* genes, respectively; GP-BB is the main GP isoform found in human and rat foetal tissues, including the muscle, although its postnatal expression is dramatically reduced in the vast majority of differentiated tissues with the exception of brain and heart, where it remains as the major isoform. We developed a cell culture model from knock-in McArdle mice that mimics the glycogen accumulation and GP-MM deficiency observed in skeletal muscle from individuals with McArdle disease. We treated mouse primary skeletal muscle cultures *in vitro* with sodium valproate (VPA), a histone deacetylase inhibitor. After VPA treatment, myotubes expressed GP-BB and a dose-dependent decrease in glycogen accumulation was also observed. Thus, this *in vitro* model could be useful for high-throughput screening of new drugs to treat this disease. The immortalization of these primary skeletal muscle cultures could provide a never-ending source of cells for this experimental model. Furthermore, VPA could be considered as a gene-expression modulator, allowing compensatory expression of GP-BB and decreased glycogen accumulation in skeletal muscle of individuals with McArdle disease.

KEY WORDS: Glycogen phosphorylase, Glycogenolysis, McArdle disease, Myotubes, Sodium valproate

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INTRODUCTION

McArdle disease, also termed glycogen storage disease type V (OMIM® number 232600), is a disorder of skeletal muscle carbohydrate metabolism caused by inherited deficiency of the skeletal-muscle isoform of glycogen phosphorylase (GP-MM). It is caused by pathogenic mutations in both copies of the GP-MM-encoding gene (*PYGM*), which is located in chromosome 11q12-11q13. Owing to their inability to use glycogen for fuelling muscle contractions, affected individuals commonly experience exercise intolerance, which typically consists of acute crises of early exertional fatigue, muscle stiffness and contractures, which, in the most severe cases, can be accompanied by rhabdomyolisis and subsequent myoglobinuria, thereby increasing the risk of renal damage (Lucia et al., 2008).

Although undetectable GP-MM activity is the common (and in fact diagnostic) observation in the differentiated cells (i.e. fibers) obtained from affected individuals' skeletal-muscle biopsies, cultured muscle cells derived from their muscle biopsies present GP activity (Martinuzzi et al., 1993; Meienhofer et al., 1977). Furthermore, human primary skeletal-muscle cultures obtained from biopsies of affected individuals do not differ from controls in that there is no excessive accumulation of periodic acid Schiff (PAS) staining material, and thus no abnormal glycogen deposits (Martinuzzi et al., 1993). However, other authors have failed to detect GP-MM in human primary skeletal-muscle cultures obtained from affected individuals or healthy controls (DiMauro et al., 1978; Sato et al., 1977).

Several types of treatments have been studied to reduce the symptoms in individuals with McArdle disease, with different and controversial results. No significant beneficial effects have been reported in patients receiving nutritional supplements such as branched-chain amino acids (MacLean et al., 1998), depot glucagon (Day and Mastaglia, 1985), dantrolene sodium (Poels et al., 1990), verapamil (Lane et al., 1984), vitamin B6 (Phoenix et al., 1998) [except in one recent case report (Sato et al., 2012)], high-dose oral D ribose (Steele et al., 1996) or high-dose creatine (Vorgerd et al., 2000). Low-dose creatine conferred a modest benefit on ischemic exercise in a few patients (Vorgerd et al., 2000). The ingestion of simple carbohydrates before engaging in strenuous exercise can alleviate their exercise-intolerance symptoms and diminish the risk of muscle rhabdomyolysis (Vissing and Haller, 2003), with supervised exercise training interventions also showing to be clinically beneficial (Haller et al., 2006; Maté-Muñoz et al., 2007; Santalla et al., 2014). The Cochrane review of pharmacological and nutritional treatment for McArdle disease includes the evidence from randomized

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TRANSLATIONAL IMPACT

Clinical issue

McArdle disease is a disorder of skeletal-muscle carbohydrate metabolism caused by inherited deficiency of muscle glycogen phosphorylase (GP-MM). This enzyme catalyzes and regulates the breakdown of glycogen into glucose-1-phosphate in muscle fibers. Thus, individuals with McArdle disease are unable to obtain energy from their muscle glycogen stores and, as such, present with exercise intolerance, typically manifested as acute crisis of undue, early exertional fatigue, muscle stiffness and contractures. In the more severe cases, these symptoms can be accompanied by rhabdomyolysis (breakdown of muscle fibers) and subsequent myoglobinuria (elevated urine levels of myoglobin, released as a muscle breakdown product). There are three *in vivo* animal models of the disease, two naturally occurring in Charolais calf and Merino sheep, respectively, and one experimentally generated in mice. The latter is a recently developed knock-in (KI) mouse model carrying the most common McArdle disease mutation (p.R50X) in the GP-MM-encoding gene (*Pygm*); these mice closely mimic the human disease phenotype. However, there is as yet no reported cellular model that mimics *in vitro* the main biochemical and histological alterations typically observed in biopsied muscle fibers from McArdle-affected individuals; that is, the absence of GP-MM and glycogen accumulation.

Results

The authors analyzed the expression of different GP isoforms [the brain (GP-BB) and liver (GP-LL) isoforms in addition to GP-MM] in cultured cells that were previously obtained from skeletal muscles of KI (GP-MM-deficient) or wild-type mice, at different stages of differentiation. They observed that GP-MM was the only GP isoform expressed *in vitro* in the differentiated cells obtained from wild-type mice, whereas GP-MM was not expressed in the differentiated cells from KI mice. In addition, only differentiated cells from KI mice accumulated glycogen in their cytoplasm, a characteristic (and, in fact, diagnostic) trait that occurs in the fibers of muscle biopsies from affected individuals. This *in vitro* model was used to assess whether sodium valproate (VPA) can reverse the muscle phenotype from a McArdle-like to a normal histological and biochemical profile. VPA activated the expression of only one GP isoform, GP-BB, in differentiated muscle cultures from KI mice, yet this isoform seemed to have beneficial functional consequences because it decreased intracellular glycogen accumulation.

Implications and future directions

These results demonstrate that primary skeletal-muscle cultures from McArdle KI mice represent a useful *in vitro* model that is able to replicate the biochemical and histological alterations associated with the human condition. In addition, the study shows that this model could be used as a high-throughput screening system for testing new drugs that aim to restore, at least partially, GP activity in the skeletal muscle tissue of individuals with McArdle disease, with potential therapeutic outcomes.

controlled trials for improving exercise performance and quality of life in McArdle disease (Quinlivan et al., 2010).

Because almost 50% of Caucasians with McArdle disease carry the nonsense p.R50X mutation, treatment with drugs that could potentially induce 'read through' of the generated premature termination codon could help to re-express GP-MM activity; however, gentamicin treatment failed to normalize phosphorus (^{31}P) magnetic resonance [indicators of GP-MM activity in the skeletal muscles of individuals with McArdle disease (Schroers et al., 2006)]. Gene-therapy strategies have also been evaluated either *in vitro*, i.e. in human and sheep myoblast cultures deficient for GP-MM, or *in vivo*, i.e. in the ovine model of McArdle disease. Whereas, in myoblast cultures, GP-MM activity was restored after transfection with *PYGM* cDNA (Pari et al., 1999), intramuscular injection of adenovirus and adeno-associated vectors containing GP-MM

expression cassettes in the ovine McArdle model only produced GP-MM functional activity in the surroundings of the injection site, and its expression diminished with time probably as a consequence of an immune response (Howell et al., 2008).

In addition to GP-MM, two other GP isoforms have been described: the liver and brain isoforms, which are encoded by the *PYGL* and *PYGB* genes, respectively. The brain isoform is the main GP isoform found in human and rat foetal tissues, including in the muscle, although its postnatal expression is dramatically reduced in the vast majority of tissues with the exception of the brain, where it remains as the major isoform. As described in the UCSC genome browser (http://genome-euro.ucsc.edu/index.html), both human, sheep and mouse *PYGB* genes present CpG islands in their promoters, and thus their expression might be regulated epigenetically through methylation of their promoters. In fact, postnatal downregulation of gene expression has been reported for many genes containing CpG promoters (Numata et al., 2012). Thus, any pharmacological treatment able to upregulate the expression of *PYGB* in the skeletal muscle of McArdle patients could theoretically alleviate the symptoms of the disease.

Valproic acid (VPA) is a short-chained fatty acid that has been used for many years in the treatment of epilepsy and bipolar disorders (McCoy et al., 1993; Sherard et al., 1980). Recent data suggest that this drug can modulate the epigenome by inhibiting histone deacetylases and activating the expression of methylated genes by stimulating active, replication-independent demethylation (Detich et al., 2003). The results of a recent study performed in a McArdle sheep model showed that enteral and intramuscular injection of VPA increased muscle expression of GP, although glycogen deposits and the expression of specific GP isoforms at the muscle tissue level were not analyzed (Howell et al., 2015).

We recently developed a p.R50X knock-in (KI) mouse model that presents with the main clinical features of the McArdle disease phenotype (Nogales-Gadea et al., 2012). Following on from this, in the present study we show that primary skeletal-muscle cultures derived from this murine model constitute a valid *in vitro* model to analyze and evaluate potential treatments for the disease because, in contrast to what occurs with muscle cultures derived from affected humans, these cells do not present GP activity and accumulate large amounts of glycogen deposits. Additionally, in these murine-derived muscle cultures, we observed how VPA treatment increased the expression of *Pygb*, providing an alternative mechanism that compensated, at least partly, for the lack of *Pygm* expression, as well as reducing polysaccharide accumulation.

RESULTS

Myotubes from primary skeletal-muscle cultures from homozygous p.R50X mice accumulate glycogen

We first analyzed *Pygm*, *Pygb* and *Pygl* expression at the undifferentiated (myoblasts) and differentiated (myotubes) stages of development in skeletal-muscle cultures derived from wild-type (WT) and KI McArdle mice. Myoblasts from WT and both myoblasts and myotubes from KI mice did not express *Pygm* mRNA (Fig. 1A). In WT muscle cultures, *Pygm* mRNA levels increased with muscle differentiation ($P<0.001$) (Fig. 1A). No significant differences were observed in *Pygb* mRNA levels among myoblasts and myotubes from WT and KI muscle cultures (Fig. 1B). Neither myoblasts nor myotubes from WT or KI mice expressed *Pygl* mRNA (data not shown). Western blot (WB) confirmed the mRNA results: GP-MM (*Pygm*) was only present in

Disease Models & Mechanisms

Fig. 1. Differential glycogen phosphorylase expression in mouse primary skeletal-muscle cultures. Only WT myotubes expressed *Pygm* mRNA (A). Myoblasts and myotubes from WT and KI mice expressed *Pygb* mRNA with a high variation and no statistically significant differences between WT and KI (B). Presence of both *Pygm* transcript and protein (GP-MM) was observed only in WT myotubes (C). PAS staining of WT myoblasts (D), WT myotubes (F), KI myoblasts (E) and KI myotubes (G): only KI myotubes accumulated high glycogen levels (G). KI, knock-in; WT, wild type; RQ, relative quantification. *$P<0.05$ for the comparison KI versus WT, **$P<0.001$ for the comparison of KI versus WT. Scale bar: 50 μm.

WT myotubes (Fig. 1C). GP-BB (*Pygb*) was not detected by western blot analysis in WT or KI myoblasts nor in WT or KI myotubes (data not shown).

We also performed a PAS staining in skeletal-muscle cultures from WT and KI mice. We observed that KI myotubes accumulated glycogen (Fig. 1G), whereas both WT and KI myoblasts and WT myotubes did not (Fig. 1D-F).

Myotubes treated with VPA for 72 h expressed *Pygb* and reversed the glycogen accumulation

WT and KI myoblasts and myotubes were treated for 72 h with VPA at 1, 2 and 5 mM. Myoblasts isolated from WT and KI mice and treated with VPA did not increase *Pygb* mRNA expression (data not shown). However, when VPA was added to the confluent myoblasts and differentiated myotubes, we observed an increased expression of *Pygb* mRNA, both in WT and in KI muscle cultures ($P<0.01$ and $P<0.05$, respectively). The highest amount of GP-BB (*Pygb*) protein expression was observed in myotubes treated with 2 mM VPA (Fig. 2). After 72 h of VPA treatment, no detachment of myotubes from the plate was observed, indicating that VPA was not toxic to the muscle culture (data not shown). The PAS staining on treated and non-treated myotubes showed a reduction in glycogen

accumulation in cultures treated with 2 and 5 mM VPA (Fig. 3A). In the heat map in Fig. 3B, hot colors indicate high glycogen concentration, whereas cold colors such as green and blue show the zones with no glycogen accumulation.

To test whether a longer period of VPA treatment could be toxic to the muscle cells, we changed the culture medium for each group (no VPA, or 1, 2 or 5 mM VPA) every 72 h over 12 days. After the 12-day period, the only muscle cultures that were maintained at a healthy myotube stage were muscle cultures treated with 2 mM of VPA (Fig. 4).

DISCUSSION

The primary skeletal-muscle cultures derived from p.R50X KI mice represent a good cellular model of McArdle disease because they mimic the glycogen accumulation that is commonly found in the skeletal muscle from individuals with McArdle disease (Lucia et al., 2008). It has been previously reported that, in human culture cells either from affected or healthy individuals, *PYGM* expression contributed little to the total GP mRNA, whereas *PYGB* expression was predominant in myoblasts and *PYGB* and *PYGL* were both expressed in myotubes (Nogales-Gadea et al., 2010). Because *PYGB* and *PYGL* were the main GP isoforms expressed in human

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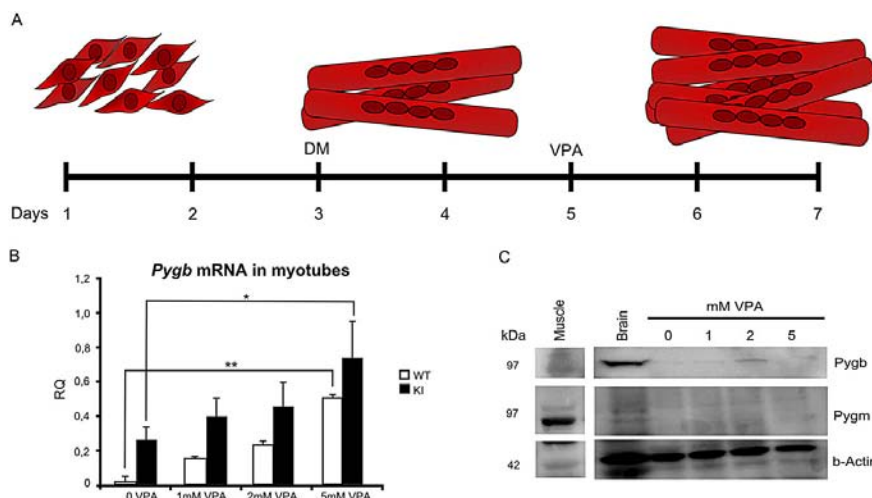
Fig. 2. VPA treatment increase *Pygb* expression in mice skeletal muscle cultures. (A) After myotubes were formed, muscle cultures were treated with different concentrations of VPA for 72 h. (B) In both cell cultures (WT and KI), *Pygb* mRNA increased in treated cultures (**$P<0.01$, *$P<0.05$). (C) GP-BB (*Pygb*) protein was also detected with western blot analysis in WT and in KI cell cultures. Abbreviations: DM, Dulbecco's modified Eagle's medium; KI, knock-in; VPA, valproic acid; WT, wild type; RQ, relative quantification.

cultures, an increase in glycogen deposits was not observed in skeletal-muscle cultures derived from individuals with McArdle disease (Nogales-Gadea et al., 2010). By contrast, neither *Pygb* nor *Pygl* were expressed at any differentiation stage in our WT or KI mouse cultures, whereas GP-MM expression was restricted to WT myotubes, similarly to what has been shown to occur in the skeletal muscle of human adult healthy individuals. Additionally, vacuolization and increased intracellular PAS staining was also observed in KI mouse cultures. Thus, primary skeletal-muscle cultures derived from KI mice might represent a useful tool to test different pharmacological therapies prior to their evaluation in *in vivo* models.

A natural ovine model of McArdle disease has been described that is characterized by an adenine-for-guanine substitution at the intron 19 acceptor splice site of the *Pygm* gene (Tan et al., 1997). These sheep exhibit clinical features and morphological changes at the muscle tissue level that are similar to those shown by humans with the disease (Tan et al., 1997). In this animal model, regeneration of muscle fibers after necrosis induced by notexin injection reduced glycogen storage in regenerating muscle fibers, which showed re-expression of non-muscle (liver and brain) isoforms of GP, thereby indicating the need to investigate the potential functional benefit of inducing at the muscle tissue level these normally latent isoforms (Howell et al., 2014).

VPA exerts significant inhibitory effects on the activity of glycogen synthase (GS) kinase 3 beta (GSK3β) both *in vitro* and also *in vivo* (i.e. on endogenous GSK3β) (Chen et al., 2000). Because the inhibition of GSK3β might generate the accumulation
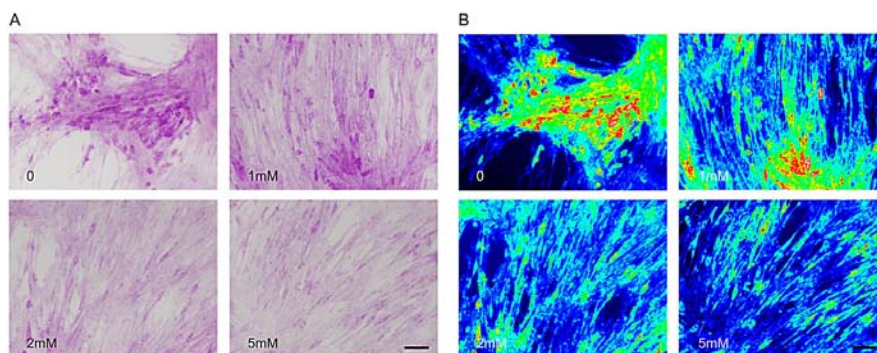
Fig. 3. Reduced glycogen accumulation in KI myotubes after treatment with VPA. (A,B) We observed a gradual reduction in glycogen content as myotubes were treated with increasing VPA concentrations. (B) Red areas correspond to sites of major glycogen accumulation, whereas 'cold' colors (green, blue) represent sites of low PAS staining in myotubes. Scale bars: 50 μm.
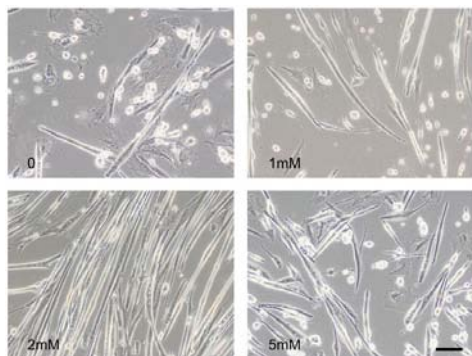
Fig. 4. Long-term VPA treatment of muscle cultures. After 12 days of treatment, only cultures treated with 2 mM VPA showed healthy myotubes.

of the more active unphosphorylated form of the GS enzyme, with its consequent increase in glycogen accumulation, we did not analyze GSK3β activity in VPA-treated or untreated KI mouse cells because, on the contrary, we observed a great reduction in glycogen accumulation *in vitro*, reaching normal levels. Additionally, it has also been observed that VPA has beneficial effects on skeletal-muscle myotubes, activating Akt signaling, stimulating gene transcription and protein synthesis, and promoting the survival of the cells via inhibition of apoptosis (Gurpur et al., 2009). We observed these 'beneficial' effects only when muscle cultures were treated with the 2 mM VPA concentration during a long period of time (12 days).

VPA induces histone acetylation of H3 histones, and is involved in the regulation of methylated genes by increasing the accessibility of the enzyme demethylase to the DNA (Detich et al., 2003; Milutinovic et al., 2006). The potential demethylation treatment of CpG islands of the *PYGB* promoter could allow the activation and transcription of the *PYGB* gene in skeletal muscle from individuals with McArdle disease as an approach to compensate for the lack of GP-MM. VPA treatment in humans could potentially have more effects on the glycogen accumulation because of the longer VPA half-life, compared with in mice (Loscher, 1978).

Our results demonstrate that mouse primary skeletal-muscle culture is a good study model of the disease because it mimics the phenotype of the muscle tissue from affected individuals. Furthermore, VPA can enhance *PYGB* expression *in vitro* and could be a candidate for the treatment of McArdle disease. Additional preclinical studies are needed to optimize the drug dosage to most effectively modulate *PYGB* gene expression.

MATERIALS AND METHODS
All experimental procedures were approved by the Animal Care and Use Committee of the Vall d'Hebron Institut de Recerca (CEEA 35/04/08), and were in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 1 2 3) and the Spanish laws (32/2007 and R.D. 1201/2005).

Mouse skeletal-muscle cultures
Myogenic precursor cells were isolated from 8-week-old KI McArdle mice carrying the p.R50X mutation in both copies of the *PYGM* gene (Nogales-Gadea et al., 2012) and from WT mice. All the muscles from lower limbs were dissected and digested in 0.2% pronase A (Calbiochem, Darmstadt, Germany) for 1 h at 37°C. After two washes with Dulbecco's modified

Eagle's medium (D-MEM, Lonza Group Ltd., Basel, Switzerland), supplemented with 10% FBS (Lonza) and 100 units/ml penicillin (Lonza), 100 µg/ml streptomycin (Lonza) and 0.25 µg/ml amphotericin (Lonza) (PSF), cells were seeded in a non-coated 100-mm petri-dish in HAM-F10 media (Lonza) supplemented with 20% FBS, 2 mM glutamine (Lonza) and PSF for 1 h to allow fibroblasts to adhere. Thereafter, supernatants were plated in 2% gelatin-coated dishes containing HAM-F10 growth media supplemented with 20 ng/ml bFGF (Peprotech, Rocky Hill, NJ, USA). 2500 cells/cm^2 were seeded in growth media to obtain myoblasts at day 7. To obtain myotubes, 12,500 cells/cm^2 were seeded in growth media and, after 48 h, the culture media was replaced with one containing D-MEM, 5% horse serum (Lonza), 2 mM glutamine and PSF to allow myoblasts to fuse and to form myotubes. Myotubes were analyzed after 5 days in differentiation medium. The medium was changed twice a week, and the muscle cultures were examined to confirm confluent growth of myoblasts and myotubes. Each condition was performed in triplicate.

VPA treatment in cell cultures
After 48 h in differentiation media in which myotubes were formed, we added VPA (Sigma-Aldrich, Madrid, Spain) at different concentrations (1, 2 and 5 mM) during an additional 72-h period.

RNA extraction and real-time polymerase-chain reaction (PCR)
Total RNA from mouse skeletal muscle cultures treated or not with VPA was extracted using Trizol (Life Technologies, Madrid, Spain). 0.5 µg of total RNA was DNAse-treated (Life Technologies, Madrid, Spain) and was then reverse-transcribed into cDNA using the high capacity cDNA RT kit (Life Technologies, Madrid, Spain).
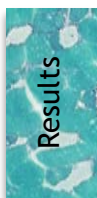
Quantification of *Pygm*, *Pygb*, *Pygl* and *Gapdh* (used as an internal standard) transcripts was performed using TaqMan Universal Master Mix technology (Life Technologies). Quantitative PCR was performed in a total reaction volume of 20 µl per well. The primers used for real-time PCR were designed by Applied Biosystems (Roche Molecular Systems) (*Pygm* Mm 00478582_m1, *Pygb* Mm 00464080_m1, *Pygl* Mm 01289790_m1 and mouse *Gapdh* endogenous control). The comparative CT method (ΔΔCT) for relative quantification of gene expression was used. The Student's *t*-test was used for statistical comparisons between the data obtained in KI versus WT mice.

Western blot
Cell and muscle samples corresponding to each experimental condition were trypsinized from the culture dish and homogenized with a lysis buffer containing 40 mM glycophosphate, 40 mM NaF, 10 mM EDTA and 20 mM of β-mercaptoethanol (final pH=6). The samples were placed in boiling water for 3 min and centrifuged at 9500 *g* for 3 min, before 100 µg of protein was applied to each lane. Unspecific binding sites on the blots were blocked by incubation in 5% low-fat dried milk powder in a phosphate buffered saline. Thereafter, primary rabbit polyclonal antibody anti-PYGB (kindly provided by Dr K. Nowak, Harry Perkins Institute of Medical Research, Nedlands, WA, Australia), the primary goat anti-PYGM [kindly provided by Dr Martinuzzi, Istituto di Ricovero e Cura a Carattere Scientifico Eugenio Medea – Associazione 'La Nostra Famiglia', Conegliano (Treviso), Italy], mouse anti-GAPDH (Ambion, Life Technologies) or anti-β-actin (Sigma-Aldrich) were added. Peroxidase-conjugated anti-rabbit (Jackson InmunoResearch, West Grove, PA, USA), peroxidase-conjugated anti-goat (Santa Cruz Biotechnology Inc., Heidelberg, Germany) and peroxidase-conjugated anti-mouse (Dako, Glostrup, Denmark) secondary antibodies were applied when using anti-PYGB antibody, anti-PYGM antibody and anti-GAPDH and anti-β-actin, respectively.

Periodic acid Schiff (PAS) staining in cultured cells
Both cell cultures treated or not treated with VPA were incubated with 1% of periodic acid (Sigma-Aldrich) in acetic acid for 30 min. Cells were washed in 0.1% sodium metabisulfite (Sigma) in 1 mM hydrochloric acid. Thereafter, they were incubated in a Schiff solution (Merck, Darmstadt, Germany) for 15 min after 0.1% sodium metabisulfite in 1 mM hydrochloric acid wash, and cells were observed in an inverted Olympus FSX100 microscope.

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3.3 Article 3

Human Mutation

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Results

McArdle Disease: Update of Reported Mutations and Polymorphisms in the *PYGM* Gene

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ABSTRACT: McArdle disease is an autosomal-recessive disorder caused by inherited deficiency of the muscle isoform of glycogen phosphorylase (or "myophosphorylase"), which catalyzes the first step of glycogen catabolism, releasing glucose-1-phosphate from glycogen deposits. As a result, muscle metabolism is impaired, leading to different degrees of exercise intolerance. Patients range from asymptomatic to severely affected, including in some cases, limitations in activities of daily living. The PYGM gene codifies myophosphorylase and to date 147 pathogenic mutations and 39 polymorphisms have been reported. Exon 1 and 17 are mutational hot-spots in PYGM and 50% of the described mutations are missense. However, c.148C>T (commonly known as p.R50X) is the most frequent mutation in the majority of the studied populations. No genotype–phenotype correlation has been reported and no mutations have been described in the myophosphorylase domains affecting the phosphorylated Ser-15, the 280's loop, the pyridoxal 5′-phosphate, and the nucleoside inhibitor binding sites. A newly generated knock-in mouse model is now available, which renders the main clinical and molecular features of the disease. Well-established methods for diagnosing patients in laboratories around the world will shorten the frequent ~20-year period stretching from first symptoms appearance to the genetic diagnosis.
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Introduction

McArdle disease, also known as glycogenosis type V (GSD5; MIM #232600), is an autosomal-recessive disorder caused by mutations in the *PYGM* gene [Lebo et al., 1984] (MIM #608455), which encodes the skeletal muscle isoform of glycogen phosphorylase, or "myophosphorylase." This enzyme catalyzes the first reaction in the catabolism of muscle glycogen [Cori and Larner, 1951] and its activity is absent in patients with McArdle disease. Patients' muscles have accumulations of subsarcolemmal glycogen, which they are unable to degrade [Nadaj-Pakleza et al., 2009]. The absence of myophosphorylase activity causes exercise intolerance, mainly in the form of acute crises of early fatigue and contractures [Lucia et al., 2008]. These symptoms are triggered mainly by static muscle contractions and dynamic exercise such as brisk walking or stair climbing [Di Mauro, 2007], although a recent report has described that even intense emotional situations could provoke crises in some cases [Brady et al., 2014].

There is phenotype heterogeneity among patients, and clinical severity can be classified into four categories, according to the Martinuzzi scale [Martinuzzi et al., 2003]: "0 = asymptomatic or virtually asymptomatic (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; no record of myoglobinuria, no muscle wasting or weakness; 2 = same as 1, plus recurrent exertional myoglobinuria, moderate restriction in exercise, and limitation in daily life activities; 3 = same as 2, plus fixed muscle weakness, with or without wasting and severe limitations on exercise and most daily life activities." In the Spanish registry, 25% of patients belong to the highest severity category 3 [Lucia et al., 2012]. Renal failure can occasionally occur but it is usually reversible [Quinlivan et al., 2010]. One pathognomonic feature of the disease is the "second wind" phenomenon [Lucia et al., 2008], which is reported by the vast majority of patients [Lucia et al., 2012]. This denotes marked improvement in tolerance to aerobic dynamic exercise (e.g., brisk walking), with disappearance of the tachycardia and undue fatigue that were

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triggered by the start of exertion [Vissing and Haller, 2003]. A common serological finding is high levels of creatine kinase activity, which is found in ~99% of patients [Lucia et al., 2012].

Symptoms usually start during childhood, yet in many cases the disease is not correctly diagnosed until the fourth decade of life [Lucia et al., 2012]. Although different methods of diagnosis can be used, the less invasive and preferred method in most laboratories is genetic analysis [Nogales-Gadea et al., 2015]. Because McArdle disease is an autosomal-recessive disorder (GenBank reference AH002957.1), all patients have a pathogenic mutation in both copies of the *PYGM* gene, being homozygous or compound heterozygotes. DNA analysis of mutations can be tedious, since the *PYGM* gene contains 20 exons, but the fact that some mutations are clearly more prevalent in most populations and some exons are highly dense in mutations can facilitate genetic diagnosis. In some cases, studies in RNA (GenBank reference NM_005609.2) are needed [Garcia-Consuegra et al., 2009] to identify mutations that cannot be detected with DNA analysis.

There is currently no treatment to restore myophosphorylase activity in McArdle patients. However, some studies have shown that supervised light-moderate intensity aerobic [Mate-Munoz et al., 2007; Perez et al., 2008; Lucia et al., 2012; Munguia-Izquierdo et al., 2015; Nogales-Gadea et al., 2015; Santalla et al., 2014b] or resistance exercise training can improve the functional capacity and/or clinical course of the disease [Garcia-Benitez et al., 2013; Santalla et al., 2014a]. Together with exercise, the most beneficial intervention for patients with McArdle disease consists of ensuring that sufficient blood glucose is constantly made available to their working muscles during daytime, especially before engaging in physical activities. This can be achieved by: (1) adopting a diet with a high proportion (65%) of complex carbohydrates (vegetables, fruits, cereals, bread, pasta, and rice) and a low proportion (20%) of fat [Andersen and Vissing, 2008] and (2) ingesting simple carbohydrates, that is, 30–40 g of glucose, fructose, or sucrose in adults some 5 min before engaging in strenuous exercise (e.g., brisk walking, hiking) [Mate-Munoz et al., 2007; Andersen et al., 2008], which translates to ~440 ml of most commercially available sport drinks, or 20 g in children before physical education classes [Perez et al., 2008]. On the other hand, no significant beneficial effects have been reported in McArdle patients receiving branched chain amino acids [MacLean et al., 1998], depot glucagon [Day and Mastaglia, 1985], dantrolene sodium [Poels et al., 1990], verapamil [Lane et al., 1986], vitamin B6 [Phoenix et al., 1998] (except in one recent case report [Sato et al., 2012]), or high-dose oral ribose [Steele et al., 1996]. More controversial are the effects of creatine supplementation: low-dose supplementation (60 mg/kg per day for 4 weeks) attenuated muscle complaints in five of nine McArdle patients [Vorgerd et al., 2000], but higher doses (150 mg/kg per day) exacerbated exercise-induced myalgia for unknown reasons [Vorgerd et al., 2002]. A 3-month treatment with the angiotensin-converting enzyme (ACE) inhibitor ramipril (2.5 mg/day) attenuated disability in McArdle patients, but the effect was more marked in those harboring the D/D genotype of the insertion(I)/deletion(D) polymorphism in the *ACE* gene [Martinuzzi et al., 2008]. A short-term trial (10 days) with a "read through" compound able to synthesize full proteins from transcripts containing premature termination stop codons (i.e., gentamicin) failed to normalize 31^P magnetic resonance spectroscopy indicators of myophosphorylase deficiency in the muscle of McArdle patients [Schroers et al., 2006].

In this review, we present an update of the reported mutations and polymorphisms in the *PYGM* gene. Type of mutation, together with gene and domain distributions are also indicated to foresee which are the "hot–spots" and the mutation consequences in terms of disease diagnosis and alteration of myophosphorylase activity. We also discuss a promising first mouse model, generated with genetic engineering and carrying the commonest mutation for the disease among Caucasians, c.148C>T or *p.R50X*.

Mutations and Polymorphisms

Mutations

To date, 147 mutations have been described in the *PYGM* gene (Supp. Table S1). All these mutations can be found in the LOVD Database (http://databases.lovd.nl/shared/variants/PYGM). In this article, we will follow the recommendations for mutation nomenclature, where nucleotide numbering uses +1 for the A of the ATG translation initiation codon in the reference sequence, and the initiation codon is "codon 1" [den Dunnen and Antonarakis, 2001]. Information on sequence variations is available regarding *PYGM* cDNA and protein reference sequence (GenBank accession numbers NM_000516.4 and NP_005600.1). Approximately 91% of the mutations are located in exonic regions and 9% in intronic regions (Fig. 1). The main type of mutations found are missense mutations that account for 50% of all described mutations (74/147). Missense mutations are contained in most of the exons, which would reflect that there are well-conserved and functionally relevant amino acid residues over the entire protein structure. Deletions are the second most common type of mutation in *PYGM* (18%; 26/147), being in the majority of cases small deletions of some nucleotides. Nonsense mutations are also frequent (13%, 19/147), followed by mutations that affect RNA splicing (11%; 16/147). In very few cases, mutations belong to the group of duplications (7/147), deletions/insertions (3/147), or missing of the starting amino acid (2/147).

Mutations have been described in all the exons. Exon 1 is the densest in mutations ($N = 17$), including the most common mutation *p.R50X*. Exon 17 is the second densest in mutations ($N = 14$), and is where the most frequent mutation in Japanese population, c.2128_2130delTTC (also p.F710del), is located [Sugie et al., 1995]. Highly mutated are also exons 10, 11, 12, 14, and 20 that comprise 32% of *PYGM* mutations. Very low in mutations is exon 7, with only one mutation described. Among the described mutations, p.R50X is by far the most frequently found in the studied populations [Bruno et al., 2006; Aquaron et al., 2007; Vieitez et al., 2011; Lucia et al., 2012; Gurgel-Giannetti et al., 2013]. In the Spanish registry of patients ($N = 239$) [Lucia et al., 2012], this mutation was found in ~55% of the mutated alleles (Fig. 2). In other cohorts, the percentage of p.R50X among all the mutated alleles was 68%–72% (France) [Aquaron et al., 2007; Vieitez et al., 2011], 58% (Germany) [Deschauer et al., 2007], 43% (Italy) [Bruno et al., 2006], 31% (Netherlands) [Martin et al., 2004], 77%–81% (UK) [el-Schahawi et al., 1996; Quinlivan et al., 2010], 63%–60% (USA) [Bartram et al., 1993; Tsujino et al., 1993], and 50% (Brazil) [Gurgel-Giannetti et al., 2013]. However, regarding patients of Asian descent, the p.R50X mutation has only been described in a Korean patient [Sohn et al., 2008] and has yet not been detected in the Japanese population [Sugie et al., 1995]. Given its high frequency in most populations, starting with screening for p.R50X is recommended, except in patients of Asian descent. An easy diagnostic approach consists of amplifying exon 1 and performing a restriction fragment length polymorphism (RFLP) analysis with the enzyme *Nla*III, which in the presence of p.R50X, cuts the polymerase chain reaction (PCR) fragment in three pieces [Tsujino et al., 1993]. One cut of *Nla*III is due to the presence of the mutation while the second cut is due to a restriction site localized in the starting codon.
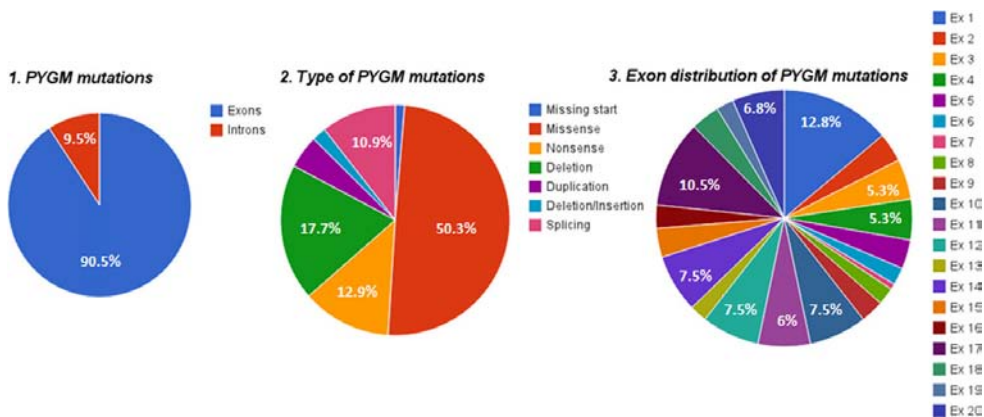
Figure 1. *PYGM* mutations types and exon distribution. **A:** Mutations located in exons and introns. **B:** Frequency of the different types of mutations. **C:** Distribution of the mutations between the 20 exons of the *PYGM* gene.
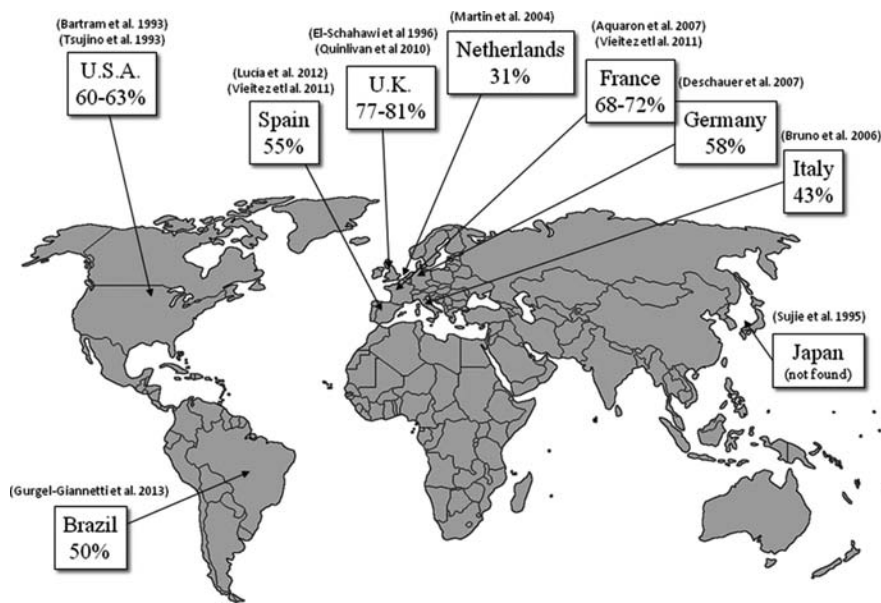
Figure 2. World map with distribution of the *p.R50X* mutation. Distribution of *p.R50X* mutation frequency, described in studied populations.

The *PYGM* gene accounts for other mutations, some of them frequently found in different geographical locations, others mostly in patients of the same country, and some only in a reduced number of patients. Depending on the population, c.613G>A (also p.G205S) is the second or third most frequent mutation, accounting for 3%–10% of the mutated alleles in some cohorts [Tsujino et al., 1993; Rubio et al., 2007; Vieitez et al., 2011; Lucia et al., 2012], but also being hardly detectable or not detectable at all in other cohorts [Sugie et al., 1995; Bruno et al., 2006; Deschauer et al., 2007; Vieitez et al., 2011]. The c.2392T>C (also p.W798R) mutation is the second most frequent mutation in Spanish population, with a frequency of 10%–17% [Lucia et al., 2012]. Accounting for 68% of the mutated alleles, p.F710del is the most frequent mutation in Japanese population [Sugie et al., 1995]. A growing number of mutations

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are only found in few patients of a given country or just in one patient or in some members of the same family. However, this does not imply that we have now a worldwide view of *PYGM* mutations: there is an urgent need to perform screening of McArdle disease in many regions, where this information is actually missing. A further knowledge of the *PYGM* mutational spectrum is needed for a better design of specific diagnostic flowcharts (see section on diagnosis).

Transcriptomic studies have unveiled the pathogenic effect of some mutations such as intronic mutations, large deletions, and silent mutations [Fernandez-Cadenas et al., 2003; Garcia-Consuegra et al., 2009]. Mutations in the donor or acceptor splicing site of *PYGM* have been described; however, splicing alterations have only been characterized in some cases. The c.1768+1G>A mutation in the donor splicing site of intron 14, causes a deletion of 67 bp at the 3′ region of exon 14 [Tsujino et al., 1994bb], and leads to an alteration in the open-reading frame and a premature termination codon (PTC) in the sequence, p.V568AfsX16. The c.528-8G>A mutation in the acceptor splicing sequence of intron 4 retains six nucleotides after splicing of intron 4, leading to the p.Q176_M177insVQ variation in the open-reading frame [Garcia-Consuegra et al., 2009]. Mutations in other introns have also been described, but their effect at the transcript level has not been studied yet. Transcriptomic studies have also revealed deletion of the full exon 17 in the transcript sequence of one patient [Garcia-Consuegra et al., 2009]. Interestingly, some silent mutations that do not alter the amino acid sequence, that is, c.645G>A (p.K215K), c.1827G>A (p.K609K), and c.2430C>T (p.G810G), have been demonstrated to affect splicing [Fernandez-Cadenas et al., 2003; Garcia-Consuegra et al., 2009].

The effect of some *PYGM* mutations at the transcription level through a mechanism known as "nonsense-mediated mRNA decay" (NMD) has been characterized [Nogales-Gadea et al., 2008; Sohn et al., 2008; Garcia-Consuegra et al., 2009; Nogales-Gadea et al., 2010]. A study of 28 Spanish McArdle patients found that NMD was acting in 92% of them [Nogales-Gadea et al., 2008]. NMD is a homeostatic mechanism that regulates the quality of the transcripts inside each cell by degrading those that contain PTCs. These PTCs can be contained in the genome and generated directly by inherited mutations such as nonsense mutations, or indirectly by mutations that alter the open-reading frame. In the case of McArdle disease, the high number of patients showing NMD of *PYGM* transcripts can be largely explained by the high frequency of the p.R50X nonsense mutation together with the fact that deletions and nonsense mutations are the second and third most frequent type of mutation in the *PYGM* gene. The action of NMD explains, at least partly, why patients typically present null myophosphorylase activity in muscle biopsies. In fact, therapies trying to control this mechanism are being considered as a potential treatment strategies in McArdle patients, since just retaining some residual enzyme activity would likely result in a clinical improvement compared with having no enzyme activity at all [Schroers et al., 2006; Birch et al., 2013].

Polymorphisms

In this review, we have selected *PYGM* polymorphisms with a minor allele frequency (MAF) $\geq$ 1% in order to identify those variants that are relatively common in the general population and as such are not likely to be pathogenic per se according to the principles of natural selection. In the data obtained from the UCSC genome browser (http://genome-euro.ucsc.edu; GRCh37/h19 assembly, chr11:64513861-64528187, dbSNP141, "Common SNPs [141]"), up to 39 different polymorphisms, with a MAF $\geq$ 1% have been described in the *PYGM* gene (Table 1). Among them,

82% (32 out of 39) are found in intronic regions, whereas only 18% (seven out of 39) are exonic. The majority of intronic polymorphisms (59%; 19 out of 39) are found in introns 5, 16, and 17, whereas the rest are equally distributed throughout the remaining introns (with the exception of introns 2, 6, 7, and 19, where no polymorphisms have been described) (Table 1). The higher presence of polymorphisms in introns 5, 16, and 17 should not be surprising, as these introns, along with intron 1, are the largest in the *PYGM* gene. With regards to their distribution, none of the polymorphisms are found in functionally relevant intronic regions such as 5′ and 3′ splice sites, the polypyrimidine tract, or the branch point. As for exonic polymorphisms, only one of them is found in the 5′UTR of the exon 1 sequence (rs483962), whereas the remaining six (rs71581787, rs11231866, rs11231865, rs114138772, rs61736659, and rs2230309) are found in the coding region of the gene (exons 10, 11, 12, 13, 16, and 17, respectively). Four of these polymorphisms cause a change in the encoded amino acid (rs71581787, rs11231866, rs61736659, and rs2230309), whereas the other two (rs11231865 and rs114138772) do not change the amino acid sequence (synonymous change). As expected, the MAF of the synonymous polymorphisms (A:3.7% in rs11231865 and C:1.2% in rs114138772) is not higher compared with those polymorphisms that produce an amino acid change (A:2.1% in rs71581787; C:9.0% in rs11231866; C:1.0% in rs61736659; and A:1.8% in rs2230309). Of note, the rs114138772 polymorphism was found in two unrelated McArdle patients, although its potential contribution to the pathogenicity of the disease was discarded, as this silent variant did not alter RNA splicing [Duno et al., 2009].

Apart from the 39 polymorphisms found in the *PYGM* gene with a MAF $\geq$ 1%, an additional list of 200+ polymorphisms can be found in the UCSC genome browser (GRCh37/h19 assembly, chr11:64513861-64528187, dbSNP141, "All SNPs [141]") for the *PYGM* gene. However, we will not refer to them in this section as their MAF is either unknown or <1%, and as such further research is needed to rule out an actual effect in myophosphorylase function. In this regard, the apparently silent *PYGM* polymorphism c.1827G>A (p.K609K) reported in one Spanish patient was actually found to severely alter mRNA splicing, and as such was finally classified as a pathogenic mutation instead of a polymorphism [Fernandez-Cadenas et al., 2003; Supp. Table S1]. Of note, two other polymorphisms were reported in the literature: c.529-22C>T and c.772-5C>T [Duno et al., 2009]. The c.529-22C>T was reported in two unrelated McArdle patients, one presenting the pathogenic mutations c.148C>T (p.R50X) and c.2385_2386delAA (p.E797VfsX18) and the other harboring the c.148C>T *(p.R50X)* and c.1466C>G (p.489R) mutations. The second reported polymorphism, c772-5C>T, was also identified in a McArdle patient in whom the pathogenic mutation for each *PYGM* allele was identified (c.107T>C [p.L36P] and c.1239+1G>A). Although both c529-22C>T and c772-5C>T variants were classified as polymorphisms, further studies are needed to evaluate the frequency of these two variants in a control population and to discard their potential pathogenicity.

Biological Relevance

Myophosphorylase Regulation and Function

Myophosphorylase (1,4 a-D-glucan: orthophosphate a-D-glucosyltransferase, E.C. 2.4.1.1) catalyzes the intracellular degradation of glycogen into glucose-1-phosphate, which is the rate-limiting step in glycogen catabolism: $(\alpha 1,4\text{-glucoside})_n + Pi \leftrightarrow (\alpha 1,4\text{-glucoside})_{n-1} + \alpha\text{-glucose-1P}$. Thus, this enzyme plays a crucial

Table 1. Described Polymorphisms in the *PYGM* Gene

Polymorphism	Position (dbSNP build 141)	Strand	DNA sequence change	Exon	Codon change	Allele frequency	HapMap populations	References
rs483962	chr11:64527751	Negative	C>T	1	5'UTR	C:28.4%; T:71.6%	MAF >5% in all 11 populations	N.A.
rs477549	chr11:64527080	Negative	c.243+48A>G	Intron 1	N.A.	A:74.8%; G:25.1%	MAF >5% in all 11 populations	N.A.
rs118038492	chr11:64526352	Positive	c.243+775C>T	Intron 1	N.A.	C:98.7%; T:1.2%	Not. Det.	N.A.
rs61884454	chr11:64525843	Positive	c424.+66A>G	Intron 3	N.A.	A:2.3%; G:97.6%	Not. Det.	N.A.
rs489192	chr11:64525644	Negative	c660.+74A>C	Intron 4	N.A.	A:71.8%; C:28.1%	MAF >5% in all 11 populations	N.A.
rs490980	chr11:64525464	Positive	c.660+254C>T	Intron 4	N.A.	C:35.8%;T:64.1%	MAF >5% in CEU, CHB, JPT, YRI	N.A.
rs589691	chr11:64525216	Positive	c.855+35C>T	Intron 5	N.A.	C:34.0%; T:66.0%	MAF >5% in all 11 populations	N.A.
rs630966	chr11:64524911	Positive	c.855+340C>G	Intron 5	N.A.	C:28.0%; G:72.0%	Not. Det.	N.A.
rs7938455	chr11:64524781	Positive	c.855+470C>T	Intron 5	N.A.	C:80.5%; T:19.4%	Not. Det.	N.A.
rs73494206	chr11:64524752	Positive	c.855+499A>G	Intron 5	N.A.	A:95.0%; G:5.0%	Not. Det.	N.A.
rs547066	chr11:64523494	Positive	c.855+1756A>C	Intron 5	N.A.	A:22.5%; C:77.5%	MAF >5% in 9 populations (A<5% in ASW and YRI)	N.A.
rs75633423	chr11:64523035	Positive	c.855+2215+C>G	Intron 5	N.A.	C:1.4%; G:98.6%	Not. Det.	N.A.
rs625172	chr11:64522066	Positive	c.999+99A>G	Intron 8	N.A.	A:30.3%; G:69.6%	MAF >5% in all 11 populations	N.A.
rs71581787	chr11:64521406	Positive	c.1184A>G	10	p.395T>M	A:2.1%; G:97.9%	Not. Det.	N.A.
rs192139668	chr11:64521279	Positive	c.1239+72C>T	Intron 10	N.A.	C:98.8; T:1.2%	Not. Det.	N.A.
rs11231866	chr11:64521154	Positive	c.1240C>G	11	p.414R>G	C:9.0%; G:91%	Not. Det.	N.A.
rs2959652	chr11:64520942	Positive	c.1240+49G>T	Intron 11	N.A.	G:35.9%; T:64.1%	MAF >5% in all 11 populations	N.A.
rs11231865	chr11:64520569	Positive	c.1494G>A	12	syn	A:3.7%; G:96.3%	MAF >5% in ASW, LWK, YRI (A<5% in CEU, MEX, MKK, TSI)	N.A.
rs565688	chr11:64520374	Positive	c.1518+171A>T	Intron 12	N.A.	A:89.0%; T:11.0%	A:100.0% in CEU, CHB, JPT, YRI	N.A.
rs7126110	chr11:64520255	Positive	c.1518+290C>G	Intron 12	N.A.	C:93.4%; G:6.5%	C:100.0% in JPT, YRI (G<5% in CEU, CHB)	N.A.
rs114138772	chr11:64519926	Positive	c.1569C>G	13	syn	C:1.2%; G:98.8%	Not. Det.	Duno et al. (2009)
rs686171	chr11:64519345	Positive	c.1768+51G>A	Intron 14	N.A.	A:17.5%; G:82.5%	Not. Det.	N.A.
rs532747	chr11:64519062	Positive	c.1827+7C>T	Intron 15	N.A.	C:16.7%; T:83.3%	MAF >5% in 10 populations (C<5% in YRI)	N.A.
rs61736659	chr11:64518809	Positive	c.1957C>G	16	p.653L>V	C:1.0%; G:99.0%	Not. Det.	N.A.
Rs111543138	Chr11:64518530-31	Positive	c.1969+267insTG	Intron 16	N.A.	(-):95.4%;TG:4.6%	Not. Det.	N.A.
rs592521	chr11:64518525	Negative	c.1969+272C>T	Intron 16	N.A.	C:14.7%; T:85.3%	Not. Det.	N.A.
rs2071320	chr11:64518520	Negative	c.1969+277A>G	Intron 16	N.A.	A:14.5%; G:85.5%	Not. Det.	N.A.
rs592532	chr11:64518517	Negative	c.1969+280A>G	Intron 16	N.A.	A:85.6%; G:14.4%	Not. Det.	N.A.
rs592546	chr11:64518511	Positive	c.1969+286C>G	Intron 16	N.A.	C:85.5%;G:14.5%	Not. Det.	N.A.
rs506354	chr11:64518504	Positive	c.1969+293C>T	Intron 16	N.A.	C:15.7%; T:84.3%	Not. Det.	N.A.
rs2230309	chr11:64517999	Negative	c.2026A>G	17	p.G676S	A:1.8%; G:98.2%	Not. Det.	N.A.
rs474006	chr11:64517326	Positive	c.2177+522A>G	Intron 17	N.A.	A:71.0%; C:29.0%	Not. Det.	N.A.
rs608261	chr11:64517317	Negative	c.2177+531A>G	Intron 17	N.A.	A:1.6%; G:98.4%	Not. Det.	N.A.
rs1207113	chr11:64517047	Positive	c.2177+801A>G	Intron 17	N.A.	A:69.8%; G:30.2%	Not. Det.	N.A.
rs9704315	chr11:64517039	Positive	c.2177+809A>G	Intron 17	N.A.	A:4.8%; G:95.2%	Not. Det.	N.A.
rs555974	chr11:64516477	Positive	c.2177+1371G>T	Intron 17	N.A.	G:72.1%; T:27.9%	Not. Det.	N.A.
rs28398896	chr11:64516308	Positive	c.2177+1540A>G	Intron 17	N.A.	A:5.7%; G:94.3%	Not. Det.	N.A.
rs566653	chr11:64515622	Positive	c.2177+2226A>G	Intron 17	N.A.	A:2.0%; G:98.0%	A<5% in CEU, YRI	N.A.
rs569602	chr11:64514506	Positive	c.2312+190A>G	Intron 18	N.A.	A:80.0%; G:20.0%	MAF >5% in all 11 populations	N.A.

Common polymorphisms(≥1%) build 141.
GenBank reference NG_013018.1.
5'UTR, 5' untranslated region; N.A., nonapplicable; A, adenosine; G, guanosine; C, cytidine; T, thymidine; (-), lack of insertion TG; MAF, minor allele frequency; CEU, Utah residents with ancestry from northern and western Europe; CHB, Han Chinese in Beijing, China; JPT, Japanese in Tokyo, Japan; YRI, Yoruba in Iabadan, Nigeria; ASW, African ancestry in SouthWestern United States; LWK, Luhya in Webuye, Kenya; MEX, Mexican ancestry in Los Angeles, CA; MKK, Masai in Kinyawa, Kenia; TSI, Toscani in Italia; Not. Det., not determined.

role for maintaining the cellular and organismal glucose homeostasis. Myophosphorylase was first described by Cori and Cori (1936), who 2 years later demonstrated that the enzyme was allosterically regulated by adenosine monophosphate (AMP) [Cori et al., 1938]. As early as 1943, it was shown that myophosphorylase is susceptible to be covalently modified, thereby generating an interconversion between the active and the inactive form [Green and Cori, 1943]. In 1955, myophosphorylase was the first protein reported to be regulated by reversible protein phosphorylation [Fischer and Krebs, 1955]. In 1973, the crystalline structures of human myophosphorylase *a* and *b* were obtained and characterized, showing that their native forms could either exist as dimers or tetramers, although the dimeric structure appeared to be preferred by both enzyme forms [Assaf and Yunis, 1973]. In the following years, the 6A X-ray crystal structure of the inactive *b* form as well as the 3-A structure of *a* form were obtained [Johnson et al., 1974; Fletterick et al., 1976]. In

1981, Withers et al. (1981) found that pyridoxal 5′-phosphate is an essential cofactor for myophosphorylase activity that participates in catalysis through a direct, noncovalent contact with the phosphate substrate or the phosphate group of the glucose-1-phosphate product.

From those early findings, it was later discovered that: (1) although myophosphorylase is activated by the binding of AMP and inosine monophosphate (IMP), this enzyme can also be inhibited by the binding of adenosine triphosphate and glucose-6-phosphate [Barford and Johnson, 1989; Barford et al., 1991; Johnson, 1992]; and (2) phosphorylase kinase is responsible for the phosphorylation of the Ser-15 of myophosphorylase, thereby converting the inactive *b* form to an active *a* form [Krebs et al., 1958]. While the nonphosphorylated form of the enzyme is activated by micromolar concentrations of AMP, the phosphorylated form does not require AMP binding for its activation [Barford et al., 1991]. In this regard, Helmreich and Cori (1964) showed that the activity of the myophosphorylase *b* form is critically dependent on AMP binding when *Pi* concentrations are low, whereas the specific activity of the *a* form barely increases 5%–10% in the presence of AMP. Additionally, they also demonstrated that AMP increases myophosphorylase *b* activity, thereby enhancing the affinity of the enzyme for *Pi* and glycogen.

Myophosphorylase Structure and Domains

The myophosphorylase dimer is made up of two identical monomers of 842 amino acids with a molecular weight of 94,500 Da. Each monomer is composed by two different domains: an N-terminal and a C-terminal domain (Fig. 3). The N-terminal domain comprises the residues 1–484 and is composed by 15 α-helices and nine β-strands, whereas the C-terminal domain comprises residues 485–842 and consists of five α-helices and six β-strands [Sprang et al., 1982; Barford et al., 1991]. The N-terminal domain contains: the phosphorylation site for Ser-15; the phosphorylated Ser-15 contacts, which are part of the subunit interphase involved in the subunits interaction to form the dimer; the AMP allosteric effector and glycogen binding sites; and part of the catalytic domain. The C-terminal domain includes the major part of the catalytic domain, as well as the pyridoxal 5′-phosphate binding and nucleoside inhibitor sites [Sprang et al., 1982].

The first 20 residues of the N-terminal domain are mainly made up of basic amino acids that are highly unstructured, and interact with a close pocket of acidic residues (Asp 110, Glu 111, Glu 121, Glu 502, Glu 506, and Glu 510) generating intrasubunit contacts when the enzyme is not phosphorylated [Johnson, 1992]. When Ser-15 is phosphorylated, the amino-terminal residues 11–23 swing ∼120° from their position and modify their contacts from intrasubunit to intersubunit; specifically, Ser-15 makes ionic contacts with Arg 70 from its own subunit and Arg 44 from the other subunit [Barford et al., 1991; Johnson and Barford, 1994; Oikonomakos et al., 2000]. Thus, through phosphorylation of the enzyme, these N-terminal residues from the subunit interphase play an important role in the subunit–subunit interaction. Additionally, Ser-15 phosphorylation displaces the N-terminal domain from the acidic residues generating a conformational change that prevents the catalytic site from being blocked by residues of the C-terminal domain [Barford et al., 1991; Johnson and Barford, 1994; Oikonomakos et al., 2000].

Another regulator of the enzyme activity found in the N-terminal domain is the AMP allosteric effector site; this site is composed by a cleft at the interphase of the two subunits [Barford et al., 1991], in which AMP and IMP binding occurs through interactions between AMP/IMP phosphate and Arg 310 and 311 [Barford and Johnson,

1989] as well as other residues from the N-terminal domain (Asn 45, Gln 73, and Tyr 76) [Barford et al., 1991]. However, in contrast with phosphorylation-mediated activation, the activation of myophosphorylase *b* by AMP does not generate structural changes in the N-terminal residues [Gusev et al., 1979]. Finally, the glycogen binding site is located at the surface of the enzyme, more than 30 amino acids away from its catalytic and allosteric sites and is mainly formed by residues 398–438 [Johnson, 1992].

The C-terminal domain, also referred to as the "catalytic domain," contains the Lys 681 that covalently binds the cofactor pyridoxal 5′-phosphate [Withers et al., 1981]. It contains residues 378–385 from the N-terminal domain and forms the catalytic site as a deep crevice between the domains of the protein [Newgard et al., 1989; Johnson, 1992]. The active site has a moveable gate constituted by the 280's loop (formed by residues 281–289). In the inactive state of the myophosphorylase *b* form (also referred to as "T-state"), the catalytic site is blocked by the 280's loop [Johnson, 1992; Buchbinder and Fletterick, 1996]. However, through allosteric activation, the 280's loop is disordered and displaced from the catalytic site, whereas Arg 570 occupies a position close to that previously occupied by Arg 284 and helps to create a higher affinity recognition site for the phosphate substrate [Johnson, 1992]. For this reason, the 280's loop has been referred to as the catalytic active site gate. The nucleoside inhibitor site is located at the entrance of the catalytic site and binds purine analogs or related heterocyclic ring compounds such as adenosine, caffeine, flavin mononucleotide, reduced form of nicotinamide adenine dinucleotide, and AMP at high concentrations (>2 mM). The fused ring compounds intercalate two aromatic rings of Phe 286 and Tyr 614 and stabilize the location of the 280's loop in the inactive state of the protein [Kasvinsky et al., 1978].

Among the 147 mutations described to date (Supp. Table S1), 133 are located in the coding region of the gene distributed 70 in the N-terminal domain of the protein and 63 are located in the C-terminal domain, making an average of 15.8 mutations per 100 residues and one mutation every 6.3 residues. The reported mutations are spread throughout the whole sequence of the monomer, starting with the c.1A>C (also p.M1L)/c.1A>G (also p.M1V) mutations and finishing with the c.2477G>C (also p.W826S) mutation. Although some mutations have been reported in the subunit interphase, the AMP allosteric effector site, the glycogen storage site and the catalytic site, no mutations affecting the phosphorylated Ser-15, the 280's loop, the pyridoxal 5′-phosphate, and the nucleoside inhibitor binding sites have been described. There are three subsequences from the monomer that present a particularly low proportion of reported mutations: residues 246–346 (six mutations), residues 398–448 (three mutations), and residues 609–648 (two mutations). Concerning the distribution of mutations per domains, at least 21 different mutations have been described in the subunit interphase from the N-terminal domain, which are involved in the subunit–subunit interaction (∼ residues 1–85). The insertion/deletions causing the c.46delGinsTT (also p.V16FfsX12) mutation affect the subdomain composed by residues 11–23, which are involved in the generation of the intersubunits contacts when Ser-15 is phosphorylated. On the other hand, no mutation has been reported in the acidic residues Asp 110, Glu 111, Glu 121, Glu 502, Glu 506, and Glu 510, which participate in the intrasubunit contacts with the basic residues of the N-terminal domain when Ser-15 is not phosphorylated. With regards to the AMP allosteric effector site, the c.212_218dupCGCAGCA mutation (also p.Q73HfsX7) generates a missense change in the residue 73 that is directly involved in AMP binding, although it also generates a frameshift in the coding sequence with the final consequence of protein disruption. Interestingly, no mutations have been reported in the 280's loop and only two (c.1193C>T or p.P398L and
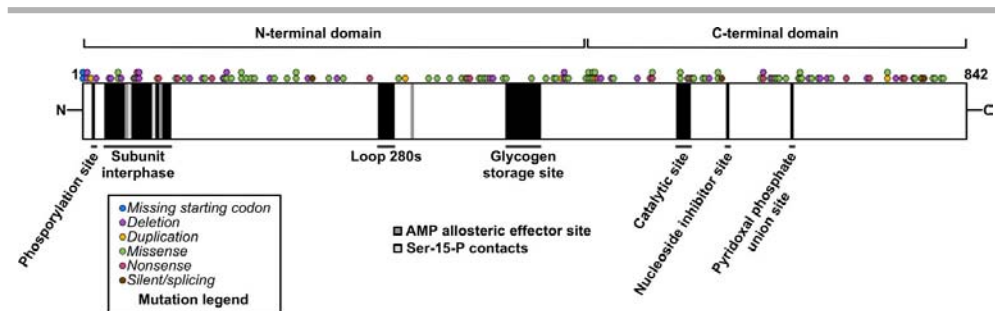
Figure 3. Graphic representation of the primary structure of the myophosphorylase subunit (monomer). The N-terminal (residues 1–484) and C-terminal (residues 485–842) are indicated as horizontal lines above the monomer representation, whereas the different functional domains are underlined and marked as colored sections (see mutation legend). The 138 coding mutations described in the *Pygm* gene are shown as colored dots (see mutation legend) above their corresponding position in the amino acid sequence of the protein.

c.1282C>T or p.R428C) are localized in the glycogen binding site of the protein. These data suggest that most mutations resulting in McArdle disease may affect the complete integrity of the myophosphorylase enzyme (nonsense mutations, mutations affecting dimer formation) rather than diminishing its activity (i.e., mutations in the 280's loop) or its efficiency in substrate binding (i.e., mutations in the glycogen binding site). Nevertheless, three mutations affecting residues from the catalytic domain (c.1147G>A also p.E383K, c.1136C>T also p.T379M, and c.1151C>A also p.A384D) have been described in McArdle patients, indicating that an alteration of these residues may cause absolute depletion of activity.

Mouse Model of McArdle Disease

A knock-in mouse model of the disease was recently generated by our group [Nogales-Gadea et al., 2012]. These mice are homozygous for the most common pathogenic mutation among Caucasians, p.R50X. This mutation was generated in the mouse genome since codon 50 of the *pygm* gene codifies for arginine and was changed to a stop codon through genetic engineering. This amino acid is conserved inside the high homology of *pygm* shared among species [Hudson et al., 1993]. Mice homozygous for this mutation have the same muscle phenotype as McArdle patients, that is, very low transcript levels of *pygm*, the absence of myophosphorylase in western blot analyses and thus total absence of myophosphorylase activity, together with massive glycogen accumulation. Further, mice exercise intolerance is evidenced by their very poor performance in wire grip and treadmill tests. In the wire grip test, the amount of time that p.R50X/p.R50X mice could stay suspended by proximal limbs was ∼90% lower compared with heterozygous or noncarrier (wild type [wt]/wt) mice. Time to exhaustion during graded treadmill running in p.R50X/p.R50X mice was only ∼41% and ∼29% of that attained by their heterozygous and wt/wt counterparts, respectively. Serum CK-activity levels were considerably elevated in p.R50X/p.R50X mice and treadmill exercise triggered myoglobinuria in these animals.

Although the mouse model mimics most of the classical features of the human disease, differences among species also cause some particularities in the animal model. Glycogen accumulation in p.R50X/p.R50X mice is 27-fold higher compared with wt/wt mice, whereas in patients it is "only" ∼twofold higher than normal [Nielsen et al., 2002]. There are also differences in the intracellular

location of glycogen: depending on the muscle, the glycogen accumulation in mice can mostly be subsarcolemmal or intrasarcoplasmic, whereas in humans it is only subsarcolemmal [Nadaj-Pakleza et al., 2009]. Another curious finding is that heterozygous mice have lower treadmill exercise capacity than the wild-types despite showing normal glycogen content in muscle biopsies. Conversely, humans who are heterozygous for *PYGM* mutations are thought to be asymptomatic, at least during daily living [Andersen et al., 2006]. Further research might determine whether partial deficiency of myophosphorylase activity caused by heterozygosity for *PYGM* mutations also impairs maximal exercise capacity in humans.

The recent knock-in mouse model might be useful in helping (1) understanding the altered pathways related to myophosphorylase deficiency, and (2) assessing new therapeutic approaches. The latter can be generic, aiming at improving muscle exercise capacity through direct or indirect target of myophosphorylase. Furthermore, given that the mouse model harbors the stop codon mutation most frequently found in patients, additional approaches include bypassing the stop codon or splice modulation therapies.

Clinical and Diagnosis Relevance

Diagnosis (Diagnostic Options)

From the genetic or transcriptomic point of view, there are different approaches that can be used for the diagnosis of McArdle disease. Among the different methods used are: PCR-RFLP, PCR studies over RNA, high melting resolution, whole-exome sequencing, and cytometry.

The traditional method of PCR-RFLP and Sanger sequencing is still extensively used in reference laboratories [Kubisch et al., 1998; Bruno et al., 2006; Deschauer et al., 2007; Rubio et al., 2007; Gurgel-Giannetti et al., 2013]. The sample needed is DNA that can be obtained from any accessible source (blood, hair follicle, buccal scraping, among others). The best approach is to first target those mutations that are more frequent in the patient's country of origin. For instance, a diagnostic flowchart was proposed for Spanish patients [Rubio et al., 2007] that is likely to make diagnosis faster and cheaper. Specific PCR-RFLP designs have been developed for identification of common *PYGM* pathogenic mutations such as p.R50X, p.G205S, or p.W798R, whose presence can be evidenced by the digestion with the enzymes *Nla*III [Tsujino et al., 1993], *Hae*III

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[Fernandez et al., 2000], and *Bsr*BI [Fernandez et al., 2000], respectively. Although obviously not all mutations can be detected with the above-mentioned approach, it is still a useful method to precede the sequencing reaction [Rubio et al., 2007; Vieitez et al., 2011]. The Sanger sequencing reactions can be accurately performed in any equipped laboratory, since a detailed description of the primers and PCR conditions is available in the literature [Kubisch et al., 1998].

Studies at the mRNA level are useful in those cases where causative mutations cannot be found with DNA analysis or in those cases where researchers want to study the pathogenic effect of the genetic variant at the transcript level: in these cases, RNA from skeletal muscle is needed. The described methods for this approach include cDNA amplification in two overlapping fragments and sequencing reactions with eight internal fragments [Garcia-Consuegra et al., 2009]. In addition, quantification of *PYGM* transcripts can be performed with specific primers and probes for quantitative PCR [Nogales-Gadea et al., 2008].

Three newer and less frequently used techniques have also been described. The first technique is high-resolution melting [Xu et al., 2004; Wu et al., 2011], which has been adapted for the study of the 20 exons of the *PYGM* gene [Wittwer et al., 2003]. With this method, the entire coding region and intronic flanking sequences are amplified in 18 different PCR fragments. If abnormalities in the melting profile of the PCR product are found, the fragment is sequenced. In this way, the sequencing costs in the screening can be reduced by 85%. The second technique is massive parallel sequencing, which has been applied for the diagnosis of patients with different glycogen storage diseases [Wang et al., 2013]. This technique was used for studying 16 genes causative of muscle or liver glycogen storage diseases, resulting in the correct diagnosis in 11 out of 17 patients, some of them harboring large deletions affecting several exons. The third potential technique is flow cytometry analysis of myophosphorylase in white blood cells (T-lymphocytes) using a fluorescent-labeled *PYGM* antibody [de Luna et al., 2014]. Although T-lymphocytes only have very low levels (1%) of *PYGM* transcripts compared with muscle tissue, diagnosis was still feasible in many Spanish patients: *PYGM* expression (as well as transcript levels) was significantly lower in the T-lymphocytes of McArdle patients compared with healthy controls, with a myophosphorylase expression value below 28.6% of normal. This technique is considered to indicate the presence of McArdle disease with 95% sensitivity and 83% specificity.

Genotype–Phenotype Correlation

No correlation between the reported individual variability in the disease severity and type of *PYGM* mutations has been found, which is likely attributable to the fact that almost all reported mutations result in complete deficiency of myophosphorylase activity, and many mutations even result in virtual absence of transcript levels owing to the above-mentioned NMD mechanism [Nogales-Gadea et al., 2008; Sohn et al., 2008]. Yet, recent studies suggest the potential existence of some residual myophosphorylase activity in a few patients. This is the case of a Japanese patient homozygous for the commonest *p.F710del* mutation in the Japanese population [Sato et al., 2012] and two North-American patients carrying two intronic mutations affecting splicing (c.425-26A>G, c.856-601G>A), and in whom 1%–2.5% of glycogen phosphorylase activity was preserved in muscle [Vissing et al., 2009]. In all three patients, the clinical presentation was moderate, which suggested maintenance of some residual myophosphorylase activity.

The patient's level of physical activity is likely the main phenotype modulator of the disease severity. Thus, active patients are much more likely to improve their clinical course (i.e., move to a lower clinical severity category of the above-mentioned Martinuzzi scale) over a 4-year period compared with their inactive peers [Lucia et al., 2012]. Levels of physical activity are also positively associated with patients' functional capacity, as assessed by peak aerobic capacity (VO_{2peak}) determination [Lucia et al., 2012]. In fact, the VO_{2peak} values of physically active patients with total absence of myophosphorylase activity are higher than those recently reported in the two above-mentioned North-American patients presenting with a "mild" form of McArdle disease because they retained some muscle myophosphorylase activity [Vissing et al., 2009]. A positive association was also recently reported between patients' physical activity levels and health-related quality of life [Munguia-Izquierdo et al., 2015].

Future Prospects

McArdle disease is one of the best studied glycogenoses. The number of reported mutations in the *PYGM* gene will increase as new or unstudied populations are reported. Because many laboratories worldwide are equipped to perform genetic diagnosis, the time of diagnosis should be reduced in the coming years. The recently generated mouse model of the disease will likely contribute to improving our understanding of the pathogenesis of this disease and assessing new therapeutic options. However, patients can already benefit from supervised exercise programs as well as from an active lifestyle, which can minimize symptoms, attenuate exercise intolerance, and improve quality of life.

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METABOLIC DISSERTATION

The pathogenomics of McArdle disease—genes, enzymes, models, and therapeutic implications

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Noemi de Luna · Alejandro Lucia · Tomàs Pinós**

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Abstract Numerous biomedical advances have been made since Carl and Gerty Cori discovered the enzyme *phosphorylase* in the 1940s and the Scottish physician Brian McArdle reported in 1951 a previously 'undescribed disorder characterized by a gross failure of the breakdown in muscle of glycogen'. Today we know that this disorder, commonly known as 'McArdle disease', is caused by inherited deficiency of the muscle isoform of *glycogen phosphorylase* (GP). Here we review the main aspects of the 'pathogenomics' of this disease including, among others: the spectrum of mutations in the gene (*PYGM*) encoding muscle GP; the interplay between

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the different tissue GP isoforms in cellular cultures and in patients; what can we learn from naturally occurring and recently laboratory-generated animal models of the disease; and potential therapies.

Introduction

Glycogenosis type V (glycogen storage disease type V [GSD V], McArdle disease or *myophosphorylase* deficiency; OMIM® database number 232600) is a disorder of skeletal-muscle carbohydrate metabolism originally described in 1951 (McArdle 1951). It is one of the most frequent genetic myopathies, *e.g.*, with a prevalence of ~1/167,000 in Spain, with both sexes similarly affected (Lucia et al 2012). This disorder is caused by pathogenic mutations in both copies of the gene (*PYGM*) encoding the muscle isoform of *glycogen phosphorylase* (hereafter abbreviated as GP), also known as *myophosphorylase* (Lucia et al 2008). Starting with a summary of the main phenotype consequences of the disease, here we review the genotype characteristics of the disease as well as available and potential therapy strategies.

Main phenotype consequences are confined to the skeletal muscle — exercise intolerance

The muscle isoform of GP (GP-MM; EC 2.4.1.1) catalyzes and regulates the breakdown of glycogen into glucose-1-phosphate in muscle fibers (by removing α-1,4 glucosyl units from the outer glycogen branches). Thus, patients are unable to obtain energy from their muscle glycogen stores and, as a result, commonly experience *exercise intolerance*. The latter typically consists of acute crises of early fatigue and muscle stiffness and contractures, especially at the start of exercise, that usually disappear if exercise is stopped or intensity is

reduced. These episodes are sometimes accompanied by marked muscle damage or rhabdomyolysis as indicated by the release of intra-muscle proteins (such as *creatine kinase*, CK, EC 2.7.3.2) to the bloodstream. Thus, high serum CK-activity (typically >1000 U/L) after exercise is a common finding that can be accompanied by myoglobinuria (or 'dark urine'), *e.g.*, ~50 % of Spanish patients report recurrent episodes of dark urine throughout their lives (Lucia, Nogales-Gadea et al 2008). The main potential danger of exertional rhabdomyolysis is acute renal failure as well as hyperkalemia, with the former eventually leading to chronic renal failure, albeit reported cases are rare (Lucia, Nogales-Gadea et al 2008). Around 25 % of patients also develop muscle fixed weakness and wasting (affecting more proximal than distal muscles) as they age (Lucia, Nogales-Gadea et al 2008). This phenomenon might be attributed, at least partly, to the cumulative effect of repeated episodes of rhabdomyolysis (Wolfe et al 2000).

Exercise intolerance is present in virtually all patients and often starts during childhood (*e.g.*, in physical education classes or in the school playground), but there is inter-individual variability in the phenotypic manifestation of the disease: in a few people symptoms are triggered only by sports participation while some individuals report functional limitations in activities of daily living, *e.g.*, lifting/carrying light weights, or simply lifting their arms during personal care (Lucia, Nogales-Gadea et al 2008).

PYGM gene and DNA diagnosis

The *PYGM* (HGNC:9726) gene was first identified by Lebo and colleagues (Lebo et al 1984). Mutations in *PYGM* leading to McArdle disease are inherited in an autosomal recessive manner. Three pseudo-dominant cases were described where the transmission was apparently dominant, yet the molecular diagnosis showed that inheritance was autosomal recessive (Hadjigeorgiou et al 2002; Isackson et al 2005; Wu et al 2011). The diagnosis of McArdle disease is currently based on the molecular analysis of DNA obtained from blood samples. This is a minimally invasive method and, given the accumulated knowledge on the genetics of this disease in different populations, it can be highly targeted (Rubio et al 2007a, b; Lucia et al 2012). Gene sequencing after PCR amplification is the most frequently utilized technique for screening the different *PYGM* mutations (Bruno et al 2006; Deschauer et al 2007; Vieitez et al 2011; Gurgel-Giannetti et al 2013). In undiagnosed patients or in patients in whom only one mutation has been found, the RNA analysis of muscle biopsies samples might help to identify mutations that cannot be detected in blood analysis and to unveil their effect at the transcriptional level (Garcia-Consuegra et al 2009).

An algorithm was developed for Spanish patients' diagnosis (Rubio et al 2007a, b). Thus, Rubio and co-workers recommended to search for the commonest p.R50X mutation first (see below) followed by the relatively frequent p.W798R and p.G205S mutations, and thereafter by the complete sequencing of exons 1, 14, 17, and 18. This approach allowed identification of the causative mutation in 75.8 % of patients in a relatively cheap and simple manner, besides avoiding the need to obtain muscle biopsies for histochemical/biochemical diagnosis (Rubio et al 2007a, b). However, this method is likely to be gradually replaced by whole gene next generation approaches.

Mutations — the common pR50X mutation and *PYGM* allelic heterogeneity

The substitution of guanine for thymine at nucleotide 148 of exon 1 (GenBank reference sequence NM_005609.1) is the commonest mutation among Caucasians. This variation, originally known as p.R49X and thereafter renamed as p.R50X (den Dunnen and Paalman 2003), shows an allele frequency above 50 % in the studied cohorts of Caucasian McArdle patients (Bartram et al 1994; el-Schahawi et al 1996; Martin et al 2004; Bruno et al 2006; Aquaron et al 2007; Deschauer et al 2007; Rubio et al 2007a, b; Gurgel-Giannetti et al 2013). This mutation is rare among Asian people (only one case, of a Korean patient, has been reported (Sohn et al 2008)), and no studies have been published in African populations. However, in mixed populations of patients from the United States, this mutation accounts for more than 50 % of the mutant alleles (Bartram et al 1993; Tsujino et al 1995). Thus, when the existence of McArdle disease is suspected, this mutation should be the first to be studied in most patient populations.

More than 150 mutations have been reported since the first *PYGM* pathogenic mutations were described in 1993 (Tsujino et al 1993; Bartram et al 1994), which reflects the genetic heterogeneity of this disease. Because mutations have been found in each of the 20 *PYGM* exons, there is likely no 'hot spot' region. However, exons 1 and 17 are especially dense in mutations. Some mutations are apparently ethnic-specific, such as p.W798R (Spain), p.F710del (Japan) or p.E451X (Finland). Numerous mutations have only been found in a single patient or in members of a family, and are thus referred to as 'private' mutations.

The number of molecular diagnosis studies using complementary DNA (cDNA) analysis has considerably increased in the last years. The first silent mutation described, c.1827G>A (formerly known as p.K609K), results in altered mRNA splicing, causing rearrangements of exons and introns through deletions and insertions (Fernandez-Cadenas et al 2003). A more recent study from our group unveiled a new silent mutation c.645G>A (formerly known as p.K215K) as well

as other mutations that produce alterations at the RNA level (Garcia-Consuegra et al 2009).

The interplay between GP isoforms

In the 1940s, Carl and Gerty Cori discovered the enzyme *phosphorylase* (Simoni et al 2002), which they showed to be responsible for degrading glycogen into glucose 1-phosphate (the latter, which they had isolated from minced frog muscle, was historically referred to as the 'Cori ester') (Simoni et al 2002). Three different GP isoforms exist: the muscle (GP-MM), the brain (GP-BB, OMIM database number 38550), and the liver isoform (GP-LL, OMIM database number 613741), which are encoded by the *PYGM*, *PYGB* (HGNC: 9723), and *PYGL* gene (HGNC: 9725), respectively (Fig. 1). The three genes are orthologs with high sequence homology (>80 % amino acid identities) (Newgard et al 1986) suggesting a common origin by gene duplication (Newgard et al 1988).

Tissue-specific expression of the three GP isoforms

Although the three isoenzymes possess the same function, that is, to initiate glycogen breakdown by removing α-1,4 glucosyl residues from the outer branches of glycogen with liberation of glucose-1-phosphate, there are tissue-specific differences. In muscle, GP expression is involved in the provision of energy for the contraction of myofibrils; in liver, GP acts to secure the maintenance of blood glucose levels; and, in other tissues such as heart and brain its helps maintain energy availability during brief periods of anoxia. The GP function in the brain might also be associated with the supply of energy to glial cells for rapid neurotransmitter clearance (Shulman et al 2001).

Although the GP-MM is mainly found in the skeletal muscle and in a minor proportion, in the heart muscle, it can be also expressed in the brain (Bresolin et al 1983; David and Crerar 1986).

Differential response of the GP isoforms to extracellular/intracellular signals

The GP isoforms exist in active (a) and inactive (b) forms, and the inter-conversion between these forms is catalyzed by *phosphorylase kinase* and *phosphorylase phosphatase* (Graves et al 1960; Lederer et al 1975; Simoni et al 2002). The GP-BB is very sensitive to activation by the allosteric factor AMP whereas GP-MM is more prone to be activated by phosphorylation of its Ser 14 amino acid through the
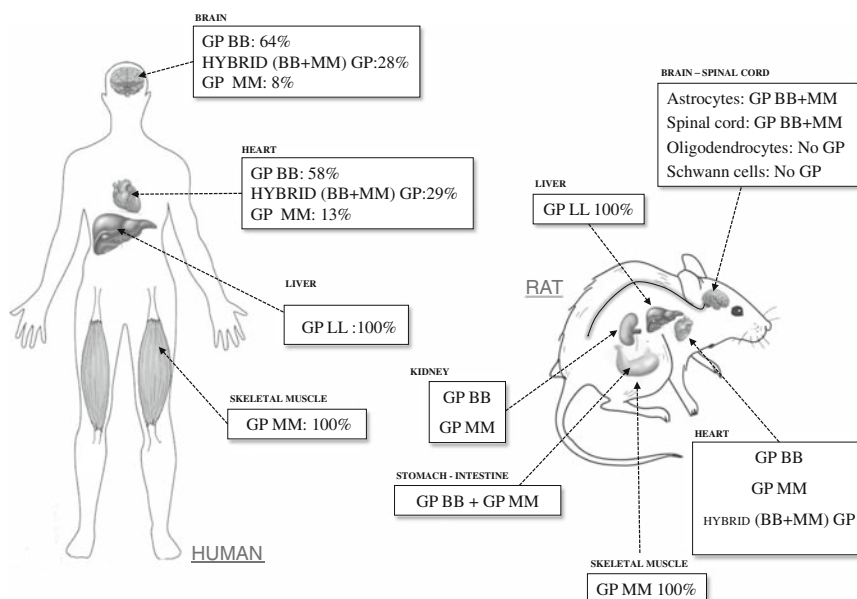
Fig. 1 Summary of the expression pattern of the different *glycogen phosphorylase* (GP) isoforms in human and rat tissues. Skeletal muscle is under 'normal conditions' (*i.e.*, with no severe episode of rhabdomyolysis in the previous hours or days inducing a marked regeneration process)

phosphorylase kinase enzyme, and is not as sensitive to intracellular changes in AMP concentration (Newgard et al 1989). Thus, the different patterns of activation of the two isoforms might result in different responses to various cell-energy demands.

The mystery of the 'reappearing enzyme' in patients' muscle

Immunohistochemical staining of GP is usually negative in the patients' muscle, with some exceptions owing to the re-expression of GP-BB in regenerating fibers (also known as fetal GP) (DiMauro et al 1978), especially after exercise-induced muscle damage (Mitsumoto 1979). Although the latter phenomenon occurs in scatter fibers of the muscle biopsy it can account for a considerable number of fibers in one tissue section (Mitsumoto 1979; Martinuzzi et al 1999). The underlying molecular signals activating the expression of the fetal GP isoform in regenerating fibers are unknown, but some developmental pathways might be involved.

The mystery of the 'reappearing enzyme' in cell cultures

Professor DiMauro and co-workers nicely described for the first time (DiMauro et al 1977) the curious fact that GP activity reappears in muscle cells cultured from McArdle patients' biopsies. At that time the existence of the different GP isoforms had already been reported (Proux et al 1974) yet the tools to differentiate the three isoforms were limited.

Thereafter different publications approached this 'mystery' and came up with a different conclusion, that is, the GP activity expressed in cultures corresponded to GP-BB (DiMauro et al 1978), GP-BB, and GP-LL (Sato et al 1977) or GP-MM (Meienhofer et al 1977; Martinuzzi et al 1993).

In 2010, our group studied cell cultures in muscle biopsies of two brothers with McArdle disease (Nogales-Gadea et al 2010). Muscle GP expression was strongly down-regulated compared to the muscle; the GP-BB was the major contributor at the beginning of the culture, whereas after 12 days of differentiation GP-BB and GP-LL were equally expressed (Fig. 2). Thus, the pattern of isoform expression changes during in vitro differentiation, a phenomenon that should be taken into account in research studies. We did not observe differences in the total GP activity of cultured muscle cells between patients and healthy controls, a finding that is in agreement with previous reports (DiMauro et al 1978; Martinuzzi et al 1993). However, expression of GP active forms was lower (~-50 %) in patients' cells compared with controls whereas there were no differences in glycogen content, which is also in line with previous research (Meienhofer et al 1977; DiMauro et al 1978).

To avoid the limitations of obtaining patients' muscle cell cultures (which inevitably requires performing unpleasant muscles biopsies), a recent *PYGM*-transfected cellular model was generated (Birch et al 2013). This model showed high levels of muscle GP mRNA and protein. Thus, it could represent a promising tool not only to study the functional consequences of the different *PYGM* mutations, but also to assess how different drugs can regulate mRNA and protein production.
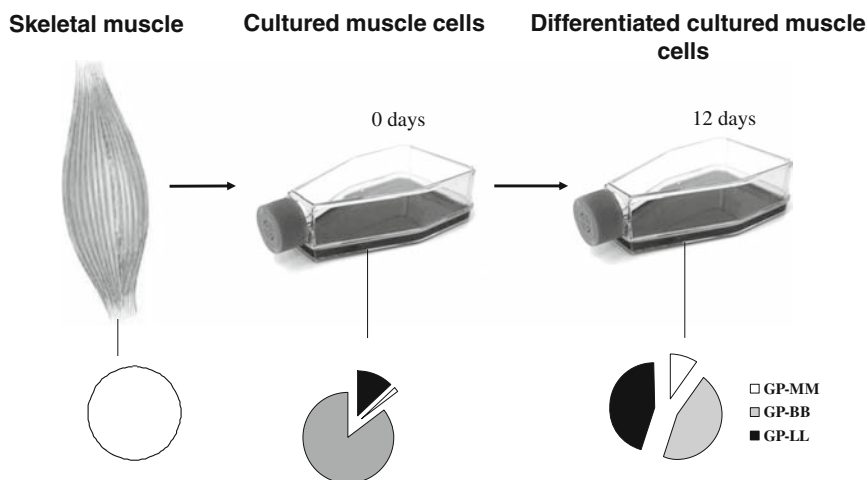
Fig. 2 GP isoforms from cultured to differentiated muscle cells. Skeletal muscle is under 'normal conditions' (*i.e.*, with no severe episode of rhabdomyolysis in the previous hours or days inducing a marked regeneration process)

Results

Naturally occurring models in McArdle disease

The first animal described was a Charolais calf, which presented with continued recumbency, severe rhabdomyolysis and electrolyte imbalance after forced exercise (Angelos et al 1995). The disease is caused by a C-to-T substitution at codon 489, which generates an arginine-to-tryptophan substitution (Tsujino et al 1996). The mutated nucleotide is highly conserved in the three different GP isoforms from various tissues/species, with immunoblot data reflecting that the mutant enzyme is rapidly degraded (Tsujino et al 1996).

The second model was identified in the Merino sheep flock of Western Australia, which showed exercise intolerance, lack of muscle GP and excess of muscle glycogen (Tan et al 1997). The disease phenotype is caused by a single base change at the 3' splice site of intron 19 in the *Pygm* gene, which activates a cryptic splice site within exon 20. This results in the disruption of the reading frame and in a premature termination codon that truncates the last 31 amino acids of the protein.

A recently generated 'knock-in' mouse model

Although both bovine and ovine models mimic the human disease phenotype, they are difficult to manipulate and to share among different research groups. These limitations prompted us to develop a 'knock-in' mouse homozygous for the p.R50X mutation (Nogales-Gadea et al 2012). Biochemical and molecular analyses of the muscle extracts from the p.R50X/p.R50X mice revealed complete absence of GP-MM protein and activity, and very low levels of *Pygm* mRNA (~90 % lower compared with wild-type mice). Muscle biopsies showed massive glycogen accumulation and negative GP-MM staining. Further characterization also confirmed the McArdle disease-like phenotype, as homozygous mice presented with 'hyper-CK-emia', myoglobinuria, and very poor performance in treadmill running and wire grip tests (~95 % lower compared with wild-type mice). Thus, we believe this new model is adequate for evaluating potential therapies.

The main characteristics and advantages and disadvantages of the different animal models are summarized in Table 1.

Potential treatment approaches

No significant beneficial effects have been reported in McArdle patients receiving branched chain amino acids (MacLean et al 1998), depot glucagon (Day and Mastaglia 1985), dantrolene sodium (Poels et al 1990), verapamil (Lane et al 1986), vitamin B6 (Phoenix et al 1998) (except in one recent case report (Sato et al 2012)), or high-dose oral ribose (Steele et al 1996). More controversial are the effects of creatine supplementation: low-dose supplementation (60 mg/kg/day for 4 weeks) attenuated muscle complaints in five out of the nine McArdle patients tested (Vorgerd et al 2000) but higher doses (150 mg/kg per day) actually exacerbated exercise-induced myalgia for unknown reasons (Vorgerd et al 2002).

The insertion (I)–deletion (D) polymorphism of the *angiotensin-converting enzyme* (*ACE*) gene is a modulator of McArdle disease severity. The *ACE* I-allele, which is associated with lower enzyme activity, improved cardiovascular function and higher uptake of blood glucose into skeletal muscle fibers (Williams et al 2000), favors a less severe clinical presentation (Martinuzzi et al 2003; Rubio et al 2007a, b) as well as higher exercise capacity (as assessed by peak oxygen consumption, VO_2peak) in McArdle patients compared with the D-allele (Gomez-Gallego et al 2008). In this regard, 12-week treatment with the ACE inhibitor ramipril (2.5 mg/day) attenuated disability in McArdle patients, and also improved VO_2peak in those harboring the D/D genotype (Martinuzzi et al 2008).

Rhabdomyolysis is a major medical concern in McArdle disease. Several mechanisms (most occurring even under basal conditions and all likely being exacerbated during exercise) are candidates to contribute to structural muscle fiber fragility and membrane disruption in McArdle patients. These include: (i) the mechanical stress imposed by high muscle glycogen stores (Di Mauro 2007), (ii) the down-regulation of Na^+-K^+ pumps in patients' muscles (Haller et al 1998) (with these pumps being responsible for maintaining cellular volume and integrity), or (iii) the elevated levels of Ca^{2+} in the sarcoplasm (owing to down-regulation of sarcoplasmic reticulum Ca^{2+} ATPase), which might activate proteases, phospholipases and other catabolic enzymes causing only structural damage (Russo et al 1992). Another factor, as indicated by recent research (Kitaoka et al 2013) could be oxidative stress. McArdle patients show increased reliance on the 'purine nucleotide cycle'; this results in the production of NH_3^+ and IMP (Brooke et al 1983; Mineo et al 1985), with IMP being metabolized to inosine and then to hypoxanthine, xanthine, and uric acid via xanthine oxidase (XO), which generates reactive oxygen species (ROS). Compared to healthy, age and physical-activity matched controls McArdle disease patients have higher basal muscle levels of a marker of oxidative stress, muscle protein carbonyls and 4-hydroxynonenal (4-HNE) (Kitaoka et al 2013). With regards to this, recent research has demonstrated that hyper-activation of the XO pathway and subsequent oxidative stress due to physical exercise is one of the main causes of muscular damage in athletes (Gomez-Cabrera et al 2003; Gomez-Cabrera et al 2006). In this regard, allopurinol, a purine hypoxanthine-based structural analogue and a well-known inhibitor of XO, has been shown to decrease muscle damage after strenuous exercise in athletes (Sanchis-Gomar et al 2014). Future trials are

Table 1 Main characteristics of the different animal models of McArdle disease

Animal model	Phenotype	Advantages	Disadvantages	Therapies evaluated
Bovine (mutation: p.W489R)	Exercise intolerance Rhabdomyolysis Blood analysis abnormalities: ↑K⁺ ↑Phosphate ↓ Ca²⁺ ↑CK Histopathology: -No staining for GP-MM activity. -Subsarcolemmal glycogen vacuoles -Some central nuclei -PAS staining *within normal limits.*	Spontaneous animal model Muscle fiber type composition (I and IIA fibers) similar to humans Mitochondria density volume per fiber volume (2-5%) similar to humans	Difficult to obtain animals for experimentation owing to: Long gestation period (270-295 days) Only 1 calf per birth Difficult to manipulate (average cow weight ~450kg) Difficult to share with other research groups High maintenance costs	None
Ovine (mutation: truncation of the last 31 amino acid of the protein)	Exercise intolerance Histopathology: No staining for GP-MM activity. PAS staining with high glycogen content.	Spontaneous animal model Animal size and muscle mass comparable to humans Muscle fiber type composition (I and IIA fibers) similar to humans Mitochondria density volume per fiber volume (2-5%) similar to humans	Difficult to obtain animals for experimentation owing to: Long gestation period (147 days) 1-3 lambs per birth Difficult to manipulate (sheep weight 45-100kg) Difficult to share with other research groups High maintenance costs	Gene therapy: AAV2/AdV5-*Pygm* vectors Pharmacologic: Valproic acid Notexin
Murine (mutation: p.R50X)	Exercise intolerance ↑ blood CK Myoglobinuria Histopathology: No staining for GP-MM activity. PAS staining with high glycogen content Molecular studies: Lack of GP-MM protein. Lack of GP-MM activity. Very low *Pygm* mRNA (10% of normal levels)	Presents with a complete McArdle disease phenotype Presents the most common mutation in Caucasian McArdle patients (p.R50X) and allows to evaluate different therapies (*e.g.* read through compounds) Easy to manipulate (mouse weight 20-45g) Easy to share with other research groups Low maintenance costs Easy to obtain animals for experimentation: -Short gestation period (19-21 days). -High litter size (7-12 pups per litter)	Genetically modified animal model: 34 bp LoxP sequence is present in intron 1 Prenatal mortality in homozygous mice ~25% mortality in homozygous mice after weaning and before 3 months of age Higher glycogen accumulation in homozygous mice muscles than in human patients Different muscle fiber type composition (predominantly IIX and IIB fibers) compared to humans (predominantly I and IIA fibers) Different mitochondria density volume per fiber volume (30%) compared to humans (2-5%)	None

Abbreviations: CK, *creatine kinase*; GP-MM, *glycogen phosphorylase*, muscle isoform; PAS, Periodic acid–Schiff.

needed to elucidate the potential benefits of this or other anti-oxidant compounds in McArdle patients.

The fact that the vast majority (~90 %) of the patients have mutations generating premature termination codons (PTC) (Nogales-Gadea et al 2008; Lucia et al 2012) makes McArdle disease a candidate to treatment with 'read through' compounds, which are able to synthesize full proteins from transcripts containing PTCs. A short-term trial (10 days) with one such compound (gentamicin) failed to normalize ^{31}P magnetic resonance spectroscopy indicators of GP deficiency in the muscle of McArdle patients (Schroers et al 2006). The lack of success of this trial might be due to the fact that aminoglycosides have strong sequence specificity depending on the nucleotide context surrounding the stop codon. Indeed, it appears that non-sense mutations are not equally sensitive to 'read through' promoting drugs. In the case of gentamicin, its

ability to suppress various premature termination codons has been reported to be highly variable (Bidou et al 2004; Bidou et al 2012). Furthermore, controversy exists on the ability of gentamicin and other 'read through' compounds (such as PTC124) to stabilize the mutant mRNAs by inhibiting their degradation through the 'non-sense mediated decay' mechanism (Tork et al 2004; Welch et al 2007). However, PTC suppression was observed in transiently transfected cells with mutated mouse *Pygm* cDNA treated with the aminoglycoside antibiotic G418 (Birch et al 2013). An important drawback of aminoglycosides is their toxicity, due to their accumulation in the proximal tubular cells of the renal cortex (causing nephrotoxicity, generally reversible) and in the cochlear hair cells (inducing ototoxicity, usually irreversible) (Bidou et al 2012). Importantly, the recently generated mouse model harboring the non-sense mutation p.R50X can be used to assess the effectiveness of the available 'read through' compounds as well as their potential side effects.

A different approach could be based on pharmacologic treatments aiming at up-regulating the expression of GP-BB and/or GP-LL in the patients' muscle. In this regard, treatment with Valproic acid induces DNA demethylation and has been used to treat bipolar disorders (Tunnicliff 1999; Johannessen 2000). In fact, after valproic acid was administered orally (in syrup form) to seven affected sheep with McArdle disease, a total of 2240 fibers were found to be GP-positive in the muscles from five sheep (Howell et al 2008a, b). In contrast, a muscle biopsy taken from each sheep before treatment did not contain GP positive fibers. More studies are needed to evaluate whether the different regulatory properties of these isoenzymes represent a limitation to this type of therapy.

Only one trial of gene therapy has been performed to date in McArdle disease, using its ovine model, where the adenovirus 5 vector (AdV5) and an adeno-associated virus serotype 2 (AAV2) containing GP-MM expression cassettes were used (Howell et al 2008a, b). Intramuscular application of both vectors produced only *local* expression of functional GP-MM, *i.e.*, confined to the surroundings of the injection site and the number of GP-MM expressing fibers diminished with time probably owing to an immune response. As a consequence of the muscular damage caused by the injection itself, expression of the non-muscle GP isoforms was also observed in regenerating muscle fibers when transduction was performed using AdV5 vector carrying only LacZ cDNA.

Useful lifestyle recommendations

Two lifestyle interventions effectively alleviate patients' exercise intolerance. Ingestion of complex carbohydrates in daily diet (Andersen and Vissing 2008) and especially of simple carbohydrates before exercise (≥30–40 g of glucose, fructose or sucrose in adults, in the form of commercially available sport drinks) does 'protect' the muscle, by ensuring that enough blood glucose is available to enter muscle fibers and by-pass the metabolic blockade, resulting in improved exercise capacity, especially during the start of exercise (Vissing and Haller 2003; Andersen et al 2008). In children, ingesting 20 g of simple carbohydrates during the warm-up period preceding any vigorous exercise bout (for instance, physical education classes) is also recommended (Perez, Mate-Munoz et al 2007). Pre-exercise ingestion of simple carbohydrates decreases perceived exertion as well as heart rate during the first minutes of exercise, which is directly attributable to an increase in the oxidative capacity of the muscles (Pernow and Saltin 1971). On the other hand, although ingestion of high amounts of sucrose (75 g) causes sucrose-induced hyperinsulinemia (which impairs the mobilization and utilization of fatty acids), the oxidative benefit of enhanced glucose availability at the start of exercise likely mitigates any potential oxidative limitation due to decreased fatty acid mobilization (Vissing and Haller 2003).

Regular, moderate-intensity aerobic exercise is also very useful for these patients, with interventional research (using non-controlled designs) showing favorable metabolic adaptations (Haller et al 2006; Mate-Munoz et al 2007). Thus, Haller et al showed a 36 % increase in the cycle-ergometer peak work capacity of eight patients after a 14-week training program (four sessions/week of cycling exercise (30-40 min duration) at 60-70 % of peak heart rate), which was also accompanied by improvements in peak cardiac output, and citrate synthase and beta-hydroxyacyl coenzyme A dehydrogenase muscle enzyme levels (Haller et al 2006). Maté-Muñoz et al reported a 44 % increase in the peak oxygen uptake (VO_{2peak}) of nine patients with an 8-month program (five weekly sessions (duration≤60 min) of walking/cycling exercise at 60 % of peak heart rate) (Mate-Munoz et al 2007). Nonetheless, adaptation to exercise training should be a gradual process and intense exercises, particularly those involving high loads on low muscle mass (*e.g.*, static muscle contractions such as handgrip exercise, carrying very heavy weights), should be discouraged (Lucia, Nogales-Gadea et al 2008; Lucia et al 2013). The importance of following an active lifestyle is reflected by the fact that individual differences in patients' physical activity levels largely explain the heterogeneity in disease severity. Physically active patients, including children (Perez et al 2008), are much more likely to improve their clinical course, that is, to move to the lowest severity group of patients (who are virtually asymptomatic and show no functional limitation in any daily life activity) over a 4-year period, compared to their inactive peers (Lucia et al 2012). Further, some physically active patients have a VO_{2peak} level≥8 metabolic equivalents (METs) (which is the minimum threshold for optimal health), with one Spanish patient reaching ~11 METs (Lucia et al 2012). Finally, McArdle disease patients with appropriate exercise habits can be almost as 'aerobically' fit as unaffected

people: after gradual, supervised training, a 38-year old patient could run regularly and cover 10 km in ~60 min with no rhabdomyolysis (Perez et al 2007a, b). Of note, the average time for recreational runners to complete a 10 km race generally falls between 75–80 min.

Conclusions

Growing information is available on the 'pathogenomics' of McArdle disease, which can help to better understand the pathophysiology of this disease, as well as to treat this and other genetic disorders. Future studies might tell us whether potential treatments have any chance to attenuate patients' symptoms and improve their functional capacity and quality of life. As of today, appropriate nutritional and exercise habits appear as the most useful strategy to mitigate the exercise intolerance of these patients.

Compliance with Ethics Guidelines

Conflict of interest None.

Informed consent and animal rights This article does not contain any studies with human or animal subjects performed by the any of the authors.

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3.5 Article 5

REVIEW ARTICLE

McArdle Disease: A Unique Study Model in Sports Medicine

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Abstract McArdle disease is arguably the paradigm of exercise intolerance in humans. This disorder is caused by inherited deficiency of myophosphorylase, the enzyme isoform that initiates glycogen breakdown in skeletal muscles. Because patients are unable to obtain energy from their muscle glycogen stores, this disease provides an interesting model of study for exercise physiologists, allowing insight to be gained into the understanding of glycogen-dependent muscle functions. Of special interest in the field of muscle physiology and sports medicine are also some specific (if not unique) characteristics of this disorder, such as the so-called 'second wind' phenomenon, the frequent exercise-induced rhabdomyolysis and myoglobinuria episodes suffered by patients (with muscle damage also occurring under basal conditions), or the early appearance of fatigue and contractures, among others. In this article we review the main pathophysiological features of this disorder leading to exercise intolerance as well as the currently available therapeutic possibilities. Patients have been traditionally advised by clinicians to refrain from exercise, yet sports medicine and careful exercise prescription are their best allies at present because no effective enzyme replacement therapy is expected to be available in the near future. As of today, although unable to restore myophosphorylase deficiency, the 'simple' use of exercise as therapy seems probably more promising and practical for patients than more 'complex' medical approaches.

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Key Points

Glycogen is an important fuel during exercise, and low glycogen availability (as occurs in McArdle disease) impairs muscle function and basic cellular events

McArdle disease is the paradigm of exercise intolerance and is an excellent model for studying the physiology of muscle fatigue

Although frequently overlooked in clinical settings, exercise interventions are among the best therapeutic options for this and other debilitating diseases

1 Introduction

Glycogenosis type V [glycogen storage disease type V (GSD V), McArdle disease or myophosphorylase deficiency; OMIM® database number 232600] is a disorder of skeletal muscle carbohydrate metabolism first described by Brian McArdle in 1951 [1]. It is one of the most frequent genetic myopathies, e.g. a prevalence of $\sim 1/167,000$ in Spain as of January 2011, with both sexes equally affected [2]. This disease is inherited in an autosomal recessive manner and produced by pathogenic mutations in both copies of the gene [phosphorylase, glycogen, muscle (*PYGM*)] encoding the muscle isoform of glycogen phosphorylase, myophosphorylase [2]. Based on our count of reported mutations, ~ 150 mutations have been described in Caucasian patients, with p.R50X being clearly the most prevalent variant, e.g. reported allele frequencies of 43 % (Italy) [3], 55 % (Spain) [2] or 77 % (UK) [4].

Because myophosphorylase catalyzes and regulates the breakdown of glycogen into glucose-1-phosphate in muscle fibers (by removing α-1,4 glucosyl units from the outer glycogen branches) [5], patients are unable to obtain energy from their muscle glycogen stores [6]. Of note, glycolysis is blocked upstream, and thus the muscle fibers of McArdle disease patients can take up glucose from the blood and convert it into glucose-6-phosphate, which then enters the downstream steps of glycolysis [6] (Fig. 1). Muscle glycolysis is not, therefore, totally impaired in these patients, yet this disorder is arguably the paradigm of exercise intolerance in humans [7], thereby providing a quite unique model of study in the field of sports medicine [8].

Exercise intolerance (as described in the next section) is present in virtually all patients and often starts during childhood (e.g. in physical education classes). For example, 58 % of patients reported that symptoms started in the
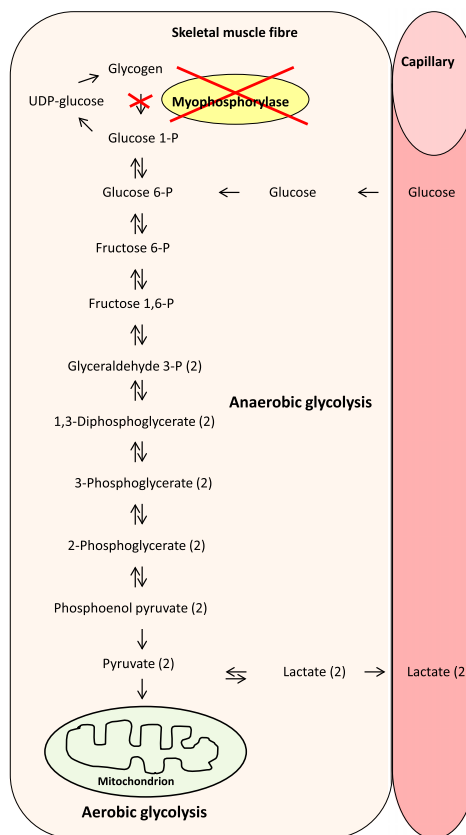
Fig. 1 Schematic representation of the metabolic blockade caused by McArdle disease. Deficiency of myophosphorylase (which catalyzes the first step of glycogen breakdown) results in patients' inability to obtain energy from muscle glycogen stores. However, because glycolysis is blocked upstream, the blood glucose that enters the muscle fibers can be converted into glucose-6-phosphate, which is metabolized in the downstream steps of glycolysis. *P* phosphate, *UDP* uridine diphosphate. *Arrows* denote the direction of the chemical reaction

first decade of life, but others reported that they appeared later in life (i.e. 28 % in the second decade and 14 % in the third or fourth decade) [2]. The intensity of the stimuli causing intolerance also shows inter-individual variability; in some rare cases (fittest patients) symptoms are triggered only by sports participation, whereas most people show intolerance to almost all types of physical exercise [2], and ~ 25 % of patients also report functional limitations during

daily life activities such as household tasks, personal care, lifting/carrying weights during shopping, or carrying children [2].

2 Main Clinical Features of the Disease

2.1 Exercise Intolerance

Exercise intolerance typically consists of acute crises of early fatigue and muscle stiffness and contractures, especially at the start of exercise, which usually disappear if exercise is stopped or intensity is reduced [9]. However, these episodes are sometimes accompanied by marked muscle damage or rhabdomyolysis, as reflected by the efflux of intramuscle proteins to the bloodstream, e.g. creatine kinase (CK) and myoglobin. Thus, high serum CK activity (typically >1,000 U/L) triggered by exercise is a common finding which can be accompanied by myoglobinuria, typically referred to as 'dark urine' [2, 10, 11]. In fact, 50 % of patients report recurrent episodes of dark urine [2]. The main potential danger of exertional rhabdomyolysis is acute renal failure as well as hyperkalemia, with the former eventually leading to chronic renal failure, although reported cases of life-threatening situations are very scarce [2]. For these potential threats, patients have been traditionally advised by clinicians to refrain from exercise practice.

2.2 The Second Wind

A unique feature of the disease is the so-called 'second wind' phenomenon [12, 13], which most patients refer to as the ability to resume dynamic, large-mass exercise (e.g. brisk walking) if they take a brief rest upon the appearance of premature fatigue early in exercise [6]. The second wind is easily detectable during a constant-load (∼40 watts or ≥65 % age-predicted maximum heart rate) cycle-ergometer test, with all adult patients showing a decrease in early exertional tachycardia (from ∼140–150 to ∼120 beats/min) after 7–8 min, and most reporting a decrease in local leg-muscle pain [2, 12]. The first few minutes of exercise act as a warm-up (e.g. inducing muscle vasodilation), after which more circulating free-fatty acids as well as glucose are available to working muscle fibers that can oxidize these substrates, leading to attenuation of exercise intolerance [9]. In fact, Haller and Vissing [9] elegantly showed that the 'second wind' phenomenon is abolished by glucose infusion or sucrose ingestion before exercise [14].

2.3 Fixed Muscle Weakness

Around 25 % of patients also develop fixed weakness and wasting (affecting more proximal than distal muscles), which

is aggravated with aging [2, 15]. This phenomenon has been attributed to the cumulative effect of repeated episodes of rhabdomyolysis [16], but this hypothesis remains to be proven.

3 Pathophysiology of Exercise Intolerance in McArdle Patients

3.1 Role of Glycogen in Human Muscle Function

Endogenous muscle glycogen represents a primary fuel source during exercise, and the relationship between glycogen and endurance exercise capacity is a fundamental concept in exercise physiology [17–19]. For more than 50 years it has been understood that alterations in pre-exercise muscle glycogen reserves by dietary or exercise manipulations, affect fatigue resistance. These observations have served to establish a close relationship between muscle glycogen and endurance and it is now generally recognized that adequate reserves of muscle carbohydrates are indispensable for sustained performance during moderate to heavy exercise. Thus, previous studies [20, 21] demonstrated a strong correlation between muscle glycogen content and endurance capacity during prolonged cycling exercise and an inability to continue such exercise when the glycogen stores were depleted [21]. As a consequence, high pre-exercise muscle and liver glycogen concentrations are believed to be essential for optimal performance [22]. While muscle fatigue is a multifactorial phenomenon depending on exercise mode, intensity and duration, the now well-established relationship between muscle glycogen content and fatigue resistance is not only evident during prolonged (>1 h) exercise, but also established during high-intensity intermittent exercise [18, 23, 24]. However, the link between glycogen depletion and the development of fatigue, as well as the precise mechanism whereby muscle glycogen affects the series of events that ultimately result in fatigue, is not yet fully understood.

Studies on both rodent single fibers and humans have pointed to a modulating role of glycogen availability on sarcoplasmic reticulum (SR) Ca^{2+} handling [25–28]. Thus, tetanic Ca^{2+} levels in the muscle fiber and the SR Ca^{2+} release rate are markedly reduced when muscle glycogen levels are low, indicating a relationship between Ca^{2+} and muscle glycogen. Furthermore, it has been revealed that the Na^+–K^+ adenosine triphosphate (ATP)-ase preferentially uses glycolytic-derived ATP, indicative of a role of glycogen in maintaining muscle excitability during repeated depolarizations [29, 30].

Although glycogen has been traditionally interpreted as if it is uniformly distributed in the fiber, providing an average concentration of this cell, electron microscopy images have revealed that glycogen is found as discrete particles in

distinct pools within the fibers [31–34]. Furthermore, each glycogen granule has its own metabolic machinery with glycolytic enzymes and regulating proteins [35, 36]. Three distinct intracellular pools of glycogen have been identified: (i) subsarcolemmal glycogen, just beneath the sarcolemma; (ii) intermyofibrillar glycogen, located between the myofibrils, mainly at the level of the I-band close to the mitochondria and the SR; and (iii) intramyofibrillar glycogen, within the myofibrils. The major glycogen pool is the intermyofibrillar, constituting ~ 75 % of the total glycogen store, whereas intramyofibrillar and subsarcolemmal glycogen accounts for 5–15 % each. However, the relative distribution of glycogen in these pools is dependent on a variety of factors, such as fiber type, training status, immobilization, acute exercise and species (rodent, man) [37]. The three spatially distinct pools of glycogen within skeletal muscle fibers have been shown to influence different events in the excitation of the muscle fiber, and independently affect contractility and fatigability (for a review see Ortenblad et al. [28]). Thus, the subcellular localization of glycogen has to be taken into consideration to fully understand the role and regulation of glycogen metabolism on skeletal muscle function. The specific pool of intramyofibrillar glycogen is the smallest pool and, in relative terms, the main utilized glycogen depot during exercise. Furthermore, the glycogen content in this depot, and not subsarcolemmal or intermyofibrillar glycogen, is correlated with the Ca^{2+} release rate in SR vesicles [27], as well as with the amount of tetanic intracellular free $[Ca^{2+}]$ during fatiguing contractions [38] and the endurance capacity of mechanically skinned fibers [39]. These observations suggest a specific importance of intramyofibrillar glycogen in muscle function and that exhaustion of this glycogen depot may explain the association of glycogen with muscle fatigue [40]. In support of this, it has been demonstrated that glycogen, associated proteins and glycolytic intermediates, interact with the SR membrane [36, 40, 41] and affect the Ca^{2+} release properties of the SR [42]. Of note, a muscle biopsy hallmark of McArdle disease is the chronically elevated glycogen store deposits, both at the high subsarcolemmal and intermyofibrillar level [7, 43, 44], but to our knowledge no information is available on the intramyofibrillar glycogen deposits in these patients.

Because patients are unable to use their muscle glycogen, McArdle disease, especially the recently generated transgenic mouse model (as discussed below, see Sect. 4), provides a unique model to understand the function of glycogen deposits in the development of fatigue.

3.2 Main Pathophysiological Features in McArdle Patients

Patients' exercise intolerance is especially evident during exercise tasks involving (aerobic/anaerobic) glycolysis for muscle ATP production [7]. Thus, 'muscle crises' are typically triggered by acute, intense, large-mass exercise tasks (e.g. sprinting to catch a bus), especially by 'static' (or isometric) contractions relying on smaller muscle groups as well as on anaerobic metabolism, e.g. lifting/carrying weights or isometric exercises such as handgrip [2, 6, 7]. During the latter, high mechanical demands are imposed on relatively low muscle mass, and the sustained muscular contraction increases the pressure inside the muscle, causing the supply of oxygenated blood to be transiently cut off or at least considerably decreased [2, 7]. This can be aggravated by the fact that excessive glycogen storage (~ 2–3 times higher than in normal people) may mechanically disrupt the contractile apparatus [6].

Dynamic exercises involving higher muscle mass and lesser mechanical loads while relying mostly on aerobic pathways (stair climbing, running, brisk walking) can also trigger acute exercise intolerance in McArdle disease patients. Indeed, their muscle oxidative capacity is usually impaired; owing to blocked glycogenolysis, their muscles' ability to produce pyruvate, a molecule that plays an anaplerotic role in the Krebs cycle, is severely reduced [7]. Impaired muscle oxidative capacity is reflected in phosphorus magnetic resonance spectroscopy (^{31}P-MRS) by (i) significantly greater phosphocreatine consumption and lower ATP concentrations compared with healthy controls after submaximal isometric calf contractions [6], or (ii) higher intracellular adenosine diphosphate (ADP) concentrations at the beginning of recovery from ischemic exercise [45]. Owing to the marked decrease in skeletal muscle phosphorylation capacity (decreased [ATP]/[ADP][inorganic phosphate, Pi] ratio), Pi and probably also ADP accumulates in patients' muscles, thereby potentially inhibiting (i) the myofibrillar ATPase, (ii) the SR Ca^{2+} ATPase (SERCA) pump, and (iii) the Na^+–K^+ ATPase (or 'pump') reactions, possibly leading to decreased contractility and premature fatigue [46–48].

3.3 Hyperkinetic Cardiorespiratory Response to Exertion

Patients often show an abnormally high cardiac output and heart rate at a given workload during dynamic exercise [49]. This 'hyperkinetic' circulation could be mediated by the local effects of K^+, Pi, or adenosine, or a combination of these substances, released excessively from working skeletal muscles on metabolically sensitive skeletal muscle afferents and vascular smooth muscle [49]. Although the abnormal cardiovascular response to exercise in McArdle disease patients could also indicate an excessive increase in sympathetic nervous system activity, previous research has shown that these patients have normal muscle sympathetic nerve responses to exertion, at least for static (handgrip)

exercise [50]. The abnormal cardiovascular response to dynamic exercise in these patients could also be due, at least in part, to an altered central motor pattern, which may manifest as exaggerated motor unit recruitment for a given workload.

3.4 Low Muscle Contractility

Using surface electromyography (EMG), which permits measurement of muscle fiber recruitment, Rae et al. recently showed that, for the same relative level of EMG activity, and thus at the same relative degree of recruitment, the muscles of McArdle disease were able to produce only about one-third of the power produced by age- and sex-matched healthy controls during a ramp cycle-ergometer test [51]. The mean $\pm$ SD peak power output at the end of the test was only 67 ± 21 W (range 27–107) in 37 adult McArdle disease patients of both sexes versus 214 ± 56 W (range 92–313) in their controls. On the other hand, the McArdle disease patients showed higher levels of EMG activity at the same relative workload during a sub-maximal constant-load cycle-ergometer test. Thus, it was hypothesized that the low capacity for producing peak muscle power as well as the high muscle mass recruitment for a given power in McArdle disease patients might reflect impaired muscle contractility.

3.5 Downregulation of Sarcoplasmic Reticulum Calcium ATPase 1 (SERCA1) Leading to Impaired Muscle Relaxation

Besides the abovementioned hypothesis of altered contractility, it could also be that muscle relaxation is delayed in patients with McArdle disease so that the muscle cannot sustain a high cadence of contraction and experiences early fatigue during exercise. Indeed, human muscle fatigue is usually accompanied by a slowing of relaxation [52] and high rates of glycolytic ATP production, since up to 80 % of the ATP consumed during contraction is required for optimum muscle relaxation, i.e. for adequate removal of Ca^{2+} from the sarcoplasm back into the SR [52, 53]. Nogales-Gadea et al. [54] recently found evidence of downregulation of SR calcium ATPase 1 (SERCA1) in the muscle of McArdle disease patients compared with healthy controls. Partial reductions in the activity or total amount of SERCA1 due to *SERCA1* gene mutations cause a disorder known as Brody myopathy [55], a rare skeletal muscle condition that shares some common clinical features with McArdle disease, i.e. patients experience progressive muscle stiffness during exercise, leading to contractures [56]. Lack of SERCA1 also causes neonatal death in mice [57]. SERCA1 is a Ca^{2+} transporter ATPase

located in the SR of fast-twitch, type II fibers that is essential for normal development of contraction–relaxation cycles in these fibers, which are mainly glycogenolytic and therefore more sensitive to myophosphorylase deficiency. Thus, SERCA1 reduction may preferentially affect those exercise tasks involving type II muscle fibers and that typically cause more severe intolerance in McArdle disease patients, such as isometric exercise or lifting weights. Additionally, concentrations of Ca^{2+}, ATP, ADP or Pi [58] can modify the phosphorylation rate of SERCA1, and some of these factors are reported to be deregulated in McArdle disease, notably increased [ADP] and [Pi], as mentioned above (Sect. 3.2).

Of note, the fact that deficient glycogen-dependent ATP supply can result in downregulation of Na^+–K^+ pumps in the skeletal muscle fibers of patients with McArdle disease might lead to contractures (sometimes referred to as 'cramps' in the sports setting) owing to loss of membrane excitability and exercise-induced hyperkalemia [46]. In addition, contrary to 'traditional beliefs', lactic acidosis does not necessarily impair muscle performance and could in fact protect against muscle fatigue (see Cairns [59] for a review), e.g. by virtue of its effect of maintaining muscle membrane excitability by decreasing chloride permeability [60] or by counteracting the depressing effects that the efflux of K^+ from working fibers has on membrane excitability [61] and on force of contraction [62].

3.6 Low Gross Mechanical Efficiency

It seems that the oxygen consumed by the muscles of McArdle disease patients is not converted into the expected power production. This is supported by the higher slope of the oxygen uptake (VO_2) to watts relationship during ramp cycle-ergometer tests [51, 63] and the very low gross mechanical efficiency values of these patients during constant, submaximal workload cycle-ergometer tests, where their muscles are only able to convert 13 % of the total oxygen they consume into mechanical work [64]; this is in contrast with the mean gross mechanical efficiency of the age- and sex-matched controls group (19 %), as well as with the normal 18–22 % range expected in non-athletic, healthy people [65]. Although more research is needed, the main reason for this phenomenon is likely the abovementioned excessive muscle recruitment (Sect. 3.4), with other potential mechanisms (which in turn can also impair contractility) being low capacity for Ca^{2+} removal from the SR, or increased dependence on fat metabolism owing to the inherited block in muscle glycogen metabolism [51]. With regards to the latter hypothesis, carbohydrates are indeed more efficient fuels than fat in terms of generating ATP per mole of oxygen [66].

Results

3.7 The Pathophysiology of Exertional Rhabdomyolysis and Basal 'Hyper-CK-Emia': Muscle Oxidative Stress Involved?

As noted above (Sect. 2.1), exercise-induced rhabdomyolysis is the main medical problem of McArdle disease, mainly due to its potential to cause renal failure [2, 4, 67–71]. The causes for exertional rhabdomyolysis to occur frequently in these patients remain to be clearly elucidated, although the mechanical stress imposed by high muscle glycogen stores [6] and particularly the documented downregulation of Na^+–K^+ pumps in patients' muscles [46] (with these pumps being responsible for maintaining cellular volume and integrity), are candidates to contribute to structural muscle fiber fragility and membrane disruption, leading to the efflux of intracytoplasmic proteins such as CK to the bloodstream. An additional proposed factor is increased oxidative stress.

Owing to reduced glycolytic flux in the working muscles of these patients, there is frequently a mismatch between ATP consumption and production. Thus, two ADP molecules can combine to regenerate ATP by the myokinase (or adenylate kinase 1) pathway in an attempt to keep up with ATP demand. In this reaction, adenosine monophosphate (AMP) is produced and removed by AMP deaminase 1, resulting in the production of NH_3^+ and inosine monophosphate (IMP), the latter being metabolized to inosine and then to hypoxanthine, xanthine, and uric acid via xanthine oxidase, which generates reactive oxygen species (ROS). There is indeed evidence of increased levels of muscle ADP [72] and plasma NH_3^+ and hypoxanthine during exercise in McArdle disease patients compared with non-patients [72, 73], suggesting high oxidative stress in their skeletal muscles. However, another characteristic of the disease is a high serum level of CK activity, even under basal conditions, i.e. in the absence of heavy exercise in the previous few hours or days, e.g. >1,000 U/L in ~80 % of all Spanish patients [2]. The reason for this striking phenomenon remains to be elucidated, although oxidative damage could also be involved. Indeed, compared with healthy age- and physical activity-matched controls, McArdle disease patients have higher basal muscle levels of a marker of oxidative stress, muscle protein carbonyls and 4-hydroxynonenal (4-HNE), together with a compensatory upregulation of the nuclear factor erythroid 2-related factor 2 (Nrf2)-mediated antioxidant response [74]. Also, the possibly elevated levels of Ca^{2+} in the sarcoplasm (owing to downregulated SERCA1, see Sect. 3.5) might activate proteases, phospholipases and other catabolic enzymes, causing not only structural damage but also initiating muscle fatigue and cramping [75].

3.8 Low-Grade Systemic Inflammation

When muscle injury occurs, e.g. typically after an exercise task with a strong eccentric component, the process of tissue repair is initiated with the induction of an acute-phase response and upregulation of a group of messenger molecules collectively known as cytokines [76]. Cytokines are released at the site of inflammation, initiating a systemic inflammatory response [77, 78]. On the other hand, several chronic diseases (e.g. cardiovascular disease, type II diabetes), as well as physical inactivity, are associated with a chronic state of systemic low-level inflammation, i.e. increases in neutrophil and natural killer cell counts as well as in circulating levels of cytokines, such as tumor necrosis factor (TNF)-α [79]. In contrast, the benefits of regular physical exercise are partly mediated by its anti-inflammatory effects; working muscles release bioactive molecules (myokines) to the bloodstream with a potential beneficial health effect at the systemic level or in other tissues, such as interleukin (IL)-6, which promotes an anti-inflammatory milieu [80].

Possibly because of the continuous state of muscle damage experienced by patients with McArdle disease, basal blood levels of neutrophil count and several cytokines [TNF-α, IL-1 receptor antagonist (IL-1ra), IL-10, IL-12 and IL-17] were shown to be significantly higher in patients than in controls [81]. In turn, non-eccentric (cycling) exercise induced a similar response in McArdle patients and healthy controls, i.e. increased serum levels of the anti-inflammatory myokine IL-6. It was thus suggested that McArdle disease is associated with low-level systemic inflammation, strengthening the rationale for prescribing carefully supervised exercise training in these patients.

3.9 Downregulation of Glycogen Synthase: A Beneficial (Protective) Mechanism?

Skeletal muscle is the major site of insulin-stimulated glucose uptake, and most of this glucose is stored as glycogen through glycogen synthase (GS), which has the opposite function of myophosphorylase but shares regulatory mechanisms with the latter enzyme, i.e. covalent modification, allosteric activation, and enzymatic translocation [82]. Evidence of deregulation in the GS pathway has been reported in McArdle disease patients [54, 83–85], who have decreased insulin action in skeletal muscle compared with healthy controls after a hyperinsulinemic clamp, resulting in lower glucose use and lower GS activity [83]. Nielsen et al. [83] elegantly showed that a group of six McArdle disease patients had normal fasting plasma glucose and plasma insulin levels, although four of them were categorized as having impaired glucose tolerance after an oral glucose tolerance test. The authors

hypothesized that the decreased glucose uptake in muscle fibers might be compensated with other mechanisms augmenting glucose uptake in extramuscular tissue, such as the liver. Studies in larger series of patients do not show a higher incidence of diabetes in McArdle disease patients despite the decreased insulin action in their skeletal muscles. For instance, the prevalence of diabetes in the Spanish registry of McArdle disease patients (10 %) [2] is similar to that reported in Spanish adults, i.e. 10–15 % [86].

McArdle disease patients have low GS activity during exercise [84] as well as higher and lower levels of inactive and active enzyme forms, respectively, than healthy controls [54, 85]. The activity of GS is regulated by a complex, multisite phosphorylation mechanism comprising several protein kinases [87]. Furthermore, GS presents a phosphorylation-dependent intracellular distribution [88] with different phosphorylation sites associated with intramyofibrillar and the two other pools of subsarcolemmal and intermyofibrillar glycogen [33, 89]. Depletion of muscle glycogen during exercise activates GS [90], and this activation is greater when muscle glycogen is lower [91], resulting in a faster rate of glycogen resynthesis. The absence of glycogen degradation during exercise in patients with McArdle disease is associated with a slight decrease in GS activity [84] and the link between glycogen and GS may be mediated by protein phosphatase 1, which is targeted to the glycogen molecule [92]. Thus, the high glycogen levels found in the muscle biopsies of these patients could be one of the contributors to GS inactivation [54], maybe playing a protective mechanism against an exaggerated, harmful accumulation of glycogen.

The current body of knowledge and postulated hypotheses on the pathophysiology of McArdle disease (explaining their exercise intolerance) is summarized in Fig. 2.

3.10 Muscle Pathology

A main finding in the muscle biopsies of McArdle disease patients is the accumulation of subsarcolemmal vacuoles that are periodic acid-Schiff (PAS) positive, due to glycogen aggregates, and the absence of staining for phosphorylase activity [5]. Some necrotic or regenerating fibers can be present. The regenerating fibers are positive for phosphorylase due to expression of the fetal isoenzyme (brain isoenzyme) in these fibers [93, 94]. Other findings have only been reported in a few studies, i.e. selective atrophy of type I fibers [95] and fiber grouping [44].

3.11 More than Just a Skeletal Muscle Disease?

McArdle disease is commonly accepted as a 'pure' myopathy, and only few studies have analyzed the potential implication of extramuscular tissues in the disease phenotype; however, myophosphorylase expression has also been reported in brain and heart tissue [96–98].

The human brain contains both brain and muscle isoforms of glycogen phosphorylase, with the latter representing ~ 25 % of the total amount of glycogen phosphorylase [96]. Thus, the total quantity of glycogen phosphorylase might be reduced in the brain of McArdle disease patients owing to their inherited myophosphorylase deficiency. However, whether this hypothetical phenomenon affects the metabolism of patients' neurons remains to be elucidated. With regards to this, some cases of patients presenting with chronic fatigue-like symptoms, seizures, anxiety or depression have been reported [4, 99, 100], and preliminary data suggest lower performance of McArdle patients in cognitive tests compared with healthy controls [101], which is in line with a published case report of impaired verbal learning, selective attention, and executive functions in a 55-year-old patient [102].

Like the brain, the heart expresses both muscle and brain isoforms of glycogen phosphorylase [96]. Therefore, the heart tissue of McArdle patients might also have diminished levels of total glycogen phosphorylase activity. Although cardiac involvement in McArdle disease is very rare, some cases of patients presenting with coronary artery disease [103], electrocardiogram changes [104], degenerative vascular disease [105] and obstructive hypertrophic cardiomiopathy have been reported [106]. However, more research is needed to clearly prove a cause:effect relationship between McArdle disease and the reported cardiac conditions.

4 Recent Generation of a Promising Murine Model of McArdle Disease

Two spontaneous animal models for McArdle disease have been identified in Charolais cattle [107] and Merino sheep [108], which, despite showing rhabdomyolysis, exercise intolerance, as well as lower force of contraction and greater fatigue in muscle contractility studies [109], have rendered a relatively limited amount of information on the pathophysiology of the disorder. This prompted our group to develop a genetically modified murine model of McArdle disease (knock-in mouse for the common *PYGM* p.R50X mutation) [110]. In contrast to available rodent models for other neuromuscular diseases, which do not show a human-like phenotype, our model presents with the main features of the patients' phenotype.

Knock-in mice homozygous for the common p.R50X human mutation show absence of myophosphorylase activity in muscle and subsequent blocked glycogenolysis, with massive glycogen accumulation in the gastrocnemius

Results
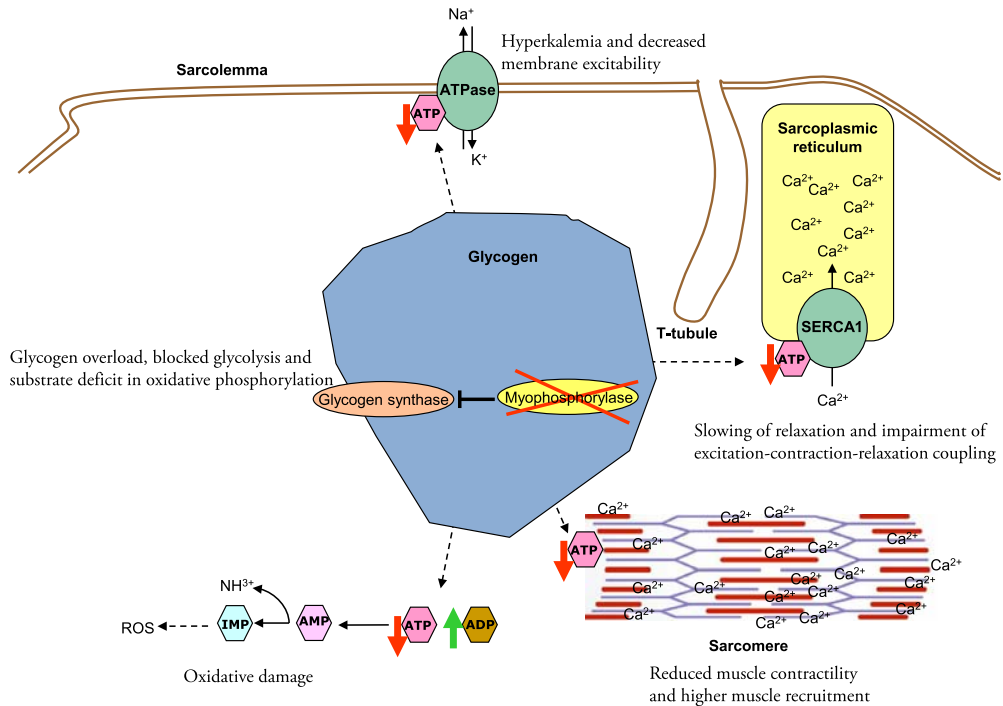
Fig. 2 The pathophysiology of exercise intolerance and muscle damage in McArdle disease. *ADP* adenosine diphosphate, *AMP* adenosine monophosphate, *ATP* adenosine triphosphate, *IMP* inosine monophosphate, *ROS* reactive oxygen species, *SERCA1* sarcoplasmic reticulum Ca^{2+} ATPase 1. *Arrows* with *dashed lines* denote inhibition

muscle, not only subsarcolemmal, as typically described in patients [15], but also intrasarcoplasmic. Interestingly, the glycogen accumulation was more pronounced in less oxidative (type II) fibers, which are more dependent on glycogen metabolism [110]. The knock-in mice also showed marked intolerance to isometric (wire grip) and intense dynamic exercise (treadmill running until exhaustion) [110]. They also had high basal 'hyper-CK-emia' and exercise-induced myoglobinuria. Thus, with the exception of the much higher glycogen stores compared with humans, this novel murine model mimics the human disease phenotype well and may represent a good tool for in-depth studies of (i) the pathophysiology of McArdle disease; (ii) the importance of glycogen deposits and its different localizations on muscle function (in a model where glycogen availability is absent); (iii) training adaptations at the muscle molecular level; and (iv) new therapeutic approaches for this and other neuromuscular diseases [because p.R50X is a nonsense mutation affecting only one tissue, muscle, whose function can be finely evaluated, our model

could also be used for exploring novel pharmacological approaches based on 'read through' strategies targeting premature termination codon (PTC) mutations; see Sect. 5.1.2].

5 Treatment Options

At present, there is no curative therapy for McArdle disease and no effective biomedical treatment is expected to be available in the foreseeable future to replace myophosphorylase deficiency in humans.

5.1 Treatments that have not yet Proven Efficient in Human Patients

5.1.1 Nutritional Supplements/Drugs

No significant beneficial effects have been reported in McArdle disease patients receiving branched chain amino

acids [111], depot glucagon [112], dantrolene sodium [113], verapamil [114], vitamin B6 [115] (except in one recent case report [116]), or high-dose oral ribose [117]. More controversial are the results for creatine supplementation, although data are not promising either; low-dose supplementation (60 mg/kg/day for 4 weeks) attenuated muscle complaints in five of nine McArdle disease patients tested [118], but higher doses (150 mg/kg/day) actually increased exercise-induced myalgia for unknown reasons [119].

5.1.2 Stop Codon-Based Therapies

Approximately one-third of all genetic inherited diseases are caused by PTC mutations [120]. This type of mutation leads to a cellular self-protective process known as 'nonsense-mediated messenger RNA decay' aiming at eliminating the majority of messenger RNA transcripts containing nonsense and frame-shift mutations [121]. The fact that several different compounds, such as aminoglycoside antibiotics [122], PTC124 [123], RTC13 [124] or amlexanox [125] might restore protein translation by inducing the ribosome to bypass a PTC, a process known as 'read through', provides a promising perspective. In fact, aminoglycoside antibiotics, PTC124 and RTC13 have been shown to partially restore dystrophin protein in the skeletal muscles of the transgenic mouse model for Duchenne muscular dystrophy [123, 124, 126]. In addition, both aminoglycoside antibiotics and PTC124 were able to suppress the PTC effects in the cystic fibrosis transmembrane conductance (CFTR) gene and to improve CFTR activity in cystic fibrosis patients [127]. Importantly, more than 90 % of patients with McArdle disease have mutations in the PYGM gene that produce a PTC, including the most common p.R50X variation [121]. However, a preliminary trial with short-term (10 days) gentamicin treatment in McArdle disease patients presenting a PTC with 'read through' failed to normalize ^{31}P-MRS indicators of myophosphorylase deficiency in muscle [128].

5.1.3 Induced Expression of Brain and Liver Isoforms of Glycogen Phosphorylase in the Muscle

The brain and liver isoforms of glycogen phosphorylase are only expressed in muscle tissue in the uterus, in neonates, and in regenerating mature fibers (i.e. after rhabdomyolysis episodes), but not in adult non-regenerating mature fibers, where only the muscle isoform, myophosphorylase, is expressed. Thus, any pharmacologic treatment able to upregulate the expression of the brain or liver isoforms of glycogen phosphorylase in the skeletal muscle of McArdle disease patients (e.g. valproate, an inhibitor of histone deacetylation) could theoretically alleviate the symptoms

of the disease by partially restoring muscle glycogen breakdown. In fact, myophosphorylase-positive fibers have been identified in five of seven sheep with McArdle disease that were treated with this drug [129]. Additionally, it has been recently published that the injection or layering of notexin (a myotoxic phospholipase) in the muscles of the ovine McArdle model resulted in necrosis followed by regeneration of muscle fibers with the expression of both liver and brain isoforms of glycogen phosphorylase, leading to reduced muscle glycogen accumulation as well as increased strength of contraction and decreased fatigability of muscle fibers [109].

5.1.4 Gene Therapy

Gene therapy has also been evaluated in the McArdle ovine model using an adenovirus 5 vector and an adeno-associated virus serotype 2 containing myophosphorylase expression cassettes [130]. Intramuscular application of both vectors produced only local expression of functional myophosphorylase, i.e. limited to the surroundings of the injection site. Furthermore, the number of myophosphorylase-expressing fibers diminished with time, probably owing to an immune response, and expression of the non-muscle isoforms of glycogen phosphorylase was also observed in regenerating muscle fibers as a consequence of the muscular damage caused by the injection itself.

A major obstacle limiting gene therapy for muscle diseases comes from the fact that systemic gene transfer to striated muscles is hampered by the vascular endothelium, which represents a barrier to distribution of vectors through the bloodstream. With regard to this, a promising recent finding is that a single intravenous administration of recombinant adeno-associated virus pseudo-type 6 vectors with the inclusion of vascular endothelium growth factor/vascular permeability enabled widespread muscle-specific expression of a functional micro-dystrophin protein in the skeletal muscles of a dystrophin-deficient mouse model of Duchenne dystrophy [131]. Future trials might be performed in McArdle disease patients.

5.2 Nutritional Interventions Aimed at Maintaining High Blood Glucose Availability to Working Muscles

A beneficial intervention for alleviating exercise intolerance symptoms and 'protecting' the muscle from rhabdomyolysis risk consists of ensuring that sufficient blood glucose (derived from high hepatic glycogen stores) is constantly made available to patients' muscles during the daytime. This can be effectively achieved by adopting a diet with a high proportion (65 %) of complex carbohydrates (such as those found in vegetables, fruits, cereals,

bread, pasta and rice) and a low proportion (20 %) of fat [132]. Another strategy is the ingestion of simple carbohydrates before engaging in strenuous exercise: (i) in adults, 75 g of sucrose 30–40 min pre-exercise [14], or lower doses (30–40 g of glucose, fructose or sucrose, which translates to 400–500 mL of most commercially available sport drinks) closer to the start of exertion (~5 min) [133]; and (ii) in children, 20 g during the warm-up period preceding any vigorous exercise bout (for instance, physical education classes) [134]. Pre-exercise ingestion of simple carbohydrates is effective only during the time when patients are highly susceptible to muscle injury [14], i.e. during the first minutes of exercise, when there is low availability of circulating fuels and absence of glycogen-derived pyruvate, both of which dramatically impair the capacity for oxidative phosphorylation [9]. Thus, this treatment abolishes the spontaneous second-wind phenomenon (as mentioned in Sect. 2.2) [9, 14]. Pre-exercise carbohydrate ingestion also decreases perceived exertion on the Borg scale as well as heart rate, which is directly attributable to an increase in the oxidative capacity of the muscles [20]. On the other hand, although ingestion of high amounts of sucrose (75 g) causes sucrose-induced hyperinsulinemia (which has a negative effect on the mobilization and utilization of fatty acids), it seems that the oxidative benefit of enhanced glucose availability at the start of exercise mitigates any potential oxidative limitation attributable to decreased fatty acid mobilization [14].

5.3 Exercise is Medicine!

5.3.1 The Paradoxical Concept of 'Healthy Patients'

Individual differences in patients' physical activity levels largely explain the heterogeneity in disease severity mentioned in Sect. 1. Physically active patients, including children [135], are much more likely to improve their clinical course, i.e. move to the lowest severity group of patients (who are virtually asymptomatic and show no functional limitation in any daily life activity) over a 4-year period compared with their inactive peers (odds ratio 225; 95 % confidence interval 20.3–2,496.7) [2]. In fact, some physically active patients have a peak oxygen uptake level (VO_{2peak}) $\geq$8 metabolic equivalents (METs) [the minimum threshold for optimal health], with one Spanish patient reaching ~11 METs despite having, like all McArdle disease patients, no myophosphorylase activity in his muscle [2]. Importantly, the VO_{2peak} values of these physically active patients are higher than those recently reported (~7 METs) in two 'atypical' patients described in the literature in whom there was a 'mild' or 'variant' form of McArdle disease, owing to some residual myophosphorylase activity (caused by the presence of an intronic

splice mutation in one of the two *PYGM* alleles) [136]. (Of note, permanent restoration of myophosphorylase activity, even at below normal levels, would be an ideal outcome of eventual gene therapy, as previously mentioned).

McArdle disease patients with appropriate exercise habits can be almost as aerobically fit as unaffected people. After gradual, supervised training, a 38-year-old patient (with no myophosphorylase activity) could run regularly and cover 10 km in ~60 min with no rhabdomyolysis [137]. The average time for recreational runners to complete a 10 km race generally falls between 75–80 min.

5.3.2 Exercise Interventions

Although evidence from randomized controlled studies is still missing, there is data from interventional research showing that patients with McArdle disease, like the vast majority of humans, adapt favorably to regular exercise, i.e. with a significant increase in VO_{2peak} after supervised aerobic exercise [64, 138]. Haller et al. showed a 36 % increase in the cycle-ergometer peak work capacity of eight patients after a 14-week training program (four sessions/week, duration 30–40 min, of cycling exercise at 60–70 % of peak heart rate), which was also accompanied by improvements in peak cardiac output (15 %), and citrate synthase (80 %) and β-hydroxyacyl coenzyme A dehydrogenase muscle enzyme levels (62 %) [138]. Mate-Munoz et al. reported a 44 % increase in the VO_{2peak} of nine patients with an 8-month program (five weekly sessions, duration $\leq$60 min, of walking/cycling exercise at 60 % of peak heart rate) [64]. No dietary intervention was included in the study by Haller et al., whereas in the report by Mate-Munoz et al. patients ingested a commercialized sports drink during warm-up before each training session [equivalent to ~30 g of simple carbohydrates (glucose and fructose)]. Pre-exercise carbohydrate ingestion might not be strictly necessary, at least in those patients who are more habituated to exercise, although in our experience it decreases their 'fear of exercise' by minimizing the risk of 'muscle crises' and attenuating the feelings of early fatigue and discomfort during the first minutes of a training session.

On the other hand, we believe pre-exercise carbohydrate is recommendable before light- to moderate-intensity weight-training exercise to prevent excessive muscle damage. Preliminary data from our group showed that a 15-year-old child with McArdle disease improved his one repetition maximum (1RM) bench press (~27 %) and multipower squat performance (~6 %) after a 6-week, supervised, light- to moderate-intensity (~65–70 % of 1RM) weight lifting training program (two sessions/week) while suffering no myoglobinuria episode during testing or training sessions [139]. Furthermore, the patient changed to

a lower disease severity class after training, i.e. he became virtually asymptomatic in terms of exercise limitations. However, more data are needed (and are in fact being currently generated by our group) in adult patients.

5.3.3 Precautions

Adaptation to exercise training should be a very gradual process, especially in those patients belonging to the highest severity class, i.e. reporting recurrent exercise-induced myoglobinuria episodes. In general, vigorous dynamic ('aerobic') exercise (i.e. at a level that does not permit normal talking) should only be performed by the more habituated patients, while very intense exercises, particularly those involving high loads on low muscle mass (e.g. static muscle contractions such as handgrip exercise, heavy weightlifting), should be generally discouraged [8].

6 Conclusion

McArdle disease provides an interesting model of study in sports medicine, allowing insight to be gained into the understanding of glycogen-dependent muscle functions and exercise fatigue. Although patients have been traditionally advised to refrain from exercise, exercise prescription carefully supervised by professionals is a promising option for these patients, especially when considering that no effective enzyme replacement therapy is expected to be available in the foreseeable future. The fact that myophosphorylase deficiency might potentially affect brain or cardiac tissue provides further support to implement exercise interventions owing to the multiorgan benefits of this therapy (including improved neurogenesis and cognitive function) that no single drug is likely to outmatch [80]. Although patients' recruitment might pose difficulties owing to the low prevalence of the disease, future intervention research using, preferably, a randomized controlled trial design is needed to accumulate evidence on the best possible exercise training strategy for these patients.

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3.6 Article 6

LETTER TO THE EDITOR

MINIMAL SYMPTOMS IN McARDLE DISEASE: A REAL *PYGM* GENOTYPE EFFECT?

Petrou *et al.* nicely reported a novel mutation in the phosphorylase, glycogen, muscle (*PYGM*) gene, c.1151C>T, in a Cypriot individual with a minimally symptomatic form of McArdle disease.[1] The disorder is caused by a deficiency in the *PYGM*-encoded, muscle-specific isoform of glycogen phosphorylase (GP-M), which catalyzes the first step of glycogenolysis in muscle. The c.1151C>T mutation is to be added to the recently updated list of pathogenic *PYGM* mutations[2] and may help identify more patients with McArdle disease. This is important because the *PYGM* gene remains largely unexplored worldwide.[3,4]

McArdle disease is arguably the paradigm of a metabolic cause for exercise intolerance, but there is great individual variability. Some patients are in fact quite fit, as beautifully exemplified by Petrou and colleagues. The patient they described suffered myalgia only during strenuous exercise; he had no limitations in activities of daily living, including his work in construction, and he reported no episodes of myoglobinuria.[1] He showed the same disease phenotype as 8% of patients in the Spanish registry who belong to the lowest severity class (= 0) in the commonly used phenotype scale.[5] These patients have intolerance to strenuous exercise as the only noticeable symptom,[5] despite having total absence of GP-M activity in their muscles.[6] Although the Cypriot individual, like 14% of Spanish patients,[6] did not report the second wind phenomenon, that is, attenuated myalgia and tachycardia after ~10 min of dynamic exercise (brisk walking or bicycling), this pathognomonic phenomenon can be observed objectively with a bicycle-ergometer test designed *ad hoc.*[7]

The authors attributed the mild, apparently rare, phenotype in their patient to the fact that some residual GP-M activity (up to ~4% of normal) was preserved in his muscle. However, the biochemical test they used does not allow differentiation of *specific* GP-M activity, and the antibody in the Western blot was not tested for recognition of other non-muscle isoforms of glycogen phosphorylase (GP), such as fetal and brain, which share >80% homology with GP-M.[8] Yet, non-muscle isoforms are known to be transiently re-expressed in regenerating fibers that are scattered randomly throughout biopsy specimens, notably following muscle injury (in up to 19% of patients).[9,10] This phenomenon can lead to some residual *total* GP activity (~3% of normal) in some patients.[9–11] This is in fact quite consistent with the findings by Petrou *et al.*, especially when one considers that their patient's daily occupation as a builder probably led to a mild degree of ongoing muscle damage.

There is likely no *PYGM* genotype–phenotype correlation (included in the Cypriot patient) despite the wide heterogeneity of disease severity reported commonly. The most common p.R50X mutation among Caucasians is also present among less affected, fitter patients. Physical activity (PA) habits, rather than *PYGM* genotype, may thus explain the individual variability in phenotypic manifestations of the disease among patients. Those who exercise daily, during leisure or in their work (e.g., construction workers), are less affected.[6] The beneficial biological adaptations in muscle induced by regular PA (e.g., increased oxidative capacity or improved muscle mass/strength triggered by a potent stimulus such as the block in muscle glycogenolysis, thereby reducing the penetrance of the disease.[4,6]

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Editor's note: Petrou, et al. have reviewed this letter and feel there is no need for additional comment.

Results

4. Discussion

The development of a knock-in specific mouse model of McArdle disease by our laboratory [193] represented a step forward to a new field of study of this pathology. Up to this point, two spontaneous animal models for McArdle disease had been identified (Charolais cattle and Merino sheep) but both models provided poor information of the disorder as a result of their difficulties in manipulation, reproduction and maintenance. Biochemical and molecular analysis of the gastrocnemius muscle from two-months-old mice revealed that those mice presented a clear McArdle disease-like phenotype [193]. In addition, exercise performance tests, assessed in the wire grip and treadmill tests, showed a very poor exercise performance with 6% and 5% of the WT values, respectively [193]. Further analyses were missing to unravel the molecular and functional consequences of lack of GP-MM, as well as, subsequent glycogen unavailability in metabolically different types of muscles, and also the consequences in the glycogen turnover metabolic pathway.

In this PhD thesis, we analyzed the molecular consequences of GP-MM deficiency in three different types of skeletal muscle with different metabolic phenotype [more oxidative/slow-twitch (soleus), intermediate (gastrocnemius) and more glycolytic/fast-twitch (EDL)], as well as, the exercise capacity and the role of the sex of the mice in the results. We also analyzed the main regulatory enzymes involved in the synthesis and degradation of glycogen, as well as, glycogen content in the three different muscles across the three *Pygm* phenotypes (WT, heterozygous and homozygous). This is the first work to study the effect of GP-MM absence in metabolically different skeletal muscles, providing a better understanding of the pathophysiology of the disease.

As previously reported in McArdle patients and in the first characterization of

Discussion

McArdle mice [126, 193], McArdle mice presented a complete absence of GP-MM, as well as, a 90% reduction of *Pygm* mRNA levels in the three muscles, suggesting that GP-MM and mRNA levels might be equally depleted in all muscles of McArdle patients. Moreover, we also observed that, in heterozygous mice, the decrease in both GP-MM and mRNA was more pronounced in soleus than in both gastrocnemius and EDL, together with GP-MM was less expressed in soleus of WT mice. In addition, soleus muscle showed the lowest glycogen content. These results might indicate that slow-twitch muscles such as soleus are not as reliant on glycogen catabolism as fast-twitch muscles, thus being less affected by GP-MM depletion.

With regard to glycogen anabolic enzymes, we also studied whether glycogen synthesis was down-regulated in homozygous mice compared to the other two groups (heterozygous and WT mice). We observed lower levels of total GS protein but higher levels of the inactive, phosphorylated form of GS in all three muscles of homozygous mice. These results were also previously observed in muscle biopsies from McArdle patients which present lower levels of mRNA, protein and activity of GS and higher levels of the phosphorylated form of the enzyme compared to healthy controls [194, 195], suggesting that a cellular mechanism aimed at preventing excessive glycogen accumulation might be occurring. It was previously reported that high glycogen content in skeletal muscles diminishes both insulin-stimulated glycogen synthesis and GS activation and increases glycolytic flux [196]. Consequently, the GS inactivation observed in the muscles of McArdle mice might be caused by the high glycogen accumulation. Moreover, it was also previously reported that high glycogen concentration does not reduce insulin signaling or glucose uptake [196], contrasting with our blood metabolites analysis, where significantly lower glucose levels in homozygous mice compared to WT controls were observed. So, is there an increment in glucose uptake because of the mismatch between the shortage of glucose from

glycogen breakdown and glycogen availability? or is glucose uptake increase due to the attempt to store glucose in the form of glycogen? Further analyses are needed to understanding the changes produced in the glucose metabolism as a consequence of the lack of glycogen as a glucose source.

We also analyzed GBE and we observed that homozygous mice presented higher levels of this enzyme in gastrocnemius and EDL but not in soleus, compared to WT and heterozygous mice. Interestingly, these results seem to indicate that the muscles which present the lowest GBE levels in healthy conditions, in homozygous mice, present the highest GBE levels in those muscles. This could probably be explained as a compensatory mechanism aimed to (1) accommodate higher amounts of glycogen in more packed granules or (2) increase GP-MM action sites by raising the number of nonreducing ends. As mentioned in section 1.4.2 (Glycogen breakdown), GP-MM only acts on the nonreducing ends of glycogen branches. Therefore, higher GBE levels in homozygous mice might indicate that: (a) glucose uptake is increased (as we observed with lower glucose in homozygous mice) with the purpose of synthesising more glycogen, therefore increased GBE levels are needed; (b) regardless of the absence of GP-MM, with a more branched glycogen, more nonreducing ends are available for the action of the enzyme in those muscles which rely on glycolytic metabolism (i.e. gastrocnemius and EDL).

Furthermore, we also observed differences in the glycogen content among muscles with distinct phenotype and different fiber composition, as well as, in the analyzed blood metabolites. Soleus presented the lowest glycogen content in both healthy and homozygous mice, suggesting as we previously reported [193], that muscles with a higher proportion of type II fibers might present a higher glycogen content than those with a higher proportion of type I fibers such as soleus. By contrast, our histochemistry results show that the largest vacuoles of glycogen

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Discussion

content present in both soleus, gastrocnemius and EDL are located in the most oxidative fibers. Further analyses are needed to determine which fiber type accumulates more glycogen.

With regard to the analyzed blood metabolites, we found that homozygous mice present lower levels of glucose (as mentioned above) and lactate, as well as, higher levels of ammonium compared to WT mice. As mentioned in section 1.3.1 (Anaerobic metabolism), ADP accumulation can lead to ATP regeneration by the myokinase pathway, generating ATP and AMP which subsequently is deaminated to IMP and NH_4^+. Therefore, the higher levels of ammonium observed in homozygous mice could be explained by the attempt to keep up with ATP demand by myokinase pathway as a result of reduced glycolytic flux in these mice. In fact, higher muscle ADP and plasma NH_4^+ levels were observed in individuals with McArdle disease compared to healthy controls [197, 198]. As consequence of the compromised glycolytic metabolism, we might also expect that oxidative metabolism of carbohydrates and lipids might be enhanced as an energy source compensatory mechanism. In fact, it was previously reported that McArdle patients presented significantly higher total FFAs levels during exercise compared to healthy controls [199], but no differences were observed at rest. According to McArdle patients, we did not observe significantly differences in plasma FFA levels among genotypes, although the results showed a trend to higher plasma FFA levels in homozygous mice compared to WT mice. Further analyses are needed to investigate whether fat oxidation in mice is enhanced during exercise as consequence of blocked muscle glycogen breakdown and whether differences on total carbohydrate oxidation are present among genotypes at rest and during exercise.

Lower blood levels of glucose might indicate a higher glucose uptake in homozygous mice compared to WT mice. Glucose is an important fuel for muscle

contraction, therefore the muscle is one of the main tissues taking glucose from the bloodstream. Glucose enters to the myocite via facilitated diffusion through GLUT4 which, in the absence of insulin or other stimuli such as exercise, is located intracellularly [200, 201]. In the presence of insulin or other stimulus, GLUT4 is translocated from intracellular vesicles to the plasma membrane and T-tubules [202]. The molecular mechanisms that allow the GLUT4 translocation are distinct depending on the stimulus (see references in [203]). Insulin stimulates glucose transport into adipocytes and myocites by regulating GLUT4 translocation [204] through Akt phosphorylation of Akt subtrate of 160 kDa (AS160, also known as TBC1D4), a Rab GTPase-activating protein [205]. In response to contraction, glucose uptake in skeletal muscle is increased by the translocation of GLUT4 to the cell membrane mediated by AMPK phosphorylation of TBC1D1, a paralog protein of TBC1D4 [206]. Phosphorylation of both TBC1D4 and TBC1D1 inhibits the Rab-GAP function, which causes GTP loading and activation of target Rabs, promoting GLUT4 translocation [207].

TBC1D1 and TBC1D4 expression differs between tissues and muscle fibers types in mouse skeletal muscle, since TBC1D1 expression is much higher in EDL than soleus, whereas the expression of TBC1D4 is much higher in soleus than in EDL [208]. By contrast, these differences are not observed in the rat [209]. Further research is needed to unravel whether these GLUT4 translocation pathways might be differently regulated in McArdle mice compared to WT mice, as well as, whether there is distinct regulation between different metabolically muscles. Furthermore, it might also be interesting to study whether in other tissues, such as liver, glycogen metabolism might be affected by GP-MM depletion.

Different from heterozygous individuals which are considered to by asymptomatic (at least, during daily living activities) [163], heterozygous mice

presented lower levels of GP-MM in the three studied muscles, as well as, reduced exercise capacity compared to WT mice. Interestingly, the reduction of GP-MM levels in heterozygous mice does not result in glycogen accumulation, suggesting that the slightly undermined exercise performance might be related to a glycolytic flux reduction, in spite of no significantly differences in plasma glucose, lactate and NH_4^+ levels were observed in heterozygous compared to WT mice.

Further studies are needed to understanding the regulation of glycogen metabolism and the importance of glycogen on muscle function, as well as, determining whether second wind phenomenon also occurs in the mouse model. In this study we only analyzed three different muscles, but it might be potentially interesting to study other muscles (e.g. diaphragm), since, although no respiratory disfunction have been reported among McArdle patients (with the exception of a unique case report where a McArdle patient presented with severe exertional dyspnea [210]), glycogen accumulation might be resulted in diaphragm as well, causing a possible impairment in respiratory function. Moreover, as I mentioned before, GP-MM is also expressed in a minor proportion in the heart muscle and it can be also expressed in the brain [36, 37]. Even though no relation between cardiac involvement or cognitive impairment with McArdle disease has been established, two case reports were reported [211, 212]. In the first case, a McArdle patient present with an incidental finding of severe obstructive hypertrophic cardiomyopathy was reported, but it is not clear whether there is a relationship between McArdle disease and this pathology [212]. In the second case, a 55-year-old woman with McArdle disease and cognitive impairment with bilateral dysfunction of prefrontal and frontal cortex was reported [211]. These cases suggest that cardiac and cognitive disfunction caused by the lack of GP-MM in heart and brain might be occurring in McArdle patients, but further studies either in McArdle patients or in the mouse model are

needed to corroborate this assumption.

Nevertheless, our results demonstrated once again that knock in McArdle mice is a great model of the disease because closely mimics the phenotype manifestations observed in McArdle patients and, therefore, might be used to study new therapeutic approaches for McArdle disease.

In 2010, Nogales-Gadea and coauthors [165] reported that GP-BB and GP-LL were both expressed in human myotubes either from McArdle patients or healthy individuals, thus glycogen accumulation was not observed in skeletal-muscle cultures derived from individuals with McArdle disease.

With the generation of the mouse model of McArdle disease [193], the idea of using primary skeletal-muscle cultures derived from this murine model as an *in vitro* model of the disease contemplated the possibility of using them to test different pharmacological therapies prior to their evaluation in *in vivo* models.

In this PhD thesis, we developed and showed that this *in vitro* model derived from the murine McArdle disease model constitutes a valid model to analyze and assess potential treatments for the disease, since, unlike muscle cultures derived from McArdle patients, these cells do not exhibit any GP activity at any differentiation stage and accumulate large amounts of glycogen deposits. However, WT mouse myotubes express GP-MM.

As mentioned above, until now, several types of treatments have been studied to reduce or eliminate the symptoms of McArdle disease but most of them obtained unsatisfactory results.

As commented before, a natural ovine model of McArdle disease has been

described [192]. It has been recently reported that the necrosis induced by injection of notexin into the muscles of affected sheep was followed by regeneration of muscle fibers. Moreover, they also observed that these regenerating muscle fibers showed re-expression of both non-muscle isoforms of GP and a reduction in glycogen accumulation [213]. In addition, in another recent publication, the authors observed increased expression of GP in muscle fibres of McArdle sheep after enteral and intramuscular treatment with VPA [180]. Therefore, we treated the recently developed mouse primary skeletal muscle cultures *in vitro* with VPA and we observed that treated myotubes expressed GP-BB. In addition, a dose-dependent diminution in glycogen accumulation was also observed. By contrast, we did not observe any beneficial effect when we treated knock-in McArdle mice *in vivo* with different doses of VPA, as well as, with distinct routes of administration (data not published). The half-life (T0.5 beta) of VPA differs among species [214], being e.g. 90-120 min in dogs [214, 215] and 15.9 ± 2.6 hours in humans [216]. In mice, VPA is willingly absorbed after oral administration and it is rapidly eliminated following intravenous administration with a half-life being around 50 min [215]. The shorted duration of VPA in the bloodstream might explain because we did not observe any effect in treated mice, so further studies using different routs of administration or with a distinct experimental design are needed to confirm VPA efficiency in mice. Moreover, the treatment of undifferentiated myoblasts with other histone deacetylase inhibitors such as trichostatin A (TSA) or phenylbutyrate (PhB) has been demonstrated to enhance muscle gene expression favouring the recruitment and fusion of myoblasts and subsequently differentiation [217-219]. And in addition, many clinical trials have been performed using other histone deacetylase that inhibit specific histone deacetylase classes, *i.e.* class I, II, III or IV [220], suggesting their potential utilization as an alternative to VPA.

Although the negative results with VPA treatment in mice, VPA treatment in humans could potentially have better effects because of the longer VPA half-life compared with mice. VPA has been used in the treatment of acute mania, epilepsy and bipolar disorders for many years [221-223], which would facilitate testing it in McArcle patients. Preclinical studies are needed to optimize the drug dosage to most effectively modulate *PYGB* expression.

During many years, apart from clinical manifestations, the diagnosis of McArdle has been based on the analysis of the two *PYGM* copies. Up to the present, 148 pathogenic mutations have been described [49, 108] which some of them being most frequent than others. Consequently, sequential analysis of these mutations must be performed, but in those patients where the most frequent mutations are not present further sequencing of *PYGM* is required and sometimes invasive muscle biopsies are needed.

Moreover, histologic studies of muscle biopsies to evaluate the grade of affectation of a disease and monitor the response to treatment are needed in many diseases such as McArdle or Pompe disease [224]. Muscle biopsies are invasive and painful procedures with a limited utilization, for these reasons, the assessment of new biomarkers in the plasma or urine for monitoring the response to treatment represent an improvement in terms of avoiding unnecessary suffering in patients with muscular pathologies.

In this PhD thesis, we have developed a potential tool for assess the GP-MM expression in WBCs by flow cytometry. Using a fluorescent-labeled GP-MM antibody, we analysed the GP-MM expression in WBCs (T-lymphocytes) of 20 healthy controls and 30 McArdle patients. Although T-lymphocytes only have very low levels (1%) of PYGM transcripts compared to muscle tissue, diagnosis was still possible and

practicable in many Spanish patients: GP-MM expression (as well as transcript levels) was significantly lower in the T-lymphocytes of McArdle patients compared to healthy controls, regardless of the type of mutation they harbored, with a GP-MM expression value below 28.6% of normal been considered to indicate the presence of McArdle disease with 95% sensitivity and 83% specificity.

As reported by Arrizabalaga *et al*. [225], we found that GP-MM is expressed in lymphocytes, specifically in T-lymphocytes (33% of T-lymphocytes expresses GP-MM) whereas B-lymphocytes did not express the enzyme.

Hence, our results suggest that flow cytometry analysis may be useful for fast, quantitative assessment of GP-MM expression in WBCs and this approach could serve as an initial screening test for McArdle disease, as well as, a monitoring tool for analysing the treatment response over time. At the beginning, parallel histological analyses using muscle biopsies might be needed to confirm the results, but once outcomes were corroborated, muscle biopsies analyses could be substituted by WBCs analysis by flow cytometry.

5. Conclusions

FIRST

McArdle mouse model closely mimics the phenotype manifestations of McArdle disease in humans and might help to provide new insights into the importance of glycogen on muscle function.

SECOND

Both complete and partial deficiency of GP-MM resulted in impaired maximal endurance capacity, thereby reflecting the key role that GP-MM plays in muscle function in mammals.

THIRD

Expression of the main muscle glycogen regulatory enzymes (as well as muscle glycogen content) differed depending on the muscle predominant phenotype (slow-*vs*. fast-twitch fibers), showing a distinct glycogen metabolism regulation among muscles.

FOURTH

The glycogen synthesis machinery was down-regulated in p.R50X/p.R50X mice, probably reflecting a protective mechanism to prevent deleterious glycogen accumulation.

FIFTH

Mouse primary skeletal-muscle culture is a good study model of the disease because it mimics the phenotype of the muscle tissue from affected individuals and constitutes a valid *in vitro* model to analyze and evaluate potential treatments for

the disease.

SIXTH

VPA can enhance GP-BB expression *in vitro* and might be a candidate for the treatment of McArdle disease.

SEVENTH

33% of human T-lymphocyte population expresses GP-MM at high levels, indicating the presence of this enzyme in another tissue different from muscle.

EIGHT

GP-MM expression was significantly lower in T-lymphocytes of McArdle patients compared to healthy controls and healthy carriers, in consonance with the expression levels observed in muscles biopsies from McArdle patients.

NINTH

Flow cytometry analysis may be useful for fast, quantitative assessment of myophosphorylase expression in WBCs and this approach could serve as an initial screening test for McArdle disease.

6. Annex 1

6.1 Insulin-stimulated glycogen synthesis pathway is down-regulated in McArdle mice

6.1.1 Introduction

McArdle disease, also termed glycogenosis type V or glycogen storage disease type V (GSD V) [OMIM® database number 232600], is an autosomal recessive disorder caused by the lack of GP-MM [75]. It is caused by pathogenic mutations in both alleles of *PYGM*, which is located in chromosome 11q12-11q13. GP-MM catalyses the breakdown of muscle glycogen into glucose-1-phosphate; thus, patients are unable to obtain energy from their muscle glycogen stores. Therefore, affected individuals typically present with exercise intolerance, in the form of acute crises of early fatigue and muscle stiffness and contractures, which can be accompanied by rhabdomyolysis, as indicated by the efflux of CK to the bloodstream or by subsequent myoglobinuria [93].

Two spontaneous animal models for McArdle disease have been reported, i.e. Charolais calf and Merino sheep [190, 192] but it was not until the development of the knock-in mouse homozygous for the most common pathogenic mutation causing McArdle disease among Caucasians (i.e. *p.R50X*) [193] that in-depth studies of the pathophysiology of this disorder could be performed. A first study revealed that knock-in mice presented a complete absence of GP-MM protein in gastrocnemius muscle extracts, as well as, a clear McArdle-like phenotype [193], whereas further research disclosed a complete absence of this enzyme and a different expression of the main regulatory enzymes of glycogen metabolism (i.e. GS, pGS, GDE and GBE) in both oxidative and glycolytic muscles [226]. It was previously reported that high

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glycogen in skeletal muscle decreases insulin-stimulated glycogen synthesis, as well as GS activation [196]. This was corroborated in McArdle mice by Brull and co-workers [226] who demonstrated a decrease in total GS expression in both oxidative and glycolytic muscles, together with an increase in the inactive phosphorylated form of GS. Therefore, further analyses were missing that might help to unravel whether other enzymes involved in the insulin-regulation pathway of glycogen synthesis are differently expressed in skeletal muscle of McArdle mice. Accordingly, in the present study, we analyzed the expression of some of the enzymes involved in the activation and inactivation of GS in young adult (2-months-old) WT, heterozygous and homozygous mice in three metabolically different skeletal muscles (i.e. soleus, gastrocnemius and EDL).

6.1.2 Materials and methods

Ethical approval. All experimental procedures were approved by the Vall d'Hebron Institutional Review Board (procedure 13/04 CEEA; 35/04/08) and were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 1 2 3) and Spanish laws (32/2007 and R.D. 1201/2005).

Animals. In all experiments described below, we used the previously developed *p.R50X/p.R50X* knock-in McArdle mice [193]. We studied 2-months-old female and male littermates and all animal were killed by cervical dislocation to collect tissue samples.

Western Blot analyses. Samples from gastrocnemius, soleus and EDL muscles were homogenized in cold homogenization buffer (40 mM ß-glycerophosphate, 40mM NaF, 10mM EDTA and 20 mM ß-mercaptoethanol, pH 6.8) supplemented with a protease and phosphatase inhibitor cocktail (Ref. 04693116001 and 04906837001,

respectively; Roche, Mannheim, Germany) and centrifuged at 10,000 g for 10 min at 4°C. Protein extracts (20 μg) were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Immun-Blot® PVDF membrane; Bio-Rad, Hercules, CA, USA) and probed with primary antibodies against phospho-protein kinase B (Thr 308) (pAkt) (ref. #9275; Cell Signaling Technology, Inc., Danvers, MA, USA), phospho-AMP-activated protein kinase subunit alpha (Thr 172) (pAMPKα) (ref. #2531; Cell Signaling Technology, Inc., Danvers, MA, USA), glycogen synthase kinase 3 α /ß protein (GSK3α/ß) (ref. NBP1-19364; Novus Biologicals EUA, Cambridge, UK), phospho-glycogen synthase kinase 3 α /ß protein (Ser21/9) (pGSK3α/ß) (ref. #9331; Cell Signaling Technology, Inc., Danvers, MA, USA) and glyceraldehyde-3-phosphate dehydrogenase protein (GAPDH) (ref. AM4300; Ambion, Austin, TX, USA). The horseradish peroxidase-conjugated secundary antibodies included rabbit anti-mouse (Dako, Glostrup, Denmark) and goat anti-rabbit (Jackson Laboratories, Baltimore Pike, PA, USA). Images were obtained with Fujifilm LAS 3000 imager (R&D Systems, Minneapolis, MN, USA) and quantified with ImageJ, version 1.48v (NIH, Bethesda, MD, USA).

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Statistical analyses. All statistical analyses were performed using the IBMS SPSS, version 15.0 for Windows (IBM Corp., Armonk, NY, USA) with α set at 0.05 and data are reported as the mean ± SD. We compared molecular phenotypes of the three study groups (WT, heterozygous or homozygous mice) using the non-parametric Mann-Whitney U test. This test allowed us to determine the statistical effect of the study group to which the mice belonged on the results, i.e. "group effect". For statistical purposes, undetectable values were considered as zero.

6.1.3 Results

We studied the protein levels of the enzymes involved in the GS regulation (pAkt (Thr 308), pAMPKα (Thr 172), GSK3α/ß and pGSK3α/ß (Ser21/9)) in three metabolically different muscles (gastrocnemius, soleus and EDL) in WT, heterozygous and homozygous mice of both sexes (Figure 10-12). For active phosphorylated form of protein kinase B (pAkt), we found a significant genotype effect in the three muscles analyzed (Figure 10-12; F). Specifically, in *post hoc* analyses, pAkt levels were lower in homozygous mice in the three muscles (Figure 10-12; F). Downstream in the pathway, once Akt is phosphorylated and thus activated, it phosphorylates GSK3α/ß and inhibits its activity; in this regard, we only found significant differences between homozygous and heterozygous mice in the gastrocnemius and soleus muscles, where total GSK3α protein levels were higher and lower in the gastrocnemius and soleus of homozygous mice, respectively (Figure 10 and 11; B), although no significant differences were observed between genotypes in the EDL (Figure 12; B). In the case of GSK3ß, we found significant higher protein levels in the gastrocnemius of homozygous mice (Figure 10; C) but no significant differences were observed in both soleus and EDL (Figure 11 and 12; C). Additionally, we also found a significant genotype effect for the inactive phosphorylated form of GSK3α/ß. In *post hoc* analyses, pGSK3α enzymes levels were significantly lower in homozygous mice compared to the other two groups (Figure 10-12; D). In the case of pGSK3ß, it presented significant lower levels in homozygous mice compared to the other two groups in EDL (Figure 12; E) but in soleus, we only found significant lower levels in homozygous compared to heterozygous mice (Figure 11; E).

When we analyzed pAMPKα protein levels, we also found a significant genotype effect in the three muscles (Figure 10-12; G); in *post hoc* analysis, significant differences between genotypes were found for the three muscles, with

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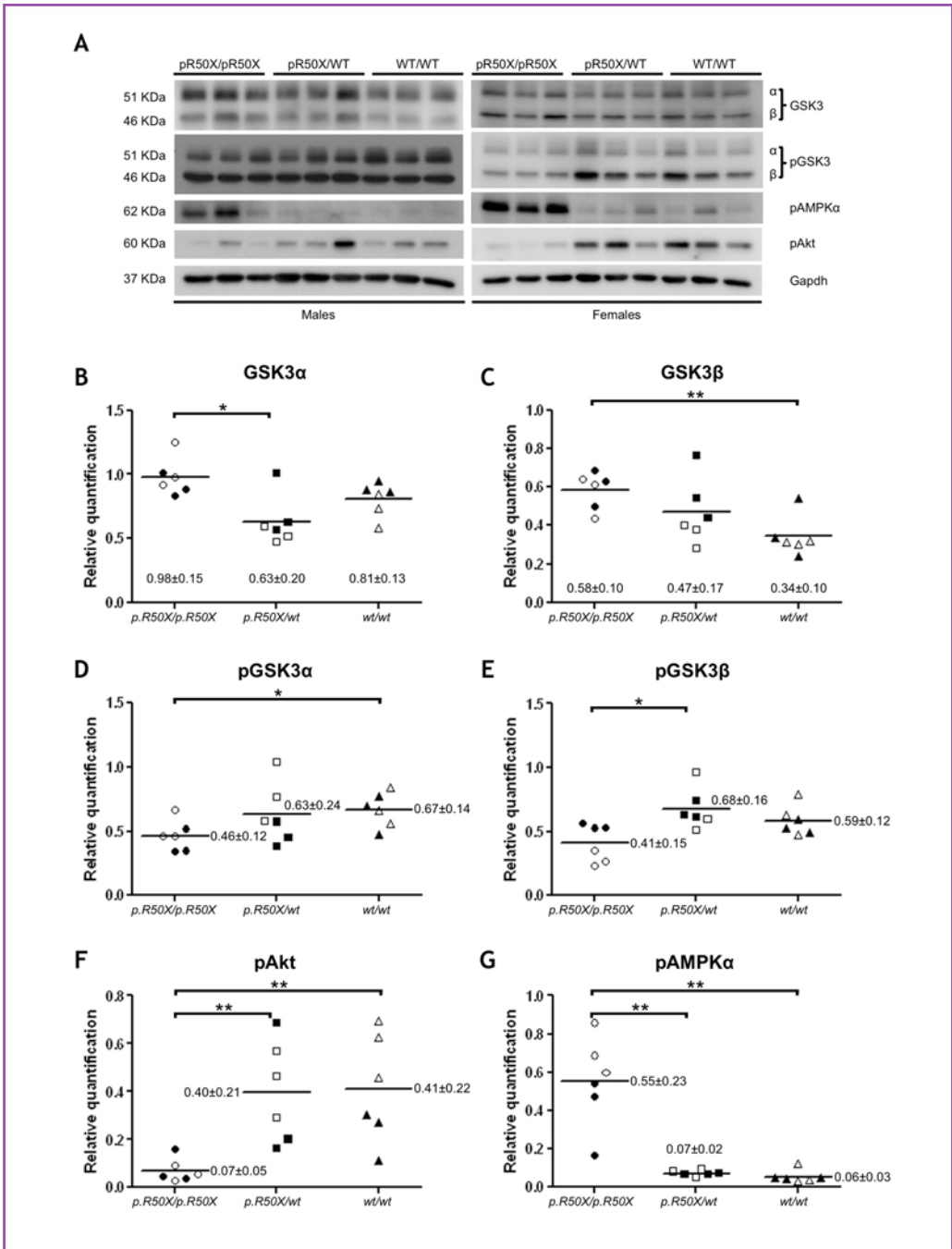
Figure 10: Effect of *Pygm* genotype on the enzymes involved in glycogen synthase regulation in gastrocnemius. A, western blot analyses. B, effect of *Pygm* genotype on GSK3α. C, effect of *Pygm* genotype on GSK3β. D, effect of *Pygm* genotype on pGSK3α. E, effect of *Pygm* genotype on pGSK3β. F, effect of *Pygm* genotype on pAkt. G, effect of *Pygm* genotype on pAMPKα. Data are shown as the mean ± SD and individual values, with males indicated by black coloured squares, dots or triangles. GSK3, glycogen synthase kinase 3; pGSK3, phospho-glycogen synthase kinase 3; pAkt, phospho-protein kinase B; pAMPKα, phospho-AMP-activated protein kinase subunit alpha; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Significant post hoc differences are indicated with asterisks ($^*P ≤ 0.05$; $^{**}P ≤ 0.01$).

Figure 11: Effect of *Pygm* genotype on the enzymes involved in glycogen synthase regulation in soleus. A, western blot analyses. B, effect of *Pygm* genotype on GSK3α. C, effect of *Pygm* genotype on GSK3β. D, effect of *Pygm* genotype on pGSK3α. E, effect of *Pygm* genotype on pGSK3β. F, effect of *Pygm* genotype on pAkt. G, effect of *Pygm* genotype on pAMPKα. Data are shown as the mean ± SD and individual values, with males indicated by black coloured squares, dots or triangles. GSK3, glycogen synthase kinase 3; pGSK3, phospho-glycogen synthase kinase 3; pAkt, phospho-protein kinase B; pAMPKα, phospho-AMP-activated protein kinase subunit alpha; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Significant post hoc differences are indicated with asterisks ($^*P \leq 0.05$; $^{**}P \leq 0.01$).
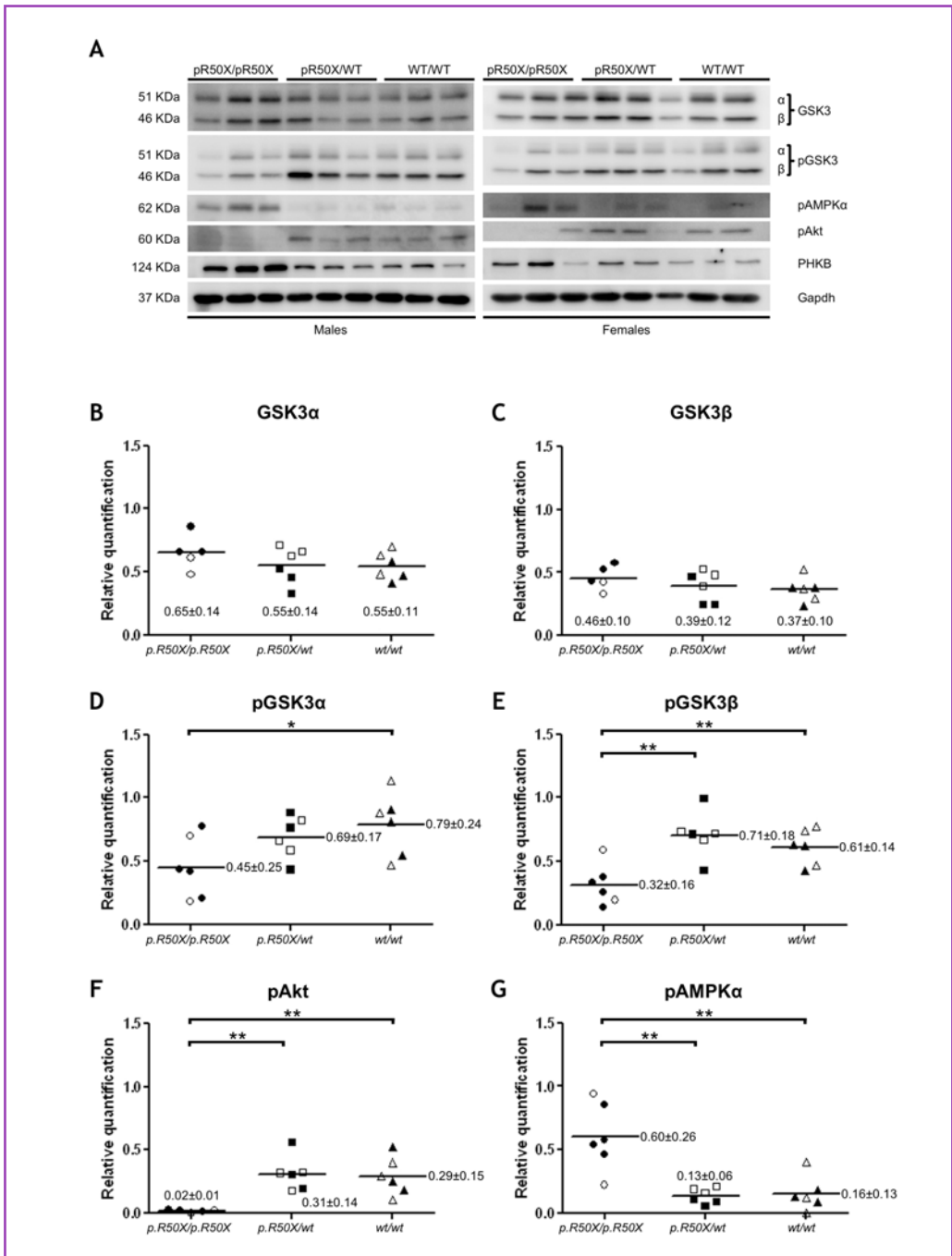
Figure 12: Effect of *Pygm* genotype on the enzymes involved in glycogen synthase regulation in EDL. A, western blot analyses. B, effect of *Pygm* genotype on GSK3α. C, effect of *Pygm* genotype on GSK3β. D, effect of *Pygm* genotype on pGSK3α. E, effect of *Pygm* genotype on pGSK3β. F, effect of *Pygm* genotype on pAkt. G, effect of *Pygm* genotype on pAMPKα. Data are shown as the mean ± SD and individual values, with males indicated by black coloured squares, dots or triangles. GSK3, glycogen synthase kinase 3; pGSK3, phospho-glycogen synthase kinase 3; pAkt, phospho-protein kinase B; pAMPKα, phospho-AMP-activated protein kinase subunit alpha; GAPDH, glyceraldehyde 3-phosphate dehydrogenase. Significant post hoc differences are indicated with asterisks ($^*P \leq 0.05$; $^{**}P \leq 0.01$).

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enzyme levels being higher in homozygous mice compared to the other two groups (Figure 10-12; G).

In all the proteins analyzed, no differences were found between sexes for any of the *Pygm* genotypes.

6.1.4 Discussion

Skeletal muscle plays an important role in maintaining blood glucose homeostasis, where glucose is stored in the form of glycogen. Glycogen accumulation in skeletal muscle is limited, thus a feedback mechanism is needed.

GS is regulated by phosphorylation and allosterically activated by glucose-6-phosphate. Stimulus such as insulin, adrenaline, glycogen content or muscle contraction may change its phosphorylation state and concentration of allosteric activators, activating GS [227]. GS has nine phosphorylation sites which are phosphorylated by different kinases: GSK3α/ß, AMPK, PKA, PHK, CaM-kinase II, casein kinase, DYRK, PAS and p38 MAP kinase (see references in [227]), but only three of them (i.e. GSK3α/ß, AMPK and PKA) have been determined to have physiological significance in the regulation of GS [228-230]. By contrast, GS is only dephosphorylated by PP1 [228].

To regulate glycogen accumulation, insulin plays an important role and it is necessary for regulation of blood glucose, being responsible for stimulating glucose uptake and increasing GS activity [196]. In the absence of insulin or other stimuli such as exercise, GLUT4 is located intracellularly [200, 201]. In the presence of insulin or other stimulus, GLUT4 is translocated from intracellular vesicles to the plasma membrane. Moreover, insulin activates GS by dephosphorylation (Figure 13; A) [231]. The binding of insulin to the transmembrane insulin receptor activates

authophosphorylation and tyrosine kinase phosphorylates the insulin receptor substrate (IRS) [232]. The phosphorylation of IRS triggers the activation of phosphatidylinositol 3-kinase (PI3K) which generates phosphatidylinositol lipid products in the plasma membrane and thereby triggers the recruitment of Akt and phosphoinositide-dependent kinase 1 (PDK1), allowing the phosphorylation and activation of Akt by PDK1 [233-235]. GSK3α/ß, which phosphorylates and inactivates GS, is inactivated by phosphorylation at Ser21 of GSK3α and Ser9 of GSK3ß by pAkt, increasing GS activity (Figure 13; A) [236].

As we previously reported, the glycogen synthesis machinery was down-regulated in *p.R50X/p.R50X* knock-in McArdle mice [226] compared to the WT and heterozygous mice. This was indicated by the lower GS protein levels and the higher levels of the inactive, pGS observed in these mice. In the present study, when we analyzed the active, phosphorylated form of Akt, we observed that compared to WT mice and heterozygous mice, pAkt was down-regulated in *p.R50X/p.R50X* knock-in McArdle mice in all muscle studied (Figure 10-12; F and Figure 13; B). Similar results were previously observed in rodent skeletal muscles preconditioned to obtain high muscle glycogen content *in vivo* [237], as well as, in McArdle patients, which present reduced insulin-induced Akt phosphorylation compared to healthy controls [194]. Prior studies have also shown that Akt phosphorylation of the Rab GTPase-activating protein, AS160 (also known as TBC1D4), triggers GLUT4 translocation [205]. A reduction of the active, phosphorylated form of Akt might also expect a reduction in GLUT4 translocation and therefore an increase in blood glucose levels. These results are controversial with results previously reported where lower blood glucose levels were observed in *p.R50X/p.R50X* knock-in McArdle mice compared to WT mice and heterozygous mice [226]. As an increase in blood glucose levels is not observed in *p.R50X/p.R50X* knock-in McArdle mice, this suggests that alternative pathways

Figure 13: Insulin signalling pathway acting via PI3K and Akt. A.-in healthy individuals. B.- in McArdle mice. IRS, insulin receptor substrate; PI3K, phosphatidylinositol 3-kinase; PIP_2, phosphatidylinositol 4,5-biphosphate; PIP_3, phosphatidylinositol 3,4,5-triphosphate; ATP, adenosine triphosphate; ADP, adenosine diphosphate; Akt, protein kinase B; GSK3, glycogen synthase kinase 3; PKA, protein kinase A; AMPK, AMP-activated protein kinase; GS, glycogen synthase; PP1, protein phosphatase 1; GDE, glycogen debranchin enzyme; GBE, glycogen branching enzyme; GPa, glycogen phosphorylase a; GPb, glycogen phosphoryase b; PHKB, phosphorylase kinase subunit beta; P, phosphate.

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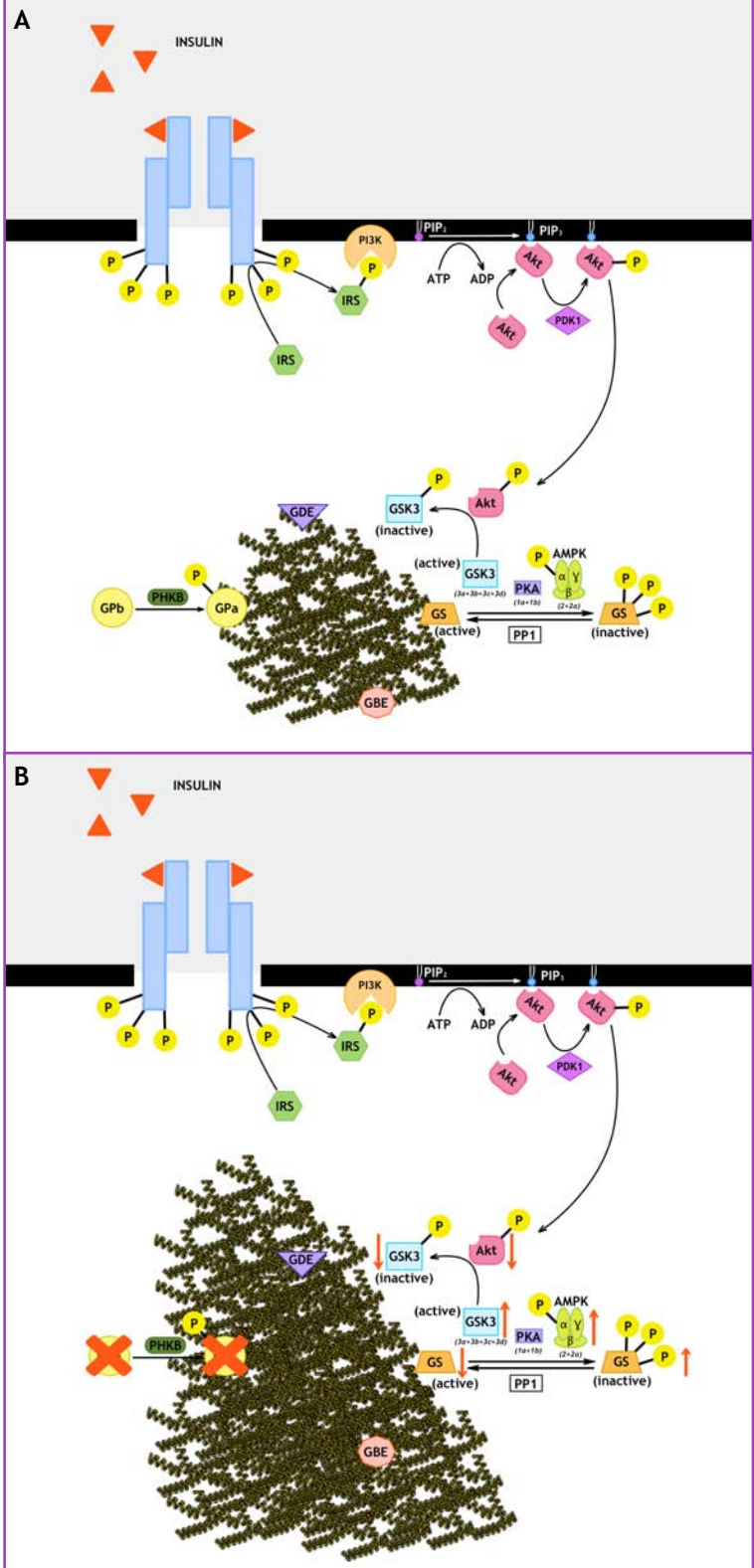
involved in GLUT4 translocation to plasma membrane, such as AMPK/TBC1D1 pathway, might be up-regulated in *p.R50X/p.R50X knock-in* McArdle mice.

It was previously reported that activity of GSK3α is reduced by insulin stimulation in human skeletal muscle [238], and the levels of GSK3α and GSK3ß were unaffected by glycogen level in human skeletal muscle from McArdle patients [194], suggesting the unlikely involvement of GSK3α and GSK3ß in the reduction of GS activity. These results are at odds with the outcomes of the present study, where even though no differences were observed in GSK3α and GSK3ß in the studied muscles (Figure 10-12; B and C), with the only exception of the gastrocnemius muscle where higher GSK3ß levels were observed in *p.R50X/p.R50X* knock-in McArdle mice compared to WT mice (Figure 10; C), we observed that pGSK3α was down-regulated in the three studied muscles (Figure 10-12; D), as well as, pGSK3ß in EDL (Figure 12; E) from *p.R50X/p.R50X* knock-in McArdle mice. This reduction in the inactive, phosphorylated form of pGSK3α might be due to the lower levels of pAkt, which is the responsible of the phosphorylation of GSK3α/ß and it might also explain the previously observed higher levels of the inactive, pGS in *p.R50X/p.R50X* knock-in McArdle mice, thus GSK3α/ß is one of the enzymes responsible of GS phosphorylation.

As mentioned above, other stimulus distinct from insulin, may also change GS phosphorylation state. High glycogen content modulates GS phosphorylation, increasing its phosphorylation at Ser^7, $Ser^{7,10}$ and Ser^{641} sites [229, 239]. GS is also regulated by adrenaline and muscle contraction. Adrenaline inhibits GS via both PKA-mediated phosphorylation and decreasing PP1 activity, as well as, blocking insulin-mediated activation [240]. By contrast, exercise decreases glycogen content and increases GS activity [241], whereas at the same time, decreases GS activity by AMPK. AMPK is mainly activated during prolonged exercise and becomes a key

enzyme in the switch from anaerobic to aerobic metabolism [21]. During muscle contraction, ATP consumption increases and other ATP-consuming processes such as biosynthesis should be switched off. The inhibition of GS by AMPK ensures that the increased flux of glucose into the cell is redirected towards catabolic breakdown of glucose rather than glycogen synthesis.

Although none of the mice which participated in the present study followed an exercise program, our results showed an increase in pAMPKα in the three studied muscles from *p.R50X/p.R50X knock-in* McArdle mice. This increase might be explained by the increment of AMP produced by myokinase pathway in the regeneration of ATP. AMP allosterically activates AMPK and facilitates its phosphorylation and activation. Therefore, AMPK increase might be produced as a) a compensatory mechanism to avoid deleterious glycogen accumulation phosphorylating and inhibiting GS or b) the attempt to redirect the flux of glucose towards catabolic breakdown of glucose rather than glycogen synthesis, favouring the switch from anaerobic to aerobic metabolism. Moreover, in response to contraction, glucose uptake in skeletal muscle is increased and this is caused by a translocation of GLUT4 to the cell membrane mediated by AMPK [206]. As we mentioned above, lower levels of blood glucose have been observed in *p.R50X/p.R50X knock-in* McArdle mice, therefore, it might be explained by the increase in pAMPKα expression in these mice compared to WT and heterozygous mice. Further studies are needed to unravel the mechanisms and pathways involved in GLUT4 translocation in this animal model.

In the present study we show that *p.R50X/p.R50X knock-in* McArdle mice represents an important tool for the study of glycogen metabolism in a high glycogen content situation, as well as, for the study of new development approaches for this pathology.

7. Annex 2

7.1 Gene therapy using a muscle-targeted AAV vector restores myophosphorylase in a murine model of McArdle disease

7.1.1 Introduction

McArdle disease (glycogenosis type V; glycogen storage disease type V (GSD V); [OMIM® database number 232600]) is an autosomal recessive disorder caused by pathogenic mutations *PYGM* encoding GP-MM. This enzyme catalyzes the first step of glycogen breakdown, thus patients are unable to obtain energy from their muscle glycogen stores and typically present with exercise intolerance with premature fatigue, muscle stiffness, contractures, high levels of CK at rest and episodic myoglobinuria [151].

Several types of treatments that include nutritional supplements [105, 172-176, 178, 179], supervised exercise training [97, 184, 242] or "read through" treatments [127] have been studied in McArdle disease with different and polemical results, but nowadays, no therapy for restoring GP-MM activity in patients exists. However, the recently generated murine model with the most common mutation (p.R50X) causing McArdle disease presents the main clinical features of the disease and represents a valid tool for evaluating potential therapies [193].

It was previously demonstrated that transfection with *PYGM* cDNA restored GP-MM activity *in vitro*, i.e. in human and sheep myoblast cultures deficient for GP-MM [181]. Whereas, intramuscular injection of adenovirus and AAV vectors containing GP-MM expression cassettes *in vivo* in the ovine model of McArdle disease only produced GP-MM activity in the vicinity of the injection site [182]. In the present study we show that gene therapy using an AAV vector containing human GP-MM

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expression cassette and targeting skeletal muscle cells is able to restore GP-MM activity in gastrocnemius muscle and reduce glycogen accumulation in this muscle in the murine model of McArdle disease.

7.1.2 Materials and methods

Vector construction and production. Human *PYGM* cDNA (hPYGM) was obtained from Source Bioscience, Nottingham, UK (Ref. IRCMp5012A1221D). Sequence was verified and cloned into AAV2/8 vector containing a modified CK promoter (CK7; GeneArt, Thermo Fisher Scientific, Waltham, MA, USA [243]) and the bovine growth hormone (BGH) polyadenylation signal (Figure 14). The virus was produced and titered by Unitat de Producció de Vectors (UPV) of the Centre de Biotecnologia Animal i Teràpia Gènica (CBATEG), Universitat Autònoma de Barcelona, Bellaterra, Spain.
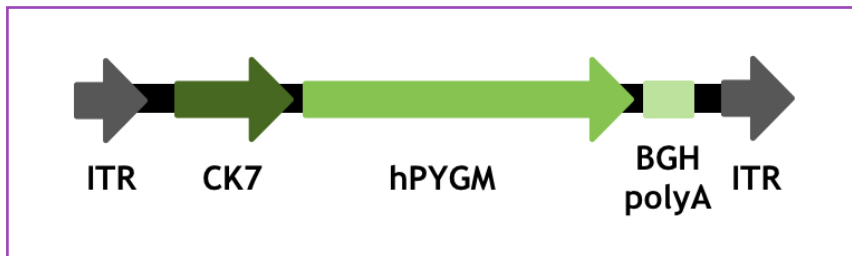
Figure 14: Schematic representation of AAV2/8-CK7-hPYGM. ITR, inverted terminal repeat; CK7, modified creatine kinase promoter 7; hPYGM, human *PYGM* cDNA; BGH poly A, bovine growth hormone polyadenylation signal.

Ethical approval. All experimental procedures were approved by the Vall d'Hebron Institutional Review Board (procedure 25/13 CEEA; 35/04/08) and were conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 1 2 3) and Spanish laws (32/2007 and R.D. 1201/2005).

Animals. In all experiments described below, we used the previously developed *p.R50X/p.R50X* knock-in McArdle mice [193]. 2-days-old *p.R50X/p.R50X* knock-in mouse was treated with 10^{12} genome copies (gc)/pup of AAV2/8-CK7-hPYGM with a single intraperitoneal injection. Eight weeks following injection, mouse was killed by cervical dislocation and tissues were collected for analysis.

PCR amplification. DNA was isolated from gastrocnemius, soleus, EDL, biceps femoris, quadriceps, tibialis anterior (TA), diaphragm, heart, liver and brain tissues following the manufacturer instructions of QIAamp DNA mini kit (Qiagen, Hilden, Germany). For PCR analysis, 50 ng of DNA was amplified for 32 cycles consisting of denaturation at 95ºC for 30 seconds, annealing at 58ºC for 30 seconds, and extension at 72ºC for 30 seconds. Initial denaturation at 95ºC was performed for 5 min and a final extension step at 72ºC for 10 minutes stopped the program. For hPYGM detection the following PCR primers were used:

hPYGM Fw: 5'-TCCAGCTCAATGACACCCAC-3'

hPYGM Rw: 5'-ATGCGCTCAGCAATGACCTC-3'

The PCR products were purified by electrophoresis in 1.5% agarose gel.

Western Blot analysis. Samples from gastrocnemius, soleus and EDL muscles were homogenized in cold homogenization buffer (40 mM ß-glycerophosphate, 40mM NaF, 10mM EDTA and 20 mM ß-mercaptoethanol, pH 6.8) supplemented with a protease and phosphatase inhibitor cocktail (Ref. 04693116001 and 04906837001, respectively; Roche, Mannheim, Germany) and centrifuged at 10,000 g for 10 min at 4ºC. Protein extracts (20 µg) were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis, transferred to a polyvinylidene difluoride membrane (Immun-Blot® PVDF membrane; Bio-Rad, Hercules, CA, USA) and probed with primary antibodies against GP-MM (kindly provided by Prof. Martinuzzi) and GAPDH (ref. AM4300; Ambion, Austin, TX, USA). The horseradish peroxidase-conjugated secundary

antibodies included rabbit anti-mouse (Dako, Glostrup, Denmark) and donkey anti-goat (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Images were obtained with Fujifilm LAS 3000 imager (R&D Systems, Minneapolis, MN,USA).

Histochemical analysis. Gastrocnemius muscle were fixed in O.C.T. medium in cold methyl butane for 30 s and samples were maintained in liquid nitrogen until analysis. To determine muscle morphology, 8 µm sections were stained with haematoxylin & eosin standard staining and slides were first incubated 5 min in haematoxylin (Merck-Millipore, Billerica, MA, USA); subsequently, after two washes with 1% hydrochloric acid and ammonia water (ammonium hydroxide), slides were incubated with 2% eosin. Glycogen content was analysed with periodic acid–Schiff (PAS) staining by sequentially incubating the sections with: periodic acid (0.5%) for 5 min, water wash, Schiff's solution for 1 min, water wash for 1 min, haematoxylin for 1 min, water wash, alcohol-xylol dehydration and DPX mounting medium (Sigma-Aldrich). Stained sections were analysed and images were obtained with an inverted microscope (IX 71 Inverted Microscope; Olympus Corp., Tokyo, Japan).

7.1.3 Results

AAV treatment restores GP-MM expression in gastrocnemius muscle

Human GP-MM protein was undetectable by western blot analysis in gastrocnemius, soleus and EDL muscles of untreated *p.R50X/p.R50X* knock-in mouse analyzed (Figure 15; B). Similar levels to untreatead WT mouse were observed in gastrocnemius of treated *p.R50X/p.R50X* knock-in mouse, whereas human GP-MM was undetectable in soleus and EDL muscles of treated mouse analyzed (Figure 15; B).

Aiming to assess if undetectable levels of GP-MM protein in soleus and EDL muscles of treated *p.R50X/p.R50X* knock-in mouse were due to inefficient AAV
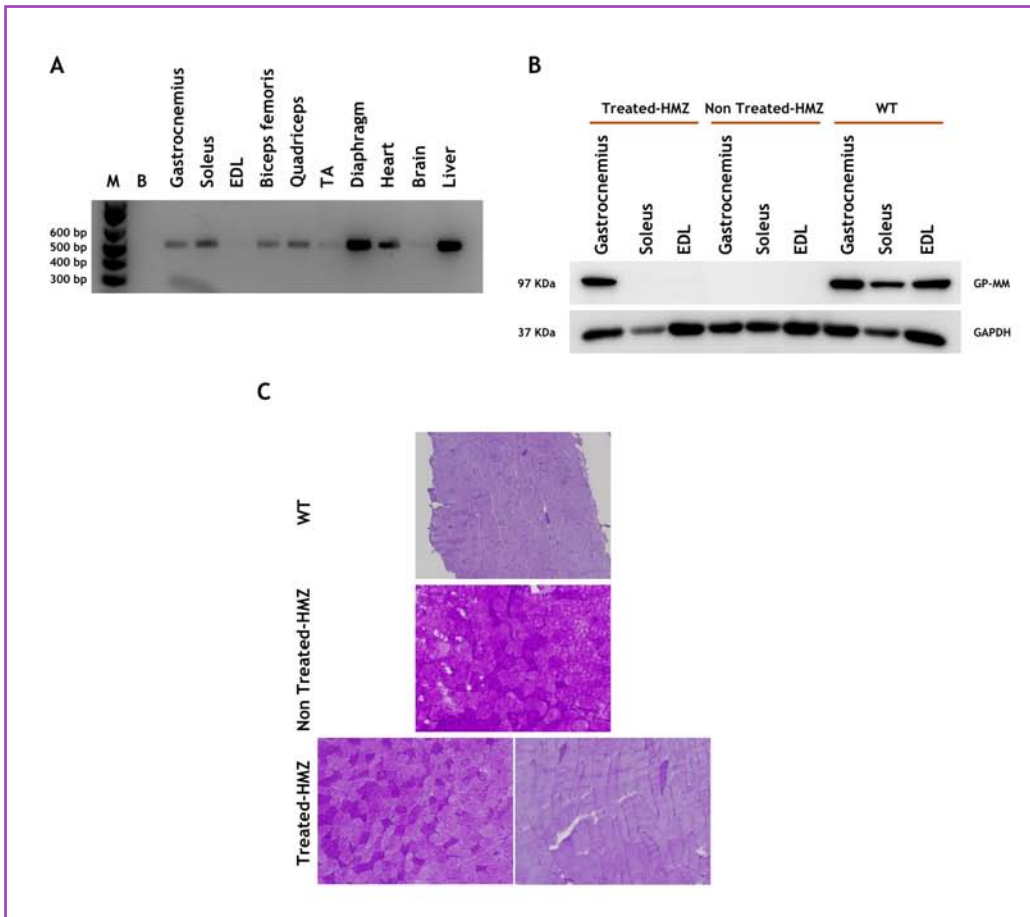
Figure 15: AAV2/8-CK7-hPYGM treatment in neonatal McArdle mice. A.- PCR amplification results showing AAV transduction in muscle and non-muscle tissues. B.- Western blot showing human GP-MM and GAPDH of gastrocnemius, soleus and EDL muscles homogenates from WT, treated and untreated homozygous mice. C.- PAS staining showing glycogen accumulation of gastrocnemius muscle from WT, treated and untreated homozygous mice. M, molecular weight marker; B, blank; bp; base pair; EDL, extensor digitorum longus muscle; TA, tibialis anterior muscle; WT, wild-type mice; GP-MM, skeletal-muscle isoform of glycogen phosphorylase protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase protein; HMZ, homozygous mice.

transduction in these muscles, we performed a PCR amplification analysis to evaluate muscle transduction among other target tissues (i.e. heart, brain and liver) (Figure 15; A). We found that AAV transduced in most of studied tissues but we observed lower levels of AAV transduction in EDL muscle compared to the other muscles. By contrast, AAV transduction in soleus muscle was higher than in gastrocnemius muscle.

AAV treatment decreases glycogen accumulation in gastrocnemius muscle from treated *p.R50X/p.R50X* knock-in mouse

To validate vector-mediated hPYGM expression, muscle sections were examined for GP-MM expression (Figure 15; C). As expected, glycogen accumulation reduction was observed in gastrocnemius muscle from treated *p.R50X/p.R50X* knock-in mouse (Figure 15; C), but not in soleus and EDL muscles (data not shown).

7.1.4 Discussion

AAV vectors are currently among the most used vectors in gene therapy, and most of the approved clinical studies are based on using these vectors. Nowadays, exist different serotypes of AAV with distinct tissue tropism and transductions efficiencies [244]. The main advantages of using AAV vectors as a therapeutic vector are: 1) no AAV has been associated with any human disease, 2) AAV infect division and quiescent cells, 3) transgen expression remains stable over long periods of time, 4) viral genome remains mainly in episomal form inside the host cell, and 5) the use of AAV is not associated with the development of tumors [245]. Therefore, AAV vectors have been already used in preclinical or even clinical studies for the treatment of cystic fibrosis [246], hemophilia [247] or Parkinson [248, 249] where transgen transmission was efficient and sustained over time. They showed a very low toxicity, but the main issue was the outbreak of an immunological reaction which differed depending on the administration route and dosage. New generations of vectors are being generated with the aim of diminishing immunological reaction against AAV.

To evaluate whether gene therapy using AAV vectors could be used for the treatment of McArdle disease, we used AAV serotype 8 (AAV8) to restore GP-MM in skeletal-muscle. AAV8 reaches a high efficiency of transduction of both skeletal and

cardiac muscles [250], as well as, being capable of transducing both slow and fast muscle fibers equally [251]. Moreover, we used a regulatory cassette that in addition to direct expression in both skeletal-muscle and heart, it also retains a high degree of tissue specificity. We used the truncated muscle CK (CK7) promoter [243], which is about 580 bp in length, and was generated by introducing three modifications to the basal promoter: a 63 bp deletion between the enhancer right E-box and MEF2 sites, a change in the sequence overlapping the transcription start site to as consensus initiator sequence and the inclusion of a highly conserved 50 bp from the murine muscle CK exon-1 5'-UTR [243]. These modifications generate a strong, muscle-specific promoter with a higher activity in cardiac muscle, diaphragm, and slow skeletal muscle (e.g. soleus) [243], even though expression in gastrocnemius and quadriceps remains higher. In this study, we evaluated therapeutic benefits of the intraperitoneal delivery of AAV2/8-CK7-hPYGM in McArdle neonatal mice.

Our results showed that AAV2/8-CK7-hPYGM is capable of transducing in all studied skeletal muscles, as well as, in heart and liver tissues (Figure 15; A). We observed the highest levels of transduction in diaphragm and liver but this might be due to the used route of administration as diaphragm and liver are close to the injection site.

In contrast to the level of AAV transduction observed in soleus (Figure 15; A), GP-MM expression was not restored in this muscle (Figure 15; B). We only observed a clear restoration of GP-MM expression in gastrocnemius of treated *p.R50X*/*p.R50X* knock-in mice (Figure 15; B). We also observed no GP-MM expression in EDL (Figure 15; B), but this might be explained by the lower AAV transduction level in this muscle. Further investigation is needed to understand the basis and mechanisms causing the differences in AAV transduction and no GP-MM expression in these muscles.

Interestingly, GP-MM restoration in gastrocnemius muscle of treated *p.R50X/p.R50X* knock-in mice was corroborated when we analyzed glycogen accumulation by PAS staining in gastrocnemius muscles sections of treated *p.R50X/p.R50X* knock-in mice. We observed a sustainable glycogen accumulation reduction in this muscle compared to WT and untreated *p.R50X/p.R50X knock-in* mice (Figure 15; C). Curiously, not all muscle areas presented the same glycogen accumulation reduction, showing parts with less glycogen than in sections from untreated mice and parts with similar amount of glycogen than WT sections (Figure 15; C). This might be explained by the different fiber composition of this muscle, but further analyses are needed to understand the variable GP-MM expression in this muscle.

In the present study we show that gene therapy using an AAV vector containing human GP-MM expression cassette and targeting skeletal muscle cells is capable of restoring GP-MM activity in gastrocnemius muscle and reducing glycogen accumulation in this muscle in the murine model of McArdle disease. Although further analyses are needed to confirm our results and study the possible immunological response against AAV, this first pilot study might provide a proof of concept of the utilization of AAV vectors as a potential treatment of McArdle disease.

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8. Annex 3

During my PhD I had the opportunity to participate in other studies about McArdle disease which resulted in the following publications:

De Luna N, Brull A , Lucia A, Santalla A, Garatachea N, Martí R, Andreu AL, Pinós T. ***PYGM expression analysis in white blood cells: A complementary tool for diagnosing McArdle disease?***. Neuromuscul Disord (2014) 24(12);1079-86.

Nogales-Gadea Y, Pinós T, Lucia A, Arenas J, Camara Y, Brull A, de Luna N, Martín MA, Garcia-Arumí E, Martí R, Andreu AL. ***Knock-in mice for the R50X mutation in the PYGM gene present with McArdle disease***. Brain, 2012. 135(Pt 7): p. 2048-57.

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PYGM expression analysis in white blood cells: A complementary tool for diagnosing McArdle disease?

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Abstract

McArdle disease is caused by an inherited deficiency of the enzyme myophosphorylase, resulting in exercise intolerance from childhood and acute crises of early fatigue and contractures. In severe cases, these manifestations can be accompanied by rhabdomyolysis, myoglobinuria, and fatal renal failure. Diagnosis of McArdle disease is based on clinical diagnostic tests, together with an absence of myophosphorylase activity in skeletal muscle biopsies and genetic analysis of the myophosphorylase-encoding gene, *PYGM*. The recently reported association between myophosphorylase and Rac1 GTPase in a T lymphocyte cell line prompted us to study myophosphorylase expression in white blood cells (WBCs) from 20 healthy donors and 30 McArdle patients by flow cytometry using a fluorescent-labeled *PYGM* antibody. We found that T lymphocytes expressed myophosphorylase in healthy donors, but expression was significantly lower in McArdle patients ($p < 0.001$). *PYGM* mRNA levels were also lower in white blood cells from McArdle patients. Nevertheless, in 13% of patients (who were either heterozygotes or homozygotes for the most common *PYGM* pathogenic mutation among Caucasians (p.R50X)), the percentage of myophosphorylase-positive white blood cells was not different compared with the control group. Our findings suggest that analysis of myophosphorylase expression in white blood cells might be a useful, less-invasive, complementary test for diagnosing McArdle disease.
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Keywords: McArdle disease; Myophosphorylase; Flow cytometry; White blood cells; Diagnosis

1. Introduction

McArdle disease, also termed *glycogen storage disease type V* or simply *glycogenosis type V* (OMIM® number

232600) is caused by an inherited deficiency of the muscle isoform of glycogen phosphorylase or myophosphorylase. The disease results from homozygous or compound heterozygous mutations in the myophosphorylase gene (*PYGM*). Myophosphorylase catalyzes the rate-limiting step of glycogenolysis by cleaving $\alpha 1 \rightarrow 4$ bonds to remove glucose molecules from the glycogen chain, leading to liberation of glucose-1-phosphate available for glycolysis [1,2]. McArdle patients typically experience exercise intolerance since childhood in the form of reversible, acute crises of early fatigue and contractures,

sometimes accompanied by rhabdomyolysis and myoglobinuria, triggered by static muscle contractions (e.g., lifting/carrying weights) or vigorous dynamic exercise (e.g., climbing stairs, brisk walking) [2,3]. Certain lifestyle recommendations have proven useful for managing the condition, such as regular moderate-intense physical activity, a diet rich in complex carbohydrates, and consumption of carbohydrate drinks prior to strenuous exercise [2].

The main tools traditionally used for diagnosing McArdle disease include clinical features; that is, exercise intolerance with or without fixed muscle weakness, and the so-called 'second-wind' phenomenon (disappearance of early exertional fatigue and tachycardia after the first ~10 min of dynamic exercise); high serum levels of total creatine kinase (CK) activity; and absence of myophosphorylase activity in muscle biopsies [4,5]. Genetic studies that identify mutant alleles in the two *PYGM* gene copies are also necessary, and because more than one hundred mutations have been described in the literature, sequential analysis of the most frequent mutations must be performed [6]. However, in some patients the most frequent mutations are not present (e.g., in ~40% of Spanish patients according to the national registry [3]) and further sequencing of the entire coding region and intron/exon boundaries of the *PYGM* gene is necessary. Thus, diagnostic genetic analysis can sometimes be difficult and time-consuming. It would be helpful to have reliable molecular tools that are easy to implement, rather than invasive muscle biopsies, to assist in the McArdle disease diagnosis until genetic confirmation is available.

Myophosphorylase is a recently described specific effector molecule for the active form of Rac1, a small GTP-ase of the Rho family that was investigated in a *T*-lymphocyte cell line, Kit225 [7]. A previously unsuspected link between Rac1 GTPase and glycogen metabolism has been reported, which implies that myophosphorylase may function downstream of Rac1 in a novel transduction pathway regulating IL2-dependent T-cell proliferation [7]. In the present study, we have characterized *PYGM* expression in white blood cells (WBCs) of McArdle patients to assess the possibility of using this parameter as a complementary tool in the diagnosis of McArdle disease.

2. Material and methods

2.1. Participants

Thirty (12 female) McArdle patients, 20 healthy volunteers, and 4 healthy carriers were recruited from June 2012 to June 2013 at the *Universidad Europea de Madrid*, Spain. In patients, the diagnosis of McArdle disease was based on a close examination of the clinical history, laboratory parameters (high baseline CK levels), objective evaluation of the second wind phenomenon

during cycle-ergometry testing, and identification of pathogenic mutations in the two copies of the *PYGM* gene [2]. Written consent for inclusion in the study was obtained from each participant. The study protocol was approved by the institutional ethics committee (*Universidad Europea de Madrid*, Spain) and was designed in accordance with the Declaration of Helsinki for Human Research of 1974 (last modified in 2008).

2.2. Myophosphorylase staining

Either quadriceps, biceps or deltoid muscle samples from patients were fixed in O.C.T. medium in cold methyl butane for 30 s in liquid nitrogen, kept at −80 °C until suitable sections on slides were obtained, and frozen at −20 °C until analysis. Myophosphorylase activity staining was performed by incubating muscle sections for 45 min with a solution containing 1% glucose-1-phosphate, 0.2% AMP, and 0.02% glycogen in 0.1 M sodium acetate buffer, pH 5.6. Sections were washed with water, Lugol's iodine was applied for 3 min, and samples were mounted with Aqua tex.

2.3. Myophosphorylase activity

Either quadriceps, biceps or deltoid muscle samples were homogenized in 20 volumes (1 ml/50 mg tissue) of cold homogenization buffer (40 mM b-glycerophosphate, 40 mM NaF, 10 mM EDTA and 20 mM b-mercaptoethanol, pH 6.8) and centrifuged at 10,000 g for 10 min, at 4 °C. Myophosphorylase activity was measured in the supernatant using a spectrophotometric kinetic method as previously described [8].

2.4. WBC isolation

WBCs from patients and controls were isolated from 5 mL of blood collected in a heparin tube. After plasma separation, buffy coat lysis buffer (10 mM NH_4HCO_3, 144 mM NH_4Cl) was added to the cell pellet, which was then incubated on ice for 30 min to lysate the erythrocytes, and centrifuged to precipitate the WBCs. The procedure was repeated until the cell pellet was free of red blood cells.

2.5. Flow cytometry analysis

One-color flow cytometry was performed for the detection of human myophosphorylase. Cells were fixed with 2% formaldehyde in phosphate buffer saline (PBS) for 10 min at room temperature, and permeabilized with 0.4% Triton-X-100 in PBS for 10 min at room temperature. Rabbit anti-*PYGM* polyclonal antibody and rabbit IgG isotype, both of them alexa-488 conjugated (Bioss, Woburn, MA, USA), were used to stain WBCs from healthy controls and patients. To analyze the different WBC subpopulations, we performed two-color

flow cytometry. In addition to *PYGM* antibody, we used mouse monoclonal IgG_1 anti-CD19 phycoerythrin (PE) conjugated antibody (clone SJ25-C1) (Becton Dickinson, Franklin Lakes, NJ, USA) to detect B-cells, mouse monoclonal IgG_1 anti-CD3 PE conjugated antibody (clone UCHT1) (Becton Dickinson; Franklin Lakes, NJ, USA) to detect T-cells, and mouse monoclonal IgG_{2a} anti-CD14 PE conjugated antibody (clone TüK4) (Becton Dickinson; Franklin Lakes, NJ, USA) to detect monocytes. IgG_1 and IgG_{2a} PE conjugated antibody (Becton Dickinson; Franklin Lakes, NJ, USA) was used as isotype control. For three-color flow cytometry, in addition to anti-*PYGM*, we used CD4-Per-Cy5.5 and CD8-PE antibodies, with their corresponding control isotypes (IgG_1 conjugated with Per Cy5.5 and PE) (Becton Dickinson; Franklin Lakes, NJ, USA) to differentiate between the two most abundant T-lymphocyte populations. Cells were ultimately acquired and analyzed in a FACScalibur cytometer (Becton Dickinson; Franklin Lakes, NJ, USA).

2.6. RNA extraction and real-time PCR

WBC RNA was extracted using Trizol (Life technologies, Madrid, Spain) according to the manufacturer's instructions. Total RNA (0.5 µg) was DNase treated (Life technologies, Madrid, Spain) and then reverse transcribed into cDNA using a high capacity cDNA RT kit (Life technologies, Madrid, Spain).

PYGM, *PYGB*, and *PYGL* gene expression was assessed by real-time PCR using Taqman fluorogenic probes in a 7500 real-time PCR system (Applied Biosystems, Foster City, CA, USA). We used a probe located in exons 6–7 for detection of *PYGM* mRNA (Hs 00194493_mL), a probe located in exons 5–6 for *PYGB* mRNA (Hs 00765686_mL), and a probe located in exons 3–4 for *PYGL* mRNA (Hs 00958087_mL). Results were normalized to ribosomal 18S RNA levels (Hs 99999901_s1).

2.7. Statistical analysis

We compared the percentage of myophosphorylase-positive WBCs between patients and controls, and between patients and healthy carriers, using the Mann–Whitney *U*-test, with α set at 0.05. Results are expressed as the mean ± standard deviation. We also evaluated the ability of the above-mentioned variable to distinguish McArdle patients from controls (0 = control, 1 = McArdle) by receiver operating characteristic (ROC) curves [9]. The area under the ROC curve (AUC) was calculated.

3. Results

The main diagnostic laboratory features of the patients included in this study are summarized in Table 1. Muscle biopsy analysis was available in 53% of patients

(16 of 30) and consistently confirmed an absence of myophosphorylase activity and histochemical detection.

To rule out a putative cross-reaction of *PYGM* antibody with the liver and brain isoforms of glycogen phosphorylase in the cytometry studies, we analyzed *PYGB* and *PYGL* mRNA levels in WBCs. No expression was found in either healthy controls or patients. On flow cytometry analysis using a specific antibody against myophosphorylase, the percentage of WBCs expressing this enzyme was significantly lower (Mann–Whitney, $p < 0.001$) in patients (18.0 ± 13.1%, range of expression, 1.9%–55.7%) compared with healthy controls (60.9% ± 19.5%; range of expression, 22.3%–89.2%) or healthy carriers ($p = 0.001$) (50.5 ± 5.3%; range of expression, 42.8%–54.7%) (Fig. 1). No statistical differences were found between healthy controls and healthy carriers. Statistical significance ($p < 0.001$) remained when comparing controls with patients having the most common genotypes in Spaniards; that is, p.R50X/p.R50X (16.3 ± 13.6%), accounting for ~36% of all *PYGM* genotypes in the Spanish patient registry [3] and for 33.3% of the present patient cohort. We also observed statistical significance in differences of myophosphorylase expression between healthy controls and patients having the following mutations: p.R50X-p.G205S (21.1 ± 6.9%, $p = 0.017$, $n = 2$), p.R50X-p.K754NfsX49 (16.1 ± 0.8%, $p = 0.009$, $n = 2$), p.R50X-p.W798R (19.4 ± 4.6, $p < 0.001$, $n = 4$) and p.G205S-E14IVS + 1A > G (10 ± 4.4%, $p = 0.001$, $n = 3$). No significant differences in myophosphorylase expression were observed between healthy controls and patients harboring p.R50X/p.A660D mutations (50.4 ± 7.4%, $p = 0.554$, $n = 2$) (Fig. 2). The percentage of myophosphorylase-positive cells in each patient is shown in Table 2.

These results enabled us to establish a threshold, in which a myophosphorylase expression value under 28.6% was considered to indicate the presence of McArdle disease with a sensitivity of 95% and specificity of 83.3% (calculated as a ROC curve, AUC 0.955).

To elucidate which subpopulation of WBCs from a healthy individual was expressing myophosphorylase, we performed a two-color flow cytometry analysis with the T-lymphocyte marker CD3, *B*-lymphocyte marker CD19, and monocyte marker CD14, together with anti-*PYGM* antibody. Two-color flow cytometry revealed that 6.5% of the monocyte population was positive for myophosphorylase, and 33% of the T-lymphocyte population expressed myophosphorylase at high levels, whereas *B* cells did not express the enzyme (Fig. 3). The CD8 and CD4 T-lymphocyte subpopulations both expressed the enzyme.

We also compared the WBC *PYGM* mRNA levels between patients and healthy volunteers. No *PYGM* gene amplification (ie, no *PYGM* expression) was found in the majority (80%) of patients, except for 6 cases (patients 7, 8, 14, 20, 22 and 23; mean mRNA 26.4% ± 8.7%). In these

Table 1
Patient demographic characteristics and main laboratory diagnostic features.

Patient #	Gender	Age	Genetic diagnosis (DNA mutation)	Genetic diagnosis (amino acid change)	Muscle biopsy: negative histochemical reaction for myophosphorylase	Muscle biopsy: non-detectable myophosphorylase activity
1	F	21	c.148C > T/c.148C > T	p.R50X/p.R50X	n.p	n.p
2	F	36	c.148C > T/c.148C > T	p.R50X/p.R50X	Yes	Yes
3	M	46	c.148C > T/c.148C > T	p.R50X/p.R50X	n.p	n.p
4	M	43	c.148C > T/c.148C > T	p.R50X/p.R50X	n.p	n.p
5	M	34	c.148C > T/c.148C > T	p.R50X/p.R50X	n.p	n.p
6	F	34	c.148C > T/c.148C > T	p.R50X/p.R50X	n.p	n.p
7	F	38	c.148C > T/c.148C > T	p.R50X/p.R50X	Yes	Yes
8	F	25	c.148C > T/c.148C > T	p.R50X/p.R50X	Yes	Yes
9	F	77	c.148C > T/c.148C > T	p.R50X/p.R50X	Yes	Yes
10	M	26	c.148C > T/c.148C > T	p.R50X/p.R50X	n.p	n.p
11	F	36	c.148C > T/c.1366G > A	p.R50X/p.V456 M	Yes	Yes
12	F	38	c.148C > T/c.13_14delCT	p.R50X/p.L5VfsX22	n.p	n.p
13	F	54	c.148C > T/c.613G > A	p.R50X/p.G205S	n.p	n.p
14	F	25	c.148C > T/c.613G > A	p.R50X/p.G205S	Yes	Yes
15	M	34	c.148C > T/c.2262delA	p.R50X/p.K754NfsX49	n.p	n.p
16	M	57	c.148C > T/c.2262delA	p.R50X/p.K754NfsX49	Yes	Yes
17	M	40	c.148C > T/c.1970_2177del	p.R50X/p.V657GfsX21	n.p	n.p
18	F	30	c.148C > T/c.2392T > C	p.R50X/p.W798R	Yes	Yes
19	M	45	c.148C > T/c.2392T > C	p.R50X/p.W798R	Yes	Yes
20	M	33	c.148C > T/c.2392T > C	p.R50X/p.W798R	Yes	Yes
21	F	34	c.148C > T/c.2392T > C	p.R50X/p.W798R	Yes	Yes
22	M	31	c.148C > T/c.1979C > A	p.R50X/p.A660D	Yes	Yes
23	M	27	c.148C > T/c.1979C > A	p.R50X/p.A660D	Yes	Yes
24	M	35	c.148C > T/c.580C > T	p.R50X/p.R194 W	n.p	n.p
25	M	12	c.148C > T/c.2083G > A	p.R50X/p.G695R	Yes	Yes
26	F	52	c.13_14delCT/c.13_14delCT	p.L5VfsX22/p.L5VfsX22	n.p	n.p
27	M	56	c.613G > A/c.613G > A	p.G205S/p.G205S	n.p	n.p
28	M	53	c.613G > A/C.1769 + 1G > A	p.G205S/E14IVS + 1A > G	n.p	n.p
29	M	28	c.613G > A/c.1769 + 1G > A	p.G205S/E14IVS + 1A > G	Yes	Yes
30	M	23	c.613G > A/c.1769 + 1G > A	p.G205S/E14IVS + 1A > G	Yes	Yes

Patients 19 and 20, 22 and 23, and 29 and 30 were brothers. The rest were unrelated. Abbreviations: F, female; M, male; n.p, not performed (access number NM_005609.1 from Genbank).
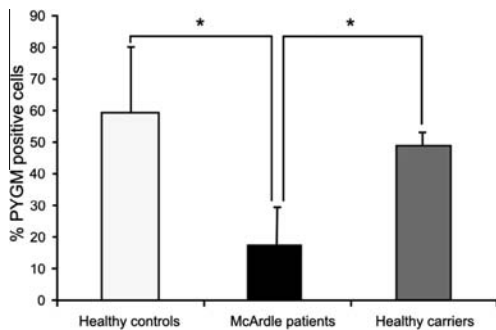
Fig. 1. Percentage of white blood cells (WBCs) expressing myophosphorylase in healthy controls (60.9 ± 19.5%) healthy carriers of one *PYGM* gene mutation (50.3 ± 5.3%), and McArdle patients (18 ± 13.1%). Significantly lower percentages of *PYGM*-positive WBCs were found in McArdle patients compared with healthy individuals ($^*p < 0.01$).

patients, muscle biopsy analysis showed no histochemical reaction for myophosphorylase and no detectable enzyme

activity. Mean percentage of myophosphorylase positive WBCs in this subgroup of patients was 34.7% ± 6.2%, showing significant differences with the control subjects ($p = 0.013$). All the healthy volunteers presented detectable amounts of *PYGM* mRNA in WBCs, although the mRNA levels corresponded to only 1% of the levels found in skeletal muscle.

4. Discussion

This is the first attempt to describe *PYGM* expression in WBCs in a representative cohort of McArdle patients (~13% of all Spanish patients diagnosed, at varying ages) as a potential complementary diagnostic tool. We used a specific *PYGM* fluorescence-labeled antibody to determine myophosphorylase expression in WBCs by flow cytometry analysis. The mean percentage of myophosphorylase-positive WBCs was significantly lower in most of the patients studied (26 of 30) than in controls, regardless of the type of mutation they harbored. There were four exceptions (patients 7, 10, 22 and 23), in whom the percentage of myophosphorylase
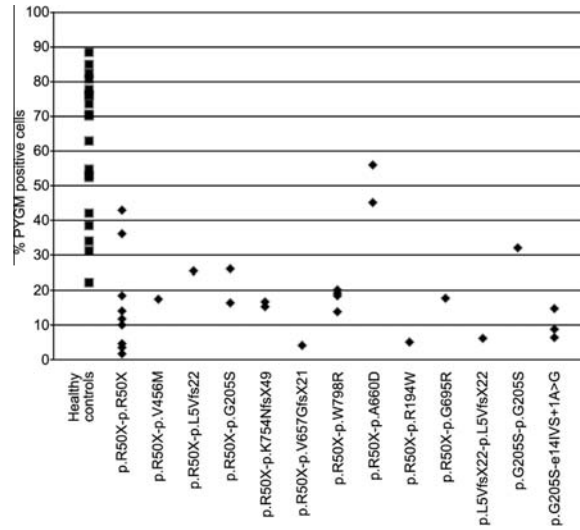
Fig. 2. Distribution of *PYGM* expression in white blood cells (WBCs) of McArdle patients, analyzed and sorted according to the mutations found.

Table 2
Percentage myophosphorylase-positive white blood cells (WBCs) in patients.

Patient #	Genetic diagnosis (amino-acid change)	% myophosphorylase-positive WBCs	mRNA (% of mean values in controls)
1	p.R50X/p.R50X	11.8	Non-detectable (0)
2	p.R50X/p.R50X	14	0
3	p.R50X/p.R50X	18.6	0
4	p.R50X/p.R50X	10.3	0
5	p.R50X/p.R50X	4.4	0
6	p.R50X/p.R50X	3.9	0
7	p.R50X/p.R50X	42.7	31
8	p.R50X/p.R50X	19	64
9	p.R50X/p.R50X	1.9	0
10	p.R50X/p.R50X	36.15	0
11	p.R50X/p.V456 M	17.4	0
12	p.R50X/p.L5VfsX22	25.4	0
13	p.R50X/p.G205S	16.3	0
14	p.R50X/p.G205S	26	34
15	p.R50X/p.K754NfsX49	15.5	0
16	p.R50X/p.K754NfsX49	16.6	0
17	p.R50X/p.V657GfsX21	4.2	0
18	p.R50X/p.W798R	13.8	0
19	p.R50X/p.W798R	19.1	0
20	p.R50X/p.W798R	19.8	11.2
21	p.R50X/p.W798R	25	0
22	p.R50X/p.A660D	45.2	5.3
23	p.R50X/p.A660D	55.7	14
24	p.R50X/p.R194 W	5	0
25	p.R50X/p.G695R	17.5	0
26	p.L5VfsX22/p.L5VfsX22	6.1	0
27	p.G205S/p.G205S	32	0
28	p.G205S/E14IVS + 1A > G	6.2	0
29	p.G205S/E14IVS + 1A > G	9	0
30	p.G205S/E14IVS + 1A > G	14.8	0

Patients 19 and 20, 22 and 23, and 29 and 30 were brothers. The rest were unrelated.

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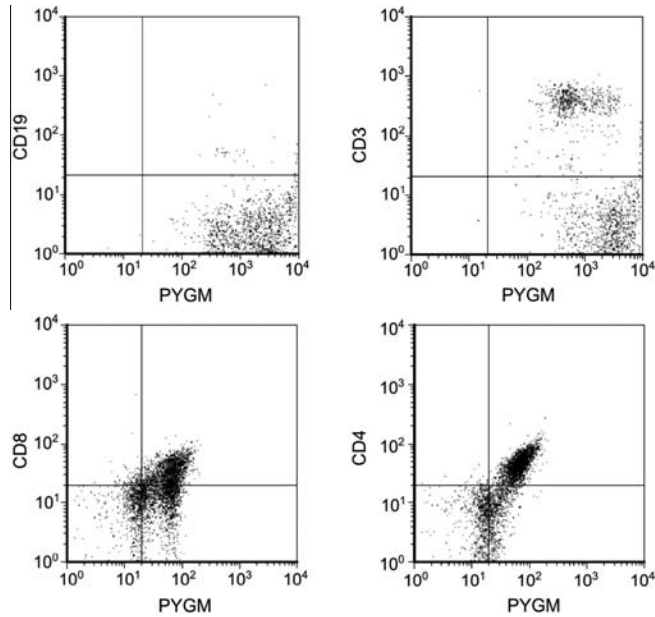
Fig. 3. Flow cytometry showed that both CD4$^+$ and CD8$^+$ T lymphocytes expressed *PYGM* in a healthy individual.

positive cells was similar to that of the control group. Two of these patients (patients 7 and 10) presented the p.R50X/p.R50X mutation, whereas the other two (patients 22 and 23) harbored the pR50X/p.A660D mutations. In patient 7, the elevated percentage of myophosphorylase-positive WBCs coincided with the relatively high levels of *PYGM* mRNA (31% of the mean control values), likely due to an undermined non-sense-mediated decay mechanism. Another explanation for the results found in this patient could be the existence of an alternative translation start site in the *PYGM* gene (as previously reported with the p.R37X mutation in the *ATRX* gene [10]). Indeed, two ATG translation sites in frame are found 125 and 149 nucleotides downstream of the c.148C > T mutation (41 and 49 codons downstream, respectively) in the cDNA sequence of the *PYGM* gene (Genbank: AK314474.1); however, in patient 10, the high percentage of myophosphorylase positive cells could not be explained by diminished action of the non-sense mediated decay mechanism or by the use of an alternative translation start site, as *PYGM* mRNA from this patient was not detected by real-time PCR, and therefore the positive staining was probably due to an unspecific signal of the *PYGM* antibody. The other two patients (patients 22 and 23), harbored a combination of a non-sense (p.R50X) with a missense mutation (p.A660D), and presented low but detectable amounts of *PYGM* mRNA (5.3% and 14% of the control mRNA levels, respectively). These results along with the detectable amounts of *PYGM* mRNA

found in patients 14 and 20 (11.2% and 34% of the control mRNA levels, respectively) which also harbored a missense mutation in one of the alleles (p.G205S in patient 14 and p.W798R in patient 20), suggested that the missense mutations found in these patients (p.G205S, p.W798R, and p.A660D) are not as deleterious as the frame-shift or non-sense mutations, enabling higher protein expression and detection. Additionally, the two patients presenting the p.A660D mutation are brothers, and their normal myophosphorylase levels may also be explained by a similar genetic background that allows higher myophosphorylase expression in WBCs. In McArdle disease, more than 100 different mutations have been described in the *PYGM* gene, and among these, the p.R50X mutation is the most common in Caucasian McArdle patients [11]. In the Spanish national registry of McArdle patients, the p.R50X/p.R50X genotype accounts for 36% of cases [3]. This mutation creates a premature termination codon in exon 1 of the *PYGM* gene, which activates the mechanism of non-sense mediated decay leading to degradation of *PYGM* mRNA. In our study, 80% of patients (8 of 10) homozygous for the p.R50X mutation presented undetectable *PYGM* mRNA levels, whereas 73% of patients (11 of 15) presenting the p.R50X mutation in heterozygosity also presented undetectable *PYGM* mRNA levels.

The presence of enzymes involved in glycogen metabolism, such as glycogen phosphorylase in blood

cells, has been previously reported [12,13]. Platelets and polymorphonuclear neutrophils contain high amounts of glycogen, monocytes and lymphocytes present lower levels of glycogen, and in erythrocytes, glycogen has not been detected [12,14–16]. In blood cell lines, such as Kit 225 cells (IL-2-dependent human T cell line) *PYGM* mRNA levels are high, with interleukin (IL)-2 inducing myophosphorylase activation by binding to the active form of Rac1, leading to T cell proliferation [7]. This potential implication of the myophosphorylase enzyme in lymphocyte proliferation was suggested in a previous study, where a pronounced increase in phosphorylase activity was found 4–5 days after addition of the mitogen agent phytohemagglutinin, and the increase coincided with termination of lymphocyte DNA replication [17]. In concordance with Arrizabalaga et al. [7], we found that *PYGM* is expressed in lymphocytes, and we have further shown that T-lymphocytes are the main WBC subpopulation expressing the gene (33% of this T-lymphocyte subset expresses *PYGM*).

Analysis of blood samples to diagnose a particular type of glycogenosis goes back to 1934 when Van Creveld showed that blood of two patients suffering from *glycogen storage disease type* III contained an increased amount of glycogen [18]. Since then, blood testing has been widely used in the diagnosis of *glycogenoses type* II, III, and VI. In glycogenosis type II, the existence of lysosomes full of PAS-positive reactive material in leukocytes was detected by electron microscopy in 1977 [19]. These vacuoles were also PAS positive in Pompe disease, although the positive material was not restricted to the vacuoles [20]. Abnormal vacuolated lymphocytes have been described in ~6% of other metabolic diseases [21].

Patients with dysferlinopathy, a disease affecting the gene encoding dysferlin (a sarcolemmal protein) do not express dysferlin in circulating monocytes, as opposed to healthy controls [22,23]. In Ulrich and Bethlem myopathy caused by mutations in collagen VI, a collagen VI defect was detected in macrophages at levels comparable to those observed in muscle biopsy samples and cultured skin fibroblasts [24].

Our results suggest that flow cytometry analysis may be useful for fast, quantitative assessment of myophosphorylase expression in WBCs. This approach could serve as an initial screening test for McArdle disease. Because symptoms usually start during childhood and adolescence, whereas an effective diagnosis is often delayed until later in life [3], this 'new method' might assist in promoting prompt adherence to lifestyle recommendations that will help McArdle patients manage the condition and improve their functional capacity.

Funding

Acknowledgement

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Annex 3

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Knock-in mice for the R50X mutation in the *PYGM* gene present with McArdle disease

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McArdle disease (glycogenosis type V), the most common muscle glycogenosis, is a recessive disorder caused by mutations in *PYGM*, the gene encoding myophosphorylase. Patients with McArdle disease typically experience exercise intolerance manifested as acute crises of early fatigue and contractures, sometimes with rhabdomyolysis and myoblobinuria, triggered by static muscle contractions or dynamic exercises. Currently, there are no therapies to restore myophosphorylase activity in patients. Although two spontaneous animal models for McArdle disease have been identified (cattle and sheep), they have rendered a limited amount of information on the pathophysiology of the disorder; therefore, there have been few opportunities for experimental research in the field. We have developed a knock-in mouse model by replacing the wild-type allele of *Pygm* with a modified allele carrying the common human mutation, *p.R50X*, which is the most frequent cause of McArdle disease. Histochemical, biochemical and molecular analyses of the phenotype, as well as exercise tests, were carried out in homozygotes, carriers and wild-type mice. *p.R50X/p.R50X* mice showed undetectable myophosphorylase protein and activity in skeletal muscle. Histochemical and biochemical analyses revealed massive muscle glycogen accumulation in homozygotes, in contrast to heterozygotes or wild-type mice, which did not show glycogen accumulation in this tissue. Additional characterization confirmed a McArdle disease-like phenotype in *p.R50X/p.R50X* mice, i.e. they had hyperCKaemia and very poor exercise performance, as assessed in the wire grip and treadmill tests (6% and 5% of the wild-type values, respectively). This model represents a powerful tool for in-depth studies of the pathophysiology of McArdle disease and other neuromuscular disorders, and for exploring new therapeutic approaches for genetic disorders caused by premature stop codon mutations.

Keywords: McArdle disease; knock-in mouse; p.R50X mutation; glycogenosis type V; neuromuscular disorders

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Introduction

Glycogenosis type V [glycogen storage disease type V (GSD V), McArdle disease or myophosphorylase deficiency; OMIM® database number 232600] is an autosomal recessive disorder of muscle glycogen metabolism described by Brian McArdle in 1951 (McArdle, 1951). Patients harbour pathogenic mutations in both alleles of the *PYGM* gene, encoding myophosphorylase, the skeletal muscle isoform of glycogen phosphorylase (Lucia *et al.*, 2008). Myophosphorylase initiates the breakdown of muscle glycogen by removing (1,4)-α-glucosyl units from its outer branches, leading to liberation of glucose-1-phosphate; thus, patients are unable to obtain energy from their muscle glycogen stores.

In virtually all patients, the clinical presentation is dominated by exercise intolerance in the form of reversible, acute crises of early fatigue and contractures, which can be accompanied (in ∼50% of cases) by rhabdomyolysis, as reflected by marked increases in serum levels of creatine kinase or myoglobinuria (Lucia *et al.*, 2012). Owing to blocked glycogenolysis, exercise intolerance is more marked during exercise tasks involving anaerobic or aerobic glycolysis for ATP production in muscles. Thus, 'muscle crises' can be triggered by: (i) 'static' (or isometric) contractions relying on small muscle groups and anaerobic glycogenolysis, e.g. carrying weights; or (ii) dynamic exercise involving large muscle mass and aerobic pathways, e.g. climbing stairs, brisk walking (DiMauro, 2007; Lucia *et al.*, 2012). A significant characteristic of the disease is a high serum level of creatine kinase under basal conditions, that is, in the absence of heavy exercise in the previous few hours or days (Lucia *et al.*, 2008, 2012). Fixed weakness, affecting mostly proximal muscles, can also be present in patients aged ⩾40 years (Nadaj-Pakleza *et al.*, 2009; Quinlivan *et al.*, 2010).

Several reports have described the *PYGM* genotype characteristics of relatively large cohorts of patients (Martin *et al.*, 2001; Bruno *et al.*, 2006; Aquaron *et al.*, 2007; Deschauer *et al.*, 2007; Rubio *et al.*, 2007*a, b*; Quinlivan *et al.*, 2010; Lucia *et al.*, 2012; Vieitez *et al.*, 2011). The most prevalent *PYGM* mutation in the Caucasian population, e.g. with an allele frequency of ∼55% among all (*n* = 239) diagnosed Spanish patients (Lucia *et al.*, 2012), is a nonsense mutation located in exon 1 (*p.R50X*). This mutation changes an arginine to a stop codon, resulting in nonsense-mediated decay of the messenger RNA (Nogales-Gadea *et al.*, 2008).

Two spontaneous animal models for McArdle disease have been identified, i.e. in Charolais cattle (Angelos *et al.*, 1995) and Merino sheep (Tan *et al.*, 1997) but they have rendered a limited amount of information on the pathophysiology of the disorder, particularly in terms of its exercise phenotype. This prompted us to develop a genetically modified murine model of McArdle disease (knock-in mouse for the common *PYGM p.R50X* mutation). Here, we present the characterization of the phenotype of this model, including biochemical, molecular and histochemical data together with exercise performance tests. In contrast to what is often observed for other rodent models of neuromuscular disease, these mice recapitulate the main features of the human disorder and therefore constitute a valuable tool for further studies and for testing therapeutic approaches.

Materials and methods

All experimental procedures were approved by the animal care and use committee of the Vall d'Hebron Institut de Recerca (procedure 13/04 CEEA; 35/04/08) and were in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS 1 2 3) and the Spanish laws (32/2007 and R.D. 1201/2005).

Generation of *Pygm* p.R50X knock-in mouse

To construct the targeting vector, a 7.3 kb fragment of the *Pygm* gene (ranging nt 6 380 746–6 388 054; GeneBank accession number NC_000085.5) was amplified by long PCR from mouse C57BL/6J DNA. The primer used were *Pygm*-F and *Pygm*-R (for primer sequences described in this section, see Supplementary Table 1). The resulting DNA fragment was cloned in the pUC19 vector. The *p.R50X* mutation (*c.148A > T* nucleotide change, GenBank accession number NM_011224.1) was introduced in exon 1 using a site-directed mutagenesis kit (Stratagene) with the primers *p.R50X*-F and *p.R50X*-R. The LoxP-flanked neomycin resistance cassette, under the human phosphoglycerate kinase promoter (LoxP-PGK-NEO), was inserted in intron 1, 214 nucleotides after exon 1, and the complete sequence of the targeting vector was verified by full sequencing. Transfection of the 129/SvPas embryonic stem cells and blastocyst injections were performed (genOway). G418 resistant mouse embryonic stem cell clones were screened for homologous recombination by long PCR in both 5′ and 3′ extremes, and confirmed by Southern blot analysis. The primers used in the long PCR screening at the 5′ site of the *Pygm* region were L5-F and L5-R; and at the 3′ site were L3-F and L3-R. Eight recombinant clones out of 227 analysed were sequenced to rule out the presence of additional mutations introduced by PCR-amplification. One recombinant clone, containing the *p.R50X* mutation but no other nucleotide changes, was selected for the injection into C57BL/6J blastocysts, which were then re-implanted into pseudo-pregnant females and allowed to develop to term. Two highly chimeric males were selected from the resulting offspring and mated to C57BL/6J females. The resulting pups were studied for germinal line transmission of the recombination event by using the PCR strategy described above. The presence of the *p.R50X* mutation was further verified by sequencing. Heterozygous males were mated with B6.FVB-Tg(EIIa-cre)C5379Lmgd/J females (Jackson Laboratories), to remove the loxP-PGK NEO cassette (this strain targets expression of Cre recombinase to the early mouse embryo). The elimination of the cassette in the offspring was analysed by PCR with the primers LoxP-F and LoxP-R. Homozygous mutant mice were obtained by inter-crossing heterozygous littermates.

In the experiments described below, animals were killed by cervical dislocation to collect tissue samples. We studied 2-month-old female and male littermates for all the experiments except the plasma creatine kinase determination, which was assessed in 5-month-old mice.

Biochemical and molecular studies

Glycogen phosphorylase activity

Gastrocnemius muscle samples were homogenized in 20 volumes (1 ml/50 mg tissue) of cold homogenization buffer (40 mM β-glycerophosphate, 40 mM NaF, 10 mM EDTA and 20 mM β-mercaptoethanol, pH 6.8) and centrifuged at 10 000 *g* for 10 min, at 4°C. Glycogen

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phosphorylase activity was measured in the supernatant using a spectrophotometric kinetic method as previously described (Miranda *et al*., 1981).

Glycogen determination

The glycogen content in muscle was measured as previously described (Lo *et al*., 1970). Briefly, $\sim$150 mg of tissue was boiled for 30 min with 30% KOH, then 1.2 volumes of 95% ethanol were added to precipitate the glycogen. After a centrifugation step (840 g, 25 min), the pelleted glycogen was resuspended in 0.1 ml of water and treated with 0.5 ml of H_2SO_4 (to hydrolyse glycogen to glucose) plus 0.1 ml of 5% phenol. The mixture was allowed to stand for 30 min at room temperature and then the released glucose was measured spectrophotometrically at 490 nm. A standard curve made with glycogen purified from rabbit liver (Sigma-Aldrich), ranging 0.1–0.8 mg/ml was processed in parallel. The results were expressed as milligrams of glycogen per gram of tissue.

Western blot analysis

Protein extracts of gastrocnemius muscle, obtained as indicated above for glycogen phosphorylase activity, were resolved on 10% SDS-PAGE gels transferred to a polyvinylidene difluoride membrane and probed with a primary antibody against myophosphorylase (kindly provided by Prof. Martinuzzi) and an anti α-tubulin mouse monoclonal antibody (Sigma-Aldrich). Suitable horseradish peroxidase-conjugated secondary antibodies were used and images were obtained with films (FujiFilm) and quantified with NIH ImageJ (version 1.37) software (Scion image, NIH).

RNA analysis

Total RNA was obtained from gastrocnemius muscle as previously described (Nogales-Gadea *et al*., 2008), and from brain and heart tissue following the manufacturer instructions of TRIzol® (Invitrogen). RNA was treated with DNase I, amplification grade (Invitrogen) to eliminate any traces of DNA. The amount and integrity of the RNA was assessed by capillary electrophoresis using the RNA 6000 Nano Chip kit (Agilent Technologies). The levels of RNA degradation were always negligible (data not shown). Complementary DNA was synthesized from RNA using the high-capacity complementary DNA archive kit (Applied Biosystems), which uses random primers. We used real-time PCR, with TaqMan® fluorogenic probes in a 7500 Real-Time PCR System (Applied Biosystems) to assess muscle, heart and liver messenger RNA levels of: (i) *Pygm* gene (Mm00478582_m1); (ii) glycogen phosphorylase, brain isoform (*Pygb*) gene (Mm00464080_m1); and (iii) glycogen phosphorylase, liver isoform (*Pygl*) gene (Mm00500078_m1). Results were normalized to peptidylprolyl isomerase A (cyclophilin A, *Ppia*) gene messenger RNA levels (probe Mm02342430_g1).

Plasma creatine kinase activity

Anticoagulated blood was collected from the saphenous vein and diluted 1:3 with PBS, centrifuged at 3000 g for 5 min at 4°C and the supernatant (diluted plasma) was immediately used to determine creatine kinase activity using a standard spectrophotometric kinetic method.

Myoglobinuria assessment

Urine ($\sim$50 μl) was collected from each mouse before and 3 h after performing on the treadmill, and myoglobin was determined by a commercially available ELISA (Mouse Myoglobin ELISA, Life Diagnostics, Inc.) following the manufacturers' instructions. Samples were 1:3 diluted, mixed with the polyclonal antibody anti-myoglobin conjugated to horseradish peroxidase and added to the plate coated with immobilized monoclonal antibody anti-myoglobin. After 1 h of incubation, wells were washed and the peroxidase reaction was developed by adding the substrate. Absorbance was measured at 450 nm and the results interpolated in a calibration curve ranging from 16 to 250 ng myoglobin/ml.

Histochemical analysis

Gastrocnemius, biceps femoris and soleus muscle samples were fixed in O.C.T. medium in cold methylbutane for 30 s in liquid nitrogen, kept at −80°C until suitable sections on slides were obtained, and frozen at −20°C until analysis. Glycogen phosphorylase activity staining was performed on gastrocnemius muscle only, by incubating muscle sections for 45 min with a solution containing 1% glucose-1-phosphate, 0.2% AMP, and 0.02% glycogen in 0.1 M sodium acetate buffer, pH 5.6. Sections were washed with water, Lugol's iodine was applied for 3 min and samples were mounted with Aquatex. Haematoxylin and eosin standard staining was performed in gastrocnemius, biceps femoris and soleus sections. Glycogen content was assessed in gastrocnemius, biceps femoris and soleus sections by periodic acid Schiff staining, by sequentially incubating the sections with: periodic acid, Schiff solution, haematoxylin and alcohol-xylol dehydration with DPX mounting medium (Sigma-Aldrich). Stained sections were analysed and images obtained with an inverted microscope (Olympus IX 71 Inverted Microscope, Olympus Corporation). Glycogen accumulation in *p.R50X/p.R50X* mice was morphometrically quantified in type I and II fibres as follows. Consecutive 20 -μm thick gastrocnemius sections were stained with periodic acid Schiff (see above) and with a type I-specific myosin heavy chain antibody, respectively. For the latter, sections were incubated for 5 min at room temperature in acetone, washed three times in PBS and incubated for 1 h at room temperature with the mouse monoclonal myosin heavy chain (slow) primary antibody (dilution 1:20; Leica Microsystems). Sections were then washed three times in PBS and incubated with rabbit anti-mouse Alexa-Fluor® 488 secondary antibody (1:200 dilution; Life Technologies). Finally, sections were washed three times in PBS and mounted with Aquatex® (Merk Chemicals). Glycogen accumulation in type I fibres (fluorescent) and type II fibres (non-fluorescent) was quantified in the corresponding fibres in the periodic acid Schiff-stained serial section using NIH ImageJ (version 1.37) software (Scion image, NIH).

Assessment of exercise capacity

Researchers in charge of assessing exercise phenotypes were blind to mice *Pygm* genotypes.

Wire grip test

Mice were picked up by the tail and placed on a metal wire suspended between two upright bars, 50 cm above a padded table (Petraglia *et al*., 2010). The time the mouse could hold onto the wire with both proximal limbs was recorded, the maximum time allowed being 180 s. When a mouse did not reach this time, it was tested twice and the maximum hanging time was recorded.

Treadmill running

Mice were exercised on an enclosed treadmill (Harvard Aparatus, Panlab) supplied with an electrified grid at the rear of the belt to provide motivation [shocks of 0.2 mA (1 Hz, 200 ms)]. Animals were familiarized with treadmill exercise the day before testing. The day of the test treadmill initial speed was 15 cm/s for 2 min, followed by 2 min at 30 cm/s, and thereafter was increased by 10 cm/s every 2 min until exhaustion, i.e. when the mouse remained more than 5 s

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on the electrified grid and was unable to continue running at the following speed load (Ayala *et al.*, 2009). During testing, treadmill inclination was kept at 25% as this inclination ensures attaining the maximum possible load for the mouse cardiorespiratory and oxidative system [i.e. attainment of maximal aerobic capacity, also termed 'maximal oxygen uptake' (VO_{2max})], while preventing injuries (Hoydal *et al.*, 2007). We determined the maximum exercise time completed by each mouse as an index of maximum aerobic capacity (Lightfoot *et al.*, 2001).

Statistical analysis

All statistical analyses were performed using the PASW (v. 19.0 for WINDOWS) with α set at 0.05. We compared molecular, biochemical and exercise phenotypes of the three study groups (wild-type, heterozygous or *p.R50X/p.R50X* mice) using the non-parametric Kruskal–Wallis test. This test allowed us to determine the statistical effect of the study group to which the mice belonged on the results, i.e. 'group effect'. Other tests used are indicated in the Results section and/or the figure legends. For statistical purposes, undetectable values were considered as zero.

Results

Generation of the p.R50X knock-in mouse

Figure 1A shows the structure of the engineered vector electroporated into mouse embryonic stem cells to generate the knock-in mutant DNA by homologous recombination (refer to 'Materials and methods'). After G418 selection in cell culture, 227 embryonic stem clones were screened and eight homologous recombinant clones were detected both by long PCR and confirmed by Southern blot analysis (Fig. 1B and C). The clone 3C1, which harboured the *p.R50X* mutation and no additional mutations, was used for blastocyst injection. Chimeric animals were obtained and germ-line transmission was later confirmed in F1. The PGK-NEO selection marker was eliminated by mating F1 heterozygous males with B6.FVB-Tg(EIIa-cre)C5379Lmgd/J females, generating F2 mice containing only the p.R50X mutation (plus the 34 bp loxP fragment) in the knock-in allele. Finally, male and female F2 heterozygotes were mated to obtain homozygote, heterozygote and wild-type mice, whose genotypes were confirmed by PCR and sequencing (Fig. 1D and E).

Absence of glycogen phosphorylase activity in the gastrocnemius of *p.R50X/p.R50X* mice

Glycogen phosphorylase activity was totally abolished in the gastrocnemius muscle of all *p.R50X/p.R50X* mice studied, while their heterozygous counterparts had 50% lower enzyme activity compared with *wt/wt* mice ($P = 0.004$ for the group effect, Fig. 2A). When the amount of protein was assessed by western blot analysis, the results closely resembled those obtained for glycogen phosphorylase activity ($P = 0.011$ for the group effect, Fig. 2B).

In skeletal muscle, the *Pygm* messenger RNA levels of *p.R50X/p.R50X* and heterozygous mice were ~7% and 60%, respectively, of those obtained in their *wt/wt* counterparts ($P = 0.005$ for the group effect), whereas we found no group effect on the messenger RNA levels of the other glycogen phosphorylase isoform genes (Fig. 3A). In the heart, *Pygm* messenger RNA levels showed the same pattern as in skeletal muscle, i.e. the transcript levels of *p.R50X/p.R50X* and heterozygous mice were ~6% and 52%, respectively, of those obtained in their *wt/wt* counterparts ($P = 0.012$ for the group effect), whereas there was no group effect for *Pygb* or *Pygl* messenger RNA levels (Fig. 3B). Interestingly, considerable messenger RNA levels of the brain isoform (*Pygb*) were observed in the murine heart (~30% as compared to the *Pygm* messenger RNA levels). This observation contrasts with the negligible *Pygb* messenger RNA levels observed in skeletal muscle. In the brain, no group effect was found for *Pygm*, *Pygb* or *Pygl* messenger RNA levels (Fig. 3C).

Histochemical analyses and glycogen accumulation in skeletal muscle

Figure 4A shows the histochemical images for glycogen phosphorylase, haematoxylin and eosin and periodic acid Schiff staining of gastrocnemius sections. This analysis confirmed the absence of glycogen phosphorylase activity in skeletal muscle in *p.R50X/p.R50X* mice and partial activity in heterozygote carriers. The haematoxylin and eosin staining in *p.R50X/p.R50X* mice showed alterations in fibre size and shape in which sub-sarcolemmal deposits could be observed (see arrows). As for periodic acid Schiff staining of glycogen, findings in *p.R50X/p.R50X* mice were similar to those reported in patients with McArdle disease, i.e. massive sub-sarcolemmal accumulation of glycogen, yet with glycogen stores also present in the sarcoplasm. Despite the lower glycogen phosphorylase activity compared with *wt/wt* mice, neither glycogen accumulation nor morphology alterations in muscle fibres were found in heterozygous mice. Similar results were observed in the other muscle groups (soleus and biceps femoris, data not shown). Glycogen accumulation in *p.R50X/p.R50X* mice was confirmed by biochemical quantitative analysis in gastrocnemius, which showed values between 48 and 58 mg of glycogen/g of tissue ($n = 3$), which, on average, indicates a 27-fold increase as compared to the glycogen found in both *wt/wt* and heterozygous mice. Interestingly, no increase of glycogen content was detected in heterozygotes (between 1.6 and 2.6 mg of glycogen/g of tissue; $n = 4$) compared with *wt/wt* mice (between 1.4 and 2.6; $n = 3$).

Morphometric analysis of serial gastrocnemius sections stained for glycogen content (periodic acid Schiff) and type I fibres (immunostaining with anti-myosin heavy chain antibody) revealed a slight but highly significant difference in glycogen accumulation between type I and type II fibres: the glycogen excess was more pronounced in type II fibres (Fig. 4B).

Exercise tests

The *p.R50X/p.R50X* mice showed a clear phenotype of exercise intolerance, both in the wire grip and exercise tests. In the wire grip, all wild-type mice tested were able to stay on the wire for

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Figure 1 Structure of the engineered vector, electroporated into mouse embryonic stem cells. (**A**) Scheme of the targeting vector, the non-targeted and the targeted *Pygm loci* (showing the location of the 3′-probe used for Southern blot analysis of the recombinant clones) and the final recombinant allele after Cre-mediated removal of the loxP-PGK-NEO fragment. Numbered black boxes indicate exons; PGK-NEO indicates the neomycin resistance cassette, under the human phosphoglycerate kinase promoter, flanked by two loxP sequences (grey triangles). Primers used for PCR screening are represented by small arrows (sequences can be found in Supplementary Table 1). Targets of restriction enzymes HindIII (H) and KpnI (K), are indicated. (**B**) Representative result of the long PCR analysis (at the 3′ and 5′ ends) of a recombinant clone. (**C**) Representative result of 3′ screening analysis of a wild-type and a recombinant clone by Southern blot. (**D**) Representative result of the PCR screening of F2 mice showing the 134 bp band (recombinant allele) and the 100 bp band (wild-type allele), due to the presence or the absence of the 34 bp lox-P fragment. (**E**) Electropherograms obtained from mouse DNAs showing the three possible *Pygm* genotypes at the c.148 position, where the A > T change was introduced (arrows).

the maximum time established in our experimental design (180 s), while none of the homozygous *p.R50X* mice tested were able to stay more than 34 s. Six out of these seven mice were unable to stay more than 7 s, which is <4% of the minimum time reached by the wild-type animals. Three out of 11 heterozygous mice did not reach the maximum time, but all resisted more than 41 s, showing a wide variability in performance times (*P* = 0.001 for the group effect) (Fig. 5A and Supplementary Videos 1 and 2).

When dynamic performance was tested with the treadmill device, the maximum exercise time in *p.R50X/p.R50X* mice was only ∼41% and ∼29% of that attained by their heterozygous and *wt/wt* counterparts, respectively (*P* = 0.001 for the group effect) (Fig. 5B and Supplementary Videos 3 and 4).

Plasma creatine kinase activity and myoglobinuria

Levels of plasma creatine kinase in *p.R50X/R50X* mice (median 1137 IU/l; range 336–1875) were much higher than in *wt/wt*
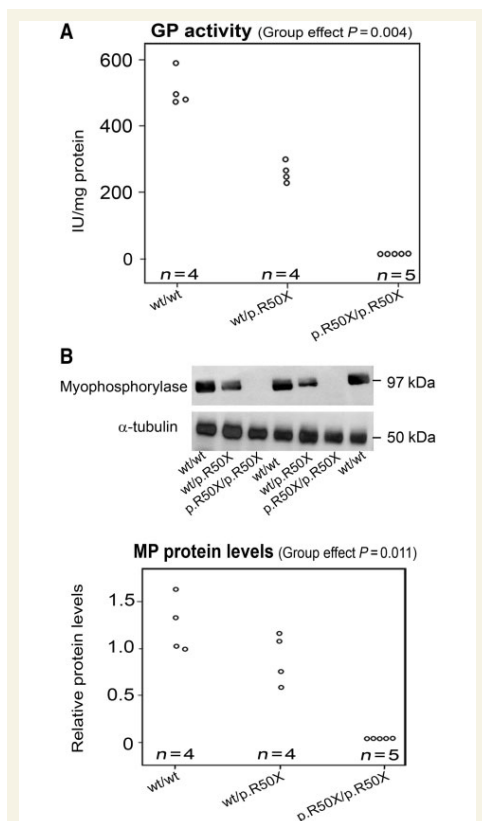
Figure 2 (**A**) Glycogen phosphorylase (GP) activity of gastrocnemius homogenates. (**B**) Representative western blot showing the amounts of myophosphorylase (MP) protein levels in gastrocnemius homogenates, and quantitative values obtained by densitometry (plot). All values were normalized to α-tubulin.

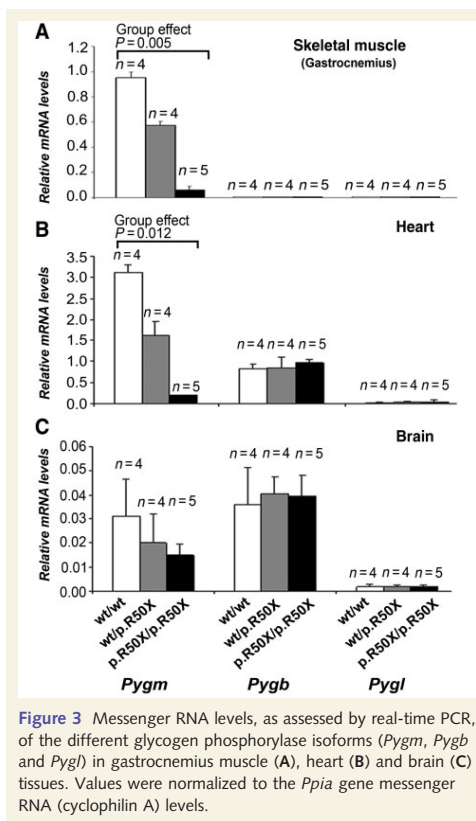**Figure 3** Messenger RNA levels, as assessed by real-time PCR, of the different glycogen phosphorylase isoforms (*Pygm*, *Pygb* and *Pygl*) in gastrocnemius muscle (**A**), heart (**B**) and brain (**C**) tissues. Values were normalized to the *Ppia* gene messenger RNA (cyclophilin A) levels.

animals (median 89 IU/l; range 37–181) (*P* = 0.009; Mann–Whitney U test, *n* = 5 for each group). In addition, preliminary results indicated that *p.R50X/p.R50X* mice develop exercise-induced myoglobinuria: two *p.R50X/p.R50X* animals tested had 375 and 1500 ng myoglobin/ml of urine after treadmill exercise, while it was undetectable in three out of three *wt/wt* mice and positive in only one (100 ng/ml) of the four heterozygous mice we tested.

Discussion

To our knowledge, this is the first successful attempt to develop a genetically modified animal model of McArdle disease. The observation that the *p.R50X* is the most prevalent mutation, at least among Caucasian patients (Martin *et al.*, 2001; Bruno *et al.*, 2006; Aquaron *et al.*, 2007; Deschauer *et al.*, 2007; Rubio *et al.*, 2007a, b;, Quinlivan *et al.*, 2010; Vieitez *et al.*, 2011; Lucia *et al.*, 2012) prompted us to develop a knock-in specific mouse model for this common defect rather than a classic strategy where the gene is partially or totally eliminated. The highly specialized function and the high evolutionary conservation of the *PYGM* gene sequences (Hudson *et al.*, 1993) made us hypothesize that the genetically modified mouse would present with a phenotype consistent with McArdle disease. This hypothesis was also based on the observation that the two spontaneous animal models identified to date, i.e. Charolais cattle (Angelos *et al.*, 1995) and Merino sheep (Tan *et al.*, 1997), showed features present in patients such as rhabdomyolysis or exercise intolerance. We thus expected our model to reflect the main characteristics present in patients.

Knock-in mice homozygous for the common *p.R50X* human mutation showed absence of glycogen phosphorylase activity in muscle and subsequent blocked glycogenolysis, which resulted in a marked exercise intolerance that closely resembles the clinical presentation of the disease. Muscle biochemical and molecular

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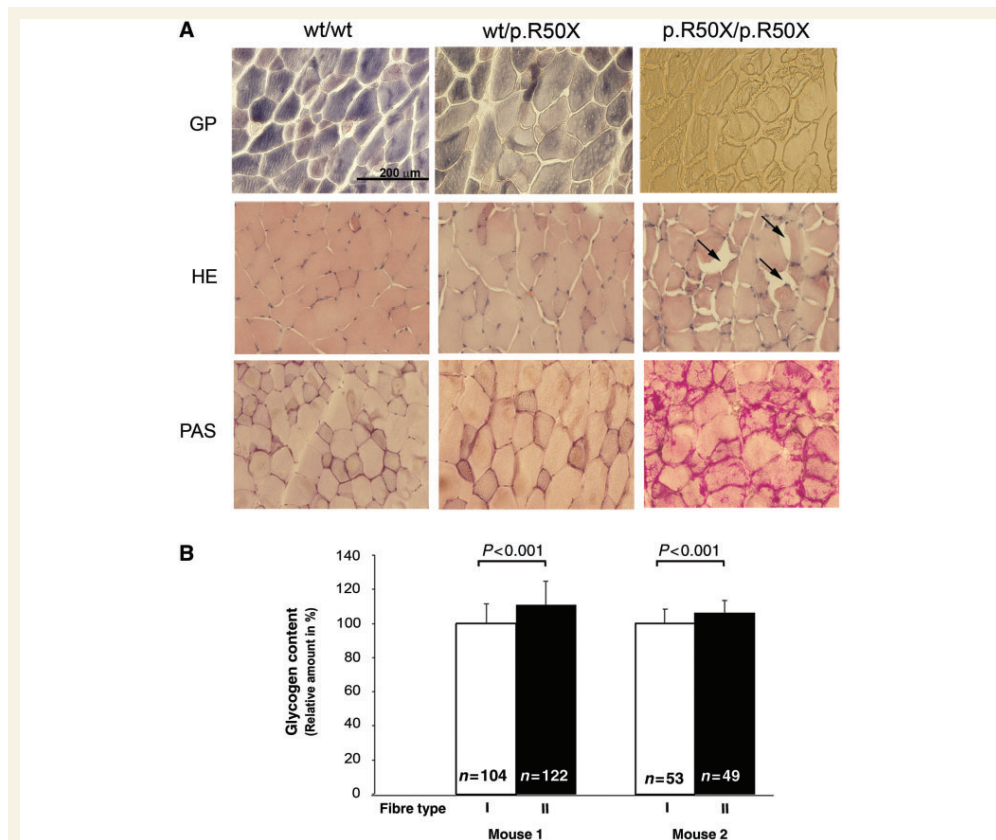
Figure 4 (**A**) Representative images obtained from histochemical analyses of gastrocnemius muscle. GP = glycogen phosphorylase activity staining; HE = haematoxylin–eosin staining and PAS = periodic acid Schiff staining. The black bar in the *wt/wt* glycogen phosphorylase image represents 200 μm; the other images were obtained at the same magnification. Arrows in the haematoxylin and eosin image of the homozygote mouse indicate glycogen accumulation. (**B**) Morphometric quantification of the periodic acid Schiff staining of type I and type II fibres in gastrocnemius sections of two different homozygous p.R50X mice. *n* indicates the total number of fibres counted for each set of values. For each mouse, the results are expressed as a percentage, taking as 100% the mean value obtained for the type I fibres. Error bars indicate standard deviations. For both mice, the mean periodic acid Schiff intensity of type II fibres (normalized by fibre surface) was significantly higher than that of the type I fibres ($P < 0.001$; Student's *t*-test). wt = wild-type.

analyses also showed the common features of the disease. As in patients harbouring the *p.R50X* mutation (Nogales-Gadea *et al.*, 2008), the muscle *Pygm* transcripts of knock-in mice undergo an extensive degree of 'nonsense-mediated decay'; indeed, messenger RNA levels of *p.R50X/p.R50X* mice were only ~10% of those found in *wt/wt* mice. Such low transcript levels are unlikely to have a translational effect on the protein levels; and, if translated, the resulting protein would be truncated and therefore unstable, as confirmed by western blot analyses showing absence of protein levels in all *p.R50X/p.R50X* mice. Blocked glycogenolysis resulted in massive accumulation of

glycogen in the gastrocnemius muscle, not only subsarcolemmal, as typically described in patients (Nadaj-Pakleza *et al.*, 2009) but also intrasarcoplasmic. In fact, the increase of muscle glycogen content observed in *p.50X/p.50X* mice (27-fold) was actually much higher than that observed in patients with McArdle disease, which rarely increases more than 2-fold (Nielsen *et al.*, 2002). This marked glycogen accumulation might be explained, at least partly, by the fact that mouse fast fibres have a greater shift towards glycolytic metabolism than humans and mouse muscle has a much higher proportion of fast, glycolytic fibres (Agbulut *et al.*, 2003) [e.g. there are only 1–8% of slow oxidative fibres in mouse

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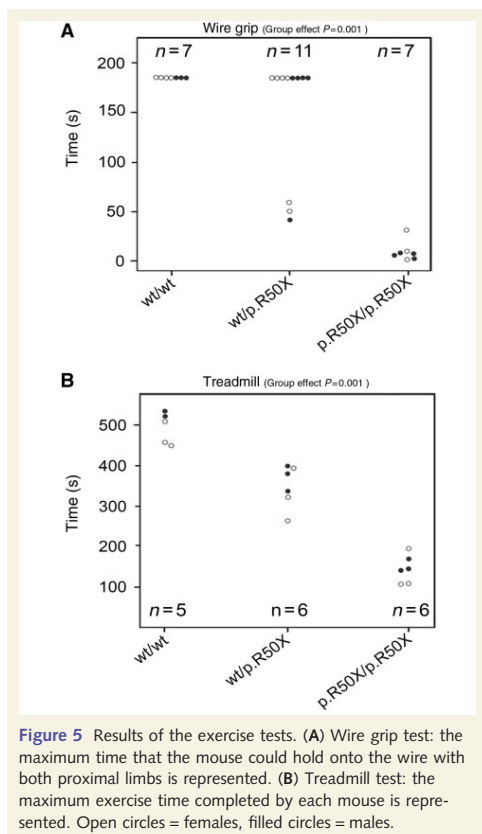
Figure 5 Results of the exercise tests. (**A**) Wire grip test: the maximum time that the mouse could hold onto the wire with both proximal limbs is represented. (**B**) Treadmill test: the maximum exercise time completed by each mouse is represented. Open circles = females, filled circles = males.

gastrocnemius muscle versus a more balanced proportion of fast versus slow twitch in humans (Kho *et al*., 2006)]. Histochemical analysis showed a similar pattern of glycogen accumulation (both at the subsarcolemmal and sarcoplasmic level in other muscles of *p.R50X/p.R50X* mice, i.e. biceps femoris or soleus muscle (the latter having 58% type I fibres in mice) (Kho *et al*., 2006). Interestingly, the glycogen accumulation was more pronounced in less-oxidative (type II) fibres, which are more dependent on glycogen metabolism.

Patients typically exhibit intolerance to static and dynamic exercises. During static or isometric exercise, e.g. in patients carrying a heavy weight, or handgrip exercise and, in rodent models hanging onto a grip, high mechanical demands are imposed on a relatively small muscle mass, and the sustained muscular contraction increases the pressure inside the muscle, causing the supply of oxygenated blood to be transiently cut off (DiMauro, 2007; Lucia *et al*., 2008). In this situation, muscles rely on an anaerobic

energy supply from intracellular glycogen stores. The exercise intolerance phenotype of *p.R50X/p.R50X* mice was in fact most marked in the wire grip test (muscle performance <10% of that reached by both heterozygous and *wt/wt* mice). On the other hand, dynamic, 'aerobic' exercises involving larger muscle mass and smaller mechanical loads, e.g. in human patients, stair-climbing or very brisk walking and in model rodents forced treadmill running, can also trigger acute exercise intolerance. Indeed, muscle oxidative capacity in patients is impaired (Zange *et al*., 2003) because glycolytic flux is reduced and thus their ability to produce pyruvate is severely reduced (Lucia *et al*., 2008). In *p.R50X/p.R50X* mice, maximal aerobic capacity (as determined by maximal treadmill exercise time) was only ~29% of the mean value obtained in *wt/wt* mice. This is consistent with recent data on the largest series of patients available (Lucia *et al*., 2012) where the mean maximal aerobic capacity (expressed as VO_{2max}) was very low, averaging 18.2 ml O_2/kg/min, which is clearly below the minimum threshold for optimal health (28 ml O_2/kg/min; Lucia *et al*., 2008).

Besides molecular genetics and biopsy analysis, basal hyperCKaemia is the main laboratory diagnostic feature indicative of McArdle disease (Lucia *et al*., 2008), as recently confirmed in a large series of Spanish patients (Lucia *et al*., 2012). HyperCKaemia is usually more marked in males than females (~two times higher mean values in the former, likely owing to their higher muscle mass), yet with large interindividual variability (Mate-Munoz *et al*., 2007). All these phenomena were corroborated in our model: despite individual variability, mean basal serum creatine kinase activity was 13-fold higher in *p.R50X/p.R50X* than in *wt/wt* mice. In addition, the occurrence of myoglobinuria after exercise in our model also points to increased muscle fragility in *p.R50X/p.R50X* mice. These and other molecular, metabolic and physical aspects of the model will have to be more extensively studied when homogeneous genetic background has been established through backcrossing, a process that is currently underway.

An interesting finding of this study is the observation of lower exercise capacity of heterozygous mice compared with *wt/wt* mice. This result contrasts with the autosomal recessive nature of the disease in humans with individuals carrying only one mutant *PYGM* allele being asymptomatic (Andersen *et al*., 2006), and with the recent observation that, compared with 'typical' patients with McArdle disease, two patients with minimal residual myophosphorylase had a much milder form of the disease (Vissing *et al.*, 2009). In our murine model there was a clear reduction of glycogen phosphorylase activity in the heterozygotes (50% reduced glycogen phosphorylase activity in muscle compared with *wt/wt* mice), but this phenomenon did not result in glycogen accumulation or fragile fibres (i.e. no exercise-induced myoglobinuria or increased basal creatine kinase). This might suggest that in heterozygous mice partially compromised exercise performance is more related to partial metabolic limitations rather than to myopathy-induced structural alterations. Further research is, however, necessary to elucidate the reasons why exercise capacity appears to be more affected in heterozygous mice compared with heterozygous humans.

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In contrast to available rodent models for other neuromuscular diseases, which do not show a human-like phenotype, here we present a murine model for the most common mutation, *p.R50X*, causing McArdle disease, which presents with the main features the human phenotype. This model may represent a valid tool for in-depth studies of the pathophysiology of this disorder, as well as for new therapeutic approaches for this and other neuromuscular diseases. Interestingly, because *p.R50X* is a nonsense mutation affecting only one tissue whose function can be finely evaluated, our model could also be used for exploring novel pharmacological approaches based on read-through strategies targeting premature termination codon mutations.

Supplementary material

Supplementary material is available at *Brain* online.

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9. Appendix

During my PhD I had the opportunity to carry out a six-months training period in the Molecular Neurogenetics Laboratory at the Harry Perkins Institute of Medical Research in Perth, Australia, working with Dr Kristen Nowak and Professor Nigel Laing AO.

During my short stay I worked on the project aimed to develop a new therapeutic approach using a CRISPR/Cas9 system to correct the *pR50X* mutation in an *"in vitro"* model of McArdle disease.

9.1 Correction of *p.R50X* mutation in an *in vitro* model of McArdle disease via use of CRISPR/Cas9-mediated gene editing by homologous recombination using an homologous donor template

9.1.1 Introduction

The modification of the genome at a precise, predetermined locus is broadly used in biomedical research, medicine and biotechnology. Targeted genome editing is built on engineered, programmable and highly specific nucleases, which can produce site-specific DNA double-strand breaks (DSBs), inducing site-specific modifications in the genomes of cellular organisms. Nuclease-induced DSBs can be repaired by two different pathways: error-prone nonhomologous end-joining (NHEJ) or homology-directed repair (HDR) (Figure 16). NHEJ does not require a template and consequently leads to the efficient introduction of insertions or deletions of various lengths, often disrupting the translational reading frame of a coding sequence or the binding sites of trans-acting factors in promoters or enhancers. HDR can be used to introduce specific point mutations or to insert sequences through recombination of

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the target locus with exogenously supplied donor DNA or a single-stranded oligodeoxynucleotide (ssODN) that acts as a template [252].
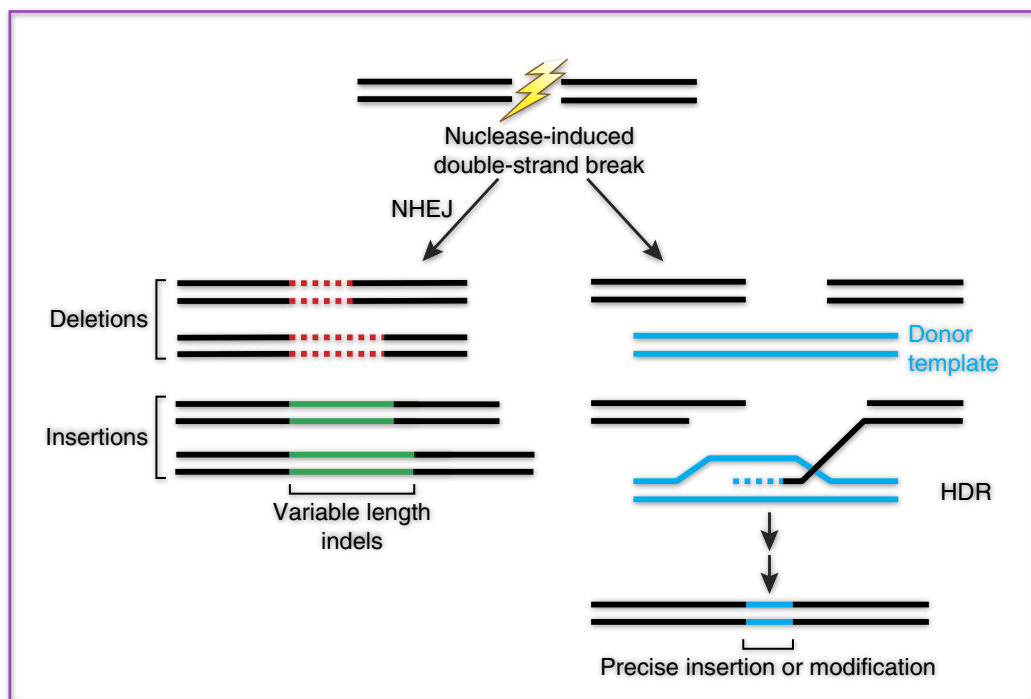
Figure 16: Nuclease-induced genome editing [252].

Multiple programmable nucleases have been developed for genome editing and each one has unique characteristics that make them appropriate for certain genome editing experiments and applications. Among them we can find zinc-finger nucleases (ZFNs), one of widely applied nucleases [253-256], that contain a common Cys_2-His_2 DNA-binding domain and a DNA cleavage domain derived from the *Fok*I restriction endonuclease [257]. Another known programmable nucleases are transcription activator-like effector nucleases (TALENs) [258, 259]. Their general structural organization is similar to ZFNs, both contain the *Fok*I nuclease domain but unlike ZFNs, TALENs use a DNA-binding domain composed of 33-35 conserved amino acids repeated motifs, each of which recognises a specific single base-pair in the

major groove by using two hypervariable residues [258, 260].

Recently, a new class of genome editing nucleases have been reported, RNA-guided CRISPR/Cas system [261-264]. Unlike ZFNs and TALENS, CRISPR/Cas genome-editing system depends on small RNA for sequence-specific cleavage [265]. Because of its simplicity and versatility, CRISPR/Cas9 genome-editing system is easily applicable and has become a remarkably tool for manipulating gene sequences in cellular organisms. This has allowed new possibilities such as efficient multiplex gene editing for simultaneously inactivating multiple genes [262, 264, 266], re-establishment of the expression of the dystrophin gene in cells carrying dystrophin mutations that cause DMD [267] or correction in mice of a dominant mutation in *Crygc* gene that causes cataracts [268].
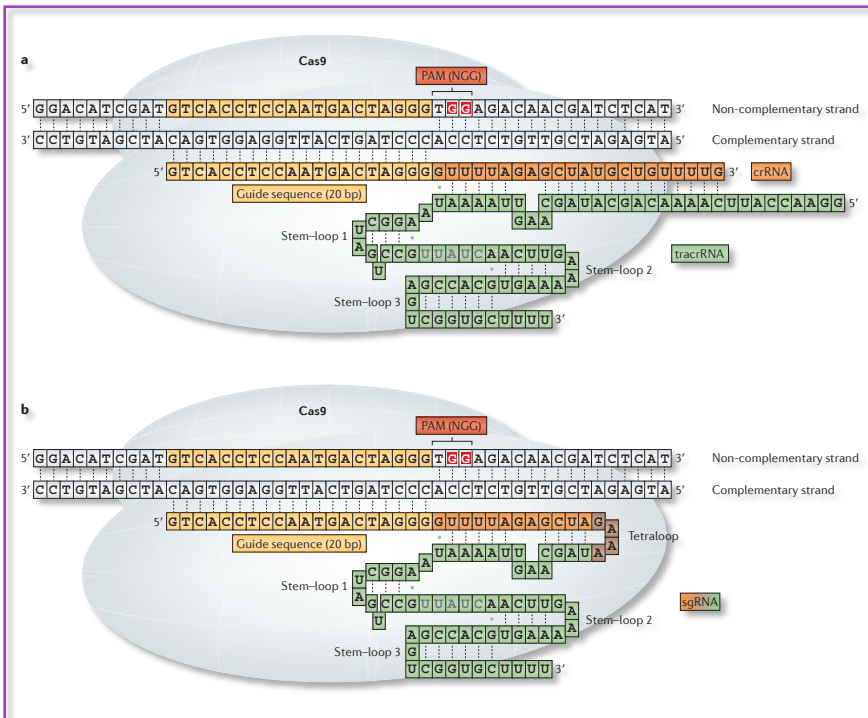
Figure 17: **Schematic representations of type II CRISPR/Cas nucleases**. a.- Type II CRISPR/Cas system is comprised of Cas9 protein, a CRISPR RNA (crRNA) and a trans-activating crRNA (tracrRNA). b.- Alternatively, Type II CRISPR/Cas system can contain Cas9 and a single-chain guide RNA (sgRNA). The guide sequence in the crRNA (part a) or sgRNA (part b) is complementary to a 20-bp target DNA sequence known as protospacer, which is next to the 5′-NGG-3′ (where N represents any nucleotide) sequence known as protospacer adjacent motif (PAM) [260].

CRISPR/Cas genome-editing system has been adapted from the RNA-based adaptative immune system of bacteria and archaea that uses CRISPR (clustered regularly interspaced short palindromic repeat) and Cas (CRISPR-associated) proteins to detect and destroy invading viruses and plasmids [269, 270]. There are three distinct types of CRISPR/Cas systems (I, II and III), based on the sequences and structures of Cas protein [271]. The type II CRISPR/Cas system from *S.pyogenes* requires a single protein, Cas9, for site-specific DNA recognition and cleavage. Cas9 is guided by a 20-nucleotide sequence (protospacer) contained in an associated CRISPR RNA (crRNA) transcript and a trans-activating crRNA (tracrRNA) that is partially complementary to the crRNA [265, 272] (Figure 17, a). The crRNA:tracrRNA complex can be fused as a single transcript (guide RNA or gRNA), which simplifies the components of CRISPR/Cas9 system [265] (Figure 17, b). Moreover, in addition to the 20-nucleotide sequence, Cas9 requires a 5'-NGG-3' sequence next to the protospacer, known as protospacer adjacent motif (PAM), which is recognised by Cas9 itself. Thus, Cas9 can be programmed to cleave double-stranded DNA at any site defined by the gRNA sequence and PAM (5'-X_{20}NGG-3') [265].

Type II CRISPR/Cas systems from other species of bacteria recognise alternative PAM sequences and have also been used for targeted genome editing [273, 274].

With this study, we attempt to develop a therapeutic approach using the type II CRISPR/Cas9 system from *S. pyogenes* with the aim of correcting the pR50X mutation in *Pygm*.

9.1.2 Materials and methods

Plasmid expressing Cas9, PYGM, (R50X)PYGM and gRNA. Six different gRNA targeting specific sites throughout the mouse *Pygm* gene were designed by software

tools (CRISPRdirect (crispr.dbcls.jp) and www.genscript.com) (Figure 18; A and B). Plasmid vectors expressing Cas9-RFP, pEGFP-C1-PYGM and pEGFP-C1-(R50X)PYGM under the citomegalovirus (CMV) promoter were obtained from Sigma-Aldrich (CAS9RFPP-1EA) and Genscript (OMu13100C, OMu13100CM_R50X), respectively. gRNAs under U6 promoter were obtained from Genscript.
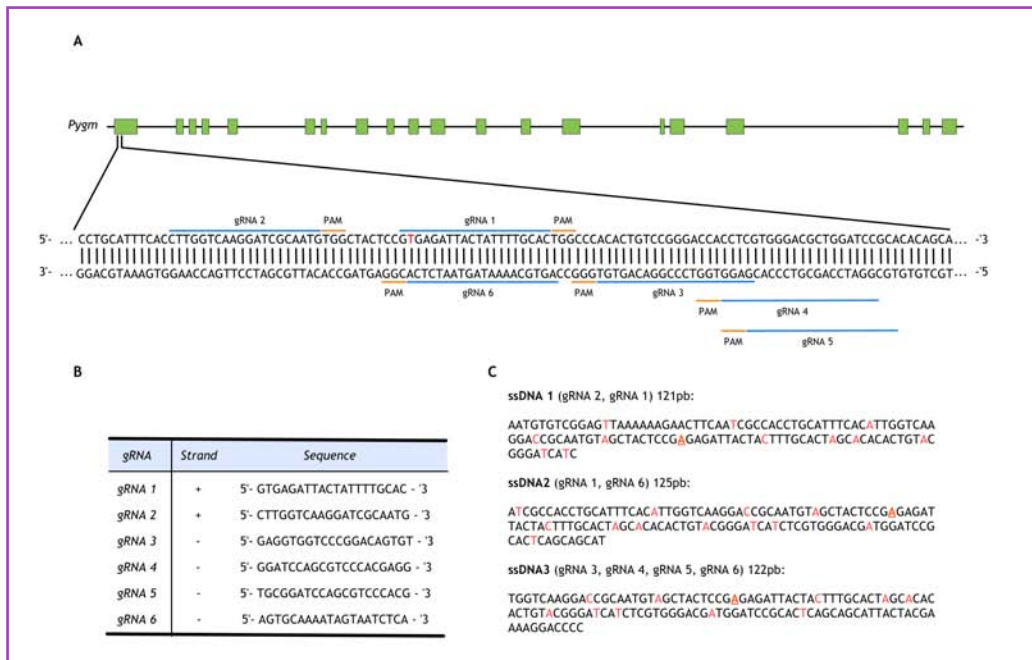
Figure 18: **CRISPR/Cas 9 design.** A.- Schematic of the Cas9-gRNA-targeting sites in mutant mouse *PYGM* gene. Blue and orange lines label the gRNA-targeting and protospacer adjacent motifs (PAM) sequences, respectively. B.- The sequences of the Cas9-gRNA-targeting sites. C.- The sequences of the three single-stranded DNA donors. Red letters indicate synonymous changes. Red and underlined letters indicate wild-type A nucleotide.

Single-stranded DNA donors. To facilitate homologous recombination (HR) and correct the T→A nonsense mutation, three different ~120-nt, single-stranded DNA (ssDNA) donors were ordered harboring the WT A nucleotide and homology arms flanking the gRNA target region, as well as, some synonymous mutations throughout the sequences (Genscript) (Figure 18; C).

Cell culture and transfection. Mouse C2C12 myoblasts were obtained from Sigma-Aldrich (Ref. 91031101) and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine calf serum (FBS) and 1% penicillin/streptomycin at 37°C and 5% CO_2. Mouse C2C12 myoblasts were seeded into 6-well plates one day prior to transfection at a density of 120,000 cells per well. Cells were transfected using Lipofectamine 3000 (Invitrogen) with 4 μg of Cas9-RFP expression vector, 6 μg of pEGFP-C1-PYGM or pEGFP-C1-(R50X)PYGM expression vector, 400 ng gRNA (1-6) expression vector and 4 μl of 10 nM ssDNA donor according to the manufacturer's protocol.

Pyrosequencing assay. Mouse C2C12 myoblasts were transfected with plasmid DNA as described above, and incubated at 37°C and 5% CO_2. Two days after transfection, cells were trypsinized and harvested and plasmidic DNA was extracted using Puralink Quick Plasmid Miniprep kit (Invitrogen). Pyrosequencing assay was designed using the algorithms built into the PyroMark Assay Design Software (Version 2.0.1, Qiagen). Briefly approximately 200-400 bp of sequence surrounding the target variant sites were input into the software. Variant sites were selected as target sites for analysis and primers designed to target these sites were chosen from a list generated by the software on the basis of the algorithms' predicted assay quality. PCR and sequencing primers for the assay are listed in Table 6.

Assay name	Forward PCR primer	Reverse PCR primer	Sequencing primer
Pygm	ATGTCCAGGCCTCTTTCAGA	/5Biosg/GGGGTCCTTTTCGTAGTAATGC	GAGTGGAAAATGTGTCG

Table 6: Primer sequences for pyrosequencing assay.

The assay regions containing the variant target sites were PCR amplified using a biotin labelled, HPLC purified primer and standard sequencing grade primer (see Table 6). All PCR amplifications were performed with the PyroMark PCR Kit

(Qiagen). Amplification reactions consisted of 12.5 μl PyroMark Mastermix, 2.5 μl Coral Load, 1 μl each of 5 μM forward and reverse primers, 2 μl of 5 ng/μl template DNA and 6 μl of water. Thermocycling conditions consisted of 15 minutes at 95°C followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C and a final extension step of 10 minutes at 72°C. All amplifications were visualised on 2% agarose gels to confirm quality and estimate concentration.

The PCR product was bound to Streptavidin Sepharose High Performance beads (GE Healthcare Life Sciences), the beads containing the immobilized PCR product were denatured and washed using proprietary solutions (Qiagen) on the Pyrosequencing Vacuum Prep Tool (Qiagen) to isolate a single stranded template. The beads were then transferred to an optically clear, 24-well sequencing plate in 0.3 μM of pyrosequencing primer. Annealing to the single-stranded template was done by

Sequence to analyze:
GAGYTAAAAAAGAACTTCAAYCGCCACCTGCATTTCACMTTGGTCAAGGAYCGCAATGTRGCTACTCCGWGAGATTACTAYTTTGCACTRGCM CACACTGTMCGGGAYCAYCTCGTGGGACGMTGGATCCGCACWCAGCA

Position	1	2	3	4	5	6	7
Name	Position 1	Position 2	Position 3	Position 4	Position 5	Position 6	Position 7
Quality	Passed	Passed	Passed	Check	Failed	Failed	Failed
A (%)	-	-	3	-	21	8	-
C (%)	100	98	97	0	-	-	19
G (%)	-	-	-	-	79	-	-
T (%)	0	2	-	100	-	92	81

Figure 19: Example of a pyrogram for the Pygm assay showing the position of the variant sites and the dispensed order used for the pyrosequencing.

heating the plate to 80ºC followed by cooling to room temperature. Pyrosequencing was performed on a PyroMark 24 Pyrosequencing System (Qiagen) as per the manufacturer's instructions. Data was analysed on the PyroMark Q24 software to give the percentage of each allele at the variant sites in the sample. An example of pyrogram for the assay showing the position of the variant sites is depicted in Figure 19.

9.1.3 Results

To utilize CRISPR/Cas9 gene-editing system for correcting the T→A nonsense mutation, we designed six gRNAs targeted to different regions in the mutant *Pygm* gene (Figure 18; A and B). gRNA 1 and gRNA 6 were designed to target regions
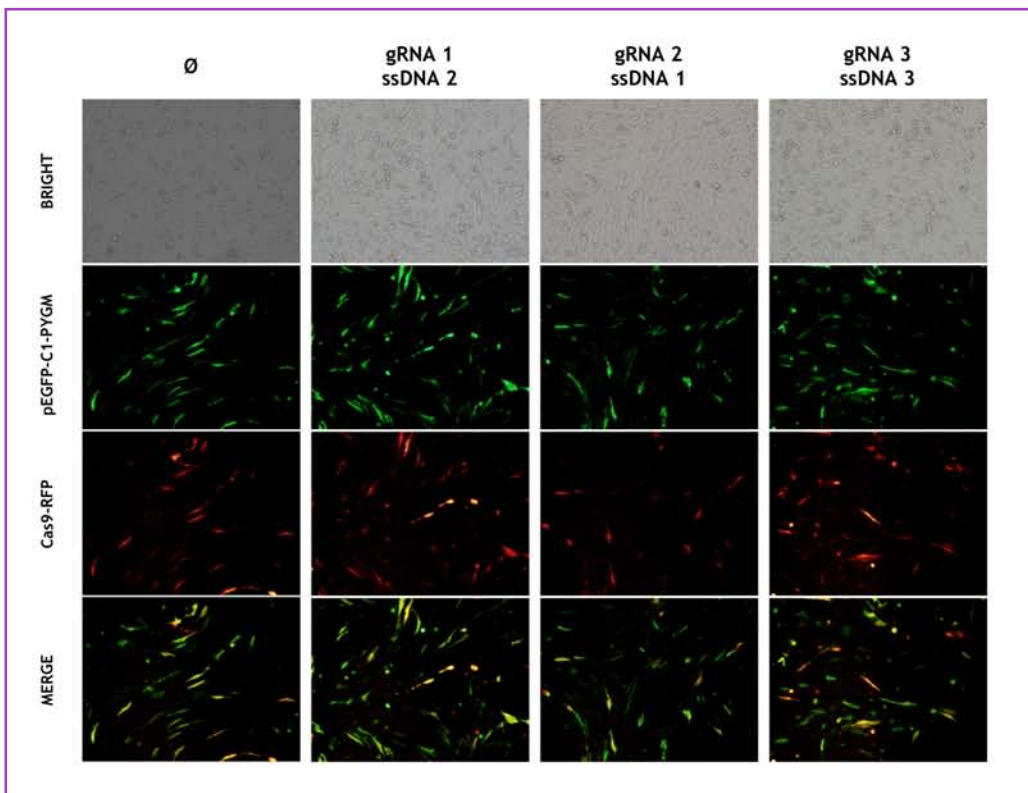
Figure 20: C2C12 cells cotransfection with Cas9-RFP, pEGFP-C1-PYGM and gRNA expression vectors, as well as, ssDNA donor. GFP: indicates green fluorescent protein; RFP: red fluorescent protein.
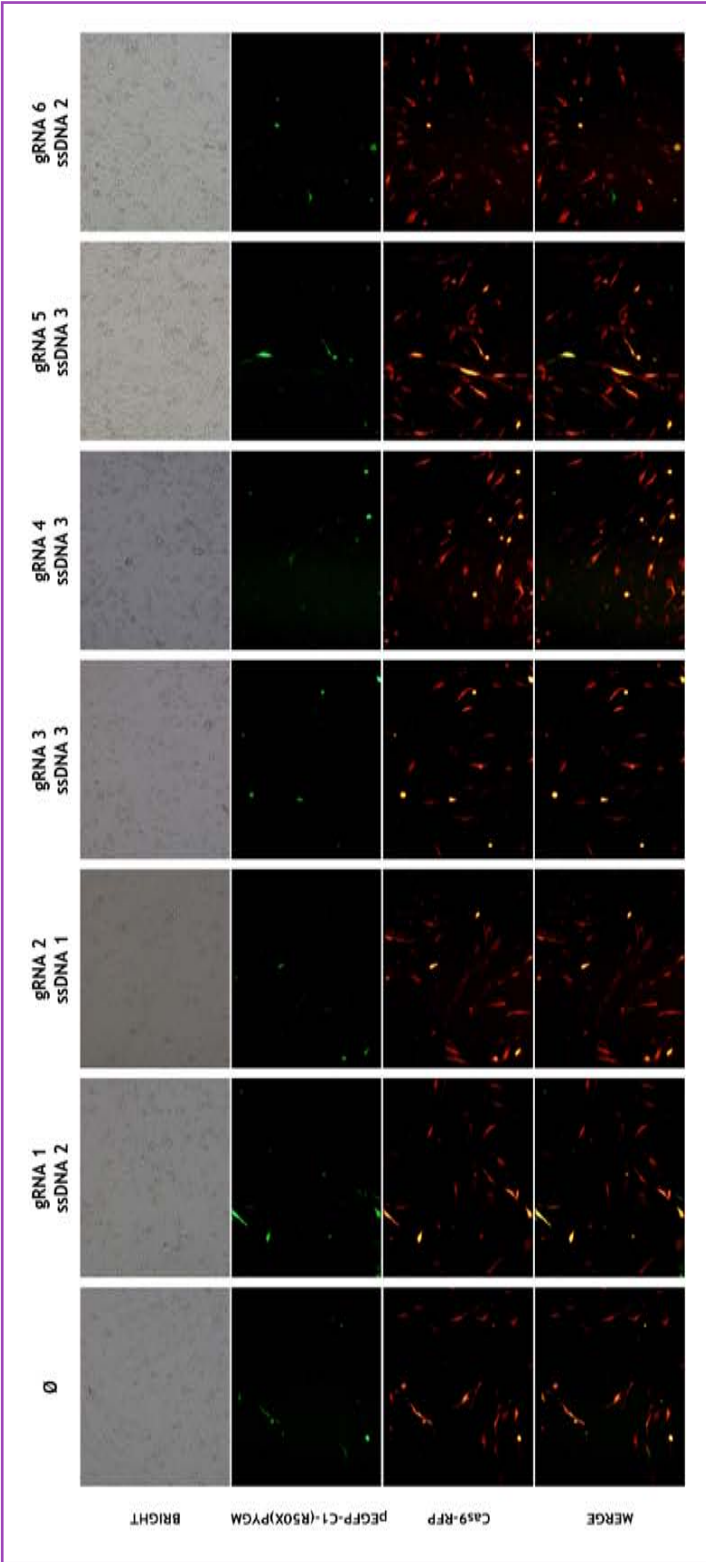
Figure 21: C2C12 cells cotransfection with Cas9-RFP, pEGFP-C1-(R50X)PYGM and gRNA expression vectors, as well as, ssDNA donor. GFP: indicates green fluorescent protein; RFP: red fluorescent protein.

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spanning the site of the nonsense mutation. gRNA 2 was designed to target the sequence upstream of the mutation and gRNA 3, gRNA 4 and gRNA 5 targeted the region immediately downstream of the mutation. Aiming to test the feasibility of CRISPR/Cas9-mediated gene editing and assess the efficiency of each gRNA, firstly we tested them (together with pEGFP-C1-(R50X)PYGM expression vector) in mouse C2C12 myoblasts. Furthermore, we also investigated the specificity of gRNAs by separately transfecting plasmids expressing both the Cas9-RFP, each of the six gRNAs and pEGFP-C1-PYGM (WT) into mouse C2C12 myoblasts. To test if both Cas9-RFP and pEGFP-C1-PYGM or pEGFP-C1-(R50X)PYGM cotransfected together, transfected cells were analyzed and images obtained with an inverted microscope (Olympus IX 71 Inverted microscope, Olympus Corporation) (Figure 20 and 21). After transfection into C2C12 myoblasts, we observed undetectable gene modification as measured by pyrosequencing. The results showed no mutation correction, as well as, no NHEJ-mediated mutations of the *Pygm* gene in transfected C2C12 myoblasts with any gRNA (see Table 7).

7.1.4 Discussion

The recent development of CRISPR/Cas9 system as a genome editing tool has become a revolution in the area. Many research groups have used this technique to try to correct genetic diseases [267, 275-278] and it has been previously demonstrated to function in human stem cells [266, 273, 279] and other human cell lines [261-264], as well as human skeletal myoblasts [267]. Furthermore, many studies have been performed with the aim of reducing off-target effects [280] and increasing the efficiency of HDR [281, 282].

In our present study we didn't achieve to modify *Pygm* gene but we cannot be totally sure that CRISPR/Cas9 system has not worked in ours cells. Our analysis was made in the bulk population of cells, and as our results showed (Figure 20 and

	Position 1 (C → T)				Position 2 (C → T)				Position 3 (C → A)				Position 4 (T → C)				Position 5 (G → A)				Position 6 (A(WT) → T(X))				Position 7 (T → C)			
	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)	A (%)	C (%)	G (%)	T (%)
Cas9/WT	-	100	-	0	-	98	-	2	3	97	-	-	-	0	-	100	21	-	79	-	79	-	-	21	-	21	-	79
	-	100	-	0	-	98	-	2	4	96	-	-	-	0	-	100	23	-	77	-	77	-	-	23	-	25	-	75
Cas9/R50X	-	100	-	0	-	98	-	2	3	97	-	-	-	0	-	100	21	-	79	-	8	-	-	92	-	19	-	81
	-	100	-	0	-	98	-	2	4	96	-	-	-	0	-	100	24	-	76	-	10	-	-	90	-	24	-	76
Cas9/WT/gRNA1/Template	-	100	-	0	-	98	-	2	3	97	-	-	-	0	-	100	21	-	79	-	79	-	-	21	-	22	-	78
	-	100	-	0	-	98	-	2	3	97	-	-	-	0	-	100	21	-	79	-	79	-	-	21	-	21	-	79
Cas9/WT/gRNA2/Template	-	100	-	0	-	98	-	2	3	97	-	-	-	0	-	100	21	-	79	-	78	-	-	22	-	22	-	78
	-	100	-	0	-	98	-	2	5	95	-	-	-	0	-	100	30	-	70	-	74	-	-	26	-	27	-	73
Cas9/WT/gRNA3/Template	-	100	-	0	-	98	-	2	3	97	-	-	-	0	-	100	22	-	78	-	76	-	-	24	-	20	-	80
Cas9/R50X/gRNA 1/Template	-	100	-	0	-	98	-	2	3	97	-	-	-	0	-	100	21	-	79	-	8	-	-	92	-	19	-	81
	-	98	-	2	-	98	-	2	5	95	-	-	-	0	-	100	26	-	74	-	10	-	-	90	-	25	-	75
Cas9/R50X/gRNA 2/Template	-	100	-	0	-	98	-	2	3	97	-	-	-	0	-	100	21	-	79	-	8	-	-	92	-	20	-	80
	-	99	-	1	-	98	-	2	4	96	-	-	-	0	-	100	23	-	77	-	10	-	-	90	-	23	-	77
Cas9/R50X/gRNA 3/Template	-	100	-	0	-	98	-	2	3	97	-	-	-	0	-	100	21	-	79	-	9	-	-	91	-	20	-	80
Cas9/R50X/gRNA 4/Template	-	100	-	0	-	98	-	2	4	96	-	-	-	0	-	100	24	-	76	-	10	-	-	90	-	24	-	76
Cas9/R50X/gRNA 5/Template	-	100	-	0	-	98	-	2	4	96	-	-	-	0	-	100	22	-	78	-	9	-	-	91	-	21	-	79
Cas9/R50X/gRNA 6/Template	-	100	-	0	-	98	-	2	3	97	-	-	-	0	-	100	21	-	79	-	9	-	-	91	-	20	-	80
	-	100	-	0	-	98	-	2	5	95	-	-	-	0	-	100	26	-	74	-	10	-	-	90	-	25	-	75
	-	100	-	0	-	98	-	2	3	97	-	-	-	0	-	100	21	-	79	-	8	-	-	92	-	19	-	81

Table 7: Pyrosequencing results.

21), the transfection efficiency rate of our cells was extremely low. In a recent study where the correction of mutations in the human dystrophin gene in Duchenne muscular dystrophy (DMD) patient cells using CRISPR/Cas9 system was shown [267], they observed no gene modification when they analyzed the bulk population of low transfected DMD myoblasts (~0.5-2%) despite observe high transfection efficiency of a control GFP expression plasmid and high efficiencies rates in HEK293T cell line. Nevertheless, after sorting the transfected DMD myoblasts, they observed a substantial increase in detectable activity at most gRNA, suggesting us that we might be unable to detect any gene modification because of lower transfections rates.

The utility of the C2C12 myoblasts in our study was to establish proof of concept for gene editing in muscular cells. The best cell type from a clinical approach is the correction of knock-in mouse myoblasts or McArdle patients myoblasts but the lack of both cell types when we carried out our study prompted us to use C2C12 myoblasts. Utilization of C2C12 myoblasts, added the goal to introduce a third plasmid containing the mutated (R50X) PYGM cDNA, hindering transfection efficiency.

Further studies using skeletal-muscle cultures derived from McArdle mouse, trying to increase the transfection efficiency rates or sorting the transfected cells are required to improve our results, as well as, the utilization of Scr7, an inhibitor of DNA ligase IV, a key enzyme in NHEJ pathway, which has been reported to promote HDR rather than NHEJ [281, 282], enabling the insertion of precise genetic modifications. Off-target analyses are also needed.

8. References

8. References

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