J Physiol 593.12 (2015) pp 2693–2706

The Journal of Physiology

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*

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DOI: 10.1113/JP270085

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.

(Received 17 December 2014; accepted after revision 10 April 2015; first published online 15 April 2015)
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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

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

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) (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	$P = 0.999$	$P = 0.901$	$P = 0.907$	$\mathbf{P < 0.001}$	$P = 0.517$	$P = 0.717$
Exercise capacity (m)	376.3 ± 81.3	600.1 ± 107.1	729.6 ± 120.5	$\mathbf{P < 0.001}$	$\mathbf{P < 0.001}$	$\mathbf{P = 0.025}$	$P = 0.365$	$\mathbf{P < 0.001}$	$P = 0.479$
[Glucose] (mg dl^{-1})	110.0 ± 22.2	153.7 ± 23.8	147.9 ± 30.1	$P = 0.004$	$\mathbf{P = 0.013}$	$P = 0.871$	$P = 0.698$	$\mathbf{P = 0.001}$	$P = 0.932$
[Lactate] (mM l^{-1})	1.9 ± 0.2	4.2 ± 0.3	4.3 ± 0.6	$\mathbf{P < 0.001}$	$\mathbf{P < 0.001}$	$P = 1.000$	$P = 0.877$	$\mathbf{P = 0.001}$	$P = 0.395$
[Ammonia] (μM l^{-1})	497.7 ± 136.1	235.0 ± 90.7	188.4 ± 78.0	$\mathbf{P < 0.001}$	$\mathbf{P < 0.001}$	$P = 0.944$	$P = 0.474$	$\mathbf{P = 0.001}$	$\mathbf{P = 0.028}$
[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 ($P < 0.001$).

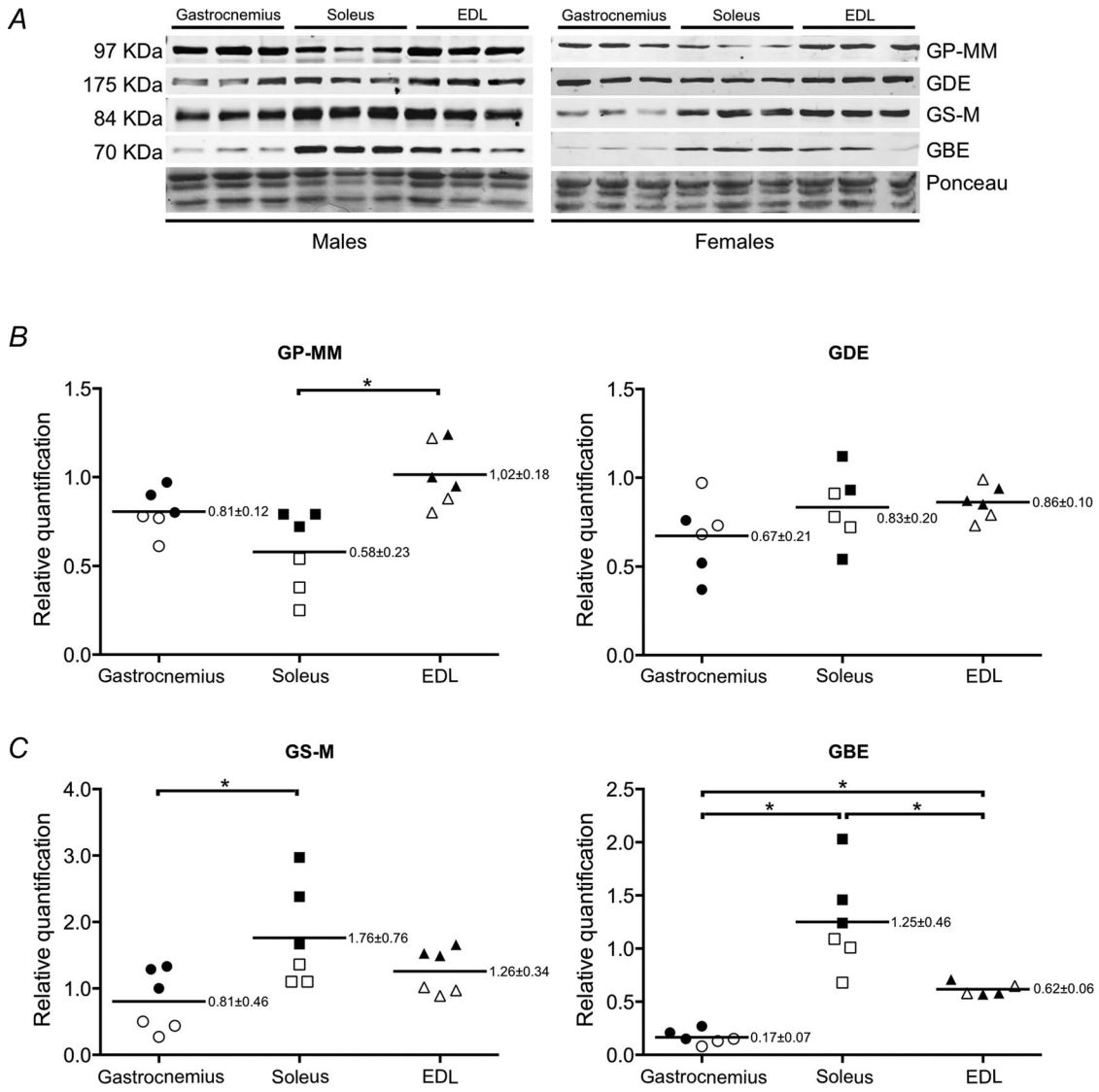

Figure 1. Levels of enzymes of glycogen metabolism enzymes in the muscles of healthy mice
A, western blot analyses. *B* and *C*, levels of the two catabolic (GP-MM and GDE) and anabolic enzymes (GS-M and GDE), respectively. Data are shown as the mean ± SD and individual values, with males indicated by 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

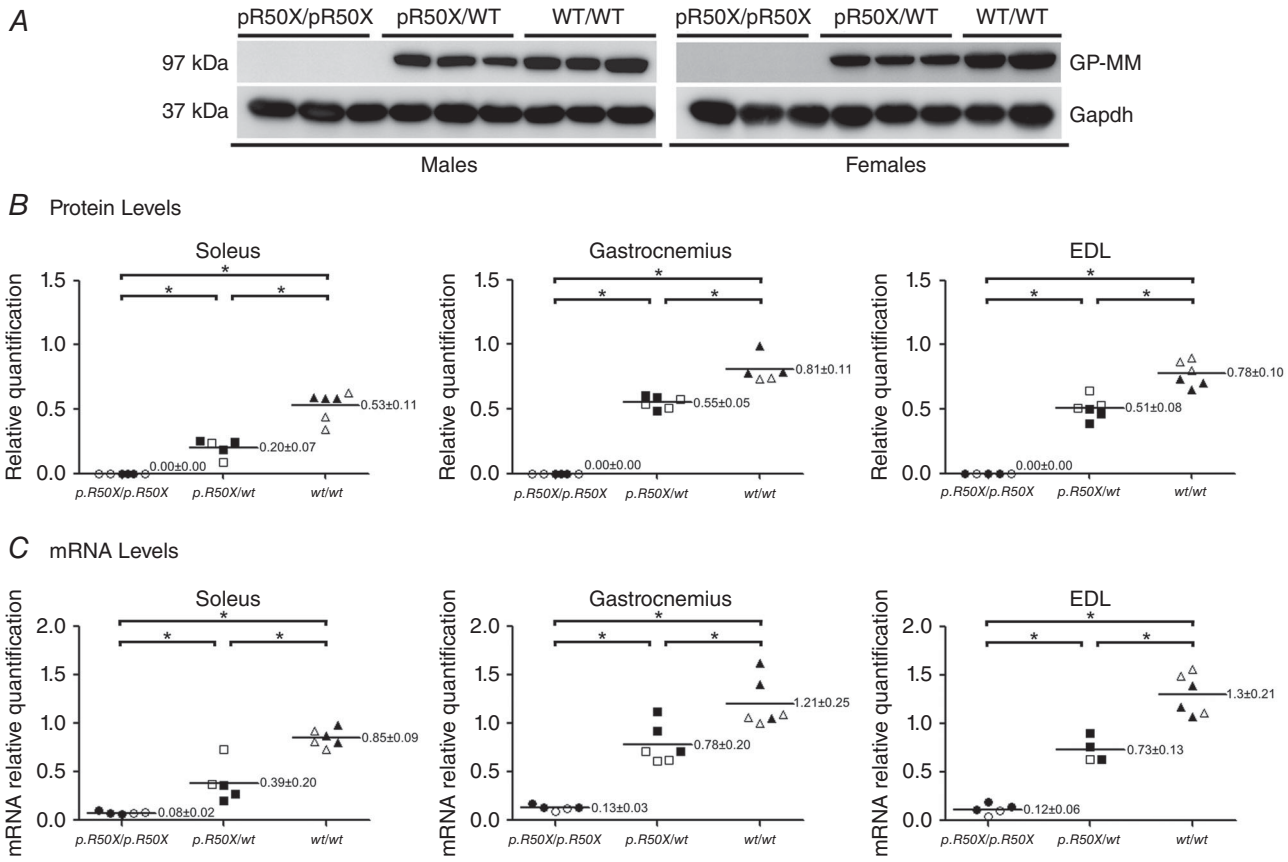

Figure 2. Comparison of protein and transcript levels of GP-MM according to muscle type and *Pygm* genotype
A, western blot analyses in *gastrocnemius* muscle. *B* and *C*, protein and transcript levels, respectively, in muscles. Data are shown as the mean ± SD and individual values, with males indicated 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

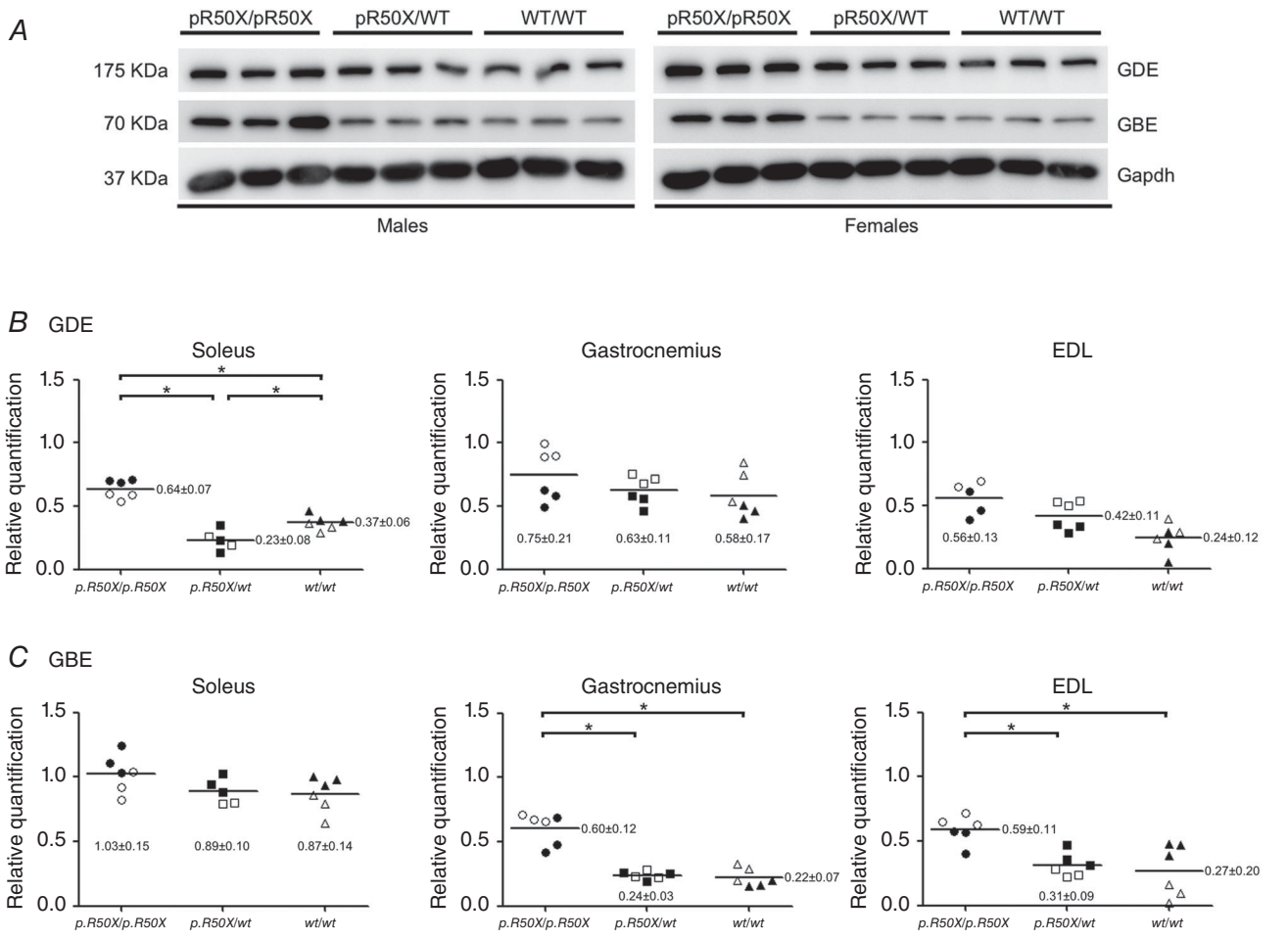

Figure 3. Effect of *Pygm* genotype and muscle type on GBE and GDE
A, western blot analyses in the *gastrocnemius* muscle. *B*, effect of *Pygm* genotype and muscle type on GBE. *C*, effect of *Pygm* genotype and muscle type on GDE. Data are shown as the mean ± SD and individual values, with males indicated 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,

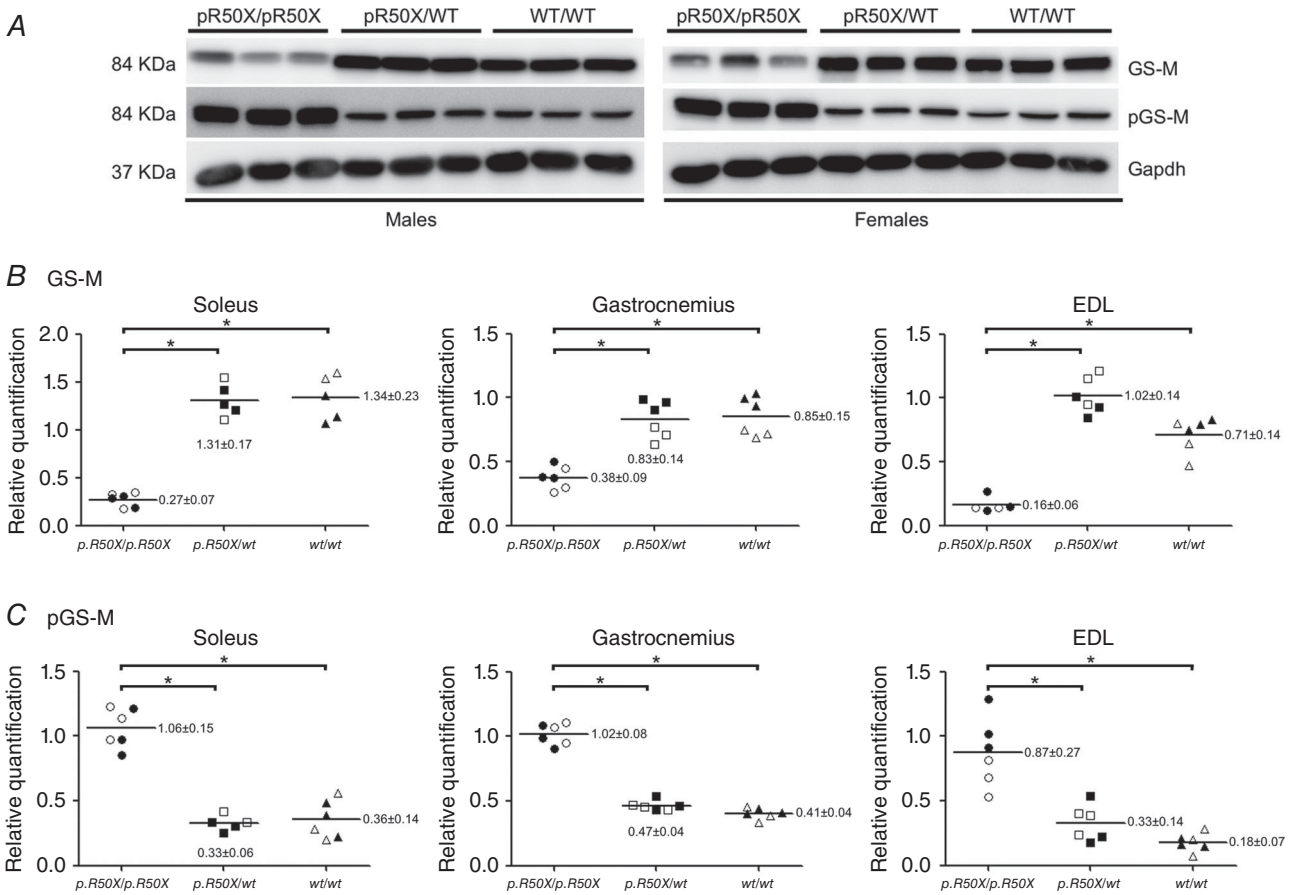

Figure 4. Effect of *Pygm* genotype and muscle type on GS-M and pGS-M
A, western blot analyses in the *gastrocnemius* muscle. *B*, effect of *Pygm* genotype and muscle type on GS-M. *C*, effect of *Pygm* genotype and muscle type on pGS-M. Data are shown as the mean ± SD and individual values, with males 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).

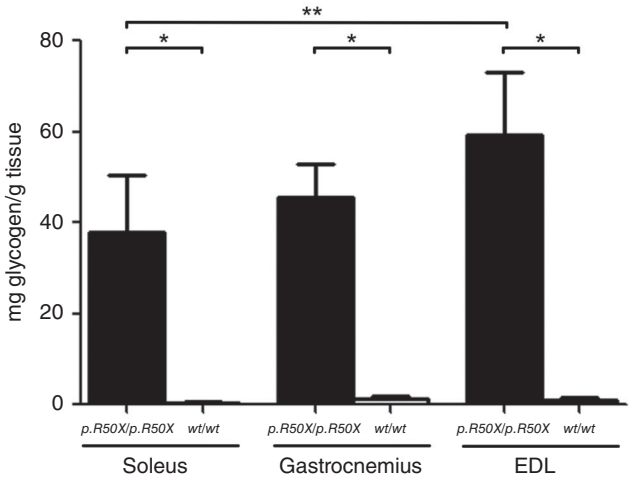

Figure 5. Effect of *Pygm* genotype and muscle type on muscle glycogen content
Data are shown as the mean ± SD. Significant *post hoc* differences are indicated in parenthesis (*$P < 0.01$; **$P < 0.05$).

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 indictating 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

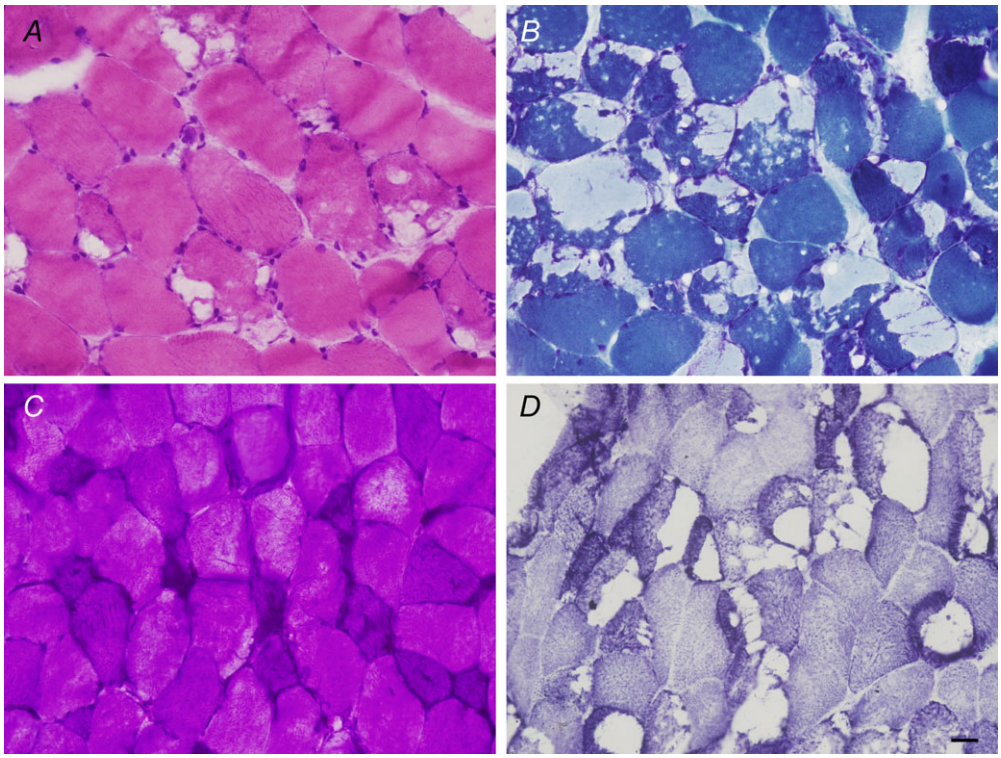

Figure 6. Histochemical analysis of a *gastrocnemius* muscle from a McArdle mouse
Haematoxylin and eosin (*A*), Gomory's trichrome (*B*), PAS (*C*) and succinate dehydrogenase (*D*) staining are shown. Scale bar = 50 μm.

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

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

The authors declare that they have no 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.

Funding

The present study was funded by grants received from the Fondo de Investigaciones Sanitarias (FIS, grant PI13/00855, PI12/00914 and PI14/00903) and cofunded by 'Fondos FEDER'. Astrid Brull is supported by a FIS grant from ISCIII FI11/00709. Noemi de Luna are supported by Sara Borrell contracts of ISCIII CD11/00060