AMERICAN COLLEGE OF SPORTS MEDICINE

Published ahead of Print

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Accepted for Publication: 15 July 2024

Medicine & Science in Sports & Exercise, Publish Ahead of Print
DOI: 10.1249/MSS.0000000000003529

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Conflicts of Interest and Funding Source: The authors declare no conflicts of interest. Research by PLV is supported by a Sara Borrell postdoctoral contract granted by Instituto de Salud Carlos III (CD21/00138). TP is funded by the Spanish Ministry of Economy and Competitiveness and Fondos Feder (grant PI22/00201). AL and DBG are funded by the Spanish Ministry of Economy and Competitiveness and Fondos Feder (PI15/00558, and PI18/00139, respectively).

ABSTRACT

Introduction: Whether cardiac impairment can be fully discarded in McArdle disease—the paradigm of 'exercise intolerance', caused by inherited deficiency of the skeletal muscle-specific glycogen phosphorylase isoform ('myophosphorylase')—remains to be determined. **Methods:** Eight patients with McArdle disease and seven age/sex-matched controls performed a 15-minute moderate, constant-load cycle-ergometer exercise bout followed by a maximal ramp test. Electrocardiographic and two-dimensional transthoracic (for cardiac dimension's assessment) and speckle tracking [for left-ventricle global longitudinal (GLS) assessments] echocardiographic evaluations were performed at baseline. Electrocardiographic and GLS assessments were also performed during constant-load exercise and immediately upon maximal exertion. Four human heart biopsies were obtained in individuals without McArdle disease, and in-depth histological/molecular analyses were performed in McArdle and wild-type mouse hearts. **Results:** Exercise intolerance was confirmed in patients ('second wind' during constant-load exercise, -55% peak power output *vs* controls). As opposed to controls, patients showed a decrease in GLS during constant-load exercise, especially upon second wind occurrence, but with no other between-group difference in cardiac structure/function. Human cardiac biopsies showed that all three glycogen phosphorylase—myophosphorylase, but also liver and especially brain—isoforms are expressed in the normal adult heart, thereby theoretically compensating for eventual myophosphorylase deficiency. No overall histological (including glycogen depots), cytoskeleton, metabolic or mitochondrial (morphology/network/distribution) differences were found between McArdle and wild-type mouse hearts, except for lower levels of pyruvate kinase M2 and translocase of outer membrane 20 kDa subunit in the former. **Conclusions:** This study provides preliminary evidence that cardiac structure and function seem to be preserved in patients with McArdle disease. However, the role for an impaired cardiac contractility associated with the second wind phenomenon should be further explored.

Key Words: GLYCOGENOSIS, GLYCOGEN, HEART, PERFORMANCE

INTRODUCTION

Glycogen storage disease (GSD) V, also known as McArdle disease, is an autosomal recessive disorder caused by pathogenic mutations in both copies of *PYGM*, the gene encoding the skeletal muscle-specific isoform of glycogen phosphorylase (GP-MM, also referred to as 'myophosphorylase'). GP-MM catalyzes the breakdown of muscle glycogen into glucose-1-phosphate and is the only GP isoform expressed in adult non-regenerating skeletal muscle fibers — of note, the inherited metabolic blockade occurs upstream blood glucose uptake by muscle fibers (1).

Patients with McArdle disease are therefore unable to obtain energy from muscle glycogen and this disorder is arguably the paradigm of 'physical activity/exercise intolerance', characterized by 'crises' of early exertional fatigue, together with muscle weakness, myalgia and contractures associated with exertion, often accompanied by episodes of rhabdomyolysis (2, 3). A characteristic feature of McArdle disease (which reflects the aforementioned exercise 'intolerance') is the so-called 'second wind' — whereby patients typically experience early fatigue, breathlessness and myalgia (and are also more prone to have contractures and potentially rhabdomyolysis) during the first minutes of dynamic exercise (*e.g.*, brisk walking, bicycling) together with undue tachycardia, which is followed by the second wind, that is, a marked improvement in symptoms and a decrease in heart rate (HR) after ~7-10 minutes have elapsed (4, 5). Although the biological underpinnings behind this phenomenon remain to be fully elucidated, at least at the cardiac level, the second wind is attributed to an increased availability of blood-borne substrates — free fatty acids and, especially, glucose — which can be subsequently metabolized by skeletal muscle fibers (4, 5).

McArdle disease has been traditionally considered as a ‘pure myopathy’ (1). Indeed, although GP-MM is also expressed in tissues other than the skeletal muscle (including the myocardium but not the liver) (6), there are other non-muscle GP — liver (GP-LL), brain (GP-BB) and perhaps hybrid (GP-BB+LL) — isoforms, which expression would theoretically compensate, at least partly, for an eventual GP-MM deficiency (7). Yet, there have been some reports of cardiac alterations associated with McArdle disease (8), including electrocardiographic (ECG) abnormalities (9) and/or hypertrophic cardiomyopathy (10, 11) or angina (12). By contrast, other previous reports suggested no cardiac alterations in these patients (normal histology in a right atrium biopsy obtained from coronary bypass surgery in one patient (12) or preservation of *total* GP activity in post-mortem cardiac samples of an affected child, as opposed to the skeletal muscle (13)). Yet, to the best of our knowledge, no study has focused in depth on the cardiac structure or GP isoform expression, nor on the cardiac function (notably, myocardial contractility) of these patients, particularly during exercise. Performing this type of study might allow discerning to what extent other non-muscle GP isoforms can compensate for GP-MM deficiency at the heart functional level, especially when metabolic demands are high. Indeed, although free fatty acid oxidation is the primary contributor to ATP production in the heart (14), this tissue responds to acute increases in energy requirements (notably during heavy exercise with subsequent adrenaline stimulation) by selective oxidation of glycogen (15-17). There is also recent evidence that glycolytic flux from myocardial glycogen increases the heart's ability to oxidize ketone bodies, ultimately increasing the free energy of ATP hydrolysis (18). Lactate is another fuel source for (but not a major byproduct of) the heart, contributing up to 15% of ATP production in this tissue (14, 19). In this regard, due to the inherited metabolic block in muscle glycogenolysis, circulating lactate levels show essentially no change (and even a slight decrease) in patients with McArdle disease during graded exercise until exhaustion, as opposed to the common exponential rise seen in unaffected people (20). Additionally, one question of interest is whether cardiac function is altered during the first minutes of exertion in these patients (*i.e.*, before the occurrence of the second wind), which as mentioned above is characterized by disproportionate tachycardia. The latter represents in fact a reason why some of them are initially seen by a cardiologist before accurate diagnosis.

The purpose of the present preliminary study was to explore whether cardiac structure and function is altered, at least partly, in McArdle disease, and to identify the potential mechanisms involved in eventual cardiac impairment. To this end, we performed three sub-studies. First, we compared the resting cardiac dimensions, as well as the electrocardiographic response and myocardial contractility in resting conditions and during exercise, of patients with McArdle disease and a group of age/gender-matched healthy controls. Second, we studied the pattern of GP isoform expression in the adult human heart in general (*i.e.*, ex vivo study in cardiac biopsies from individuals without McArdle disease). Third, we performed in-depth metabolic molecular analyses in the myocardium of mice with McArdle disease [which are known to mimic the human phenotype closely (21) compared with wild-type (WT) controls] in order to determine whether there are molecular alterations in this tissue as a result of GP-MM deficiency.

METHODS

Study in patients with McArdle disease

All the evaluations were performed in the Exercise Physiology laboratory at the Universidad Europea de Madrid (Madrid, Spain). In order to be included in the study, participants had to be aged ≥18 years and free of any condition contraindicating maximal exercise testing as well as free of any type of regular medication (excluding painkillers in patients). Individuals diagnosed with McArdle disease were recruited through the Spanish association of patients with this condition [*Asociación Española de Enfermos de Glucogenosis* (AEEG); www.glucogenosis.org]. The presence of McArdle disease was previously ascertained by genetic diagnosis, as per international recommendations (3). In addition, patients had to be familiarized with exercise testing in our laboratory. This included having done one or more sessions of cycle-ergometer exercise testing at a constant submaximal workload to pass the 'second wind' (5) and having previously experienced the feeling of myalgia associated with heavy exertion. Finally, in order to ensure safety of maximal exercise testing, patients had to pertain to the two lowest classes (0 or 1) of clinical severity scale for McArdle disease — the 'Martinuzzi scale' (ranging from 0 [lowest] to 3 [highest]) (22); where: 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. An age- and sex-matched control group of healthy subjects (nonathletic, but familiarized with exercise testing in our laboratory) was recruited from university students and staff.

The study adhered to the ethics guidelines of the Declaration of Helsinki, and was approved by the relevant Ethics Committee (approval number 16/081). All participants were informed about the study procedures and signed a written informed consent.

Participants

Eight patients with McArdle disease and a group of seven age/sex-matched healthy controls (three female in each group) were studied. The presence of McArdle disease was confirmed by genetic diagnosis in one of the three ‘reference’ centers in Spain for genetic analysis of this condition (Hospital 12 de Octubre, Madrid; Hospital Vall d'Hebron, Barcelona; or Hospital Meixoeiro, Vigo). This implied identification of a documented pathogenic mutation causing McArdle disease in both *PYGM* alleles (either the same mutation in homozygosis or two different mutations in heterozygosis), as per international recommendations (3). Thus, mutant *PYGM* alleles were identified in blood samples using SNaPShot minisequencing (Thermo Fisher; Waltham, MA) (23), followed by Sanger sequencing of the entire coding region and intron/exon boundaries (24). Alternatively, a next-generation sequencing-customized gene panel on a PGM-IonTorrent platform (Thermo Fisher), consisting of 35 genes (including *PYGM*) associated with metabolic myopathies, was used.

There were no between-group (Mann-Whitney *U* test) differences in age (patients: 35 ± 9 years; controls: 34 ± 9 years, p=0.602), body mass index (22.5 ± 3.0 and 23.3 ± 1.3 kg/m^2, *p*=0.729), or body surface area (1.85 ± 0.15 and 1.85 ± 0.16 m^2, *p*=0.953). The individual demographic data of the participants, together with patients' *PYGM* genotypes, are shown in Table 1. Each of the patients' *PYGM* mutations is pathogenic and has been previously reported in the last update of the Spanish registry of patients with McArdle disease (25). Likewise, each of these *PYGM* genotypes is known to result in complete deficiency of GP-MM activity (26-28).

Exercise tests

After an overnight fast, participants consumed a standardized, caffeine-free, low-carbohydrate breakfast [~300 kcal; 11 g carbohydrate, 22 g fat, 17 g protein, and 4 g fiber (*i.e.*, 30 g York ham, 125 g low-fat yogurt, 25 g almonds, and 1 egg)] 2.5 hours before the exercise protocol. In addition to this low-carbohydrate breakfast, participants did not take any carbohydrate drink prior to exercise as this strategy attenuates the second wind (29) and one of our main study goals was to study potential cardiac dysfunction associated with this phenomenon. They were instructed to refrain from any type of strenuous exercise or physical activity in the 48 hours prior to assessment, which started with cardiac evaluation at baseline (see section below).

All the exercise tests were done in an electrically-braked cycle-ergometer (800S, Ergoline; Bitz, Germany) with a pedal cadence of 60-80 rpm. Participants first performed a 15-minute constant-load moderate-intensity test to induce the second wind in the patients, as recently described by our group (5). This test is considered to be of diagnostic value in McArdle disease (4), as stated in the recent international clinical guidelines for the management this condition (3). Thus, the workload was progressively increased until participants reached 60% of their estimated maximum HR (220 *minus* age in years) in the first three minutes of the test, and this workload was maintained for a total of 15 minutes. Immediately after the constant test, they performed a ramp test until exhaustion. The ramp test started with the workload applied in the constant test, which was subsequently increased by 5 watts every 30 seconds until volitional exhaustion or when one of the following two criteria was met: contracture or inability to keep a pedal cadence ≥ 60 rpm (30).

Gas exchange data were recorded during exercise (CPX Ultima, Medical Graphics Corporation; St Paul, NM) to determine the peak oxygen uptake ($\dot{V}O_{2peak}$, *i.e.*, highest 20-second average $\dot{V}O_2$ value recorded during the ramp tests) (20). The workload eliciting the ventilatory threshold (VT) was visually identified by two independent investigators (or by a third one in case of disagreement) as the workload eliciting an increase in the 20-second average value of the ventilatory equivalent for oxygen, with no concomitant increase in the ventilatory equivalent for carbon dioxide (20).

Blood lactate (Lactate Plus, Nova Biomedical; Waltham, MA), glucose (FreeStyle Freedom Lite, Abbott; Chicago, IL) and ketone levels (GKI, Keto-Mojo; Emeryville, CA) were assessed in capillary blood samples obtained from fingertips at baseline and immediately after the ramp test.

The rating of perceived exertion (RPE, where 0=‘rest’, 1=‘very, very easy’, 2=‘easy’, 3=‘moderate’, 4=‘somewhat hard’, 5-6=‘hard’, 7-9=‘very hard’, and 10=‘maximal’) and the rating of pain perception [RPP, from ‘no pain’ (=0) to ‘worst possible pain’ (=10) and with

5=‘moderate pain’] were determined on a numerical rate at the seventh minute of the constant-load test (which is the time point that usually corresponds to maximum pain in patients with McArdle disease, after which the second wind starts), as well as at the end of the ramp test. The use of the aforementioned RPP and RPE scales has been recommended for monitoring exercise intensity in the clinical guidelines (see Supplemental Digital Content, http://links.lww.com/MSS/D73) for these patients (3) and has been previously reported by our group (30).

Cardiac assessments

Subjects rested for 15 minutes in the supine position prior to the following assessment: 12-lead ECG (CPX Ultima, Medical Graphics Corporation; St Paul, NM) (which was also recorded continuously during exercise) and both transthoracic two-dimensional conventional and two-dimensional speckle tracking echocardiography, with the latter allowing to determine left ventricle (LV) global longitudinal strain (GLS). Additionally, GLS was also determined at the following three time points in all participants (while sitting in the bicycle-ergometer): at the third (*i.e.*, upon the start of undue tachycardia in patients, before the occurrence of the second wind) and seventh minute of the constant-load test (*i.e.*, start of the second wind in patients) and upon maximal exertion (immediately after the end of the ramp test).

Transthoracic two-dimensional echocardiography measures at baseline. All dimensions were measured in the left-lateral supine position following the recommendations of the American Society of Echocardiography (31) using an EPIQ CVx system (Philips; Amsterdan, the Netherlands). All the echocardiographic measures corresponding to diastole and systole were obtained coinciding with the start of the QRS complex and with the maximal posterior displacement of the interventricular septum, respectively. Cardiac dimensions were obtained using the mean of three (or five, in case of doubt) cardiac cycles (31).

Transthoracic two-dimensional speckle tracking echocardiography measures (baseline and exercise). The GLS of the LV was determined with participants at rest in the supine position (at baseline) or when sitting on the cycle-ergometer (for the measurements corresponding to exercise and immediately thereafter). The AutoStrain feature within the Philips EPIQ CVx ultrasound system was employed for the off-cart assessment of strain in archived examinations. GLS values were derived through the analysis of multi-chamber perspectives, including two, three, and four-chamber views. The software seamlessly produced longitudinal strain curves, and the GLS was subsequently computed by averaging the values obtained across all myocardial segments. Additionally, a visual inspection of the AutoStrain analysis was conducted by a second cardiologist to confirm — or rectify, if necessary — the strain values. Of note, the results of GLS are herein reported without the negative sign for the sake of simplicity.

Prior to conducting the tests, the reliability of GLS measures during cycle-ergometer exercise was assessed in our laboratory by taking measurements (25 in total, five per each exercise intensity level) at different work intensities based on HR levels (*i.e.*, 112–118, 126–130, 140–149, 159–165, and 175–190 beats/min) in an adult healthy subject. The results demonstrated a high level of measurement reliability (Cronbach's alpha= 0.83, and coefficient of variation for the aforementioned HR levels of 11.4%, 0.6%, 4.4%, 2.8%, and 8.1%, respectively) (Supplemental Table 1, supplemental Digital Content, Reliability of left ventricular GLS measures obtained with transthoracic two-dimensional speckle tracking echocardiography during cycle-ergometer exercise in a healthy subject, http://links.lww.com/MSS/D73).

Ex vivo study in human hearts (without McArdle disease)

Right atrial appendage samples were obtained from four adults **(**two women) without known McArdle disease (and effectively discarded by the presence of *PYGM* expression, as shown below) who underwent cardiac surgery with extracorporeal circulation at the Vall d'Hebron University Hospital (Barcelona, Spain). The reason for surgery was valve replacement. None of these individuals had a clinical history of right heart failure with dilatation of right atrium or ventricle, and none had severe pulmonary hypertension. All were >18-year-old and signed a written informed consent from before surgery. Samples were immersed in cold sterile saline buffer and carried on ice to the laboratory, where they were stored at –80ºC. The study protocol for the collection, storage and use of cardiac biopsies for scientific purposes followed the Declaration of Helsinki and was approved by the Ethical Committee of the Vall d'Hebron University Hospital (updated PR(AG)136/2010).

Study in hearts from McArdle mice

All the mouse studies were performed at the Vall d'Hebron Institute of Research (VHIR, Barcelona, Spain). Previously developed p.R50*/p.R50* mice, back-crossed for 10 generations to C57/6 J background, were used in this study (21, 33). A group of 27 WT (p.R50R/p.R50R genotype, 18 female, average age 16.5±10.3 weeks) and 25 McArdle (p. R50*/ p.R50*, 12 female, 21.3 ± 12.4 weeks) mice were studied. Six heterozygous (p.R50R/p.R50*, three female, average age eight weeks) mice were also studied for analysis of the mRNA levels of the three GP isoforms. They were euthanized by cervical dislocation immediately before heart dissection. All experimental procedures were approved by the institutional review board (protocol number 58/17 CEEA; 35/04/08) and conducted in accordance with the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (ETS1 2 3) and Spanish laws (32/2007 and R.D. 1201/2005).

Histochemistry. Formalin fixed paraffin-embedded hearts from WT and McArdle mice were used. Three-µm cryosections were stained with hematoxylin and eosin (H&E) for general histopathological evaluation as previously described (21). Glycogen content was analyzed with Periodic Acid-Schiff (PAS) staining by sequentially incubating the sections with periodic acid (Fisher Scientific; Hampton, NH) (0.5%) for five minutes, water wash, Schiff ‘s solution (Merck-Millipore; Burlington, MA) for 15 minutes, water for one minute, alcohol-xylol dehydration, and dibutylphthalate polystyrene xylene (DPX) mounting (SigmaeAldrich; St. Louis, MO). Masson’s trichrome stainings were also performed (pathological anatomy department, VHIR), with all images obtained using the Pannoramic 250 Flash III (Sysmex; Kobe, Japan).

Immunofluorescence. To analyze the cytoskeleton structure [actin α cardiac muscle 1 (ACTC1), α and βII tubulin] as well as the mitochondrial subcellular distribution [voltage-dependent anion-selective channel 1 (VDAC1)] the mouse heart sections were stained with the following primary antibodies: ACTC1 (reference DF2269, Affinity Biosciences; Melbourne, Australia); α (reference GTX628802; GeneTex, Irvine, CA) and βII-tubulin (reference ab179512, Abcam, Cambridge, UK); desmin (reference ab32362, Abcam); and VADC1 (reference ab15895, Abcam; and reference MABN504, Sigma-Aldrich, Saint Louis, MO). After overnight incubation at 4ºC with primary antibodies, three five-minute washes in PBS were performed, and sections were then incubated during three hours at room temperature with the following secondary antibodies: goat anti-Rabbit IgG (H+L) Superclonal™ Secondary Antibody, Alexa Fluor® 488 conjugated and goat anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor® 594 conjugate (both from Thermo Scientific, Waltham, MA). Finally, sections were stained with 40,6-diamidino-2-phenylin- dole (DAPI) nucleic acid stain reactive (Invitrogen; Carlsbad, CA), for five minutes and mounted with ProLongTM Gold Antifade reagent (Molecular probes; Eugene, OR). Images were taken using a Zeiss LSM980 fully-spectral confocal microscope with AiryScan 2 confocal super-resolution detector (Zeiss Microscopy; Jena, Germany). Super-resolution images were analyzed with the Zeiss-ZEN software (Zeiss Microscopy).

Gene expression. Total RNA was obtained from the mouse hearts as previously described (26) following the manufacturer's instructions (TRIzol, Invitrogen). RNA was treated with DNase I, amplification grade (Invitrogen) to eliminate any DNA trace. Complementary DNA was synthesized from RNA using the high-capacity complementary DNA archive kit (Applied Biosystems; Foster City, CA), which uses random primers. We used real-time polymerase chain reaction (PCR), with TaqMan fluorogenic probes in a 7900 real-time PCR System (Applied Biosystems) to assess mRNA levels in heart samples of genes directly involved in the synthesis [glycogen synthase 1 (*Gys1*) and 1,4-alpha-glucan branching enzyme 1 (*Gbe1*)] or degradation [GP-MM (*Pygm*, Mm00478582_m1), GP-BB (*Pygb*, Mm00464080_m1), GP-LL (*Pygl*) and amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase (*Agl*)] of glycogen. Results were normalized to peptidylprolyl isomerase A (cyclophilin A, Ppia) gene messenger RNA levels and quantified using 7900 SDS v2.4.1 software (Applied Biosystems).

Western blots. Mouse heart samples were homogenized using Pellet pestles Cordless motor (Sigma-Aldrich) in cold homogenization radioimmunoprecipitation (RIPA) buffer and centrifuged (10 000 g) for 10 minutes at 4ºC. The samples were placed in boiling water for three minutes, before 20 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. Membranes were incubated in primary antibodies overnight at 4ºC (Supplemental Table 2, Supplemental Digital Content, List of antibodies used in Western blot analyses in mouse hearts, http://links.lww.com/MSS/D73) and in secondary antibodies for three hours at room temperature. Amido Black staining solution (Sigma-Aldrich) was used as a loading control for all the membranes. Membranes were developed with Immobilon Western Chemiluminiscent horseradish peroxidase (HRP) substrate (Merck-Millipore) and images obtained with a LiCor Odyssey® Fc Imaging System (LICOR Biosciences; Lincoln, NE) and quantified with Image Studio™ Lite software, version 5.2 (LICOR Biosciences). We analyzed the levels of proteins involved in glycogen synthesis (glycogen synthase and its phosphorylated form in Ser641), cytoskeleton structure (ACTC1, α-actinin, α-tubulin, and desmin), glycolysis [phosphofructokinase, muscle isoform (PFKM), pyruvate kinase M2 (PKM2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] as well as in mitochondrial and oxidative metabolism [VDAC1, translocase of outer membrane 20 kDa subunit (TOMM20), citrate synthase (CS), NADH:ubiquinone oxidoreductase subunit A9 (NDUAF9), succinate dehydrogenase complex flavoprotein subunit A (SDHA), ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), cytochrome oxidase subunit II (COXII), and perilipin (PLIN 5)].

Statistical analyses

Data are shown as mean ± SD. Differences between patients with McArdle disease and controls in those variables assessed at a single time point, as well as between WT and McArdle mice, were assessed with the Mann-Whitney *U* test, whereas a two-factor mixed (group, time) ANOVA with repeated measures on time was used for those variables (GLS, blood parameters) assessed at two or more time points in patients with McArdle disease and controls. To minimize the risk of statistical type I error, post hoc comparisons were only performed (using the Bonferroni test) when a significant group by time interaction effect was found. The Kruskal-Wallis test was used to determine: *(a)* whether an ‘isoform’ (muscle, brain, or liver) effect existed in GP expression levels in ‘normal’ (non-McArdle) human and in mouse hearts (in the latter, in both McArdle and WT animals, respectively), with eventual post hoc pairwise comparisons performed with the Wilcoxon test; and *(b)* whether a genotype effect (WT, heterozygous, McArdle) existed in the expression levels of the three GP isoforms in the mouse heart, with eventual post hoc unpaired genotype comparisons assessed with the Mann-Whitney *U* test. Given the low sample size, the statistical power was reported for all the results. Statistical analyses were conducted using statistical software package (*Stata Statistical Software: Release 14*. College Station, TX: StataCorp LLC) setting the significance level at 0.05.

RESULTS

Study in patients with McArdle disease

Exercise tests. The average power output and HR value in the submaximal constant tests [*i.e.*, from the third to the last (15th) minute] was significantly lower and higher, respectively, in patients than in controls (41 ± 13 *vs* 86 ± 37 watts, respectively, p=0.007, statistical power=86.4%; and 124 ± 1.5 *vs* 110 ± 1 beats/min, p<0.001, power=100%). The relative load of these tests for second wind determination corresponded to a significantly (p=0.003, power=100%) higher percentage of the VO_{2peak} reached by patients in the subsequent ramp test (75.8 ± 7.0 %) compared with controls (60.9 ± 2.1 %), yet with no between-group differences when expressed relative to the peak power output (PPO) reached in these tests (43.1 ± 5.5 and 42.4 ± 5.2 %, p=0.908, power=4.3%). On the other hand, RPE (6 ± 2) and RPP (6 ± 3) values in the patients at the seventh minute of the submaximal constant-load bout were indicative of ‘hard’ intensity and ‘moderate-to-high’ pain, respectively, and significantly higher compared with the controls [1 ± 1 (p=0.003, power=100%) and 0 ± 1 (p< 0.001, power=100%), respectively].

The second wind (*i.e.*, as determined by a decrease in HR values after previous excess tachycardia) started in patients at 7.3 ± 1.7 minutes (range 6 to 11) and was evident in each of them (*i.e.*, decrease by 35 ± 3 beats/min on average from the seventh to the last minute of the constant-load test) thereby corroborating that this phenomenon is a characteristic feature of McArdle disease with respect to nonpatients (individual and mean ± SD results shown in Figure 1). By contrast, HR tended to show the typical mild, gradual increase (*i.e.*, ‘cardiac drift’) in controls.

Concerning the maximal ramp test — the gas exchange data from one control participant could not be analyzed due to technical errors, and therefore six participants in this group were analyzed for $\dot{V}O_{2peak}$ data — patients showed considerably lower values of $\dot{V}O_{2peak}$ (21.8 ± 6.5 *vs* 40.1 ± 10.2 mL/kg/min, respectively, *p*=0.001, power=98.3%), PPO (95 ± 24 *vs* 211 ± 58 watts, *p*<0.001, power=99.9%), and absolute workload at the VT (60 ± 20 *vs* 121 ± 42 watts, *p*=0.003, power=93.9%) than controls. By contrast, no significant differences were found for the relative workload at the VT (63.2 ± 15.6 vs 57.3 ± 9.4 % of PPO**,** *p*=0.400**,** power=14.4%) or for the peak values of HR (166 ± 23 *vs* 169 ± 27 beats/min, *p*=0.788, power=4.2%), RPE (8 ± 3 *vs* 8 ± 3, *p*=0.928, power=2.9%) or RPP (4 ± 5 *vs* 6 ± 4, *p*=0.358**,** power=13.6%) recorded at the end of the test, thereby indicating a comparable effort level at exhaustion in both groups.

Despite no significant time effect (*p*=0.526, statistical power=9.3%), a significant group (*p*=0.017, power=71.4%) and group by time interaction effect (*p*=0.018, power=70.9%) was found for blood glucose, with post hoc analyses showing significantly lower values in patients at post-exercise (*p*=0.007, power=84%), but no between-group differences at baseline (*p*=0.436, power=11.6%), and thus a similar availability of this substrate for the skeletal or myocardial tissue prior to exertion in the two groups) (Figure 2). A significant time (*p*<0.001, power=100%), group (*p*<0.001, power=100%) and group by time interaction effect (*p*<0.001, power=100%) was found for blood lactate levels, with post hoc analyses showing the expected significant differences at post-exercise (*i.e.*, much lower values in patients, *p*<0.001, power=100%) but not before exercise (*p*=0.248, power=20.2%). No significant time (*p*=0.356, power=14.4%), group (*p*=0.822, power=5.5%), or group by time interaction effect (*p*=0.356, power=14.4%) was found for blood ketones.

Cardiac assessments. No ECG abnormality (*i.e.*, no sign of arrhythmia or ischemia) was found at rest or during/after exercise in patients or controls. The echocardiographic evaluations at baseline (*i.e.*, lying supine under resting conditions) showed no significant between-group differences in cardiac dimensions, except for a lower absolute (*p*=0.020) — but not body surface area-indexed (*p*=0.093) — left-ventricular mass in patients than in controls (individual and mean ± SD results shown in Table 2).

No significant group [*p*=0.215, statistical power =22.7%) or time effect (*p*=0.074, power =58.2%) was found for GLS, but a significant group by time interaction effect was found (*p*=0.026; power=72.9%) (individual and mean ± SD results shown in Figure 3). Post hoc analyses showed a lower GLS (*p*=0.002, power=94%) in patients compared controls in the measurement corresponding to the second wind phenomenon.

Ex vivo study in human hearts (without McArdle disease)

In adult human heart biopsies, *PYGB* mRNA was clearly the most abundantly expressed GP isoform (accounting for ~78% of total GP mRNA) compared to both *PYGM* and *PYGL* (~11% each) [*p*=0.013 (power=9.9%) for GP isoform effect with the Kruskal-Wallis test,; *p*=0.021 for the post hoc comparison of *PYGM vs* both *PYGB* and *PYGL* (power=91.8% and 89.4%, respectively)] (Figure 4).

Study in hearts from McArdle mice

Histopathology. Using H&E staining, no significant histological differences were observed between the heart from WT and McArdle mice (Figure 5A, *p*=0.278, power=76.1%). Glycogen content analysis using PAS staining (Figure 5B; *p*=0.280, power=82.6%) and biochemical quantification (Figure 5C; *p*=0.833, power=7.9%) showed no differences between both genotypes. Additionally, Masson trichrome staining demonstrated no between-group differences in collagen type I staining, thereby not supporting the existence of more cardiac fibrosis in the McArdle mouse heart (Supplemental Figure 1, Supplemental Digital Content, Masson trichrome staining of myocardial collagen type I by group or wild-type; *p*=0.343, power=52.0%, http://links.lww.com/MSS/D73).

Immunofluorescence. No between-group differences were found in cytoskeleton structure (ACTC1, α and β-II-tubulin, desmin) or in mitochondrial morphology, network, or distribution (VDAC1) (Supplemental Figure 2, Supplemental Digital Content, Immunofluorescence results of myocardial cytoskeleton structure as well as in mitochondrial morphology, network and distribution by group or wild-type, http://links.lww.com/MSS/D73).

Gene expression studies. As opposed to the adult human heart, *Pygm* appeared to most abundantly expressed GP isoform in WT mice, with a lower expression of *Pygb* and *Pygl* (especially the latter) (Figure 6A). Nonetheless, no significant genotype (McArdle, WT or heterozygosity) effect was found for *Pygb* (Kruskal Wallis *p*-value=0.412, power=42.8%) or *Pygl* (*p*=0.462, power=42.2%). By contrast, a significant genotype effect was found for *Pygm* (*p*<0.001, power=43.1%) with mRNA levels significantly lower in McArdle than in WT (-93%, *p*<0.001, power=99.0%) or heterozygous (-78%, *p*=0.002, power=70.0%) mice, and also lower in heterozygous mice compared with WT animals (-66%, *p*=0.002, power=99.0%). In turn, there was a quasi-significant trend towards a lower expression of *Gys1* in McArdle mice compared to the other two groups (*p*=0.057 for the *Gys1* genotype effect, power=43.0%), but no between-group differences were found for *Agl* (*p*=0.429, power=52.0%) or *Gbe1* (*p*=0.430, power=23.3%).

There were no between-group differences in the protein levels of GS and its phosphorylated form (Figure 6B), and overall no between-group differences for markers of cytoskeleton structure (Figure 6C), glycolysis (Figure 6D) or mitochondrial oxidative metabolism (Figure 6E), except for significantly lower levels of PKM2 (*p*=0.012, power=97.0%) and TOMM20 (*p*=0.021, power=96.8%) in the McArdle mice compared with the WT controls.

DISCUSSION

The main findings of our study were three-fold (as graphically summarized in Supplemental Figure 3, Supplemental Digital Content, Graphic summary of the main study findings, http://links.lww.com/MSS/D73). As for the study in patients and controls, although cardiac dimensions and function seem to be overall unaltered in patients with McArdle disease, a reduced GLS was observed during moderate, constant-load exercise (eliciting the ‘second wind’), which deserves attention. Second, the brain GP isoform, GP-BB, seems to be the main GP isoform in the heart of human adults without McArdle disease, at least at the mRNA level, which should overall compensate for GP-MM deficiency in the context of McArdle disease (of note, this finding is in contrast with the results in the mouse heart, where *Pygm* is the most expressed isoform). Third, the study in McArdle mice showed that myocardial expression of *Pygl*, and especially *Pygb*, would seem to overall compensate for eventual GP-MM deficiency in the context of McArdle disease, since no higher glycogen accumulation and no major histological or functional consequences were found in the hearts of McArdle mice compared to WT animals, and essentially no differences in key metabolic enzymes.

Study in patients with McArdle disease

An important finding of our study pertains to the results of GLS — or deformation of the LV, which is considered a good proxy for myocardial contractility (34, 35) — in patients with McArdle disease. While keeping in mind that it was not possible to match the relative load of the two groups (at least in terms of % of VO_{2peak}, albeit with no differences in % of PPO) during moderate, constant-load exercise, GLS tended to decrease in patients as opposed to controls, with between-group differences reaching statistical significance upon the onset of the second wind phenomenon. However, GLS in patients showed the normal, expected improvement during the maximal (ramp) exercise testing compared to baseline (*i.e.*, raise from ~17% on average at baseline to ~20% immediately post-exertion), a response that has been previously reported in healthy adults (36-39). By contrast, an inability to improve LV deformation (no change in GLS) during maximal exertion compared to baseline is considered a sign of reduced LV contractile reserve and, for instance, can allow to discriminate patients with unstable angina pectoris who also have significant coronary artery disease from those with normal myocardium and coronary arteries (40). Thus, LV contractile reserve seemed to be overall preserved during maximal exercise in patients with McArdle disease and any eventual impairment in cardiac contractility does not seem to be a major contributor for the very low maximal aerobic capacity of these patients (*i.e.*, their mean $\dot{V}O_{2peak}$ levels were 46% lower compared to controls)]. Peripheral (*i.e.*, mitochondrial network and OXPHOS alterations at the skeletal muscle tissue level, as discussed below) rather than central (cardiac) limitations would thus be the main contributors to the impairment in maximal aerobic capacity that characterizes McArdle disease. The impairment of skeletal muscle oxidative metabolism in these patients was detected by Grassi et al. through slower VO_2 kinetics during transitions to constant-load moderate-intensity cycle-ergometer exercise (41). This group also found an impaired peak capacity for oxygen extraction in the skeletal muscle of patients with McArdle disease (42). Besides, the ability of the skeletal muscle to produce pyruvate — a molecule that plays an anaplerotic role in the Krebs cycle — is severely reduced as a result of the inherited metabolic block (1) and the OXPHOS impairment in this tissue has been reflected on phosphorus magnetic resonance spectroscopy by remarkably greater phosphocreatine consumption and lower ATP concentrations compared to healthy controls after submaximal isometric calf contractions (43). The resultant marked decrease in skeletal muscle phosphorylation potential ([ATP][ADP][phosphate]) leads to the accumulation of phosphate, and probably also ADP, in patients' muscles, thereby inhibiting myofibrillar ATPase, calcium pump, and sodium-potassium pump reactions and leading to premature muscle fatigue and contractures (2, 44).

Although baseline (resting) mean GLS values in these patients (averaging ~17%) were within the normal range for healthy adults (15.8 to 23.4%) (45), they were lower than normal during the initial exercise phase preceding the second wind, characterized by undue tachycardia (*i.e.*, averaging 14.8±4.9% with two individual values barely reaching 10%, albeit with no significant difference *vs* controls). Moreover, after the peak tachycardia (*i.e.*, minute ~7 of the test, but with HR values still higher than in controls) patients showed a significantly impaired GLS (13% on average) compared to controls (19%). This finding is, at least apparently, counterintuitive given that catecholamine surges stimulate the coupling of myocardial β1 and β2-

adrenergic receptors with intracellular stimulatory G protein (Gs), which increases cyclic adenosine monophosphate (cAMP), thereby leading to subsequent stimulation of several downstream intracellular targets resulting in an increased contractile response (*i.e.*, inotropic effect) (46). Excess levels of adrenaline, however, could also exert a negative inotropic effect — notably in the so-called 'Stress (or Takotsubo) cardiomyopathy' — by switching β2-adrenoceptor coupling in ventricular cardiomyocytes from the Gs to the Gi protein signaling via the β2-adrenoceptor, which protects the myocardium against the proapoptotic effects of intense activation of β1-adrenoceptors at the expense of an acute, transient apical ventricular dysfunction (46). In any case, GLS after maximal exertion did not differ between patients and controls despite the lower availability of blood lactate in the former, with circulating lactate potentially contributing up to 15% of ATP production in this tissue (14, 19).

Ex vivo study in human hearts

The brain GP isoform, GP-BB, seems to be the main GP isoform in the human heart, which is also able to express GP-LL. This should therefore compensate for GP-MM deficiency in the context of McArdle disease. These findings, together with the rest of results, would confirm the notion that, at the clinical level, McArdle disease is overall a 'pure' myopathy (1). It would thus seem that previous reports of cardiac alterations associated with this condition (8-12) were not directly caused by GP-MM deficiency per se. This is in contrast with several other GSDs. For instance *Gys1*$^{-/-}$ mice (GSD 0), which lack the *Gys1* gene-encoded muscle glycogen synthase (leading to undetectable glycogen depots in cardiac muscle) show defects in heart development and function (47). The classical infantile-onset form GSD II (Pompe disease, caused by deficiency or absence of acid alpha-glucosidase and subsequent progressive expansion of glycogen-filled lysosomes in cardiac and skeletal muscle) manifests early in life with progressive and severe hypertrophic cardiomyopathy (48-50) and mouse models of GSD-II show cardiomegaly and ECG alterations (51) or hypertrophic cardiomyopathy (52). Increased LV mass and wall thickness is prevalent in patients with GSD IIIa (a condition caused by deficiency of the glycogen debranching enzyme in hepatic, cardiac and skeletal muscle tissue) (53), eventually leading to severe LV hypertrophy (54), although ventricular systolic function can be preserved (53). Cardiomyopathy can also be found in patients with GSD IV, a rare autosomal recessive disorder caused by deficiency of the glycogen branching enzyme that leads to the accumulation of amylopectin-like structures in affected tissues (55).

Study in mice

Our findings suggest that eventual GP-MM deficiency in the heart of McArdle mice is not necessarily accompanied by a ‘compensatory’ increase in the expression of the two other GP isoform genes. However, we found normal glycogen depots in the McArdle mouse myocardium and subsequently no overall histological or biochemical alterations at the cytoskeleton, metabolic and mitochondrial level in this tissue. This is in contrast with our recent findings on the skeletal muscle of these mice, a tissue unable to express *Pygb* or *Pygl* and which, likely as a result of massive glycogen deposits, shows major disruption of the three main cytoskeleton components (actin microfilaments, microtubules, and intermediate filaments) (56). This in turn might contribute to mitochondrial network disruption in skeletal muscle fibers, together with an altered subcellular localization of mitochondrial fission/fusion proteins and of the sarcoplasmic reticulum protein calsequestrin, with subsequent alteration in mitochondrial dynamics/function, impairment in mitochondrial content/biogenesis and of several oxidative phosphorylation

(OXPHOS)-related complex proteins/activities (56). Nevertheless, significantly lower levels of PKM2 and TOMM20 were found in the myocardium of McArdle mice compared with the WT controls, which suggests that a certain (mild) degree of cardiac function impairment cannot be totally ruled out in the former, at least at the molecular level. On the one hand, as opposed to the adult (PKM1) isoform, the fetal isoform PKM2 reduces pyruvate kinase activity and promotes the alternate anabolic glycolytic route, the pentose phosphate pathway, which prevents oxidative stress while also promoting cardiac regeneration (57). For instance, a shift from the 'catabolic' M1 (involved in glycolytic-derived energy generation) to the 'anabolic' M2 isoform of pyruvate kinase is found in the context of cardiac failure (58). On the other hand, TOMM20, a translocase of the outer mitochondrial membrane, plays an important role in enabling protein-transporting ATPase activity and unfolded protein binding activity, with its expression levels reduced in ischemic or dilated cardiomyopathy (59). However, whether any eventual cardiac function impairment in McArdle mice is due to the disease itself or to the chronically lower spontaneous activity levels of these animals as a result of their documented severe exercise intolerance — with even partial GP-MM deficiency (*i.e.*, in heterozygous mice) affecting exercise capacity (33) — remains to be determined.

Limitations

A major limitation of our study is the low sample size, especially in the human study, which we think is justifiable given the very low prevalence of McArdle disease. Additionally, we assessed patients previously familiarized with exercise testing in our laboratory, who tend to be fitter and less affected (*i.e.*, belonging to severity classes 0 and 1) than those in higher severity classes (2 and 3). It therefore remains to be determined whether cardiac function is preserved irrespective of clinical severity. It would also be interesting to study the cardiac function of McArdle (versus WT) mice. Another limitation is that we assessed exertional GLS with the participants sitting in the bicycle-ergometer (instead of lying supine) and we did not study whether the GLS impairment associated with the second wind disappears (or not) upon the documented attenuation of this phenomenon with pre-exercise carbohydrate ingestion (29). Additionally, more refined imaging or molecular techniques should be used in future studies, such as cardiac magnetic resonance with late gadolinium enhancement (to fully discard the presence of actual structural myocardial damage in patients with McArdle disease) or single-cell omics approaches (*e.g.*, to discern whether there are distinct expression patterns of GP or other molecules in cardiomyocytes versus electrical conducting cells). On the other hand, we believe there are major strengths in our work, including the integration of preclinical and clinical studies as well as the collection of human heart biopsies in vivo, or the use of different methodological approaches (from molecular to physiology or state-of-art cardiology techniques). We also used a mouse model that is known to mimic quite closely the clinical manifestations of McArdle disease (21).

CONCLUSIONS AND CLINICAL PERSPECTIVES

Barring some transient degree of cardiac impairment around the second wind (which deserves further consideration), our study provides preliminary evidence that cardiac function seems to be overall preserved in patients with McArdle disease at baseline and in response to maximal exertion. On the other hand, the normal human adult heart expresses GP-MM but also GP-LL and especially GP-BB (which should compensate, at least partly, for an eventual GP-MM deficiency in the context of McArdle disease), and no major histochemical/molecular alterations were found in the heart of the mouse model for this condition.

Routine cardiac examination (including echocardiography) every 5 or 10 years might be useful in patients with McArdle disease as a preventive means. Furthermore, future research is needed to determine whether there are more severe cardiac alterations associated with McArdle disease later in life (*i.e.*, by comparing older patients with age-matched controls). The question posed here should also be addressed at the epidemiological level (for instance, in the European Registry of patients with McArdle disease (60).

Acknowledgements

Data will be made available upon request to the corresponding author. Research by PLV is supported by a Sara Borrell postdoctoral contract granted by Instituto de Salud Carlos III (CD21/00138). TP is funded by the Spanish Ministry of Economy and Competitiveness and Fondos Feder (grant PI22/00201). AL and DBG are funded by the Spanish Ministry of Economy and Competitiveness and Fondos Feder (PI15/00558, and PI18/00139, respectively). The authors sincerely thank all the participants. The results of the study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation. The results of the present study do not constitute endorsement by the American College of Sports Medicine. All authors contributed substantially to either the conception or design of the work or the acquisition or analysis or interpretation of data for the work. All authors approved the final version of the manuscript and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy and integrity of any part of the work are appropriately investigated and resolved. All persons listed as authors qualify for authorship, and all those who qualify for authorship are listed.

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FIGURE LEGENDS

Figure 1. Heart rate response during the constant-load bout in patients with McArdle disease and controls (individual and mean ± SD values are shown for each group and minute). Of note, for the sake of clarity heart rate values are shown from the start of the third minute, which is where the load was kept constant after gradual increases in the first three minutes.

Figure 2. Results (mean ± SD) of blood variables at baseline (before the start of the constant-load test) and upon exertion (immediately after the maximal ramp test) in patients with McArdle disease and controls. Symbols: ** *p*=0.007 (power=84%) for patients *vs* controls upon exertion; *** *p*<0.001 (power=100%) for patients *vs* controls upon exertion.

Figure 3. Results (individual and mean ± SD values) of left ventricular global longitudinal strain (GLS) in patients with McArdle disease and controls. Assessments were performed using trans-thoracic two-dimensional speckle tracking echocardiography in the supine position (baseline, resting conditions) or with participants sitting in the cycle-ergometer [during the moderate-intensity constant-load test (minutes 3 and 7), and upon maximal exertion (i.e., immediately after the end of the ramp test)]. Of note, GLS values are shown as positive (instead of negative) values for simplicity purposes. Symbol: * p=0.002 for the between-group comparison at the relevant time point.

Figure 4. Results (individual and mean ± SD values) of mRNA expression levels of the muscle (*PYGM*), brain (*PYGB*), and liver (*PYGL*) isoforms of glycogen phosphorylase in human heart

(right atrium) biopsies from individuals without McArdle disease. Symbol: * *p*=0.021 (power=91.8% and 89.4%, respectively) for *PYGM vs* both *PYGB* and *PYGL* [the *p*-value for *PYGB vs PYGL* was 0.248 (power=40.9%)].

Figure 5. Histological analysis by group [McArdle (McA, p.R50* / p.R50*) or wild-type (WT, p.R50R / p.R50R) mice] using hematoxylin-eosin staining **(A)** and myocardial glycogen as determined with PAS staining **(B)**, or through biochemical quantification (mean± SD and individual values also shown, for the comparison between groups) **(C)**. Detailed values in the quantification of the images: hematoxylin-eosin (40x), number of muscle fibers (mean ± SD) 98±31 (McArdle) *vs* 117±21 (WT) (*p*=0.278, power=76.1%) **(A)**; and PAS (40x), glycogen accumulation 61±8% (McArdle) *vs* 53±12% (WT) (*p*=0.280, power=82.6%) **(B)**. No statistical between-group difference was noted either for biochemical glycogen quantification (*p*=0.833, power=7.9%).

Figure 6. Results (individual and mean± SD values) by genotype [p.R50*/ p.R50* (McArdle, McA), p.R50R / p.R50* (heterozygous, HTZ), or p.R50R / p.R50R (wild-type, WT)] or group (McA, WT) of the following variables: expression levels of genes directly involved in the degradation [muscle (*Pygm*), brain (*Pygb*) and liver (*Pygl*) isoform of glycogen phosphorylase, and amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase (*Agl*)] or synthesis [glycogen synthase 1 (*Gys1*) and 1,4-alpha-glucan branching enzyme 1 (*Gbe1*)] of glycogen (**A**); protein levels of glycogen synthase (GS) and its phosphorylated form in Ser 641 (GSp, as well as GS/GSp ratio) (**B**); and protein levels of markers of cytoskeleton structure [actin α cardiac muscle 1 (ACTC1), α-actinin, α-tubulin and desmin] (**C**), glycolysis [phosphofructokinase, muscle isoform (PFKM),

pyruvate kinase M2 (PKM2), and glyceraldehyde 3-phosphate dehydrogenase (GAPDH)] (**D**) and mitochondrial oxidative metabolism [voltage-dependent anion-selective channel (VDAC1), translocase of outer membrane 20 kDa subunit (TOMM20), citrate synthase (CS), NADH:ubiquinone oxidoreductase subunit A9 (NDUAF9), succinate dehydrogenase complex flavoprotein subunit A (SDHA), ubiquinol-cytochrome c reductase core protein 2 (UQCRC2), cytochrome oxidase subunit II (COXII) and perilipin (PLIN 5)] (**E**). Symbols: † p<0.001 for *Pygm* genotype effect [Kruskal Wallis *p*-value <0.001, power=43.1%; with post hoc comparisons showing significantly lower mRNA levels in McArdle than in WT (*p*<0.001, power=99.0%) or heterozygous (*p*=0.002, power=70.0%) mice, and also lower in HTZ mice compared with WT animals (*p*=0.002, power=99.0%); * *p*=0.021 (power=96.8%) for the between-group comparison of TOMM20; ** *p*=0.012, (power=97.0%) for the between-group comparison of PKM2. The following non-significant *p*-values we found for genotype effect: *Pygb*, *p*=0.462 (power=42.8%); *Pygl*, *p*=0.905 (power=42.2%); and *Gys1*, *p*=0.057 (power=43.0%). The following non-significant *p*-values we found for between-group (McA *vs* WT) comparisons: *Agl*, *p*=0.429 (power=52.0%); *Gbe1*, *p*=0.430 (power=23.3%); GS, *p*=0.327 (power=92.3%); GSp, *p*=0.841 (power=38.6%); pGS/GS ratio, *p*=0.772 (power=4.2%); ACTC1, *p*=0.211 (power=98.2%); α-actinin, *p*=1.000 (power=7.0%); α-tubulin, *p*=0.060 (power=99.0%); desmin, *p*=0.116 (power=97.7%); PFKM, *p*=0.674 (power=11.9%); GAPDH, *p*=0.114 (power=4.9%); VDAC1, *p*=0.211 (power=26.8%); CS, *p*=0.529 (power=10.9%); NDUAF9, *p*=0.401 (power=38.6%); SDHA, *p*=0.834 (power=3.5%); UQCRC2, *p*=0.211 (power=92.6%); COXII, *p*=0.144 (power=13%); and PLIN 5, *p*=0.400 (power=77.3%).

SUPPLEMENTAL DIGITAL CONTENT

SDC 1: Supplemental Digital Content.docx

Figure 1

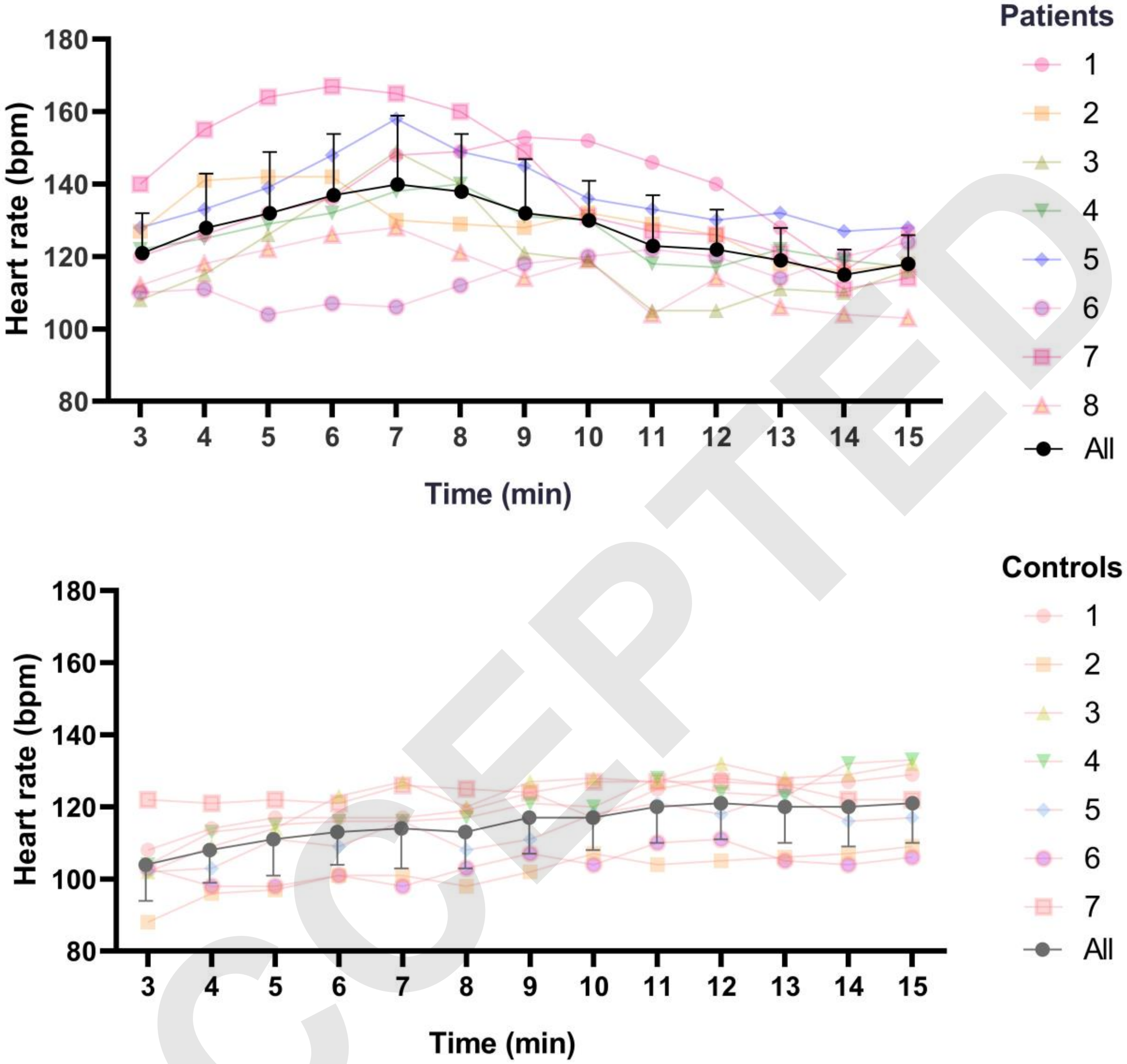

Patients
1
2
3
4
5
6
7
8
All
Heart rate (bpm)
180
160
140
120
100
80
3
4
5
6
7
8
9
10
11
12
13
14
15
Time (min)
Controls
1
2
3
4
5
6
7
All
Heart rate (bpm)
180
160
140
120
100
80
3
4
5
6
7
8
9
10
11
12
13
14
15
Time (min)

Figure 2

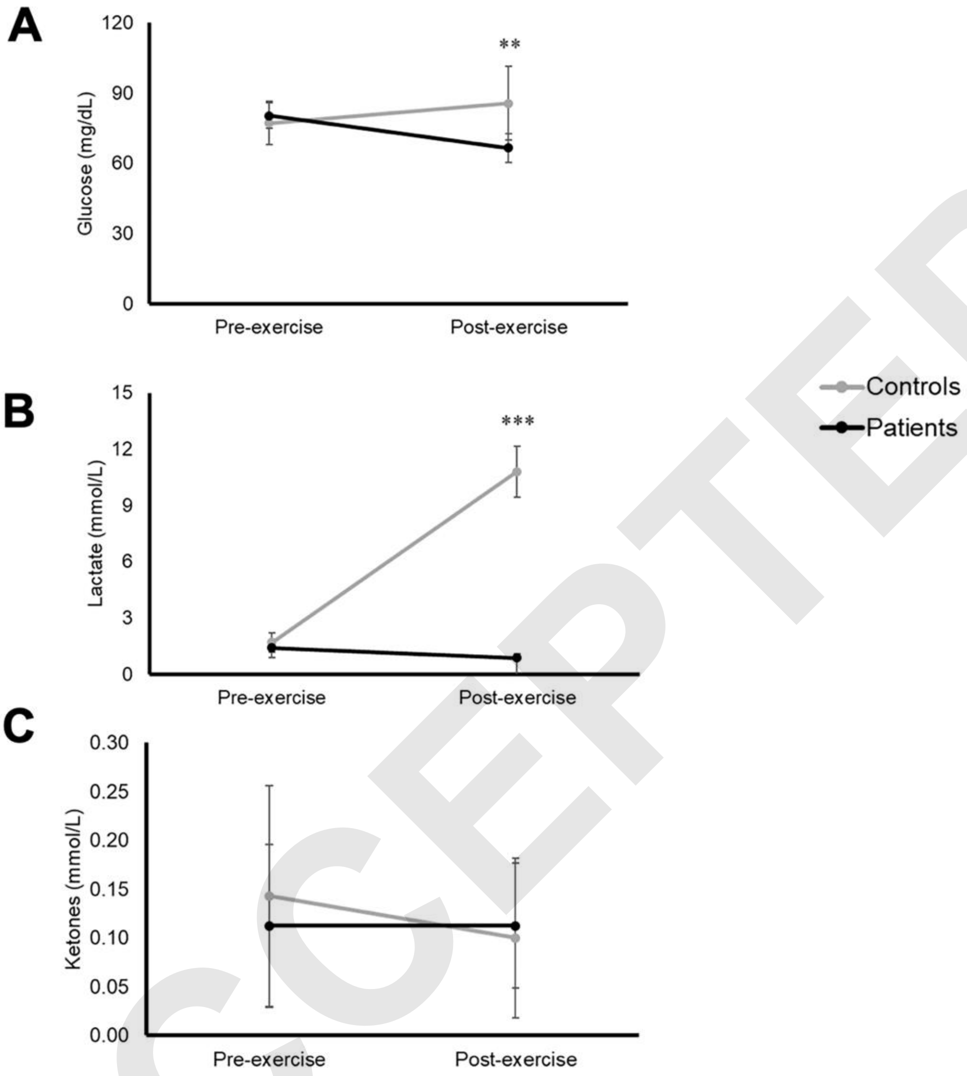
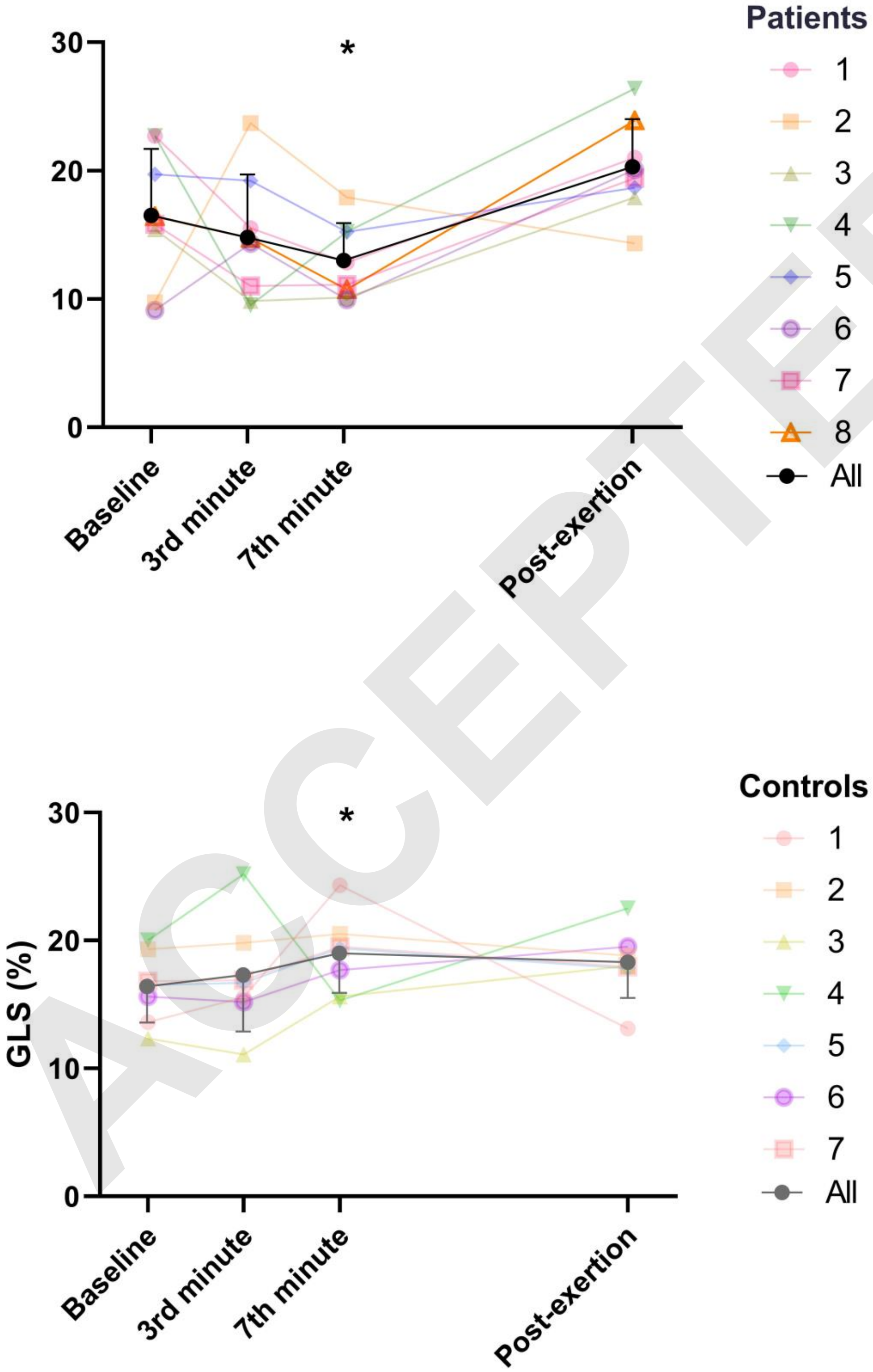

A
120
90
60
30
0
Glucose (mg/dL)
**
Pre-exercise
Post-exercise
B
15
12
9
6
3
0
Lactate (mmol/L)
***
Pre-exercise
Post-exercise
Controls
Patients
C
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Ketones (mmol/L)
Pre-exercise
Post-exercise

Figure 3



Patients
1
2
3
4
5
6
7
8
All
30
20
10
0
*
Baseline
3rd minute
7th minute
Post-exertion
Controls
1
2
3
4
5
6
7
All
GLS (%)
30
20
10
0
*
Baseline
3rd minute
7th minute
Post-exertion

Figure 4

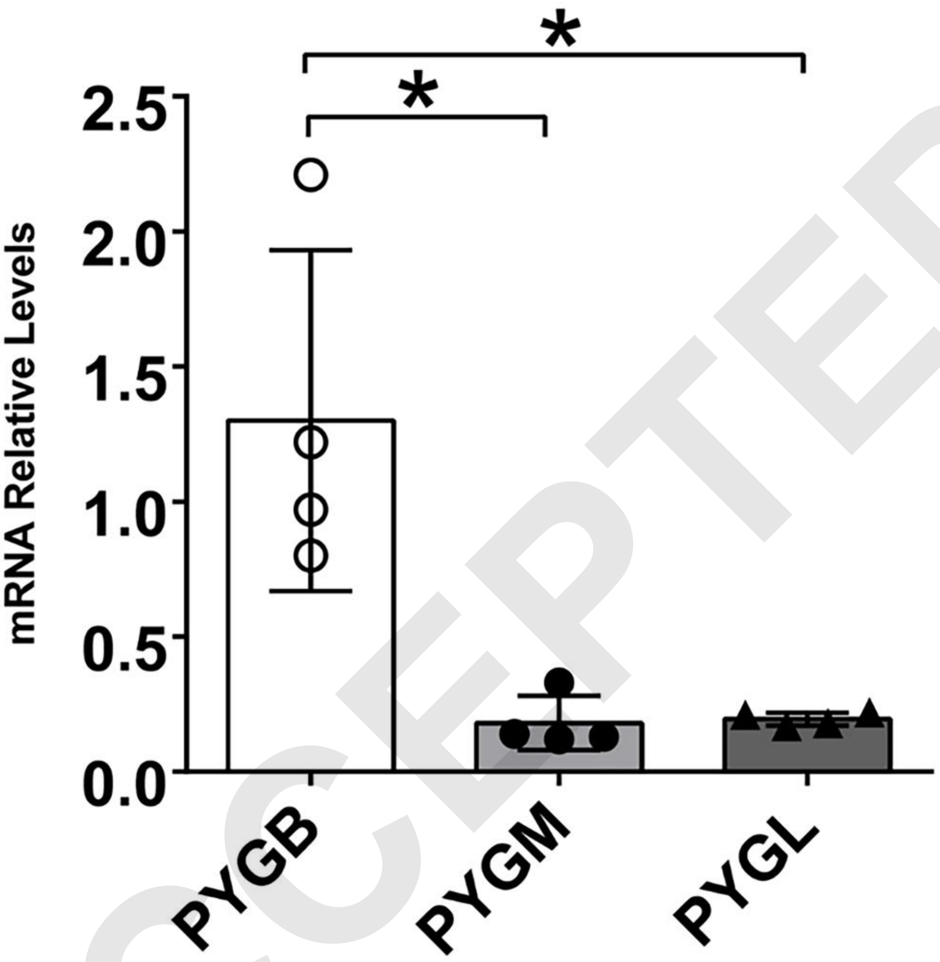


Figure 5

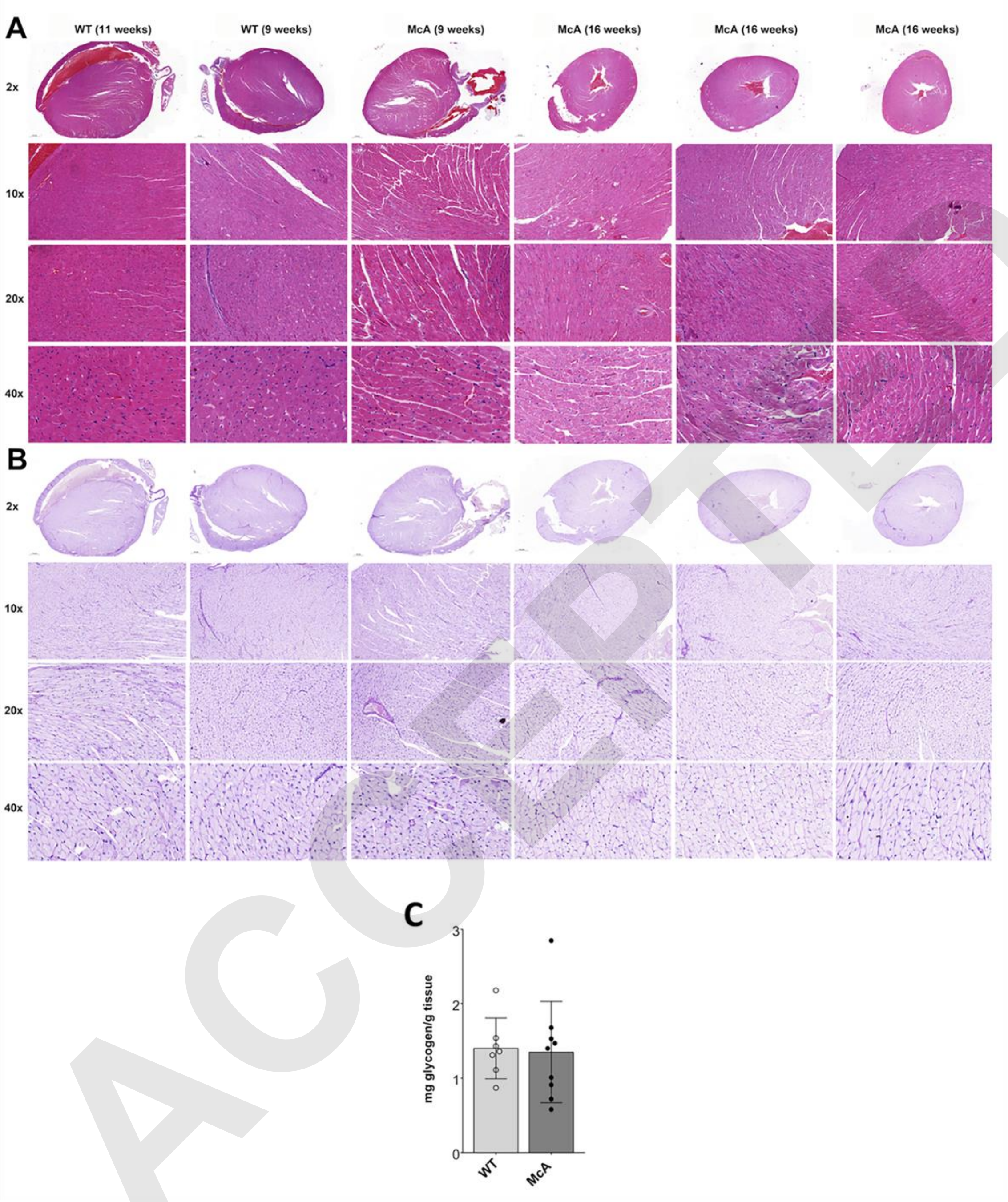
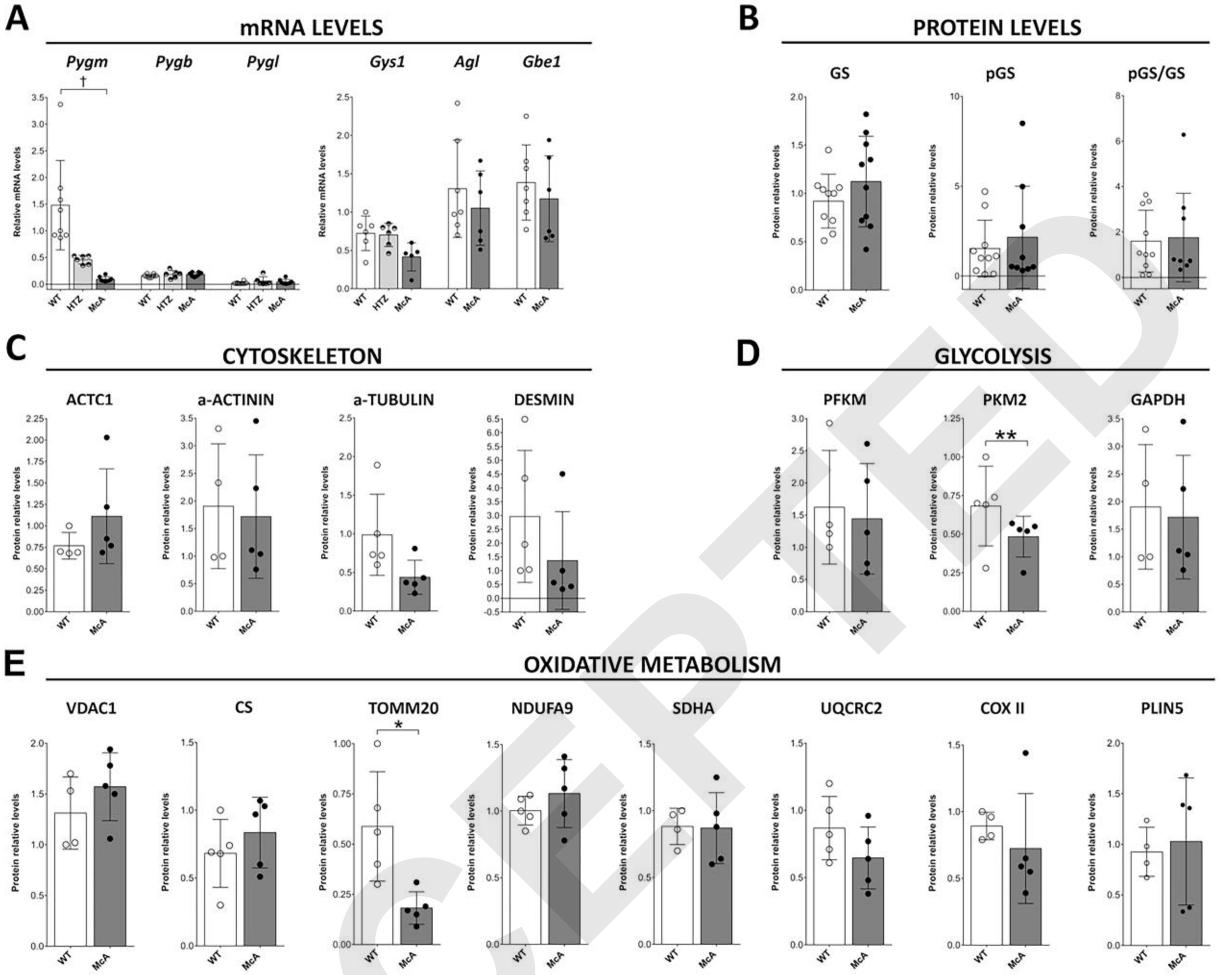

A
WT (11 weeks)
WT (9 weeks)
McA (9 weeks)
McA (16 weeks)
McA (16 weeks)
McA (16 weeks)
2x
10x
20x
40x
B
2x
10x
20x
40x
C
mg glycogen/g tissue
0
1
2
3
WT
McA

Figure 6



A
mRNA LEVELS
Pygm
Pygb
Pygl
Gys1
Agl
Gbe1
†
Relative mRNA levels
WT
HTZ
McA
B
PROTEIN LEVELS
GS
pGS
pGS/GS
Protein relative levels
C
CYTOSKELETON
ACTC1
a-ACTININ
a-TUBULIN
DESMIN
D
GLYCOLYSIS
PFKM
PKM2
**
GAPDH
E
OXIDATIVE METABOLISM
VDAC1
CS
TOMM20
*
NDUFA9
SDHA
UQCRC2
COX II
PLIN5

Table 1. Demographic characteristics by group

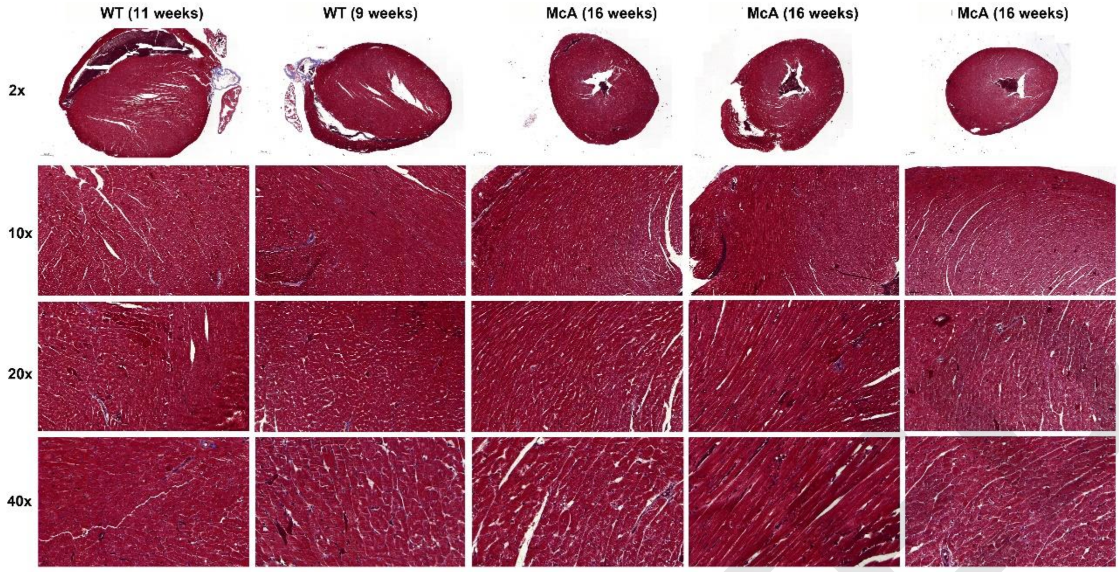
	Sex	**Age (years)**	**BMI (kg/m^2)**	**BSA (m^2)**	***Physically active (Yes/No)***	***PYGM* genotype**	
						Allele 1	***Allele 2***
Patients							
1	Female	35	20.0	1.84	Yes	p.R50* (c.148C > T)	p.W798R (c.2392 T > C)
2	Male	30	27.2	1.89	Yes	p.R50* (c.148C > T)	p.R50* (c.148C > T)
3	Female	41	18.2	2.16	Yes	p.R50* (c.148C > T)	p.A660D (c.1979C > A)
4	Male	46	25.5	1.82	No	p.R50* (c.148C > T)	p.R50* (c.148C > T)
5	Male	18	21.5	1.84	No	p.R50* (c.148C > T)	p.R50* (c.148C > T)
6	Female	45	20.3	1.81	Yes	p.R50* (c.148C > T)	p.W798R (c.2392 T > C)
7	Male	36	23.8	1.61	No	p.G205S (c.613G > A)	p.G205S (c.613G > A)
8	Male	32	23.7	1.84	Yes	p.R50* (c.148C > T)	p.R602W (c.1804C > T)
Mean ± SD		35 ± 9	22.5 ± 3.0	1.85 ± 0.15			
Controls							
1	Male	29	22.4	1.84	Yes	-	
2	Male	49	22.7	1.89	No	-	
3	Male	30	25.2	2.16	Yes	-	
4	Male	26	23.1	1.82	Yes	-	
5	Female	39	24.7	1.84	No	-	
6	Female	43	23.3	1.81	Yes	-	
7	Female	24	21.4	1.61	Yes	-	
Mean ± SD		34 ± 9	23.3 ± 1.3	1.85 ± 0.16			

Abbreviations: BMI, body surface are; BSA, body surface area (determined as detailed elsewhere (32)); *PYGM*, symbol for the gene encoding the skeletal muscle-specific isoform of glycogen phosphorylase. Participants were categorized as physically active or inactive during the prior 6 months as done recently by us in another cohort of patients with McArdle disease (5) [*i.e.*, active = reporting ≥ 150 min/week in moderate-intensity aerobic physical activity or ≥75 min/week in vigorous-intensity aerobic physical activity].

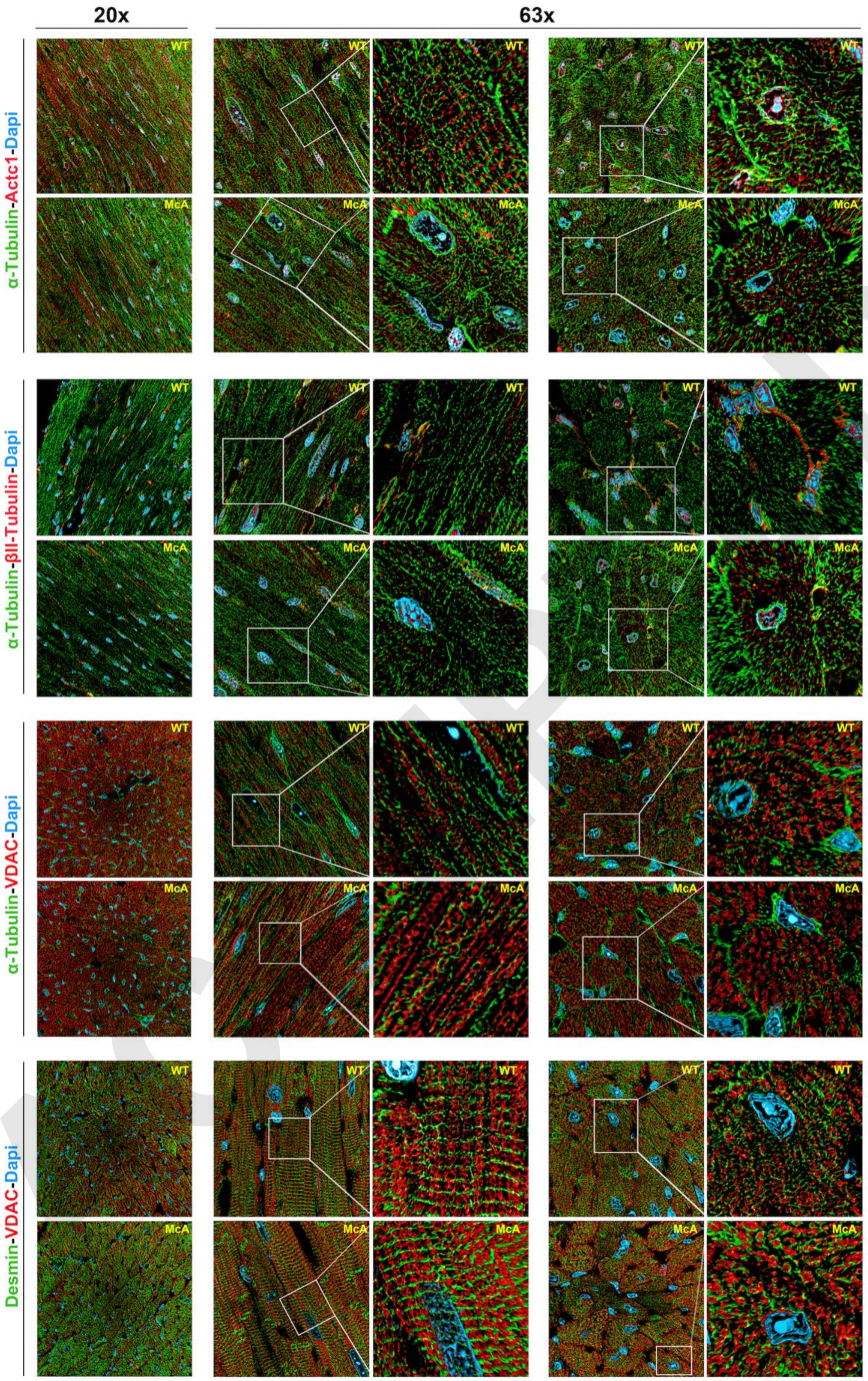
Table 2. Individual and mean ±SD results of cardiac measures (assessed with transthoracic two-dimensional echocardiography in the supine position) at rest by group.

	Sex	**RVEDV (mL)**	**RVEDV / BSA (mL/m^2)**	**RVEF (%)**	**LVEDV (mL)**	**LVEDV / BSA (mL/m^2)**	**LV mass (g)**	**LV mass / BSA (g/m^2)**	**LVEF (%)**
Patients with McArdle disease									
1	Female	91	54	62	100	59	97	58	65
2	Male	138	72	50	144	75	146	76	60
3	Female	129	81	44	135	84	136	85	71
4	Male	140	74	56	129	68	130	68	66
5	Male	115	64	52	125	70	127	71	66
6	Female	63	39	65	77	48	78	49	69
7	Male	113	58	55	111	57	110	56	73
8	Male	98	58	57	126	74	124	73	60
Mean ± SD		**111 ± 26**	**63 ± 13**	**55 ± 7**	**118 ± 22**	**67 ± 12**	**119 ± 22**	**67 ± 12**	**66 ± 5**
Controls									
1	Male	180	98	57	139	83	145	79	62
2	Male	126	67	51	118	63	135	71	60
3	Male	155	72	43	121	76	140	65	68
4	Male	192	105	52	120	63	165	91	71
5	Female	137	75	45	121	68	137	75	69
6	Female	96	53	53	125	78	135	75	61
7	Female	87	54	52	100	51	140	87	65
Mean ± SD		**139 ± 40**	**75 ± 20**	**50 ± 5**	**121 ± 11**	**69 ± 11**	**142 ± 11**	**78 ± 9**	**65 ± 4**
***P*-value between groups**		0.247	0.353	0.181	0.643	0.685	0.020	0.093	0.683
Statistical power		35.3%	27.3%	36.2%	5.0%	5.2%	71.9%	52.5%	6.3%

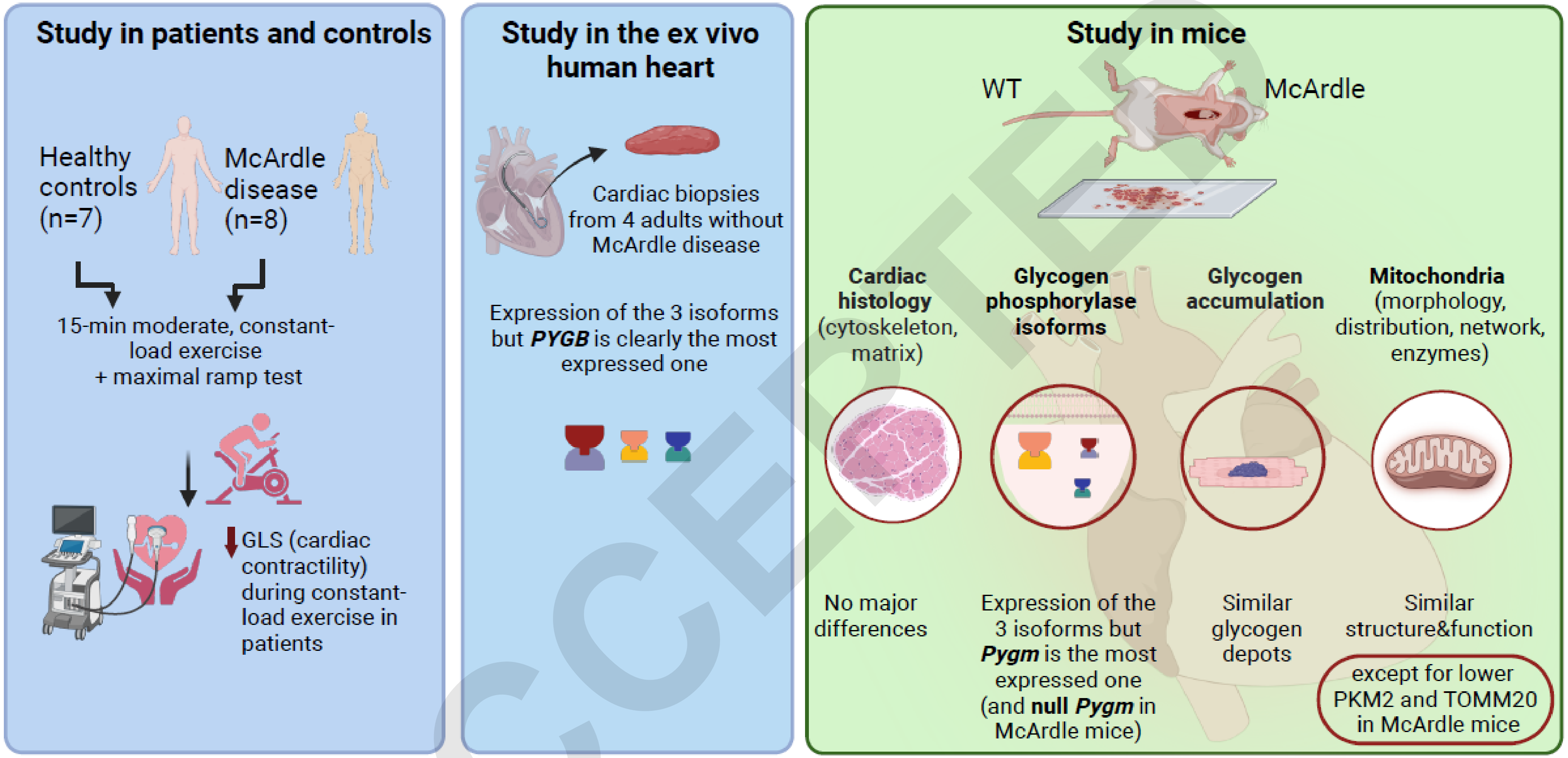
Abbreviations: Abbreviations: BSA, body surface area (determined as detailed elsewhere (32)); LV, left ventricle; LVEDV, left ventricle end-diastolic volume; LVEF, left ventricle ejection fraction; RVEDV, right ventricle end-diastolic volume; RVEF, right ventricle ejection fraction. *P-values* were determined with the nonparametric Mann-Whitney *U* test.



Supplemental Figure 1. Masson trichrome staining of myocardial collagen type I by group [McArdle (McA, p.R50* / p.R50*) or wild-type (WT, p.R50R / p.R50R) mice]. Detailed values in the quantification of the image: trichrome (20x), collagen fibers 12±5% (McArdle) *vs* 15±6% (WT) (*p*=0.434, power=52.0%).



Supplemental Figure 2. Immunofluorescence results of myocardial cytoskeleton structure [actin α cardiac muscle 1 (ACTC1), α and β II-tubulin, desmin] as well as in mitochondrial morphology, network and distribution (voltage-dependent anion-selective channel (VADC1)] by group [McArdle (McA, p.R50* / p.R50*) or wild-type (WT, p.R50R / p.R50R) mice]. Of note, due to the qualitative nature of immunofluorescence data, no p-values (and thus no statistical power) are provided.



Supplemental Figure 3. Graphic summary of the main study findings. Abbreviations: GLS, global longitudinal strain; PKM2, pyruvate kinase M2; *PYGB*, human gene encoding for the brain isoform of glycogen phosphorylase; *Pygm*, mouse gene encoding for the muscle isoform of glycogen phosphorylase (also known as 'myophosphorylase'); TOMM20, translocase of outer membrane 20 kDa subunit; WT, wild type.

Supplemental Table 1. Reliability of left ventricular global longitudinal strain (GLS) measures obtained with transthoracic two-dimensional speckle tracking echocardiography during cycle-ergometer exercise in a healthy subject (male, 42 years, very physically active).

Intensity	Measurement	Heart rate (bpm) ranges	Left-ventricle GLS	Coefficient of variation for each intensity
1	1	112-118	27.7	11.4%
1	2	112-118	27.1	
1	3	112-118	20.6	
1	4	112-118	26.3	
1	5	112-118	27.2	
2	1	126-130	27.1	0.6%
2	2	126-130	27.4	
2	3	126-130	27.5	
2	4	126-130	27.4	
2	5	126-130	27.2	
3	1	140-149	27.2	4.4%
3	2	140-149	25.2	
3	3	140-149	27.2	
3	4	140-149	25.3	
3	5	140-149	24.9	
4	1	159-165	26.2	2.8%
4	2	159-165	27.1	
4	3	159-165	28.1	
4	4	159-165	26.4	
4	5	159-165	27.3	
5	1	175-190	21.2	8.1%
5	2	175-190	20.3	
5	3	175-190	23.2	
5	4	175-190	22.3	

5	5	175-190	25.0	

Table footnote: GLS data are shown in positive values for the sake of simplicity.

Supplemental Table 2. List of antibodies used in Western blot analyses in mouse hearts.

ID	Reference	Dilution	Diluent	ID	Dilution	Diluent
Glycogen synthase	Cell Signaling/#3893	1/2000	TTBS 0.1% + 5% Skim milk	Goat to Rabbit HRP (DAKO P0448)	1/5000	TTBS 0.1% + 5% Skim milk
p-glycogen synthase Ser 640	GeneTex/GTX22479	1/2000	TTBS 0.1% + 5% Skim milk	Goat to Rabbit HRP (DAKO P0448)	1/5000	TTBS 0.1% + 5% Skim milk
a-actinin	Sigma/A7811	1/1000	TTBS 0.1% + 5% Skim milk	Rabbit to Mouse HRP (DAKO P0260)	1/5000	TTBS 0.1% + 5% Skim milk
a-actin (ACTC1)	Invitrogen/MA5-14084	1/1000	TTBS 0.1% + 5% Skim milk	Rabbit to Mouse HRP (DAKO P0260)	1/5000	TTBS 0.1% + 5% Skim milk
a-tubulin	GeneTex/GTX628802	1/2000	TTBS 0.1% + 5% Skim milk	Rabbit to Mouse HRP (DAKO P0260)	1/5000	TTBS 0.1% + 5% Skim milk
Desmin	Abcam/ab32362	1/750	TTBS 0.1% + 5% Skim milk	Goat to Rabbit HRP (DAKO P0448)	1/5000	TTBS 0.1% + 5% Skim milk
Pfkm	Abcam/ab154804	1/2000	TTBS 0.1% + 5% Skim milk	Goat to Rabbit HRP (DAKO P0448)	1/5000	TTBS 0.1% + 5% Skim milk
Pkm2	Abcam/ab137791	1/2000	TTBS 0.1% + 5% Skim milk	Goat to Rabbit HRP (DAKO P0448)	1/5000	TTBS 0.1% + 5% Skim milk
Gapdh	Origene/TA802519	1/10000	TTBS 0.1% + 5% Skim milk	Rabbit to Mouse HRP (DAKO P0260)	1/5000	TTBS 0.1% + 5% Skim milk
Vdac1	Abcam/ab15895	1/2000	TTBS 0.1% + 5% Skim milk	Goat to Rabbit HRP (DAKO P0448)	1/5000	TTBS 0.1% + 5% Skim milk
Cytrate synthase	Cell Signaling/#14309	1/2000	TTBS 0.1% + 5% Skim milk	Goat to Rabbit HRP (DAKO P0448)	1/5000	TTBS 0.1% + 5% Skim milk
TOMM20	Cell Signaling/#42406	1/750	TTBS 0.1% + 5% Skim milk	Goat to Rabbit HRP (DAKO P0448)	1/5000	TTBS 0.1% + 5% Skim milk
Ndufa9	Invitrogen/459100	1/1000	TTBS 0.1% + 5% Skim milk	Rabbit to Mouse HRP (DAKO P0260)	1/5000	TTBS 0.1% + 5% Skim milk
Sdha	Abcam/ab147115	1/1000	TTBS 0.1% + 5% Skim milk	Rabbit to Mouse HRP (DAKO P0260)	1/5000	TTBS 0.1% + 5% Skim milk
Uqcrc2	Molecular Probes/A11143	1/1000	TTBS 0.1% + 5% Skim milk	Rabbit to Mouse HRP (DAKO P0260)	1/5000	TTBS 0.1% + 5% Skim milk
CoxII	GeneTex/GTX33329	1/2000	TTBS 0.1% + 5% Skim milk	Goat to Rabbit HRP (DAKO P0448)	1/5000	TTBS 0.1% + 5% Skim milk
Plin 5	Thermo/PA1-46215	1/1000	TTBS 0.1% + 5% Skim milk	Goat to Rabbit HRP (DAKO P0448)	1/5000	TTBS 0.1% + 5% Skim milk