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2 **model throughout ageing**

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25 **Key words:** *ageing; exercise performance; mitochondrial creatine kinase; skeletal*

26 *muscle; efficiency*

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31 **Abstract**

32 **Purpose:** The objective of the present study was to establish the role of sarcomeric
33 mitochondrial creatine kinase (Mt-CK) in muscle energy output during exercise in a murine
34 model of ageing (the Mt-CK knock-out mouse, Mt-CK^{-/-}).

35 **Methods:** Three age groups of Mt-CK^{-/-} mice and control male mice (6, 9, and 18 months of age)
36 underwent incremental treadmill running tests. The maximum speed (V_{peak}) and maximal
37 oxygen consumption (VO_{2peak}) values were recorded. Urine samples were analyzed using
38 metabolomic techniques. The skeletal muscle (quadriceps) expression of proteins involved in
39 mitochondria biogenesis, peroxisome proliferator-activated receptor gamma coactivator 1-alpha
40 (PGC-1α) and dynamin-related GTPase mitofusin 2 (Mnf2) were quantified.

41 **Results:** The VO₂ peak (normalized to heart weight: HW) of 18-month-old (mo) Mt-CK^{-/-} mice
42 was 27% (p<0.001) lower than in 18-mo control mice. The VO_{2peak}/HW ratio was 29% (p<0.001)
43 lower in 18-mo Mt-CK^{-/-} mice than in 6-mo (p<0.001) and 32% (p<0.001) than 9-mo Mt-CK^{-/-}
44 mice. With a 0° slope, V_{peak} was 10% (p<0.05) lower in 18-mo Mt-CK^{-/-} mice than in 6-mo Mt-
45 CK^{-/-} mice but did not differ when comparing the 18-mo and 6-mo control groups. The skeletal
46 muscles weight normalized on body weight in 6-mo Mt-CK^{-/-} were 13 to 14% (p< 0.001, p<0.05)
47 lower versus the 6-mo control, in addition, the presence of branched-chain amino acids in the
48 urine of 6-mo Mt-CK^{-/-} mice suggests an imbalance in protein turnover (catabolism rather than
49 anabolism) but we did not observe any age-related differences. The expression of PGC-1α and
50 Mnf2 proteins in the quadriceps showed that age-related effects were more prominent than
51 genotype effects.

52 **Conclusion:** The present study showed ageing is potentialized by Mt-CK deficiency with regard to
53 VO_{2peak}, V_{peak} and mitochondrial protein expression. Our results support that Mt-CK^{-/-} mice
54 undergo physiological adaptations, enabling them to survive and to perform as well as wild-type

55 mice. Furthermore, it is possible that these adaptations in Mt-CK^{-/-} mice have a high energy cost
56 and might trigger premature ageing.

57 **Abbreviations:**

58 **AK:** Adenylate Kinase

59 **BCAAs:** Branched-chain amino acids

60 **BW:** Body Weight

61 **CK:** Creatine kinase

62 **CK^{-/-}:** Cytosolic creatine kinase, Mitochondrial creatine kinase knock out

63 **Cr:** Creatine

64 **CS:** Citrate synthase

65 **Drp-1:** Dynamin-related protein 1

66 **EDL:** Extensor digitorum longus

67 **HW:** Heart Weight

68 **KO:** Knock-out

69 **MM-CK:** Cytosolic creatine kinase

70 **MM-CK^{-/-}:** Cytosolic creatine kinase knock out

71 **Mt-CK:** Mitochondrial creatine kinase

72 **Mt-CK^{-/-}:** Mitochondrial creatine kinase knock out

73 **Mfn2:** mitochondrial membrane fusion factors dynamin-related GTPase mitofusin 2

74 **mo:** Month

75 **OPLS:** Orthogonal projection on latent structure

76 **Opa1:** Optic atrophy protein 1

77 **PCr:** Phosphocreatine

78 **PGC-1 α :** Peroxisome proliferator-activated receptor gamma coactivator 1-alpha

79 **TA:** Tibialis

80 **VO₂:** Oxygen uptake

81 **VO₂peak:** Maximal oxygen uptake

82 **Vpeak:** Maximal speed

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86 **1. Introduction**

87 Of the various mechanisms that contribute to metabolic homeostasis, energy transfer
88 pathways have important roles in reacting rapidly to changes in energy requirements and the
89 intensity of physical activity. In skeletal muscles and the heart, creatine kinase (CK) is required
90 during rapid energy transitions. A decrease in gene expression and enzymatic activity of the
91 sarcomeric mitochondrial creatine kinase (Mt-CK) and the cytosolic isoform (MM-CK) has been
92 observed in senescent skeletal or cardiac muscles in both humans and rodents (Bodyak et al.
93 2002; Kaczor et al. 2006; Nemetlu et al. 2015; Nuss et al. 2009; Tepp et al. 2016). This decline in
94 CK activity might be caused by oxidative stress (Nuss et al. 2009) and is one of the key factors in
95 the loss of muscle function with age (Kaczor et al. 2006; Nuss et al. 2009; Pastoris et al. 2000).

96 The phosphocreatine/creatine (PCr/Cr) shuttle contributes significantly to energy transfer
97 by regenerating PCr and increasing the availability of ADP for mitochondrial respiration (Miotto
98 and Holloway 2016; Tepp et al. 2016; Ydfors et al. 2016). The PCr and Cr diffuse around 2000
99 times faster than ADP and ATP through the mitochondrial outer membrane and the cytosol
100 (Kaldis et al. 1997), which allows efficient energy transfer - especially during intense physical
101 activity.

102 Among the different isoforms of CK, the sarcomeric Mt-CK is bound to the outer surface
103 of the inner mitochondrial membrane, so that the ATP generated by oxidative phosphorylation
104 can be transphosphorylated to PCr. The Mt-CK forms a complex with voltage-dependent anion
105 channels and adenine nucleotide translocase within the mitochondria membrane (Schlattner et
106 al. 2001; Schlattner et al. 1998)

107 Creatine kinase knock-out mouse models Mt-CK^{-/-}, MM-CK^{-/-} or both CK^{-/-} were created
108 by Wieringa's research group (Steeghs et al. 1998). This targeted mutagenesis made it possible
109 to study respective roles of the two isoforms in exercise adaptations in these mice (Momken et

110 al. 2005; van Deursen et al. 1993; Veksler et al. 1995). A few studies proposed that CK^{-/-} mice
111 have alternative mechanisms or cytoarchitectural rearrangements for maintaining efficient
112 energy transfer and signal transduction between ATP synthesis sites and ATPases (Kaasik et al.
113 2003; Novotova et al. 2006; Wallimann 2015). The muscles and hearts of MM-CK^{-/-} mice have
114 normal levels of PCr, ATP, and Cr because of the presence of Mt-CK in the interspace of
115 mitochondria membranes. However, the limb muscles are unable to contract efficiently at the
116 beginning of a stimulation period, and an electron microscopy analysis has shown a greater
117 number of mitochondria and a higher mitochondrial volume in skeletal muscle fast-twitch fibres
118 (Kaasik et al. 2003; Novotova et al. 2006). Furthermore, both MM-CK^{-/-} and CK^{-/-} mice showed
119 worse voluntary exercise performance, relative to wild-type mice (Lygate et al. 2009; Momken et
120 al. 2005). Muscles that can no longer function properly with regard to energy reserves and buffer
121 systems adapt (at least partly) by increasing their oxidative and glycolytic potentials and by
122 operating in a tense-flow mode (Veksler et al. 1995; Ventura-Clapier et al. 2004). For heart, it has
123 been proposed that the increase in cardiac work become more “energetically costly” when the
124 activity of the CK falls below a certain level (Saupe et al. 1998) and it seems that lack of Mt-CK
125 induces lower MM-CK activity in heart (Boehm et al. 1998).

126 Mt-CK^{-/-} mice have been less frequently studied than MM-CK^{-/-} and CK^{-/-} mice because
127 their phenotype is less abnormal. At the age of 3 months, Mt-CK^{-/-} mice and control mice shown
128 similar levels of performance in a moderate- and high-intensity incremental exercise test (Miotto
129 and Holloway 2016). However, Lygate and al. (2009) showed that older (7- to 8-months) Mt-CK^{-/-}
130 mice had a lower voluntary exercise capacity (Lygate et al. 2009); this could be related to the fact
131 that at 7-8 months PCr and ATP levels were reduced while ADP level was increased in the left
132 ventricle, despite normal cardiac phenotype and function parameters measured (Spindler et al.
133 2002), therefore that Mt-CK is necessary for normal metabolic homeostasis.

134 Hence, the objectives of the present work were to assess the performance of 6-, 9- and
135 18-months (mo) Mt-CK^{-/-} male mice in an incremental exercise test and to characterize the
136 associated age-related metabolic adaptations. Furthermore, we hypothesized that ageing
137 contribute to impairments in mitochondrial biogenesis and mitochondria dynamics. It is well
138 known that mitochondria are highly dynamic organelles and are constantly being remodelled by
139 biogenesis, fusion, and fission. Alterations in mitochondria dynamics contribute to impairment
140 energy generation and more recently, studies proposed that alteration of mitochondrial dynamic
141 factors could affect muscle atrophy (Romanello et al. 2010; Tezze et al. 2017). It appears that
142 mitochondrial dynamics change with age, even though causes, regulation mechanisms and
143 consequences of these processes have not been elucidated (Liu et al. 2020). Indeed, the MM-
144 CK^{-/-} mouse displays high levels of citrate synthase (CS) activity in glycolytic skeletal muscle and
145 higher mRNA expression levels of the mitochondrial fission factor dynamin-related protein 1
146 (Drp-1) (Vaarmann et al. 2008). We therefore investigated the mitochondrial factors involved in
147 mitochondrial biogenesis and dynamics in the skeletal muscle (quadriceps) of Mt-CK^{-/-} mice.

148 **2. Material and method**

149 **2.1 Animals**

150 The Mt-CK^{-/-} mice had a mixed C57Bl/6–129/Sv background and were produced from
151 heterozygous mice donated by Professor Stefan Neubauer laboratory's (Department of
152 Cardiovascular Medicine, University of Oxford, Oxford, UK). These mice were originally created in
153 Professor Bé Wieringa's laboratory (Nijmegen University, Nijmegen, The Netherlands) (Steeghs et
154 al. 1998). Breeding was monitored in an animal facility. The mice were produced by
155 heterozygous mating and the Mt-CK^{-/-} and Mt-CK^{+/+} offsprings were used for experimentation.
156 The mice were genotyped to confirm sarcomeric ablation of the Mt-CK isoenzyme, using the
157 protocol described by the originating laboratory (Nahrendorf et al. 2005). A total of 72 male mice

158 were obtained: 38 Mt-CK^{-/-} mice and 34 control mice Mt-CK^{+/+}. The two types were divided into
159 three age groups, referred to henceforth as 6-mo, 9-mo, and 18-mo. These mice were housed (in
160 subgroups of three or four per cage) in a specific and opportunistic pathogen-free environment
161 at a temperature of 22°C, with 12-hour light-dark cycles and a standard *ad libitum* diet.

162 All protocols were approved by our institution's Animal Care and Use Committee and
163 complied with the Council of Europe's convention on the protection of vertebrate animals used
164 for experimental and other scientific purposes.

165 **2.2 The incremental exercise test**

166 All groups underwent an incremental exercise test on a treadmill with a slope of 0° or 25°.
167 Before testing, all mice were familiarized with the one-lane treadmill equipped for gas exchange
168 measurements (Modular Enclosed Metabolic Treadmill for Mice, Columbus Instruments,
169 Columbus, OH, USA) over a one-week period. The familiarization started on the first day at 0
170 m.min⁻¹ for 10 minutes, and then with a 10-min run at 3 m.min⁻¹. On day 2, the mice ran at 3
171 m.min⁻¹ for 5 min and then at 6 m.min⁻¹ for 5 min. On day 3, they ran at 6 m.min⁻¹ for 10
172 minutes. On day 4, they ran at 6 m.min⁻¹ for 5 min and then at 10 m.min⁻¹ for 5 min. Lastly, on
173 day 5, the mice ran at 10 m.min⁻¹ for 10 min. After 48h the mice underwent the test at 0° and
174 then the same mice were tested after one week at 25°.

175 In the test, the mice were first recorded at rest for 8 min. The mice then started to run at
176 10 m.min⁻¹, and the treadmill velocity was then increased by 3 m.min⁻¹ every 3 min until the mice
177 were exhausted (defined as the moment when the mouse was in contact with the electric grid
178 for 5 seconds). Gas samples were taken every 5 seconds and dried prior to measurement of the
179 oxygen fraction with a gas analyser (Columbus Instruments). Oxygen uptake (VO₂) was calculated
180 as described previously (Ayachi et al. 2016; Taylor et al. 1981). To enable a comparison with

181 human data, VO_2 was expressed relative to the BW raised to the power 0.75 (supplementary
182 figure).

183 We presented the absolute highest oxygen consumption (VO_{2peak}) values and
184 VO_{2peak}/HW ratio, since the oxygen consumption capacity and the cardiac output are directly
185 related (according to the Fick equation: $Q = VO_2 / a-vO_2$ difference).

186 Performance was evaluated as the maximum running speed (V_{peak}). The blood lactate
187 concentration was measured in a drop of blood from the tail vein 5 minutes after each
188 incremental test, using the Lactate Pro LT-1710 meter (ARKRAY Europe, B.V., Amstelveen, the
189 Netherlands). For exercise with a 25° slope, only those results that differed from the 0° slope
190 setting are presented.

191 **2.3 Mice sacrifice and sampling**

192 Forty-eight hours after the last incremental exercise test, mice were sacrificed by
193 intraperitoneal infusion of sodium pentobarbital (100 mg/kg; Sanofi Santé Animale, Paris,
194 France). Samples of urine, heart muscle, skeletal muscles (the gastrocnemius, EDL, soleus, TA,
195 and quadriceps) and liver were collected. The absolute weights of all skeletal muscles were
196 normalized against the BW. We used the quadriceps of one leg for enzyme activity assays and
197 the quadriceps of the other leg for Western blots. The urine samples were directly syringed from
198 the bladder. All samples were stored at -80°C prior to analysis.

199 **2.4 Enzyme assays**

200 The quadriceps were weighed, homogenized (50 mg wet weight per 1 ml) in ice-cold
201 buffer containing HEPES 5 mM (pH 8.7), EGTA 1 mM, dithiothreitol 1 mM and Triton X-100
202 (0.1%), and incubated for 60 min at 4 °C for complete enzyme extraction. The total activities of
203 CK and CS were assayed (30 °C, pH 7.5) with coupled enzyme systems, as described previously
204 (De Sousa et al. 2000). Citrate synthase activity was measured in terms of the production of 2-

205 nitro-5-thiobenzoate (measured spectrophotometrically at 412 nm) by the reaction between
206 5',5'-dithiobis-2-nitrobenzoic acid and CoA-SH. Total adenylate kinase and CK activities were
207 determined using a coupled glucose-6-phosphate dehydrogenase/hexokinase enzyme assay,
208 which produced NADPH (measured spectrophotometrically at 340 nm).

209 **2.5 Western blots**

210 The quadriceps muscles were homogenized in CellLytic™ MT Cell Lysis Reagent (Sigma-
211 Aldrich, France) and then centrifuged at 11000 rpm (15000g) for 15 min at 4°C. The supernatant
212 was removed and protease inhibitor cocktail was added. The protein concentration was
213 quantified in a Bradford assay. Samples were denatured in SDS Laemmli 2× concentrate (Sigma-
214 Aldrich) at 90°C for 5 min. Next, 10 µg aliquots of protein in 30 µl were loaded into each well of
215 an SDS polyacrylamide gel (12%). The same protein standards were loaded on all gels to avoid
216 variations from one membrane to another. The gels were run with running buffer at 140 V for 1
217 hour and then transferred onto a nitrocellulose membrane (pore size: 0.2 µm). After staining
218 with Ponceau S reagent, the membranes were blocked with 5% bovine serum albumin (BSA) in
219 Tris-buffered saline containing 0.1% Tween-20 (TBST) for 1 hour. The membranes were
220 incubated with primary antibodies overnight at 4°C. The following primary antibodies were
221 purchased from Cell Signaling (Netherlands) and Abcam (France) and were diluted in TBST-BSA:
222 Mfn2 rabbit mAb (9482 Cell Signaling), 80 kDa dilution 1:1000; OPA-1 Rabbit mAb (80471 Cell
223 Signaling), 80-100 kDa, 1:1000, Drp-1 rabbit mAb, 78-82 kDa (8570 Cell Signaling) dilution
224 1:1000; β-actin rabbit mAb (horse radish peroxidase (HRP) conjugate), 45 kDa, 1:1000 and PGC-
225 1α-N-terminal rabbit polyclonal 91 kDa (ab54481 abcam), 1 µg/ml TBST-BSA. Anti-rabbit HRP-
226 conjugated secondary antibody (Sigma Aldrich) diluted 1:10 000 in TBST-BSA was used to detect
227 the primary antibodies. All protein expressions were normalized against β-actin (5125 Cell
228 Signaling).

229 The membranes were then incubated with secondary antibodies (anti- rabbit Sigma) for 1
230 hour at room temperature and then washed in TBST four times prior to incubation with the
231 secondary antibody for 1 hour at room temperature. The bands were detected using an
232 enhanced chemiluminescence detection reagent kit (Bio-Rad France). Densitometry was
233 performed using a Fusion imaging system (Viber Lourmat Deutschland GmbH, Eberhardzell,
234 Germany).

235 **2.6 NMR spectrometry**

236 Urine samples were thawed at room temperature. A 100 μ L aliquot of urine QS 600 μ L
237 PBS/D₂O was placed in a 5 mm NMR tube. An aliquot of D₂O (for field locking) was placed in the
238 capillary tube holder. The proton spectra were acquired at 600 MHz on a Bruker Avance
239 spectrometer (Bruker, France), with a reversed cryoprobe. The temperature was set to 294 K.
240 The free induction decays (FIDs) were acquired using a NOESY1D sequence for water
241 suppression, with a preacquisition delay of 2 seconds, a 100 ms mixing time, and a 90° pulse. The
242 FIDs were collected to 64K complex points in a spectral window of 6600 Hz and 64 transients,
243 after four silent scans. The FIDs were processed with NMRpipe software (Delaglio et al. 1995).
244 The dataset was Fourier-transformed with an exponential function, producing 1 Hz line
245 broadening. The spectra were phased, and the baseline was corrected using the segment
246 method and three points at 0, 5, and 9 ppm. Each dataset was calibrated using the creatinine
247 signal at 3.05 ppm. The spectrum between 0 ppm and 9.5 ppm was divided into 9500 spectral
248 buckets of 0.001 ppm, using an in-house program written with R software. Each bucket was
249 labelled with its median chemical shift value. The water region (between 4.6 and 5 ppm) was
250 excluded from the data matrix. The bucket intensities were normalized using the probabilistic
251 quotient technique (Meyer and Peters 2003) to obtain the X matrix for statistical analysis. Unit
252 variance scaling was performed on all variables prior to a multivariate statistical analysis, and

253 spectra were aligned using the icoshift method (Savorani et al. 2010) to correct for the effect of
254 pH on the metabolites' chemical shifts.

255 **2.7 Statistical analyses**

256 Statistical analyses were performed using SigmaStat software (version 3.5). Different age
257 or genotype groups were compared in a two-way analysis of variance followed by a Student-
258 Newman-Keuls post-hoc test. Whenever the normality test did not pass, for type effect we used
259 Mann-Whitney analysis and for age a Kruskal-Wallis by ranks analysis was performed.

260 **Multivariate statistical analysis**

261 An unsupervised principal component analysis and a supervised orthogonal projection on
262 latent structure (OPLS) analysis were performed using an in-house MATLAB routine (The
263 MathWorks, Natick, MA, USA) based on the method described by Trygg and Wold (Trygg and
264 Wold 2002). The principal component analysis was first applied to the X matrix data, in order to
265 detect any separation between groups on the basis of the NMR signal variability. The O-PLS
266 analysis was performed to identify differences between sample spectra as a function of the type
267 of mice (control vs. Mt-CK^{-/-}). A model was computed for each age group (i.e. 6-, 9- and 18-mo).
268 The quality of the O-PLS model was assessed by calculating the R²_Y fit parameter (the variance
269 explained) and the Q²_Y cross-validated coefficient (the model's predictability). In our cases, only
270 the model obtained with 6-mo mice urine samples gave satisfactory, valid R²_Y and Q²_Y
271 parameters. A score plot and a loading plot were computed to illustrate the results of the O-PLS
272 model. Each point in the score plot represented the projection of an NMR spectrum on the
273 model's predictive component. The metabolites responsible for the classification obtained in the
274 score plot were taken into account when their correlation coefficients between the NMR data
275 and the model were higher than 0.5. In this case, these metabolites were considered as

276 discriminant metabolites. A heatmap summarizes these results, considering the discriminant
277 metabolites only.

278 **Results**

279 **3.1 Body weight and organ weights**

280 The control and Mt-CK^{-/-} groups did not differ with regard to body weight (BW) at 6 and 9
281 mo. However, at 18 months of age, the BW was 19% higher (p<0.001) in the control group than
282 in the Mt-CK^{-/-} group (Figure 1A). The BW was respectively 27% (p<0.001) and 21% higher in the
283 18-mo control group when compared with the 6-mo and 9-mo groups. When considering both
284 types of mouse, a significant effect of age on BW (p <0.001) and a significant interaction between
285 age and type (p<0.001) were seen (Figure 1A).

286 In both types of mouse, the absolute weight of the heart (Figure 1B) was significantly
287 higher in the 18-mo group than in the 6-mo (p<0.001) and 9-mo (p<0.001) groups. However,
288 once the heart weight (HW) was normalized to BW (Figure 1B), only the 18-mo Mt-CK^{-/-} group
289 had a significant higher value than the 6-mo Mt-CK^{-/-} group (p<0.001) and the 9-mo Mt-CK^{-/-}
290 group (p<0.001). Furthermore, the ratio of HW/BW was significantly higher in the 18-mo Mt-
291 CK^{-/-} group than in the 18-mo control group (p<0.01). An overall effect of age was seen for both
292 types at 6 months (p=0.03) and 9 months (p=0.016), relative to 18 months. Furthermore, the
293 interaction between age and type was highly significant (p=0.002).

294 The absolute weight of EDL and TA did not differ by age or type, and only soleus weight
295 was significantly lower in 18-mo Mt-CK^{-/-} mice than in 18-mo control mice (p<0.05) (Table 1).

296 Once the soleus weight was normalized against BW (Figure 1C), a significant effect of age
297 (p<0.001) was detected. Moreover, the soleus/BW ratio was significantly lower (p<0.001) in 6-
298 mo Mt-CK^{-/-} mice than in 6-mo control mice. Age had no impact on the soleus weight in the Mt-
299 CK^{-/-} groups. In contrast, the soleus/BW ratio was significantly lower in the 9-mo (p<0.05) and

300 18-mo ($p<0.001$) control groups than in the 6-mo control group. A significant age-type
301 interaction was detected for the soleus/BW ratio ($p=0.012$). This difference is mainly related to
302 the absence of BW gain in the oldest group of Mt-CK^{-/-} mouse.

303 Once the EDL weight was normalized against BW (Figure 1C), a significant age effect
304 appeared ($p<0.001$). Furthermore, the EDL weight was lower in the 6-mo Mt-CK^{-/-} group than in
305 the 6-mo control group ($p<0.05$). The EDL/BW ratio was significantly lower in the 9-mo control
306 ($p<0.05$) and 18-mo control ($p<0.001$) groups than in the 6-mo control. Age had no impact on the
307 EDL/BW ratio in Mt-CK^{-/-} mice.

308 The ratio of TA weight on BW (Figure 1C) was lower in 6-mo Mt-CK^{-/-} mice than in 6-mo
309 control mice ($p<0.05$). In control mice, the TA/BW ratio in 9-mo and 18-mo control mice were
310 lower when compared to 6-mo control ($p<0.01$ at 9-mo; $p<0.001$ at 18-mo), while for Mt-CK^{-/-},
311 the TA/BW ratio remained unaffected by age. However, the interaction between age and type
312 was significant ($p=0.032$).

313 In overall, for all skeletal muscles/BW ratio, the difference between control and Mt-CK^{-/-},
314 were only observed at 6-mo and not between 9 or 18-mo mice.

315 Finally, to make sure that ageing had no impact on the liver as an important organ for the
316 regulation of glucose and lipid homeostasis, and in parallel to our metabolomics analysis, we
317 measured the liver weight and controlled the general appearance. Comparison of absolute liver
318 weight and liver weight/BW ratio showed no significant differences between mice types and
319 between different ages in Mt-CK^{-/-} mice. However, in control mice, the absolute liver weight was
320 higher in 18-mo mice (1957.3 ± 122.5) than in 6-mo (1466.27 ± 116) and 9-mo (1349.7 ± 122.5) mice
321 ($p<0.01$ for both). This difference disappeared once the liver weight was normalized on BW.

322 **3.2 VO₂peak & performance**

323 *VO₂peak*

324 We measured the maximal oxygen uptake (VO_2 peak) with 0° and 25° slopes, as an index
325 of the mice aerobic capacity. In the control mice, the absolute VO_2 peak ($L \cdot \text{min}^{-1}$) was higher in
326 the 18-mo group than in the 6- and 9-mo groups (Figure 2A). When comparing the absolute
327 VO_2 peak (Figure 2 A) in the two types of mice of the same age, VO_2 peak value was significant
328 lower for 18-mo Mt-CK^{-/-} mice ($p < 0.001$) than for 18-mo controls. There was a significant effect
329 of age in both control and Mt-CK^{-/-} mice ($p = 0.026$ at 0°).

330 At both slopes the VO_2 peak/BW ratio (Figure 2B) was lower in the 18-mo Mt-CK^{-/-} group than in
331 the 6 and 9-mo Mt-CK^{-/-} groups; however, no difference was detected between 18-mo control
332 and Mt-CK^{-/-} at the same age. This might also be related to the absence of BW gain in the oldest
333 group of Mt-CK^{-/-} mouse.

334 Furthermore, when VO_2 peak was normalized to HW, the VO_2 peak/HW ratio was 27%
335 ($p < 0.001$) lower in the 18-mo Mt-CK^{-/-} group than in the 18-mo control group. Moreover, The
336 VO_2 peak /HW ratio was 29% ($p < 0.001$) lower in 18-mo Mt-CK^{-/-} mice than in 6-mo ($p < 0.001$) and
337 32% ($p < 0.001$) than 9-mo Mt-CK^{-/-} mice (Figure 2C).

338 *Maximal speed (Vpeak)*

339 With a slope of 0° and 25° no differences were found between types of the same age. At
340 0° slope, there were no age differences in V_{peak} among the control mice. However, V_{peak} was
341 13% lower ($p < 0.05$) in the 18-mo Mt-CK^{-/-} group than in the 6-mo Mt-CK^{-/-} group (Figure 3A).
342 With a slope of 25° , the V_{peak} was 22% lower ($p < 0.05$) in the 18-mo group than in the 6-mo
343 group for both control and Mt-CK^{-/-} mice (Figure 3 B). Likewise, the V_{peak} in the 18-mo groups
344 was lower than in the 9-mo groups (Figure 3B). Hence, a significant effect of age was present at
345 both slopes ($p = 0.012$ at 0° and $p = 0.001$ at 25°).

346 **3.3 Blood lactate level 5 minutes after the incremental test**

347 To evaluate the mice's recovery after the incremental test, we measured the clearance of
348 accumulated blood lactate during 5 min of recovery after the end of the exercise bout. With a
349 treadmill slope of 0°, the blood lactate concentration was significantly lower in the Mt-CK^{-/-}
350 group than in the control group at the age of 9 months (p<0.05) and 18 months (p<0.01). The
351 blood lactate concentration was significantly higher in the 18-mo control group than in the 6-mo
352 control group (p=0.001) and the 9-mo control group (p<0.05). In contrast, blood lactate did not
353 appear to be related to age in the Mt-CK^{-/-} mice (Figure 4). Hence, we observed an overall
354 significant effect of age (p=0.021) and genotype (p<0.001), and an interaction between age and
355 type (p=0.03) (Figure 4 A). The differences seen at a slope of 0° were not detected at a slope of
356 25° (Figure 4B).

357 **3.4 Citrate synthase, creatine kinase and adenylate kinase activity in skeletal muscle**

358 The enzyme activities in extracts from frozen quadriceps were determined
359 spectrophotometrically. The assay results showed that neither age nor genotype influenced the
360 activity values (Table 2). An analysis of the total CK activity in the quadriceps only revealed a
361 significant interaction between age and genotype (p= 0.048).

362 **3.5 Metabolomic**

363 In an analysis of the urine metabolome, the OPLS-based comparison of the Mt-CK^{-/-}
364 group and the control group (Figure 5 A) showed that the R2Y and Q2Y values (0.980 and 0.771,
365 respectively) were acceptable in 6-mo mice only. There was no control vs. Mt-CK^{-/-} differences in
366 mice at 9-mo or 18 mo. The discriminant metabolites responsible for this classification are
367 represented as a heatmap (Figure 5B) showing that one of the most discriminant metabolites
368 were branched-chain amino acids (BCAA) with other amino-acids such as glutamate and lysine.
369 On the opposite, alanine was less eliminated in the urine of Mt-CK^{-/-} when compared to control

370 mice. Besides, several other metabolites demonstrate that these two genotypes differ also from
371 a metabolic thus phenotypic point of view.

372 **3.6 Mitochondrial dynamics**

373 The Western blot analysis showed that PGC-1 α expression in the quadriceps muscle was
374 lower in 18-mo control and Mt-CK^{-/-} mice than in the corresponding 6-mo groups. An effect of
375 age was detected (p= 0.021) (Figure 6A). There was also an overall effect of age on Mfn-2
376 expression in the quadriceps (p=0.017); expression of Mfn-2 in the quadriceps was significantly
377 higher in the 18-mo groups than in the 6-mo groups (p=0.013) and the 9-mo groups (p=0.034)
378 (Figure 6 B). A *post hoc* test revealed that the expression of Mfn-2 in the skeletal muscle of 18-
379 mo Mt-CK^{-/-} was significantly higher than in both the 6-mo Mt-CK^{-/-} mice (p<0.01) and the 9-mo
380 Mt-CK^{-/-} mice (p<0.05). The expression of OPA-1 and Drp-1 in the quadriceps remained the same
381 in both types of mouse and for all ages (6 C, D).

382 **3. Discussion**

383 Taken as a whole, the present study showed ageing is potentialized by Mt-CK deficiency
384 with regard to VO₂peak, Vpeak and mitochondrial protein expression. The Vpeak for 18-mo Mt-
385 CK^{-/-} mice decreased at 0° and 25° while for control group of the same age the Vpeak decreased
386 only at 25°. The main effects of genotype included a smaller BW gain with age and a greater
387 HW/BW in Mt-CK^{-/-} mice than in control mice. The skeletal muscle weights/BWs were lower in 6-
388 mo Mt-CK^{-/-} mice than in 6-mo control. In the same line, the metabolites eliminated in urine
389 (notably higher levels of BCAAs) discriminated between Mt-CK^{-/-} mice from control mice at the
390 age of 6 months only. It is noteworthy that the Mt-CK^{-/-} and control groups did not differ with
391 regard to levels of lipid metabolites.

392 Although we did not measure energy expenditure or body temperature in the present
393 study, research on other models related to creatine metabolism such as mice lacking creatine

394 transporters (SLC6A8), guanidinoacetate methyltransferase (GAMT) or CK KO mice (lacking brain-
395 type CK and mitochondrial ubiquitous CK) displayed a low BW, muscle weakness, muscle
396 atrophy, low amounts of white adipose tissue or impaired body temperature maintenance (Kan
397 et al. 2005; Russell et al. 2014; Stockebrand et al. 2018; Streijger et al. 2009). In addition, as it
398 was aforementioned, the increase in cardiac work become more “energetically costly” when the
399 activity of the CK fall below a certain level (Saupe et al. 1998) and it seems that lack of Mt-CK
400 induces lower MM-CK activity in heart (Boehm et al. 1998), that might explain a higher substrate
401 utilisation by heart in Mt-CK^{-/-} mice.

402 The absolute HWs were significantly greater in both control and Mt-CK^{-/-} mice at 18
403 months of age than at 6 and 9 months of age. After normalization against the BW, this difference
404 disappeared for control mice but remained for Mt-CK^{-/-} because of the absence of BW gain.
405 Lygate et al. (2009) showed that Mt-CK^{-/-} mice had normal cardiac function; hypertrophy was
406 not detected at the age of 20 to 40 weeks (Lygate et al. 2009). However, the Mt-CK^{-/-} mice had a
407 higher heart rate (Lygate et al. 2009), and it has been reported that Mt-CK is required to maintain
408 normal high energy phosphate metabolite levels in heart (Spindler et al 2002).

409 With a treadmill slope of 0°, the absolute values of VO₂peak were the same in all Mt-CK^{-/-}
410 age groups. However, this was not the case for control mice, where the absolute VO₂peak was
411 higher at the age of 18 months than at 6 and 9 months. This might correspond to a response or
412 adaptation to a higher BW. We decided to normalize the VO₂peak against HW because the BW
413 curves differed for controls vs. Mt-CK^{-/-} mice while the absolute HWs followed the same pattern
414 in both types of mouse. Once VO₂peak was normalized against HW, the VO₂peak/HW ratio with
415 treadmill slopes of 0° and 25° were significantly lower for 18-mo Mt-CK^{-/-} mice than in 18-mo
416 control mice and in 6-mo and 9-mo Mt-CK^{-/-} mice. We hypothesize that even after cardiac

417 adaptation at 18 months of age, the oldest Mt-CK^{-/-} mice presented a lower aerobic capacity (as
418 indicated by the VO₂peak value).

419 We observed a significant effect of age on Vpeak at slopes of 0° and 25°. However, the
420 difference at 0° was mainly related to a lower Vpeak in 18-mo Mt-CK^{-/-} than in 6-mo mice, which
421 might be related to a greater age-related decline in performance in the Mt-CK^{-/-} mice. With a 25°
422 slope, the two genotypes showed the same decline in Vpeak with ageing. Our results are in line
423 with Miotto and Holloway's (2016) report on run times to exhaustion in younger (4-month-old)
424 control and Mt-CK^{-/-} mice with treadmill slopes of 5° and 20° (Miotto and Holloway 2016).
425 However, in a study of voluntary wheel running over 3 weeks, Lygate et al. (2009) found that Mt-
426 CK^{-/-} mice aged 30 weeks performed less well than control mice (Lygate et al. 2009). This might
427 have been related to the level of motivation of Mt-CK^{-/-} mice, which opens up perspectives for
428 the behavioural evaluation of this strain.

429 The two types of mouse differed with regard to the age-related change in blood lactate
430 during the incremental test with a 0° slope (i.e. an age-type interaction). The blood lactate
431 concentration was significant lower in the 9-mo and 18-mo Mt-CK^{-/-} mice than in the
432 corresponding control groups. This difference disappeared when the slope was 25°, that is more
433 comparable to the results found by Miotto et al (2016), comparing 12 weeks Mt-CK^{-/-} mice blood
434 lactate with wild type mice after exhaustive exercise (Miotto and Holloway 2016) . For the 18-mo
435 Mt-CK^{-/-} mice, the lower blood lactate might be related to lower performance or less work load
436 because of lower body weight. However, the two types of mouse achieved the same maximal
437 speed at 9 months of age but the blood lactate level was lower in the 9-mo Mt-CK^{-/-} group. The
438 lower blood lactate level in Mt-CK^{-/-} mice might be also related to adaptation of the heart in
439 order to compensate for the energy deficit; the Mt-CK^{-/-} heart tissue might use a higher
440 concentration of lactic acid than the heart tissue of control mice at the same age. Consequently,

441 the Mt-CK^{-/-} mice might have a more efficient lactate uptake system, as has been shown in some
442 tissues (such as the liver) in starved mice (Schutkowski et al. 2014). In addition, probably these
443 mice had other compensatory adaptations, such as increasing the production of ATP through the
444 cofactor nicotinamide adenine dinucleotide (NAD⁺) production to boost oxidative
445 phosphorylation, that needs further studies in Mt-CK^{-/-} mice. Further, as metabolomic results
446 (discussed below) showed the Mt-CK^{-/-} at 6mo, had increased BCAAs, glutamate and lysine in
447 urine while alanine was less present in the urine of the 6-mo Mt-CK^{-/-} mice, probably suggesting
448 that alanine was mainly converted to pyruvate through gluconeogenesis.

449 The presence of methionine (an anabolic stimulant) and BCAAs and other amino acids in
450 the urine of 6-mo Mt-CK^{-/-} mice (compared with 6-mo control mice) might suggest an imbalance
451 in protein turnover, i.e. more protein catabolism rather than protein anabolism in these animals.
452 These Mt-CK^{-/-} vs. control differences were not detected at the ages of 9 and 18 months, and the
453 difference appeared to have been lost with age. Protein turnover may lead to protein catabolism
454 to a similar extent in both the Mt-CK^{-/-} and control mice at 9 and 18 months of age. Other
455 studies have suggested that muscle mass loss during ageing is due to impairment of the cell's
456 energy status (Hiona et al. 2010; Neelakantan et al. 2019; Nuss et al. 2009; Tepp et al. 2016). At
457 11 months of age, a mouse model of premature ageing (the mitochondrial DNA polymerase γ
458 knock-out mouse) displays the degree of skeletal muscle sarcopenia usually observed at the age
459 of 30 months in control mice. This sarcopenia was associated with low levels of electron
460 transport chain complex components and impaired mitochondrial bioenergetics (Hiona et al.
461 2010).

462 The protein expression of factors involved in mitochondrial biogenesis and dynamics in
463 the quadriceps revealed an effect of age. We found that in both types of mouse, protein
464 expression of PGC-1 was lower at 18 months of age and protein expression of Mfn-2 was higher

465 at 18 months of age than in younger counterparts. The expression of Opa1 did not differ with
466 age, and we did not observe any differences of Drp-1 expression as a function of genotype or
467 age. The impact of ageing on muscle mitochondrial content is subject to debate (Chabi et al.
468 2008; Lanza and Nair 2009; Leduc-Gaudet et al. 2015). Indeed, protein synthesis is an
469 energetically demanding process. A number of studies suggest that alterations in mitochondrial
470 function and structure (i) are involved in sarcopenia and the loss of muscle function during
471 ageing, and (ii) start before muscle sarcopenia becomes apparent (Del Campo et al. 2018;
472 Figueiredo et al. 2009; Joseph et al. 2013). Our results showed that ageing impacts the protein
473 expression of PGC-1 α , as has been observed in skeletal muscle from older adults (Joseph et al.
474 2012). This probably suggests that mitochondrial biogenesis in the quadriceps was lower in the
475 18-mo group than in the younger mice. Furthermore, greater skeletal muscle levels of both Mfn1
476 and Mfn2 were reported in elderly mice and monkeys (Mercken et al. 2017). However, Leduc-
477 Gaudet et al. (2015) reported that ageing does not alter the expression levels of proteins related
478 to mitochondrial fission or fusion (Leduc-Gaudet et al. 2015). Nevertheless, they observed a
479 higher Mfn2/Drp-1 ratio in the skeletal muscle of older mice (Leduc-Gaudet et al. 2015). It is
480 possible that with age, adaptation of mitochondria in the skeletal muscles (through fusion and
481 fission) is more prominent than mitochondrial biogenesis.

482 It has been reported that muscle disuse is associated with lower levels of the inner and
483 outer mitochondrial membrane fusion factors Mfn2 and Opa1 (Tezze et al. 2017). Furthermore,
484 the overexpression of Drp-1 is reportedly involved in skeletal muscle atrophy and muscle disuse
485 in mice (Romanello et al. 2010). However, we did not find intergroup differences in Drp-1
486 expression in the quadriceps. In contrast, other studies reported the overexpression of Drp-1 in
487 the gastrocnemius muscle of 9-mo rats (Faitg et al. 2019). It is probable that not all muscle types
488 age at the same speed (Crupi et al. 2018).

489 The skeletal muscle enzyme activity assay did not reveal any significant differences in
490 total CK and citrate synthase activities. However, a significant interaction between age and type
491 was observed. This might be related to compensation by MM-CK in Mt-CK^{-/-} mice. However, we
492 did not measure the enzyme activity in skeletal muscles other than the quadriceps that is a
493 mixed muscle. Lygate and al. showed that Mt-CK^{-/-} mice at 30 weeks, had higher MM-CK activity
494 in the soleus muscle but not in glycolytic (gastrocnemius) muscle (Lygate et al. 2009). The higher
495 expression of MM-CK might be in favour of maintaining muscle power, and probably the reason
496 that at 6 mo and 9 mo the Mt-CK^{-/-} mice present the same performance as control mice;
497 however, this compensation might not be enough at older age.

498 A large body of evidence shows that the CK phosphotransfer pathway in rodent heart and
499 skeletal muscle becomes significantly less efficient with age (Kanski et al. 2005; Nuss et al. 2009;
500 Tepp et al. 2017; Tepp et al. 2016). On the same lines, most of our results suggest that the effect
501 of age was greater than the effect of genotype. The observed effect of genotype and the
502 presence of a lower BW and higher HW in Mt-CK^{-/-} mice suggest that physiological adaptations
503 enable the KO mice to maintain the same level of performance as control mice. However, the
504 fact that VO₂peak and Vpeak are lower in older Mt-CK^{-/-} mice than in younger Mt-CK^{-/-} mice
505 suggests that this strain ages more quickly than control mice. In future research, it would be
506 interesting to use the cre-lox system to create targeted Mt-CK^{-/-} mice and this to avoid the
507 physiological adaptations that are present in most transgenic mice. It would also be interesting
508 to perform the same study in Mt-CK^{-/-} at older age such as 24 months.

509 The present study had several strengths, this was the first time that the performance of
510 Mt-CK^{-/-} mice had been evaluated at such an advance age (18 months). A relatively large number
511 of mice were used. The study also had several limitations. Firstly, we did not normalize the organ
512 weight against the length of the tibia bone. Secondly, we did not measure energy expenditure

513 and cardiac function. Finally, the protein assays and enzyme activity assay were performed on
514 the quadriceps only. In future research, it would be better to study these parameters in the heart
515 and in a broader range of skeletal muscles. These observations might provide more information
516 on putative adaptation mechanisms during ageing in Mt-CK^{-/-} mice.

517 **4. Conclusion**

518 In conclusion, the present study showed ageing is potentialized by Mt-CK deficiency with
519 regard to VO₂peak, Vpeak and mitochondrial protein expression. The genotype effect in our
520 study were mainly reflected in smaller BW gain, a greater HW/BW in Mt-CK^{-/-} mice through
521 ageing. The skeletal muscle weights/BW and urine metabolomics analysis could explain protein
522 wasting in these mice at younger age when compared to their counterparts in control but this
523 difference disappeared at older age probably due to the loss of muscle mass in control mice.
524 There is no doubt that Mt-CK^{-/-} mice undergo physiological adaptations, enabling them to
525 survive and to performance as well as wild-type mice. However, it is possible that these
526 adaptations in Mt-CK^{-/-} mice have a high energy cost and might trigger premature ageing.
527 Studies of energy metabolism pathways would be required to confirm this.

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535 **Authors contribution statements**

536 In the present study: Niel R: performed all the incremental exercise test and analyzed the
537 data and participated in writing the manuscript. Le Moyec L: supervised all mice urine sample
538 analysis by NMR and worked on the manuscript. Launay T: participated in experimentation and
539 analysis of the western blots and worked on the manuscript. Hamard Mille L: supervised the
540 incremental tests and worked on the manuscript. Triba MN: contributed to the statistical analysis
541 of NMR data. Maciejak O: carried out the NMR experimentation. Billat V: developed the idea and
542 worked on the manuscript. Momken I: conducted and directed the project, performed
543 genotyping of mice and the enzyme activity assay and wrote the manuscript. All authors,
544 discussed the results and the manuscript.

545

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Table Legend

Table 1: Anatomic data. For the two-way ANOVA and post hoc test: * $p < 0.05$, ** $p < 0.01$, indicate a significant difference vs. controls of the same age; † $p < 0.05$ and †† $p < 0.01$ indicate a significant difference vs. the 6-mo group of the same genotype, and \$\$\$ $p < 0.01$, indicate a significant difference vs. the 9-mo group of the same genotype; Extensor digitorum longus (EDL), Anterior tibialis (TA).

Table 2. The quadriceps citrate synthase, creatine kinase and adenylate kinase activity, the enzymes activities are expressed in international unit per gram wet weight (IU/g WW).

Table 1.

	6 mo Cont n=12	6 mo Mt-CK ^{-/-} n=13	9 mo Cont n=11	9 mo Mt-CK ^{-/-} n=13	18 mo Cont n=10	18 mo Mt-CK ^{-/-} n=9	Interaction Age x type
Soleus (mg)	9.91±0.4	9.11±0.35	9.3±0.34	9.45±0.15	9.85±0.25	8.61±0.57*	-----
EDL (mg)	10±0.38	9.09±0.37	9.13±0.42	9.45±0.42	9.56±0.47	8.43±0.66	-----
TA (mg)	46.1±1.33	42.5±1.96	41.7±2.46	40.6±1.58	43.9±1.87	42.4±1.75	-----

Table 2.

IU/g WW	6 mo Cont n=7	6 mo Mt-CK ^{-/-} n=8	9 mo Cont n=8	9 mo Mt-CK ^{-/-} n=8	18 mo Cont n=7	18 mo Mt-CK ^{-/-} n=7	Interaction Age x type
CS	681.9±28	757.3±15	678.5±18	737.2±20.5	697.9±23	656.7±17	0.059
Total CK	1118.6±53	1192.7±49.6	1064.15±49.6	1185±49.6	1190.9±53	1061.3±53	0.048

AK	838±31.5	852.5±29.4	827.85±29.4	835.35±29.4	870.3±31.5	830.65±31.5	0.641
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Figure Legend

Figure 1. (A) Body weight at 6, 9 and 18 months of age; (B) Absolute heart weight (HW) and HW normalized to BW; (C) Skeletal muscles: Soleus, Extensor digitorum longus (EDL), Anterior tibialis (TA) normalised to body weight (BW). In the *post hoc* test, * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ indicate a significant difference vs. controls of the same age, † $p < 0.05$, †† $p < 0.01$, ††† $p < 0.001$ indicate a significant difference vs. the 6-mo group of the same genotype. The age-type interactions were apparent. 6-mo control $n = 12$, 9-mo control $n = 10$, 18-mo control $n = 10$; 6-mo Mt-CK^{-/-} $n = 13$, 9-mo Mt-CK^{-/-} $n = 13$, 18-mo Mt-CK^{-/-} $n = 9$.

Figure 2. (A) Net VO₂peak at a slope of 0°. (B) VO₂peak/BW (C) VO₂peak/Heart weight (HW) at a slope of 0°. In a *post hoc* test; ** $p < 0.01$ *** $p < 0.001$ indicates a significant difference vs. controls of the same age, † $p < 0.05$, ††† $p < 0.001$ indicates a significant difference vs. the 6-mo group of the same genotype and \$ $p < 0.05$, \$\$ $p < 0.01$ and indicate a significant difference vs. the 9-mo group of the same genotype. 6-mo control $n = 12$, 9-mo control $n = 10$, 18-mo control $n = 10$; 6-mo Mt-CK^{-/-} $n = 13$, 9-mo Mt-CK^{-/-} $n = 13$, 18-mo Mt-CK^{-/-} $n = 8$

Figure 3. Maximum running speed (V_{peak}) (A) at a slope of 0° and (B) at a slope of 25° slope. A, Kruskal-Wallis by ranks analysis was performed for V_{peak} . † $p < 0.05$, indicates a significant difference vs. the 6-mo group of the same genotype, and \$ $p < 0.05$ indicates a significant difference vs. the 9-mo group of the same genotype. V_{peak} at 0° , Number of the test (NT) at 6mo=31, NT at 9-mo control = 20, NT at 18-mo control =11; NT at 6-mo Mt-CK^{-/-} =38, NT 9-mo Mt-CK^{-/-} =26, NT 18-mo Mt-CK^{-/-} =10.

V_{peak} at 25° , Number of the test (NT) at 6mo=24 NT at 9-mo control = 13, NT at 18-mo control =11; NT at 6-mo Mt-CK^{-/-} =10, NT 9-mo Mt-CK^{-/-} =18, NT 18-mo Mt-CK^{-/-} =9.

Figure 4. Assay of the blood lactate level 5 min after the end of the incremental test (A) at a slope of 0° slope, (B) At a slope of 25° . In a *post hoc* test, * $p < 0.05$ and ** $p < 0.01$ indicate a significant difference vs. controls of the same age, †† $p < 0.01$ and ††† $p < 0.001$ indicate a significant difference vs. the 6-mo group of the same genotype and \$ $p < 0.05$, indicates a significant difference vs. the 9-mo group of the same genotype. 6-mo control n=12, 9-mo control n=10, 18-mo control n=10; 6-mo Mt-CK^{-/-} n=13, 9-mo Mt-CK^{-/-} n=13, 18-mo Mt-CK^{-/-} n=8

Figure 5. (A) The score plot from the OPLS model were obtained for urine samples collected in the control group (blue dots, n=7; at 6-mo) and the Mt-CK^{-/-} group (red dots, n=8; at 6-mo). Each score plot is given with its Q^2_Y and R^2_Y values (B) Heatmap of the correlation coefficients between metabolites and group classification. Only metabolites with $R > 0.5$ are represented (discriminant metabolites); trimethylamine N-oxide (TMAO), trimethylamine (TMA), Dimethylamine (DMA); 6-mo control n=7, 6-mo Mt-CK^{-/-} n=8.

Figure 6. Protein expression in the quadriceps: (A) PGC-1 α . (B) Mnf2. (C) OPA-1. (D) Drp-1. In a *post hoc* test, ††p<0.01 indicates a significant difference vs. the 6-mo group of the same genotype, and \$p<0.05 indicates a significant difference vs. the 9-mo group of the same genotype . 6-mo control n=9, 9-mo control n=7, 18-mo control n=6, 6-mo Mt-CK^{-/-} n=8, 9-mo Mt-CK^{-/-}, n=7, 18-mo Mt-CK^{-/-} n= 6.

Figure 1.

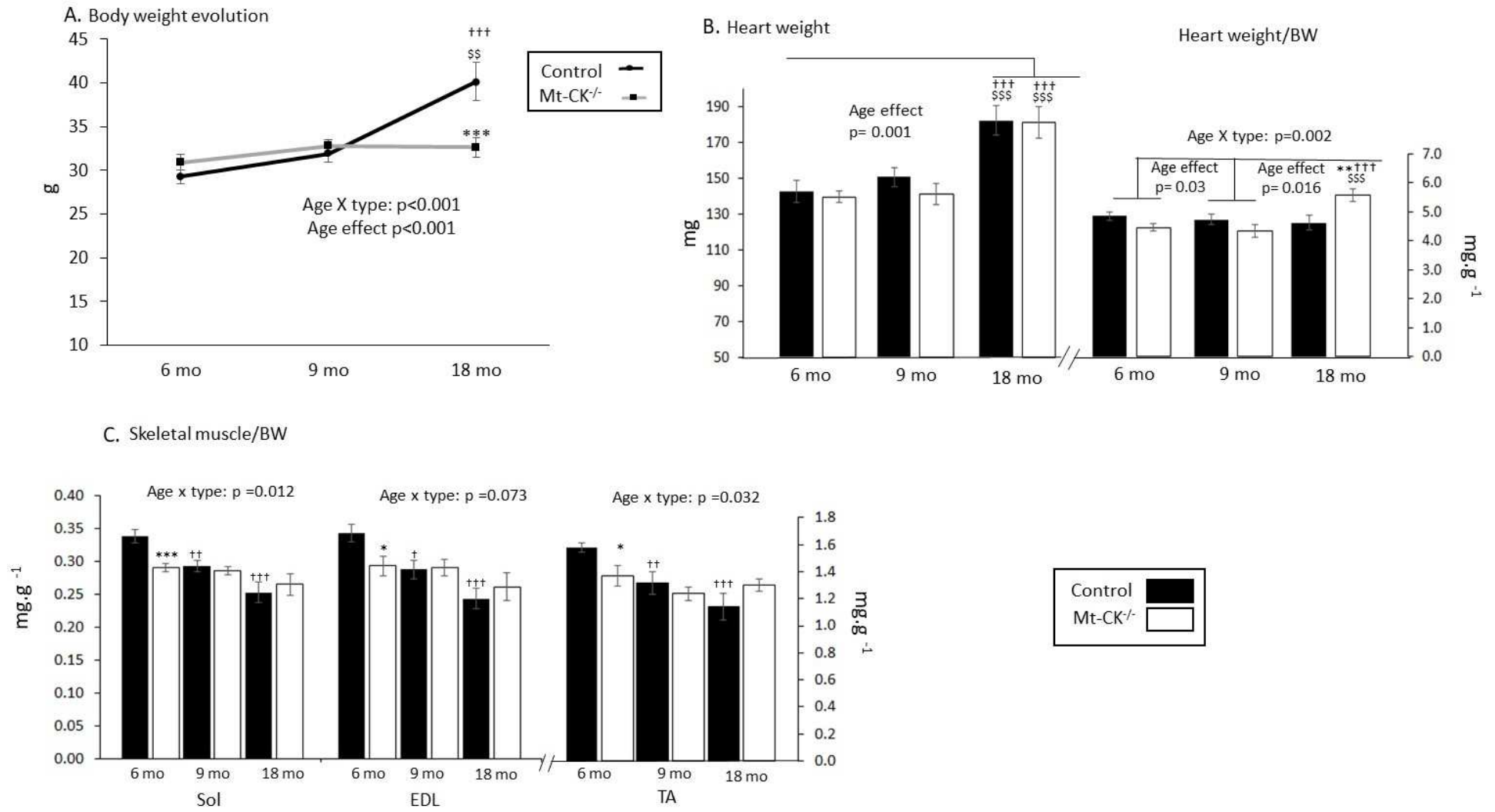


Figure 2

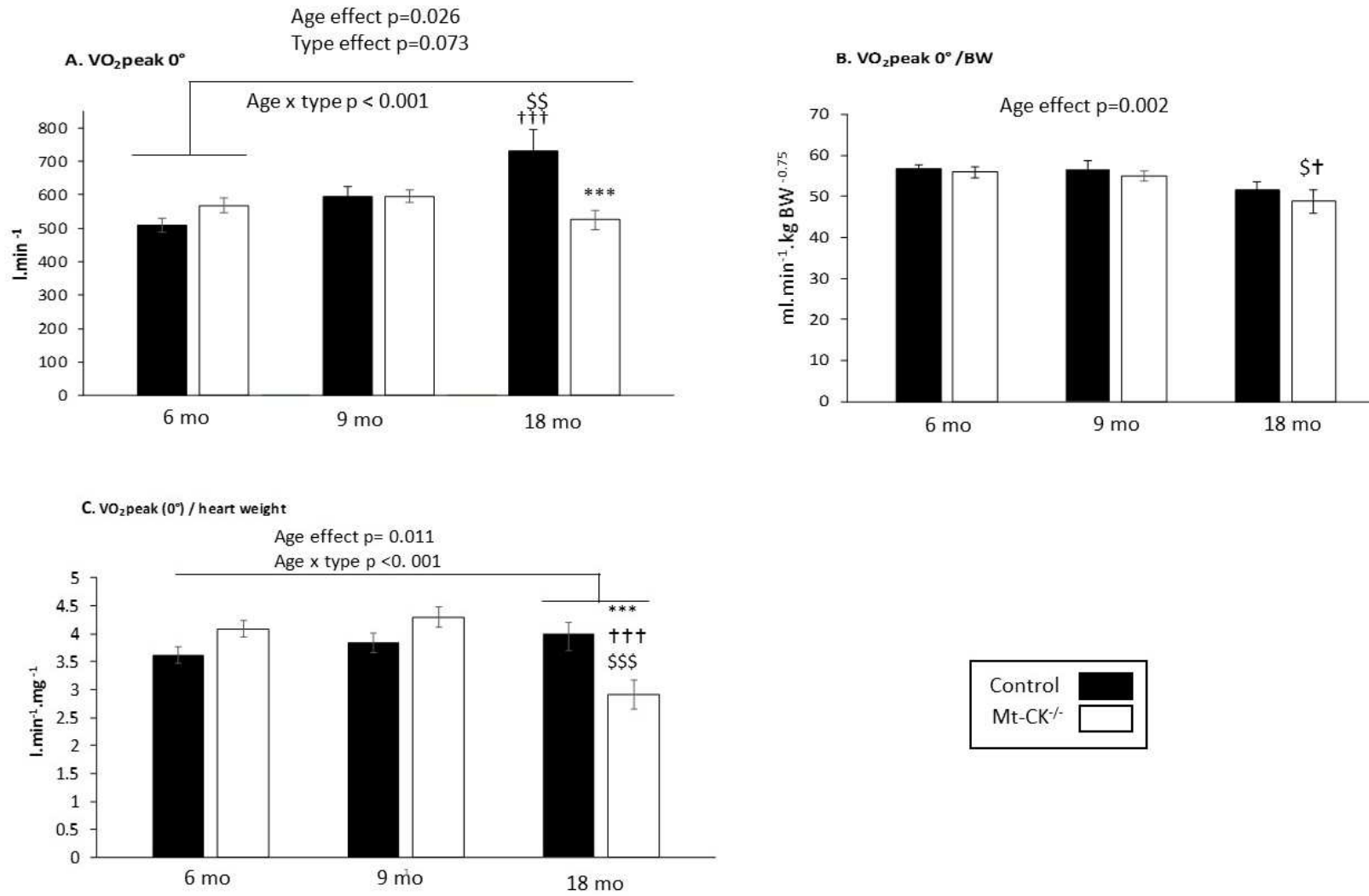


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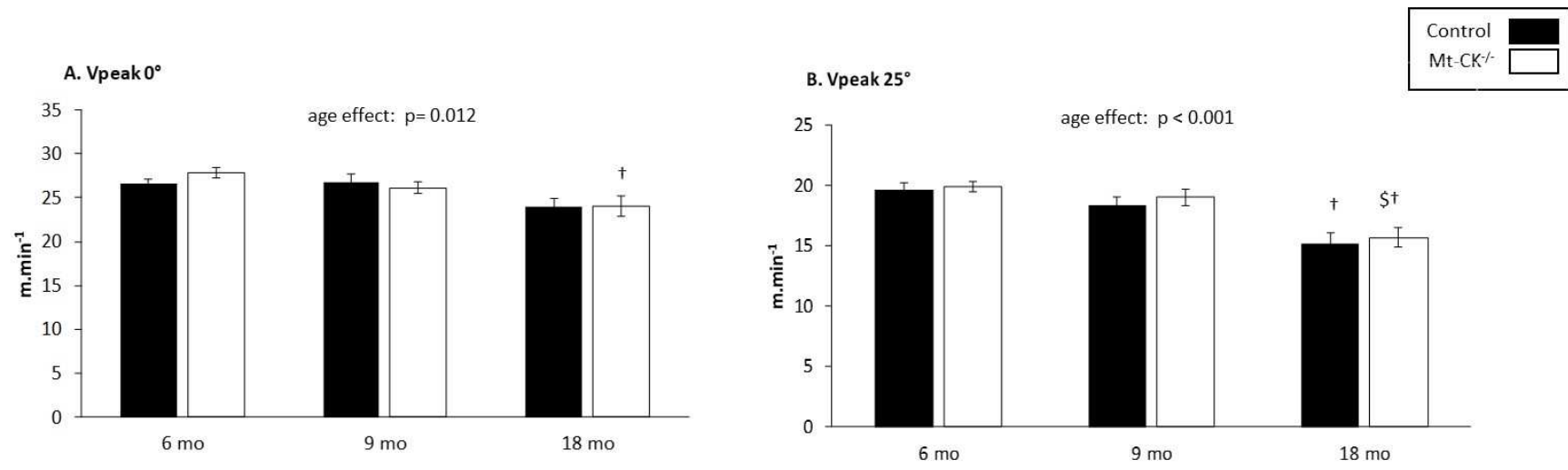


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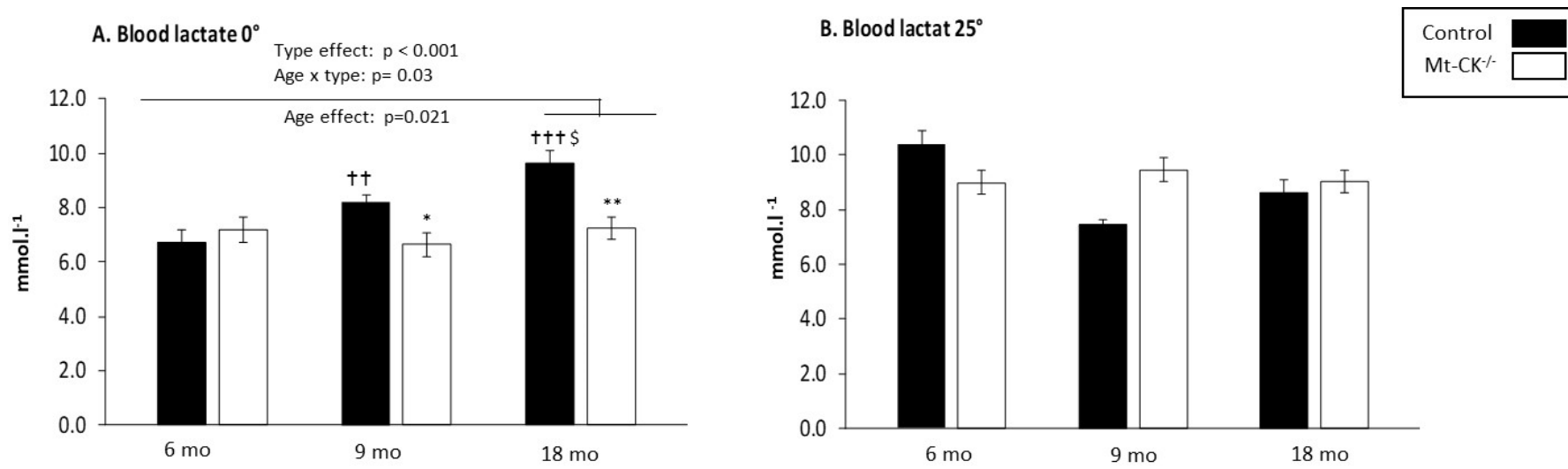
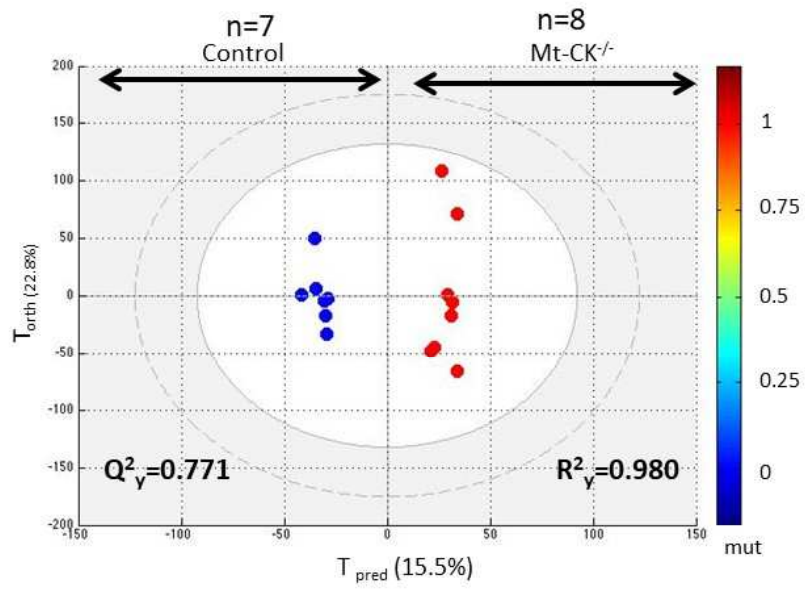


Figure 5.

A.



B.

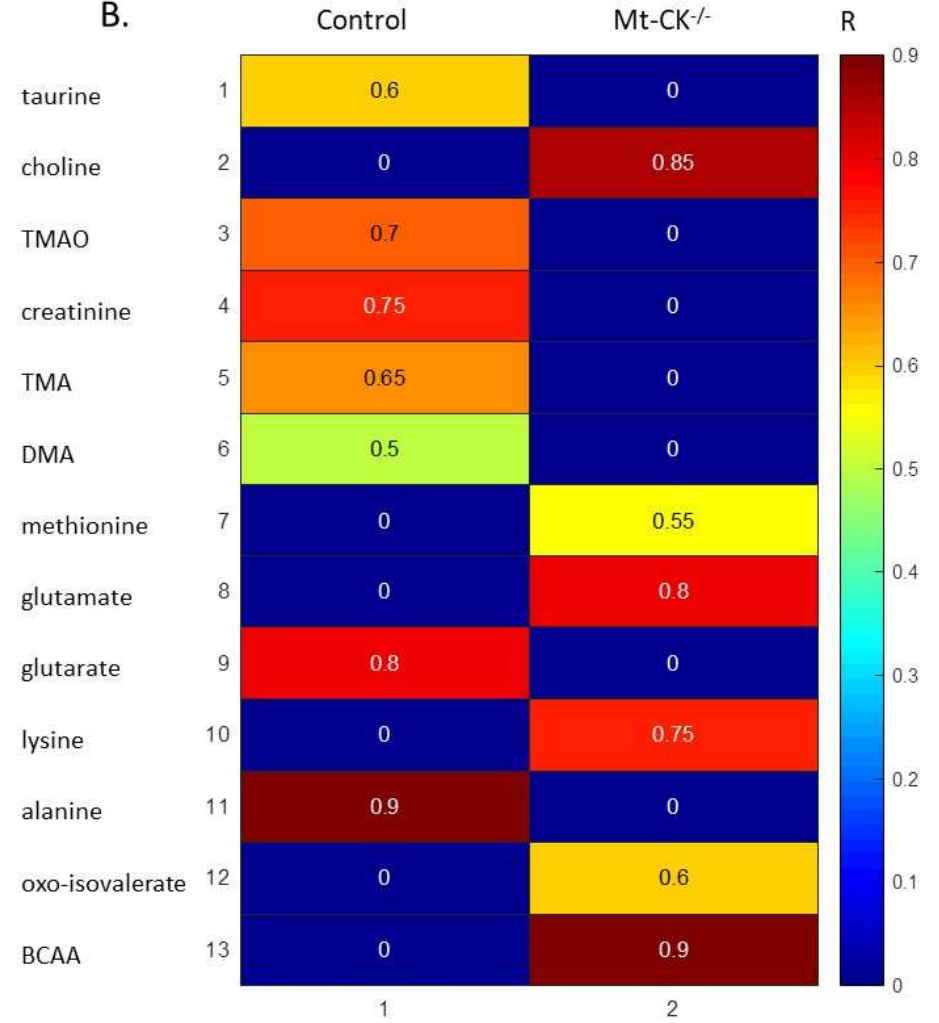
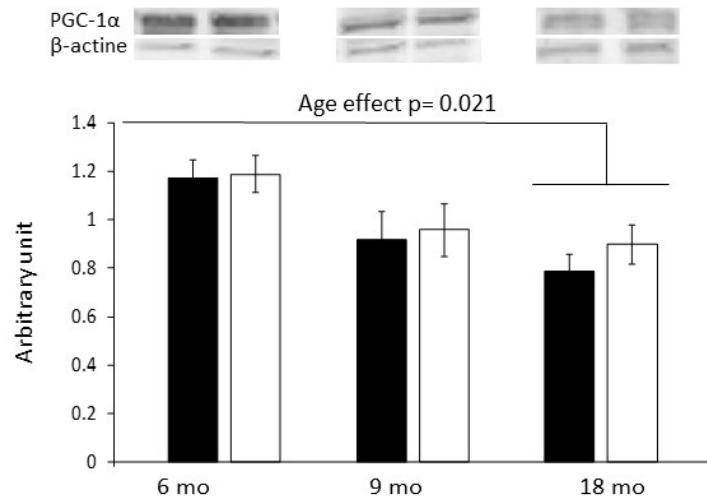
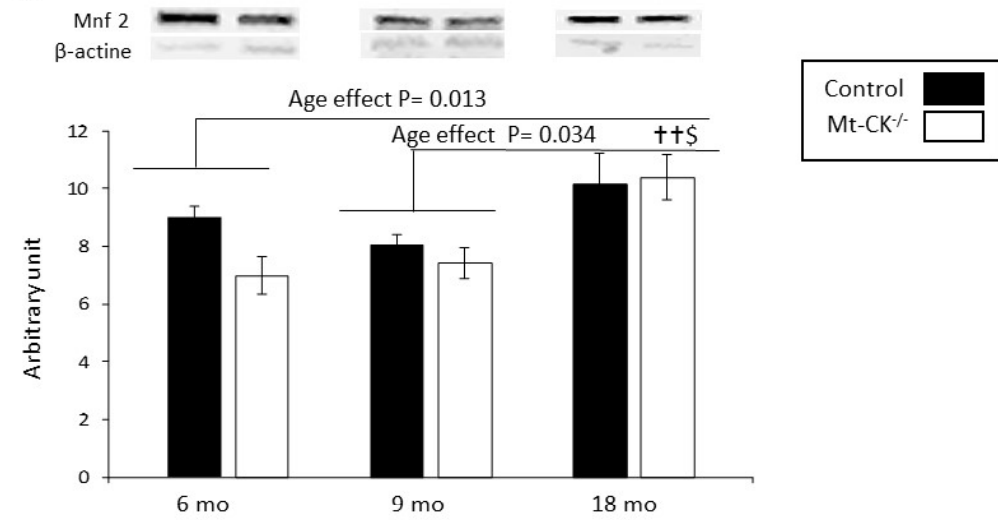


Figure 6.

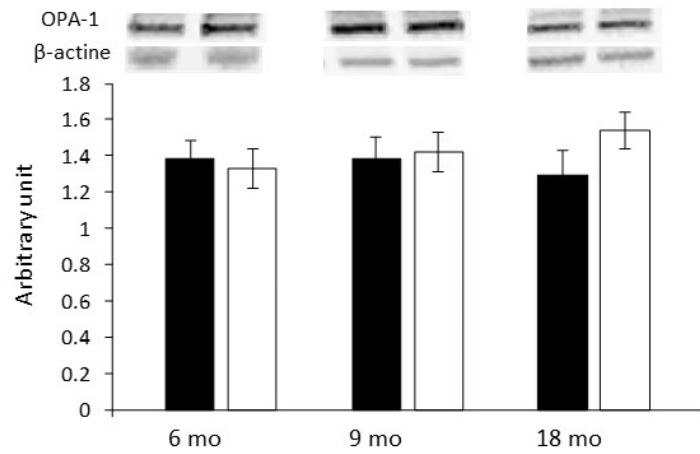
A.



B.



C.



D.

