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1 **Endurance training and Hydroxyurea have synergistic effects**  
2 **on muscle function and energetics in sickle cell disease mice**

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17  
18  
*Abbreviations*: SCD: Sickle cell disease, HU: Hydroxyurea, <sup>31</sup>P-MRS: 31-Phosphorus Magnetic Resonance Spectroscopy, MRI: Magnetic Resonance Imaging, ATP: Adenosine triphosphate, PCr: Phosphocreatine, Pi: inorganic phosphate, TFP: Total force production, Pf: peak force, VPCr<sub>stim</sub>: rate of PCr consumption, VPCr<sub>rec</sub>: rate of PCr recovery, V<sub>max</sub>: Maximal rate of oxidative ATP resynthesis

## Abstract

19

20 Sickle cell disease (SCD) is an hemoglobinopathy resulting in the production of an abnormal Hb (HbS)  
21 which can polymerize in deoxygenated conditions, leading to the sickling of red blood cells (RBC). These  
22 alterations can decrease the oxygen-carrying capacity leading to impaired function and energetics of skeletal  
23 muscle. Any strategy which could reverse the corresponding defects could be of interest. In SCD, endurance  
24 training is known to improve multiples muscle properties which restores patient's exercise capacity but  
25 present reduced effects in anemic patients. Hydroxyurea (HU) can increase fetal hemoglobin production  
26 which can reduce anemia in patients. The present study was conducted to determine whether HU can  
27 improve the effects of endurance training to improve muscle function and energetics. Twenty SCD Townes  
28 mice have been trained for 8 weeks with (n = 11) or without (n = 9) HU. SCD mice muscle function and  
29 energetics were analyzed during a standardized rest-exercise-recovery protocol, using Phosphorus-31  
30 Magnetic resonance spectroscopy (<sup>31</sup>P-MRS) and transcutaneous stimulation. The combination of training  
31 and HU specifically decreased fatigue index and PCr consumption while muscle oxidative capacity was  
32 improved. These results illustrate the potential synergistic effects of endurance training and HU on muscle  
33 function and energetics in sickle cell disease.

34 Keyword: Endurance training, Hydroxyurea, Sickle Cell Disease, Skeletal muscle, <sup>31</sup>P-Magnetic  
35 Resonance Spectroscopy

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## Introduction

45

46 Sickle cell disease (SCD) is an inherited hemoglobinopathy resulting in the production of abnormal  
47 hemoglobin (HbS). In deoxygenated conditions, HbS can polymerize eventually leading to the sickling of  
48 the red blood cells (RBC) [1]. SCD patients suffer from severe chronic hemolytic anemia [2] and arterial  
49 desaturation [3,4], resulting in deficient blood oxygen carriage and ultimately in tissue hypoxia [5,6]. This  
50 decreased blood oxygen-carrying capacity may account, at least partly, for alterations in muscle structure,  
51 function and energetics [7–14]. Such alterations have been reported in both patients [7–10] and mice with  
52 SCD [11–14]. Because muscle structure, function and energetics are determinant for the quality of life of  
53 patients [15], any strategy which could mitigate these muscular defects could be of interest.

54 Recent studies have suggested that regular physical activity could be considered as an interesting  
55 complementary therapeutic strategy in SCD. In patients, moderate-intensity endurance-exercise has been  
56 reported as safe and has been shown to improve patients' physical ability, capillary network and oxidative  
57 enzymes activities [16–18]. Functional and metabolic improvements after endurance training have been also  
58 observed in 7-month-old Townes SCD mice [19]. In addition, in another SCD mice model (SAD mice),  
59 endurance training has been shown to mitigate systemic inflammation [20], a potential trigger of main SCD-  
60 related complications [21], and severe organ damages [22]. Yet, in a context of anemia, the effects of  
61 endurance training on muscle function and energetics were reported as limited compared to the  
62 corresponding effects in healthy individuals [23] so that muscle function and energetics of trained anemic  
63 individuals did not reach the level of non-anemic untrained subjects [23].

64 Hydroxyurea (HU) is a pharmacological treatment commonly used in SCD. HU can reactivate fetal  
65 hemoglobin (HbF) production [24,25] thereby alleviating some SCD-related symptoms [26]. Indeed, HU  
66 can lead to an increased total Hb content ultimately reducing the relative HbS content [26–31], enhancing  
67 oxygen saturation [32] and hemodynamics [33] and increasing skeletal muscle perfusion [34]. In this  
68 context, HU seems a perfect match for endurance training in SCD patients to maximize the metabolic  
69 adaptations of a training intervention.

70 Additionally, HU treatment has also been related to an improved physical fitness in patients [35] and  
71 has been shown to prevent muscle alterations over time in SCD mice [36]. Considering the HU-mediated  
72 increase muscle perfusion and oxygen supply, one could expect some positive effects of HU on muscle  
73 energetics. To the best of our knowledge, such an effect has never been assessed. Of interest, hyperoxia has  
74 been shown to increase skeletal muscle oxygenation and improve oxidative capacity in trained but not in  
75 sedentary subjects [37,38]. On that basis, it could be hypothesized that endurance training combined with  
76 the HU-mediated muscle perfusion improvement could be translated into amplified positive effects.

77 Overall, endurance training is known to have considerable potential for the restoration of skeletal muscle  
78 function and energetics although this is limited in SCD due to anemia. For this reason, its combination with  
79 HU, which is known to also have its own positive effects, can be a perfect fit to achieve significant  
80 improvement through an enhancement of their individual effectiveness. Indeed, on the one hand, HU can  
81 reduce anemia thereby enhancing skeletal muscle adaptations to endurance training. On the other hand, HU  
82 can increase oxygen supply which can be optimally used in endurance trained individuals. Overall, a  
83 synergistic effect between endurance training and HU may be expected on skeletal muscle function and  
84 energetics. Therefore, the purpose of the present study was to investigate the effects of endurance training  
85 in association with HU treatment on skeletal muscle energetics and function in SCD mice.

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87

88

## Animals and Methods

### 89 **Animal model, care & feeding**

90 Twenty HbSS Townes mice (Jackson Laboratories, Bar Harbor, USA) of 2-month-old were  
91 investigated. Townes mice are a murine model of SCD which express the human hemoglobin gene and  
92 display the typical clinical phenotype of SCD patients including anemia, sickling and organs dysfunction  
93 [39]. The study was performed in conformity with the French guidelines for animal care and in conformity  
94 with the European convention for the protection of vertebrate animals used for experimental purposes and  
95 institutional guidelines n° 86/609/ CEE November 24, 1986. All animal experiments were approved by the  
96 Institutional Animal Care Committee of Aix-Marseille University (permit number n°27800-  
97 2020112517474997). Mice were housed collectively (4-5 mice per cage) in a controlled-environment  
98 facility (12–12 h light–dark cycle, 22 °C) and received standard food *ad libitum*. Mice were also provided  
99 with either water *ad libitum* or an HU treatment. For the latter and as previously described, HU was added  
100 into drinking water at a concentration of 50 mg/kg/day [31,40] which was adjusted daily according to water  
101 intake.

102

### 103 **Study design**

104 In order to assess the effectiveness of the addition of HU to endurance training and because the individual  
105 effects of endurance training on the one hand [19] and HU on the other hand [36] have been already  
106 documented, we presently analyzed the 2-month evolution of HU-supplemented trained and untrained SCD  
107 mice. In that purpose, muscle function and energetics of the whole set of mice were initially assessed at 2-  
108 month-old during a standardized rest-stimulation-recovery protocol. Mice were then randomly assigned to  
109 two groups: SCD<sub>TR</sub> (n = 9) and SCD<sub>TR-HU</sub> (n = 11). Both groups were submitted to an 8-week endurance  
110 training program. While SCD<sub>TR</sub> did not receive any treatment, SCD<sub>TR-HU</sub> received the HU-treatment during  
111 the training period. Muscle function and energetics were then assessed a second time in response to the same  
112 standardized protocol. Each assessment was identified with number 1 and 2 respectively, e.g. SCD<sub>TR1</sub> and  
113 SCD<sub>TR2</sub>. One week after the second assessment, mice were anesthetized with vetflurane in an induction  
114 chamber and then euthanized by cervical dislocation.

### 115 **Animal training**

116 SCD<sub>TR</sub> and SCD<sub>TR-HU</sub> mice were trained 5 days (D) a week for 8 weeks (W) on a treadmill (Columbus  
117 Instruments, Columbus, OH, USA) as previously described [19]. Each training session started with a 5-min

118 warm-up period in order to progressively reach the target speed (*vide infra*). Mice were first submitted to an  
119 acclimatization period over week 1 (W1). At D1, the target speed was 11 m/min for 10 minutes. Running  
120 duration and target speed were then increased by 10 minutes and 1 m/min respectively on a daily basis until  
121 D5. During W2, W3 and W4, mice ran 50 minutes at 15 m/min and the target speed was increased to 16  
122 m/min at W5 and W6 and to 17 m/min at W7 and W8.

### 123 **Non-invasive investigation of posterior hindlimb muscle function and bioenergetics**

124 Investigations were conducted using an in-house experimental setup which has been designed to be  
125 operational inside a PharmaScan® AVANCETM III HD 70/16 US equipped with a 90 mm BGA09S (760  
126 mT/m) gradient insert (Bruker BioSpin MRI GmbH, Ettlinge, Germany). This setup is adapted from a  
127 previously described model used on a 4.7 T MR scanner [41]. It allows (i) to get muscle volumetric  
128 information using MR imaging (MRI), (ii) to assess its corresponding mechanical performance with a  
129 dedicated ergometer consisting of a foot pedal coupled to a force transducer, and (iii) to monitor dynamically  
130 its levels of high-energy phosphorylated compounds and acidosis using <sup>31</sup>P-MRS. The posterior hindlimb  
131 muscles, which form the belly of the calf, were studied because this muscle group i) is clearly distinct from  
132 the other muscles of the leg, ii) is easily accessible for MR coils and large enough to give <sup>31</sup>P-MR spectra in  
133 a short time and with a good signal to noise ratio, and iii) has been shown to be preferentially activated using  
134 a standardized experimental transcutaneous stimulation protocol [41].

### 135 **Animal preparation**

136 Mice were anesthetized in an induction chamber (Equipement vétérinaire Minerve, Esternay, France)  
137 using an air flow (3 L/min) containing 4 % vetflurane. The left hindlimb was shaved and an electrode cream  
138 was applied at the knee and heel regions so as to optimize electrical contacts. Each anesthetized animal was  
139 then positioned supine within the experimental setup [41]. Corneas were protected from drying by applying  
140 ophthalmic cream, and the animal's head was equipped with a facemask continuously supplied with 1-2 %  
141 vetflurane mixed in 66 % room air (1 L/min) and 33 % O<sub>2</sub> (0.5 L/min). Breathing rate was monitored during  
142 the protocol and maintained between 100 and 130 breaths/min thanks to vetflurane concentration  
143 adjustments. The mouse's foot was positioned on the ergometer's pedal and the hindlimb was centered inside  
144 a 16-mm diameter <sup>1</sup>H Helmholtz coil while the belly of the posterior hindlimb muscles was located above  
145 an elliptic (8 x 6 mm<sup>2</sup>) <sup>31</sup>P surface coil. Body temperature was controlled and maintained at a physiological  
146 level throughout the experiment using a feedback loop including an electrical heating blanket, a temperature  
147 control unit (ref. 507137, Harvard Apparatus, Holliston, MA, USA) and a homemade rectal temperature  
148 probe as previously described [41].

## 149 **Muscle contraction and force measurement**

150 Muscle contractions were induced by electrostimulation using two transcutaneous surface electrodes  
151 connected to a constant-current stimulator (DS7A, Digitimer, Hertfordshire, United Kingdom). One  
152 electrode was placed at the heel level and the other one was located just above the knee joint. Electrical  
153 signal coming out from the force transducer of the ergometer was amplified (Two operational amplifiers:  
154 AD620 and OP497, Analog Devices, Norwood, MA, USA, with a total dB gain: 70dB @ 0-5 kHz  
155 bandwidth), continuously monitored and recorded on a personal computer using a dedicated data acquisition  
156 system (Powerlab 16/36, AD Instruments, Sydney, Australia) and a dedicated software (LabChart 8, AD  
157 Instruments, Sydney, Australia). The digital signal was converted to force (mN) according to a linear  
158 calibration curve.

## 159 **Moderate exercise protocol**

160 Function and energetics of the posterior hindlimb muscles were evaluated throughout a 6-min  
161 standardized moderate-intensity exercise consisting of maximal isometric contractions repeated at a  
162 frequency of 1 Hz induced with square wave pulses (0.5 ms duration). Force production was acquired  
163 continuously with the LabChart software (AD Instruments, Oxford, UK) and then processed by a custom-  
164 written MATLAB routine computing mechanical performance parameters. Individual maximal stimulation  
165 intensity was determined by a progressive increase of the stimulus intensity until no further force increase  
166 was observed. The corresponding force was referred to as peak force (Pf, mN). Total force production (TFP,  
167 N) was computed as the sum of the whole set of twitches. Pf and TFP were normalized to the posterior  
168 hindlimb muscles volume ( $\text{mm}^3$ ), as these muscles are preferentially activated during our exercise protocol.  
169 The corresponding volume was computed from anatomic hindlimb MR images and the normalized metrics  
170 were referred to as specific Pf and specific TFP (sPf and sTFP, respectively). Force was analyzed at specific  
171 time points i.e. initial and final (initial and final sPf, respectively) while the maximal recorded value was  
172 referred to as maximal sPf. Additionally, the relative force increase, and the fatigue index were computed.  
173 The relative force increase was measured as the ratio between the maximal and initial force measurements  
174 and the fatigue index was computed as the ratio between the maximal and the final force measurements.  
175 Both values were expressed as percentage (%).

176

## 177 **Multimodal MR data acquisition**

178 Ten consecutive non-contiguous axial slices (1 mm thickness, slice gap = 0.25 mm) were recorded at  
179 rest in order to assess muscle volume. The corresponding region of interest covering the region from the



180 knee to the ankle was selected across the lower hindlimb. RARE images (Rare factor = 8, effective echo  
181 time = 35.29 ms, actual echo time = 11.76 ms, repetition time = 5000 ms, one accumulation, 20 x 20 mm  
182 field of view, 256 x 192 matrix size, acquisition time = 3 min 14 s). Posterior hindlimb muscles region was  
183 manually delineated in each of the 6 largest slices. Then, muscle volume of each slice was extracted using  
184 FSLeaves [42]. Total muscle volume (mm<sup>3</sup>) was calculated using the truncated cone volume formula  
185 considering slice thickness and gap volumes. Multiecho T2-weighted images (16 echo times equally spaced  
186 from 7.28 ms to 116.59 ms, 2000-ms repetition time, one accumulation, 20 x 30 mm field of view, 256 x  
187 256 matrix size, total acquisition time = 8.32 min, slice thickness = 1 mm, slice gap = 1 mm) were recorded  
188 at rest before the rest-exercise-recovery protocol. The T2-weighted images were processed to generate T2  
189 maps on a pixel by pixel basis by fitting the corresponding data with a single exponential function. Mean  
190 T2 values of posterior hindlimb muscles were measured on T2 maps and averaged on the 2 largest  
191 consecutive slices from the outlined regions of interest. The corresponding values were quantified in the  
192 gastrocnemius (GA) and the tibialis anterior (TA) muscles. <sup>31</sup>P-MR spectra (8 kHz sweep width; 2048 data  
193 points) from posterior hindlimb muscles were continuously acquired before (rest period; 4.7 min duration),  
194 during and after (recovery period; 16 min duration) the 6-min moderate stimulation protocol. Spectra  
195 acquisition was gated to muscle electrostimulation to reduce potential motion artifacts due to contraction. A  
196 total of 800 saturated free induction decays (FIDs, 1.875 s repetition time) were acquired. The first 140 FIDs  
197 were acquired in resting muscle and summed together. The next 450 FIDs were acquired during exercise  
198 and were summed as blocks of 15, allowing a 30 s temporal resolution to assess the PCr time-dependent  
199 changes. Due to reduced signal to noise ratio for the Pi signal, FIDs were also summed as block of 30 to  
200 assess pH<sub>i</sub> changes with respect to time (1 min temporal resolution). The remaining 210 FIDs were recorded  
201 during the recovery period and were summed as blocks of 30 FIDs.

## 202 **MRS data processing**

203 MRS data were processed using Python custom-written routines [43] integrating the AMARES Fortran  
204 code [44] from jMRUI (<http://www.jmrui.eu/>). Relative concentrations of phosphocreatine (PCr), inorganic  
205 phosphate (Pi) and ATP were obtained from <sup>31</sup>P-MR spectra using the AMARES-MRUI-based time domain  
206 fitting routine including appropriate prior knowledge for the ATP multiplets [43]. Absolute amounts of  
207 phosphorylated compounds were expressed taking into account a 5-mM β-ATP basal concentration [45] and  
208 were corrected taking into account T1 saturation effects for PCr and ATP. The PCr relative concentration  
209 was expressed relative to the PCr content at rest which was set at 100%. Intracellular pH was calculated  
210 from the chemical shift of the Pi signal relative to PCr according to the formula [46]:  $pH_i = 6.75 + \log[(3.27 - \delta Pi) / (\delta Pi - 5.69)]$ . Cytosolic ADP concentration was calculated, as previously described [47,48]:  $[ADP] = ([Cr] \times [ATP]) / ([PCr] \times 10^{-pH} \times K_{CK})$ , using the creatine kinase equilibrium constant ( $K_{CK} = 1.66 \times 10^9$  M<sup>-1</sup>

213 <sup>1</sup>) [49] and total creatine [Cr] calculated by assuming that PCr represents ~85% of the total Cr content [50].  
214 The rate of PCr consumption ( $V_{PCr_{stim}}$ , in mM/min) at the onset of exercise was calculated as  $V_{PCr_{stim}} =$   
215  $\Delta PCr / \tau PCr_{stim}$ , where  $\Delta PCr$  is the extent of PCr depletion measured at the end of stimulation (relative to  
216 basal value) and  $\tau PCr_{stim}$  is the time constant of PCr degradation.  $\tau PCr_{stim}$  was determined by fitting the time  
217 course of PCr to a mono-exponential function using a least-means-squared algorithm. sPf-normalized non-  
218 oxidative cost of contraction ( $PCr_{cost}$  in  $\mu M/min/mN/cm^3$ ) was calculated as the ratio between  $V_{PCr_{stim}}$  and  
219 the amount of specific force produced at the beginning of exercise, considering that at that time PCr is the  
220 only source of ATP production. Similarly, the PCr recovery parameter ( $\tau PCr_{rec}$ ) was quantified during the  
221 post-exercise period by fitting the time course of PCr resynthesis to a mono-exponential function. As  
222 previously suggested,  $V_{PCr_{rec}}$  (in mM/min) was considered as the rate of oxidative ATP production at the  
223 end of exercise as  $V_{PCr_{rec}} = \Delta PCr / \tau PCr_{rec}$  [51,52]. sPf-normalized oxidative cost of contraction ( $PCr_{rec}$  in  
224  $\mu M/min/mN/cm^3$ ) was calculated as the ratio between  $V_{PCr_{rec}}$  and the amount of specific force produced at  
225 the end of exercise. The maximal rate of oxidative ATP resynthesis ( $V_{max}$ , mM/min), identified as the muscle  
226 oxidative capacity, was calculated as previously described:  $V_{max} = V_{PCr_{rec}} (1 + (K_m / [ADP_6]))$  [53], where  
227  $K_m$  is the affinity constant of the mitochondria for ADP (30  $\mu M$  in gastrocnemius muscle of the mice), and  
228  $[ADP_6]$  is the ADP concentration at the end of exercise.

229

## 230 **Statistics**

231 All values are presented as means  $\pm$  SD except on graphics for which SE are displayed for the sake of  
232 clarity. Sample distribution was tested with the Shapiro-Wilk test. Considering that our main purpose was  
233 to identify the improvements HU could achieve in SCD trained mice compared to training alone, the  
234 statistical analyses were performed to identify the 8-week effects of each intervention i.e.,  $SCD_{TR1}$  vs.  
235  $SCD_{TR2}$  and  $SCD_{TR-HU1}$  vs.  $SCD_{TR-HU2}$ . The additional or synergistic effect has been recognized when a given  
236 significant change occurred in the  $SCD_{TR-HU}$  group but not in the  $SCD_{TR}$  group. Differences were assessed  
237 using paired non-parametric Wilcoxon tests or parametric Student's t-tests according to the result of a  
238 Shapiro-Wilk normality test. All statistics were performed using R (RStudio). The significance level was set  
239 at  $p < 0.05$ . Exact p-values are reported, except for results applying to multiple groups where a combined p-  
240 value was given for the sake of clarity (NS,  $p > 0.05$  or  $p < 0.05$ ).

241

242

243

## Results

244

### 245 **Muscle volume and T2 relaxation times**

246 As summarized in table 1, body weight increased in each group over the 8-week period ( $p < 0.05$ ).  
247 Muscle volume also significantly increased over the 8-week period in SCD<sub>TR</sub> mice ( $p < 0.001$ ) but did not  
248 change in SCD<sub>TR-HU</sub> mice (NS,  $p = 0.17$ ). The relative muscle volume calculated as the muscle volume scaled  
249 to body weight did not change over the 8-week period in SCD<sub>TR</sub> mice (NS,  $p > 0.05$ ) but did decrease for  
250 the combined SCD<sub>TR-HU</sub> experimental condition ( $p < 0.001$ ).

251 Over the same period, the average T2 value of tibialis anterior (TA) and gastrocnemius (GA) muscles  
252 significantly decreased in each condition (table 1,  $p < 0.05$ ).

253

### 254 **Muscle function**

255 The sPf time-course was classically biphasic with an initial increase followed by a progressive decrease  
256 until the end of the stimulation period (figure 1A-B). sPf reached its maximum value at around 2 minutes of  
257 stimulation.

258 The initial, maximal, and final force production sPf were significantly increased after the 8-week period  
259 in each group ( $p < 0.05$ , table 1). The fatigue index was unchanged during the training intervention (SCD<sub>TR2</sub>,  
260  $-2 \% \pm 14$ ,  $p = 1.00$ ). On the contrary, the combined HU and endurance training intervention resulted in a  
261 significant decrease of the fatigue index (SCD<sub>TR-HU2</sub>,  $-12 \% \pm 16$ ,  $p = 0.02$ , figure 1D).

262 As illustrated in figure 1C, sTFP was significantly higher after the 8-week period in both SCD<sub>TR2</sub> ( $+52$   
263  $\% \pm 42$ ,  $p = 0.0013$ ) and SCD<sub>TR-HU2</sub> ( $+34 \% \pm 26$ ,  $p = 0.0007$ ) compared to their initial measurements  
264 (SCD<sub>TR1</sub> and SCD<sub>TR-HU1</sub>, respectively).

265

### 266 **Posterior hindlimb muscle bioenergetics**

#### 267 *PCr*

268 As illustrated in figure 2A-B, during the standardized rest-exercise protocol, the relative PCr time course  
269 classically displayed 3 different phases i.e. first, an initial progressive decrease during the first 4 min of  
270 exercise followed by a second slower one ending by a last period during which a progressive return towards  
271 baseline values could be observed.

272 PCr consumption ( $\Delta$ PCr) was unchanged by the training intervention only. On the contrary, the  
273 corresponding parameter was significantly decreased in the SCD<sub>TR-HU</sub> group over the 8-week TR and HU  
274 co-intervention period (table 1).

275 After the 8-week period, the ADP concentration at the end of exercise was significantly increased in  
276 both the SCD<sub>TR</sub> and SCD<sub>TR-HU</sub> groups ( $+50 \pm 72$  %,  $p = 0.033$  and  $+40 \pm 54$  %,  $p = 0.048$ , respectively).

277 The rate of PCr consumption ( $V_{PCr_{stim}}$ ) remained unchanged after the 8-week period in both groups  
278 (with an average of  $+0.05 \pm 1.73$  mM/min, table 1). On the contrary, the rate of PCr recovery ( $V_{PCr_{rec}}$ ) was  
279 significantly accelerated after TR ( $+1.17 \pm 1.25$  mM/min,  $p = 0.0078$ ) and TR-HU intervention ( $+1.47 \pm$   
280  $1.32$  mM/min,  $p = 0.0049$ , table 1).

281 The maximal rate of PCr recovery,  $V_{max}$ , remained unchanged over the 8-week period in SCD<sub>TR</sub> mice  
282 ( $+1.18 \pm 2.29$  mM/min,  $p > 0.05$ ) but increased significantly in SCD<sub>TR-HU</sub> over the same period ( $+2.11 \pm$   
283  $2.38$  mM/min,  $p = 0.027$ ).

## 284 $pH_i$

285  $pH_i$  at rest was similar before and after the intervention for both conditions ( $p > 0.05$ , table 1).

286 As illustrated in figure 2C-D, during the stimulation period, a transient alkalinization was observed,  
287 followed by a  $pH_i$  drop. The acidotic phase was initially fast and then slower. When the stimulation ceased,  
288  $pH_i$  progressively increased toward resting values.

289 The extent of intracellular acidosis ( $\Delta pH_i$ ) in response to the stimulation period was not significantly  
290 different between the groups (table 1). The  $pH_i$  at end of the recovery period,  $pH_{i_{end}}$ , was not significantly  
291 different over the 8-week period in SCD<sub>TR</sub> group ( $+1.32 \pm 2.76$ ,  $p = 0.88$ ). On the contrary,  $pH_{i_{end}}$  was  
292 significantly higher after the combined training-HU intervention ( $+2.01 \pm 2.20$  %,  $p = 0.025$ ).

293

294

## Discussion

295

296 In the present study, we aimed at determining whether HU can improve endurance training effects by  
297 addition or synergia on skeletal muscle function and energetics in a sickle cell disease murine model.  
298 According to our initial hypotheses, a synergistic effect has been identified in SCD<sub>TR-HU</sub> for multiple  
299 parameters. During the standardized exercise part, both the fatigue index and PCr breakdown were reduced.  
300 During the post-exercise recovery period, the maximal aerobic capacity ( $V_{max}$ ) was improved. Of note, mice  
301 submitted to the combined intervention did not show any increase in muscle volume over the 8-week period  
302 whereas the trained mice did.

303 The present results are supportive of the expected effects of endurance training. Indeed, oxidative ATP  
304 production was improved in SCD<sub>TR</sub> mice as illustrated by the increased  $VPCr_{rec}$  and so in agreement with  
305 the previously reported improved oxidative capacity [54,55] and faster PCr resynthesis [56–59]. At the same  
306 time, an increased force production was measured. This increased performance is in agreement with previous  
307 results showing an increased force production in SCD<sub>mice</sub> after an 8-week endurance training program [19].  
308 One could have also expected an increased  $V_{max}$ , a recognized index of oxidative function and maximal  
309 aerobic capacity [53] as previously reported [54,55]. This was not observed in the present study. Overall,  
310 these results further support the positive effects of training on oxidative muscle function of SCD mice  
311 [54,55].

312 The rationale of this study was based on the fact that endurance training is known to improve muscle  
313 function and energetics in SCD mice [19] with limited effects in anemic individuals. We hypothesized that  
314 the positive effects of HU could overcome this issue [36] given that previous results were supporting this  
315 idea [26–30,34]. We aimed at identifying whether synergistic effects could be identified when using both  
316 interventions at the same time. This synergy was confirmed when changes occurred as a result of the  
317 combined intervention whereas no change was identified for a single condition and when literature does not  
318 support any effects of HU on the considered parameters.

319 The combined interventions led to changes during the standardized exercise i.e. a reduced fatigue index  
320 and a reduced PCr consumption and the following recovery period i.e. an increased  $V_{max}$ . Different  
321 interactions between endurance training and HU could account for these synergetic effects. HU has been  
322 recognized to alleviate anemia and improve muscle perfusion and oxygen supply [26–30,34], both of which  
323 contributing to improve the condition of SCD patients. On the one hand, training has been reported as less  
324 effective in anemic rats than in control rats [23]. The recognized HU-mediated effects on anemia would be  
325 expected to enhance the beneficial effects of training. On the other hand, it has been previously evidenced

326 that endurance-trained individuals were more responsive to an increased oxygen supply [60]. More  
327 specifically, the beneficial effect of hyperoxia on mitochondrial function has been identified in trained  
328 subjects only and not in sedentary counterparts [37,38]. Therefore, the HU-mediated increased oxygen  
329 supply could then be optimally used in trained individuals. Overall, these synergetic effects would strongly  
330 support the hypothesis that hydroxyurea (HU) supplementation enhances training-induced adaptations in  
331 skeletal muscle and can most likely be explained through the two mentioned effects.

332 More particularly, our study indicated a fatigue index reduction which has been previously related to  
333 endurance training [61,62]. Endurance training could partly restore muscle oxygenation through capillary  
334 supply [63,64] thereby contributing to lowering muscle fatigue [65]. The effects of HU on fatigue are still a  
335 matter of discussion [36,66]. In a protocol similar to the one presented here, no effect of HU has been  
336 reported on fatigue in SCD mice [36] whereas in SCD patients, HU has been reported to increase the time  
337 before exhaustion and so likely as a result of the higher Hb level [66]. In the present study, endurance training  
338 alone was not sufficient to reduce fatigue. However, as the combination with HU did, one can hypothesize  
339 that the combination of HU with training may have potentiated the training effects and led to a reduced  
340 fatigue through an anemia reduction and an increased oxygen supply [26–30,34].

341 Regarding the reduced PCr consumption, one must keep in mind that PCr changes illustrate the balance  
342 between the ongoing PCr consumption and resynthesis. On that basis, the corresponding reduction can be  
343 related to a reduction of the catabolic process i.e. PCr demand or as an increase of PCr resynthesis. A reduced  
344 PCr consumption has been previously reported in SCD mice submitted to an endurance training program  
345 [19,67] further supporting an enhanced contractile efficiency although the exact mechanisms have not been  
346 identified. An improved PCr resynthesis rate illustrates a higher oxidative contribution to ATP production  
347 as previously described as a result of training [56–59,68,69]. In the present study,  $V_{PCr_{rec}}$  was increased in  
348 both SCD<sub>TR</sub> and SCD<sub>TR-HU</sub> thereby indicating no synergistic effect. On the contrary,  $V_{max}$ , an index of the  
349 maximal aerobic capacity [53], was only increased in the SCD<sub>TR-HU</sub> group.  $V_{max}$  can be understood as the rate  
350 of aerobic ATP production in a given condition of end-of-exercise ADP, a known activator of aerobic ATP  
351 production. Overall, the combination of HU with endurance training maximized the metabolic adaptations  
352 contributing to a significant improvement of the maximal aerobic capacity,  $V_{max}$ . Indeed,  $V_{max}$  is related to  
353 both  $V_{PCr_{rec}}$  and  $[ADP]_{end}$ . Although  $V_{PCr_{rec}}$  and  $[ADP]_{end}$  increased significantly in both SCD<sub>TR</sub> and  
354 SCD<sub>TR-HU</sub>, the improvement of  $V_{max}$  only in SCD<sub>TR-HU</sub> highlights the fact that the combination of HU and  
355 endurance training allowed different modifications and ultimately improved the maximal aerobic capacity.

356 Over the 8-week period, muscle volume increased as a result of training (SCD<sub>TR</sub>) but not when training  
357 and HU supplementation were combined (SCD<sub>TR-HU</sub>) whereas body weight increased in both groups. In a

358 recent study, Hill *et al* (2020) reported that muscle cross sectional area and muscle mass increased up to 52  
359 weeks of age in control mice [70]. Our results might indicate that, in SCD mice, the combination of  
360 endurance training and HU would interfere with growth whereas training only would not. However, it has  
361 been shown that repeated high dose (205 mg/kg) of HU did not prevent muscle growth *in vitro* [71] and that  
362 patients with and without HU displayed the same lean mass over 12 months [35]. Overall, the absence of  
363 muscle growth in SCD<sub>TR-HU</sub> mice can hardly be related to a negative effect of HU. Taking into account that  
364 both groups reached comparable muscle volume after the 8-week period, one can think that the reported  
365 differences in muscle volume evolution might originate from an earlier muscle growth in SCD<sub>TR-HU</sub> mice.  
366 Another unknown synergistic effect might also have occurred.

### 367 ***Limitations***

368 We have to acknowledge the absence of Hb measurement and of an untrained group as a limitation of  
369 the present study. First, Townes mice are largely acknowledged as anemic with a low hemoglobin  
370 concentration ( $7.1 \pm 0.9$  g/dL) [39]. A large range of Hb concentrations has been reported in this model  
371 (from  $\sim 6 \pm 1$  g/dL to  $10.06 \pm 0.7$  g/dL) [31,72]. Nevertheless, thanks to our longitudinal assessment where  
372 each mouse is his own control, we can consider any severity of anemia and focus on the analysis of the  
373 individual evolution of a mouse in response to endurance training and an additional HU supplementation.  
374 We considered that such a group would not have been informative regarding the synergistic effects of  
375 training and HU supplementation. For obvious ethical reasons, we have decided to keep the number of  
376 animals investigated as small as possible. We did not assess the time effects which could also partly account  
377 for the results reported as a result of endurance training.

378

379

## **Conclusion**

380 Synergistic effects of endurance training and hydroxyurea treatment have been observed for skeletal  
381 muscle function and energetics in a sickle cell disease mice model. The co-intervention minimized fatigue,  
382 reduced the PCr consumption and improved the muscle maximal aerobic capacity. Such an enhanced aerobic  
383 capacity in patients would be expected to improve daily life and quality of life. The reduced fatigue should  
384 also be a key factor for patients given that fatigue has been described as a major symptom of the disease  
385 affecting daily life.

386

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392 **Data availability**

393 Data will be made available upon reasonable request.

394



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## 613 Figures

614 **Figure 1:** Muscle function parameters with: time-dependent changes in specific peak force throughout  
615 the 6-min moderate stimulation protocol for SCD<sub>TR</sub> (A) and SCD<sub>TR-HU</sub> mice (B), presented as mean ± SEM,  
616 and specific total force production quantified throughout the 6-minute moderate stimulation protocol of each  
617 condition before and after intervention (C) and fatigue index (%) in each condition (D), presented as  
618 boxplots. SCD<sub>TR</sub>: mice before (1) and after (2) an 8-week endurance training period. SCD<sub>TR-HU</sub>: mice before  
619 (1) and after (2) an 8-week endurance training combined to an HU treatment period.

620 **Figure 2:** Muscle metabolic parameters with time-dependent changes in phosphocreatine (PCr) during  
621 the 6-min moderate stimulation protocol for endurance training (A) and combined intervention (B) and the  
622 time-dependent changes in intracellular pH (pHi) for SCD<sub>TR</sub> (C) and SCD<sub>TR-HU</sub> mice (D) presented as means  
623 ± SEM. SCD<sub>TR</sub>: mice before (1) and after (2) an 8-week endurance training period. SCD<sub>TR-HU</sub>: mice before  
624 (1) and after (2) an 8-week endurance training combined to an HU treatment period.

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## 626 Tables

627 Table 1: Anatomical, functional and metabolic characteristics in SCD<sub>TR</sub> and SCD<sub>TR-HU</sub> groups.

	SCD <sub>TR1</sub>	SCD <sub>TR2</sub>	SCD <sub>TR-HU1</sub>	SCD <sub>TR-HU2</sub>
<i>Anatomical characteristics</i>				
Body weight (g)	30.70 ± 1.42	33.77 ± 1.89***	29.68 ± 1.49	34.07 ± 2.19***
Hindlimb muscle volume (mm <sup>3</sup> )	124.08 ± 6.54	140.22 ± 6.49***	133.40 ± 9.00	138.45 ± 11.54

Relative hindlimb muscle volume (mm <sup>3</sup> /g)	4.04 ± 0.18	4.16 ± 0.28	4.50 ± 0.25	4.06 ± 0.16***
<b><i>T2 muscles values</i></b>				
Tibialis Anterior (ms)	26.98 ± 0.88	26.28 ± 0.44*	28.52 ± 1.75	25.36 ± 3.69*
Gastrocnemius (ms)	32.30 ± 1.41	31.08 ± 1.12*	35.14 ± 3.43	32.17 ± 0.96*
<b><i>Muscle function characteristic</i></b>				
Initial force (mN/cm <sup>3</sup> )	524.98 ± 113.52	787.47 ± 235.02*	488.98 ± 111.07	612.51 ± 138.75**
Force increase (%)	17.22 ± 8.47	18.66 ± 14.86	18.94 ± 8.76	18.02 ± 15.73
Maximal force (mN/cm <sup>3</sup> )	630.50 ± 131.67	907.92 ± 172.44**	658.72 ± 129.98	708.76 ± 121.71*
Final force (mN/cm <sup>3</sup> )	501.27 ± 156.35	725.26 ± 117.32**	414.08 ± 98.57	590.74 ± 108.59**
Fatigue index (%)	-21.62 ± 12.43	-19.47 ± 7.69	-28.39 ± 8.71	-16.46 ± 7.74*
sTFP (N/cm <sup>3</sup> )	205.03 ± 47.53	297.25 ± 56.15**	180.33 ± 39.92	232.06 ± 42.20*
<b><i>Muscle metabolic indices</i></b>				
pH <sub>i rest</sub>	7.17 ± 0.18	7.22 ± 0.11	7.19 ± 0.14	7.17 ± 0.17
Δ pH <sub>i</sub>	-0.24 ± 0.11	-0.26 ± 0.12	-0.30 ± 0.11	-0.23 ± 0.15
Δ pH <sub>i</sub> / sTFP	-0.0012 ± 0.0007	-0.0009 ± 0.0005	-0.0020 ± 0.0007	-0.0010 ± 0.0006*
pH <sub>i end</sub>	7.17 ± 0.18	7.26 ± 0.04	7.13 ± 0.15	7.27 ± 0.10*
Δ PCr (%)	-68.27 ± 6.22	-68.91 ± 4.91	-72.46 ± 5.04	-67.84 ± 5.71**
Δ PCr / sTFP	-0.36 ± 0.12	-0.24 ± 0.04*	-0.43 ± 0.12	-0.30 ± 0.05*

ADP <sub>6</sub> (μM)	41.03 ± 16.69	52.25 ± 10.56*	42.97 ± 10.20	59.44 ± 23.20*
VPCr <sub>stim</sub> (mM/min)	4.59 ± 1.66	4.56 ± 1.22	4.91 ± 1.57	5.41 ± 1.58
VPCr <sub>rec</sub> (mM/min)	2.01 ± 0.60	3.18 ± 1.03**	2.16 ± 0.59	3.63 ± 1.05*
V <sub>max</sub> (mM/min)	3.81 ± 1.62	4.99 ± 1.33	3.70 ± 0.88	5.81 ± 2.14*

628 *Data are presented as means ± SD, \* Significantly different from the pre-intervention condition \*(p < 0.05), \*\**  
629 *(p < 0.005), \*\*\*(p < 0.0005). SCD<sub>TR</sub>: mice before (1) and after (2) an 8-week endurance training period. SCD<sub>TR-HU</sub>:*  
630 *mice before (1) and after (2) an 8-week endurance training combined to an HU treatment period. sTFP, specific total*  
631 *force production; VPCr<sub>stim</sub>, rate of PCr consumption; VPCr<sub>rec</sub>, rate of PCr resynthesis; pH<sub>iend</sub> : intracellular pH at*  
632 *the end of the recovery period; ADP<sub>6</sub>: ADP quantity at the end of the exercise i.e. 6 minutes of stimulation.*

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Table 1: Anatomical, functional and metabolic characteristics in SCD<sub>CON</sub>, SCD<sub>TR-HU</sub> and SCD<sub>TR</sub> groups.

	SCD <sub>CON1</sub>	SCD <sub>CON2</sub>	SCD <sub>TR1</sub>	SCD <sub>TR2</sub>	SCD <sub>TR-HU1</sub>	SCD <sub>TR-HU2</sub>
<b>Anatomical characteristics</b>						
Body weight (g)	27.48 SD 2.08	33.08 SD 1.98*	30.70 SD 1.42	33.77 SD 1.89***	29.68 SD 1.49	34.07 SD 2.19***
Hindlimb muscle volume (mm <sup>3</sup> )	111.83 SD 15.82	134.17 SD 13.98*	124.08 SD 6.54	140.22 SD 6.49***	133.40 SD 9.00	138.45 SD 11.54
Relative hindlimb muscle volume (mm <sup>3</sup> /g)	4.05 SD 0.31	4.05 SD 0.30	4.04 SD 0.18	4.16 SD 0.28	4.50 SD 0.25	4.06 SD 0.16***
<b>T2 muscles values</b>						
Tibialis Anterior (ms)	26.69 SD 0.44	25.93 SD 0.69*	26.98 SD 0.88	26.28 SD 0.44*	28.52 SD 1.75	25.36 SD 3.69*
Gastrocnemius (ms)	32.27 SD 0.80	30.81 SD 1.36*	32.30 SD 1.41	31.08 SD 1.12*	35.14 SD 3.43	32.17 SD 0.96*
<b>Muscle function characteristic</b>						
Initial force (mN/cm <sup>3</sup> )	484.20 SD 141.49	765.61 SD 189.94**	524.98 SD 113.52	787.47 SD 235.02*	488.98 SD 111.07	612.51 SD 138.75**
Force increase (%)	41.21 SD 37.54	16.44 SD 12.56	17.22 SD 8.47	18.66 SD 14.86	18.94 SD 8.76	18.02 SD 15.73
Maximal force (mN/cm <sup>3</sup> )	655.41 SD 161.42	882.98 SD 189.10**	630.50 SD 131.67	907.92 SD 172.44**	658.72 SD 129.98	708.76 SD 121.71*
Final force (mN/cm <sup>3</sup> )	535.15 SD 144.14	653.58 SD 167.53	501.27 SD 156.35	725.26 SD 117.32**	414.08 SD 98.57	590.74 SD 108.59**
Fatigue index (%)	-18.12 SD 12.05	-26.65 SD 4.38	-21.62 SD 12.43	-21.62 SD 12.43	-28.39 SD 8.71	-16.46 SD 7.74*
sTFP (N/cm <sup>3</sup> )	210.67 SD 48.60	276.40 SD 62.12*	205.03 SD 47.53	297.25 SD 56.15**	180.33 SD 39.92	232.06 SD 42.20*
<b>Muscle metabolic indices</b>						
pH <sub>i rest</sub>	7.16 SD 0.12	7.12 SD 0.19	7.17 SD 0.18	7.22 SD 0.11	7.19 SD 0.14	7.17 SD 0.17
Δ pH <sub>i</sub>	-0.27 SD 0.10	-0.27 SD 0.09	-0.24 SD 0.11	-0.26 SD 0.12	-0.30 SD 0.11	-0.23 SD 0.15



$\Delta \text{pH}_i / \text{sTFP}$	-0.0013 SD 0.0003	-0.0010 SD 0.0002	-0.0012 SD 0.0007	-0.0009 SD 0.0005	-0.0020 SD 0.0007	-0.0010 SD 0.0006*
$\text{pH}_{i \text{ end}}$	7.19 SD 0.18	7.34 SD 0.08	7.17 SD 0.18	7.26 SD 0.04	7.13 SD 0.15	7.27 SD 0.10*
$\Delta \text{PCr} (\%)$	-71.76 SD 8.65	-79.79 SD 5.60	-68.27 SD 6.22	-68.91 SD 4.91	-72.46 SD 5.04	-67.84 SD 5.71**
$\Delta \text{PCr} / \text{sTFP}$	-0.36 SD 0.12	-0.31 SD 0.11	-0.36 SD 0.12	-0.24 SD 0.04*	-0.43 SD 0.12	-0.30 SD 0.05*
$\text{ADP}_6 (\mu\text{M})$	52.75 SD 24.02	81.66 SD 36.34	41.03 SD 16.69	52.25 SD 10.56*	42.97 SD 10.20	59.44 SD 23.20*
$\text{VPCr}_{\text{stim}} (\text{mM}/\text{min})$	5.13 SD 1.43	4.63 SD 0.54	4.59 SD 1.66	4.56 SD 1.22	4.91 SD 1.57	5.41 SD 1.58
$\text{VPCr}_{\text{rec}} (\text{mM}/\text{min})$	2.34 SD 0.80	3.17 SD 0.50	2.01 SD 0.60	3.18 SD 1.03**	2.16 SD 0.59	3.63 SD 1.05*
$V_{\text{max}} (\text{mM}/\text{min})$	3.80 SD 1.01	4.57 SD 1.14	3.81 SD 1.62	4.99 SD 1.33	3.70 SD 0.88	5.81 SD 2.14*

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*Data are presented as means SD, \* Significantly different from the pre-intervention condition \*( $p < 0.05$ ), \*\* ( $p < 0.005$ ), \*\*\*( $p < 0.0005$ ).  $\text{SCD}_{\text{CON}}$ : mice before (1) and after (2) an 8-week control period.  $\text{SCD}_{\text{TR}}$ : mice before (1) and after (2) an 8-week endurance training period.  $\text{SCD}_{\text{TR-HU}}$ : mice before (1) and after (2) an 8-week endurance training combined to an HU treatment period.  $\text{sTFP}$ , specific total force production;  $\text{VPCr}_{\text{stim}}$ , rate of PCr consumption;  $\text{VPCr}_{\text{rec}}$ , rate of PCr resynthesis;  $\text{pH}_{i \text{ end}}$  : intracellular pH at the end of the recovery period;  $\text{ADP}_6$ : ADP quantity at the end of the exercise i.e. 6 minutes of stimulation.*

Table 2: Relative indexes variations over the 8-week period in each group.

	SCD <sub>CON</sub>	SCD <sub>TR</sub>	SCD <sub>TR-HU</sub>
Body weight (%)	+ 20.75 SD 10.23	+ 10.07 SD 5.63	+ 14.74 SD 3.21
T2 values in TA (%)	- 2.79 SD 3.77	- 2.53 SD 2.79	- 10.35 SD 18.03
T2 values in GA (%)	- 4.40 SD 6.24	- 3.65 SD 4.39	- 7.85 SD 7.45
Muscle volume (%)	+ 20.93 SD 10.40	+ 13.07 SD 3.08	
Initial force (%)	+ 68.30 SD 59.62	+ 53.45 SD 41.11	26.74 SD 20.90
Maximal force (%)	+ 36.90 SD 20.61	49.62 SD 40.48	25.74 SD 24.88
Final force (%)		58.49 SD 56.73	49.14 SD 38.65
sTFP (%)	+ 32.30 SD 18.51	51.63 SD 42.34	32.45 SD 25.79
VPC <sub>rec</sub> (mM/min)		+1.17 SD 1.25	+1.47 + 1.32
Δ PCr / sTFP		+ 0.12 SD 0.12	+ 0.13 SD 0.09

*Results are presented as means SD. No significant variation was found. TFP, total force production; TA: tibialis anterior muscle; GA: gastrocnemius muscle; SCD<sub>CON</sub>: mice submitted to a control period; SCD<sub>TR</sub>: mice submitted to a training period; SCD<sub>TR-HU</sub>: mice submitted to a combined training and HU-treatment period.*



