



**HAL**  
open science

## Effects of hydroxyurea on skeletal muscle energetics and force production in a sickle cell disease murine model

Constance Michel, David Bendahan, Benoit Giannesini, Christophe Vilmen, Yann Le Fur, Laurent Messonnier

### ► To cite this version:

Constance Michel, David Bendahan, Benoit Giannesini, Christophe Vilmen, Yann Le Fur, et al.. Effects of hydroxyurea on skeletal muscle energetics and force production in a sickle cell disease murine model. *Journal of Applied Physiology*, 2023, 134 (2), pp.415-425. 10.1152/jappphysiol.00333.2022 . hal-04250804

**HAL Id: hal-04250804**

**<https://hal.science/hal-04250804>**

Submitted on 2 May 2024

**HAL** is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

1 **Effects of hydroxyurea on skeletal muscle energetics and force**  
2 **production in a sickle cell disease murine model.**

3 Constance P Michel<sup>1\*</sup>, David Bendahan<sup>1</sup>, Benoit Giannesini<sup>1</sup>, Christophe Vilmen<sup>1</sup>, Yann Le Fur<sup>1</sup>,  
4 Laurent A Messonnier<sup>2</sup>

5 <sup>1</sup>Aix-Marseille Université, CNRS, CRMBM, Marseille, France.

6 <sup>2</sup>Université Savoie Mont Blanc, Laboratoire Interuniversitaire de Biologie de la Motricité EA7424,  
7 Chambéry, France.

8 \*corresponding author:

9 Email: [constance.michel@univ-amu.fr](mailto:constance.michel@univ-amu.fr)

10 Postal address:

11 CRMBM, UMR 7339

12 Faculté de Médecine

13 27 boulevard Jean Moulin

14 13005, Marseille, France

15 Declarations of interest: none

16

## 17 **Abstract**

18 Hydroxyurea (HU) is commonly used as a treatment for Sickle Cell Disease (SCD) patients in order to  
19 enhance fetal hemoglobin production. This increased production is expected to reduce anemia (which  
20 depresses oxygen transport) and abnormal Hb content alleviating clinical symptoms such as vaso-occlusive  
21 crisis and acute chest syndrome. The effects of HU on skeletal muscle bioenergetics *in vivo* are still  
22 unknown. Due to the beneficial effects of HU upon oxygen delivery, improved skeletal muscle energetics  
23 and function in response to a HU treatment have been hypothesized. Muscle energetics and function were  
24 analyzed during a standardized rest-exercise-recovery protocol, using <sup>31</sup>P-Magnetic Resonance  
25 Spectroscopy in Townes SCD mice. Measurements were performed in three groups of mice: one group of  
26 2-month-old mice (SCD<sub>2m</sub>, n = 8), another one of 4-month-old mice (SCD<sub>4m</sub>, n = 8) and a last group of 4-  
27 month-old mice that have been treated from 2 months of age with HU at 50 mg/kg/day (SCD<sub>4m-HU</sub>, n = 8).  
28 As compared to SCD<sub>2m</sub> mice, SCD<sub>4m</sub> mice were heavier and displayed a lower acidosis. As lower specific  
29 forces were developed by SCD<sub>4m</sub> compared to SCD<sub>2m</sub>, greater force-normalized phosphocreatine  
30 consumption and oxidative and non-oxidative costs of contraction were also reported. HU treated mice  
31 (SCD<sub>4m-HU</sub>) displayed a significantly higher specific force production as compared to untreated mice  
32 (SCD<sub>4m</sub>) whereas muscle energetics was unchanged. Overall, our results support a beneficial effect of HU  
33 on muscle function.

34

35 Keyword: Sickle Cell Disease, Hydroxyurea, MR Spectroscopy, Skeletal muscle bioenergetics, force

36 New & Noteworthy: Our results highlighted that force production decreases between two and four  
37 months of age in SCD mice thereby indicating a decrease of muscle function during this period. Of interest,  
38 HU treatment seemed to blunt the observed age-effect given that SCD<sub>4m-HU</sub> mice displayed a higher specific  
39 force production as compared to SCD<sub>4m</sub> mice. In that respect, HU treatment would help to maintain a higher  
40 capacity of force production during aging in SCD.

41

42

## 43 **Introduction**

44 Sickle cell disease (SCD) is an inherited hemoglobinopathy resulting from a single point mutation in the  
45  $\beta$ -globin chain gene causing the substitution of valine for glutamic acid at the sixth amino acid position,  
46 hence leading to abnormal hemoglobin (HbS) synthesis (1). SCD includes homozygous HbSS genotype but  
47 also combination of HbS with less common variants such as HbC or other hemoglobinopathies such as  $\beta$ -  
48 thalassemia (such as HbS $\beta^0$ ). The primary pathological event in SCD is the polymerization of deoxygenated  
49 HbS during which the normal biconcave disc-shaped red blood cell (RBC) turns into a rigid and fragile  
50 sickle-shaped RBC. Vaso-occlusive crises (VOCs) and hemolytic anemia constitute the major complications  
51 of SCD (1). VOCs result from the entrapment of adherent sickled RBCs and leucocytes in the  
52 microcirculation, leading to transient ischemia (2). Ischemia and the subsequent reperfusion both  
53 compromise organ integrity and function (3). Sickle RBCs break easily thereby resulting in hemolysis which  
54 is accompanied by a marked reduction in nitric oxide (NO) bioavailability (4). Hemolytic anemia affects  
55 oxygen transport, delivery and utilization, which ultimately lead to limitations of exercise capacity (5).  
56 Accordingly, impaired skeletal muscle bioenergetics and function have been reported in patients (6–10) as  
57 well as SCD mouse models (11–14).

58 SCD patients undergo a skeletal muscle remodeling including a rarefied microvasculature and a  
59 decreased activity of oxidative enzymes, such as the citrate synthase (CS) and cytochrome *c* oxidase (COx)  
60 (10). At the skeletal muscle level, this remodeling most likely indicates a reduction in oxygen supply (8, 15)  
61 and utilization for oxidative metabolism, respectively (10). The increased blood lactate concentration  
62 reported at rest (16) and during exercise (17, 18) in SCD patients compared to healthy controls would support  
63 a compensatory mechanism leading to an increased contribution of non-oxidative pathways in energy  
64 supply. Accordingly, an exacerbated exercise-induced acidosis together with a higher PCr depletion have  
65 been reported in SCD mice (19). The higher Enolase/CS enzyme activity ratio reported in SCD mice  
66 compared to their control counterparts (HbAA genotype) is also supportive of a higher contribution of non-  
67 oxidative processes (19). From a functional point of view, a reduced force production has been reported in

68 SCD patients using handgrip tests (7–9, 20) and dynamometric assessments (6). Similar results have been  
69 reported in SCD mice (11, 12, 14).

70 Hydroxyurea (HU) is commonly used as a treatment in SCD and is mainly recommended for HbSS and  
71 HbS $\beta^0$  genotypes. HU acts as a ribonucleotide reductase inhibitor leading to a reduced availability of  
72 intracellular deoxynucleotide triphosphate which is needed for DNA synthesis (21). As a result, HU triggers  
73 an erythropoietic stress resulting in the induction of  $\gamma$ -globin expression and consequently fetal hemoglobin  
74 (HbF) synthesis (21–23). An increased level of HbF directly reduces the HbS relative level, alleviates SCD-  
75 related symptoms and decreases transfusion requirement (24). HU improves oxygen saturation (25) but also  
76 enhances hemodynamics (26) and thanks to its NO donor function, local blood flow (22, 27). Although the  
77 beneficial effects of HU on exercise capacity and absolute force production have been reported in patients  
78 (28, 29), to the best of our knowledge, the underlying impacts on skeletal muscle bioenergetics *in vivo* have  
79 not been described so far. Considering the beneficial effects of HU on hematological and hemodynamic  
80 parameters and consequently on oxygen delivery, it can be hypothesized that HU treatment could improve  
81 skeletal muscle bioenergetics and function.

82 In order to test this hypothesis, we have investigated the effect of 2-month HU supplementation upon  
83 muscle energy metabolism and mechanical performance in Townes SCD mouse model using magnetic  
84 resonance (MR) imaging and <sup>31</sup>Phosphorus MR spectroscopy (<sup>31</sup>P-MRS).

85

86

87

88

89

90

91

## 92 **Materials and Methods**

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

94 Twenty-four humanized  $h\alpha/h\alpha::\beta s/\beta s$  Townes mice aged  $2.0 \pm 0.5$  month-old (Jackson Laboratories, Bar  
95 Harbord, USA (30)) were assessed. The present study was performed in conformity with the French  
96 guidelines for animal care and in conformity with the European convention for the protection of vertebrate  
97 animals used for experimental purposes and institutional guidelines n° 86/609/ CEE November 24, 1986.  
98 All animal experiments were approved by the Institutional Animal Care Committee of Aix-Marseille  
99 University (permit number n°27800-2020112517474997). Mice of a same experimental group (*vide infra*)  
100 were housed (4-5 mice per cage) in a controlled-environment facility (12–12 h light–dark cycle, 22 °C) and  
101 received water and standard food *ad libitum*.

102

### 103 **Study design**

104 Mice have been randomly assigned to three groups. Eight mice were investigated at  $2.0 \pm 0.5$  month-old  
105 without any intervention ( $SCD_{2m}$ ). Another group of 2-month-old mice ( $n = 8$ ) was supplemented with HU  
106 for 8 weeks and then investigated at 4 months of age ( $SCD_{4m-HU}$ ). Hydroxyurea was given every day (50  
107 mg/kg/day) (31) in drinking water and the concentration was adjusted daily according to water consumption.  
108 Previous studies have reported a positive effect of a similar dose provided acutely or for a 6-week period on  
109 hematological characteristics (23, 31). A last group of mice without treatment ( $n = 8$ ) was investigated at 4  
110 months of age ( $SCD_{4m}$ ). Each animal was studied in a noninvasive manner in order to assess posterior  
111 hindlimb muscles function and bioenergetics throughout a standardized rest-stimulation-recovery protocol  
112 as previously described (32). One week after the investigation, mice were anesthetized in an induction  
113 chamber with isoflurane and euthanized by cervical dislocation.

114

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

116 Investigations were conducted using an in-house experimental setup which has been designed to be  
117 operational inside the PharmaScan® AVANCETM III HD 70/16 US equipped with a 90 mm BGA09S  
118 (760mT/m) gradient insert (Bruker BioSpin MRI GmbH, Ettlinge, Germany). This setup has been adapted  
119 from a previously described model used on a 4.7T MR scanner (32). It allows (i) to get anatomical  
120 information about the hindlimb using MR imaging (MRI), (ii) to assess posterior hindlimb muscles  
121 mechanical performance with a dedicated ergometer consisting of a foot pedal coupled to a force transducer,  
122 and (iii) to monitor dynamically the levels of high-energy phosphorylated compound and acidosis in this  
123 muscle using <sup>31</sup>P-MRS. We have chosen to study the posterior hindlimb muscles, which forms the belly of  
124 the calf, because it is clearly distinct from the other muscles of the leg, easily accessible for MR coils and  
125 large enough to give <sup>31</sup>P-MR spectra in a short time and with good signal to noise ratio. In addition, it has  
126 been shown that gastrocnemius muscle was preferentially activated using our experimental setup (32).

127

### 128 **Animal preparation**

129 Mice were anesthetized in an induction chamber (Equipement vétérinaire Minerve, Esternay, France)  
130 using an air flow (3 L/min) containing 4% isoflurane. The left hindlimb was shaved and an electrode cream  
131 was applied at the knee and heel regions to optimize electrical contacts. The anesthetized animal was then  
132 placed supine in the experimental setup. Corneas were protected from drying by applying ophthalmic cream,  
133 and the animal's head was equipped with a facemask continuously supplied with 1-2% isoflurane mixed in  
134 66% room air (1 L/min) and 33% O<sub>2</sub> (0.5 L/min). Breathing rate was monitored throughout the experiment  
135 with a MR-compatible monitoring system (SA Instruments, Stony Brook, NY, USA) and kept between 100  
136 and 130 breaths/min thanks to isoflurane concentration adjustments. The foot was positioned on the  
137 ergometer pedal and the hindlimb was centered inside a 16-mm diameter <sup>1</sup>H Helmholtz coil while the belly  
138 of the gastrocnemius muscle was located above an elliptic (8 x 6 mm<sup>2</sup>) <sup>31</sup>P surface coil. Body temperature  
139 was controlled and maintained at a physiological level throughout the experiment using a feedback loop  
140 including an electrical heating blanket, a temperature control unit (ref. 507137, Harvard Apparatus,  
141 Holliston, MA, USA) and a homemade rectal thermometer.

142

143 **Induction of muscle contraction and contractile force measurement**

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

153

154 **Moderate-intensity exercise protocol**

155 Function and bioenergetics of the posterior hindlimb muscles were evaluated throughout a 6-min  
156 moderate-intensity exercise consisting of maximal isometric contractions repeated at a frequency of 1 Hz,  
157 induced with square wave pulses (0.5-ms duration). Individual maximal stimulation intensity was  
158 determined by a progressive increase of the stimulus intensity until there was no further force increase. Force  
159 parameters were acquired with LabChart software (AD Instruments, Oxford, UK). Peak force (Pf, mN) was  
160 quantified. Total force production (TFP) was computed as the sum of each twitch. Specific parameters (sPf,  
161 sTFP) were normalized to posterior hindlimb muscles volume (cm<sup>3</sup>) computed from anatomic hindlimb MR  
162 images. Force increase (%) was defined as the ratio between the maximal and initial force values. Fatigue  
163 index (%) was measured as the ratio between final and initial force measurements.

164

165 **Multimodal MR data acquisition**

166 Ten consecutive non-contiguous axial slices (1-mm thickness, spaced 0.25 mm) covering the region  
167 from the knee to the ankle were selected across the lower hindlimb. RARE images (Rare factor = 8, effective



168 echo time = 35.29 ms, actual echo time = 11.76 ms, repetition time = 5000 ms, one accumulation, 20 x 20  
169 mm field of view, 256 x 192 matrix size, acquisition time = 3 min 14 s) were recorded at rest in order to  
170 assess muscle volume. Posterior hindlimb muscles region was manually delineated on each of the 6 largest  
171 slices. Then, muscle volume of each slice was extracted using the FSLEyes software (33). Total muscle  
172 volume (cm<sup>3</sup>) was calculated using the truncated cone volume formula considering slice and gap volumes.

173 Multiecho T2-weighted images (16 echo times equally spaced from 7.28 ms to 116.59 ms, 2000-ms  
174 repetition time, one accumulation, 20 x 30 mm field of view, 256 x 256 matrix size, total acquisition time =  
175 8.32 min, slice thickness = 1 mm, slice gap = 1 mm) were recorded at rest. T2-weighted images were  
176 processed to generate T2-maps on a pixel by pixel basis by fitting the corresponding data with a single  
177 exponential function. Mean T2 values of hindlimb muscles were measured on T2 maps and averaged on the  
178 2 largest consecutive slices from the outlined regions of interest. The corresponding values were quantified  
179 for total gastrocnemius (GA) muscle and its right, central and left parts. Measurements were also performed  
180 for the tibialis anterior (TA). The averaged T2 of water protons illustrates the molecular environment  
181 together with the water mobility. <sup>31</sup>P-MR spectra (8 kHz sweep width; 2048 data points) from the posterior  
182 hindlimb muscles were continuously acquired before (rest period; 4.7 min duration), during (stimulation; 6  
183 min duration) and after (recovery period; 16 min duration) the standardized stimulation bout. Spectra  
184 acquisition was gated to muscle electrostimulation to reduce potential motion artifacts due to contraction. A  
185 total of 800 saturated free induction decays (FID, 1.875 s repetition time) were acquired. The first 140 FID  
186 were acquired in resting muscle and summed together. The next 450 FIDs were acquired during exercise  
187 and were summed as blocks of 15, allowing a 30-s temporal resolution. The remaining 210 FIDS were  
188 obtained during the recovery period and were summed as blocks of 30 FIDs.

189

### 190 **MR Spectroscopy data processing**

191 MRS data were processed using custom-written routines in Python (34) integrating the AMARES  
192 Fortran code (35) from jMRUI (<http://www.jmrui.eu/>). Relative concentrations of phosphocreatine (PCr),  
193 inorganic phosphate (Pi) and ATP were obtained from <sup>31</sup>P-MR spectra using the AMARES-MRUI-based

194 time domain fitting routine including appropriate prior knowledge for the ATP multiplets (34). Absolute  
 195 amounts of phosphorylated compounds were expressed taking into account a 5-mM  $\beta$ -ATP basal  
 196 concentration (36). The PCr relative concentration was expressed relative to the PCr content at rest which  
 197 was set at 100%. Intracellular pH was calculated from the chemical shift of the Pi signal relative to PCr  
 198 according to the formula (37):  $pHi = 6.75 + \log[(3.27 - \delta Pi) / (\delta Pi - 5.69)]$ . Cytosolic ADP concentration was  
 199 calculated, as previously described (38, 39):  $[ADP] = ([Cr] \times [ATP]) / ([PCr] \times 10^{-pHi} \times K_{CK})$ , using the creatine  
 200 kinase equilibrium constant ( $K_{CK} = 1.66 \times 10^9 \text{ M}^{-1}$ ) (40) and total creatine [Cr] calculated by assuming that  
 201 PCr represents ~85% of total Cr (41). The rate of PCr degradation ( $V_{PCr_{stim}}$ , in mM/min) at the onset of  
 202 exercise was calculated as  $V_{PCr_{stim}} = \Delta PCr / \tau_{PCr_{stim}}$ , where  $\Delta PCr$  is the extent of PCr depletion measured at  
 203 the end of stimulation (relative to basal value) and  $\tau_{PCr_{stim}}$  is the time constant of PCr degradation.  $\tau_{PCr_{stim}}$   
 204 was determined by fitting the time course of PCr to a mono-exponential function using a least-means-  
 205 squared algorithm. sPf-normalized non-oxidative cost of contraction ( $PCr_{cost}$  in  $\mu\text{M}/\text{min}/\text{mN}/\text{cm}^3$ ) was  
 206 calculated as the ratio between  $V_{PCr_{stim}}$  and the amount of specific force produced at the beginning of  
 207 exercise, considering that at that time PCr is the only source of ATP production. Similarly, the PCr recovery  
 208 parameters ( $\tau_{PCr_{rec}}$ ) were quantified during the post-exercise period by fitting the time course of PCr  
 209 resynthesis to a mono-exponential function. As previously suggested,  $V_{PCr_{rec}}$  was considered as the rate of  
 210 oxidative ATP production at the end of exercise as  $V_{PCr_{rec}} = \Delta PCr / \tau_{PCr_{rec}}$  (42, 43). sPf-normalized oxidative  
 211 cost of contraction ( $PCr_{rec}$  in  $\mu\text{M}/\text{min}/\text{mN}/\text{cm}^3$ ) was calculated as the ratio between  $V_{PCr_{rec}}$  and the amount  
 212 of specific force produced at the end of exercise. The maximal rate of PCr recovery ( $V_{max}$ , mM/min) was  
 213 calculated as  $V_{max} = V_{PCr_{rec}} (1 + (K_m / [ADP_{end}]))$  (44), where  $K_m$  is the affinity constant of the mitochondria  
 214 for ADP (30  $\mu\text{M}$  in gastrocnemius muscle of the mice), and  $[ADP_{end}]$  is the ADP concentration at the end  
 215 of exercise.

216 The rate net of proton efflux ( $\text{H}^+$  efflux, mM/min) was computed as previously described using the  
 217 stoichiometric coefficient  $\phi = 1 / [1 + 10^{(pHi - 6.75)}]$  and the apparent buffering capacity  $\beta_{tot} = \beta_{tissue} + \beta^{Pi}$  such  
 218 as  $\text{H}^+$  efflux =  $\phi dPCr/dt + \beta_{tot} dpHi/dt$  (45). The apparent buffering capacity takes into account  $\beta_{tissue} = -21$   
 219  $pHi + 163$  and  $\beta^{Pi} = 2.3[Pi] / \{ [1 + 10^{(pHi - 6.75)}][1 + 10^{(pHi - 6.75)}] \}$ .

220

## 221 **Statistics**

222 A power analysis (power.t.test, significance = 0.05, power = 0.8, type = “two-sample”) has been  
223 conducted considering the maximal Pf. This test indicated that a minimum of 7 mice should be included in  
224 each group.

225 All values are presented as means (SD) except on graphical representations for which SE are displayed  
226 for the sake of clarity. The effects of the disease evolution were assessed from the comparative analysis  
227 between SCD<sub>2m</sub> and SCD<sub>4m</sub> mice while the effects of HU treatment were determined based on the  
228 comparative analysis between SCD<sub>4m</sub> and SCD<sub>4m-HU</sub> mice. Normality of sample distribution was tested with  
229 the Shapiro-Wilk test.

230 To analyze the time-course of force, PCr and pHi, a two-way ANOVA (group, experimentation time)  
231 with repeated measures was used. In case of positive interaction, pairwise t-tests (significance level was set  
232 at  $p < 0.05$ ) with Bonferroni correction were performed in order to compare groups at the corresponding  
233 timepoints.

234 For non-time course variables, statistical differences between groups were assessed using non-  
235 parametric Wilcoxon tests or parametric Student’s t-test according to the result of the normality test. A  
236 Bonferroni correction was applied to take into account the multiple comparison of SCD<sub>4m</sub>, consequently  
237 setting the significance level at  $p < 0.025$ . All statistics were performed using RStudio (4.0.2).

238

## 239 **Results**

### 240 **Morphological characteristics & T2 measurements**

241 As indicated in table 1, SCD<sub>4m</sub> mice were heavier than SCD<sub>2m</sub> whereas body mass in SCD<sub>4m-HU</sub> mice and  
242 SCD<sub>4m</sub> mice was similar. Neither posterior hindlimb muscle volume nor the corresponding ratio to body  
243 mass was significantly different between groups.

244 T2 values were similar in the three groups whatever the muscles considered, or the part of the  
245 gastrocnemius muscle considered (table 2).

246

### 247 **Muscle function**

248 At the onset of stimulation, sPf was significantly higher in SCD<sub>2m</sub> (1287.86 SD 288.91 mN/cm<sup>3</sup>) as  
249 compared to SCD<sub>4m</sub> (591.99 SD 186.27 mN/cm<sup>3</sup>, Student's t-test, p < 0.001) mice whereas SCD<sub>4m</sub> and  
250 SCD<sub>4m-HU</sub> (717.75 SD 157.12 mN/cm<sup>3</sup>, Student's t-test, p = 0.17) mice had similar values (table 3). As  
251 illustrated in figure 1, the sPf time-course was classically biphasic. sPf initially increases to reach an apex  
252 at 135 s, 175 s and 150 s of stimulation for SCD<sub>2m</sub>, SCD<sub>4m</sub> and SCD<sub>4m-HU</sub>, respectively. No significant  
253 difference in the relative force increase between initial and maximal force was detected between SCD<sub>2m</sub>  
254 (17.35 SD 8.28 %) and SCD<sub>4m</sub> mice (40.55 SD 35.40 %, Wilcoxon test, p = 0.09) and between SCD<sub>4m</sub> and  
255 SCD<sub>4m-HU</sub> mice (38.99 SD 21.71 %, Student's t-test, p = 0.92). Then, sPf progressively decreased until the  
256 end of the stimulation period. For the stimulation bout, significant time, group and time x group interaction  
257 effects were found between SCD<sub>2m</sub> and SCD<sub>4m</sub> mice (Two-way ANOVA, p < 0.005). Overall, SCD<sub>2m</sub> mice  
258 produced a significantly larger amount of specific force at all times of the stimulation period as compared  
259 to SCD<sub>4m</sub> (Pairwise t-test, p < 0.0001). In addition, even if no group effect was observed, significant time  
260 and time x group interaction effects were found between SCD<sub>4m</sub> and SCD<sub>4m-HU</sub> mice (Two-way ANOVA, p  
261 < 0.0001). The sPf produced during the period ranging from minute 1 to minute 5.25 was significantly higher  
262 in SCD<sub>4m-HU</sub> as compared to SCD<sub>4m</sub> (Pairwise t-test, p < 0.05, figure 1). At the end of stimulation, sPf was  
263 significantly higher in SCD<sub>2m</sub> (1189.37 SD 358.21 mN/cm<sup>3</sup>) as compared to SCD<sub>4m</sub> mice (678.63 SD 80.01  
264 mN/cm<sup>3</sup>, Student's t-test, p = 0.001, table 3) but was not different between SCD<sub>4m</sub> and SCD<sub>4m-HU</sub> (813.16  
265 SD 211.45 mN/cm<sup>3</sup>, Student's t-test, p = 0.11, table 3).

266 The sTFP of the SCD<sub>2m</sub> group (484.76 SD 130.80 N/cm<sup>3</sup>) was significantly larger than the corresponding  
267 value in the SCD<sub>4m</sub> group whereas SCD<sub>4m</sub> (254.60 SD 35.80 N/cm<sup>3</sup>) and SCD<sub>4m-HU</sub> mice (320.32 SD 67.36  
268 N/cm<sup>3</sup>, figure 2 and table 3) displayed similar values. The absolute TFP was significantly larger in SCD<sub>2m</sub>

269 (3909.62 SD 829.01 N) compared to SCD<sub>4m</sub> (2040.87 SD 560.70 N,  $p < 0.001$ ). Additionally, TFP of SCD<sub>4m</sub>-  
270 *HU* (2930.76 SD 766.55 N,  $p = 0.019$ ) was also higher as compared to SCD<sub>4m</sub>.

271 The fatigue index was neither different between SCD<sub>2m</sub> (-6.10 SD 9.86 %) and SCD<sub>4m</sub> (24.73 SD 37.32  
272 %, Student's t-test,  $p = 0.04$ ) nor between SCD<sub>4m</sub> and SCD<sub>4m-HU</sub> mice (14.86 SD 26.42 %, Student's t-test,  $p$   
273 = 0.05, table 3).

274

### 275 **Muscle bioenergetics**

276 At rest,  $pHi$  was similar in SCD<sub>2m</sub>, SCD<sub>4m</sub> and SCD<sub>4m-HU</sub> mice (table 4). As illustrated in figure 3A,  
277 during the 6-min moderate stimulation period, a transient alkalinization was observed, followed by a  $pHi$   
278 drop. This acidotic phase was initially fast and then slower. As the stimulation ceased,  $pHi$  initially stabilized  
279 and then continuously increased towards the resting value. While a significant time effect was observed  
280 (Two-way ANOVA,  $p < 0.0001$ ), no group or time x group effects could be identified between groups.  $pHi$   
281 decrease ( $\Delta pHi$ ) was significantly lower in SCD<sub>4m</sub> as compared to SCD<sub>2m</sub> mice (Student t-test,  $p = 0.014$ ,  
282 table 4) and not significantly different from SCD<sub>4m-HU</sub> mice (Student t-test,  $p = 0.029$ ). However, considering  
283 the sTFP-normalized  $pHi$  decrease, no difference was found between groups (table 4). The absolute  $pHi$  at  
284 the end of the stimulation period ( $pHi_{end}$ ) was significantly higher in SCD<sub>4m</sub> as compared to SCD<sub>2m</sub> (Student  
285 t-test,  $p = 0.024$ ) whereas it was not significantly different between SCD<sub>4m</sub> and SCD<sub>4m-HU</sub> (Student t-test,  $p$   
286 = 0.049, table 4). At the beginning of the recovery,  $H^+$  efflux was similar among groups (table 4).

287 As illustrated in figure 3B, the PCr time course could be divided in 3 phases. An initial progressive  
288 decrease occurred during the first 4 min of exercise followed by a slower decrease. The recovery period was  
289 characterized by a progressive return towards baseline values. A significant time effect was found ( $p > 0.05$ ),  
290 whereas neither group nor time x group interaction effects were found. The  $\Delta PCr$  was similar in the three  
291 groups, whereas the sTFP-normalized PCr decrease was larger in SCD<sub>4m</sub> as compared to SCD<sub>2m</sub> mice ( $p =$   
292 0.024) and similar in SCD<sub>4m</sub> and SCD<sub>4m-HU</sub> mice (table 4,  $p = 0.56$ ).

293 At the end of the stimulation period,  $Pi_{end}$  and  $ADP_{end}$  were similar in the three groups (table 4).  $Pi_{end}$   
294 normalized to the corresponding sPf ( $Pi_{end}/sPf_{end}$ ) was significantly higher in  $SCD_{4m}$  compared to  $SCD_{2m}$   
295 mice (Student t-test,  $p = 0.019$ ) but was not different compared to  $SCD_{4m-HU}$  mice (table 4).

296  $VPCr_{stim}$ ,  $VPCr_{rec}$  and  $V_{max}$  were similar in the three groups (table 4). The sPf-normalized non-oxidative  
297 and oxidative costs of contraction, assessed by  $PCr_{cost}$  and  $PCr_{rec}$ , respectively were significantly higher in  
298  $SCD_{4m}$  as compared to  $SCD_{2m}$  mice (table 4,  $p = 0.0019$  and  $p = 0.0006$ , respectively).  $PCr_{rec}$  and  $PCr_{cost}$  were  
299 similar in  $SCD_{4m}$  and  $SCD_{4m-HU}$  mice.

300

## 301 **Discussion**

302 In the present study, we aimed at characterizing changes in skeletal muscle bioenergetics and function  
303 following an 8-week HU treatment in sickle cell disease mice. The results obtained in the 3 groups assessed  
304 in the present study allowed us to distinguish the effects related with HU treatment from those associated to  
305 a 2-month period without treatment. As compared to  $SCD_{2m}$  mice,  $SCD_{4m}$  were heavier, produced a smaller  
306 specific force while the corresponding acidosis and sTFP-normalized PCr decrease were lower. Both non-  
307 oxidative and oxidative costs of contraction were also higher in  $SCD_{4m}$  than in  $SCD_{2m}$  mice. Interestingly,  
308  $SCD_{4m-HU}$  produced higher specific peak force over a part of stimulation than  $SCD_{4m}$  mice whereas metabolic  
309 changes were not different between groups. As a whole, it appeared that HU treatment was beneficial for  
310 muscle function in SCD mice.

311

### 312 **Morphological characteristics & T2 measurements**

313 The greater body mass in  $SCD_{4m}$  mice compared to their  $SCD_{2m}$  counterparts is in accordance with the  
314 literature showing that weight can increase in mice up to 52 weeks of age (46). The similar body mass in  
315  $SCD_{4m-HU}$  as compared to  $SCD_{4m}$  mice indicates that the HU treatment did not affect body mass in the present  
316 study. This result in mice differs from what was observed in children or adults with (28, 47). To date, no  
317 satisfactory explanation for this discrepancy between the studies and the species can be provided.

318 Previous studies have considered elevated T2 values to be a reflection of inflammatory phenomena in a  
319 variety of physiological conditions (48–52). Given the previously reported muscle VOC and myonecrosis  
320 in SCD patients (53–56), one could have expected muscle T2 changes through age and treatment. As no  
321 difference in T2 values were observed between mice in the present study, one can exclude an inflammatory  
322 phenomenon or its aggravation in the studied SCD mice muscles. This result is in accordance with the fact  
323 that muscle complications (such as myonecrosis) are rare in patients with SCD (10, 57).

324

### 325 **Effects of an 8-week period on skeletal muscle function and energetics**

326 SCD<sub>4m</sub> mice displayed a significantly lower specific force production during the entire stimulation bout  
327 as compared to SCD<sub>2m</sub> and consequently a reduced sTFP. This reduction might be related to a time effect  
328 and/or the disease progression.

329 It has recently been reported in control mice that specific force production significantly increased from  
330 3 to 10 weeks of age (+ 33%) whereas it tended to decrease (- 13%) from 10 to 30 weeks of age (46). On  
331 that basis, one may hypothesize that the prominent specific force reduction (- 45%) observed in the present  
332 study between SCD mice of 8 and 16 weeks of age (SCD<sub>2m</sub> and SCD<sub>4m</sub>, respectively) was unlikely due to a  
333 time effect but rather to the disease progression. While it has largely been recognized that skeletal muscle  
334 function can be impaired in both SCD patients (6–9) and mouse models (11, 13, 14), the present results  
335 extend our knowledge by suggesting that disease progression can affect muscle function. Of note, the present  
336 groups of mice were rather fragile (7 deaths over 31 mice in this litter after muscle function and energetics  
337 investigation) and one cannot exclude that this fragility may at least partly account for such a low specific  
338 force production in SCD<sub>4m</sub>.

339 The impaired capacity of force production previously reported in SCD has partly been related to  
340 reductions in posterior hindlimb muscle mass (7, 20), electromyographic activity (8), and daily activity (58),  
341 as well as to a deep tissue hyperalgesia (11, 13). Yet, the similar muscle volumes reported between SCD<sub>2m</sub>  
342 and SCD<sub>4m</sub> mice exclude the implication of divergent muscle mass as an accounting factor of the lower force  
343 production in SCD<sub>4m</sub> mice. In the same way, central nervous factors of muscle activation can be ruled out

344 given that muscle contraction was evoked by transcutaneous electrostimulation in anesthetized mice. One  
345 needs to keep in mind that the stimulation intensity used in the present study was normalized for each animal  
346 so that the corresponding mechanical and metabolic changes should be comparable. Considering that muscle  
347 volume could be a potential confounding factor, absolute force has been normalized to muscle volume in  
348 order to compute the specific force. The lower specific force production recorded in SCD<sub>4m</sub> mice clearly  
349 illustrates a contractile defect in this group compared to SCD<sub>2m</sub> mice. In other words, for a given muscle  
350 volume and a comparable standardized exercise intensity, SCD<sub>4m</sub> mice produced less force than SCD<sub>2m</sub> mice.

351 There is a link between force production and bioenergetic parameter. In order to illustrate this idea,  
352 Chatel *et al.* used a stimulation frequency twice the one used here and obtained larger force production  
353 associated with more pronounced metabolic changes (19). In the present study, the different force production  
354 in SCD<sub>4m</sub> and SCD<sub>2m</sub> mice should be associated with divergent metabolic response. To investigate the  
355 metabolic response, absolute terms and values normalized to force has been expressed.

356 During the standardized stimulation conditions, SCD<sub>4m</sub> mice displayed similar PCr consumption but a  
357 lower acidosis as compared to SCD<sub>2m</sub> mice. Considering the well-known alkalotic effect of PCr breakdown,  
358 these results indicate that alkalosis was similar in both groups and so could not account for the reduced  
359 acidosis in SCD<sub>4m</sub> mice. Most likely, this reduced pH change might be linked to the lower specific force  
360 production. Indeed, the sTFP-normalized acidosis was similar in SCD<sub>4m</sub> and SCD<sub>2m</sub> mice.

361 Although the extent of PCr consumption was similar, the specific force production was lower in SCD<sub>4m</sub>  
362 than in SCD<sub>2m</sub> mice. As a result, the sTFP-normalized consumption of PCr was higher in the former group,  
363 indicating that muscle of SCD<sub>4m</sub> mice would consume more PCr to produce a given amount of force.  
364 Supportive of this idea, the oxidative and non-oxidative costs of contraction (expressed by PCr<sub>rec</sub> and PCr<sub>cost</sub>,  
365 respectively) were significantly greater in SCD<sub>4m</sub> than in SCD<sub>2m</sub> mice. As a whole, our results seem to  
366 indicate that muscle bioenergetics is altered between 2 and 4 months of age SCD mice, and that this  
367 impairment likely results from disrupted costs of contraction. The costs of contraction are the resultant of  
368 the rates of ATP consumption (including contractile and non-contractile processes such as ions pumping)  
369 and resynthesis. Therefore, higher (oxidative and non-oxidative) costs of contraction would indicate either



370 a higher ATP consumption and/or a lower ATP resynthesis via oxidative and non-oxidative processes for a  
371 given amount of force produced in 4-month-old SCD mice.

372 In multiple studies conducted in humans, an increased cost of contraction has been related to a lower  
373 contractile efficiency (59–61). Several accounting factors such as acidosis and Pi accumulation have been  
374 put forth (62). On the one hand, acidosis could inhibit oxidative phosphorylation, force and shortening  
375 velocity (63–65). However, the similar sTFP-normalized acidosis obtained in SCD<sub>2m</sub> and SCD<sub>4m</sub> mice rules  
376 out acidosis as a potential accounting factor of the disturbed costs of contraction found in the latter group.  
377 On the other hand, Pi has been shown to disrupt some steps of the cross-bridge cycle (62), including a  
378 reduction of the myofibrillar sensitivity to calcium (66, 67). Although the absolute amount of Pi at the end  
379 of exercise was similar in SCD<sub>2m</sub> and SCD<sub>4m</sub>, the sPf-normalized Pi content at the end of exercise was higher  
380 in SCD<sub>4m</sub> group as compared to SCD<sub>2m</sub> group thereby illustrating a larger Pi content for a given force.  
381 Overall, the higher sPf-normalized Pi content might have been involved in the lower values of force  
382 production as well as in the higher costs of contraction reported in the SCD<sub>4m</sub> mice of present study.

383 Finally, one cannot exclude that the diminished specific force production can be related to disrupted  
384 mechanisms of ATP utilization and/or resynthesis by processes unexplored in the present study. Further  
385 studies are warranted in order to address this issue.

386

### 387 **Effects of an 8-week HU treatment upon muscle function and energetics**

388 In the present study, the specific force production of SCD<sub>4m-HU</sub> mice was greater compared to the SCD<sub>4m</sub>  
389 mice from 1 to 5.25 min of stimulation. Hence, in the present study, HU seemed to act as a protective agent  
390 against force loss over time in SCD mice. The accounting factors of this increased force production are still  
391 not well understood. Indeed, although HU is commonly used as a treatment in SCD, its impact on muscle  
392 function remains poorly investigated. Nevertheless, a study tends to support that, in young SCD patients,  
393 HU treatment would improve physical fitness (28). More specifically, in these patients, an 18-month HU  
394 treatment was associated with a reduced peak heart rate at 75 W as well as an increased performance in  
395 response to a short high-intensity exercise (Wingate test) (28). The authors attributed these improvements

396 to hematological (e.g., increased fetal Hb level and decreased HbS level), hemorheological (e.g., increased  
397 red blood cell deformability) and hemodynamical (e.g., increased circulating NO level) benefits associated  
398 with HU treatment (68). It is interesting to note that a supplementation in nitrate, a NO precursor, has been  
399 reported to enhance local blood flow but also muscle function, including in SCD (69–72). This is especially  
400 interesting taking into account that i) NO bioavailability is reduced in SCD patients (4) and ii) that HU can  
401 act like nitrate supplementation by promoting NO production (73). Along this line of reasoning, the  
402 increased force production observed in HU-treated mice might be associated with a partial restoration of  
403 NO levels. Additionally, considering the beneficial effects of HU on clinical and functional outcomes in  
404 SCD patients (24, 28, 29, 74), one could have expected an increased cage activity in HU-treated mice which  
405 could be partly responsible for the improved muscle function (75). However, this index has not been  
406 measured in the present study. Further studies are warranted in order to address the potential involvement  
407 of NO and increased cage activity in the benefits of HU on skeletal muscle function.

408 An improved force production in response to HU treatment may be related to a better contractile function  
409 and/or a better ATP production for contraction. Because none of the energetic parameters explored in the  
410 present study were different between  $SCD_{4m}$  and  $SCD_{4m-HU}$  mice, our results clearly exclude that the  
411 improved force production with HU treatment was due to i) a protective effect of muscle function in relation  
412 to lower intracellular Pi or acidosis, or ii) a better energy (ATP) supply for contractile processes in relation  
413 to a higher PCr breakdown, glycolytic rate (indirectly assessed by acidosis and  $H^+$  efflux) or mitochondrial  
414 oxidative processes (indirectly assessed by  $V_{max}$ ). Therefore, the improved force production in response to  
415 HU treatment remains unexplained to date. One possible explanation might be related to oxidative stress.  
416 While oxidative stress has been reported to be higher in SCD patients (76, 77), HU-treated SCD patients  
417 display a reduced degree of oxidative perturbation compared to untreated counterparts (77). Considering the  
418 well-established effect of oxidative stress on muscle function (78), it is not excluded that the better muscle  
419 function in HU-treated mice may be explained a lower oxidative stress. Since oxidative stress was not  
420 explored in the present study, this possibility will deserve to be investigated in the near future.

421

## 422 **Conclusion**

423 In conclusion, this study confirmed our hypothesis that an 8-week HU treatment was beneficial for  
424 skeletal muscle function of SCD mice. Considering that HU treatment did not significantly modify i) the  
425 non-oxidative (PCr breakdown, acidosis, proton efflux) or oxidative ( $V_{max}$ ) metabolism and ii) the non-  
426 oxidative and oxidative costs of contraction, our results suggest that the used HU treatment did not impact  
427 muscle bioenergetics. Of note, the present results disclosed progressive disturbances of skeletal muscle  
428 function and bioenergetics between 2 and 4 months of age in SCD mice.

429

## 430 **Acknowledgments**

431 Hydroxyurea has been kindly offered by addmedica.

## 432 References

- 433 1. **Stuart MJ, Nagel RL.** Sickle-cell disease. *The Lancet* 364: 1343–1360, 2004. doi: 10.1016/S0140-  
434 6736(04)17192-4.
- 435 2. **Rees DC, Williams TN, Gladwin MT.** Sickle-cell disease. *The Lancet* 376: 2018–2031, 2010. doi:  
436 10.1016/S0140-6736(10)61029-X.
- 437 3. **Piel FB, Steinberg MH, Rees DC.** Sickle Cell Disease. *N Engl J Med* 376: 1561–1573, 2017. doi:  
438 10.1056/NEJMra1510865.
- 439 4. **Reiter CD, Wang X, Tanus-Santos JE, Hogg N, Cannon RO, Schechter AN, Gladwin MT.** Cell-  
440 free hemoglobin limits nitric oxide bioavailability in sickle-cell disease. *Nat Med* 8: 1383–1389, 2002.  
441 doi: 10.1038/nm1202-799.
- 442 5. **Woodson RD, Wills RE, Lenfant C.** Effect of acute and established anemia on O<sub>2</sub> transport at rest,  
443 submaximal and maximal work. *J Appl Physiol* 44: 36–43, 1978. doi: 10.1152/jappl.1978.44.1.36.
- 444 6. **Moheeb H, Wali YA, El-Sayed MS.** Physical fitness indices and anthropometrics profiles in  
445 schoolchildren with sickle cell trait/disease. *Am J Hematol* 82: 91–97, 2007. doi: 10.1002/ajh.20755.
- 446 7. **Dougherty KA, Schall JI, Rovner AJ, Stallings VA, Zemel BS.** Attenuated Maximal Muscle  
447 Strength and Peak Power in Children With Sickle Cell Disease. *J Pediatr Hematol Oncol* 33: 93–97,  
448 2011. doi: 10.1097/MPH.0b013e318200ef49.
- 449 8. **Waltz X, Pichon A, Lemonne N, Mougénel D, Lalanne-Mistrih M-L, Lamarre Y, Tarer V,  
450 Tressières B, Etienne-Julan M, Hardy-Dessources M-D, Hue O, Connes P.** Normal Muscle  
451 Oxygen Consumption and Fatigability in Sickle Cell Patients Despite Reduced Microvascular  
452 Oxygenation and Hemorheological Abnormalities. *PLoS ONE* 7: e52471, 2012. doi:  
453 10.1371/journal.pone.0052471.
- 454 9. **Ohara DG, Ruas G, Walsh IAP, Castro SS, Jamami M.** Lung function and six-minute walk test  
455 performance in individuals with sickle cell disease. *Braz J Phys Ther* 18: 79–87, 2014. doi:  
456 10.1590/S1413-35552012005000139.
- 457 10. **Ravelojaona M, Féasson L, Oyono-Enguélé S, Vincent L, Djoubairou B, Ewa'Sama Essoue C,  
458 Messonnier LA.** Evidence for a Profound Remodeling of Skeletal Muscle and Its Microvasculature  
459 in Sickle Cell Anemia. *Am J Pathol* 185: 1448–1456, 2015. doi: 10.1016/j.ajpath.2015.01.023.
- 460 11. **Kohli DR, Li Y, Khasabov SG, Gupta P, Kehl LJ, Ericson ME, Nguyen J, Gupta V, Hebbel RP,  
461 Simone DA, Gupta K.** Pain-related behaviors and neurochemical alterations in mice expressing sickle  
462 hemoglobin: modulation by cannabinoids. *Blood* 116: 456–465, 2010. doi: 10.1182/blood-2010-01-  
463 260372.
- 464 12. **Capers PL, Hyacinth HI, Cue S, Chappa P, Vikulina T, Roser-Page S, Weitzmann MN, Archer  
465 DR, Newman GW, Quarshie A, Stiles JK, Hibbert JM.** Body composition and grip strength are  
466 improved in transgenic sickle mice fed a high-protein diet. *J Nutr Sci* 4: e6, 2015. doi:  
467 10.1017/jns.2014.63.
- 468 13. **Vang D, Paul JA, Nguyen J, Tran H, Vincent L, Yasuda D, Zaveri NT, Gupta K.** Small-molecule  
469 nociceptin receptor agonist ameliorates mast cell activation and pain in sickle mice. *Haematologica*  
470 100: 1517–1525, 2015. doi: 10.3324/haematol.2015.128736.

- 471 14. **Chatel B, Hourdé C, Gondin J, Fouré A, Le Fur Y, Vilmen C, Bernard M, Messonnier LA,**  
472 **Bendahan D.** Impaired muscle force production and higher fatigability in a mouse model of sickle  
473 cell disease. *Blood Cells Mol Dis* 63: 37–44, 2017. doi: 10.1016/j.bcmd.2017.01.004.
- 474 15. **Charlot K, Antoine-Jonville S, Moeckesch B, Jumet S, Romana M, Waltz X, Divialle-Doumbo**  
475 **L, Hardy-Dessources M-D, Petras M, Tressières B, Tarer V, Hue O, Etienne-Julan M, Connes**  
476 **P.** Cerebral and muscle microvascular oxygenation in children with sickle cell disease: Influence of  
477 hematology, hemorheology and vasomotion. *Blood Cells Mol Dis* 65: 23–28, 2017. doi:  
478 10.1016/j.bcmd.2017.03.015.
- 479 16. **Petto J, de Jesus JB, Vasques LMR, Pinheiro RLS, Oliveira AM, Spinola KAB, Silva WDS.**  
480 Resting blood lactate in individuals with sickle cell disease. *Rev Bras Hematol E Hemoter* 33: 26–30,  
481 2011. doi: 10.5581/1516-8484.20110010.
- 482 17. **Miller GJ, Serjeant GR, Sivapragasam S, Petch MC.** Cardio-Pulmonary Responses and Gas  
483 Exchange during Exercise in Adults with Homozygous Sickle-Cell Disease (Sickle-Cell Anaemia).  
484 *Clin Sci* 44: 113–128, 1973. doi: 10.1042/cs0440113.
- 485 18. **Freund H, Lonsdorfer J, Oyono-Enguelle S, Lonsdorfer A, Bogui P.** Lactate exchange and  
486 removal abilities in sickle cell patients and in untrained and trained healthy humans. *J Appl Physiol*  
487 73: 2580–2587, 1992. doi: 10.1152/jappl.1992.73.6.2580.
- 488 19. **Chatel B, Messonnier LA, Hourdé C, Vilmen C, Bernard M, Bendahan D.** Moderate and intense  
489 muscular exercises induce marked intramyocellular metabolic acidosis in sickle cell disease mice. *J*  
490 *Appl Physiol* 122: 1362–1369, 2017. doi: 10.1152/jappphysiol.01099.2016.
- 491 20. **Dougherty KA, Bertolaso C, Schall JI, Smith-Whitley K, Stallings VA.** Muscle Strength, Power,  
492 and Torque Deficits in Children With Type SS Sickle Cell Disease. *J Pediatr Hematol Oncol* 40: 348–  
493 354, 2018. doi: 10.1097/MPH.0000000000001143.
- 494 21. **Krakoff IH, Brown NC, Reichard P.** Inhibition of ribonucleoside diphosphate reductase by  
495 hydroxyurea. *Cancer Res* 28: 1559–1565, 1968.
- 496 22. **Cokic VP, Smith RD, Beleslin-Cokic BB, Njoroge JM, Miller JL, Gladwin MT, Schechter AN.**  
497 Hydroxyurea induces fetal hemoglobin by the nitric oxide-dependent activation of soluble guanylyl  
498 cyclase. *J Clin Invest* 111: 231–239, 2003. doi: 10.1172/JCI200316672.
- 499 23. **Platt O, Orkin S, Dover G, Beardsley G, Miller B, Nathan D.** Hydroxyurea enhances fetal  
500 hemoglobin production in sickle cell anemia. *J Clin Invest* 74: 652–6, 1984. doi: 10.1172/JCI11464.
- 501 24. **Charache S, Terrin ML, Moore RD, Dover GJ, Barton FB, Eckert SV, McMahon RP, Bonds**  
502 **DR.** Effect of Hydroxyurea on the Frequency of Painful Crises in Sickle Cell Anemia. *N Engl J Med*  
503 332: 1317–1322, 1995. doi: 10.1056/NEJM199505183322001.
- 504 25. **van Geysel L, Arigliani M, Inusa B, Singh B, Kozłowska W, Chakravorty S, Bossley CJ, Ruiz**  
505 **G, Rees D, Gupta A.** Higher oxygen saturation with hydroxyurea in paediatric sickle cell disease.  
506 *Arch Dis Child* 105: 575–579, 2020. doi: 10.1136/archdischild-2019-317862.
- 507 26. **Lemonne N, Charlot K, Waltz X, Ballas SK, Lamarre Y, Lee K, Hierso R, Connes C, Etienne-**  
508 **Julan M, Romana M, Connes P.** Hydroxyurea treatment does not increase blood viscosity and  
509 improves red blood cell rheology in sickle cell anemia. *Haematologica* 100: e383–e386, 2015. doi:  
510 10.3324/haematol.2015.130435.

- 511 27. **Kobayashi J, Uchida H, Kofuji A, Ito J, Shimizu M, Kim H, Sekiguchi Y, Kushibe S.** Molecular  
512 regulation of skeletal muscle mass and the contribution of nitric oxide: A review. .
- 513 28. **Hackney AC, Hezier W, Gulledge TP, Jones S, Strayhorn D, Busby M, Hoffman E, Orringer**  
514 **EP.** Effects of Hydroxyurea Administration on the Body Weight, Body Composition and Exercise  
515 Performance of Patients with Sickle-Cell Anaemia. *Clin Sci* 92: 481–486, 1997. doi:  
516 10.1042/cs0920481.
- 517 29. **Wali YA, Moheeb H.** Effect of Hydroxyurea on Physical Fitness Indices in Children with Sickle Cell  
518 Anemia. *Pediatr Hematol Oncol* 28: 43–50, 2011. doi: 10.3109/08880018.2010.524278.
- 519 30. **Wu L-C, Sun C-W, Ryan TM, Pawlik KM, Ren J, Townes TM.** Correction of sickle cell disease  
520 by homologous recombination in embryonic stem cells. *Blood* 108: 1183–1188, 2006. doi:  
521 10.1182/blood-2006-02-004812.
- 522 31. **Lebensburger JD, Pestina TI, Ware RE, Boyd KL, Persons DA.** Hydroxyurea therapy requires  
523 HbF induction for clinical benefit in a sickle cell mouse model. *Haematologica* 95: 1599–1603, 2010.  
524 doi: 10.3324/haematol.2010.023325.
- 525 32. **Giannesini B, Vilmen C, Le Fur Y, Dalmaso C, Cozzone PJ, Bendahan D.** A strictly noninvasive  
526 MR setup dedicated to longitudinal studies of mechanical performance, bioenergetics, anatomy, and  
527 muscle recruitment in contracting mouse skeletal muscle. *Magn Reson Med* 64: 262–270, 2010. doi:  
528 10.1002/mrm.22386.
- 529 33. **McCarthy, Paul.** FSLEyes. Zenodo: 2020.
- 530 34. **Le Fur Y, Nicoli F, Guye M, Confort-Gouny S, Cozzone PJ, Kober F.** Grid-free interactive and  
531 automated data processing for MR chemical shift imaging data. *Magn Reson Mater Phys Biol Med*  
532 23: 23–30, 2010. doi: 10.1007/s10334-009-0186-y.
- 533 35. **Vanhamme L, van den Boogaart A, Van Huffel S.** Improved Method for Accurate and Efficient  
534 Quantification of MRS Data with Use of Prior Knowledge. *J Magn Reson* 129: 35–43, 1997. doi:  
535 10.1006/jmre.1997.1244.
- 536 36. **Giannesini B, Vilmen C, Amthor H, Bernard M, Bendahan D.** Lack of myostatin impairs  
537 mechanical performance and ATP cost of contraction in exercising mouse gastrocnemius muscle in  
538 vivo. *Am J Physiol-Endocrinol Metab* 305: E33–E40, 2013. doi: 10.1152/ajpendo.00651.2012.
- 539 37. **Arnold DL, Matthews PM, Radda GK.** metabolic recovery after exercise and the assessment of  
540 mitochondrial function in Vivo in human skeletal muscle by means of <sup>31</sup>P NMR. *Magn Reson Med* 1:  
541 307–315, 1984. doi: 10.1002/mrm.1910010303.
- 542 38. **Harkema SJ, Meyer RA.** Effect of acidosis on control of respiration in skeletal muscle. *Am J Physiol-*  
543 *Cell Physiol* 272: C491–C500, 1997. doi: 10.1152/ajpcell.1997.272.2.C491.
- 544 39. **Kemp GJ, Taylor DJ, Radda GK.** Control of phosphocreatine resynthesis during recovery from  
545 exercise in human skeletal muscle. *NMR Biomed* 6: 66–72, 1993. doi: 10.1002/nbm.1940060111.
- 546 40. **Kemp GJ, Roussel M, Bendahan D, Fur YL, Cozzone PJ.** Interrelations of ATP synthesis and  
547 proton handling in ischaemically exercising human forearm muscle studied by <sup>31</sup>P magnetic resonance  
548 spectroscopy. *J Physiol* 535: 901–928, 2001. doi: 10.1111/j.1469-7793.2001.00901.x.

- 549 41. **Kemp GJ, Meyerspeer M, Moser E.** Absolute quantification of phosphorus metabolite  
550 concentrations in human muscle in vivo by <sup>31</sup>P MRS: a quantitative review. *NMR Biomed* 20: 555–  
551 565, 2007. doi: 10.1002/nbm.1192.
- 552 42. **Boska M.** Estimating the ATP cost of force production in the human gastrocnemius/soleus muscle  
553 group using <sup>31</sup>P MRS and <sup>1</sup>H MRI. *NMR Biomed* 4: 173–181, 1991. doi: 10.1002/nbm.1940040404.
- 554 43. **Prompers JJ, Wessels B, Kemp GJ, Nicolay K.** MITOCHONDRIA: Investigation of in vivo muscle  
555 mitochondrial function by <sup>31</sup>P magnetic resonance spectroscopy. *Int J Biochem Cell Biol* 50: 67–72,  
556 2014. doi: 10.1016/j.biocel.2014.02.014.
- 557 44. **Kemp GJ, Radda GK.** Quantitative interpretation of bioenergetic data from <sup>31</sup>P and <sup>1</sup>H magnetic  
558 resonance spectroscopic studies of skeletal muscle: an analytical review. *Magn Reson Q* 10: 43–63,  
559 1994.
- 560 45. **Wolfe CL, Gilbert HF, Brindle KM, Radda GK.** Determination of buffering capacity of rat  
561 myocardium during ischemia. *Biochim Biophys Acta BBA - Mol Cell Res* 971: 9–20, 1988. doi:  
562 10.1016/0167-4889(88)90156-5.
- 563 46. **Hill C, James RS, Cox ValM, Seebacher F, Tallis J.** Age-related changes in isolated mouse skeletal  
564 muscle function are dependent on sex, muscle, and contractility mode. *Am J Physiol-Regul Integr*  
565 *Comp Physiol* 319: R296–R314, 2020. doi: 10.1152/ajpregu.00073.2020.
- 566 47. **Adegoke SA, Braga JAP, D. Adekile A, Figueiredo MS.** Impact of Hydroxyurea on Anthropometry  
567 and Serum 25-Hydroxyvitamin D Among Children With Sickle Cell Disease. *J Pediatr Hematol*  
568 *Oncol* 40: e243–e247, 2018. doi: 10.1097/MPH.0000000000001002.
- 569 48. **Zaccagnini G, Palmisano A, Canu T, Maimone B, Lo Russo FM, Ambrogi F, Gaetano C, De**  
570 **Cobelli F, Del Maschio A, Esposito A, Martelli F.** Magnetic Resonance Imaging Allows the  
571 Evaluation of Tissue Damage and Regeneration in a Mouse Model of Critical Limb Ischemia. *PLOS*  
572 *ONE* 10: e0142111, 2015. doi: 10.1371/journal.pone.0142111.
- 573 49. **Marqueste T, Giannesini B, Fur YL, Cozzone PJ, Bendahan D.** Comparative MRI analysis of T2  
574 changes associated with single and repeated bouts of downhill running leading to eccentric-induced  
575 muscle damage. *J Appl Physiol* 105: 299–307, 2008. doi: 10.1152/jappphysiol.00738.2007.
- 576 50. **Esposito A, Campana L, Palmisano A, De Cobelli F, Canu T, Santarella F, Colantoni C, Monno**  
577 **A, Vezzoli M, Pezzetti G, Manfredi AA, Rovere-Querini P, Maschio AD.** Magnetic Resonance  
578 Imaging at 7T Reveals Common Events in Age-Related Sarcopenia and in the Homeostatic Response  
579 to Muscle Sterile Injury. *PLoS ONE* 8: e59308, 2013. doi: 10.1371/journal.pone.0059308.
- 580 51. **Bryant ND, Li K, Does MD, Barnes S, Gochberg DF, Yankeelov TE, Park JH, Damon BM.**  
581 Multi-parametric MRI characterization of inflammation in murine skeletal muscle. *NMR Biomed* 27:  
582 716–725, 2014. doi: 10.1002/nbm.3113.
- 583 52. **Fisher MJ, Meyer RA, Adams GR, Foley JM, Potchen EJ.** Direct Relationship Between Proton T2  
584 and Exercise Intensity in Skeletal Muscle MR Images: *Invest Radiol* 25: 480–485, 1990. doi:  
585 10.1097/00004424-199005000-00003.
- 586 53. **Mankad VN, Williams JP, Harpen MD, Mancini E, Longenecker G, Moore RB, Shah A, Yang**  
587 **YM, Brogdon BG.** Magnetic resonance imaging of bone marrow in sickle cell disease: clinical,  
588 hematologic, and pathologic correlations. *Blood* 75: 274–283, 1990.

- 589 54. **Ejindu VC, Hine AL, Mashayekhi M, Shorvon PJ, Misra RR.** Musculoskeletal Manifestations of  
590 Sickle Cell Disease. *RadioGraphics* 27: 1005–1021, 2007. doi: 10.1148/rg.274065142.
- 591 55. **Malekgoudarzi B, Feffer S.** Myonecrosis in Sickle Cell Anemia. *N Engl J Med* 340: 483–483, 1999.  
592 doi: 10.1056/NEJM199902113400615.
- 593 56. **Mani S, Duffy TP.** Sickle myonecrosis revisited. *Am J Med* 95: 525–530, 1993. doi: 10.1016/0002-  
594 9343(93)90336-N.
- 595 57. **Vicari P, Achkar R, Oliveira KRB, Miszpupten ML, Fernandes ARC, Figueiredo MS, Bordin  
596 JO.** Myonecrosis in Sickle Cell Anemia: Case Report and Review of the Literature: *South Med J* 97:  
597 894–896, 2004. doi: 10.1097/01.SMJ.0000125172.95454.B7.
- 598 58. **Melo HN, Stoots SJ-M, Pool MA, Carvalho VO, Aragão MLDC, Gurgel RQ, Agyemang C,  
599 Cipolotti R.** Objectively measured physical activity levels and sedentary time in children and  
600 adolescents with sickle cell anemia. *PLOS ONE* 13: e0208916, 2018. doi:  
601 10.1371/journal.pone.0208916.
- 602 59. **Bangsbo J, Krstrup P, González-Alonso J, Saltin B.** ATP production and efficiency of human  
603 skeletal muscle during intense exercise: effect of previous exercise. *Am J Physiol-Endocrinol Metab*  
604 280: E956–E964, 2001. doi: 10.1152/ajpendo.2001.280.6.E956.
- 605 60. **Rossiter HB, Ward SA, Kowalchuk JM, Howe FA, Griffiths JR, Whipp BJ.** Dynamic asymmetry  
606 of phosphocreatine concentration and O<sub>2</sub> uptake between the on- and off-transients of moderate- and  
607 high-intensity exercise in humans. *J Physiol* 541: 991–1002, 2002. doi:  
608 10.1113/jphysiol.2001.012910.
- 609 61. **Krstrup P, Jones AM, Wilkerson DP, Calbet JAL, Bangsbo J.** Muscular and pulmonary O<sub>2</sub>  
610 uptake kinetics during moderate- and high-intensity sub-maximal knee-extensor exercise in humans:  
611 V<sub>O<sub>2</sub></sub> kinetics during knee-extensor exercise. *J Physiol* 587: 1843–1856, 2009. doi:  
612 10.1113/jphysiol.2008.166397.
- 613 62. **Debold EP, Walcott S, Woodward M, Turner MA.** Direct observation of phosphate inhibiting the  
614 force-generating capacity of a miniensemble of Myosin molecules. *Biophys J* 105: 2374–2384, 2013.  
615 doi: 10.1016/j.bpj.2013.09.046.
- 616 63. **Jubrias SA, Crowther GJ, Shankland EG, Gronka RK, Conley KE.** Acidosis inhibits oxidative  
617 phosphorylation in contracting human skeletal muscle in vivo. *J Physiol* 553: 589–599, 2003.
- 618 64. **Westerblad H, Bruton JD, Lännergren J.** The effect of intracellular pH on contractile function of  
619 intact, single fibres of mouse muscle declines with increasing temperature. *J Physiol* 500: 193–204,  
620 1997.
- 621 65. **Knuth ST, Dave H, Peters JR, Fitts RH.** Low cell pH depresses peak power in rat skeletal muscle  
622 fibres at both 30°C and 15°C: implications for muscle fatigue: Low cell pH depresses power. *J Physiol*  
623 575: 887–899, 2006. doi: 10.1113/jphysiol.2006.106732.
- 624 66. **Allen DG, Lamb GD, Westerblad H.** Skeletal Muscle Fatigue: Cellular Mechanisms. *Physiol Rev*  
625 88: 287–332, 2008. doi: 10.1152/physrev.00015.2007.
- 626 67. **Bruton JD, Wretman C, Katz A, Westerblad H.** Increased tetanic force and reduced myoplasmic  
627 [P(i)] following a brief series of tetani in mouse soleus muscle. *Am J Physiol-Cell Physiol* 272: C870–  
628 C874, 1997. doi: 10.1152/ajpcell.1997.272.3.C870.



- 629 68. **McGann PT, Ware RE.** Hydroxyurea therapy for sickle cell anemia. *Expert Opin Drug Saf* 14: 1749–  
630 1758, 2015. doi: 10.1517/14740338.2015.1088827.
- 631 69. **Hernández A, Schiffer TA, Ivarsson N, Cheng AJ, Bruton JD, Lundberg JO, Weitzberg E,**  
632 **Westerblad H.** Dietary nitrate increases tetanic  $[Ca^{2+}]_i$  and contractile force in mouse fast-twitch  
633 muscle. *J Physiol* 590: 3575–3583, 2012. doi: 10.1113/jphysiol.2012.232777.
- 634 70. **Jones AM, Wilkerson DP, Koppo K, Wilmshurst S, Campbell IT.** Inhibition of Nitric Oxide  
635 Synthase by L-NAME Speeds Phase II Pulmonary  $\dot{V}_{O_2}$  Kinetics in the Transition to Moderate-  
636 Intensity Exercise in Man. *J Physiol* 552: 265–272, 2003. doi: 10.1113/jphysiol.2003.045799.
- 637 71. **Ferguson SK, Hirai DM, Copp SW, Holdsworth CT, Allen JD, Jones AM, Musch TI, Poole DC.**  
638 Impact of dietary nitrate supplementation via beetroot juice on exercising muscle vascular control in  
639 rats: Beetroot juice and exercising muscle blood flow. *J Physiol* 591: 547–557, 2013. doi:  
640 10.1113/jphysiol.2012.243121.
- 641 72. **Ferguson SK, Redinius KM, Harral JW, Pak DI, Swindle DC, Hirai DM, Blackwell JR, Jones**  
642 **AM, Stenmark KR, Buehler PW, Irwin DC.** The effect of dietary nitrate supplementation on the  
643 speed-duration relationship in mice with sickle cell disease. *J Appl Physiol* 129: 474–482, 2020. doi:  
644 10.1152/jappphysiol.00122.2020.
- 645 73. **Gladwin MT, Shelhamer JH, Ognibene FP, Pease-Fye ME, Nichols JS, Link B, Patel DB,**  
646 **Jankowski MA, Pannell LK, Schechter AN, Rodgers GP.** Nitric oxide donor properties of  
647 hydroxyurea in patients with sickle cell disease: NO Donor Properties of Hydroxyurea. *Br J Haematol*  
648 116: 436–444, 2002. doi: 10.1046/j.1365-2141.2002.03274.x.
- 649 74. **Ballas SK, Barton FB, Waclawiw MA, Swerdlow P, Eckman JR, Pegelow CH, Koshy M, Barton**  
650 **BA, Bonds DR.** Hydroxyurea and sickle cell anemia: effect on quality of life. *Health Qual Life*  
651 *Outcomes* 4: 59, 2006. doi: 10.1186/1477-7525-4-59.
- 652 75. **Bowden Davies KA, Pickles S, Sprung VS, Kemp GJ, Alam U, Moore DR, Tahrani AA,**  
653 **Cuthbertson DJ.** Reduced physical activity in young and older adults: metabolic and musculoskeletal  
654 implications. *Ther Adv Endocrinol Metab* 10: 204201881988882, 2019. doi:  
655 10.1177/2042018819888824.
- 656 76. **Nur E, Biemond BJ, Otten H-M, Brandjes DP, Schnog J-JB, the CURAMA Study Group.**  
657 Oxidative stress in sickle cell disease; pathophysiology and potential implications for disease  
658 management. *Am J Hematol* 86: 484–489, 2011. doi: 10.1002/ajh.22012.
- 659 77. **Vinhaes CL, Teixeira RS, Monteiro-Júnior JAS, Tibúrcio R, Cubillos-Angulo JM, Arriaga MB,**  
660 **Sabarin AG, de Souza AJ, Silva JJ, Lyra IM, Ladeia AM, Andrade BB.** Hydroxyurea treatment  
661 is associated with reduced degree of oxidative perturbation in children and adolescents with sickle cell  
662 anemia. *Sci Rep* 10: 18982, 2020. doi: 10.1038/s41598-020-76075-5.
- 663 78. **Powers SK, Jackson MJ.** Exercise-Induced Oxidative Stress: Cellular Mechanisms and Impact on  
664 Muscle Force Production. *Physiol Rev* 88: 1243–1276, 2008. doi: 10.1152/physrev.00031.2007.
- 665
- 666

## 667 **Figures**

668 Figure 1: Time dependent changes in specific peak force throughout the moderate-intensity exercise in  
669 SCD<sub>2m</sub> (grey, n = 8), SCD<sub>4m</sub> (black, n = 8) and SCD<sub>4m-HU</sub> mice (white, n = 8). Results are presented as means  
670  $\pm$  SEM. \* significantly different from SCD<sub>4m</sub> (p < 0.05).

671

672 Figure 2: Specific total force production in SCD<sub>2m</sub> (grey, n = 8), SCD<sub>4m</sub> (black, n = 8) and SCD<sub>4m-HU</sub>  
673 (white, n = 8) mice. All data presented a normal distribution, Student's t-test was used.

674

675 Figure 3: Time courses of pHi (A) and relative PCr concentration (B) during the 6-min stimulation period  
676 corresponding to a moderate-intensity exercise and the subsequent recovery in SCD<sub>2m</sub> (grey, n = 8), SCD<sub>4m</sub>  
677 (black, n = 8) and SCD<sub>4m-HU</sub> (white, n = 8) mice. Results are presented as means  $\pm$  SEM. The grey area  
678 represents the stimulation bout.

679

Table 1: Body weight and muscle volume of SCD<sub>2m</sub>, SCD<sub>4m</sub> and SCD<sub>4m-HU</sub> mice.

	SCD <sub>2m</sub> (n = 8)	SCD <sub>4m</sub> (n = 8)	SCD <sub>4m-HU</sub> (n = 8)	p-value	
				2m vs. 4m	4m vs. 4m- HU
Body weight (g)	28.14 (SD 2.67)	32.74 (SD 4.25)	30.50 (SD 2.21)	0.021	0.21
Posterior hindlimb muscle volume (mm <sup>3</sup> )	123.23 (SD 14.82)	128.31 (SD 9.20)	138.02 (SD 21.67)	0.43	0.26
Relative posterior hindlimb muscle volume (mm <sup>3</sup> /g)	4.39 (SD 0.44)	3.97 (SD 0.50)	4.51 (SD 0.51)	0.097	0.049

*Results are presented as means (SD). All data presented a normal distribution, Student's t-test was used.*

Table 2: Muscle T2 values in SCD<sub>2m</sub>, SCD<sub>4m</sub> and SCD<sub>4m-HU</sub> mice.

	SCD <sub>2m</sub> (n = 8)	SCD <sub>4m</sub> (n = 8)	SCD <sub>4m-HU</sub> (n = 8)	p-value	
				2m vs. 4m	4m vs. 4m-HU
Tibialis anterior (ms)	27.09 (SD 1.14)	25.83 (SD 1.26)	27.89 (SD 4.31)	0.055 <sup>s</sup>	0.51 <sup>w</sup>
Whole GA (ms)	33.20 (SD 0.91)	33.85 (SD 2.94)	34.20 (SD 4.92)	0.56 <sup>s</sup>	0.87 <sup>s</sup>
Right part of GA (ms)	29.54 (SD 2.19)	29.69 (SD 1.21)	30.60 (SD 4.47)	0.87 <sup>s</sup>	1 <sup>w</sup>
Center part of GA (ms)	31.55 (SD 2.80)	29.45 (SD 3.05)	29.92 (SD 5.02)	0.17 <sup>s</sup>	0.8 <sup>w</sup>
Left part of GA (ms)	32.32 (SD 1.85)	30.65 (SD 2.73)	32.37 (SD 6.00)	0.17 <sup>s</sup>	0.47 <sup>s</sup>

*Results are presented as means (SD). GA, gastrocnemius. <sup>s</sup> Student's t-test was used. <sup>w</sup> Wilcoxon test was used.*

Table 3: Muscle force production parameters in SCD<sub>2m</sub>, SCD<sub>4m</sub> and SCD<sub>4m-HU</sub> mice.

	SCD <sub>2m</sub> (n = 8)	SCD <sub>4m</sub> (n = 8)	SCD <sub>4m-HU</sub> (n = 8)	p-value	
				2m vs. 4m	4m vs. 4m- HU
sPf <sub>initial</sub> (mN/cm <sup>3</sup> )	1287.86 (SD 288.91)	591.99 (SD 186.27)	717.75 (SD 157.12)	< 0.001	0.17
Force increase (%)	17.35 (SD 8.28)	40.55 (SD 35.40)	38.99 (SD 21.71)	0.09	0.92
sTFP (N/cm <sup>3</sup> )	484.76 (SD 130.80)	254.60 (SD 35.80)	320.32 (SD 67.36)	< 0.001	0.029
TFP (N)	3909.62 (SD 829.01)	2040.87 (SD 560.07)	2930.76 (SD 766.55)	< 0.001	0.019
sPf <sub>final</sub> (mN/cm <sup>3</sup> )	1198.37 (SD 358.21)	678.63 (SD 80.01)	813.16 (SD 211.45)	0.001	0.11
Fatigue index (%)	-6.10 (SD 9.86)	24.73 (SD 37.32)	14.86 (SD 26.42)	0.04	0.05

*Values are presented as means (SD). sPf: Specific peak force. sPf<sub>initial</sub>: sPf at the beginning of exercise. sPf<sub>final</sub>: sPf at the end of exercise. sTFP: Specific total force production. All data presented a normal distribution, Student's t-test was used.*





