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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/japplphysiol.00333.2022 . hal-04250804

# HAL Id: hal-04250804 https://hal.science/hal-04250804

Submitted on 2 May 2024  $\,$ 

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1	Effects of hydroxyurea on skeletal muscle energetics and force
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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
10	Postal address:
11	CRMBM, UMR 7339
12	Faculté de Medicine
13	27 boulevard Jean Moulin
14	13005, Marseille, France
15	Declarations of interest: none
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### 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>4*m*-HU</sub>, n = 8). 28 As compared to  $SCD_{2m}$  mice,  $SCD_{4m}$  mice were heavier and displayed a lower acidosis. As lower specific forces were developed by SCD4m compared to SCD2m, greater force-normalized phosphocreatine 29 30 consumption and oxidative and non-oxidative costs of contraction were also reported. HU treated mice 31  $(SCD_{4m-HU})$  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.

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

41

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

70 Hydroxyurea (HU) is commonly used as a treatment in SCD and in 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 it 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.

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

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### 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 ± 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<sub>2m</sub>). 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<sub>4m-HU</sub>). 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<sub>4m</sub>). 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 <u>Non-invasive investigation of posterior hindlimb muscle function and bioenergetics</u>

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 muscle using <sup>31</sup>P-MRS. We have chosen to study the posterior hindlimb muscles, which forms the belly of 123 124 the calf, because it is clearly distinct from the other muscles of the leg, easily accessible for MR coils and 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 125 126 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%  $O_2$  (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 of the gastrocnemius muscle was located above an elliptic (8 x 6 mm<sup>2</sup>) <sup>31</sup>P surface coil. Body temperature 138 139 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, Herthfordshire, 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 acquisition system (AD Instruments, Sydney, Australia) and a dedicated software (LabChart 8, AD 150 151 Instruments, Sydney, Australia). The digital signal was converted to force (mN) according to a linear 152 calibration curve.

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### 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,  $20 \ge 20$ 169 mm field of view,  $256 \ge 192$  matrix size, acquisition time = 3 = 3 = 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,  $20 \times 30$  mm field of view,  $256 \times 256$  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

MRS data were processed using custom-written routines in Python (34) integrating the AMARES
Fortran code (35) from jMRUI (http://www.jmrui.eu/). Relative concentrations of phosphocreatine (PCr),
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 calculated, as previously described (38, 39):  $[ADP] = ([Cr] \times [ATP])/([PCr] \times 10^{-pH} \times K_{CK})$ , using the creatine 199 kinase equilibrium constant ( $K_{CK} = 1.66 \times 10^9 \text{ M}^{-1}$ ) (40) and total creatine [Cr] calculated by assuming that 200 201 PCr represents ~85% of total Cr (41). The rate of PCr degradation (VPCr<sub>stim</sub>, in mM/min) at the onset of 202 exercise was calculated as VPCr<sub>stim</sub> =  $\Delta PCr/\tau PCr_{stim}$ , 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<sub>cost</sub> in  $\mu$ M/min/mN/cm<sup>3</sup>) was 206 calculated as the ratio between VPCr<sub>stim</sub> 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, VPCr<sub>rec</sub> was considered as the rate of 210 oxidative ATP production at the end of exercise as  $VPCr_{rec} = \Delta PCr/\tau PCr_{rec}$  (42, 43). sPf-normalized oxidative 211 cost of contraction (PCr<sub>rec</sub> in  $\mu$ M/min/mN/cm<sup>3</sup>) was calculated as the ratio between VPCr<sub>rec</sub> and the amount 212 of specific force produced at the end of exercise. The maximal rate of PCr recovery (V<sub>max</sub>, mM/min) was 213 calculated as  $V_{max} = VPCr_{rec} (1 + (K_m / [ADP_{end}])) (44)$ , where  $K_m$  is the affinity constant of the mitochondria for ADP (30 µM in gastrocnemius muscle of the mice), and [ADPend] is the ADP concentration at the end 214 215 of exercise.

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

220

### 221 Statistics

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

All values are presented as means (SD) except on graphical representations for which SE are displayed for the sake of clarity. The effects of the disease evolution were assessed from the comparative analysis between  $SCD_{2m}$  and  $SCD_{4m}$  mice while the effects of HU treatment were determined based on the comparative analysis between  $SCD_{4m}$  and  $SCD_{4m-HU}$  mice. Normality of sample distribution was tested with the Shapiro-Wilk test.

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

For non-time course variables, statistical differences between groups were assessed using nonparametric Wilcoxon tests or parametric Student's t-test according to the result of the normality test. A Bonferroni correction was applied to take into account the multiple comparison of  $SCD_{4m}$ , consequently 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

As indicated in table 1,  $SCD_{4m}$  mice were heavier than  $SCD_{2m}$  whereas body mass in  $SCD_{4m-HU}$  mice and SCD<sub>4m</sub> mice was similar. Neither posterior hindlimb muscle volume nor the corresponding ratio to body mass was significantly different between groups. T2 values were similar in the three groups whatever the muscles considered, or the part of the 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  $SCD_{4m-HU}$  (717.75 SD 157.12 mN/cm<sup>3</sup>, Student's t-test, p = 0.17) mice had similar values (table 3). As 250 251 illustrated in figure 1, the sPf time-course was classically biphasic. sPf initially increases to reach an apex 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 252 253 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  $\text{SCD}_{4m}$  mice (40.55 SD 35.40 %, Wilcoxon test, p = 0.09) and between  $\text{SCD}_{4m}$  and 255  $SCD_{4m-HU}$  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  $SCD_{2m}$  and  $SCD_{4m}$  mice (Two-way ANOVA, p < 0.005). Overall,  $SCD_{2m}$  mice 258 produced a significantly larger amount of specific force at all times of the stimulation period as compared 259 to  $SCD_{4m}$  (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  $SCD_{4m}$  and  $SCD_{4m-HU}$  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>4*m*-HU</sub> as compared to SCD<sub>4*m*</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).

The sTFP of the  $SCD_{2m}$  group (484.76 SD 130.80 N/cm<sup>3</sup>) was significantly larger than the corresponding value in the  $SCD_{4m}$  group whereas  $SCD_{4m}$  (254.60 SD 35.80 N/cm<sup>3</sup>) and  $SCD_{4m-HU}$  mice (320.32 SD 67.36 N/cm<sup>3</sup>, figure 2 and table 3) displayed similar values. The absolute TFP was significantly larger in  $SCD_{2m}$  269 (3909.62 SD 829.01 N) compared to  $SCD_{4m}$  (2040.87 SD 560.70 N, p < 0.001). Additionally, TFP of  $SCD_{4m}$ . 270 <sub>*HU*</sub> (2930.76 SD 766.55 N, p = 0.019) was also higher as compared to  $SCD_{4m}$ .

The fatigue index was neither different between  $SCD_{2m}$  (-6.10 SD 9.86 %) and  $SCD_{4m}$  (24.73 SD 37.32 %, Student's t-test, p = 0.04) nor between  $SCD_{4m}$  and  $SCD_{4m-HU}$  mice (14.86 SD 26.42 %, Student's t-test, p = 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 pHi278 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.  $pH_i$ 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  $SCD_{4m-HU}$  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).

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

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

296 VPCr<sub>stim</sub>, VPCr<sub>rec</sub> and V<sub>max</sub> were similar in the three groups (table 4). The sPf-normalized non-oxidative 297 and oxidative costs of contraction, assessed by PCr<sub>cost</sub> and PCr<sub>rec</sub>, respectively were significantly higher in 298 SCD<sub>4m</sub> as compared to SCD<sub>2m</sub> mice (table 4, p = 0.0019 and p = 0.0006, respectively). PCr<sub>rec</sub> and PCr<sub>cost</sub> were 299 similar in SCD<sub>4m</sub> and SCD<sub>4m-HU</sub> 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

The greater body mass in  $SCD_{4m}$  mice compared to their  $SCD_{2m}$  counterparts is in accordance with the literature showing that weight can increase in mice up to 52 weeks of age (46). The similar body mass in  $SCD_{4m-HU}$  as compared to  $SCD_{4m}$  mice indicates that the HU treatment did not affect body mass in the present study. This result in mice differs from what was observed in children or adults with (28, 47). To date, no satisfactory explanation for this discrepancy between the studies and the species can be provided. Previous studies have considered elevated T2 values to be a reflection of inflammatory phenomena in a variety of physiological conditions (48–52). Given the previously reported muscle VOC and myonecrosis in SCD patients (53–56), one could have expected muscle T2 changes through age and treatment. As no difference in T2 values were observed between mice in the present study, one can exclude an inflammatory phenomenon or its aggravation in the studied SCD mice muscles. This result is in accordance with the fact that muscle complications (such as myonecrosis) are rare in patients with SCD (10, 57).

324

#### 325 <u>Effects of an 8-week period on skeletal muscle function and energetics</u>

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

The impaired capacity of force production previously reported in SCD has partly been related to reductions in posterior hindlimb muscle mass (7, 20), electromyographic activity (8), and daily activity (58), as well as to a deep tissue hyperalgesia (11, 13). Yet, the similar muscle volumes reported between  $SCD_{2m}$ and  $SCD_{4m}$  mice exclude the implication of divergent muscle mass as an accounting factor of the lower force production in  $SCD_{4m}$  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  $SCD_{4m}$  mice clearly 349 illustrates a contractile defect in this group compared to  $SCD_{2m}$  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  $SCD_{4m}$  and  $SCD_{2m}$  mice should be associated with divergent metabolic response. To investigate the 355 metabolic response, absolute terms and values normalized to force has been expressed.

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

Although the extent of PCr consumption was similar, the specific force production was lower in SCD<sub>4m</sub> 361 than in SCD<sub>2m</sub> mice. As a result, the sTFP-normalized consumption of PCr was higher in the former group, 362 363 indicating that muscle of  $SCD_{4m}$  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>, respectively) were significantly greater in SCD4m than in SCD2m mice. As a whole, our results seem to 365 366 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

a higher ATP consumption and/or a lower ATP resynthesis via oxidative and non-oxidative processes for a
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  $SCD_{4m}$  group as compared to  $SCD_{2m}$  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  $SCD_{4m}$  mice of present study.

Finally, one cannot exclude that the diminished specific force production can be related to disrupted mechanisms of ATP utilization and/or resynthesis by processes unexplored in the present study. Further 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  $SCD_{4m-HU}$  mice was greater compared to the  $SCD_{4m}$ 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<sub>4m</sub> and SCD<sub>4m-HU</sub> 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<sup>+</sup> 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.

## 422 **Conclusion**

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

429

### 430 Acknowledgments

431 Hydroxyurea has been kindly offered by addmedica.

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## 667 Figures

668 Figure 1: Time dependent changes in specific peak force throughout the moderate-intensity exercise in 669  $SCD_{2m}$  (grey, n = 8),  $SCD_{4m}$  (black, n = 8) and  $SCD_{4m-HU}$  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  $SCD_{2m}$  (grey, n = 8),  $SCD_{4m}$  (black, n = 8) and  $SCD_{4m-HU}$ 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  $SCD_{2m}$  (grey, n = 8),  $SCD_{4m}$ 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.

	$\mathrm{SCD}_{2m}$	$\mathrm{SCD}_{4m}$	$\mathrm{SCD}_{4m\text{-}HU}$	p-value	
	(n = 8)	(n = 8)	(n = 8)	2 <i>m</i> vs. 4 <i>m</i>	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	123.23 (SD 14.82)	128.31 (SD 9.20)	138.02 (SD 21.67)	0.43	0.26
volume (mm <sup>3</sup> )					
Relative posterior hindlimb	4 30 (SD 0 44)	3 97 (SD 0 50)	4 51 (SD 0 51)	0.097	0.049
muscle volume (mm <sup>3</sup> /g)	4.37 (3D 0.44)	5.77 (SD 0.50)	4.51 (50 0.51)	0.097	0.049

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

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

	$\mathrm{SCD}_{2m}$	$SCD_{4m}$	SCD <sub>4m-HU</sub>	p-value	
	(n = 8)	(n = 8)	(n = 8)	2 <i>m</i> vs. 4 <i>m</i>	4 <i>m</i> vs. 4 <i>m-HU</i>
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>	$1^{w}$
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>	$0.8^{w}$
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>

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

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

	SCD <sub>2m</sub>	$SCD_{4m}$	SCD <sub>4m-HU</sub>	p-'	value
	(n = 8)	(n = 8)	(n = 8)	2 <i>m</i> vs. 4 <i>m</i>	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 (SD67.36)	< 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

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

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.



Time (min)



