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2 **Simulated microgravity disturbs iron metabolism and distribution in humans: lessons**
3 **from dry immersion, an innovative ground-based human model**

4
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20
21 **Running head:** DI-5-Cuffs - Dry immersion affects iron metabolism

22 **Key words:** erythropoiesis, iron misdistribution, bedrest, spaceflight, muscle atrophy

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34 **NONSTANDARD ABBREVIATIONS**

35 ANSM : French National Agency for Medicines and Health Products Safety

36 BDC: baseline data collection

37 DI : dry immersion

38 EPO: erythropoietin

39 HIC : hepatic iron store

40 HU : hindlimb unloading

41 SIC : splenic iron store

42

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49

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51 No conflict of interest, financial or otherwise related to this work, is declared by the author(s).

52

53 **AUTHOR CONTRIBUTIONS**

54 FD, GG-K and OL designed the clinical study. KN, SR, M-LI, LO, M-PB, AB, MR, and FD
55 performed experiments; KN, SR, and FD analyzed data; KN, SR, MR, CK-R, OL, and FD
56 interpreted results of experiments; KN, and FD prepared figures; KN, OL, and FD drafted
57 manuscript; KN, MR, CK-R, OL, and FD edited and revised manuscript; KN, SR, M-LI, LO,
58 MR, CK-R, GG-K, OL, and FD approved final version of manuscript.

59

60 **ABSTRACT**

61 The objective of the present study was to determine the effects of dry immersion, an
62 innovative ground-based human model of simulated microgravity and extreme physical
63 inactivity, on iron homeostasis and distribution. Twenty young healthy men were recruited
64 and submitted to 5 days of dry immersion (DI). Fasting blood samples and MRI were
65 performed before and after DI exposure to assess iron status, as well as hematological
66 responses. DI increased spleen iron concentrations (SIC), whereas hepatic iron store (HIC)
67 was not affected. Spleen iron sequestration could be due to the concomitant increase in serum
68 hepcidin levels ($p < 0.001$). Increased serum unconjugated bilirubin, as well as the rise of
69 serum myoglobin levels support that DI may promote hemolysis and myolysis. These
70 phenomena could contribute to the concomitant increase of serum iron and transferrin
71 saturation levels ($p < 0.001$). As HIC remained unchanged, increased serum hepcidin levels
72 could be due both to higher transferrin saturation level, and to low-grade pro-inflammatory as
73 suggested by the significant rise of serum ferritin and haptoglobin levels after DI ($p = 0.003$
74 and $p = 0.003$, respectively). These observations highlight the need for better assessment of
75 iron metabolism in bedridden patients, and an optimization of the diet currently proposed to
76 astronauts.

77 **Keywords:** erythropoiesis, iron misdistribution, bedrest, spaceflight, muscle atrophy

78

79 **INTRODUCTION**

80 Physical function decreases in astronauts and patients in hospital, especially due to exposure
81 to extreme physical inactivity. In both cases, microgravity and bedrest reduce work capacity
82 and complicate recovery (1, 2). Iron is required due to its role in many biological functions
83 including protein, lipid, carbohydrates and nucleic acid metabolisms. However, abnormally
84 accumulated in organs, iron promotes oxidative stress through the Fenton reaction, and thus
85 may play a role in the development of osteoporosis and muscle atrophy (3, 4). In addition,
86 when abnormally distributed in the body, iron could also affect erythropoiesis, and so
87 contribute to anemia (1, 5).

88 Previous studies reported that serum ferritin levels are higher in astronauts and bedridden
89 subjects, suggesting increased body iron stores (6, 7). Using the hind limb unloading (HU)
90 model to simulate microgravity in rats, we recently showed that short-term exposure to HU
91 causes iron misdistribution, characterized by increased splenic iron store (8). Interestingly,
92 hepatic hepcidin expression and blood hepcidin levels increase in rodents exposed to
93 simulated microgravity (8–10). As hepcidin controls iron release from cells, especially spleen
94 macrophages, it could play a key role in microgravity-induced iron misdistribution by limiting
95 the expression and activity of ferroportin, the cell iron exporter that allows iron recycling
96 from macrophages in plasma (11). Notably, it has been suggested that iron metabolism has
97 strong interactions with other essential metals, especially due to the fact that some iron
98 proteins could also participate in the metabolism of other metals (12–16). Except few data
99 collected in astronauts during spaceflight (17), the effects of microgravity on hepcidin
100 regulation in humans remains poorly understood despite its major role as an integrative signal
101 in the control of systemic iron metabolism.

102 Therefore, our objective was to characterize the effects of simulated microgravity and extreme
103 physical inactivity on iron metabolism and distribution in relation with hepcidin modulation.
104 For this purpose, we used the dry immersion (DI) model that accurately reproduces the effects
105 of microgravity (18), and exposed healthy young volunteers for 5 days to this innovative
106 ground-based human model. In addition, a group of participants underwent venoconstrictive
107 thigh cuffs protocol, a countermeasure to microgravity used to sequester fluids in the lower
108 limbs (Figure 1).

109

110 **METHODS**

111 Participants

112 20 healthy men were recruited to be exposed to 5 days of dry immersion. Two subjects were
113 excluded before BDC-5 for reasons unrelated to protocol. A total of eighteen subjects were
114 included in the study and randomly divided at BDC-2 into Control or Cuffs group (9/9 split).
115 Participants were anonymized and designated in data sets by single letters. Subjects B, E, F, I,
116 K, M, O, Q and S were included in control group while A, C, D, G, H, J, N, P and R were
117 included in Cuffs group. All subjects were informed about the experimental procedures and
118 gave their written consent. The experimental protocol conformed to the standards set by the
119 Declaration of Helsinki and was approved by the local Ethic Committee (CPP Est III: October
120 2, 2018, n° ID RCB 2018-A01470-55) and French Health Authorities (ANSM: August 13,
121 2018, ClinicalTrials.gov Identifier: NCT03915457). Baseline group characteristics are
122 detailed in Table 1. There was no significant difference between groups at baseline (Table 1).

123 Overall study design

124 The study was conducted at the MEDES space clinic, Toulouse, France from 19/11/2018 to
125 23/03/2019. Subjects arrived in the evening of BDC-5 and left in the morning of R+2. The
126 experimental protocol included four days of ambulatory baseline measurements before
127 immersion (BDC-4 to BDC-1), five days (120 hours) of dry immersion (DI1 to DI5) and two
128 days of ambulatory recovery (R0, R+1). Besides, a week prior to beginning the protocol the
129 subjects went to MEDES for pre-immersion muscle biopsy and resting metabolic rate
130 measurement. General protocol of strict DI was conducted according to methodology detailed
131 in De Abreu et al. 2017 (19). Two subjects, one from Control group and one from Cuff
132 group, underwent dry immersion simultaneously in the same room, in two separate baths
133 (except for two subjects, one Cuffs and one Control, C and M, who had no mate).
134 Thermoneutral water temperature was continuously maintained. Light-off period was set at
135 23:00-07:00. Daily hygiene, weighing and some specific measurements required extraction
136 from the bath. During these out-of-bath periods, subjects maintained the -6° head-down
137 position, a reliable position to maintain physiological effects of microgravity used in bedrest
138 studies (20). Total out-of-bath supine time for the 120 h of immersion was 9.7 ± 1.3 h. On DI1-
139 DI4 out-of-bath time was 1.1 ± 0.6 h/day. On DI5 out-of-bath time was 5.3 ± 1.1 h, because of
140 muscle biopsy and Magnetic resonance imaging (MRI). Otherwise, during DI, subjects
141 remained immersed in a supine position for all activities and were continuously observed by
142 video monitoring. Body weight, blood pressure, heart rate and tympanic body temperature

143 were measured daily. The range of adequate water intake was fixed at 35-60 ml/kg/day;
144 within this range water intake throughout the protocol was ad libitum (measured). The menu
145 composition of each experiment day was identical for all participants and dietary intake was
146 individually tailored and controlled during the study. The individual energy intake was
147 calculated by multiplying resting metabolic rate with physical activity levels of respectively
148 1.6 and 1.3 before and during DI. Daily caloric intake was around 2625 kcal for baseline and
149 2160 kcal for the dry immersion period. Daily intake for iron was approximately 10 and 15
150 mg. Subject N was finally excluded from all statistical analysis for the present study due to
151 higher abnormal values before and after DI for transferrin saturation (48.7 and 65.3%), serum
152 ferritin levels (449.8 and 615 µg/l) and iron liver content (70.0 and 71.1 µmol/g).

153 Cuff

154 Subjects randomized to Cuff group wore the thigh cuffs during the 5 days of DI, from 10h to
155 18h at DI1 and from 8h to 18h at DI2-DI5. Thigh cuffs are elastic strips, adapted to each
156 subject to have the same effects on lower limb distensibility as at counterpressure of about 30
157 mmHg. Individual adjustment was determined for each subject with calf plethysmography,
158 performed in the supine position at BDC-2. At DI1, thigh cuffs were put on immediately prior
159 to the onset of immersion at 10h.

160 Body composition

161 Body mass (kg) was measured on a weighing trolley at baseline and every morning during dry
162 immersion. Fat and fat free mass were measured by DEXA (Hologic, QDR 4500 C, MA,
163 USA) 4 days before DI (BDC-4), and after 5 days of DI (DI5).

164 Hematology and iron status

165 Venous blood was sampled at 7 a.m. in fasting state from a forearm vein 4 days before dry
166 immersion (BDC-4), 2 hours before subject reambulation (R+0) and 2 days after reambulation
167 (R+2). White blood cells (WBC) and red blood cells (RBC) counts, hemoglobin, hematocrit,
168 reticulocytes by the Advia 2120 (Siemens Healthcare Diagnostics, Deerfield, Illinois, USA),
169 an automated hematology analyzer. Serum iron, transferrin, bilirubin, myoglobin, haptoglobin
170 concentrations were measured in the biochemistry laboratory (Rennes Pontchaillou Hospital)
171 on Cobas 8000 analyser Roche® (Roche Diagnostics, Meylan, France). Serum iron and
172 bilirubin concentrations were measured by standard colorimetric methods; serum transferrin
173 and haptoglobin levels were determined by immunoturbidimetry; serum ferritin and
174 myoglobin concentrations were measured by ECLIA (Electro-Chemiluminescence
175 ImmunoAssay). Total iron binding capacity (TIBC) was calculated using the formula:

176 (transferrin in g/l x 25) and transferrin saturation (%) was calculated as follows: (iron in
177 $\mu\text{mol/l} / \text{TIBC}) \times 100$. Reference interval of local laboratory values of serum iron,
178 transferrin saturation, ferritin, haptoglobin, myoglobin were 12.5-25 $\mu\text{mol/L}$, 23-46%, 55-
179 345 $\mu\text{g/L}$, 0,3-2g/l, 10-92ng/ml respectively.

180 MRI protocol

181 MRI is a sensitive technique to quantify iron variations in tissues. Indeed, iron is a
182 paramagnetic substance that becomes superparamagnetic (with a strong decrease of the T2*),
183 when several atoms are grouped. Using this concept, Gandon et al. developed algorithms to
184 quantify iron content in spleen and liver (21). For this study, a 1.5-T Siemens-Avanto
185 magnetic resonance imaging system was used in Toulouse Hospital (CHU Rangueil). MRI
186 sessions were performed before DI at BDC-3 and at DI-5 evening. Subjects were transferred
187 from DI bath to MRI apparatus in head-down bed rest -6° in order to maintain the effects of
188 simulated microgravity. Subjects were in supine rest position during all the MRI acquisition.
189 Five gradient echo sequences were performed, following the protocol proposed by Gandon et
190 al. (21). A typical T1-weighted sequence with an in-phase echo time of 4 ms and a 90° pulse
191 angle was followed by four sequences with a 20° pulse angle and in-phase echo times ranging
192 from 4 to 21 ms, to progressively increase T2-weighting. Every sequence had an acquisition
193 time of 15 s. Magnetic resonance gradient echo sequences used are summarized in Table 2.
194 For each sequence three circular regions of interest (ROIs) were drawn in the right lobe of the
195 liver, one in the spleen and two in the left and right paraspinal muscles. The liver-to-muscle
196 and spleen-to-liver SI ratio (SIR) were then calculated for each sequence using the algorithm
197 provided by Gandon et al. (21), which is available online
198 (<http://www.radio.univrennes1.fr/Sources/EN/HemoCalc15.html>). Due to anxiety attack
199 during MRI protocol for the subject M, these data are missing on the analyses.

200 Serum EPO, wr-CRP and hepcidin levels

201 Measurement of serum EPO and hepcidin levels was performed according to manufacturer's
202 instructions of the LEGEND MAX™ Human Erythropoietin (EPO) ELISA Kit (BioLegend,
203 USA) and Hpcidin-25 (human) – Lyophilized Antiserum for EIA/ELISA Kit (catalog
204 number : S-1337, Peninsula Laboratories International, Inc, USA), respectively. Serum wr-
205 CRP was measured on Cobas 8000 analyzer Roche® by a latex-enhanced
206 immunoturbidimetry method (C-Reactive Protein Gen.3, catalog no. 05172373 190, Roche
207 Diagnostics, Meylan, France) with a detection limit of 0.3 mg/L and extended measuring
208 range of 0.3–350 mg/L.

209

210 Statistical analysis

211 All data are presented in plots (individual data) or as mean \pm SD (group data). The average
212 variation observed in participants after DI compared to before DI is expressed in percentage
213 (%). The normality of each distribution and homogeneity of variance were assessed with the
214 Kolmogorov-Smirnov and Bartlett test, respectively. Statistical significance was checked
215 using a two-way ANOVA for repeated measures. Data were analyzed using GraphPad Prism
216 version 6.02 for Windows (GraphPad Software, La Jolla, California).

217 **RESULTS**

218 *Participant Flow*

219 The participant flow is represented in Figure 2. This clinical trial was originally designed to
220 assess the effects of thigh cuffs on the hematological, cardiovascular and musculoskeletal
221 system responses induced by 5 days of DI. As cuffs did not have any significant effects on
222 iron metabolism parameters (Figure 3) during simulated microgravity, the results and
223 discussion section are only focused on the effects of DI on iron metabolism.

224 *Dry immersion affects both body weight and composition*

225 After 5 days of DI, body lean mass decreased significantly in all participants (-2.5%, $p < 0.001$;
226 Table. 3 and Supplemental Fig. 2B), as well as the leg lean mass (-2.9%, $p < 0.001$; Table. 3).
227 Total body mass and total fat mass concomitantly decreased (-2.5% and -0.1% respectively,
228 $p < 0.001$ for both; Table 3 and Supplemental Fig. 2A and 2C), so the fat mass proportion of
229 the participants remained unchanged (Table. 3 and Supplemental Fig. 2D). Thigh cuffs had no
230 significant impact on body weight and body composition (Table. 3).

231 *Dry immersion alters iron homeostasis and distribution in the body*

232 Serum ferritin levels significantly increased in response to DI (+25%, $p = 0.003$; Figure 3I).
233 MRI analysis of body iron stores showed that 5 days of DI induced iron accumulation in the
234 spleen (+29%; $p = 0.009$; Figure 3A and 3C), but not in the liver (Figure 3B and 3C). We did
235 not observe any correlation between spleen iron and serum ferritin levels ($r = 0.04$, $p = 0.81$). DI
236 also increased serum iron concentration, and transferrin saturation level (+22% and +25%
237 respectively, $p < 0.001$ for both; Figure 3D and 3E), whereas serum transferrin concentration
238 remained unchanged (Figure 3F). The serum hepcidin level significantly increased after DI

239 (+66%, $p < 0.001$, Figure 3G). We did not observe any significant effect of thigh cuffs on iron
240 homeostasis and body iron distribution (Figure 3).

241 *Effects of dry immersion on erythropoiesis, hemolysis and myolysis parameters*

242 Dry immersion increased red blood cells (RBC) and white blood cells (WBC) counts (+13%
243 and +25% respectively, $p < 0.001$ for both; Table 4), as well as hemoglobin and hematocrit
244 levels (+12% and +13% respectively, $p < 0.001$ for both; Table. 4). To explore both
245 hematologic and iron homeostasis responses to dry immersion, erythropoiesis, hemolysis and
246 myolysis biomarkers were assessed in blood compartment. Serum EPO levels and blood
247 reticulocyte fraction were significantly decreased after 5 days of DI (-22% and -17%
248 respectively, $p = 0.009$ and $p = 0.003$ respectively; Table. 4). Unconjugated bilirubin and
249 haptoglobin levels were significantly increased in the bloodstream after 5 days of DI (+24%
250 and +21% respectively, $p = 0.037$ and $p < 0.001$, Table. 4 and Figure 3H, respectively). Serum
251 myoglobin levels were also higher after 5 days of DI compared to baseline (+15%, $p = 0.011$;
252 Table. 4). Wide range C-reactive protein (wr-CRP) level remained unchanged by DI (Table.
253 4). Thigh cuffs participants exhibited lower RBC count compared to CTL participants, both
254 before and after exposure to DI (-7% before DI and -10% after DI, Cuff effect: $p = 0.009$;
255 Table. 4)

256 **DISCUSSION**

257 The present study was designed to explore in humans the consequences of simulated
258 microgravity and extreme physical inactivity on iron metabolism and distribution in relation
259 with the modulation of hepcidin, the central regulator of systemic iron homeostasis. Using an
260 innovative ground-based model of DI and MRI, we demonstrated that short-term exposure to
261 simulated microgravity causes iron sequestration in the spleen. Such spleen iron accumulation
262 could be related to the increase of serum hepcidin levels we observed, since this peptide limits
263 iron egress from macrophages. Our data also support that hemolysis and skeletal muscle
264 atrophy could contribute to this iron misdistribution.

265 The dry immersion model allows scientists to accurately and rapidly mimic most of
266 physiological effects of microgravity and extreme physical inactivity (19, 22, 23). Short-term
267 DI studies are sufficient to induce significant changes in fluid volume redistribution (19, 24),
268 cardiorespiratory responses (18, 25) and muscle plasticity (22, 26) similarly to those observed
269 during spaceflight or prolonged bed rest. The morphometric and hematologic data we
270 observed in the present study, including skeletal muscle atrophy and pseudopolycythaemia

271 (i.e. hemoglobin and hematocrit increases), agree with those previously reported during
272 spaceflight, and in bedrest and DI studies (22, 27–29). These results suggest that the iron
273 metabolism parameters we studied should truly reflect conditions related to microgravity and
274 physical inactivity.

275 The increase of serum ferritin levels in response to DI agrees fully with previous reports in
276 real and simulated microgravity studies (6, 7). Such a rise in ferritin levels could indicate that
277 iron overload develops during DI. However, a ferritin increase can also be related to other
278 etiologies including acute and chronic inflammation (30). To clarify the situation, we used
279 MRI that allows a non-invasive quantification of iron concentration variations before and
280 after DI, both in the liver and the spleen, two key organs involved in iron metabolism and
281 storage (21). Here, for the first time according to our knowledge, we found in humans that
282 short-term exposure to simulated microgravity causes an increase in iron concentration
283 increase in the spleen, whereas the liver remains spared from iron excess. Importantly, these
284 data are in agreement with our previous findings obtained in rats, in which 7 days of hindlimb
285 unloading increased spleen iron concentrations, but did not affect liver iron store (8). These
286 results suggest that spleen iron sequestration occurring during the first days of microgravity
287 could be a potential mechanism to protect other organs from iron excess. However, even if we
288 were not able to determine spleen volume variations, we cannot exclude that microgravity
289 could affect this parameter, and so spleen iron concentrations. It would be thus interesting to
290 explore in humans the effects of long-duration exposure to microgravity on iron content in
291 other organs known to be functionally affected by this condition (e.g. liver, bone, heart). In
292 this way, preclinical studies performed on mice support that iron starts to accumulate in the
293 liver and bone when hindlimb unloading is prolonged from 3 to 4 weeks (9, 31) suggesting
294 that the risks of iron-related disorders need to be considered for long-duration space travel.

295 Hepcidin, the gatekeeper of systemic iron metabolism, is known to promote ferroportin
296 degradation, thus inhibiting iron export from enterocytes and macrophages, and leading to
297 iron sequestration in cells, especially in the spleen which is a macrophage-rich organ (11).
298 Our data showing that DI increased serum hepcidin levels support that increased spleen iron
299 concentrations would be due to iron sequestration in this organ more than to a variation in
300 spleen volume. The main inducers of hepcidin expression are the increase of transferrin
301 saturation and iron stores levels, especially in the liver, but also the inflammatory process
302 (32).

303 Our data supports that the hepatic iron accumulation is likely not involved in the hepcidin
304 concentration increase, as MRI did not reveal any significant change in hepatic iron
305 concentrations after DI period. Conversely, both the increase of serum iron and transferrin
306 saturation levels could have played a role in promoting hepcidin synthesis as suggested by
307 reports linking serum transferrin saturation level and hepcidin expression (33). However,
308 microgravity could rapidly induce a low-grade pro-inflammatory state that could also
309 participate in the hepcidin level increase (34). Indeed, despite the normal wr-CRP levels, we
310 found higher levels of serum haptoglobin and ferritin concentrations, both proteins known to
311 be induced during inflammation. The increase of serum iron and transferrin saturation levels
312 were not expected, as an increase in hepcidin concentration is reported to decrease both
313 parameters by limiting iron egress. Therefore, it must be considered whether the rise of
314 transferrin saturation is the initial element leading to iron metabolism disturbance in our
315 conditions. The mechanisms leading to such an increase are likely multifactorial. The first one
316 could be the hemoconcentration due to the decrease of plasma volume. In the present study, a
317 decrease of 15 to 20% of plasma volume has been reported between the first and the fifth day
318 of DI (24). This reduction of plasma volume remain quite close to those observed during bed
319 rest studies and spaceflight for the same duration (35–37). Even if this decrease in plasma
320 volume could slightly contribute to increase of some blood analytes, it cannot explain the rise
321 of transferrin saturation calculated from a ratio of serum iron and transferrin concentrations.
322 Furthermore, our data suggest that hemolysis and myolysis could also be involved by
323 promoting iron release in blood compartment.

324 Regarding hemolysis, it is well known that 65-70% of the total body iron is contained in
325 hemoglobin of RBC (38, 39). Noteworthy, senescent RBC are engulfed by macrophages in
326 the reticuloendothelial system, especially in spleen macrophages during the
327 erythrophagocytosis process. Specifically, the hemoglobin they contained is catabolized by
328 the cytosolic hemoxygenase-1 to release the heme-bound iron, whereas biliverdin is
329 converted in unconjugated bilirubin (40, 41). Hemolysis phenomenon is biologically
330 characterized by increased serum unconjugated bilirubin levels, combined in case of
331 intravascular hemolysis, to a reduction of serum haptoglobin level (42). Previous studies
332 evaluating these markers observed that serum bilirubin levels remained unaffected in humans
333 exposed to real or simulated microgravity, thus suggesting that significant hemolysis did
334 occur under microgravity (36, 43, 44). On contrary, we observed that 5 days of DI increased
335 both serum unconjugated bilirubin and haptoglobin levels, suggesting that if hemolysis occurs

336 under DI, this hemolysis is extravascular (45). The slight hemolysis we observed could
337 ultimately be due to the rapid destruction of young red blood cells (i.e. neocytolysis), a
338 phenomenon previously described in astronauts during the first days of spaceflight (1, 46).
339 Interestingly, neocytolysis is triggered when a decrease in EPO production occurs, splenic
340 endothelial cells responding to this withdrawal by increasing permeability and promoting
341 phagocytosis of young red cells by macrophages (47). Thus, the decrease of serum EPO levels
342 we observed after 5 days of DI supports that this EPO withdrawal could favor iron uptake by
343 splenic macrophages. Combined with the increase of serum hepcidin levels limiting iron
344 export from macrophages, this decrease in EPO levels probably contribute to splenic iron
345 sequestration observed in the present study.

346 Myolysis may also play a role in the increases of serum iron and transferrin saturation levels.
347 Indeed, skeletal muscle contains 10 to 15% of body iron, mainly as heme-bound iron of
348 myoglobin (38, 39). In agreement with previous short-term bedrest and DI studies (22, 27,
349 28), our data showed that total and leg lean mass are significantly reduced after 5 days of DI
350 compared with baseline. These data strongly support the development of skeletal muscle
351 atrophy during DI period. Importantly, at the same time, the serum myoglobin levels
352 significantly increased after 5 days of DI. Similarly to hemoglobin, macrophages are able to
353 phagocyte myoglobin to degrade myoglobin-associated heme, and to recycle the heme-bound
354 iron (48). Thus, we cannot exclude that phagocytosis of myoglobin by splenic macrophages
355 could also promote both spleen iron sequestration and the increase of unconjugated bilirubin
356 release to bloodstream.

357 In summary, our study demonstrates for the first time that in young healthy men, dry
358 immersion rapidly induces iron misdistribution characterized by increased spleen iron level.
359 Our data suggest that hemolysis and myolysis combined with the increase the hepcidin level
360 are involved in iron sequestration in spleen. Consequently, these results question the long-
361 term effects of microgravity and extreme physical inactivity on iron metabolism, and their
362 potential deleterious consequences on the health of astronauts, especially as iron-induced
363 oxidative stress could be facilitated by radiation exposure in space (49). Whereas the current
364 Dietary Reference Intake for iron is 8 mg/day for men (50), the mean iron content of the
365 standard International Space Station menu is around 20 ± 6 mg/day (50). These values can
366 even reach 35 mg/day for some crewmembers due to consumption of commercial food items
367 fortified with iron (51). Our results finally indicate that high iron content in the astronaut's
368 food, or iron supplementation during periods of extreme physical inactivity, are questionable

369 and must be carefully monitored to avoid the potentially deleterious impact of iron
370 misdistribution.

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377 **CONFLICT OF INTEREST STATEMENT**

378 No conflict of interest, financial or otherwise related to this work, is declared by the author(s).

379 **AUTHOR CONTRIBUTIONS**

380 FD, GG-K and OL designed the clinical study. KN, SR, M-LI, LO, M-PB, AB, MR, and FD
381 performed experiments; KN, SR, and FD analyzed data; KN, SR, MR, CK-R, OL, and FD
382 interpreted results of experiments; KN, and FD prepared figures; KN, OL, and FD drafted
383 manuscript; KN, MR, CK-R, OL, and FD edited and revised manuscript; KN, SR, M-LI, LO,
384 MR, CK-R, GG-K, OL, and FD approved final version of manuscript.

385

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526

527 **TABLES**

	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m ²)	VO ₂ max (ml/min/kg)	Morning HR (bpm)	Morning T (°C)	Morning SBP (mmHg)	Morning DBP (mmHg)
Control (n=9)	33.4±7.1	176±6	73.9±7.5	23.9±1.7	46.5±8.1	57±6	36.4±0.3	115±11	68±5
Cuffs (n=9)	33.8±3.7	180±4	74.3±8.8	22.7±1.8	46.9±5.8	58±8	36.4±0.5	117±10	68±9
Unpaired T-test	p=0.90	p=0.08	p=0.97	p=0.11	p=0.91	p=0.6	p=0.71	p=0.78	p=0.92
All (n=18)	33.6±5.5	178±6	74.4±8.0	23.5±1.9	46.7±6.9	58±7	36.4±0.4	116±10	68±7

528 **Table 1. Baseline group characteristics at BDC-2.** Data are the mean ± SD. Statistical
529 significance was checked using an unpaired T-test.

530

Sequence	TR (msec)	FA(°)	TE (msec)	TA (s)
GRE-T1	120	90	2.4	15
GRE-PD	120	20	4.8	15
GRE-T2	120	20	9.6	15
GRE-T2+	120	20	14	15
GRE-T2++	120	20	21	15

531 **Table 2. Magnetic resonance gradient echo sequences used in MRI protocol.** TR,
532 repetition time; TE, echo time; FA, flip angle; PD, proton density; TA, acquisition time.

533

	CTL (n=9)		Cuff (n=9)		Source of Variation		
	BDC-4	DI5	BDC-4	DI5	Time	Cuff	Interaction
Total body mass (kg)	73.79 ± 7.24	71.37 ± 7.09	73.41 ± 9.12	71.48 ± 8.68	p<0.001	NS	NS
Body lean mass (kg)	55.50 ± 4.74	54.16 ± 4.54	55.78 ± 6.73	54.33 ± 6.47	p<0.001	NS	NS
Leg lean mass (kg)	18.42 ± 1.93	17.74 ± 1.71	18.29 ± 2.53	17.62 ± 2.16	p<0.001	NS	NS
Body fat mass (kg)	17.67 ± 3.34	17.22 ± 3.31	17.62 ± 3.69	17.14 ± 3.63	p<0.001	NS	NS
Body fat mass (%)	24.0 ± 2.8	24.0 ± 2.8	23.9 ± 3.2	23.9 ± 3.4	NS	NS	NS

534

535 **Table 3.** Impact of 5 days of DI on body weight and composition. Data are the mean ± SD.
536 Statistical significance was checked using a two-way ANOVA, sources of variation (time,
537 Cuff and interaction) are reported, and the significance level was set at 0.05 (CTL, n=9; Cuff,
538 n=8). DI, dry immersion; CTL, controls (no thigh cuffs).

539

540

541

542 **TABLES**

	CTL		Cuff		Variation		
	Before DI	After DI	Before DI	After DI	Time	Cuff	Interaction
WBC (mm³)	5421 ± 930	7034 ± 1704	5567 ± 805	6613 ± 1534	p<0.001	NS	NS
RBC (mm³)	5.02x10 ⁶ ± 3.70x10 ⁵	5.75x10 ⁶ ± 3.98x10 ⁵	4.65x10 ⁶ ± 2,61x10 ⁵	5.01x10 ⁶ ± 3.50x10 ⁵	p<0.001	p=0.009	NS
Hemoglobin (g/100ml)	14.6 ± 0.7	16.6 ± 0.8	14.5 ± 1.1	15.9 ± 1.1	p<0.001	NS	NS
Hematocrit (%)	42.2 ± 1.7	48.4 ± 2.4	41.7 ± 3.0	45.9 ± 3.5	p<0.001	NS	NS
EPO (mU/ml)	6.1 ± 2.3	4.6 ± 2.0	9.5 ± 1.9	6.4 ± 2.2	p=0.009	NS	NS
Reticulocytes (%)	1.1 ± 0.2	0.9 ± 0.2	1.2 ± 0.2	1.0 ± 0.3	p=0.003	NS	NS
Bilirubin (µmol/l)	8.0 ± 1.9	9.3 ± 2.5	8.0 ± 2.7	8.5 ± 2.1	p=0.037	NS	NS
Conjugated bilirubin (µmol/l)	3.4 ± 0.9	3.5 ± 0.9	3.3 ± 1.0	3.3 ± 0.8	NS	NS	NS
Unconjugated bilirubin (µmol/l)	4.6 ± 1.2	5.75 ± 1.8	4.7 ± 1.9	5.2 ± 1.5	p=0.034	NS	NS
Myoglobin (ng/ml)	29.3 ± 5.5	34.2 ± 5.0	26.6 ± 6.2	28.7 ± 6.4	p=0.011	NS	NS
wr-CRP (mg/l)	0.4 ± 0.3	0.5 ± 0.6	0.8 ± 1.0	0.6 ± 0.6	NS	NS	NS

543

544 **Table 4. Laboratory work-up before and after 5 days of dry immersion.** Data are the
545 mean ± SD. Statistical significance was checked using a two-way ANOVA, sources of
546 variation (time, Cuff and interaction) are reported, and the significance level was set at 0.05
547 (CTL, n=9; Cuff, n=8). DI, dry immersion; CTL, controls (no thigh cuffs); WBC, white blood
548 cells; RBC, red blood cells; EPO, erythropoietin; wr-CRP, wide range C-reactive protein.

549

550 **FIGURE LEGENDS**

551 **Figure 1. Dry immersion method: the subject is immersed in thermoneutral water**
552 **covered with an elastic waterproof fabric (Photo MEDES)**

553 **Figure 2. Flow chart showing the progress of participants in the study**

554 **Figure 3. Iron distribution and regulation before and after 5 days of dry immersion.**

555 Estimated spleen iron concentrations (A), estimated liver iron concentrations (B),
556 representative MRI images to determine liver and spleen iron concentrations (C), serum iron
557 concentrations (D), transferrin saturation level (E), serum transferrin concentrations (F),
558 serum hepcidin concentrations (G), serum haptoglobin concentrations (H), and serum ferritin
559 concentrations (I) in controls (CTL) and participants with cuffs (Cuff). Values are shown as
560 individual values and the mean of each group. Significance was checked using two-way
561 ANOVA tests and sources of variation (time, cuffs and interaction are reported). Time effects:
562 * $p < 0.05$ **, $p < 0.01$, *** $p < 0.001$. (n=8 for each group for MRI analyses, n=7-9 for all serum
563 analyses).

564

Figure 1

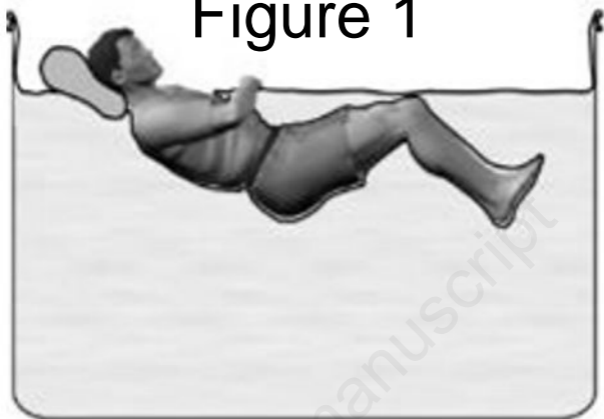


Figure 2

Participant flow chart diagram

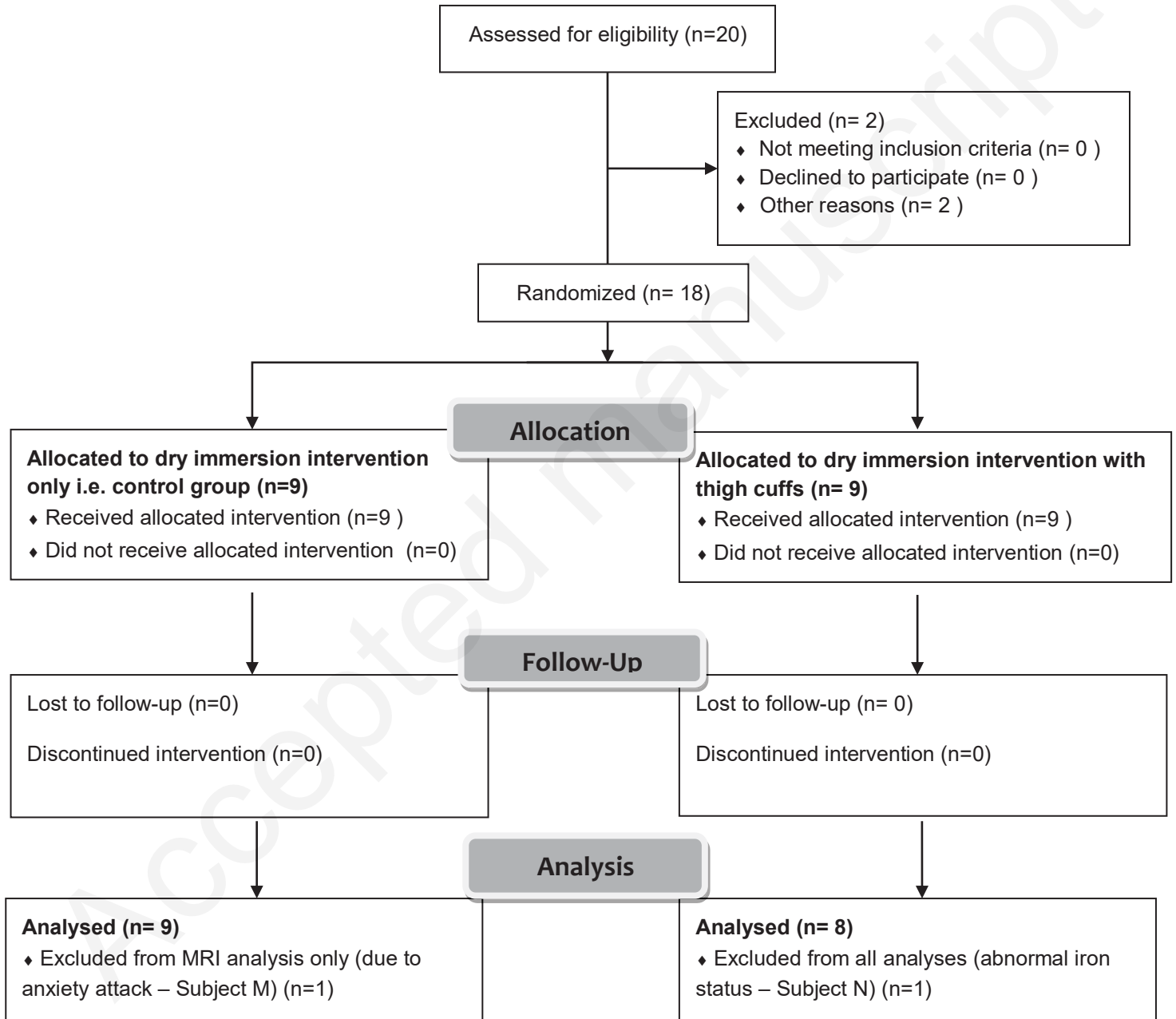
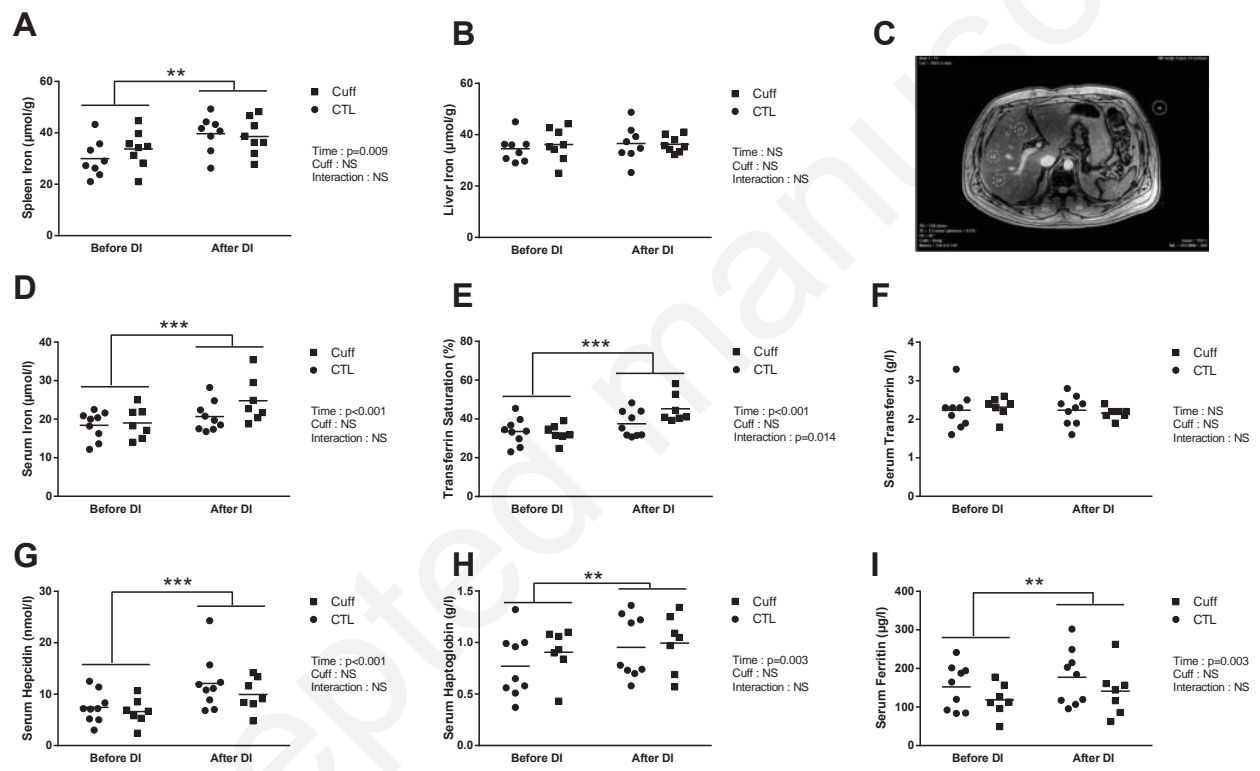
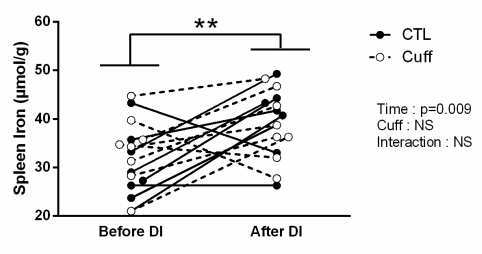
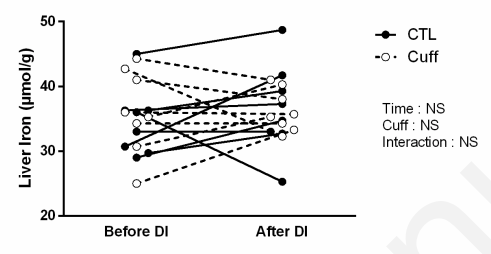
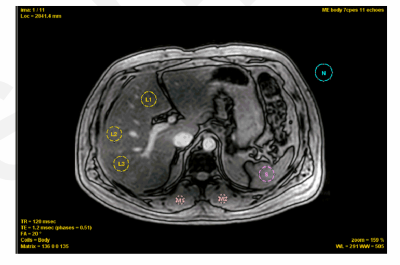
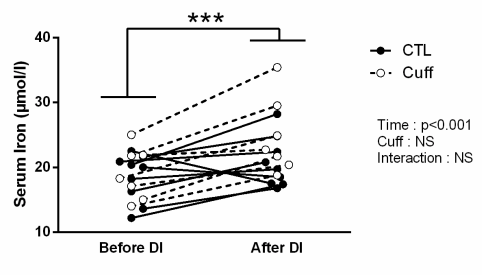
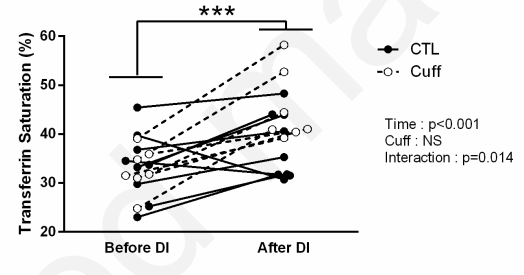
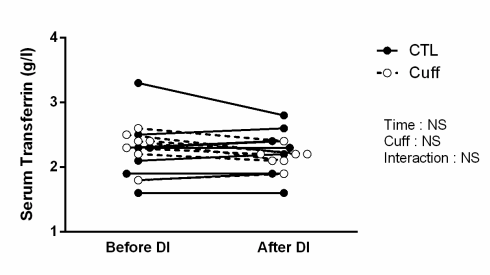
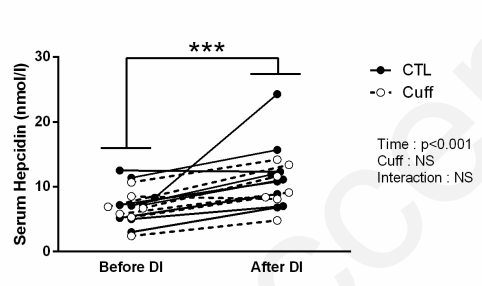
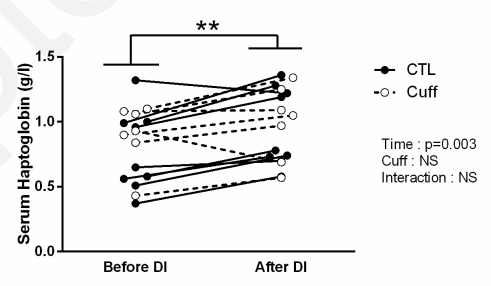
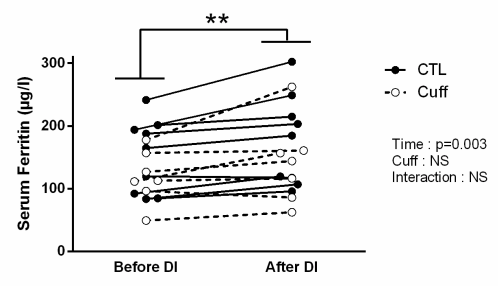
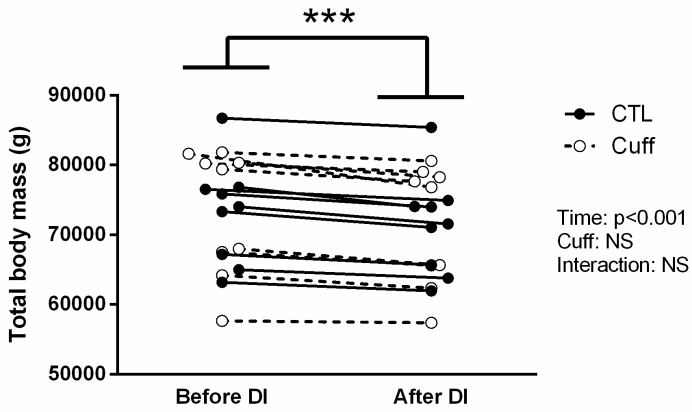
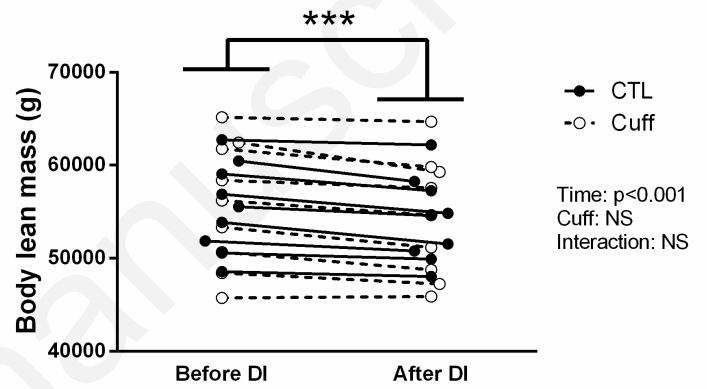
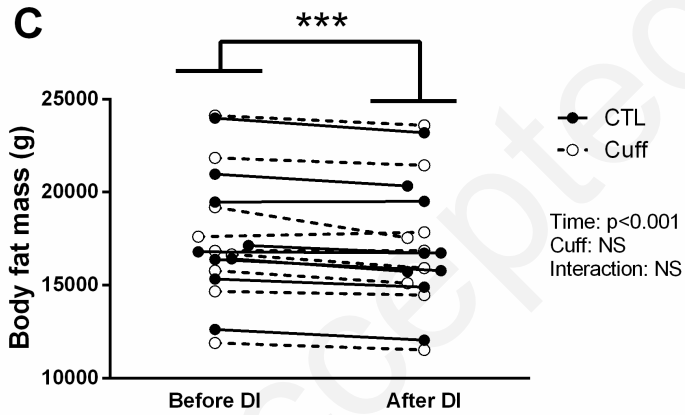
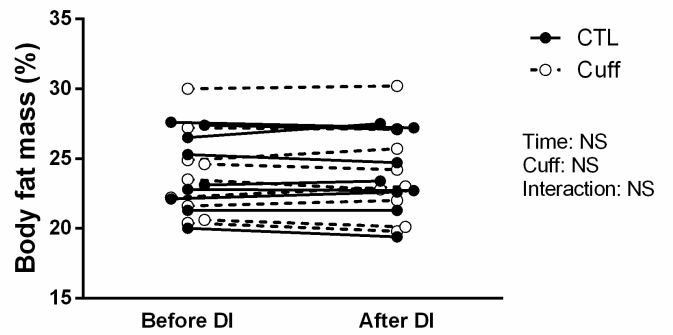


Figure 3



A**B****C****D****E****F****G****H****I**

A**B****C****D**

Supplemental Table 1. Inclusion/exclusion criteria of the study

Inclusion criteria	<ul style="list-style-type: none"> • Healthy male volunteer (see below the description of medical tests and laboratory analysis performed at the selection visit), • Age 20 to 45, • No overweight nor excessive thinness with BMI (weight Kg/ height m²) between 20 and 26, • Height between 158 and 185 cm, • No personal nor family past record of chronic or acute disease or psychological disturbances which could affect the physiological data and/or create a risk for the subject during the experiment, • Fitness level assessment: <ul style="list-style-type: none"> ○ if age < 35 years: 35 ml/min/kg < VO₂max < 60ml/min/kg ○ if age > 35 years: 30 ml/min/kg < VO₂max < 60ml/min/kg • Walking between 7000 - 8000 steps/day, • Active and free from any orthopedic (in particular no vertebral fracture, scoliosis or herniated disc) musculoskeletal and cardiovascular disorders, • Non smokers, • No alcohol, no drug dependence and no medical treatment, • No antibiotic treatment in the 2 previous months before the beginning of the study, • Covered by a Social Security system, • Have signed the information consent, • Free of any engagement during the study.
Exclusion criteria	<ul style="list-style-type: none"> • Past record of orthostatic intolerance, • Cardiac rhythm disorders, • Chronic back pains, • Vertebral fracture, scoliosis or herniated disc, • Glaucoma, • HTA, • History of migraines, • History of hiatus hernia or gastro-esophageal reflux, • History of thyroid dysfunction, renal stones, diabetes, • History of head trauma, • Past records of thrombophlebitis, family history of thrombosis or positive response in thrombosis screening procedure (anti thrombin III, S-protein, C-protein, factor V Leiden mutation and the mutation 20210 of the prothrombin gene), • Individuals exhibiting mutations involved in hereditary hemochromatosis (HAMP, HFE, HFE2, SCL40A1 and TRF2), • Abnormal result for lower limbs echo-doppler, • History or active claustrophobia, • History of genetic muscle and bone diseases of any kind, • Bone mineral density: T-score ≤ -1.5 on the hip, • Osteosynthesis material, presence of metallic implants or any other contra-indication for MRI, • Poor tolerance to blood sampling, • Having given blood (more than 8ml/kg) in a period of 8 weeks or less before the start of the experiment, • Antibiotic treatment in the 2 previous months before the beginning of the study, • Vegetarian or vegan, • History of food allergy, • Positive reaction to any of the following tests: HVA IgM (hepatitis A), HBs antigen (hepatitis B), anti-HVC antibodies (hepatitis C), anti-HIV1+2 antibodies, • Subject already participating or in the exclusion period of a clinical research, • Refusal to give permission to contact his general practitioner, • Incarcerated persons, • Subject who, in the judgment of the investigator, is likely to be non-compliant during the study, or unable to cooperate because of a language problem or poor mental development, • Subject who has received more than 4500 Euros within 12 months for being a research subject. • Subject under guardianship or trusteeship.

Supplemental Figure 1. Iron distribution and regulation before and after 5 days of dry immersion.

Estimated spleen iron concentrations (A), estimated liver iron concentrations (B), representative MRI images to determine liver and spleen iron concentrations (C), serum iron concentrations (D), transferrin saturation level (E), serum transferrin concentrations (F), serum hepcidin concentrations (G), serum haptoglobin concentrations (H), and serum ferritin concentrations (I) in controls (CTL) and participants with cuffs (Cuff). Values are shown as paired individual values. Significance was checked using two-way ANOVA tests and sources of variation (time, cuffs and interaction are reported). Time effects: * $p < 0.05$ **, $p < 0.01$, *** $p < 0.001$. (n=8 for each group for MRI analyses, n=7-9 for all serum analyses).

Supplemental Figure 2. Impact of 5 days of DI on body weight and composition. Individual profile.

Total body mass (A), Body lean mass (B), Body fat mass (C) and percentage of body fat mass (D) in controls (CTL) and participants with cuffs (Cuff). Values are shown as paired individual values. Significance was checked using two-way ANOVA tests and sources of variation (time, cuffs and interaction are reported). Time effects: * $p < 0.05$ **, $p < 0.01$, *** $p < 0.001$. (n=8-9).