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

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Running head: DI-5-Cuffs - Dry immersion affects iron metabolism

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

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NONSTANDARD ABBREVIATIONS

ANSM : French National Agency for Medicines and Health Products Safety

BDC: baseline data collection

DI : dry immersion

EPO: erythropoietin

HIC : hepatic iron store

HU : hindlimb unloading

SIC : splenic iron store

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CONFLICT OF INTEREST STATEMENT

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

AUTHOR CONTRIBUTIONS

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

ABSTRACT

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

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

INTRODUCTION

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

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

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

METHODS

Participants

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

Overall study design

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

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

Cuff

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

Body composition

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

Hematology and iron status

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

(transferrin in g/l x 25) and transferrin saturation (%) was calculated as follows: (iron in µmol/l / TIBC) × 100. Reference interval of local laboratory values of serum iron, transferrin saturation, ferritin, haptoglobin, myoglobin were 12.5-25 µmol/L, 23-46%, 55-345 µg/L, 0,3-2g/l, 10-92ng/ml respectively.

MRI protocol

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

Serum EPO, wr-CRP and hepcidin levels

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

Statistical analysis

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

RESULTS

Participant Flow

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

Dry immersion affects both body weight and composition

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

Dry immersion alters iron homeostasis and distribution in the body

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

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

Effects of dry immersion on erythropoiesis, hemolysis and myolysis parameters

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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AUTHOR CONTRIBUTIONS

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

REFERENCES

1. De Santo, N. G., Cirillo, M., Kirsch, K. A., Correale, G., Drummer, C., Frassl, W., Perna, A. F., Di Stazio, E., Bellini, L., and Gunga, H.-C. (2005) Anemia and erythropoietin in space flights. *Semin. Nephrol.* **25**, 379–387
2. Vico, L., Collet, P., Guignandon, A., Lafage-Proust, M. H., Thomas, T., Rehaillia, M., and Alexandre, C. (2000) Effects of long-term microgravity exposure on cancellous and cortical weight-bearing bones of cosmonauts. *Lancet* **355**, 1607–1611
3. Reardon, T. F. and Allen, D. G. (2009) Iron injections in mice increase skeletal muscle iron content, induce oxidative stress and reduce exercise performance. *Exp. Physiol.* **94**, 720–730
4. Tsay, J., Yang, Z., Ross, F. P., Cunningham-Rundles, S., Lin, H., Coleman, R., Mayer-Kuckuk, P., Doty, S. B., Grady, R. W., Giardina, P. J., Boskey, A. L., and Vogiatzi, M. G. (2010) Bone loss caused by iron overload in a murine model: importance of oxidative stress. *Blood* **116**, 2582–2589
5. Smith, S. M. (2002) Red blood cell and iron metabolism during space flight. *Nutrition* **18**, 864–866
6. Smith, S. M., Zwart, S. R., Block, G., Rice, B. L., and Davis-Street, J. E. (2005) The nutritional status of astronauts is altered after long-term space flight aboard the International Space Station. *J. Nutr.* **135**, 437–443
7. Morgan, J. L. L., Zwart, S. R., Heer, M., Ploutz-Snyder, R., Ericson, K., and Smith, S. M. (2012) Bone metabolism and nutritional status during 30-day head-down-tilt bed rest. *J. Appl. Physiol.* **113**, 1519–1529
8. Cavey, T., Pierre, N., Nay, K., Allain, C., Ropert, M., Loréal, O., and Derbré, F. (2017) Simulated microgravity decreases circulating iron in rats: role of inflammation-induced hepcidin upregulation. *Exp. Physiol.* **102**, 291–298
9. Xu, Z., Sun, W., Li, Y., Ling, S., Zhao, C., Zhong, G., Zhao, D., Song, J., Song, H., Li, J., You, L., Nie, G., Chang, Y., and Li, Y. (2017) The regulation of iron metabolism by hepcidin contributes to unloading-induced bone loss. *Bone* **94**, 152–161
10. Nay, K., Martin, D., Orfila, L., Saligaut, D., Martin, B., Horeau, M., Cavey, T., Kenawi, M., Island, M.-L., Ropert, M., Loréal, O., Koechlin-Ramonatxo, C., and Derbré, F. (2020) Intermittent reloading does not prevent iron availability decrease and hepcidin upregulation caused by hindlimb unloading. *Exp. Physiol.*
11. Zhang, A.-S. (2010) Control of Systemic Iron Homeostasis by the Hemojuvelin-Hepcidin Axis12. *Adv Nutr* **1**, 38–45
12. Loréal, O., Cavey, T., Bardou-Jacquet, E., Guggenbuhl, P., Ropert, M., and Brissot, P. (2014) Iron, hepcidin, and the metal connection. *Front Pharmacol* **5**, 128
13. Cavey, T., Latour, C., Island, M.-L., Leroyer, P., Guggenbuhl, P., Coppin, H., Roth, M.-P., Bendavid, C., Brissot, P., Ropert, M., and Loréal, O. (2019) Spleen iron, molybdenum, and manganese concentrations are coregulated in hepcidin-deficient and secondary iron overload models in mice. *FASEB J.* **33**, 11072–11081
14. Cavey, T., Ropert, M., de Tayrac, M., Bardou-Jacquet, E., Island, M.-L., Leroyer, P., Bendavid, C., Brissot, P., and Loréal, O. (2015) Mouse genetic background impacts both on iron and non-iron metals parameters and on their relationships. *Biometals* **28**, 733–743
15. Valberg, L. S., Ludwig, J., and Olatunbosun, D. (1969) Alteration in cobalt absorption in patients with disorders of iron metabolism. *Gastroenterology* **56**, 241–251
16. Hansen, S. L., Trakooljul, N., Liu, H.-C. S., Hicks, J. A., Ashwell, M. S., and Spears, J. W. (2010) Proteins involved in iron metabolism in beef cattle are affected by copper

deficiency in combination with high dietary manganese, but not by copper deficiency alone. *J. Anim. Sci.* **88**, 275–283
17. Zwart, S. R., Morgan, J. L. L., and Smith, S. M. (2013) Iron status and its relations with oxidative damage and bone loss during long-duration space flight on the International Space Station. *Am. J. Clin. Nutr.* **98**, 217–223
18. Navasiolava, N. M., Custaud, M.-A., Tomilovskaya, E. S., Larina, I. M., Mano, T., Gauquelin-Koch, G., Gharib, C., and Kozlovskaya, I. B. (2011) Long-term dry immersion: review and prospects. *Eur. J. Appl. Physiol.* **111**, 1235–1260
19. De Abreu, S., Amirova, L., Murphy, R., Wallace, R., Twomey, L., Gauquelin-Koch, G., Raverot, V., Larcher, F., Custaud, M.-A., and Navasiolava, N. (2017) Multi-System Deconditioning in 3-Day Dry Immersion without Daily Raise. *Front Physiol* **8**, 799
20. Pavy-Le Traon, A., Heer, M., Narici, M. V., Rittweger, J., and Vernikos, J. (2007) From space to Earth: advances in human physiology from 20 years of bed rest studies (1986-2006). *Eur. J. Appl. Physiol.* **101**, 143–194
21. Gandon, Y., Olivié, D., Guyader, D., Aubé, C., Oberti, F., Sebille, V., and Deugnier, Y. (2004) Non-invasive assessment of hepatic iron stores by MRI. *Lancet* **363**, 357–362
22. Demangel, R., Treffel, L., Py, G., Brioche, T., Pagano, A. F., Bareille, M.-P., Beck, A., Pessemesse, L., Candau, R., Gharib, C., Chopard, A., and Millet, C. (2017) Early structural and functional signature of 3-day human skeletal muscle disuse using the dry immersion model. *J. Physiol. (Lond.)* **595**, 4301–4315
23. Treffel, L., Dmitrieva, L., Gauquelin-Koch, G., Custaud, M.-A., Blanc, S., Gharib, C., and Millet, C. (2016) Craniomandibular System and Postural Balance after 3-Day Dry Immersion. *PLoS ONE* **11**, e0150052
24. Robin, A., Auvinet, A., Degryse, B., Murphy, R., Bareille, M.-P., Beck, A., Gharib, C., Gauquelin-Koch, G., Daviet, A., Larcher, F., Custaud, M.-A., and Navasiolava, N. (2020) DI-5-CUFFS: Venoconstrictive Thigh Cuffs Limit Body Fluid Changes but Not Orthostatic Intolerance Induced by a 5-Day Dry Immersion. *Front Physiol* **11**
25. Tomilovskaya, E., Shigueva, T., Sayenko, D., Rukavishnikov, I., and Kozlovskaya, I. (2019) Dry Immersion as a Ground-Based Model of Microgravity Physiological Effects. *Front Physiol* **10**, 284
26. Shenkman, B. S., Kozlovskaya, I. B., Nemirovskaya, T. L., and Tcheglova, I. A. (1997) Human muscle atrophy in supportlessness: effects of short-term exposure to dry immersion. *J Gravit Physiol* **4**, P137-138
27. Fitts, R. H., Riley, D. R., and Widrick, J. J. (2001) Functional and structural adaptations of skeletal muscle to microgravity. *J. Exp. Biol.* **204**, 3201–3208
28. Dirks, M. L., Smeets, J. S. J., Holwerda, A. M., Kouw, I. W. K., Marzuca-Nassr, G. N., Gijsen, A. P., Holloway, G. P., Verdijk, L. B., and van Loon, L. J. C. (2019) Dietary feeding pattern does not modulate the loss of muscle mass or the decline in metabolic health during short-term bed rest. *Am. J. Physiol. Endocrinol. Metab.* **316**, E536–E545
29. Risso, A., Ciana, A., Achilli, C., Antonutto, G., and Minetti, G. (2014) Neocytolysis: none, one or many? A reappraisal and future perspectives. *Front Physiol* **5**
30. Kohgo, Y., Ikuta, K., Ohtake, T., Torimoto, Y., and Kato, J. (2008) Body iron metabolism and pathophysiology of iron overload. *Int. J. Hematol.* **88**, 7–15
31. Yang, J., Meng, X., Dong, D., Xue, Y., Chen, X., Wang, S., Shen, Y., Zhang, G., and Shang, P. (2018) Iron overload involved in the enhancement of unloading-induced bone loss by hypomagnetic field. *Bone* **114**, 235–245
32. Fung, E. and Nemeth, E. (2013) Manipulation of the hepcidin pathway for therapeutic purposes. *Haematologica* **98**, 1667–1676
33. Hentze, M. W., Muckenthaler, M. U., Galy, B., and Camaschella, C. (2010) Two to tango: regulation of Mammalian iron metabolism. *Cell* **142**, 24–38

34. Bosutti, A., Malaponte, G., Zanetti, M., Castellino, P., Heer, M., Guarnieri, G., and Biolo, G. (2008) Calorie restriction modulates inactivity-induced changes in the inflammatory markers C-reactive protein and pentraxin-3. *J. Clin. Endocrinol. Metab.* **93**, 3226–3229
35. Convertino, V. A. (1996) Clinical aspects of the control of plasma volume at microgravity and during return to one gravity. *Med Sci Sports Exerc* **28**, S45-52
36. Alfrey, C. P., Udden, M. M., Leach-Huntoon, C., Driscoll, T., and Pickett, M. H. (1996) Control of red blood cell mass in spaceflight. *J. Appl. Physiol.* **81**, 98–104
37. Leach, C. S., Alfrey, C. P., Suki, W. N., Leonard, J. I., Rambaut, P. C., Inners, L. D., Smith, S. M., Lane, H. W., and Krauhs, J. M. (1996) Regulation of body fluid compartments during short-term spaceflight. *J. Appl. Physiol.* **81**, 105–116
38. Andrews, N. C. (1999) Disorders of iron metabolism. *N. Engl. J. Med.* **341**, 1986–1995
39. Muñoz, M., García-Erce, J. A., and Remacha, A. F. (2011) Disorders of iron metabolism. Part 1: molecular basis of iron homoeostasis. *J. Clin. Pathol.* **64**, 281–286
40. Zhang, A.-S. and Enns, C. A. (2009) Molecular mechanisms of normal iron homeostasis. *Hematology Am Soc Hematol Educ Program* 207–214
41. Nairz, M., Theurl, I., Swirski, F. K., and Weiss, G. (2017) "Pumping iron"-how macrophages handle iron at the systemic, microenvironmental, and cellular levels. *Pflugers Arch.* **469**, 397–418
42. Barcellini, W. and Fattizzo, B. (2015) Clinical Applications of Hemolytic Markers in the Differential Diagnosis and Management of Hemolytic Anemia. *Dis. Markers* **2015**, 635670
43. Ryan, B. J., Goodrich, J. A., Schmidt, W. F., Stothard, E. R., Wright, K. P., and Byrnes, W. C. (2016) Haemoglobin mass alterations in healthy humans following four-day head-down tilt bed rest. *Exp. Physiol.* **101**, 628–640
44. Trudel, G., Uhthoff, H. K., and Laneuville, O. (2017) Hemolysis during and after 21 days of head-down-tilt bed rest. *Physiol Rep* **5**
45. Mebius, R. E. and Kraal, G. (2005) Structure and function of the spleen. *Nat. Rev. Immunol.* **5**, 606–616
46. Alfrey, C. P., Rice, L., Udden, M. M., and Driscoll, T. B. (1997) Neocytolysis: physiological down-regulator of red-cell mass. *Lancet* **349**, 1389–1390
47. Trial, J., Rice, L., and Alfrey, C. P. (2001) Erythropoietin Withdrawal Alters Interactions Between Young Red Blood Cells, Splenic Endothelial Cells, and Macrophages: An In Vitro Model of Neocytolysis. *Journal of Investigative Medicine* **49**, 335–345
48. Corna, G., Caserta, I., Monno, A., Apostoli, P., Manfredi, A. A., Camaschella, C., and Rovere-Querini, P. (2016) The Repair of Skeletal Muscle Requires Iron Recycling through Macrophage Ferroportin. *J. Immunol.* **197**, 1914–1925
49. Yang, J., Zhang, G., Dong, D., and Shang, P. (2018) Effects of Iron Overload and Oxidative Damage on the Musculoskeletal System in the Space Environment: Data from Spaceflights and Ground-Based Simulation Models. *Int J Mol Sci* **19**
50. Smith, S. M., Rice, B. L., Dlouhy, H., and Zwart, S. R. (2013) Assessment of Nutritional Intake During Space Flight and Space Flight Analogs. *Procedia Food Science* **2**, 27–34

TABLES

	Age (years)	Height (cm)	Weight (kg)	BMI (kg/m^2)	VO_2max (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

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

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

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

	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

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

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

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

FIGURE LEGENDS

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

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

Figure 3. 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 individual values and the mean of each group. 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).

Figure 1

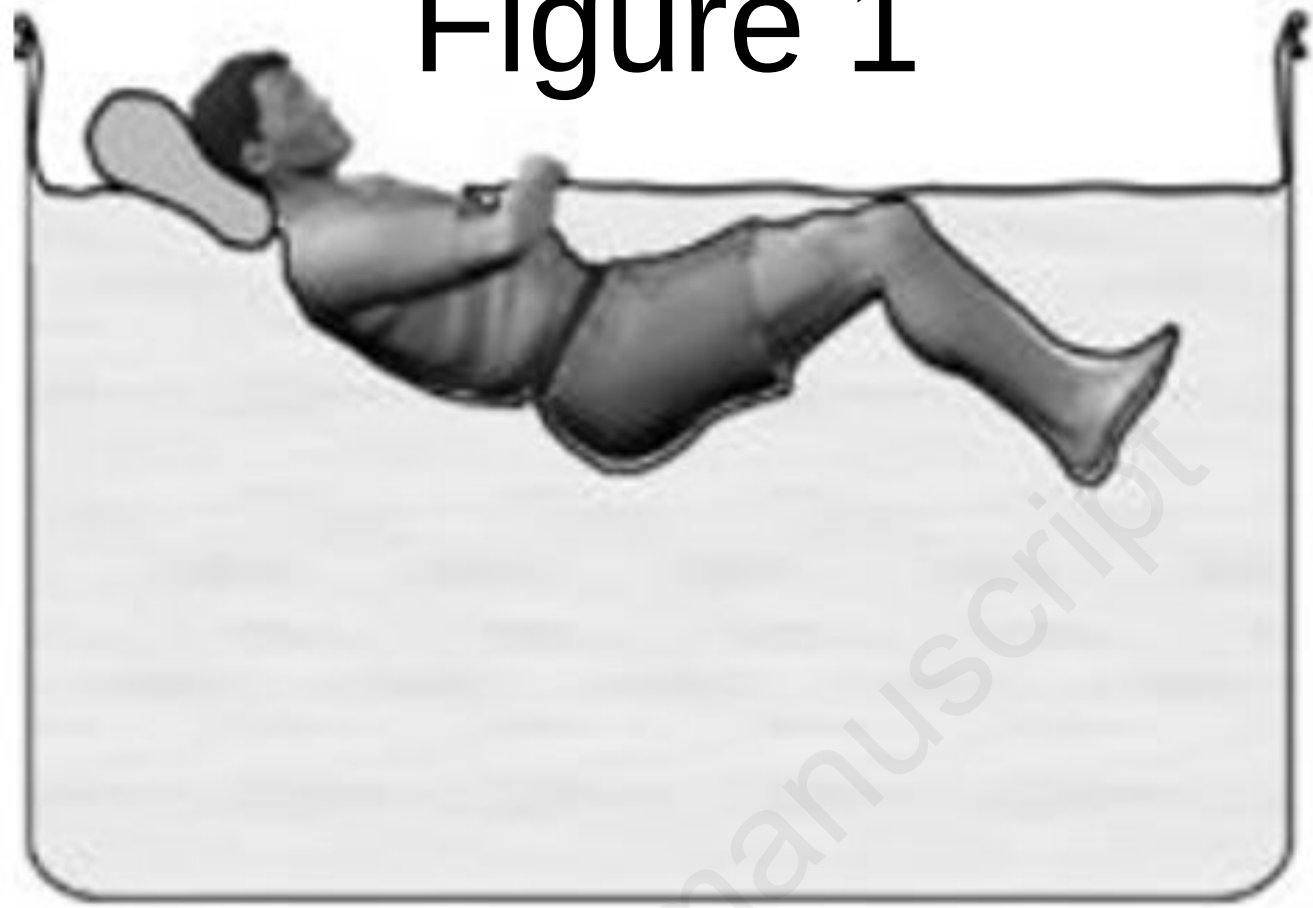


Figure 2

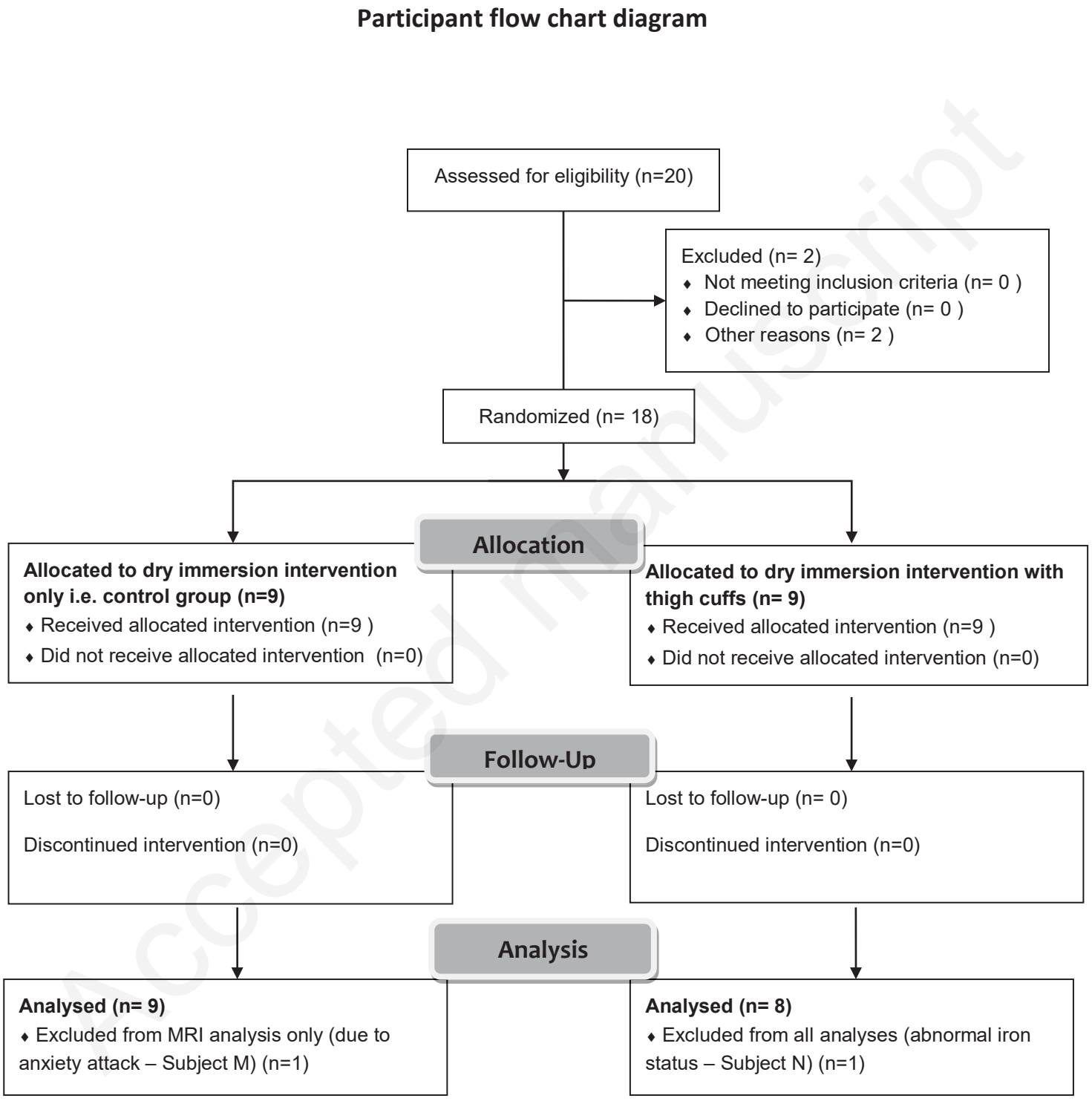
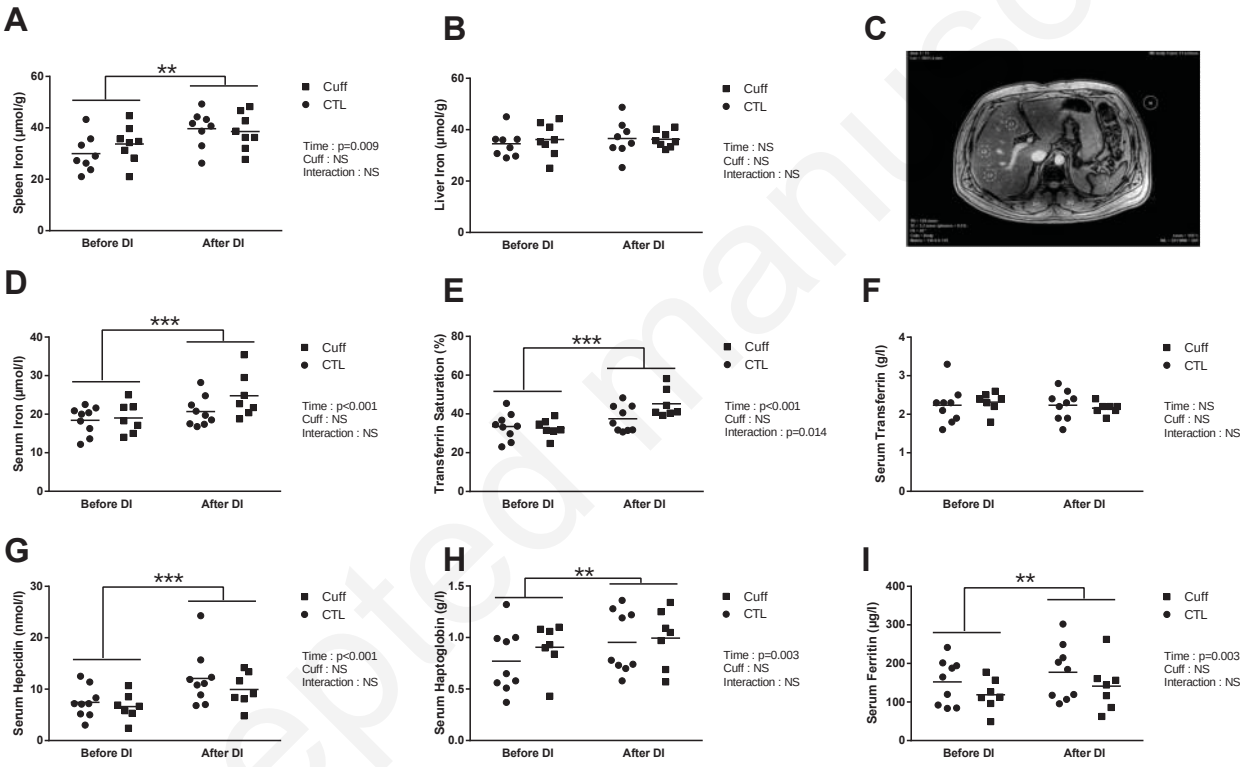
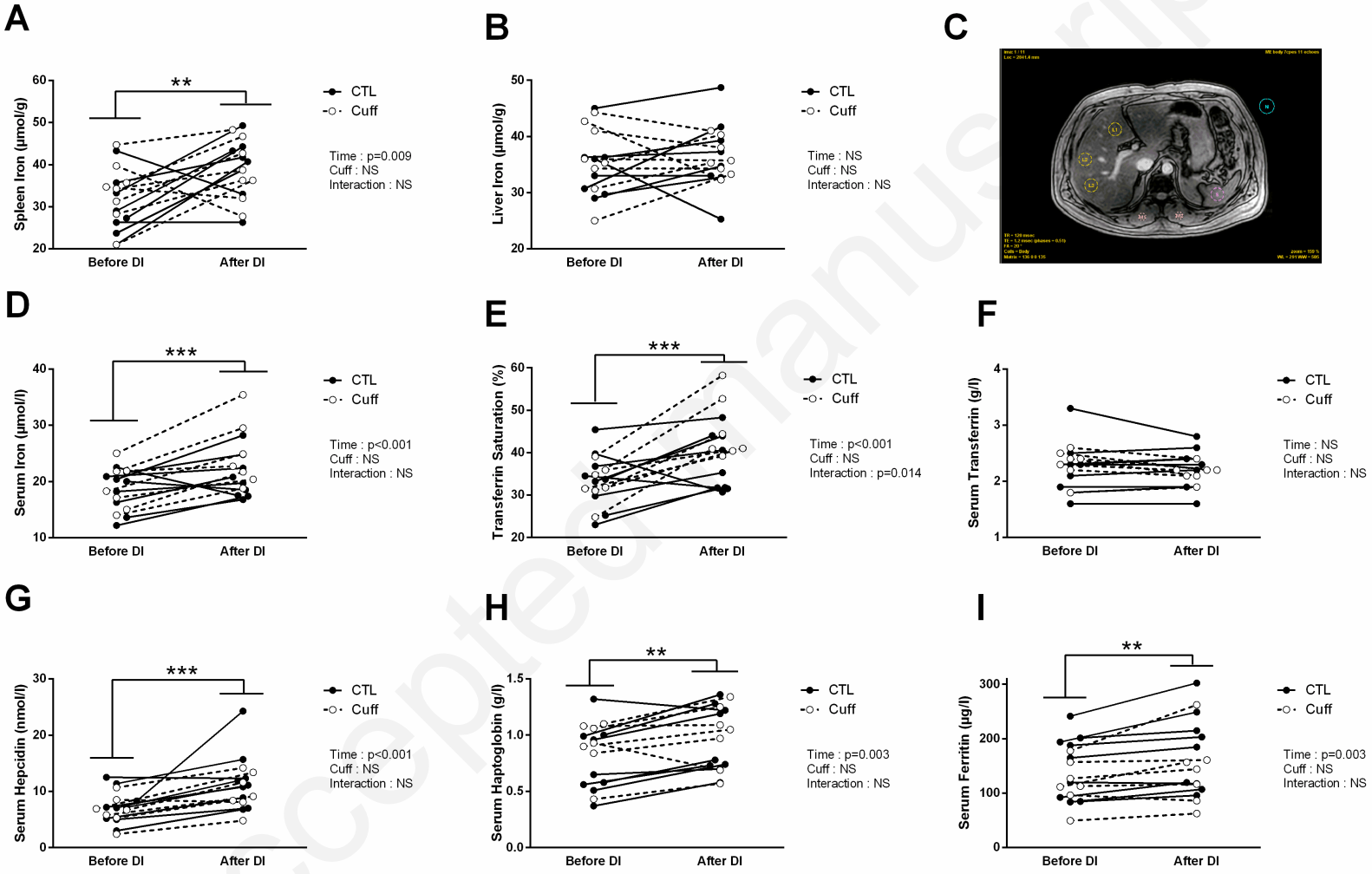
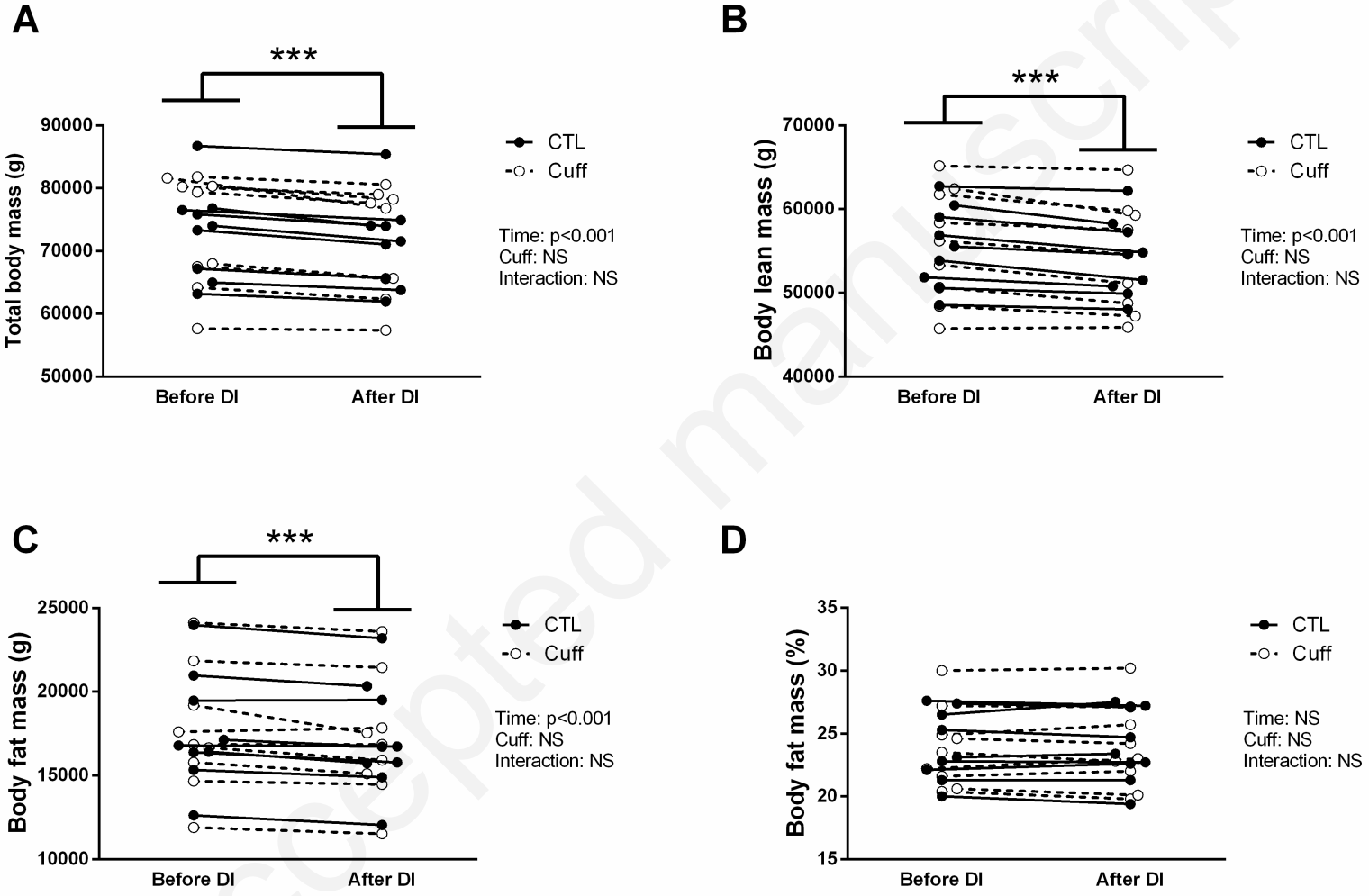


Figure 3







Supplemental Table 1. Inclusion/exclusion criteria of the study

Inclusion criteria	• 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:
	  o if age < 35 years: 35 ml/min/kg < VO2max < 60ml/min/kg
	  o 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	• 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).