Iron metabolism regulation in females and males exposed to simulated microgravity: results from the randomized trial AGBRESA

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NON-STANDARD ABBREVIATIONS

AG: Artificial Gravity; AGBRESA: Artificial Gravity Bedrest – European Space Agency; BDC: Baseline Data Collection; BMI: Body Mass Index; BV: Blood Volume; cAG: continuous Artificial Gravity; CO: Carbon monoxide; CRP: C-Reactive Protein; DLR: German Aerospace Center; HDT: Head-Down Tilt; hsCRP: high-sensitivity CRP; iAG: intermittent Artificial Gravity; IL-6: Interleukin-6; MCH: Mean Corpuscular Hemoglobin; MCHC: Mean Corpuscular Hemoglobin Concentration; MCV: Mean Corpuscular Volume; ND: non detectable; oCORM: optimized CO Rebreathing Method; PV: Plasma Volume; RBC: Red Blood Cell; RBCv: Red Blood Cell volume; RDW: Red cell Distribution Width; sTfR: soluble Transferrin Receptor; tHb mass: total hemoglobin mass; TIBC: Total Iron Binding Capacity; %HbCO: carboxyhemoglobin percentage.

CONFLICT OF INTEREST STATEMENT

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

AUTHOR CONTRIBUTIONS

EM, FR, and OL designed the experimental study. MH, MR, JT, and GA performed the experiments; MH, EM, JT, GA, and FD analyzed the data; MH, OL and FD interpreted the experiment results; MH prepared the figures; MH, OL, and FD wrote the draft manuscript. All authors critically revised the manuscript draft. All authors approved the final version of the manuscript.

ABSTRACT

Background: Iron metabolism imbalance could contribute to physical deconditioning experienced by astronauts due to its essential role in energy metabolism, cellular respiration, and oxygen transport.

Objective: In this clinical exploratory study, we wanted to determine whether artificial gravity (AG) training modulated iron metabolism, red blood cell indices, and body lean mass in male and female healthy participants exposed to head-down tilt (HDT) bed rest, the reference ground-based model of microgravity.

Methods: We recruited 8 female and 16 male healthy participants who were all exposed to HDT bed rest for 60 days. In addition, they were assigned to three experimental groups (n=8/each): controls, continuous AG training in a short-arm centrifuge (1 x 30 min/day), and intermittent AG training (6 × 5 min/day).

Results: The iron metabolism responses to simulated microgravity of AG training groups do not significantly differ from the responses of controls. Independently from AG, we found that both serum iron (+31.3%, p=0.027) and transferrin saturation levels (+28.4%, p=0.009) increased in males after 6 days of HDT bed rest, as well as serum hepcidin levels (+36.9% p=0.005). The increase of transferrin saturation levels persisted after 57 days of HDT bed rest (+13.5%, p=0.026), suggesting that long-term exposure to microgravity sustainably increases serum iron availability in males, and consequently the risk of iron excess or misdistribution. In females, 6 and 57 days of HDT bed rest did not significantly change serum iron, transferrin saturation, and hepcidin levels.

Conclusions: The data from this exploratory study suggest that 1) AG training does not influence the iron metabolism responses to microgravity; 2) iron metabolism parameters, especially iron availability for cells, are significantly increased in males, but not in females,

exposed to long-term simulated microgravity. Due to the small sample size of females, we nevertheless must be cautious before concluding that iron metabolism could differently respond to microgravity in females.

Keywords: Spaceflight, inflammation, disuse, trace elements, metals

INTRODUCTION

Extreme physical inactivity, as experienced by astronauts in microgravity conditions and by patients restricted to bed rest, induces skeletal muscle wasting, osteoporosis, and anemia (1–3). Alterations in iron metabolism could play a major role in these imbalances, due to its implication in energy metabolism, cell respiration, oxygen transport, and muscle metabolism (4). Previous studies highlighted that in young males, short-term exposure to real or simulated microgravity leads to iron misdistribution, characterized by higher serum iron availability and splenic iron excess (5,6). Concomitantly with iron misdistribution, hepcidin expression is increased in liver and in the circulation in human and rodents males (5,7–9). By limiting the expression and activity of the cellular iron exporter ferroportin (10), especially in splenic macrophages and enterocytes, hepcidin could contribute to or counteract the early iron misdistribution observed in astronauts and bedridden patients. However, all these studies were carried out in male rodents and humans exposed to extreme physical inactivity only for few days. Therefore, it is not known whether these iron metabolism changes are transient and adaptive, or chronic and potentially harmful for astronauts and bedridden patients. Moreover, the short and long-term effects of microgravity on iron metabolism regulation in females have been rarely studied (6), although the number of female astronauts has progressively increased since the 1980s (11). All these questions need to be addressed, also for the management of long-term spaceflights to Mars in the next decades. Indeed, iron excess could promote

oxidative stress in some organs, thus increasing the risk of organ damage, particularly osteoporosis, muscle atrophy, cancer, or liver injuries (12–14). In addition, better understanding microgravity effects on iron metabolism could improve also the follow-up of patients confined to bed rest (i.e. another example of extreme physical inactivity).

To mitigate the effects of microgravity, space agencies have developed several countermeasures, including artificial gravity (AG). The principle of AG training is to intermittently mimic the gravitational forces that naturally occur on Earth, with the aim of preventing the physiological deconditioning observed in microgravity conditions (15). AG has some beneficial effects on cardiovascular deconditioning, orthostatic tolerance, and maintenance of physical capacities (15,16). AG might also partially limit muscle wasting and muscle protein synthesis decrease in astronauts (17,18). As 70% of the body iron is stored in hemoglobin and 15-20% in muscle myoglobin, limiting muscle atrophy in microgravity conditions could also prevent iron metabolism alterations in astronauts.

In this context, the aim of the present clinical exploratory study was to determine whether AG could prevent iron metabolism alterations in participants exposed to head-down tilt (HDT) bed rest, the reference model to explore the effects of extreme physical inactivity and microgravity. The study focused particularly on the impact of short- (5-6 days) and long-term (56-60 days) HDT bed rest on iron metabolism in males and females healthy participants.

METHODS

Study Design

The Artificial Gravity Bedrest – European Space Agency (AGBRESA) study is a randomized controlled clinical trial that took place at the Envihab facility of the Aerospace Medicine, German Aerospace Center (DLR), Cologne (Germany), in 2019. The study was divided into

two successive campaigns with 12 participants/each. Each campaign included 14 days of baseline data collection (BDC-14 to BDC-1) and 60 days of -6° HDT bed rest (HDT1 to HDT60). The study was registered in the German Clinical Trials Register (DRKS00015677).

Participants

This exploratory study enrolled 8 female and 16 male healthy participants between January and December 2019. Inclusion criteria were: being healthy, between 24 and 55 years of age, height between 1.53 and 1.90 m, body mass index between 19 and 30 kg/m^2, non-smoker for at least 6 months before the study start, and covered by a medical insurance. Exclusion criteria were: vegetarian or vegan, requirement of prescription drugs (including contraception), substance abuse, criminal record, or health conditions that would preclude participation. These health conditions included existing or history of cardiovascular diseases, musculoskeletal, ophthalmological, neurological and psychiatric conditions, metabolic or endocrine disorders (e.g. diabetes mellitus), blood clotting disorders, current or history of pulmonary disease, sleep and pain disorders, gastroesophageal reflux, renal stones, infectious or inflammatory diseases. Before inclusion in the study, all participants gave their written informed consent to the experimental procedures that were approved by the Northern Rhine Medical Association ethics committee (Ärztekammer Nordrhein, application #2018143), Duesseldorf, Germany, and the Federal Office for Radiation Protection (Bundesamt für Strahlenschutz).

At the beginning of the HDT bed rest period (HDT1), participants were randomly assigned to three experimental groups: control group (n=8), continuous centrifugation group (cAG, n=8), and intermittent centrifugation group (iAG, n=8). The number of female participants was lower that than of male participants (n=8 vs n=16), resulting in an imbalanced sex distribution among groups. The Project Leader assigned participants to one of the three groups in a semi-random fashion. For campaign 1, group assignment was based on sex distribution. For

campaign 2, we wanted to balance age, sex, height, and weight distribution among groups to complement the demographic information collected during campaign 1. The participants' baseline characteristics are summarized in Table 1 and Supplemental Table 1. The female participant G (iAG group), who had abnormally low serum iron concentration (5.0 µmol/l), transferrin saturation (5.7%), and serum ferritin concentration (6.0 µg/g) at baseline, was excluded from the study statistical analyses, despite the absence of exclusion criteria. The participants' inclusion flowchart is presented in Supplemental Figure 1.

HDT bed rest protocol

During the HDT bed rest period (starting at 9 a.m. of HDT1), beds were tilted 6° head-down. All daily routine activities, including eating, personal hygiene and leisure activities (e.g. reading, watching TV), were performed in this position. The use of pillows was prohibited throughout the HDT phase of the study. To control the adherence to the HDT position, participants were instructed to continuously touch the mattress with one shoulder and they were monitored (24h/7 days) by staff and a closed video system. Participants were not allowed to raise, contract, and stretch their legs to minimize mechanical stimuli. Static and dynamic muscle contractions were prohibited, but key muscle groups and joints were stretched daily by physiotherapists to avoid muscle contractures, and to alleviate muscle stiffness and back pain. Fluid and food intakes were strictly controlled, and were predefined and standardized according to the National Aeronautics and Space Administration and European Space Agency standardization plan. On BDC-14, each participant's energy intake was calculated by multiplying their resting metabolic rate by the physical activity level before and during HDT bed rest (1.6 and 1.3, respectively). The daily caloric intake was approximately 2610 kcal before and 2304 kcal during the HDT bed rest period. The daily iron intake from diet was ~15 and 25 mg before and during the HDT bed rest period. Data on the daily nutrient intake are presented in Supplemental Table 2.

AG protocol

Participants in the two intervention groups (cAG and iAG) underwent a daily 30-min session of centrifugation throughout the HDT bed rest period (60 days). In the cAG group, participants underwent continuous centrifugation for 30 minutes, while in the iAG group they underwent 6 × 5-min centrifugation sessions spaced by 3 minutes of rest. During centrifugation, participants were in supine position and immediately after the centrifuge final halt, they returned in the -6° HDT position. The centrifugation protocol is fully described in the study by Kramer and colleagues (19).

Iron metabolism and hemolysis parameters

Iron metabolism parameters were measured in samples collected at BDC-6, HDT6, and HDT57 at the biochemistry laboratory (Rennes University Hospital, France). Serum iron and bilirubin concentrations were measured using standard colorimetric methods, serum transferrin and haptoglobin levels by immunoturbidimetry, and serum ferritin and myoglobin concentrations by electro-chemiluminescence immunoassay. The total (or transferrin) iron binding capacity (TIBC in µmol/L) was calculated (transferrin in g/l x 25) to determine transferrin saturation (%) (iron in µmol/L/TIBC) × 100.

Red blood cell parameters

Red blood cell (RBC) and reticulocyte counts, mean corpuscular hemoglobin (MCH), mean corpuscular volume (MCV), red cell distribution width (RDW), and mean corpuscular hemoglobin concentration (MCHC) were measured in blood samples collected at BDC-3 and HDT60 at the Laboratory Quade (Köln, Germany) using the Sysmex XN-9000 Automated Hematology System (Sysmex Europe, Norderstedt, Germany).

Total hemoglobin mass (tHb), total red blood cell volume (RBCv), total plasma volume (PV) and total blood volume (BV) were measured using the optimized carbon monoxide (CO)

rebreathing method (oCORM) at BDC-12, HDT5, HDT21 and HDT56. After an initial resting period in supine position (0° during BDC and 6° during HDT bed rest), a baseline 2.6 ml EDTA blood sample was taken from an antecubital vein (S-Monovette®, Sarstaedt AG & Co., Nümbrecht, Germany) and the percentage of carboxyhemoglobin (%HbCO) was analyzed immediately using a hemoximeter (ABL 825, Radiometer, Denmark). The blood sample was transferred to a capillary and centrifuged, and the ratio was measured and expressed as a decimal or percentage fraction. Participants initially breathed 100% oxygen for 4 min to flush the nitrogen from the airways, followed by administration of a bolus (1.5 ml/kg for males and 1.0 for females) of 99.997% chemically pure CO (CO N47, Air Liquide, France). Participants rebreathed this gas mixture for 10 min. Then, another 2.6 ml blood sample was collected to measure again the %HbCO. The change between %HbCO values was used to calculate the tHb mass, by taking into account the CO amount that remained in the rebreathing circuit.

Hormones and cytokines

Serum hepcidin was measured using the Hepcidin-25 (human) – Lyophilized Antiserum for EIA/ELISA Kit (catalog number: S-1337, Peninsula Laboratories International, Inc, USA) according to the manufacturer's instructions. Serum estradiol and testosterone were measured using the ADVIA Centaur Enhanced Estradiol and TSTII assay, respectively. Serum interleukin-6 (IL-6) was quantified with the Elecsys IL-6 immunoassay (IL-6 CalSet, for 4 x 2.0 mL). Serum high-sensitivity C reactive protein (hsCRP) was measured on a Cobas 8000 analyzer Roche® using the 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 an extended measuring range of 0.3-350 mg/L.

Body Composition

Body composition was evaluated by dual-energy X-ray absorptiometry, using the whole-body scan feature of the Prodigy Full Pro system (GE Healthcare GmbH, Solingen, Germany), at BDC-4, HDT15, HDT30, HDT45, and HDT60. The manufacturer's enCORE software (version 16.10.151) was used to generate automated reports of total lean mass (g) and lower limb mass (g).

Statistical analyses

All data are presented as mean ± standard deviation (SD). The data distribution normality was checked using the Shapiro-Wilk test. To determine AG effect on the time responses to HDT bed rest, each experimental group was compared using two-way ANOVA (i.e. general linear model) for repeated measures with the Greenhouse-Geisser correction followed by the Tukey's multiple comparison test with adjusted p-value. To assess the responses to HDT bed rest in male and female participants, values obtained in this exploratory study were compared relative to baseline in each sex group using one-way ANOVA for repeated measures with the Greenhouse-Geisser correction followed by the Tukey's multiple comparison test with adjusted p-value. If the normality assumption was rejected, values were analyzed using the Friedman test followed by the Dunn's multiple comparison test with adjusted p-value. Data were analyzed using GraphPad Prism, version 8.20 (GraphPad Software, La Jolla, California).

Sample size was calculated using the difference in transferrin saturation (i.e. serum iron availability) as primary outcome measure. On the basis of our previous study on the simulated microgravity effects on iron metabolism (5), we used a difference between groups in transferrin saturation of 25% (within-group SD: 20%). Using a power of 80%, for a significance level of 0.05, seven participants per group needed to be included.

RESULTS

Effects of continuous and intermittent artificial gravity on iron metabolism parameters during short- and long-term head-down tilt bed rest

All iron metabolism parameters (i.e. serum iron, transferrin, transferrin saturation, serum hepcidin, ferritin and soluble transferrin receptor) were not different in the cAG and iAG groups compared with the control group at HDT6 and HDT57 (Figure 1). Similarly, we did not find any significant effect of cAG and iAG on red blood cell indices (Supplemental Table 3) and body lean mass (Supplemental Table 4). As AG (both modalities) did not have any significant effect on iron metabolism parameters, red blood cell indices and lean mass, we pooled the three groups to analyze the global effects of HDT bed rest in male and female participants.

Short- and long-term effects of head-down tilt bed rest on iron metabolism parameters in males and females

The short- and long-term effects of HDT bed rest on iron metabolism parameters in males are presented in Figure 2 and Supplemental Table 5. In males, short-term bed rest (HDT6) exposure significantly increased serum iron levels (+31.3%, p=0.027; Figure 2A) and decreased transferrin levels (-4.9%, p=0.019; Figure 2B), leading to a significant increase of transferrin saturation (+28.4%, p=0.009; Figure 2C). Serum hepcidin levels were significantly increased at HDT6 in males (+36.9 % p=0.005; Figure 2D). At HDT57, serum transferrin levels were decreased compared with baseline (-8.2%, p=0.002; Figure 2B), whereas serum iron levels returned to levels that were comparable to those observed at baseline (Figure 2A). However, transferrin saturation levels at HDT57 remained significantly higher compared with baseline (+13.5%, p=0.026, Figure 2C). Serum hepcidin levels returned to baseline values

(Figure 2D). Serum ferritin and soluble transferrin receptor levels in males were not affected by short and long-term exposure to HDT bed rest (Figure 2E and F).

In female participants, serum iron and transferrin saturation were not affected by short- and long-term exposure to HDT bed rest (Figure 3A and C). Similarly, serum transferrin levels at HDT6 and HDT57 were comparable with baseline (Figure 3B), as well as serum hepcidin, ferritin, and soluble transferrin receptor levels (Figure 3D, E and F and Supplemental Table 5).

Short and long-term effects of head-down tilt bed rest on blood volume and red blood cell indices

The global effects of HDT bed rest on red blood cell indices, blood and plasma volume are presented in Table 2 and Figure 4.

In males, BV, PV, RBCv and tHb mass at HDT5 were not changed compared with baseline (Figure 4 and Table 2). Conversely, they were decreased at HDT21 (-10.17%, $p<0.001$; -10.39%, $p<0.001$; -9.83%, $p=0.003$; -6.89%, $p<0.001$, respectively, Figure 4) and also at HDT56 (-7.39%, $p<0.001$; -7.24%, $p<0.001$; -7.63%, $p=0.042$; -6.57%, $p=0.012$, respectively, Figure 4 and Table 2). At HDT60, MCV was reduced (-1.86%, $p<0.001$; Table 2) and MCHC was increased compared with baseline (+1.15%, $p=0.004$; Table 2). Reticulocyte and serum unconjugated bilirubin levels remained unchanged between baseline (BDC-3) and the study end (HDT60) (Table 2).

In female participants, at HDT5, BV (-15.5%, $p=0.011$; Figure 5A), PV (-16.7%, $p=0.026$; Figure 5B), RBCv (-13.7%, $p=0.003$; Figure 5C) and tHb mass (-13.1% $p=0.039$; Figure 5D) were significantly decreased compared with baseline. At HDT60, MCV was decreased (-1.03%, $p=0.049$, Table 2) and MCHC increased (+2.95%, $p=0.003$, Table 2) compared with

baseline (BDC-3). Conversely, reticulocyte levels (p=0.403; Table 2) and serum unconjugated bilirubin levels remained unchanged between baseline (BDC-3) and HDT60 (Table 2).

Short and long-term effects of head-down tilt bed rest on serum markers of inflammation and sex hormones

In male and female participants, short and long-term exposures to HDT bed rest did not significantly affect hsCRP levels (Table 2) and also serum IL-6 levels, which remained below the detection threshold for all samples (Table 2). Long-term exposure to HDT bed rest (HDT60) increased serum testosterone and estradiol levels (+17.6%, p=0.026; +15.5% p=0.047, respectively, Table 2) in males, whereas it decreased serum estradiol levels (-37.2%, p=0.047, Table 2) in females.

Effects of head-down tilt bed rest on body and lower limb lean mass

Table 3 and Figure 6 show the global effect of HDT bed rest on body and lower limb lean mass. In males, short-term exposure to bed rest (HDT15) reduced total lean mass compared with baseline (-1.7%, p<0.001, Table 3), mainly due to a decrease of the lower limb lean mass (-3.8%, p<0.001; Table 3). Total body weight and total lean mass were decreased in males at HDT60 compared with baseline (-2.0%, p<0.001; -5.1%, p<0.001; respectively, Table 3). In females, total body weight and total lean mass were lower at HDT15 and HDT60 than at baseline (p<0.001, Table 3), as well as limb lean mass (p<0.001; Table 3). In females, the relative body lean mass was significantly decreased already at HDT30 (-3.36%, p=0.011, Figure 6), but in males only from HDT45 (-2.86% p=0.013, Figure 6).

DISCUSSION

This clinical study was initially designed to assess the effects of long-term HDT bed rest, combined or not with AG, on iron metabolism parameters in healthy males and females. As

iron atoms are predominantly associated with hemoglobin in red blood cells and myoglobin in skeletal muscles, we hypothesized that AG, by preventing anemia and muscle atrophy, could also modulate iron metabolism. However, we did not find any significant evidence that AG (either continuous or intermittent) influence the iron metabolism responses to simulated microgravity. This is in agreement with the data collected by other teams during the AGBRESA study showing that AG does not prevent muscle atrophy (20) and cardiorespiratory and vascular systems alterations (22–24), and does not affect the neuromuscular secretome (21). Therefore, ours and these previous findings question the interest of using AG to counteract microgravity-related physical deconditioning.

Importantly, independently from the analysis of AG effects in conditions of simulated microgravity, we found that in males, iron availability in plasma, reflected by the transferrin saturation level, and serum hepcidin levels increased after short-term exposure to HDT bed rest. The increase of transferrin saturation persisted at HDT60, indicating that long-term exposure to microgravity durably increases serum iron availability in males. Similarly, previous studies showed that short-term exposure to real microgravity or dry immersion causes iron misdistribution in males, with an increase of serum iron availability and spleen iron content (5,6). Moreover, the concomitant increase of serum hepcidin levels, compared with baseline, confirmed our previous finding in males exposed to dry immersion (5). As transferrin saturation is related to hepcidin levels (23), in the absence of a significant increase of inflammatory markers, these findings suggest that the increase of circulating hepcidin levels in simulated microgravity conditions might be an adaptive response to the increased serum iron availability. Serum transferrin concentration in males was decreased at HDT60, and this explained why transferrin saturation level remained high, although serum iron concentration at HDT60 was almost back to baseline levels. It is known that transferrin saturation increase promotes iron accumulation in parenchymal cells. Moreover, iron excess

has been observed in bone and liver after several weeks of simulated microgravity in rodents (26,27). Therefore, we hypothesize that after long-term exposure to microgravity, a relative iron excess occurs in males, exposing them to oxidative stress and its complications.

The heme present in hemoglobin of RBCs contains 65-70% of the total body iron that is needed for oxygen binding (28). Iron is constantly recycled from senescent RBCs by reticuloendothelial macrophages, especially in spleen, and may remain sequestered in these cells in conditions of secondary iron overload or during inflammatory processes (25,29). Therefore, the tHb mass decrease we observed in males could affect body iron distribution in microgravity. This anemia occurring in males in microgravity (30) could result from an increase of the hemolysis rate, assessed by quantifying serum unconjugated bilirubin and haptoglobin levels (31). However, we did not observe any modulation of these serum hemolysis parameters in males exposed to HDT bed rest, unlike a recent study (1). More studies are required to determine whether the anemia observed in microgravity contributes to iron redistribution and by which mechanism(s), including erythropoietic activity among others. As previously reported (32,33), short and long-term exposure to HDT bed rest also promoted muscle atrophy in males, as indicated by the decrease of total body lean mass. As muscle myoglobin contains 20-25% of the total body iron, muscle atrophy also could contribute to body iron distribution changes in males exposed to microgravity.

Although the number of women astronauts is constantly increasing (11), many physiological and metabolic adaptations to microgravity, including iron metabolism responses, remain poorly understood in this population. Here, we observed that short- and long-term exposure to HDT bed rest did not affect serum iron and transferrin saturation levels in females. In accordance with these findings, serum hepcidin levels remained unchanged in females in response to HDT bed rest, supporting the hypothesis that the upregulation of hepcidin expression in liver observed in males could be related to the increase of serum iron

availability. In addition, we observed a decrease of tHb mass and body lean mass in females. Anemia and muscle atrophy have been previously reported in females exposed to real or simulated microgravity (34,35); however, the distribution in the female organism of the iron previously contained in the lost erythrocytes and atrophied muscle fibers is unknown. Indeed, the prevalence of severe iron accumulation forms during hemochromatosis related to the homozygous p.Cys282Tyr HFE protein mutation is lower in females than in males (36,37). It has been proposed that the hormonal status and menstrual bleeding are protective mechanisms against iron overload in young females (38,39). Therefore, we cannot exclude that menstrual bleeding could have contributed to the absence of serum iron availability increase in female participants at HDT60, despite the observed anemia and muscle atrophy. The small sample size (n=7) of this exploratory study constitutes nevertheless a limitation that could contribute to mask potentially significant variations in females, especially concerning the short-term effects of HDT on serum iron availability. Finally, all these findings highlight the need 1) to conduct future studies with larger sample size in females, and 2) to thoroughly investigate iron levels in some key organs implicated in iron metabolism (spleen, skeletal muscle, liver, bone). These kind of perspectives will allow to especially determine whether the lack of changes in serum iron availability in response to long-term exposure to microgravity in females is a defense mechanism against iron excess or whether it is deleterious for some organs by causing abnormal iron accumulation.

In summary, this study, performed in a small but very well controlled group of participants, showed that AG does not affect iron metabolism in males and females exposed to prolonged simulated microgravity. Independently from AG, our data also highlighted that iron metabolism parameters, especially iron availability for cells, are significantly increased in males after short- and long-term exposure to simulated microgravity. In females, iron metabolism parameters were not affected by microgravity despite the important decrease of

tHb mass and muscle mass. This may suggest an adaptation of iron metabolism and iron fluxes in this group in microgravity conditions. Nevertheless, we must be cautious before concluding that females could differently respond to males under microgravity, due to the small sample size of females in this exploratory study. Our findings open promising research perspectives to optimize nutrition for males and females exposed to microgravity or to extreme physical inactivity, such as astronauts and bedridden patients, respectively.

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	Males	Females
	n=16	**n=7**
Age (years)	33.06 ± 9.89	33.75 ± 7.63
Height (cm)	178.69 ± 4.96	166.24 ± 7.79
Weight (kg)	78.44 ± 7.33	65.71 ± 8.95
BMI (kg/m^2)	24.56 ± 1.96	23.70 ± 2.05

Table 1. Baseline characteristics at BDC-13[1] in male and female participants

[1] Values are presented as mean ± SD for both sexes, (n=16 males, n=7 females).

[2]*BDC: Baseline Data Collection; BMI: Body Mass Index*

Table 2. Effects of HDT bed rest on red blood cells, hemolysis, and sex hormones in male and female participants[1].

	MALES (n=16)				FEMALES (n=7)			
	Baseline[5]	**Short-term[6]**	**Long-term[7]**	**One-way RM ANOVA [2]**	**Baseline[5]**	**Short-term[6]**	**Long-term[7]**	**One-way RM ANOVA [2]**
Blood volume (ml)	7269 ± 1602	6830.4 ± 1405.4	6732 ± 1637.3 ***	**P<0.001**	5985.3 ± 885.3	5055.9 ± 815.7 #	5201.4 ± 915.9	**P=0.001**
Plasma volume (ml)	4405.6 ± 1302.9	4126.3 ± 1218.4	4086.7±1291.4***	**P<0.001**	3683.5 ± 551	3068.7 ± 619.6 #	3149 ± 648.2 #	**P<0.005**
RBC volume (ml)[3]	2863.7 ± 400.2	2704.1 ± 282.1	2645.3 ± 423.5 *	**P<0.001**	2301.8 ± 364.4	1987.2 ± 227.2 ##	2052.4 ± 274.5	**P=0.001**
tHbmass (g)[4]	893.6 ± 96.7	868.4 ± 115.2	834.9 ± 106.8 *	**P=0.002**	630.7 ± 170.6	548.1 ± 115.6 #	535.6 ± 123.7 ##	**P=0.004**
Hemoglobin (g/dl)	15.6 ± 1.18	15.9± 1.03	15.8 ± 1.01	**P=0.008**	13.5 ± 0.3	14.1 ± 0.87 #	13.48 ± 1.1	**P=0.117**
Hematocrit (%)	40.3 ± 5.6	40.6 ± 6.0	40.3 ± 5.9	**P=0.45**	38.5 ± 1.7	39.7 ± 3.3	39.7± 2.2	**P=0.32**
RBC (10^6/pL)	4.9 ± 0.37	-	5.2 ± 0.43***	**P<0.001**	4.2 ± 0.28	-	4.3 ± 0.07	**P=0.75**
Reticulocyte count (%)	0.83 ± 0.22	-	0.84 ± 0.24	**P=0.95**	1.23 ± 0.07	-	1.22 ± 0.04	**P=0.40**
MCV (fL)	85.9 ± 3.9	-	84.3 ± 3.5***	**P<0.001**	87.5 ± 5.4	-	86.6 ± 4.3 #	**P=0.049**
RDW (%)	23.23 ± 14.3	-	23.61 ± 15.31	**P=0.89**	12.4 ± 0.7	-	12.2 ± 0.4	**P=0.40**
MCH (pg)	29.7 ± 1.5	-	29.5 ± 1.4	**P=0.21**	29.7 ± 2.0	-	30.2 ± 1.6	**P=0.23**
MCHC (g/dL)	34.6 ± 0.5	-	35.0 ± 0.6**	**P=0.04**	33.9 ± 0.9	-	34.9 ± 0.7##	**P=0.003**
Bilirubin (µmol/l)	13.6 ± 7.6	14.3 ± 7.3	12.3 ± 7.0	**P=0.012**	7.5 ± 2.2	7.38 ± 2.7	6.13 ± 1.6	**P=0.157**
Conjugated bilirubin (µmol/l)	4.75 ± 1.8	4.69 ± 2.0	4.31 ± 1.7	**P=0.098**	3.25 ± 0.5	3.38 ± 0.7	3.0 ± 0.0	**P=0.444**
Unconjugated bilirubin (µmol/l)	13.1 ± 7.7	13.7 ± 7.7	11.8 ± 7.4	**P=0.185**	7.5 ± 2.2	7.0 ± 2.9	6.1 ± 1.55	**P=0.191**
Haptoglobin (g/l)	1.04 ± 0.4	1.14 ± 0.3	1.16 ± 0.5	**P=0.181**	0.93 ± 0.4	1.03 ± 0.4	1.06 ± 0.4	**P=0.054**
IL-6 (ng/ml)[8]	ND	ND	ND	**-**	ND	ND	ND	**-**
hsCRP (mg/l)	1.08 ± 0.4	1.03 ± 0.1	1.03 ± 0.1	**P=0.584**	<1	<1	<1	**-**
Testosterone (ng/ml)	4.42 ± 1.2	-	5.20 ± 1.3 *	**P=0.026**	0.30 ± 0.1	-	0.35 ± 0.1	**P=0.13**
Estradiol (ng/L)	32.8 ± 7.4	-	37.9 ± 7.8 *	**P=0.047**	163.0 ± 116.1	-	102.3 ± 56.8 #	**P=0.047**

[1] Values are presented as mean ± SD for both sexes (n=16 males, n=7 females).

[2] Data were analyzed with one-way ANOVA for repeated measures in each group

For all significant effects, the Tukey's post hoc test was used to detect differences between baseline and each time point

[3] Males compared with baseline: *P <0.05; **P <0.01; ***P <0.001

[4]Females compared with baseline: #P <0.05; ##P <0.01; ###P <0.001

[5]Baseline: BDC-12, BDC-6 or BDC-3, depending on the parameter

[6]Short-term: HDT5 or HDT6, depending on the parameter

[7]Long-term: HDT56, HDT57 or HDT60, in function of the parameter

[8] *BDC: Baseline Data Collection; CRP: C-reactive protein; tHb mass: total hemoglobin mass; HDT: Head-down tilt bed rest; hsCRP: high-sensitivity CRP; IL-6: Interleukin-6; MCH: Mean corpuscular hemoglobin; MCHC: Mean corpuscular hemoglobin concentration; MCV: Mean corpuscular volume; ND: non detectable; RBC: Red blood cell; RDW: Red cell Distribution Width.*

Table 3. Effects of HDT bed rest on body lean mass in male and female participants[1]

	MALES (n=16)				FEMALES (n=7)			
	BDC-4	**HDT15**	**HDT60**	**One-way RM ANOVA [2]**	**BDC-4**	**HDT15**	**HDT60**	**One-way RM ANOVA [2]**
Total body weight (kg)[3,4]	79.17 ± 7.2	78.28 ± 7.2	77.61 ± 7.2***	**P<0.001**	67.76 ± 8.7	66.63 ± 8.1#	65.41 ± 8.3###	**P<0.001**
Absolute total lean mass (kg)	58.45 ± 3.9	57.44 ± 4.4***	55.47 ± 4.3***	**P<0.001**	41.18 ± 5.8	39.49 ± 5.3###	37.87 ± 5.5###	**P<0.001**
Relative total lean mass (g/kg)	745.4 ± 93.5	740.9 ± 96.9	721.5 ± 94.2**	**P<0.001**	616.9 ± 121.6	600.8 ± 113.3	587.5 ± 116.6 ###	**P<0.001**
Absolute lower limb lean mass (kg)	19.64 ± 1.3	18.89 ± 1.5*	17.70 ± 1.4***	**P<0.001**	14.11 ± 1.9	13.11 ± 1.8##	11.9 ± 1.5###	**P<0.001**
Relative lower limb lean mass (g/kg)	234.3 ± 37.5	225.9 ± 38.8**	211.1 ± 38.0***	**P<0.001**	255.4 ± 41.4	244.6 ± 41.8##	233.2 ± 40.7###	**P<0.001**

[1] Values are presented as mean ± SD for both sexes (n=16 male, n=7 female)

[2] Data were analyzed with one-way repeated measures ANOVA in each group

For all significant effects, the Tukey's post hoc test was used to detect differences between baseline and each time point

[3] Males compared with baseline: *P <0.05; **P <0.01; ***P <0.001.

[4]Females compared with baseline: #P <0.05; ##P <0.01; ###P <0.001

[5]*BDC: Baseline Data Collection; HDT: Head-down tilt bed rest*

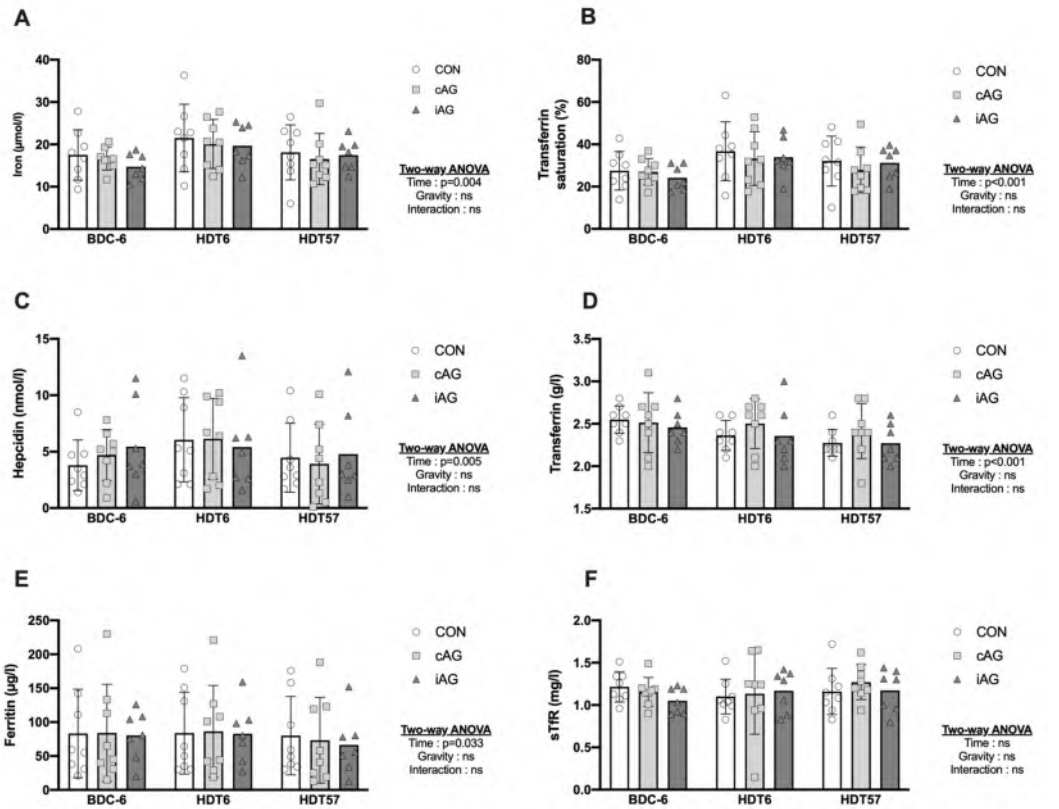

Figure 1. Impact of artificial gravity on iron metabolism parameters before (BDC-6), and after 6 and 57 days of HDT bed rest (HDT6 and HDT57). Serum iron concentration (A), transferrin saturation level (B), serum hepcidin concentration (C), serum transferrin concentration (D), serum ferritin concentration (E), and soluble transferrin receptor concentration (F). Data are presented as individual values and the mean ± SD for each group (CON: n=8 ; cAG: n=8 ; iAG: n=7). Data were analyzed with two-way ANOVA for repeated measures followed by the Tukey's post hoc test to detect differences between mean values for all significant interactions. *cAG: continuous Artificial Gravity; CON: Control; BDC: Baseline Data Collection; HDT: Head-Down Tilt Bed Rest; iAG: intermittent Artificial Gravity; sTfR: soluble Transferrin Receptor.*

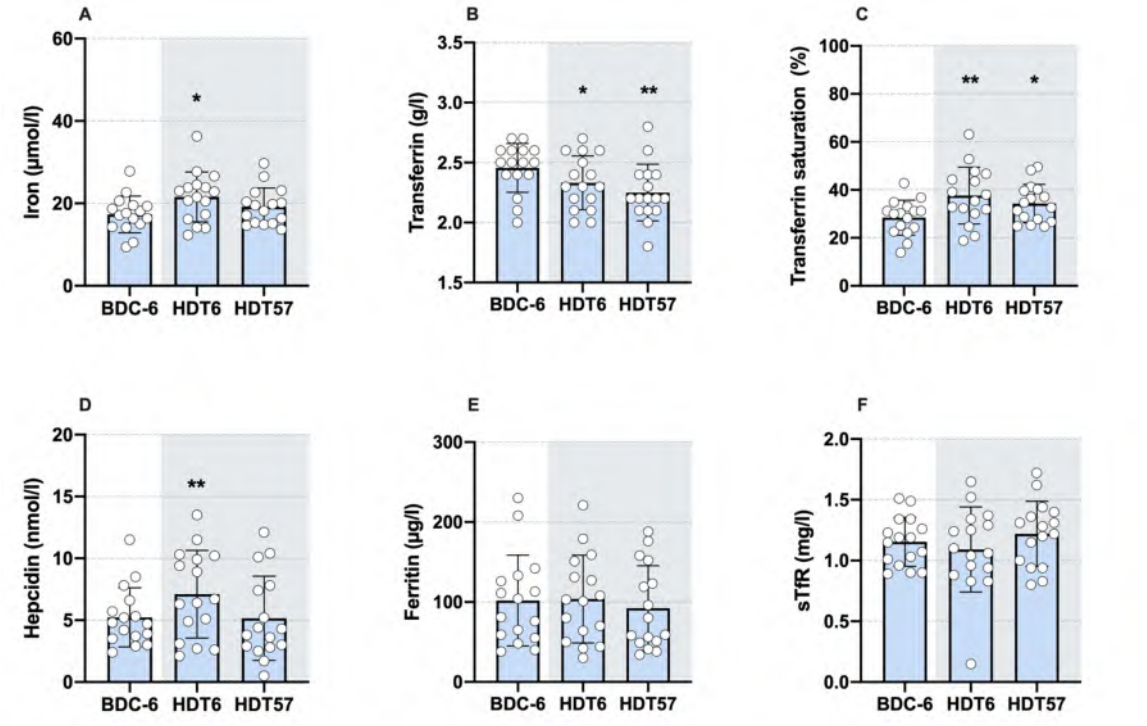

Figure 2. Iron regulation before (BDC-6), and after 6 and 57 days of HDT bed rest (HDT6 and HDT57) in male participants. Serum iron concentration (A), serum transferrin concentration (B), serum transferrin saturation percentage (C), hepcidin concentration (D), serum ferritin concentration (E), and serum transferrin receptor concentration (F). Data are presented as individual values and the mean ± SD for each group. Data were analyzed with one-way repeated measures ANOVA for each group, followed by the Tukey's post hoc test to detect differences between baseline and each time point for all significant effects. Males compared with baseline (n=16): *P <0.05; **P <0.01. *BDC: Baseline Data Collection; HDT: Head-Down Tilt Bed Rest; sTfR: soluble Transferrin Receptor.*

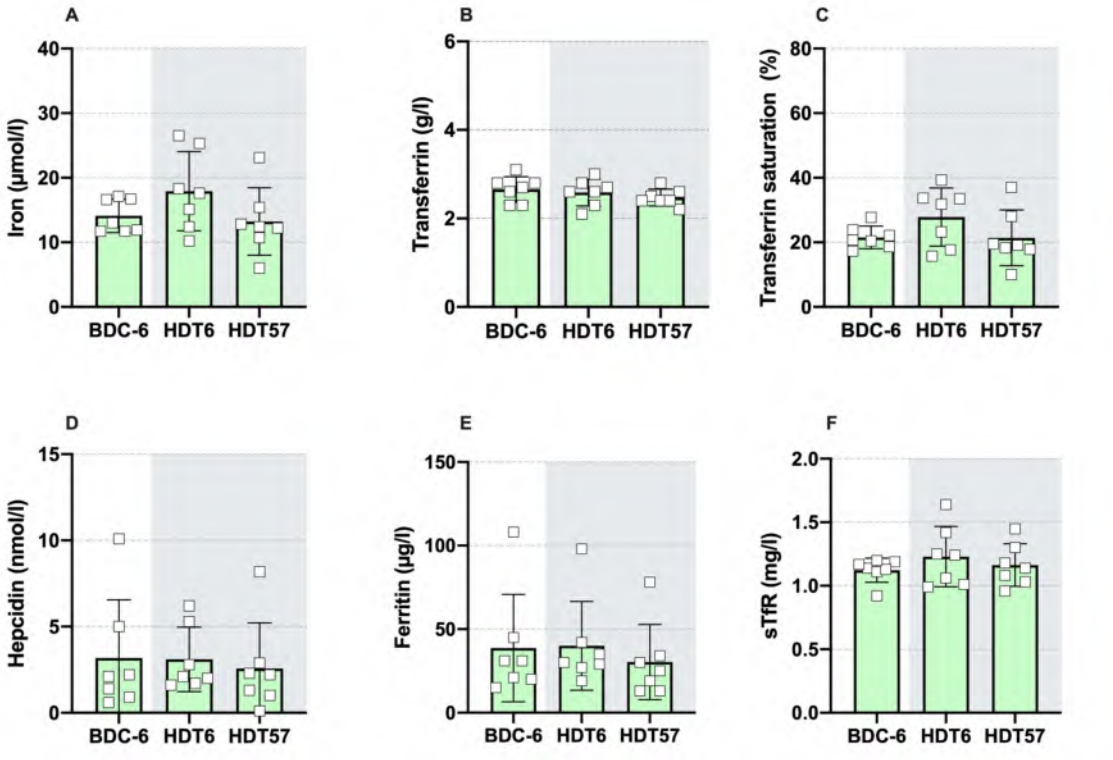

Figure 3. Iron regulation before (BDC-6), and after 6 and 57 days of HDT bed rest (HDT6 and HDT57) in female participants. Serum iron concentration (A), serum transferrin concentration (B), serum transferrin saturation percentage (C), hepcidin concentration (D), serum ferritin concentration (E), and serum transferrin receptor concentration (F). Data are presented as individual values and the mean ± SD (n=7). Data were analyzed with one-way repeated measures ANOVA followed by the Tukey's post hoc test to detect differences between baseline and each time point for all significant effects. *BDC: Baseline Data Collection; HDT: Head-Down Tilt Bed Rest; sTfR: soluble Transferrin Receptor.*

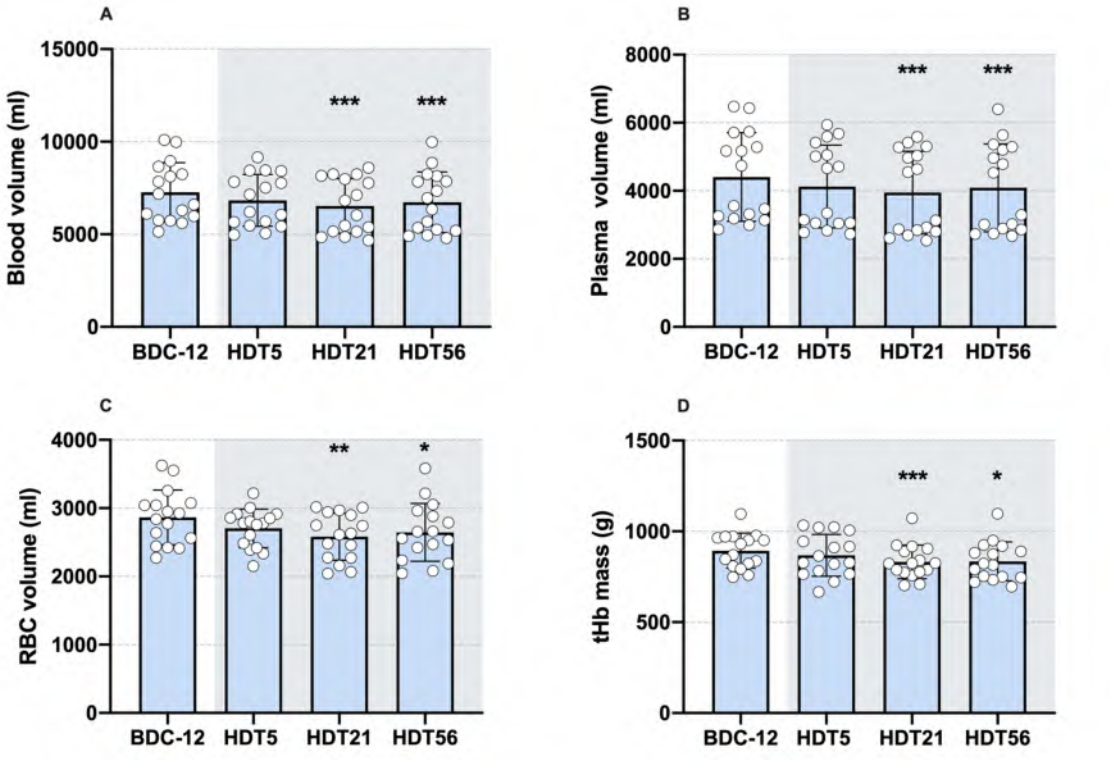

Figure 4. Effect of HDT bed rest on blood and RBC parameters in male participants. Blood volume (A), plasma volume (B), red blood cell volume (C), and hemoglobin mass (D). Data are presented as individual values and the mean ± SD. Data were analyzed with one-way repeated measures ANOVA followed by the Tukey's post hoc test to detect differences between baseline and each time point for all significant effects. Males (n=16) compared with baseline: *p <0.05; **p <0.01; ***p <0.001. *BDC: Baseline Data Collection; HDT: Head-Down Tilt Bed Rest; tHb mass: total hemoglobin mass; RBC: Red Blood Cell.*

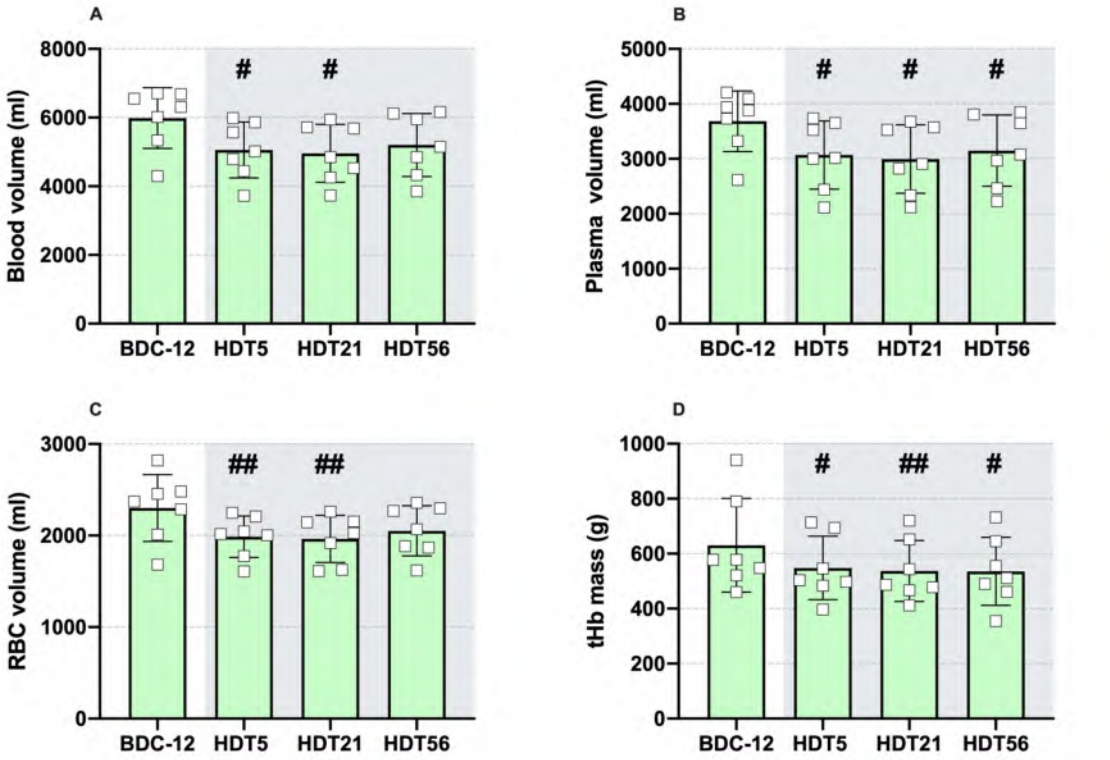

Figure 5. Effect of HDT bed rest on blood and RBC parameters in female participants. Blood volume (A), plasma volume (B), red blood cell volume (C), and hemoglobin mass (D). Data are presented as individual values and the mean ± SD. Data were analyzed with one-way repeated measures ANOVA followed by the Tukey's post hoc test to detect differences between baseline and each time point for all significant effects. Females (n=7) compared with baseline: $^{\#}P < 0.05$; $^{\#\#}P < 0.01$; $^{\#\#\#}P < 0.001$. *BDC: Baseline Data Collection; HDT: Head-Down Tilt Bed Rest; tHb mass: total hemoglobin mass; RBC: Red blood cell.*

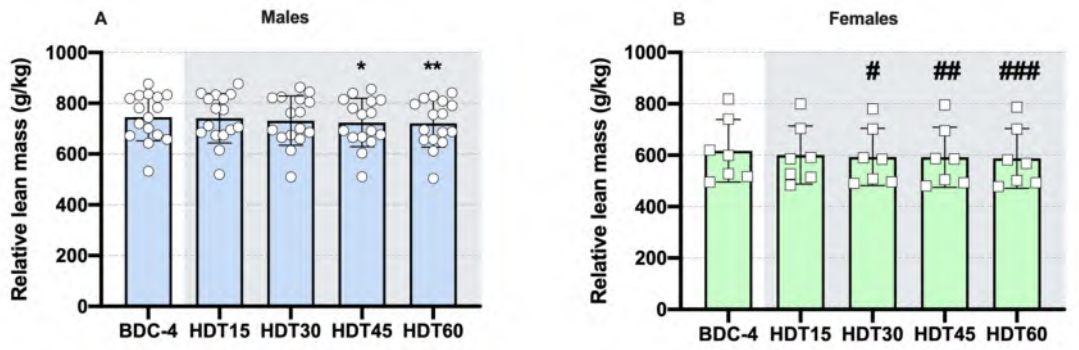

Figure 6. Effect of HDT bed rest on relative lean mass in male and female participants. Values are the mean ± SD for both sexes. Data were analyzed with one-way repeated measures ANOVA for each group followed by the Tukey's post hoc test to detect differences between baseline and each time point for all significant effects. Males (n=16) compared with baseline: *P <0.05; **P <0.01; and females (n=7) compared with baseline: $^{\#}$P <0.05; $^{\#\#}$P <0.01; $^{\#\#\#}$P <0.001. *BDC: Baseline Data Collection; HDT: Head-Down Tilt Bed Rest.*