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International standard measures during the AGBRESA bed rest study

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Abstract

The *Artificial Gravity Bed Rest with European Space Agency* (AGBRESA) study involved 2 campaigns of 60-day bed rest at 6° head down tilt in the :envihab facility in Cologne, Germany, in 2019. The objective was to determine whether centrifugation of supine subjects mitigates physiological changes that occur due to the exposure to the spaceflight analog of 6° head down tilt. A set of international standard measures was used to evaluate bone, muscle, nutritional status, ocular changes, and psychological state and to assess cardiovascular, sensorimotor, and immune systems functions. After 60 days of bed rest, the subjects who were not exposed to centrifugation had significant decreases in mineral density at the hip and lumbar spine, decreased muscle strength of the knee and ankle, reduced maximal aerobic capacity, orthostatic intolerance, and impaired balance and locomotion, as well as significant biochemical, immunological, and psychological alterations. A daily 30-min exposure to 1 Gz supine centrifugation at the center of mass (0.3 Gz at the head, 2 Gz at the feet) mitigated mineral density loss in the femur neck and lumbar spine, and lessened the effects of bed rest on aerobic capacity, biochemistry, and immunology. However, low statistical power could contribute to the non-significant effects that are reported.

Keywords: Bed rest; Microgravity; Artificial gravity; Centrifugation; Adaptation; Physiology; Psychology

1. Introduction

The *Artificial Gravity Bed Rest* (AGBRESA) study was conducted on 24 subjects in the :envihab facility of the German Aerospace Center (DLR) in Cologne, Germany, during 2 campaigns: from 25 March to 2 September 2019 and from 2 September 2020 to 23 December 2020. The primary aim of the AGBRESA study was to comprehensively evaluate the effects of a 60 days of strict 6° head down tilt (HDT) bed rest (BR) on bone, muscle, cardiovascular, sensorimotor, biochemical, immune, and psychological functions, and on ocular anatomy and physiology. The secondary aim of the AGBRESA study was to determine whether centrifugation in the supine position for 30 min a day mitigates BR-induced deconditioning of these functions.

It is possible that artificial gravity (AG) generated by centrifugation could mitigate physiological deconditioning caused by prolonged exposure to weightlessness during spaceflight [1]. Long-duration BR is used extensively to test measures for mitigating the effects of spaceflight because BR simulates the effects of weightlessness on bone, muscle, and the cardiovascular system [2]. Previous BR studies ranging from 5-21 days have shown that daily exposure to centrifugation generating 1-2 Gz at heart level for 0.5-2 hours effectively mitigated orthostatic intolerance and maintained exercise capacity [3-8]. A more recent study, called BRAG-1, has compared the effects of daily centrifuge sessions generating +1 Gz at the heart for 30 min continuously (1 x 30 min) to the effects from 6 bouts of 5 min (6 x 5 min) separated by 5 min of rest [9]. The 6 x 5 min +Gz intervention was more effective in preserving orthostatic tolerance after BR and the positive effects appeared equivalent to those seen after continuous 60-min exposure to +Gz stimulation in other studies [7]. The intermittent exposures to centrifugation were also better tolerated by the subjects. However, this BR was only 5 days, so it was too short to evaluate how effectively daily centrifugation protects the structure and strength of bone and muscle, or mitigate sensorimotor deconditioning. Also, proper ocular function will be essential for the success of exploration-type space missions, but changes to the physiology and function of the eye were not tested during these previous BR studies.

The AGBRESA study was an international collaborative effort between the National Aeronautics and Space Administration (NASA), the European Space Agency, and DLR to extend the knowledge gained from these previous BR studies. During the AGBRESA study, a set of measures collectively called *International Standard Measures* (ISM) were gathered to supplement measures collected during the individual investigators' studies, and to allow the responses and the effects of countermeasures to be compared for BR studies of different durations, conducted at various locations. The ISMs are defined in the *International Guidelines for Standardization of Bed Rest Studies in the Spaceflight Context* [10,11], issued by the International Academy of Astronautics Study Group, which includes representatives of all participating space agencies. We have provided detailed descriptions of the ISMs and their timeline during BR studies in a recent paper [12]. Here, we report the results of the ISMs obtained during AGBRESA to assess the effects of continuous and intermittent centrifugation (daily 30-min of 1 Gz at the center of mass, 0.3 Gz at the head, and 2 Gz at the feet) on bone, muscle, cardiovascular, sensorimotor, ocular, immune, and psychological functions.

2. Methods

2.1. Subjects

Twenty-four subjects participated in the AGBRESA study: 16 men (M), 8 women (F); 33.3 ± 9.0 years (mean ± SD) (Table 1). NASA institutional review board approved the experimental protocol for this study (Pro2219). The experimental procedures were approved by the Ethics Committee of the Northern Rhine Medical Association (Ärztekammer Nordrhein, application No. 2018143) in Düsseldorf, Germany, as well as the Federal Office for Radiation Protection (Bundesamt für Strahlenschutz, application No. 22464/2018-074-R-G). The study is registered at the German Clinical Trials Register (No. DRKS00015677). All subjects gave their written informed consent before participating in the study.

[Table 1 about here]

Each subject underwent a 14-day ambulatory baseline data collection (BDC-) phase; a 60-day bed rest phase in head down tilt (HDT); and a 14-day ambulatory recovery phase (R+). R+0 was the day the subjects stood up after HDT. During the BR period, the test subjects were tilted 6° head-down, except during the centrifugation runs. Daily routine activities such as eating, washing, showering and toilet, and leisure activities (e.g., reading, watching TV) were performed in the HDT position. Subjects were continuously supervised by dedicated staff via a closed video system that could also be used to provide immediate feedback to the subjects. Subjects did not use a pillow to support their head, and, to minimize mechanical stimuli, they were instructed not to raise, contract, or stretch their legs [12].

2.2. Countermeasures

The subjects were assigned to one of the following 3 experimental groups: passive control group (Ctrl): no centrifugation (n=8); continuous AG group (cAG): 1 x 30 min of centrifugation daily (n=8); or intermittent AG group (iAG): 6 × 5 min of centrifugation daily (n=8).

Subjects assigned to the cAG and iAG groups were exposed to 30 min a day of supine centrifugation with 1 Gz (i.e., in the head-to-foot direction) at their center of mass (COM) for 60 days during the HDT phase. The COM for each subject was calculated using a ratio of COM to body height of 56% for males and 54% for females [13]. This corresponded to distances from the axis of rotation (radius) of 2.34–2.42 m and rotation rates of 19.6–19.2 rpm. Because the magnitude of the centripetal acceleration is function of the radius (and the square of angular velocity), there is a gravity gradient along the subject's body. In this study, subjects were exposed at approximately 1.4-1.6 Gz at feet level and 0.3 Gz at head level. The direction of the rotation was alternated each day between clockwise and counterclockwise. The subjects who were exposed to the AG protocol during HDT were familiarized with the protocol during 2 sessions before HDT began.

Subjects were maintained in HDT while preparing for the centrifugation (donning of equipment and safety checks) and were placed in the horizontal position just before the centrifugation commenced. Immediately after the final halt of the centrifuge (either after 30 min or after the final 5-min run), the subjects were placed in HDT while still on the centrifuge. Subjects in the iAG group remained in the horizontal position between centrifuge runs; consequently, they had 4 x 3 min = 12 min of horizontal rest daily in addition to the 30 min of centrifugation. To promote subject engagement and relaxation during the AG session, the subjects could listen to music or an audiobook of their choice.

Of the total 960 AG sessions from both BR campaigns, 11 runs (1.14%) of 6 different individuals (2M, 4F) were interrupted; and 8 cAG runs and 1 iAG run were aborted prematurely. Reasons for discontinuation included medical stops due to presyncopal signs (n=7), subjects reporting high levels of motion sickness including nausea and dizziness (n=3), and pain in bilateral lower legs (n=1). Age was not a strong predictor of intolerance, and no trends were evident regarding the time at which the subject showed signs or symptoms during the 30 min AG runs. None of the symptoms occurred for the same subject on consecutive days. All symptoms resolved quickly after the centrifuge stopped rotating.

2.3. Data analysis

Data were evaluated to determine whether daily exposure to AG delivered by means of supine centrifugation could mitigate expected physiological deconditioning induced by 60 days of strict 6° HDT bed rest. Statistical analyses were performed with repeated measures analysis of variance and *post-hoc* test, or regression analyses using linear mixed-effects models. Analyses were performed using GraphPad ver. 9.1, Matlab ver. R2019, SPSS ver. 22.0, and R ver. 4.0.2, with a significant level of 0.05. Given the many different measurements that were assessed in this study, the methods used for recording and analyzing these data are included in the Results section.

3. Results

3.1. Bone mineral density and body composition

Measures of 2-dimensional bone mineral density (BMD, g/cm^2) and body composition (mass, g) were obtained by dual-energy X-ray absorptiometry (DXA) using a whole-body densitometer (GE Lunar Prodigy, enCORE software version 16) on BDC-13 (the pre-HDT measurement) and R+11. Single scans were obtained from the following sites: whole body, lumbar spine, and hip. BMD variables were derived from the hip and spine scans, and body composition variables were derived from the whole body scans. The percent changes from before to after HDT were then calculated for each skeletal site or body composition region. As seen in Figure 1, most within-group changes in BMD were not significant, with the exception of the trochanter in the cAG group (-3.2%), and the femur neck and lumbar spine in the Ctrl group (-1.3% and -1.9%, respectively). In contrast with the Ctrl group, no significant changes from before to after HDT were observed in the iAG and cAG groups for the femur neck and lumbar spine.

[Fig. 1 about here]

Figure 2 clearly shows a gradation of changes in lean mass that are consistent with prolonged HDT, i.e., the greatest increases were toward the head and the greatest decreases were in the legs. In the cAG group, statistically significant mean changes of were detected in lean mass of the legs (-2.5%), lean total mean mass (-1.5%), and lead head mass (+2%). Note that lean mass is defined as fat-free, bone-free mass, and therefore contains not only muscle but fluid and other fat-free tissue. Presumably, the increase in lean mass in the head reflects cephalad fluid shifts. For all 3 groups, the within-group changes in lean mass of the trunk and arms were not significant. The mean changes in lean mass were not significant between the 3 subject groups for all body regions.

[Fig. 2 about here]

Fat mass, on the other hand, increased in one or more body all regions across all groups. Statistically significant mean increases were seen in the Ctrl group (head +4%, legs +4.3%), in the cAG group (head +6.8%, legs +9.1%, trunk +8.1%, total body +7.6%), and in the iAG group (trunk +11%). The iAG group was the only group that had a statistically significant mean *loss* of fat in any region (-9.3% in the arms). As with lean mass, all between-group differences for mean change in fat mass were not significant for all body regions.

3.2. Muscle strength and peak aerobic capacity

Isometric maximum voluntary contractions were measured in knee and ankle muscles. Isokinetic muscle testing was used to assess strength of muscles in the knee, ankle, and trunk. These tests were described in detail in a previous publication [12]. Comparison of values before HDT (BDC-5) and after HDT (R+2) indicated significant decreases in peak torque of isometric and isokinetic ankle plantar flexion, and knee isometric and isokinetic extension (Fig. 3). Similar changes from BDC-5 to R+2 were observed for the other muscle strength measures. Repeated measures analysis of variance (2 time points, 3 subject groups) using Holm-Sidak *post-hoc* test indicated no differences between the cAG, iAG, and Ctrl groups.

[Fig. 3 about here]

Peak aerobic capacity ($\dot{V}O_2$ peak) was assessed on BDC-3 and R+0 by measuring respiratory gas exchange (Innocor, Innovision, Odense, Denmark) during a graded exercise protocol on an electronically braked cycle ergometer (Lode, Gronigen, the Netherlands). Subjects wore a nose clip and breathed through a respiratory valve so gases could be accurately collected. Gas and volume flow were calibrated prior to the exercise test. The graded exercise protocol provided an individualized approach for achieving each subject's $\dot{V}O_2$ peak using small increments in workload. During testing, subjects maintained a pedaling cadence of 70-75 rpm. Workload began at 50 W for 3 min and then increased by 25 W every minute until volitional exhaustion.

Increasing workload in small increments with each minute of exercise allows ventilatory threshold and $\dot{V}O_2$ peak to be evaluated. Using this protocol, maximal exercise was achieved in approximately 8 to 15 min.

Heart rate was monitored continuously via 12-lead ECG (Padsy, Medset Medizintechnik, Germany) during resting. Blood pressure was measured using a sphygmomanometer and stethoscope during each of the first 3 exercise stages, and every 2 min during the subsequent 1-min stages. Spiroergometry data were filtered by calculating the median of 5 breaths and subsequently the moving average over 30 seconds [14]. Afterwards, the peak values for the following parameters were extracted: maximal workload, heart rate, breathing rate, minute ventilation (VE), oxygen elimination ($\dot{V}O_2$), and carbon dioxide elimination ($\dot{V}CO_2$).

Comparison between pre- and post-HDT measurements for all groups combined showed significant decreases in maximal workload (-26%), ventilation (-13%), $\dot{V}CO_2$ (-24%), and $\dot{V}O_2$ (-23%) (Fig. 4). Similar changes from BDC-3 to R+0 were observed for the $\dot{V}CO_2$ peak measures. Repeated measures analysis of variance (2 time points, 3 subject groups) using Holm-Sidak *post-hoc* test indicated significant differences between the Ctrl group and the cAG and iAG groups for heart rate, and between the Ctrl group and the iAG group for minute ventilation.

[Fig. 4 about here]

3.3. Orthostatic intolerance

Orthostatic tolerance was measured in all subjects on BDC-5 and R+0. Heart rate along with beat-to-beat finger blood pressure were collected during 5 min of supine rest, 15 min of 80° head-up tilt, and when lower body negative pressure (LBNP) was applied while the subject was tilted in -10 mmHg increments every 3 min until presyncope [15]. In this report only the data collected during tilt without LBNP were considered.

Brachial blood pressure data were acquired only every 2 to 3 min; however, in 9 instances during R+0 testing the brachial blood pressures were not recorded through presyncope or through the end of tilt. The available brachial blood pressure data were summarized, averaging 2 to 3 measurements obtained during supine rest and at 2-min intervals during tilt (e.g., min 1-2, min 3-4, etc.), except for the final interval of 3 min (i.e., min 13-15).

Although only the centrifugation groups were balanced by sex (Ctrl: 2F, 6M; cAG: 3F, 5M; iAG: 3F, 5M), it is striking that the pre-HDT tolerance times appear different between the groups (Fig. 5). Specifically, all the control subjects completed at least 12 min of head-up tilt before presyncope, and mean tilt tolerance time before HDT was 14.3 ± 1.4 min (mean ± SD). In contrast, the cAG group included 3 subjects who completed only 8 min or less of tilt, and the iAG included 3 subjects who completed 6.5 min or less of tilt. Mean tilt tolerance time before HDT was 11.5 ± 4.8 and 11.1 ± 5.5 min in the cAG and iAG groups, respectively.

[Fig. 5 about here]

After HDT, all but one of the control subjects were unable to complete the full 15 min of head-up tilt, and the average tilt tolerance time decreased by 5.4 ± 3.4 min. Three of the subjects (1F, 2M) in the cAG group completed 15 min of head-up tilt before and after HDT, but of the remaining 5 subjects, the longer tilt tolerance time after HDT ranged from 1.5 to 7.7 min. Mean tilt tolerance time in the cAG group decreased by 2.7 ± 3.2 min. The 3 cAG subjects with lower tolerance times before HDT tolerated head-up tilt for less time after HDT. Two subjects in the iAG group (2M) completed 15 min of head-up tilt before and after HDT. The remaining 3 iAG subjects (2F, 1M) who tolerated 15 min of tilt before HDT demonstrated a decrease in post-HDT tilt tolerance of more than half their pre-HDT tolerance. Interestingly, the 3 iAG subjects who had lower tilt tolerance before HDT (< 7 min) had higher tilt tolerance after HDT, ranging from an improvement of 0.3 to 4.1 min; however, none of these subjects could tolerate more than 10 min of head-up tilt after HDT. There was no difference between the groups post-HDT in presyncope-free survival time during tilting (Fig. 6).

[Fig. 6 about here]

3.4. Postural equilibrium control

Computerized dynamic posturography with the *Equitest* (NeuroCom International, Clackamas, OR) and sensory organization tests (SOT) were used to objectively assess the subjects' ability to effectively use (or suppress inappropriate) visual, vestibular, and proprioceptive information for balance control [16]. The more challenging SOT conditions involved disrupting proprioceptive and visual feedback by rotating the support surface and the visual surround in proportion to body sway, which is referred to as sway-referencing. The standard SOT protocol with head erect comprised 6 conditions involving 2 support surface conditions (fixed and sway-referenced) and 3 visual conditions (eyes open, eyes closed, and sway-referenced surround). Two modified SOT conditions were also used to increase sensitivity by including dynamic head tilts with eyes closed on either fixed (SOT2M) or sway-referenced support surface (SOT5M). The dynamic tilts involved pitching the head at 0.33 Hz (± 20°) paced by an audible tone. For each SOT trial, data were recorded for 20 s or until there was a fall. Three trials were conducted in each condition. The order of conditions was randomized.

The motor control tests (MCT) assessed the subjects' ability to quickly and automatically recover from unexpected perturbations of the support surface. Large forward and backward platform translations (400 ms, amplitude scaled to 3.2 deg equivalent sway for the subject height, e.g., approximately 5.6 cm for a 180-cm tall subject) were performed to elicit automatic postural responses.

Throughout each SOT and MCT trial, subjects were instructed to maintain a stable upright posture with their arms folded across their chest. External auditory orientation cues were masked by white noise supplied through headphones. Center-of mass sway angles were estimated from instantaneous anterior-posterior (AP) and medial-lateral center-of-force

positions computed from force transducers mounted within the *Equitest* force plates. The AP peak-to-peak sway angle (A, in deg), was used to compute a continuous equilibrium score (EQ) as follows:

$$EQ = (1-(A/12.5)) \times \% \text{ trial completed},$$

where 12.5° is the maximum theoretical peak-to-peak AP sway and the range of normalized values was between 0 and 100 [17].

Figure 7 shows the EQ scores during the SOT2M and SOT5M conditions on BDC-1 and on R+0 for the 3 subject groups. A significant pre- to post-HDT difference in EQ scores was detected for both SOT conditions (Wilcoxon Signed Rank, $p < 0.01$). Similar effects were seen with the other SOT conditions. Half of subjects experienced falls on R+0 on SOT5M. Nevertheless, no statistically significant differences were detected between the iAG, cAG, and Ctrl groups (Mann-Whitney U tests).

[Fig. 7 about here]

Highly significant differences were detected in the MCT measures obtained before and after HDT for the 3 subject groups. The time-to-stability during the forward and backward perturbations increased from 1.78 ± 0.1 s (mean ± SEM) before HDT to 2.78 ± 0.3 s on R+0 (Wilcoxon Signed Rank, $p = 0.001$). Likewise, the sway path length increased from 15.75 ± 1.6 cm before HDT to 22.69 ± 3.0 cm on R+0 (Wilcoxon Signed Rank, $p = 0.03$). According to independent samples Mann-Whitney U-tests, the MCT measures were not significantly different between the 3 subject groups.

3.5. Biochemistry and nutritional status

Blood and urine samples were obtained from the AGBRESA subjects before (BR-3) and after HDT (R+0), from which 179 parameters were analyzed [18]. Data were evaluated to determine

whether daily exposure to AG delivered by supine centrifugation mitigated potential HDT-induced changes in general chemistry and levels of vitamins, minerals, and bone markers. Because men and women differ in the normal concentration of some of these biochemical markers, sex was included as a covariate in the statistical models. Regression analyses were completed for all biochemistry data using linear mixed-effects models. Three models were considered for each measure: BR phase + sex (ignoring countermeasure); BR phase + countermeasure + sex (main effects only); and BR phase x countermeasure + sex (interaction model).

No major differences were detected in the dietary intake of the Ctrl, cAG, and iAG groups. However, the percent of carbohydrates relative to the total energy and the triglycerides level significantly increased in the cAG and iAG groups (Table 2). The testosterone levels also increased in the iAG and cAG groups after HDT compared to pre-HDT. Blood pH decreased in control subjects after HDT compared to pre-HDT, but blood pH did not change during HDT in both the iAG and cAG groups. Protein total increased after HDT in the Ctrl group, but not in the iAG and cAG groups. After HDT, albumin in the Ctrl group increased in male subjects and decreased in female subjects, but no changes were seen in the iAG and cAG groups. In the iAG group, triglyceride decreased after HDT in females and increased in males. The opposite was observed for the cAG group: post-HDT triglyceride concentration increased in females and decreased in males. Urine sodium decreased after HDT in the Ctrl group, but not in the iAG and cAG groups. Glucose concentration increased after HDT in Ctrl and iAG groups, but not in the cAG group. After HDT, vitamin B12 concentration increased in the Ctrl group for both male and female subjects. However, in the iAG group, vitamin B12 concentration increased in females and decreased in males. Bilirubin and creatine kinase decreased after HDT compared to pre-HDT in all 3 subject groups. (Table 2).

[Table 2 about here]

Calcium, vitamin D, and markers of bone turnover expressed as a percent change from before to after HDT during the AGBRESA study are shown in Figure 8. No differences were detected between the iAG, cAG, and Ctrl groups.

[Fig. 8 about here]

3.6. Immunology and hematology

Blood samples were obtained from the AGBRESA subjects before (BDC-3) and after HDT (R+0). Forty immune parameters were measured including leukocyte subsets, red cells, plasma cytokines, plasma antibodies, stress hormones, and viral antibodies [19]. Regression analyses using linear mixed-models considering BR phase, countermeasure, and sex indicated a significant decrease in the number of monocytes, and increased concentrations of alpha2-, beta-, and gamma-globulin after HDT compared to before HDT in the Ctrl group (Table 3). The subjects in the iAG and cAG groups did not show changes in monocytes and alpha2-globulin post-HDT relative to pre-HDT. The beta globulin concentration did not change after HDT in the iAG group, whereas the gamma globulin concentration did not change after HDT in the cAG group (Table 3).

[Table 3 about here]

3.7. Psychological state

Two tests were used to monitor the subjects' psychological health throughout the BR phases: the positive and negative affect scale (PANAS) and a general health questionnaire (GHQ). PANAS is a 20-item self-evaluation questionnaire that measures affect, or indicators, of emotional states. The PANAS separately assesses positive and negative affect. Positive affect reflects the extent to which a person feels enthusiastic, active, and alert. Negative affect reflects the degree to which a person feels subjective distress and unpleasant engagement. The

GHQ is a self-evaluation questionnaire measuring recent behavioral health states for 4 dimensions: anxiety and insomnia (somatic symptoms), social role dysfunction, and symptoms of severe depression.

All PANAS and GHQ scales were normalized to a 0-100% scale for analysis. Forty-eight percent of responses to item 28 of the GHQ were missing, likely due to cultural variation in the acceptability of answering questions surrounding suicidal ideation among international participants [20]. Therefore, item 28 of the GHQ was excluded from analysis, reducing the number of total GHQ items to 27 and reducing the severe depression subscale from 7 items to 6.

PANAS and GHQ questionnaires were administered on BDC-10 and BDC-4, weekly during HDT, and then on R+2 and R+10. Linear mixed-model analysis was employed to determine the fixed effects of countermeasure group and timepoint on PANAS and GHQ scales. Bonferroni-corrected paired *t*-tests were applied for *post-hoc* analyses. Statistical significance for all analyses was set at $\alpha = 0.05$.

For PANAS, overall, positive affect scores were higher than negative affect scores, with mean levels of 56.5% and 9.56% of scale maximum, respectively, for all timepoints (Fig. 9). Linear mixed model for positive affect indicated a significant effect of BR phase ($p < 0.001$). Post-hoc paired-sample *t*-tests revealed lower levels of positive affect post-HDT compared to pre-HDT (estimated marginal means < 32.4% of scale maximum vs. 35.7% at BDC-10). Although statistically significant, the absolute difference from before HDT is minimal. No statistically significant positive affect changes were detected between the countermeasure groups ($p = 0.13$). No significant changes in negative affect were observed between subject groups ($p = 0.53$) nor a main effect of time ($p = 0.15$).

[Fig. 9 about here]

Overall, scores on the GHQ scales were low to moderate for all BR phases: mean levels of severe depression were 5.5% of scale maximum, anxiety and insomnia were 16.8%, somatic problems were 27.8%, and social dysfunction (e.g., role functioning interference) were 54.9%. Mixed models for the somatic ($p < 0.001$), severe depression ($p = 0.03$), and social dysfunction scales (e.g., interference to productivity) ($p < 0.001$) all indicated a significant main effect of BR phase (Fig. 10). Post-hoc paired-sample *t*-tests revealed higher levels of somatic symptoms during and after HDT compared to levels before HDT (estimated marginal means = 16.4% and 15.4% of scale maximum at BDC-10 and BDC-4 respectively vs. ≥ 28.2% throughout HDT and after HDT). Subjects self-reported higher levels of social dysfunction during middle and end of HDT and at R+10 than they did before HDT (estimated marginal means ≥ 55.7% of scale maximum vs. 46.9% at BDC-4). Severe depression scores were higher at the conclusion of HDT (HDT56 estimated marginal mean = 7.64%) than baseline scores (BDC-10 estimated marginal mean = 4.17%). Similar changes in PANAS and GHQ responses were previously observed after the VAPER 30-day BR study with elevated ambient CO2 [12].

[Fig. 10 about here]

3.8. Ocular changes

3.8.1. Intraocular pressure

Intraocular Pressure (IOP) is the fluid pressure of the aqueous humor inside the anterior chamber of the eye. Tonometry was performed on the right eye (OD) and left eye (OS) using a commercial tonometer (iCare PRO, Nava Ophthalmic, Glendale, USA)) on BDC-3 and on R+0 with subjects in the seated posture. The operator stabilized the subject's head and activated the device for it to gently tape the tonometer tip to the clear surface of the open eye directly over pupil to obtain the measurement. Each measurement consisted of 6 successive taps and the tonometer automatically eliminated the highest and lowest values and reported the mean

of the remaining 4 values; this constituted one measurement. Three good measurements that fell within a range of 1.5 mmHg were collected on both the OD and the OS.

Baseline IOP on BDC-3 was 14.2 ± 1.7 mmHg OD and 13.5 ± 1.7 mmHg OS for the Ctrl group; 15.5 ± 2.1 mmHg OD and 15.0 ± 1.7 mmHg OS for the cAG group; and 14.7 ± 1.5 mmHg OD and 14.0 ± 1.8 mmHg OS for the iAG group. No significant pre- to post-HDT change was detected in IOP in either eye for any subject group (cAG, iAG, Ctrl). For the Ctrl group, IOP increased an average of 1.5 mmHg OS after HDT. However, the repeatability coefficient for the iCare PRO instrument is approximately 3 mmHg [21], suggesting this was not a physiologically meaningful or real change.

3.8.2. Optic nerve diameter

Ocular images were obtained with the ultrasound probe in the axial position. To acquire the optic nerve and nerve sheath, the subject was instructed to adjust their gaze upward to move the lens of the eye out of the path of the sound wave, removing the interference the lens could cause. The diameter of the optic nerve and nerve sheath (ONSD) was measured on BDC-3 and R+0. ONSD was measured 3 mm behind the globe using axial scans, with the standard technique for this measure that has the highest correlation with intracranial pressure values. Baseline ONSD was 6.1 ± 0.5, 6.4 ± 0.6, and 6.2 ± 0.6 mm for Ctrl, cAG, and iAG groups, respectively; there was no consistent HDT-induced change in ONSD.

3.8.3. Retinal thickness

Optical coherence tomography (OCT) was performed on BDC-3 and R+0 to measure changes in retina anatomical using a method of quantitative cross-sectional analysis (Spectralis OCT2, Heidelberg Engineering, Heidelberg, Germany). On R+0, ocular tests were scheduled during 2 different sessions that occurred ~6-11 hours after subjects sat up that morning. Subjects were seated and they placed their chin on a chinrest while fixing their gaze on a blue light in the

camera. The camera performed a scan using light in various scan patterns that allow for the measurement of tissue thickness at various locations on the retina and surrounding the optic nerve head. The 3.5-mm diameter circular scan from the anatomic positioning system-based scan patterns was used to quantify circum-papillary retinal nerve fiber layer (RNFL) thickness, total retinal thickness, and choroid thickness. Before HDT, RNFL thicknesses for Ctrl, cAG, and iAG groups were 106.2 ± 9.9, 104.2 ± 10.7, and 103.6 ± 8.1 µm, respectively. Similarly, before HDT total retinal thickness and choroid thickness (quantified from the circle scan) were 329.2 ± 23.6 and 331.1 ± 17.9 for the Ctrl group; 325.8 ± 13.0 µm and 225.3 ± 57.0 for the cAG group; and 200.9 ± 43.0 and 230.4 ± 49.9 µm for the AG group, respectively. After HDT, a significant increase in RNFL thickness and total retinal thickness was detected in all 3 subject-groups, as was a decrease in choroidal thickness (Fig. 11).

[Fig. 11 about here]

The standard *Early Treatment of Diabetic Retinopathy Study* (ETDRS) grid was centered on the block scans over the macula and used to quantify total retinal thickness and volume within a 1 mm diameter circle and in 1-3 mm and 3-6 mm annuli. For the 2 annular regions, these metrics were quantified in each of 4 quadrants: temporal, superior, nasal, inferior surrounding the macula. No significant changes were observed in any region throughout the BR phases. The ETDRS grid placed on the block scans centered on the optic nerve head (ONH) was used to quantify total retinal thickness and volume in an annulus extending 1-3 mm around the ONH [22]. Global retinal thickness before HDT was 378.8 ± 49.3, 370.6 ± 48.8, and 376.7 ± 41.0 µm for Ctrl, cAG, and iAG groups, respectively. Total retinal thickness increased after HDT compared to pre-HDT, but there were no significant differences between Ctrl, cAG, and iAG groups (Fig. 12). Note, the ETDRS region of interest surrounding the optic nerve head used to quantify retinal thickness values reported here is different than that quantified from radial OCT patterns previously reported in this cohort [23].

[Fig. 12 about here]

4. Discussion

4.1. Effects of 60 days of HDT on the control group

Bone Mineral Density. Data from the AGBRESA study can be compared to data from previous BR studies and from spaceflight. Site-specific DXA BMD losses in the AGBRESA controls averaged ~-0.5 to -1.0% per month, which is smaller in magnitude relative to previous HDT bed rest studies [24,25] and to long-duration spaceflight (~-1 to -1.5% per month) [26]. At least 60 days may be the minimum duration of HDT for the optimal evaluation of countermeasures for declines in DXA-measured BMD.

Muscle Strength. The decreases in peak torque of isometric and isokinetic ankle plantar flexion, and knee isometric flexion and extension in the Ctrl subjects during the AGBRESA HDT phase are comparable to those of the control subjects in 70-day [27] and 30-day [12] BR studies. Similarly, the decreases in aerobic capacity for maximal workload, peak ventilation, $\dot{V}CO_2$ peak, and $\dot{V}O_2$ peak agree with those of the control subjects in BR studies lasting 30-70 days [12,27,28].

Orthostatic Intolerance. Orthostatic intolerance is commonly observed when standing immediately after BR [29-32]. Multiple factors contribute to this orthostatic intolerance, including decreased plasma volume, changes in autonomic control, altered systemic vascular and hemodynamic responses, and impaired cerebral vascular autoregulation [31]. The number of subjects who completed 15 min of tilt after HDT in the present study is similar to numbers previously reported in a mixed-sex group of BR subjects [12] and in astronauts after long-duration missions [33,34].

Postural Equilibrium Control. Decreases in equilibrium scores after 30-70 days of BR have previously been reported [35-37]. During HDT, the vestibular system is less challenged because

of the inactivity, and there is negligible stimulation of the proprioceptors in the legs and ankle joints and foot cutaneous mechanoreceptors. Although in strict bed rest, subjects are allowed to move and change position during lying. Except during these movements, somatosensory receptors receive constant input like in free-floating body condition during spaceflight. Consistent with our previous study, greater decrements in the sway-referenced conditions are consistent with down-weighting of proprioceptive feedback and in eyes closed conditions of upweighting of visual feedback [38]. It has also been hypothesized that HDT results in bottom-up modifications in posture and locomotion due to unloading with subsequent postural and locomotor instability [35].

Biochemistry. The differences in biochemistry between the intervention groups were small and likely not clinically significant. We have previously documented nutritional status during BR studies [12,39,40], and the expectation is that the diet should maintain nutritional status. Nonetheless, the data included herein are an important part of understanding broad physiological changes and their biochemical underpinnings.

Ocular Changes. Our results confirm that strict 6˚ HDT bed rest without exposure to elevated CO_2 can produce a significant increase in retinal thickness in healthy test subjects [12]. This increase in retinal thickness after a 60-day BR is similar to the increase previously measured after a 30-day *strict* HDT BR study [22]. It is also greater than the increase seen after a previous 70-day of BR when HDT was not strictly maintained [41] and in astronauts after a 6-month spaceflight [42-44]. This sign of optic disc edema has been attributed to the chronic headward fluid shift. Greenwald et al. [45] hypothesized that the resulting elevated venous pressure in the head may increase capillary filtration in the optic nerve head (a region that lacks blood-brain barrier markers), thereby contributing to the development of optic disc edema. However, in agreement with previous BR studies [22,41], the results of the present study indicate no change in upright-seated measured of IOP after HDT.

4.2. Effectiveness of supine centrifugation

Our results indicate that a daily 30-min exposure to continuous or intermittent supine centrifugation did not prevent the development of optic disc edema. The short-arm centrifuge used in this study generated 1 g at the COM, resulting in only ~0.3 g at the level of the eye. Greater g acceleration at the level of the eye may be needed to reverse the impact of the chronic headward fluid shift. Also, the daily 30-min AG exposure may not have been long enough to allow for a reabsorption of extravascular fluid within the retinal microcirculation at the optic nerve head [23].

We have reviewed the results of 12 studies that used short-radius centrifugation applying 1-2 g at the heart during BR ranging from 4 to 21 days [46]. In these previous studies, supine centrifugation attenuated orthostatic intolerance and reduced alterations in exercise capacity and postural stability after HDT. This is in contrast to the results of the present AGBRESA study. However, in these previous BR studies, the duration of daily AG sessions was typically longer (1.0 ± 0.9 h, mean ± SD). Also, in some studies the subjects performed aerobic exercise, which may have contributed to the mitigation of decreases in exercise capacity and postural instability after HDT. In the AGBRESA study, the duration of daily AG was chosen to match the duration of actual physical exercise performed by crewmembers on board the International Space Station [47] to compare the effects of AG with the effects of traditional countermeasures. While the duration of centrifugation matched the duration of exercise on board the ISS, the forces exerted on the body during centrifugation were different from ISS exercise (i.e., constant forces during centrifugation versus pounding on the bottom of feet while running on the ISS treadmill). These differences might explain the physiological changes observed in the present study compared to those seen in astronauts after 2 months in space. Nevertheless, these daily 30 min of continuous or intermittent AG did not provide an adequate countermeasure to prevent the development of orthostatic intolerance after 60 days of strict 6˚ HDT bed rest. However, interpretation of these results is complicated by apparent pre-HDT differences in tilt tolerance time between the groups.

In the previous BR studies mentioned above [46], supine centrifugation did not prevent immune system deficiency, and the effects on bone loss were inconclusive, presumably because of the limited duration of HDT (4-21 days). The AGBRESA study, on the other hand, with HDT lasting 60 days, demonstrated a protective effect of AG for BMD loss, immunological alterations, and maximal aerobic capacity. Some HDT-induced alterations in biochemical markers were also mitigated by intermittent or continuous AG.

Prior to the AGBRESA study, only one BR study (BRAG-1) had studied the effects of multiple daily AG sessions vs. a single bout of AG [9]. This 5-day HDT study indicated that iAG (6 x 5 min) was more effective than cAG (1 x 30 min), suggesting that the frequency of g transitions was a contributing factor. The AGBRESA ISM results that agree with this observation are the following:

a. iAG was more effective at mitigating the increase in beta globulin relative to no centrifugation (Ctrl), an efficacy that was not observed with cAG. (On the other hand, cAG was more effective than iAG at mitigating the changes in glucose, vitamin B12, and gamma globulin).
b. iAG was more effective for minute ventilation that cAG.
c. The 2 subjects with the greatest increase in total retinal thickness were in the cAG group.
d. Three subjects in the iAG group showed improved orthostatic tolerance after HDT, whereas none of the cAG subjects or Ctrl subjects did (although the difference between groups is not significant).
e. iAG was better tolerated than cAG by the subjects (9 cAG runs were aborted prematurely, compared to 2 iAG runs).

It is possible, however, that the extended period (12 min daily) the iAG subject group spent in the horizontal position (in both the AGBRESA and BRAG-1 studies) contributed to the different effects induced by the iAG and cAG protocols.

Our results also showed that a 30-min daily exposure to centrifugation had no protective effect against loss of muscle strength and decreased aerobic capacity induced by HDT BR. A previous 21-day BR study reported positive effects of 60-min daily centrifugation on physical performance and the cardiovascular system [8]. The 30-min AG protocols during the AGBRESA study were not able to maintain aerobic exercise capacity, probably due to the lower cardiorespiratory demand of this intervention [48].

4.3. Limitations and outlook

Due to the high operational cost and the challenging nature of integrated bed rest studies, the number of participants is kept to the bare minimum required for addressing primary outcomes. The main objective of this study was to evaluate the effectiveness of AG for a potential countermeasure during spaceflight. Implementing a human-rated centrifuge inside a spacecraft has considerable implications in terms of mass, power, and cost. It was considered that if the mitigating effects of the AG regimens tested in this bed rest study were not significant for 8 subjects across all physiological systems, then it would not be worth implementing this protocol during spaceflight. Consequently, the low sample size, in this case 8 subjects per experimental group, may have limited the statistical power to detect significant effects with an alpha level of 0.05. Also, the data suffer from imbalanced sex distribution among the experimental groups, limiting the opportunity to adequately assess potential sex differences in AG efficacy.

Further investigation is needed to optimize the effectiveness of supine centrifugation as a countermeasure for physiological deconditioning during BR. For example, future studies might consider increasing the duration of the AG protocol or the number of centrifuge runs during each iAG session (e.g., 30 x 1 min) and placing the subjects in HDT between runs. Also, using a centrifuge with a larger radius would increase the g acceleration at the head, while decreasing the gravity gradient from head to foot. In addition, the effectiveness of centrifugation would presumably be enhanced if the participants performed some forms of exercise (e.g., cycling or squatting) while rotating, as shown in previous BR studies [5,27]. Higher g acceleration applied

more frequently may induce a greater reversal of fluid shift at the level of the eye to prevent the development of optic disc edema, while exercise may help mitigate orthostatic intolerance and the decreases in muscle strength and postural stability. However, higher g acceleration would need to be balanced with the increased risk of syncope during the countermeasure. Future AG studies should also consider the optimal time of day for applying intermittent centrifugation because studies have shown that exposure to altered gravity levels changed homeostatic parameters [49], which may influence behavioral responses as well.

5. Conclusion

Sixty-day strict head-down bed rest induces significant decrease in bone mineral density, muscle strength, maximal aerobic capacity, orthostatic tolerance, balance performance, biochemistry, immunological alterations, psychological state, and ocular changes. A daily 30-min exposure to 1 Gz supine centrifugation mitigates bone loss in the femur and lumbar spine and lessens the effects of bed rest on aerobic capacity, biochemistry, and immunology.

Data statement

NASA Human Research Program Standard Measures Cross-Cutting Project is the source of the data. The ISM data are stored in the NASA *Life Science Data Archive* (LSDA) and can be requested at the following website: https://lsda.jsc.nasa.gov/StandardMeasures/Home#envihab.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figure Caption

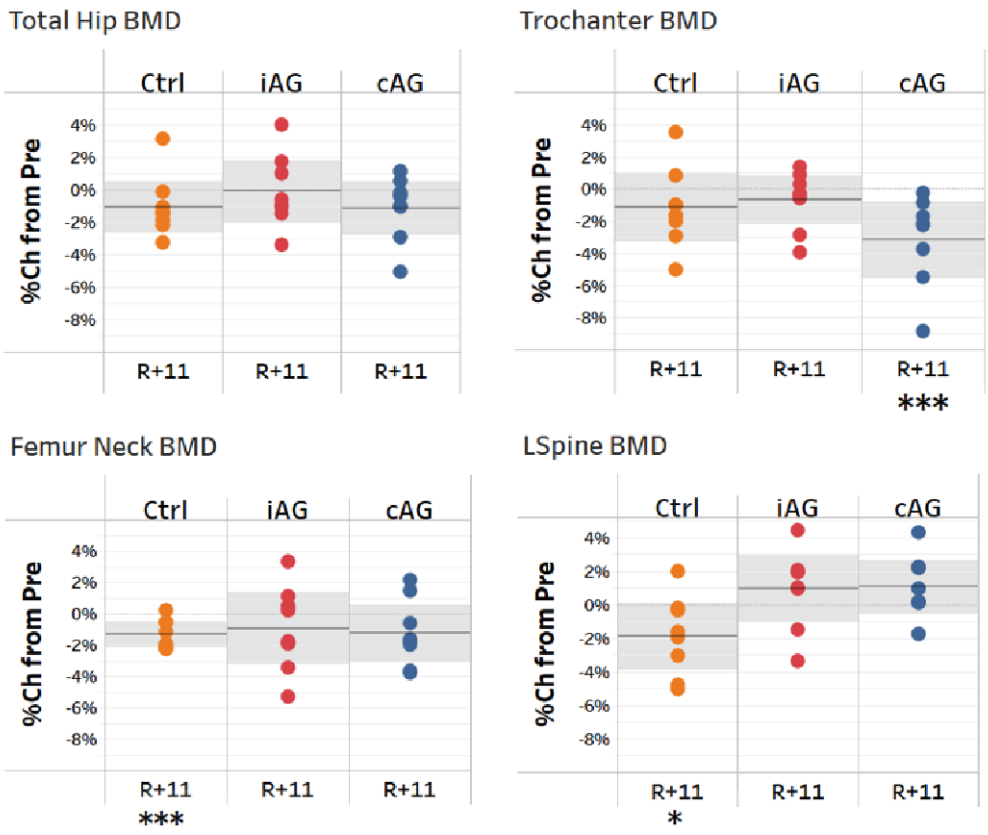


Fig. 1. Regional (total hip, trochanter, femur neck, lumber spine) bone mineral density (BMD) after (R+11) head down tilt (HDT) expressed as a percent change (%Ch) from before HDT in the 3 groups of subjects: Ctrl, control group, no centrifugation; iAG, group subjected to 30 min a day of intermittent bouts of artificial gravity; cAG, group subjected to 30 min of continuous artificial gravity a day. Horizontal bars represent group means; gray shaded boxes represent 95% confidence intervals around the means. Statistics were performed using a linear mixed effects model on absolute BMD values (not percent change). All between-group differences were not significant. For within-group differences (Pre vs. R+11): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

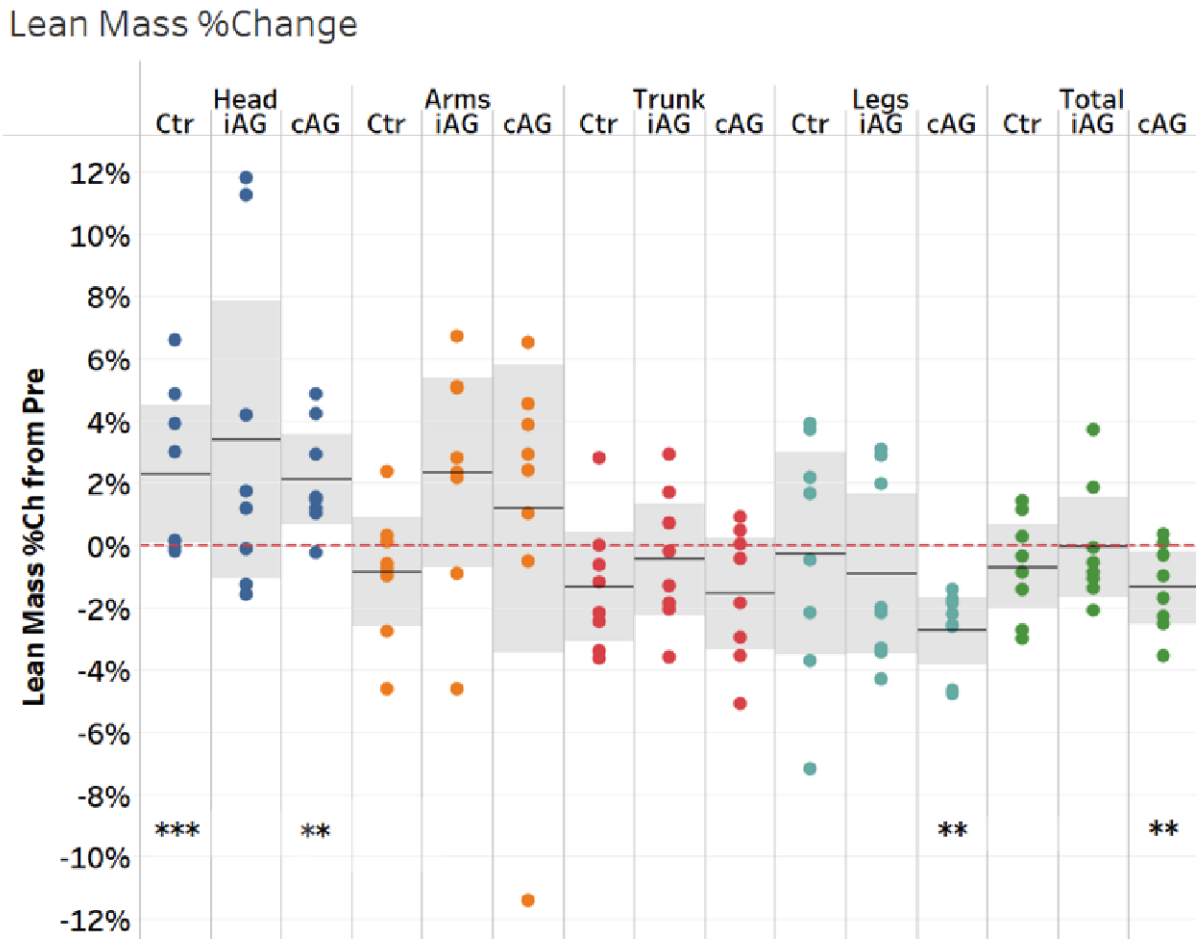


Fig. 2. Percent changes in regional (head, arms, trunk, legs) and total lean body mass acquired from DXA whole body scans before HDT and at R+11 in the 3 subject groups (Ctr, iAG, cAG). Horizontal bars represent group means; gray shaded boxes represent 95% confidence intervals around the means. Statistics were performed using a linear mixed effects model on absolute BMD values (not percent change). All between-group differences were not significant ($p > 0.05$). For within-group differences (Pre vs. R+11): * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

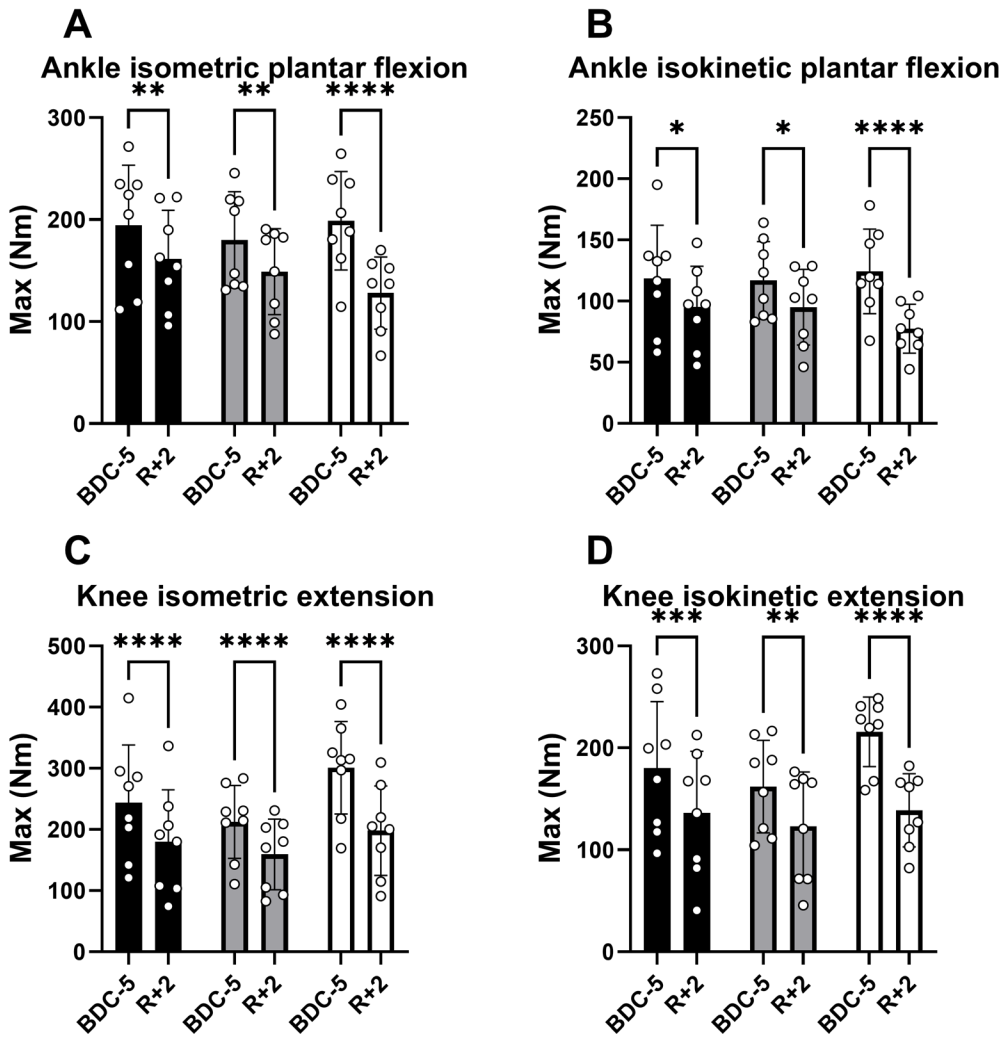


Fig. 3. Mean ± SD of ankle isometric plantar flexion (A), ankle isokinetic ankle plantar flexion (B), knee isometric extension (C), and knee isokinetic extension (D) before (BDC-5) and after (R+2) HDT in 3 groups of subjects: Control (open bars), iAG (grey bars), and cAG (black bars). N=8 per condition. Significant $p < 0.05$, Time x Condition interaction and Main Effect for Time (BDC-5 to R+2) for each. Test differences within each condition: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

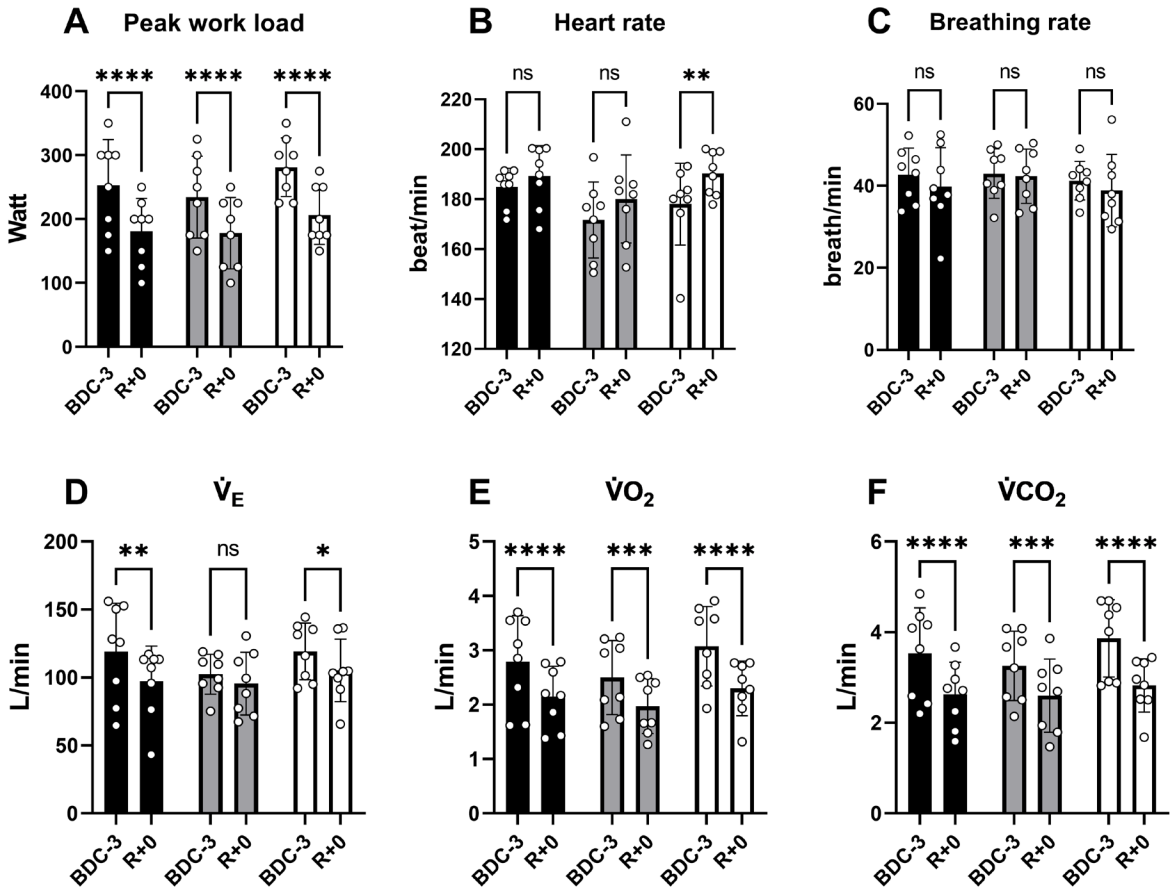


Fig. 4. Mean ± SD of maximal workload (A), heart rate (B), breathing rate (C), minute ventilation ($\dot{V}E$) (D), $\dot{V}O_2$ peak (E), and $\dot{V}CO_2$ peak (F) before (BDC-3) and after (R+0) HDT in the 3 subject groups: Control (open bars), iAG (grey bars), and cAG (black bars). N = 8 per condition. Significant $p < 0.001$, Main Effect for Time (BDC-3 to R+0) for peak workload, heart rate, $\dot{V}E$, $\dot{V}O2$, and $\dot{V}CO2$. Test differences within each condition: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$.

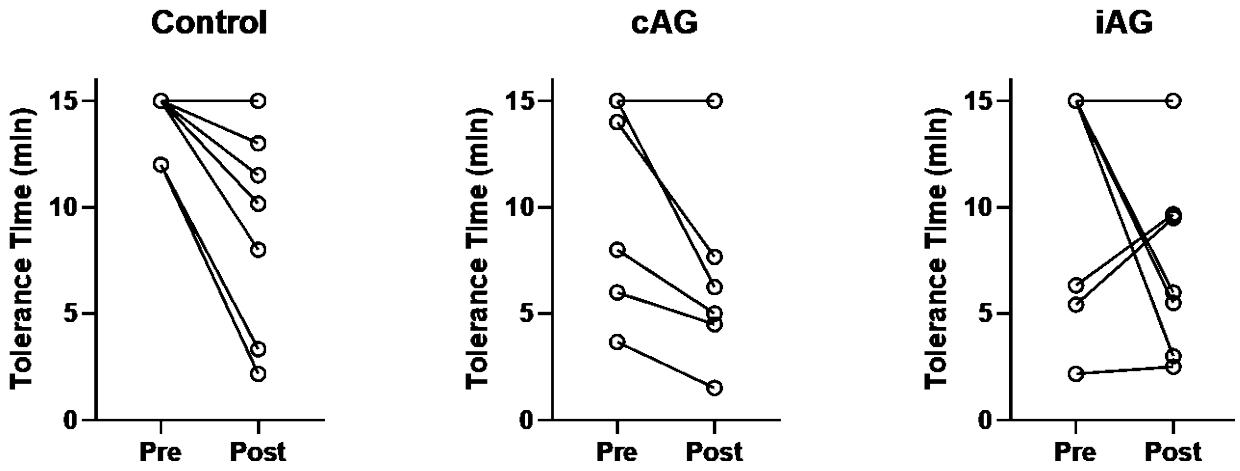


Fig. 5. Tolerance time (min) to 80° head-up tilt during the pre-HDT (Pre) and post-HDT (Post) phases in each of the 3 groups of subjects (Control, cAG, iAG). Each symbol represents results from individual subjects with a solid line connecting pre- and post-HDT tilt test tolerance time. In some cases, it is not possible to see data from subjects who had the same results (e.g., subjects who completed the entire 15-min tilt test before and after HDT). See text for details.

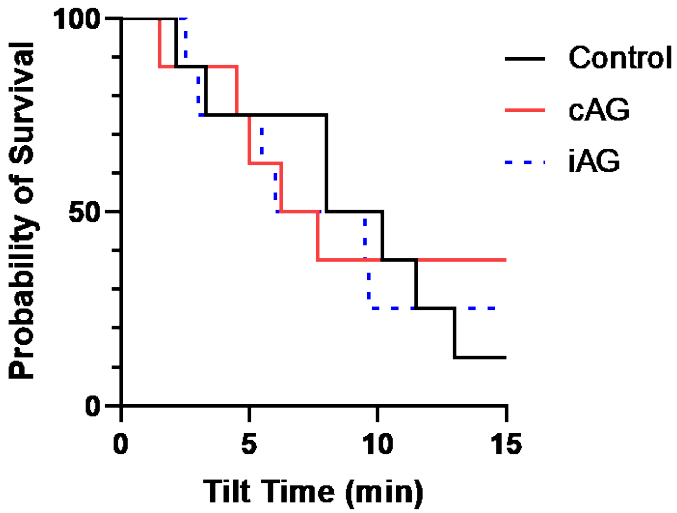


Fig. 6. Post-HDT syncope-free "survival time" during the 80° head-up tilt test for the 3 groups of subjects: Control (black solid line), cAG (red solid line), iAG (blue broken line).

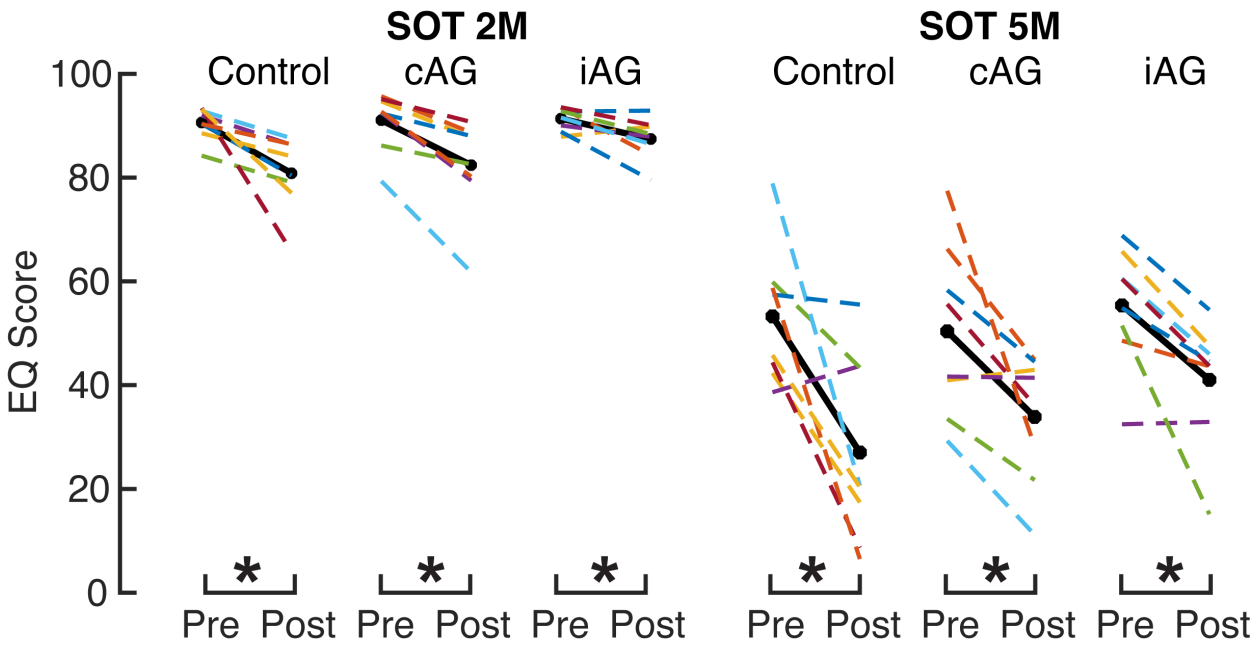


Fig. 7. Equilibrium (EQ) scores during standing posture with the eyes closed and with dynamic head movements on fixed referenced support surface (SOT 2M) and sway-referenced support surface (SOT 5M) in the 3 subject groups (Control, cAG, iAG). The dashed lines are the mean of 3 trials before the HDT (Pre) and 3 HDT trials after HDT (Post) for each of the 8 subjects per group. The black symbols/line represents the mean of the 8 subjects per group. Comparison of Pre vs. Post EQ scores were significant for all subject groups (* $p < 0.01$).

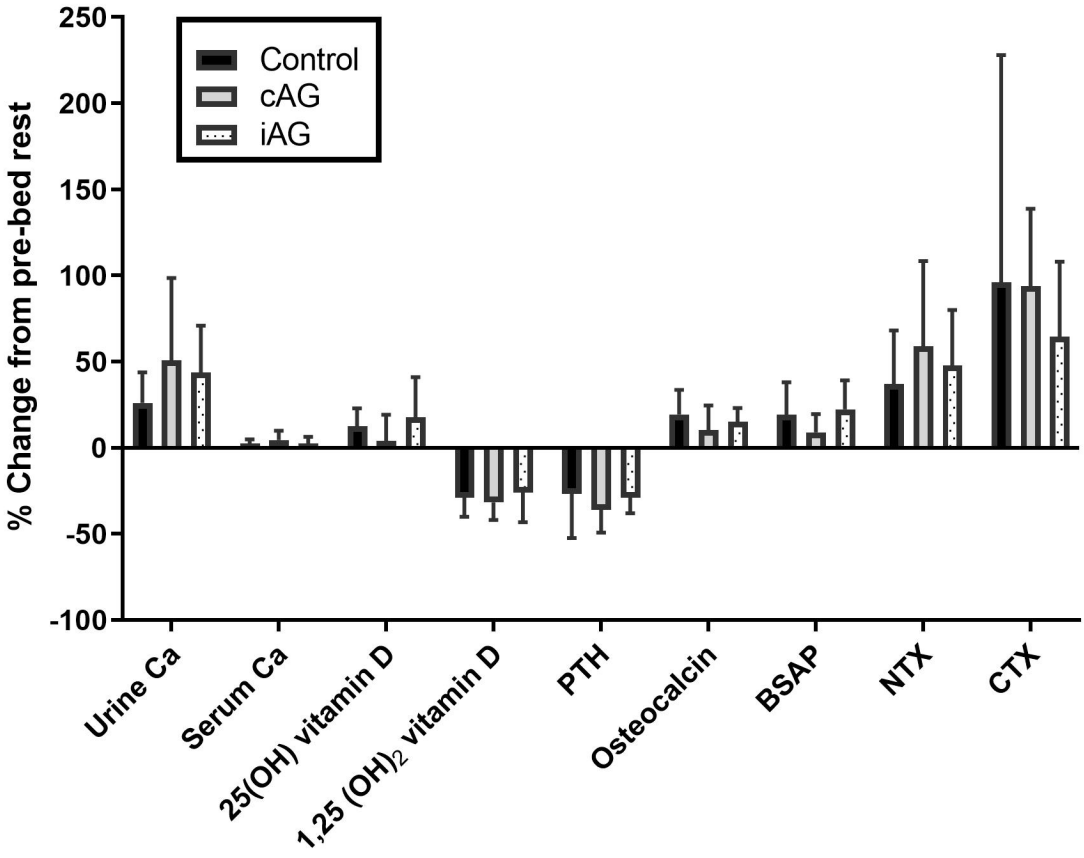


Fig. 8. Calcium, vitamin D, and markers of bone turnover expressed as a percent change from before to after HDT in the 3 subject groups (Control, cAG, iAG). PTH: parathyroid hormone; BSAP: bone-specific alkaline phosphatase; NTX: n-telopeptide; CTX: c-telopeptide. There were no statistical differences between groups.

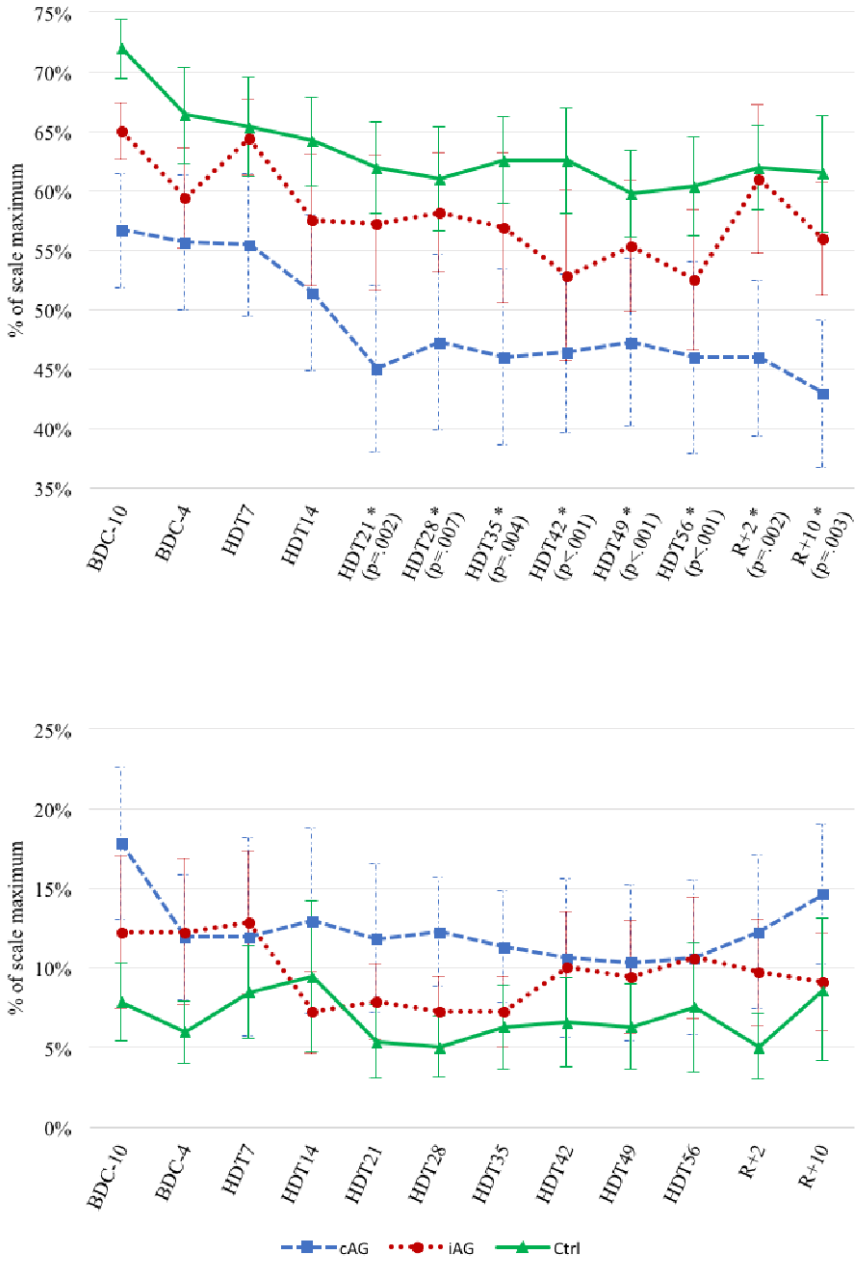
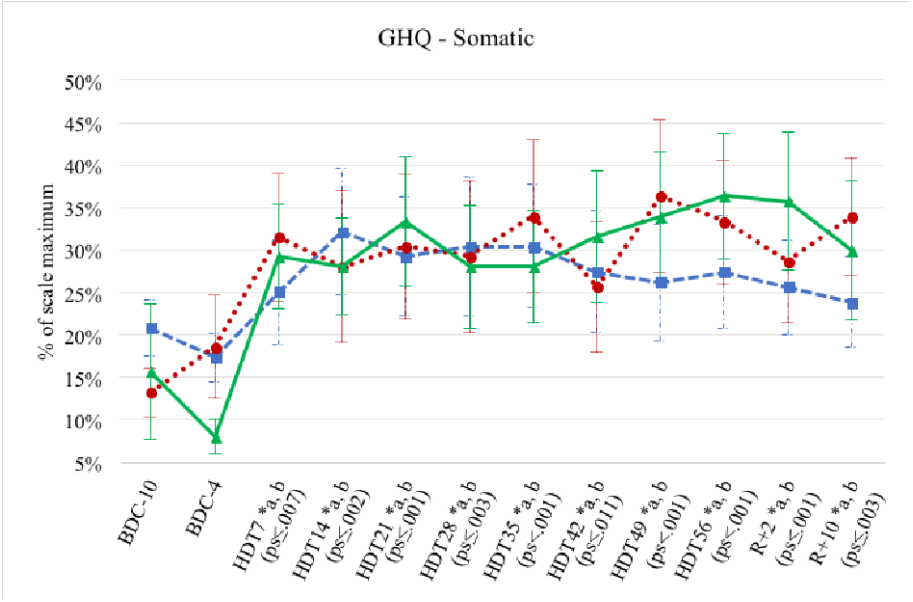
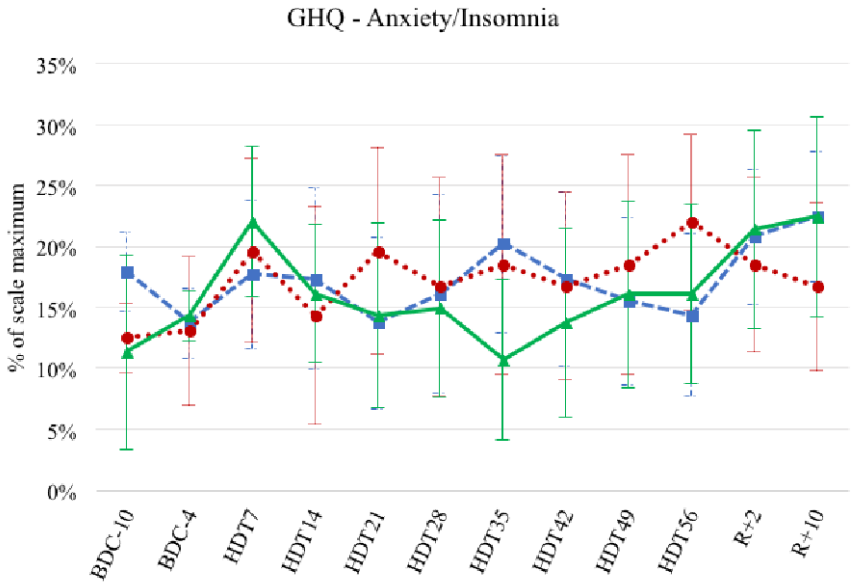


Fig. 9. The positive and negative affect scale (PANAS): positive affect (top) and negative (bottom) affect before (BDC-), during (HDT), and after (R+) head down tilt (HDT) in the 3 subject groups (Ctrl, iAG, cAG). Mean ± SEM. * = statistically significant difference compared to BDC-10 (main effect of time).


GHQ - Somatic
% of scale maximum
50%
45%
40%
35%
30%
25%
20%
15%
10%
5%
BDC-10
BDC-4
HDT7 *a, b (ps≤.007)
HDT14 *a, b (ps≤.002)
HDT21 *a, b (ps≤.001)
HDT28 *a, b (ps≤.003)
HDT35 *a, b (ps<.001)
HDT42 *a, b (ps≤.011)
HDT49 *a, b (ps<.001)
HDT56 *a, b (ps<.001)
R+2 *a, b (ps≤.001)
R+10 *a, b (ps≤.003)


GHQ - Anxiety/Insomnia
% of scale maximum
35%
30%
25%
20%
15%
10%
5%
0%
BDC-10
BDC-4
HDT7
HDT14
HDT21
HDT28
HDT35
HDT42
HDT49
HDT56
R+2
R+10

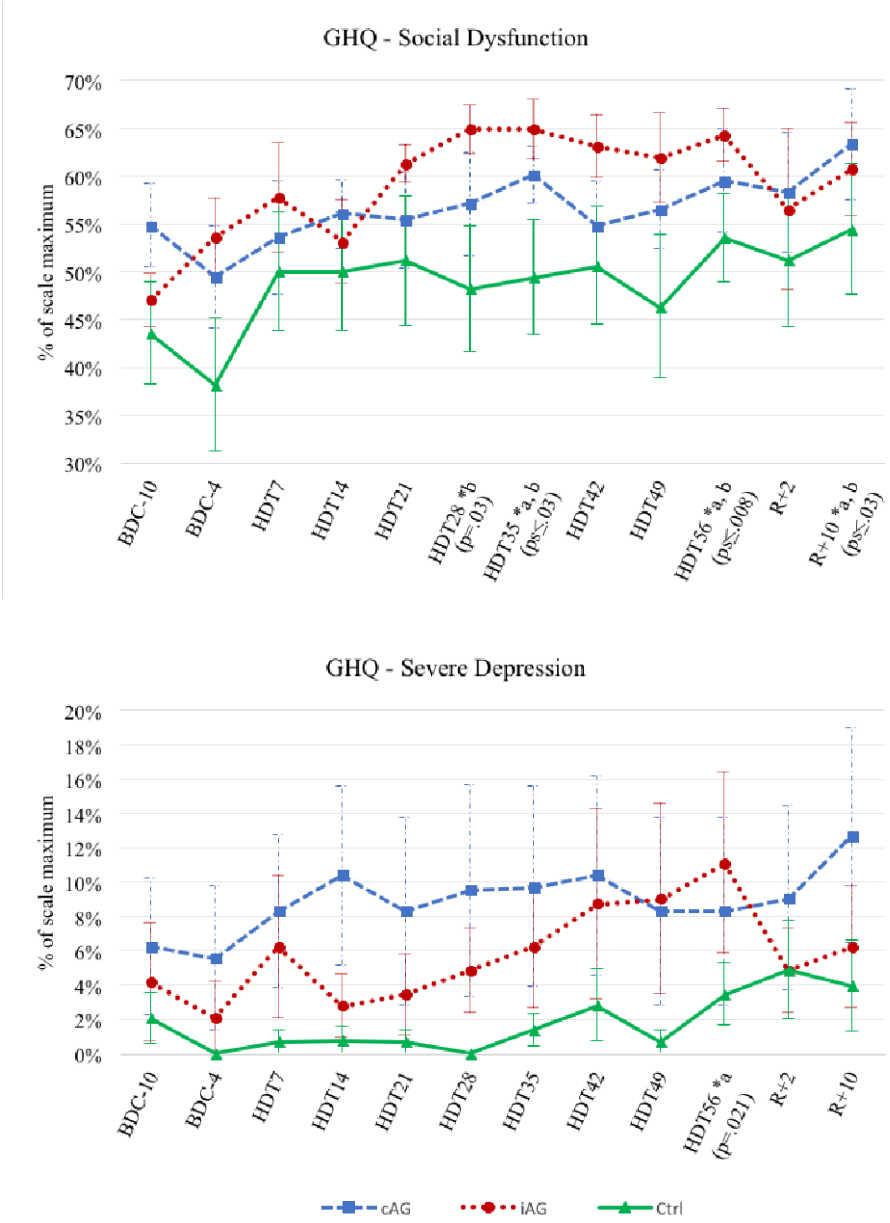


Fig. 10. Levels of somatic, anxiety/insomnia, social dysfunction, and severe depression before (BDC-), during (HDT), and after (R+) head down tilt (HDT) in the 3 subject groups (Ctrl, iAG, cAG). Mean ± SEM. *a = statistically significant difference from BDC-10; *b = statistically significant difference from BDC-4 (main effects of time).

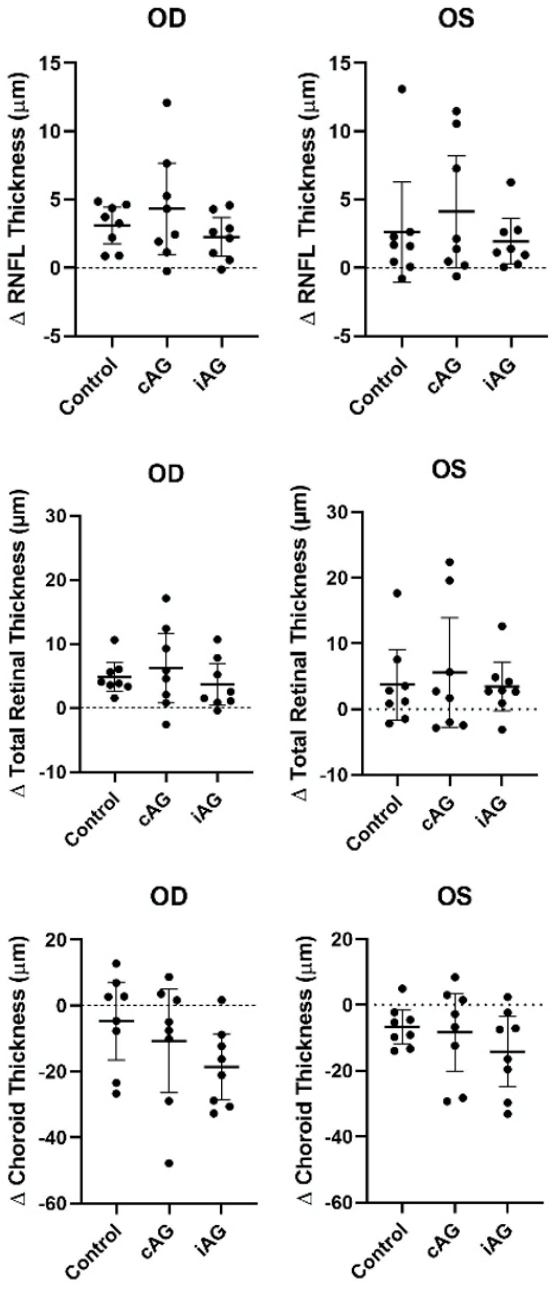


Fig. 11. Head down tilt-induced change in RNFL thickness (top panels), total retinal thickness (middle panels), and choroid thickness (bottom panels) at 1.75 mm from the optic nerve head center for right eye (OD) and left eye (OS) in the 3 subject groups (Control, iAG, cAG). Horizontal bars represent mean and SD of each subject group.

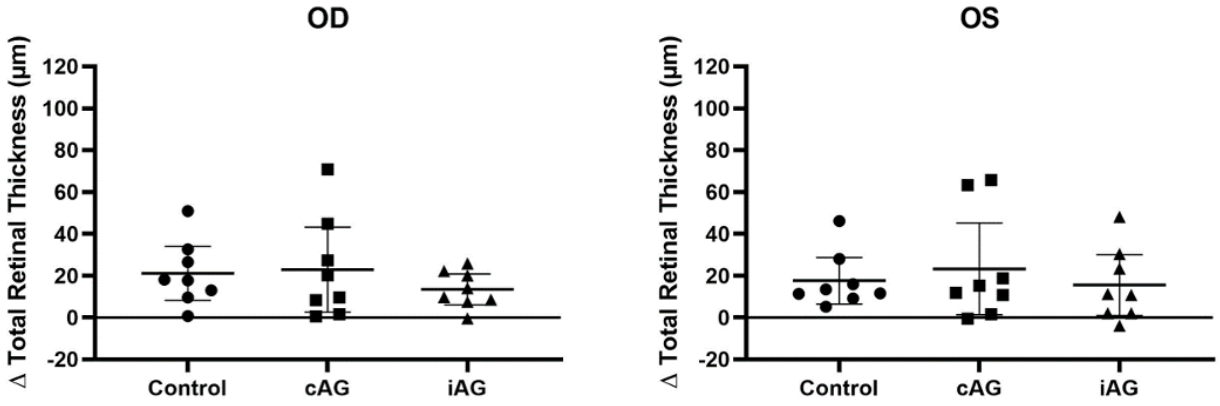


Fig. 12. Head down tilt-induced change in global total retinal thickness 1-3 mm from the center of the optic nerve head in the 3 subject groups (Control, iAG, cAG). Right eye (OD) and left eye (OS).

Table Caption

Table 1. Demographic information of the AGBRESA participants. All measures were recorded upon admission and are presented as Mean (SD). No statistically significant difference was detected for any of the group measures ($p < 0.05$). Ctrl: Control group; cAG: continuous AG; iAG: intermittent AG; BMI: body mass index; m: males, f: females.

	Ctrl	cAG	iAG	Total
N	8 (6 m; 2 f)	8 (5 m; 3 f)	8 (5 m; 3 f)	24 (16 m; 8 f)
Age (years)	34.25 (7.85)	31.88 (9.75)	33.75 (10.78)	33.29 (9.17)
Height (cm)	177.04 (7.27)	172.50 (8.05)	174.10 (10.52)	174.55 (8.55)
Weight (kg)	79.40 (12.67)	71.80 (10.15)	71.40 (4.51)	74.20 (10.03)
BMI (kg/m2)	25.18 (2.58)	24.00 (1.71)	23.64 (1.61)	24.27 (2.04)

Table 2. Head down tilt-induced significant changes in the concentrations of blood and urine components in the 3 subject groups (Ctrl, iAG, cAG). Colors denote significant *p*-values ($p < 0.05$) associated with model 0 (variable: phase + sex), model 1 (variable: phase + treatment + sex), or model 2 (variable: phase x treatment + sex). Mean (SD).

Component	Sex	Pre-HDT			Post-HDT		
		Ctrl	iAG	cAG	Ctrl	iAG	cAG
Albumin (g/dl)	F	3.90 (0.4)	3.49 (0.21)	3.94 (0.52)	**3.88 (0.14)**	3.44 (0.34)	3.92 (0.1)
	M	3.84 (0.21)	3.94 (0.24)	4.08 (0.16)	**4.15 (0.25)**	3.92 (0.27)	4.06 (0.35)
Bilirubin (mg/dl)	F	0.76 (0.12)	0.56 (0.16)	0.89 (0.4)	**0.45 (0.01)**	**0.41 (0.06)**	**0.50 (0.16)**
	M	0.86 (0.22)	0.94 (0.31)	1.26 (0.38)	**0.71 (0.24)**	**0.64 (0.12)**	**1.14 (0.55)**
Blood pH (Finger stick)	F	7.42 (0.01)	7.42 (0.01)	7.42 (0)	**7.41 (0.02)**	7.42 (0.02)	7.42 (0.02)
	M	7.43 (0.03)	7.42 (0.01)	7.41 (0.01)	**7.41 (0.02)**	7.41 (0.01)	7.42 (0.01)
Carbohydrates (% tot energy)	F	50.13 (1.62)	49.93 (1.67)	50.45 (1.07)	50.11 (0.76)	**50.49 (1.96)**	**51.6 (1.23)**
	M	51.20 (1.33)	51.86 (1.36)	51.98 (1.4)	51.49 (1.58)	**52.25 (0.96)**	**52.31 (1.42)**
Creatine Kinase (ul)	F	136.5 (21.6)	206.7 (166)	189.3 (73.1)	**62.5 (9.9)**	**46.1 (6.8)**	**77.5 (38.6)**
	M	187.8 (48.4)	172.4 (67.6)	717.3 (1082)	**107.0 (29.4)**	**72.0 (17.1)**	**99.9 (63.1)**
Glucose (mg/dl)	F	80.12 (5.55)	77.98 (5.11)	82.57 (4.06)	**85.3 (8.13)**	**83.6 (5.63)**	83.7 (3.74)
	M	81.17 (4.77)	76.37 (5.24)	81.09 (3.69)	**84.38 (4.69)**	**80.82 (6.53)**	81.95 (3.83)
Protein Total (g/dl)	F	6.03 (0.88)	5.97 (0.1)	*6.5 (0.61)*	**6.2 (0.14)**	5.9 (0.36)	6.53 (0.1)
	M	6.05 (0.19)	6.28 (0.39)	*6.31 (0.29)*	**6.54 (0.27)**	6.31 (0.41)	6.38 (0.4)
Testosterone (ng/ml)	F	0.29 (0.04)	0.26 (0.08)	0.34 (0.02)	0.36 (0)	0.33 (0.09)	0.36 (0.08)
	M	4.03 (0.75)	5.46 (1.39)	4.84 (0.82)	3.93 (0.7)	**5.87 (0.94)**	**6.03 (1.05)**
Triglyceride (mg/dl)	F	68.2 (24.4)	55.3 (18.4)	58.0 (19.5)	90.2 (39.2)	**44.6 (10.9)**	**60.6 (16.1)**
	M	92.0 (40.0)	60.6 (7.59)	66.4 (23.2)	96.1 (34.9)	**67.7 (12.1)**	**64.4 (20.7)**
Urine Sodium (mmol/g)	F	67.36 (7.36)	66.3 (12.43)	78.43 (28.6)	**28.66 (10.5)**	37.26 (1.77)	41.15 (12.9)
	M	50.36 (10.0)	60.55 (8.78)	53.68 (13.6)	**26.75 (4.05)**	35.03 (8.83)	34.47 (8.08)
Vitamin B12 (ng/l)	F	475 (47)	350 (77)	444 (47)	**571 (102)**	**398 (54)**	449 (62)
	M	424 (70)	365 (40)	419 (73)	**473 (88)**	**350 (112)**	472 (83)

Model 0 Model 1 Model 2

Table 3. Significant immunological changes in blood components post-HDT compared to pre-HDT in the 3 subject groups (Ctrl, iAG, cAG). Colors denote significant *p*-values ($p < 0.05$) associated with model 0 (variable: phase + sex), model 1 (variable: phase + treatment + sex), or model 2 (variable: phase x treatment + sex). Mean (SD).

Component	**Sex**	**Pre-HDT**			**Post-HDT**		
		Ctrl	iAG	cAG	Ctrl	iAG	cAG
Alpha2 Globulin (g/dl)	F	0.50 (0.12)	0.56 (0.05)	0.56 (0.03)	**0.54 (0.07)**	0.53 (0.03)	0.58 (0.02)
	M	0.49 (0.06)	0.53 (0.07)	0.55 (0.08)	**0.52 (0.06)**	0.51 (0.04)	0.57 (0.10)
Beta Globulin (g/dl)	F	0.57 (0.08)	*0.76 (0.09)*	0.71 (0.03)	**0.64 (0.02)**	0.73 (0.05)	**0.74 (0.03)**
	M	0.64 (0.09)	*0.65 (0.05)*	0.60 (0.03)	**0.69 (0.09)**	0.68 (0.03)	**0.63 (0.05)**
Gamma Globulin (g/dl)	F	13.9 (2.05)	15.82 (1.86)	16.52 (1.94)	**14.40 (1.91)**	**16.57 (1.14)**	16.35 (1.89)
	M	14.06 (2.02)	14.83 (1.98)	13.72 (0.95)	**14.43 (1.84)**	**15.62 (2.34)**	13.98 (0.93)
Monocytes (n/ul)	F	11.68 (0.46)	9.88 (1.5)	*8.93 (0.85)*	**9.15 (1.48)**	9.18 (3.25)	9.68 (0.98)
	M	9.93 (1.87)	8.85 (2.24)	*8.02 (1.29)*	**8.94 (1.80)**	8.99 (2.130	8.17 (1.17)

Model 0 Model 1 Model 2