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Locomotion replacement exercise cannot counteract cartilage biomarker response to 5 days of immobilization in healthy adults

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AML (anna-maria.liphardt@uk-erlangen.de) conceived the experiment and was responsible for conception, study design, data collection, analysis and interpretation, and wrote the manuscript. She also takes responsibility for the integrity of the work as a whole, from inception to finished article. AM was involved in study design, data analysis and interpretation. AM and AN critically revised the article for important

intellectual content of the manuscript; SA performed ELISA analysis and contributed to data interpretation; MH and JM contributed to study design and data interpretation. All authors have read, critically revised and approved the final submitted manuscript.

Abstract (250 words): Biomarkers of cartilage metabolism are sensitive to changes in the biological and mechanical environment and can indicate early changes in cartilage homeostasis. The purpose of this study was to determine if a daily locomotion replacement program can serve as a countermeasure for changes in cartilage biomarker serum concentration caused by immobilization. Ten healthy male subjects (mean ± 1 standard deviation, age: 29.4±5.9 years; body mass: 77.7±4.1 kg) participated in the cross-over 5 days bed rest study with three interventions: control (CON), standing (STA) and locomotion replacement training (LRT). Serum samples were taken before, during, and after bed rest. Biomarker concentrations were measured using commercial enzyme-linked immunosorbent assays. Cartilage oligomeric matrix protein (COMP) levels after 24hrs of bed rest decreased independently of the intervention (-16.8 to -9.8%) and continued to decreased until 72hrs of bed rest (minimum, -23.2 to -20.6%). LRT and STA did not affect COMP during bed rests ($p=0.056$) but there was a strong tendency for a slower decrease with LRT (-9.4%) and STA (-11.7%) compared to CON (-16.8%). MMP-3 levels decreased within the first 24hrs of bed rest (CON: -22.3%; STA: -14.7%; LRT: -17%) without intervention effect. Both COMP and MMP-3 levels recovered to baseline levels during the 6 days recovery period. MMP-1, MMP-9 and TNF-alpha levels were not affected by immobilization or intervention. COMP and MMP-3 are mechanosensitive cartilage biomarkers affected by immobilization, and simple interventions such as standing upright or LRT during bed rest cannot prevent these

changes. **Clinical significance: Simple locomotion interventions cannot prevent cartilage biomarker change during bed rest.**

Keywords: Cartilage biomarkers; immobilization; bed rest; cartilage oligomeric matrix protein; cartilage degeneration

Running title: Cartilage biomarkers during bed rest

Introduction

Healthy articular cartilage is a prerequisite for proper joint function, and thus for unrestricted physical activity. In synovial joints articular cartilage provides joint congruency, and transfers and distributes force between articulating surfaces thus facilitating joint motion. Mechano-biological factors cause changes in articular cartilage thickness distributions or cartilage morphology in a joint throughout life .[1] Because of the bradytrophic nature of articular cartilage, joint motion is an important factor for ensuring nutrient supply and metabolite transport.[2] A change in the biological and/or mechanical environment as a result of injury or disease can lead to cartilage degeneration or osteoarthritis, most frequently at the knee.[3] While the role of mechano-biological factors for healthy cartilage development and maintenance has received much attention [4; 5], the effects of immobilization or a reduction in mechanical load on cartilage biology and morphology in humans are largely unknown. Disuse induced changes in cartilage morphology and biology have been reported in previous studies on animals [6-9] as well as patients [10; 11] suggesting that cartilage morphology is sensitive to unloading.

The status of cartilage metabolism can be measured indirectly by biological markers of tissue turnover that can identify individuals at risk of developing osteoarthritis.[12] Moreover, the role of biomarkers is becoming increasingly important in the

diagnostics of diseases and treatment monitoring.[13] Cartilage oligomeric matrix protein (COMP) is one candidate marker that has been extensively researched in this context by our group and others. COMP is a structural protein primarily found in cartilage [14] and contributes to the stabilization of the extracellular matrix through its interaction with collagen fibrils and other matrix components.[15] Changes in serum COMP concentration have been associated with cartilage degradation [16] and may be an indicator of cartilage catabolism because the turnover of COMP fragments may be an important mechanism for regulating tissue synthesis and degradation.[5; 17] Serum COMP concentrations are elevated in patients with knee osteoarthritis [18], rheumatoid arthritis [19], and in response to physical exercise.[14; 17; 20-23] Moreover, immobilization of healthy individuals using bed rest has led to a decrease in serum COMP concentration.[24]

Matrix metalloproteinases (MMP) are a multi-member family of proteases that are involved in the degradation of the cartilage matrix components[25] and represent the main proteolytic enzyme group involved in remodeling the extracellular matrix and modifying cell-cell and cell-matrix interactions.[26] Previous studies have suggested that MMPs play a key role in regulating the balance of structural proteins of the articular cartilage matrix according to local mechanical demands.[27] While collagenase-1 (MMP-1) cleaves collagens such as collagen I, II, III, VII, and X, stromelysin-1 (MMP-3) cleaves a variety of extracellular matrix components, including certain proteoglycans, collagens, and procollagens. MMP-9 belongs to the gelatinases (MMP-2, -9) targeting type IV collagen in basement membrane. MMP serum levels are usually low in healthy individuals and increasing levels have been reported in arthritic patients.[28] Serum concentrations of MMP-1, MMP-3 and MMP-9 are sensitive to loading with increasing values after for example resistive exercise (MMP-1) [29],

walking (MMP-3 only), half-marathon, marathon and ultra-marathon running.[30-32] The expression of MMPs is usually regulated by cytokines such as tumor necrosis factor alpha (TNF-alpha) which can be elevated after exercise.[33] Leong et al. [34] reported an increase in MMP-3 mRNA expression as early as 6 hours after immobilization using hind-limb unloading in rats followed by increased enzyme activity starting after one day of immobilization and both lasting until 21-days of immobilization. This suggests that immobilization may trigger a catabolic gene expression and enzyme activity response early after immobilization and that reduced load and overloading may share a common progression of cartilage degradation.[35; 36]

In humans, investigating the effect of unloading on articular cartilage is difficult especially in healthy individuals, and space flight related models provide a unique possibility to investigate this particular response. For instance, serum concentrations of COMP [24; 37], MMP-3 and MMP-9 [37] are sensitive to immobilization using bed rest. However, although space flight is not only a good model for investigating the effects of immobilization on a healthy human body and associated effects are highly relevant for crew health, to date space flight research has not tackled issues related to cartilage health. This may be due to the shorter crew times during the Space Shuttle missions and the fact, that cartilage degeneration is not visible and cannot be felt by the crew members. In recent years, imaging techniques and availability of biochemical methods for analyzing serum biomarker concentration have advanced. Previous work by our group and others suggests that immobilization can initiate cartilage degeneration [10; 11; 24; 37-42] leading to an increased health risk for human space flight [7] and that current countermeasure regiments may not be capable of addressing cartilage health.[24] Moreover, there is evidence from animal models to suggest that radiation exposure

may enhance the deteriorating effects of immobilization on cartilage health, which needs to be further investigated in humans.[7; 43]

While exercise countermeasures are currently implemented on the International Space Station (ISS) the optimal training regimen has yet to be found. Countermeasure optimization is an important component of successful human space flight with regards to crew health.

Nutritional countermeasures including whey protein and bicarbonate did not affect serum concentration of these cartilage biomarkers.[37] Moreover, a physical exercise intervention during bed rest comprising whole body vibration without additional resistance training could not prevent the immobilization effects on serum COMP during 14 days of bed rest.[24]

The purpose of this study was to determine if a defined locomotion replacement program corresponding to the biomechanical loading of normal locomotion during daily living can serve as a countermeasure for changes in serum concentration of cartilage biomarker caused by immobilization and would be more beneficial than simply standing upright. We tested the hypothesis that (a) a training program replacing normal daily locomotion with vertical jumping and squats of different intensities and duration or (b) a simple standing upright intervention counteracts the effects of 5 days of head down tilt bed rest on cartilage degradation as shown by serum concentrations of COMP, MMP-1, MMP-3, MMP-9 and TNF- alpha.

Methods

Subjects

Ten young healthy male subjects (mean ± 1 standard deviation, age: 29.4 ± 5.9 years (range: 23.4 – 43.5 years); body mass: 77.7 ± 4.1 kg; height: 1.79 ± 0.04 m; all values at the start of the study) were included in the study after medical and psychological screening and randomized to the order of interventions (Level of evidence II). The exclusion criteria were: history of hypertension, diabetes, obesity, rheumatic disease, hyperlipidemia, hepatic disease, or bone disease; physical exercise more than four times per week; smoking, drug consumption; excessive alcohol consumption; normal range of thrombophilia screening panel (AT III, S-Akt, Lupus-PTT, ferritin, Factor V Leiden, Factor IV, and Factor II). All subjects provided their written informed consent prior to participation. This study was approved by the Ärztekammer Nordrhein, Germany, and conducted in accordance with the Declaration of Helsinki. The trial was registered as NCT01820702 at www.clinicaltrials.gov.

Study Design

The ESA (European Space Agency) STBR-AG2 (short-term bed rest artificial gravity 2)-study was performed in a randomized cross-over design with three study campaigns that were completed within a 7 months' time window (Figure 1). Subjects had to participate in all three interventions; the order of the interventions was randomized (see Supplement 1). Study campaigns were divided into three periods: 5-days baseline data collection (BDC-5 to BDC-1), 5-days head down-tilt bed (HDT1 to HDT5) and 6-days recovery (R+0 to R+5). The respective interventions were applied during the HDT period. A physiotherapy session was scheduled on HDT4 of each campaign consisting of passive treatment.

Location and Laboratory Environment

The study was performed at the metabolic ward (Arbeitsmedizinische Simulationsanlage, AMSAN) and the physiology laboratory of the Institute of Aerospace Medicine at the German Aerospace Center (DLR e.V.) in Cologne, Germany. Room temperature ranged from 18.0 to 24.5°C (mean, 22°C).

Interventions

During the HDT period, one of three interventions was applied daily to each of the subjects: locomotion replacement training (LRT), standing intervention (STA) or control intervention (CON). The countermeasure protocols were predefined by ESA and an exercise expert groups consulting ESA regarding the exercise intervention. Countermeasure design was based on previous findings and aimed to find a minimal relevant training load. Interventions were scheduled in the afternoon between 2 pm and 5 pm. All sessions were supervised by an exercise physiologist and a medical doctor.

LRT

For the daily LRT session, subjects were transported in HDT position from the ward to the exercise physiology laboratory. After 5 minutes of supine rest in HDT, subjects sat for 2 minutes on the gurney, then rose to a standing position and executed the LRT program comprising successive exercises (Table 1). A smith machine with fixed rails (PTS-1000 Dual Action Smith ™ Cage, Hoist Fitness Systems, San Diego, USA) was used to guide the heel rise and squat exercises in the vertical axis. A metronome was used to direct the speed of the exercises (except for the static squat). Squats and heel rises were performed against body weight plus the additional weight of a barbell (15 kg). Heel rises were performed with straight knees from a 90° ankle angle to the

maximal plantar flexed position. Slight squats were performed continuously for 3 minutes. Bilateral hops and cross-hopping exercises were performed without the smith machine. Bilateral hops were performed with 15s rests between each set, cross-hopping was performed continuously for 3 minutes at around 1.3 repetitions per second (metronome: 152 bpm). The duration of the static squats increased from 45 s at HDT1 to 70 s at HDT5. Including the scheduled breaks between exercises, the LRT sessions lasted 24 to 25 min.

STA

The STA intervention was performed once daily in the subject's room and each session started with 5 minutes of supine rest in HDT, followed by 2 minutes of sitting on the bedside and 25 minutes of standing next to the bed and concluded with final 5 minutes of supine rest in HDT. Subjects were allowed to quietly talk to the supervising medical doctor but were not allowed to perform any other activity during the session. Instructions on actively minimizing or counteracting normal postural sway were not provided.

CON

During the CON intervention subjects remained in bed and in their rooms and no physical activity was allowed.

Diet

During all three 15-day campaigns of the study, volunteers received a strictly controlled individually tailored diet as specified in the ESA standardization plan. The energy intake (total energy expenditure, TEE) was calculated by multiplying resting metabolic rate (measured by indirect calorimetry with the Delta Trac device, Deltatrac

II MBH 200 metabolic monitor, Datex-Ohmeda) with a physical activity level of 1.4 (for light physical activities) for the intervention phases (LRT and STA) and 1.1 during bed rest alone (CON). 10% of TEE was added for energy expenditure associated with thermogenesis from food and beverages. 29.7 ± 0.2% of the daily energy intake was consumed as fat and 55.2 ± 0.2% as carbohydrates, and subjects consumed 1.19 ± 0.05 g/kg body weight (BW)/d protein. The daily meal content was for calcium 1078 ± 59 mg, potassium 3.9 ± 0.3 g, and sodium 2.3 ± 0.1 mmol/kgBW, and water intake was 49 ± 2.0 mL/kgBW. A dose of 1000 IU/day of Vitamin D3 was administered. Vitamins and elements were controlled to meet the *Recommended Daily Allowances* (RDAs). Intake of caffeine, methylxanthine and alcohol consumption was prohibited. Additional fluid and energy intake was administered as water and apple juice following physically demanding experiments to compensate for fluid and energy loss. All meals were prepared in a metabolic kitchen. Ingredients were weighed with an accuracy of ± 0.1 g using laboratory scales and the nutrient content of each prepared meal was calculated using the PRODI 5.2 software (Kluthe, Prodi 5® expert, [44]).

Blood samples and analysis

Blood samples were collected on the following study days: BDC-3, BDC-1, HDT2, HDT3, HDT5, R+1 and R+4. Blood sampling conditions were identical during all three study phases. During the ambulatory BDC and recovery periods, fasting blood samples were taken in the morning after subjects had rested supine for 30 minutes. During the HDT period, fasting blood samples were taken in the morning. Samples rested for 30 minutes to allow clotting and were then centrifuged at 2000g for 10 minutes and frozen at -80°C until analysis. Serum concentrations of all biomarkers were determined using the following commercially available enzyme-linked

immunosorbent assays (ELISA): COMP – COMP® ELISA, AnaMar Medical AB, Lund, Sweden; MMP-1 – R&D Systems, Human Pro-MMP-1 Quantikine ELISA Kit (# DMP 100) minimum detectable dose: 0.006-0.095 ng/mL; MMP-3 – R&D Systems, Human Total MMP-3 Quantikine ELISA Kit (# DMP 300), minimum detectable dose: 0.002-0.045 ng/mL, intra-assay precision (coefficient of variation, CV%): 5.7-6.4, inter-assay precision (CV%): 7.0-8.6; MMP-9 – R&D Systems, Human MMP-9 Quantikine ELISA Kit (# DMP 900), minimum detectable dose: < 0.156 ng/mL, intra-assay precision (CV%): 1.9-2.9, inter-assay precision (CV%): 7.9-7.9; TNF-α – R&D Systems, Human TNF-alpha Quantikine HS ELISA (#HSTA00D), minimum detectable dose: 0.038-0.191 pg/mL, intra-assay precision (CV%): 3.1-8.7, Inter-Assay precision (CV%): 7.2-10.4. All R&D Kits: Minneapolis, MN, US. All samples of the same person were analyzed in duplicates on the same ELISA plate. ELISA kits for each respective biomarker were from the same lot.

Statistical analysis

Values are presented as means ± 95% confidence interval (CI) if not otherwise stated. Statistical analysis was performed with IBM SPSS Version 19.0 (IBM Corporation, Amonk, IL, USA) using repeated measures ANOVA with intervention and time as within-subject factors. Greenhouse-Geisser correction was applied when necessary. The level of significance was a priori set to $p \leq 0.05$.

Results

The anthropometric data of all subjects for the three study phases are summarized in Table 2. Body mass did not change throughout the study.

Absolute biomarker levels of all analyzed molecules are presented in Table 3. For all biomarkers, baseline concentrations were not different across all campaigns over the 7 months' time interval

($p > 0.05$ for all).

COMP levels were significantly affected by time ($p < 0.001$; effect size 0.787) and decreased significantly with the start of bed rest by -9.8 to -16.8% after 24 h of bed rest during the three interventions (Figure 2, $p < 0.05$; effect size 0.811). Serum COMP levels during the control intervention decreased further until 72 hours of bed rest and then remained stable. All values measured during the bed rest period were significantly lower compared to BDC-1. Both interventions, the locomotion replacement training and the standing intervention did not affect serum COMP kinetics during bed rests ($p=0.056$) but there was a strong tendency for serum COMP to decrease not as rapidly in the initial days of bed rest when subjects received either the locomotion replacement training or the standing intervention as compared to the control intervention (CON: -16.8%; STA: -11.7%; LRT: -9.4%; Figure 2). Serum COMP concentrations reached a minimum of -20.6 to -23.2% at 72 hours after the start of bed rest. On R+1 serum COMP was significantly higher ($p < 0.05$; effect size 0.661) compared to BDC-1 and recovered to baseline levels on R+5.

MMP-3 values of one subject were excluded from the analysis because the baseline values exceeded the normal range for the assay described by the manufacturer (normal mean values: 18.1 ng/ml; measured mean values: 94.6 ng/ml). MMP-3 levels were significantly affected by time ($p = 0.020$; effect size 0.782) and decreased significantly compared to mean BDC values within the first 24h of bed rest during all three interventions (CON: -22.3%; STA: -14.7%; LRT: -17%; Figure 2, $p < 0.001$;

effect size 0.757). Values were significantly lower compared to BDC-1 and stable during all measured time point within the bed rest period. There was no difference in the response to bed rest between the different interventions ($p = 0.365$). MMP-3 serum concentrations recovered to baseline levels when individuals were ambulatory again but serum concentrations show a tendency to decrease again during the recovery period (R+5) with respect to BDC values (CON: -11.6%; STA: -6.4%; LRT: -12.9%).

We did not observe a bed rest or intervention effect in serum levels of MMP-1 (intervention: $p = 0.815$, time: 0.172), MMP-9 (intervention: $p = 0.312$, time: 0.278) and TNF-alpha (intervention: $p = 0.206$, time: 0.195) (Table 3). TNF-α values of four participants were below the detectable range of the assay resulting in complete data sets for only 6 subjects.

Discussion

Healthy articular cartilage is an essential component for unconfined movement, and to date the effect of immobilization on articular cartilage health is not well described. However, for situations were immobilization is inevitable, such as for example in injury or disease, and in an extreme environment as microgravity, a better understanding of the effects of immobilization on articular cartilage and the development of potential countermeasures is important. The purpose of our study was to investigate the effects of a locomotion replacement intervention during 5-days of immobilization in HDT bed rest to counteract the effects of immobilization on serum concentration of biomarkers indicative of cartilage metabolism. The study was successfully conducted with all 10 participating subjects completing all three study campaigns. Body mass was constant throughout the study emphasizing the relevance of a controlled energy balanced diet. Hence, loss of body mass as an important

confounding factor for potential changes in the musculoskeletal system can be excluded from affecting the outcome of the study.

The study confirmed for COMP and showed for MMP-3 that individual resting serum concentration of the biomarkers are well reproducible even over the course of one year (from start of study campaign 1 until completion of study campaign 3) when standardized experimental conditions are applied. The results of this study further confirmed also that serum COMP concentration is strongly affected by immobilization using bed rest shown by a significant decrease 24hrs after the start of the immobilization [24; 37] and that the values decrease further until 72hrs of bed rest. New in this trial was the application of a submaximal exercise intervention with the intention to mimic daily physical activity levels. COMP values had a tendency to decrease more slowly during the standing and locomotion replacement interventions suggesting that even minimally loading the joint in a standing position (STA) or a 25-minute exercise program (LRT) may lead to a release of COMP fragments, which is detectable systemically even around 18hrs after the intervention. This observation highlights the sensitivity of serum COMP levels to mechanical loading and stresses the importance of standardized procedures, such as exercise constraints, when investigating the response of cartilage biomarkers to changes in the mechanical environment. The load applied with the LRT and STA interventions did not show a clear effect on serum marker concentrations.

According to the current understanding, greater serum COMP levels are associated with greater degree of cartilage degradation while lower levels are reported in healthy individuals.[16; 18] Reference values for healthy and diseased individuals are based on fasting blood samples in the morning.[45] Low serum levels may also be caused by a reduced transport of metabolites out of the joint because of the lack of cyclic

loading.[46] Piscoya et al. [47] found in their in vitro study that at load magnitudes above 0.025 MPa more COMP was released into the media with increasing magnitude of mechanical stress. The authors stated that increased COMP in the medium may have resulted from facilitated release in response to dynamic loading or as a result of degradation of COMP. Piscoya et al. [47] doubted that it reflects an increased biosynthesis as the overall matrix biosynthetic rates in their experiments did not increase with increasing stress and concluded that the synthesis and release of COMP in cartilage can be altered by mechanical compression. Thus, the initial drop of serum COMP levels in our study may reflect a reduced release of COMP into the system as a result of the lacking mechanical loading, while further reduction over the bed rest period may reflect a reduced availability of metabolites because of lower cartilage turn-over rates. Furthermore, since nutrient transport in this avascular tissue happens either through diffusion from the synovial fluid [48] or from subchondral bone [46], reduced cyclic loading may reduce nutrient transport into the joint [2; 49] thus also affecting the local tissue metabolism.

We have previously shown similar patterns of serum MMP-3 and COMP kinetics in response to immobilization with bed rest.[37] In that 21-day bed rest study, serum MMP-3 decreased in the first 24 hours after initiation of bed rest by -25% compared to pre bed rest baseline. A protein based nutrition countermeasure did not alter this effect. The present study confirms these results and adds that a daily exercise intervention is not enough to counteract this effect. The observation that both, MMP-3 and COMP respond in similar patterns supports the hypothesis that the reduced serum levels may be a result of slower metabolite transport out of the joint. While reduced serum COMP concentration in response to unloading can be explained with lower tissue turn-over rates in an unloading condition, reduced MMP-3 levels seem counter-

intuitive. As a matrix degrading enzyme, lower MMP-3 levels can mirror a lower tissue turn-over rate that can potentially prevent tissue degradation. But while acutely less tissue degradation may have a positive effect on tissue health, lower cartilage-turnover may also result in higher tissue age because of less exchange of old for new tissue components and thus in deteriorating tissue properties [50]. Previous studies investigating the effects of immobilization on cartilage homeostasis have reported cartilage softening [6; 9], thinning of the articulating surface [6; 11] and a change in tissue composition.[51] In a rat model, Leong et al. [34] found an upregulation of MMP-3 mRNA expression already 6 hours after immobilization and also an increased protein expression. While these observations seem contradictory to the reduced MMP-3 serum concentrations in the current study, it is unclear from our results which processes are initiated at the tissue level.

The observation that MMP-1 and TNF-alpha did not respond to immobilization in this study is in agreement with results of our previous study.[37] It is possible that during immobilization matrix degradation occurs independently of cytokine mediation. In contrast to our previous study where we observed decreased MMP-9 levels after 3 days of bed rest followed by increased values compared to baseline or recovery later during the immobilization period [37], in the current study MMP-9 did not respond systematically to immobilization, which may be a result of shorter trial length (5 days versus 14 or 21 days of HDT). Because of the long response window and low regeneration capacity of articular cartilage, persisting differences after a period of immobilization may become more pronounced several weeks or months after the bed rest intervention.

The main strength of this study is the highly controlled experimental environment with strict boundary conditions. However, the study design also has some limitation.

First, the exercise load applied in this study was rather low as it was designed to mimic the physical activity level performed during daily living and not meant to reflect a typical exercise protocol. The goal of the study was to answer the question whether replacing normal daily physical activity by an exercise session could prevent deteriorative effects in response to immobilization. Higher training volumes with different loading modalities may be required for a countermeasure relevant to cartilage health. While the duration of the LRT intervention in this study was only 24–25 minutes per day, most healthy people are usually mobile for 16 hours per day. To date it is still unclear if longer loading interventions (e.g. several hours/day) simulating daily life more closely would result in different outcomes. Future countermeasure programs should consider the typical frequency of joint loading in daily life. For instance, repetitive cyclic loading is important for preserving cartilage homeostasis [2]. Second, the short follow-up period of only 5 days was rather short, and resting serum concentrations of the mechano-sensitive biomarkers returned to baseline when the subjects were mobile again within the bed rest facility, reflecting a rather sedentary lifestyle. We did not investigate changes in resting serum COMP and MMP3 concentration several weeks after immobilization, which would reflect the chronic effect of immobilization on cartilage biomarker concentration. Finally, this investigation included only a small number of subjects because bed rest studies are very costly experiments. This limitation is partially accounted for by employing a cross-over study design hence reducing the impact of inter-individual differences on the study outcome.

In conclusion, this study supports the finding that serum COMP and MMP-3 are sensitive to immobilization and that values are well reproducible in standardized study conditions. Further, during 5 days of bed rest the locomotion replacement training

(LRT) and the standing control (STA) did not clearly change the effect of immobilization on serum COMP and MMP-3. Hence, the deteriorating effects of immobility on articular cartilage health have to be further investigated and current countermeasure and rehabilitation concepts need to be revised to be potentially effective for articular cartilage health. Nonetheless, this study provides further evidence for the mechanosensitivity of serum COMP and serum MMP-3 and that immobilization may initiate catabolic processes in cartilage tissue in healthy individuals that may even be enhanced by radiation exposure present during space flight. Thus, deterioration of joint health should be considered as a risk factor for long-term space flight, and countermeasure design for the prevention and rehabilitation of musculoskeletal degeneration should incorporate articular cartilage health in addition to skeletal muscle and bone health. Finally, in a clinical context immobilization has to be considered as additional risk factor for cartilage degeneration in injury and disease.

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bed rest study and conducting sample collection. The authors declare no conflict of interest regarding the content of the manuscript.

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Figure legends

Figure 1: Cross-over study design consisting of three campaigns each lasting 16 days. BDC = baseline data collection, HDT = head-down-tilt, R = recovery, CON = control intervention, LRT = locomotion replacement training, STA = standing intervention. Campaign 1-3 = three study campaigns.

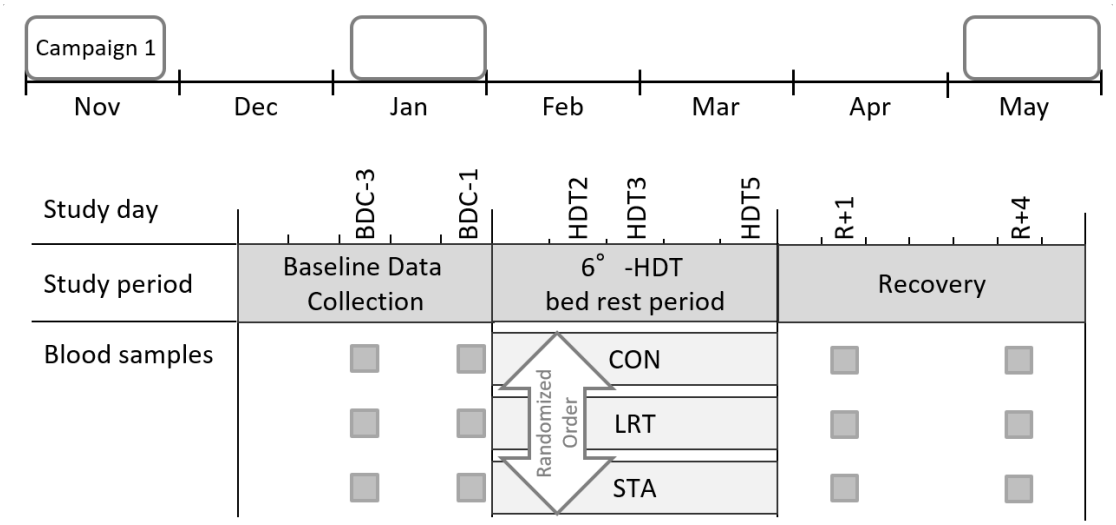

Figure 1: Cross-over study design ESA STBR-AG2 study consisting of three campaigns each lasting 16 days. ESA = European Space Agency, STBR = Short Term Bed Rest, AG2 = Artificial Gravity 2, BDC = baseline data collection, HDT = head-down-tilt, R = recovery, CON = control intervention, LRT = locomotion replacement training, STA = standing intervention. Campaign 1-3 = three study campaigns.

Figure 2: Relative change (%) to mean baseline for **(A)** cartilage oligomeric matrix protein (COMP) and **(B)** Matrix metalloproteinase (MMP)-3 for all time points in the three different interventions. Asterisks (*) indicate a significant ($p \leq 0.05$) difference of the respective time point compared to the mean BDC value. BDC = baseline data collection, HDT = head-down-tilt, R = recovery, CON = control, STA = standing, LRT = locomotion replacement training.

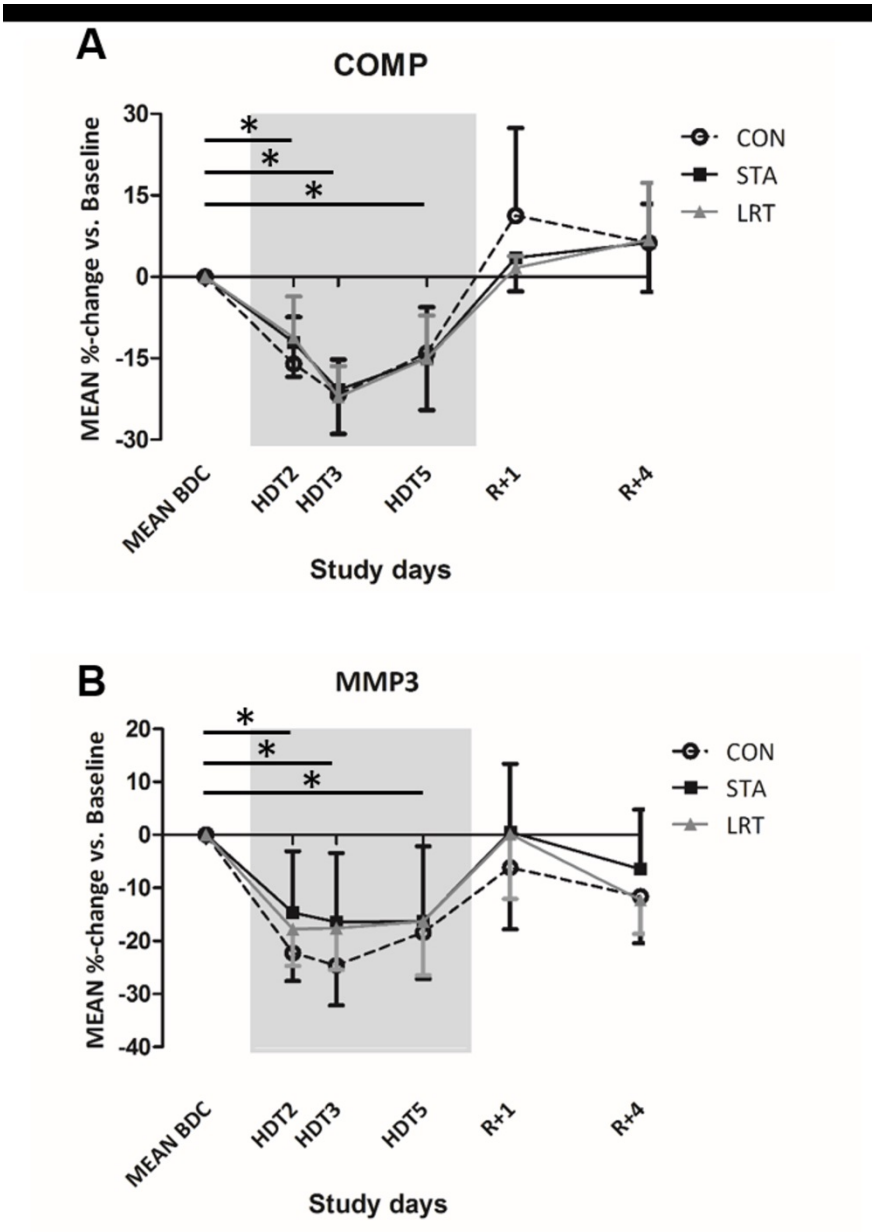

Figure 2

Table 1: Locomotion replacement training scheme

Exercise type	**Repetition rate**	**Repetition**
Bilateral heel raise	1 per 6 s	20
Bilateral squatting (90°):	1 per 6 s	20
Bilateral hopping	3 per s	24 (4 x 6)
60 s pause (standing)		
Unilateral heel raise left	1 per 6 s	12
Unilateral heel raise right	1 per 6 s	12
Bilateral deep squatting (60°):	1 per 6 s	12
Bilateral hopping	3 per s	24 (4 x 6)
60 s pause (standing)		
Unilateral heel raise left	1 per 6 s	12
Unilateral heel raise right	1 per 6 s	12
Bilateral shallow squatting (120°)	1 per 4 s	45
Bilateral cross-hopping	1.3 per s	228
Static squat (90°):	--	1

Table 2: Mean anthropometric data for all subjects (n=10) at the first day of each of the three study phases.

Parameter	Phase 1	Phase 2	Phase 3
	Mean ± SD	Mean ± SD	Mean ± SD
Height [cm]	179 ± 3.7	179 ± 3.7	179 ± 3.7
Body Mass [kg]	77.7 ± 4.1	77.8 ± 5.4	78.4 ± 5.0
Age [yrs]	29.4 ± 5.9	29.6 ± 5.9	29.9 ± 5.9

Table 3: Absolute concentrations (mean ± 95% confidence intervall (CI)) of all biomarkers analyzed in this trial for all study days during the three different interventions (CON = Control, STA = standing, LRT = locomotion replacement training). Repeated measures ANONVA repeated measures ANOVA with intervention and time as within-subject factors. Geisser correction was applied when necessary. The level of significance was a priori set to $p \leq 0.05$. COMP = Cartilage Oligomeric Matrix Protein, MMP = Matrix Metalloproteinase, ng/mL = nanograms / Milliliter, U/L = Units/Litre.

		Intervention						
	Study day	**CON** Mean (95%CI)		**STA** Mean (95%CI)		**LRT** Mean (95%CI)		**Statistical results**
COMP (U/L, N=10)	BDC -3	7.43	(6.42 to 8.44)	7.21	(6.68 to 7.73)	8.04	(7.01 to 9.08)	intervention : p= 0.056 time: p<0.001 *posthoc vs. BDC-1: p < 0.05 intervention *time:
	BDC -1	7.61	(6.65 to 8.56)	7.22	(6.40 to 8.03)	7.70	(6.68 to 8.72)	
	HDT 2*	6.28	(5.49 to 7.07)	6.37	(5.62 to 7.12)	6.98	(6.01 to 8.03)	
	HDT 3*	5.84	(5.20 to 6.48)	5.72	(5.10 to 6.34)	6.11	(5.37 to 6.85)	
	HDT 5*	6.43	(5.64 to 7.22)	6.13	(5.38 to 6.91)	6.69	(5.68 to 7.70)	

	R+1 *	8.40	(7.10 to 9.70)	7.50	(6.67 to 8.33)	8.03	(6.96 to 9.10)	p=0.136
	R+4	7.92	(7.16 to 8.68)	7.71	(6.81 to 8.60)	7.75	(6.64 to 8.86)	
MMP-1 (ng/mL, N=10)	BDC -3	4.03	(2.07.to 5.99)	3.53	(1.55 to 5.51)	3.73	(1.65 to 5.82)	intervention : p=0.815 time: p=0.172 intervention *time: p=0.670
	BDC -1	3.53	(1.59 to 5.47)	3.47	(1.37 to 5.58)	3.51	(1.44 to 5.58)	
	HDT 2	3.56	(1.73 to 5.38)	3.75	(1.30 to 6.21)	3.61	(1.48 to 5.74)	
	HDT 3	3.60	(1.68 to 5.52)	3.52	(1.49 to 5.54)	3.42	(1.57 to 5.27)	
	HDT 5	3.71	(1.87 to 5.56)	3.76	(1.83 to 5.69)	3.54	(1.60 to 5.48)	
	R+1	3.20	(1.56 to 4.84)	3.45	(1.63 to 5.26)	3.29	(1.50 to 5.08)	
	R+4	3.49	(1.69 to 5.29)	3.38	(1.57 to 5.18)	3.37	(1.43 to 5.32)	
MMP-3 (ng/mL, N=9)	BDC -3	13.97	(11.61 to 16.34)	14.07	(10.82 to 17.33)	15.11	(12.81 to 17.41)	intervention : p=0.365 time: p<0.001 *posthoc vs. BDC-1: < 0.05 intervention *time: p=0.255
	BDC -1	14.43	(11.84 to 17.01)	13.71	(10.73 to 16.69)	14.05	(12.09 to 16.02)	
	HDT 2*	11.03	(9.06 to 13.01)	11.42	(9.60 to 13.25)	11.94	(10.16 to 13.72)	
	HDT 3*	10.74	(8.60 to 12.88)	11.24	(9.19 to 13.29)	12.04	(9.97 to 14.11)	
	HDT 5*	11.55	(9.36 to 13.73)	11.21	(9.19 to 13.22)	12.34	(9.87 to 14.80)	
	R+1	13.50	(10.61 to 16.39)	13.69	(11.08 to 16.30)	14.65	(12.06 to 17.25)	
	R+4	12.60	(10.04 to 15.17)	12.75	(10.25 to 15.25)	12.82	(10.69 to 14.95)	
MMP-9 (ng/mL, N=10)	BDC -3	202.12	(159.99 to 244.24)	209.05	(171.82 to 246.27)	224.86	(166.53 to 283.19)	intervention : p=0.312 time: p=0.278
	BDC -1	209.48	(173.84 to 245.12)	220.31	(175.96 to 264.66)	228.06	(175.61 to 280.51)	

	HDT 2	223.30	(168.85 to 277.75)	276.98	(204.25 to 349.70)	241.67	(189.39 to 293.95)	intervention *time: p=0.429
	HDT 3	203.66	(157.82 to 249.51)	226.76	(176.15 to 277.37)	204.77	(160.55 to 248.99)	
	HDT 5	247.87	(176.99 to 318.75)	247.07	(193.90 to 300.24)	226.47	(172.19 to 280.74)	
	R+1	205.41	(164.79 to 246.03)	224.70	(187.18 to 262.21)	268.11	(157.99 to 378.23)	
	R+4	179.22	(148.80 to 209.64)	190.80	(142.62 to 238.98)	200.22	(130.69 to 269.75)	
TNF-α (ng/mL, N=6)	BDC -3	2.03	(1.00 to 3.06)	2.90	(1.19 to 4.61)	2.32	(0.98 to 3.65)	intervention : p=0.206 time: p=0.195 intervention *time: p=0.305
	BDC -1	2.37	(0.86 to 3.88)	3.58	(-.12 to 7.28)	1.95	(0.39 to 3.52)	
	HDT 2	2.22	(1.16 to 3.28)	2.21	(0.92 to 3.50)	1.65	(0.80 to 2.51)	
	HDT 3	1.82	(1.19 to 2.45)	1.68	(1.19 to 2.67)	1.92	(1.03 to 2.81)	
	HDT 5	1.92	(1.30 to 2.55)	1.67	(1.22 to 2.13)	1.57	(1.15 to 2.00)	
	R+1	1.69	(1.25 to 2.23)	1.53	(1.19 to 1.86)	1.28	(0.97 to 1.59)	
	R+4	1.84	(1.20 to 2.48)	1.55	(1.17 to 1.93)	1.33	(0.94 to 1.72)	