BONE LOSS RECOVERY IN MICE FOLLOWING MICROGRAVITY WITH CONCURRENT BONE-COMPARTMENT-SPECIFIC OSTEOCYTE CHARACTERISTICS

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Abstract

Space missions provide the opportunity to investigate the influence of gravity on the dynamic remodelling processes in bone. Mice were examined following space flight and subsequent recovery to determine the effects on bone compartment-specific microstructure and composition. The resulting bone loss following microgravity recovered only in trabecular bone, while in cortical bone the tissue mineral density was restored after only one week on Earth. Detection of TRAP-positive bone surface cells in the trabecular compartment indicated increased resorption following space flight. In cortical bone, a persistent reduced viability of osteocytes suggested an impaired sensitivity to mechanical stresses. A compartment-dependent structural recovery from microgravity-induced bone loss was shown, with a direct osteocytic contribution to persistent low bone volume in the cortical region even after a recovery period. Trabecular recovery was not accompanied by changes in osteocyte characteristics. These post-space-flight findings will contribute to the understanding of compositional changes that compromise bone quality caused by unloading, immobilisation, or disuse.

Keywords: Microgravity, mineralisation, bone compartments, osteocyte, bone remodelling.

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List of Abbreviations

2D	2 dimensional
3D	3 dimensional
aBMD	areal bone mineral density
BMDD	bone mineral density distribution
BPm	bone perimeter
BS	bone surface
BV	bone volume
CaMean	mean calcium content
Cell-S	cellular surface
Ct	cortical
DXA	dual energy x-ray absorptiometry
e	empty
G	on the ground
G_{rec}	on the ground following recovery period
ID	inner diameter
IQR	interquartile range
Lc	lacuna
LCN	lacuno-canalicular network
MCT	mean cortex thickness

N	number
OD	outer diameter
Ot	osteocyte
PMMA	polymethylmethacrylate
qBEI	quantitative backscattered electron imaging
RANKL	receptor activator of nuclear factor kappa-B ligand
S	in space group
S_{rec}	in space group following recovery period
Sp	spacing
Tb	trabecular
Th	thickness
TMD	tissue mineral density
TRAP	tartrate-resistant acid phosphatase
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
TV	tissue volume
V	volume
Wnt	Wingless and Int-1
µCT	micro-computed tomography

Introduction

Missions to space have long been a dream of mankind. However, the burden they impose on the human body is known since the first missions in 1961-63 (Smith *et al.*, 2014). On Earth, a constant gravitational force acts on the human body. In space, microgravity-induced weightlessness causes detrimental effects on all tissues, including the musculoskeletal system. These are caused by the effects of large forces during take-off and landing, radiation exposure, reduced exercise, and limited social interactions, as well as the loss of the light-induced circadian rhythm (Buravkova *et al.*, 2010; Collet *et al.* 1997; Vico *et al.*, 2000). Bone, as an essential biomaterial assuring mobility, protection, and a mineral stock in a healthy adult, *e.g.* a candidate astronaut, is constantly remodelled by the activity of matrix-building and mineralising osteoblasts as well as resorbing osteoclasts. Space missions lead to weight loss, contributed to by a rapid loss of muscle mass and a significantly reduced bone volume (Caillot-Augusseau *et al.*, 1998; Grimm *et al.*, 2016; Stein 2013; Vico *et al.*, 2000). It is important to either prevent such changes, or allow post-mission recovery; however, understanding their mechanistics could aid bone research on Earth, *e.g.* for the treatment of immobilisation-induced bone loss.

The nano-composition of bone is constantly being remodelled and has been shown to be imbalanced, favouring increased bone resorption with unchanged or reduced bone formation and a rapid decrease in bone-matrix-stored minerals during space missions. This is also observed following prolonged periods of immobilisation (Smith *et al.*, 1999). Reduced bone-mineral content during space missions is site-specific, occurring predominantly in load-bearing bones (Orwoll *et al.*, 2013; Vico *et al.*, 2000). This suggests that unloading of skeletal regions to be the main cause of bone mineral loss. Reports on readaptation following unloading or microgravity indicate at least a 2.5× longer recovery period for bone mass, which could be dependent on mission duration, skeletal site, and bone compartment (Hargens and Vico 2016; Leblanc *et al.*, 1990; Orwoll *et al.*, 2013; Rolvien *et al.*, 2020; Smith *et al.*, 1999; Sotnezova *et al.*, 2017).

Residing in the bone matrix, osteocytes – which are terminally differentiated osteoblasts – orchestrate the coupling of bone remodelling. By forming an interconnected fluid-filled network through the LCN, osteocytes allow mechanosensitivity within the dense bone matrix and communication to surface cells. Specifically, simulated microgravity induces osteocyte expression of the Wnt inhibitor sclerostin, repressing bone formation by osteoblasts, and of the RANKL, activating bone-resorbing osteoclasts (Spatz *et al.*, 2015). Additionally, osteocytes directly contribute to mineral homeostasis by osteocytic osteolysis in situations of extreme calcium demand, *e.g.* during lactation (Jähn *et al.* 2017) or vitamin D deficiency (Rolvien *et al.*, 2017). Bone resorption by osteocytes under microgravity is not consistently reported and may depend upon a variety of factors, including which bone compartment is studied (Blaber *et al.*, 2013; Gerbaix *et al.*, 2017). In addition, osteocyte viability and network connectivity are the basis of functional mechano-regulated bone matrix turnover. Osteocyte apoptosis was evident in a murine model of immobilisation using tail suspension (Aguirre *et al.*, 2006) and has been suggested by some, but not all, studies to accompany microgravity-induced bone loss (Blaber *et al.*, 2013; Gerbaix *et al.*, 2017). Recently, lower numbers of osteocyte canaliculi in human Ct-bone from immobilised individuals were reported (Rolvien *et al.*, 2020), suggesting impaired lacuno-canalicular characteristics due to mechanical unloading, which remains to be determined regarding microgravity.

The first studies of the impact of space missions on osteocytes were performed; however, controversial data have been found regarding osteocyte viability and osteocytic osteolysis (Blaber *et al.*, 2013). With Ct-bone being metabolically less active while participating in load bearing and fracture resistance, Tb-bone is more often subject to bone remodelling (Allen and Burr, 2014), suggesting that post space mission differential changes would be expected in these bone compartments. For better preventive strategies during space flight or long-term immobilisation, an understanding of compartment-specific bone responses – including determining osteocyte characteristics – are needed during and following space missions in order to understand the influence of gravitational forces on bone quality.

In this study, the tibiae of mice that went on the Russian BION-M1 mission in 2013 were analysed to determine the effects of a one-month space flight and a 7 d recovery on Earth on Tb- and Ct-bone. Specifically, a multiscale nano-

composite characterisation, including μCT, qBEI, immunohistochemistry, and histology were performed to evaluate compartment-specific changes in bone microstructure, mineralisation and osteocyte viability and activity during and following space missions. Previous studies suggest that, in addition to a reduced amount of bone, microgravity might induce long-term alterations within the bone matrix (Sibonga *et al.*, 2007; Vico *et al.*, 2000). Therefore, the working hypothesis was that a distinct response of Tb- and Ct-bone during recovery – following space flight – might lead to compartment-specific changes in bone quality. Furthermore, compartment-specific negative effects of unloading on osteocyte viability and canalicular connections, impacting osteocyte regulation of bone remodelling following space flight are suggested.

Materials and Methods

Experimental design

Male wild-type C57BL/6N mice were obtained from the animal nursery of the Shemyakina-Ovchinnikov Institute of Bioorganic Chemistry (Moscow, Russia) at the age of 8-9 weeks, with specified pathogen-free status. As pre-adaptation for flight and ground experiments, mice were split into small groups of 3 animals and kept in 1.7 L cylindrical habitats on a 12 h/12 h day/night cycle, mimicking experimental conditions. Two weeks prior to the start of the experiment, the regular diet was changed to a special flight-paste food – based on pelleted food, water and casein – that was provided at a daily dosage of 54 g per 3 mice. The habitats ensured the same climatic conditions for all mice throughout both flight and ground experiments. More detailed information on selection process, housing and feeding conditions are described elsewhere (Andreev-Andrievskiy *et al.*, 2014). For the flight and ground experiments, animal housing groups were randomly divided into 4 experimental groups. Two groups were sent to space on the BION-M1 biosatellite mission for 4 weeks and subsequently sacrificed (S, n = 5) or kept on the ground for 7 d to recover post space mission (S_{rec}, n = 5). A recovery period of 7 d was chosen in order to determine bone-related changes directly following a space flight. This was done to provide new information and create evidence for optimal treatment strategies following space flight. The apposition rate and bone remodelling cycle in 3 months old C57BL/6N mice are documented as 2 μm/d and 2 weeks, respectively (Allen & Burr, 2014; Jilka, 2013). This suggests detection of the first potential bone formation after 7 d of re-ambulation. As controls, 2 groups of mice were housed on the ground for 4 weeks (G, n = 6) and 5 weeks (G_{rec}, n = 6). Over the course of the experiments, a similar locomotive behaviour – with frequent animal aggregations at the habitat walls – was observed in all groups. The differences in bodyweight between space-flight and ground-control groups were not significant (Andreev-Andrievskiy *et al.*, 2014). A bioethical examination of the study protocol was carried out by the Bioethics Commission of the Research Institute of Mitoengineering of Moscow State University (protocol # 35 of November 1, 2012) and the Commission on Biomedical Ethics of the State Scientific Centre of the Russian Federation – Institute of Biomedical Problems of the Russian Academy of Sciences (protocol # 319 of April 4, 2013) (Andreev-Andrievsky *et al.*, 2014). The experiments were carried out in accordance with the European Convention for the Protection of Vertebrate Animals Used for Experiments or for Other Scientific Purposes (Strasbourg, March 18, 1986) and order # 742 of the USSR Ministry of Higher and Secondary Special Education "on approving the rules for work with the use of experimental animals" from 13.11.1984.

Skeletal microstructure

Following the space mission, 9 h post landing, and recovery time, the tibiae of the mice were dissected and frozen in the laboratory of the Institute of Biomedical Problems, Moscow, Russia. All skeletal analyses were performed at the Institute of Osteology and Biomechanics, UKE, Hamburg, Germany. Tibiae were fixed in 3.5 % neutral buffered formalin and scanned using a μCT (μCT 40, Scanco Medical AG, Switzerland) at 55 kV, 145 μA, a voxel size of 10 μm, and an integration time of 200 ms. Three-dimensional reconstruction was evaluated using the software provided by the manufacturer to assess Tb-BV/TV (%), Tb-Th (mm), Tb-N (1/mm), Tb-Sp (mm), and BS/BV (mm). Ct parameters assessed were Ct-Th (mm), Ct-BS/BV (1/mm), and TMD (mgHA/cm^3). μCT image slides were used to determine the OD (mm), ID (mm), and MCT (OD-ID/2, mm) using the interosseous crest as an anatomical landmark to measure the thickness of the cortex on both sides divided by 2 in the medial lateral direction.

In accordance with international guidelines (Bouxsein *et al.*, 2010), the Tb microstructure at 0.5 mm to 1.5 mm distal to the growth plate was assessed, avoiding the primary spongiosa. The Ct microstructure was determined at 1 mm to 1.5 mm proximal to the tibia-fibula fusion landmark.

BMDD

Tibiae were embedded into PMMA and cut with a microtome (Leica Microsystems, Wetzlar, Germany) to obtain 4 μm bone sections for histological assessment (Zimmermann *et al.*, 2015). qBEI using a scanning electron microscope (LEO 435 VP; LEO Electron Microscopy Ltd., Cambridge, UK) with a backscattered electron detector (Type 202; K.E. Developments Ltd., Cambridge, UK) was performed at 20 kV, 680 pA, and with a constant working distance of 20 mm on the longitudinal midsection and proximal spongiosa as previously described (Busse

et al. 2009; Rolvien *et al*. 2019). With a customised MATLAB routine (MATLAB R2014a, MathWorks®, MA, USA), the mean calcium content (CaMean, wt %) in the proximal region for Tb and in the midshaft region for Ct-bone was determined.

Tb-TMD and osteocyte lacunar analysis

Embedded proximal tibiae were further scanned using µCT (Skyscan 1272, Bruker, Kontich, Belgium) at a voxel size of 0.8 µm, 80 kV accelerating voltage, 124 µA tube current, 3,800 ms exposure time, and 0.2 ° rotation step to three-dimensionally characterise both Tb-TMD (mgHA/ccm) by precalibration using hydroxyapatite phantoms and Tb and Ct osteocyte lacunar morphology. The region of interest was defined from 0.5 to 1.5 mm distal to the growth zone to avoid the inclusion of primary spongiosa, and postprocessing was performed as described previously by Hemmatian *et al.* (2017) to determine the lacuna number density (N-Lc/TV) and the mean Lc-V. In detail, a global grey value threshold, beam-hardening correction and ring-artifact reduction was applied to all samples and evaluated automatically using CTAn software (Bruker, Kontich, Belgium). A histogram-based global threshold was applied to segment mineralised from non-mineralised tissue. Images were inverted using the 3D despeckle filter and analysed using Individual Object Analysis

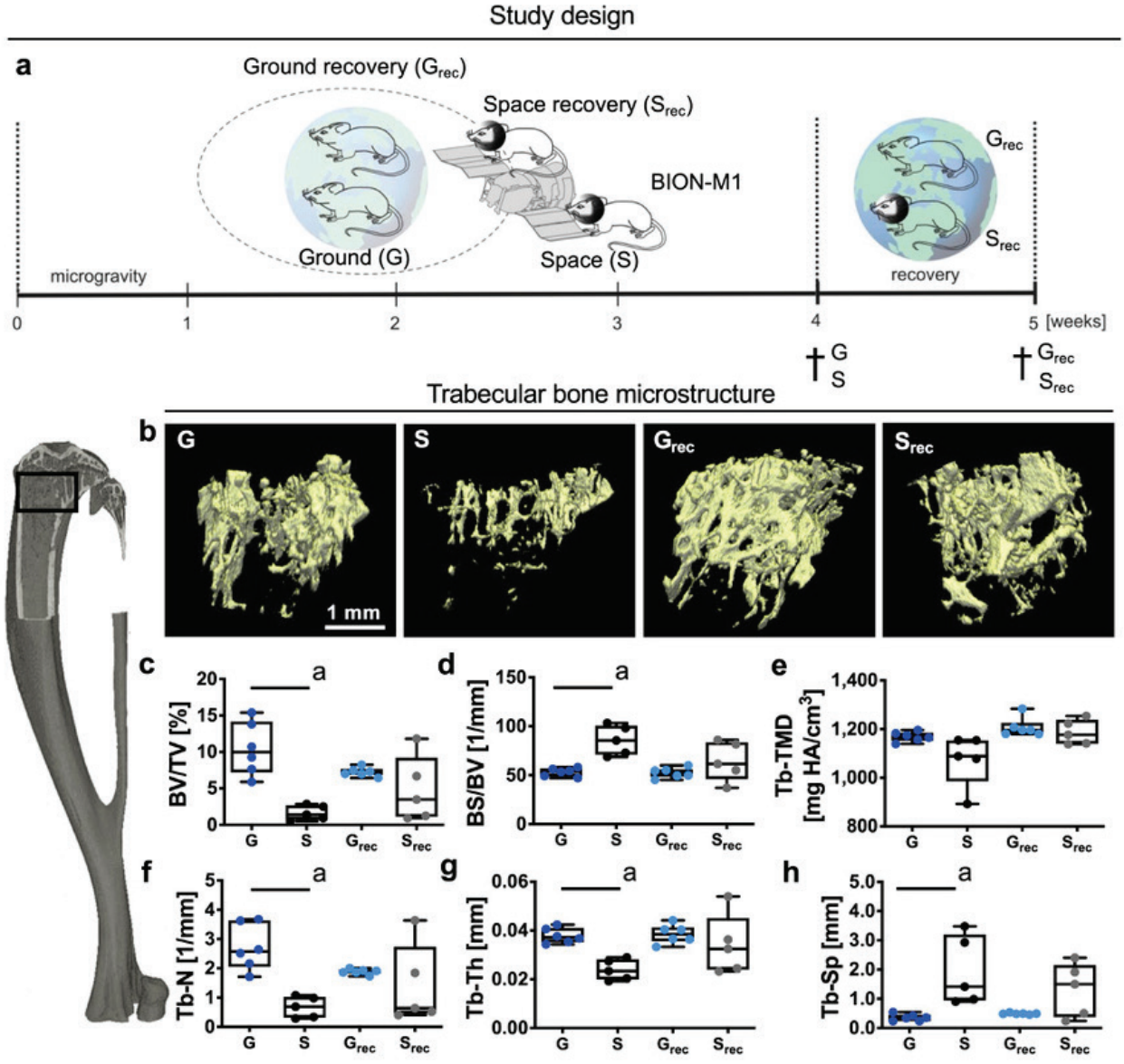

Fig. 1. µCT evaluation of Tb morphology showed reduced bone volume in mice after space flight and 7 d of recovery. (**a**) Study design, analysing mice that were in space for 4 weeks on the BION-M1 mission (S), followed by 1 week of recovery on Earth (S_{rec}), compared to control mice kept on the ground for 4 (G) and 5 weeks (G_{rec}), respectively. (**b**) 3D µCT images of Tb-bone in the proximal tibia analysis revealed (**c**) reduced Tb-BV/TV in S compared to G and (**d**) a higher ratio of bone surface to bone volume (BS/BV) in S than in G. (**e**) While Tb-TMD was similar in all groups, Tb microstructural analysis showed that (**f**) Tb-N and (**g**) Tb-Th were significantly lower and (**h**) Tb-Sp was higher in S than in G. Groups: S, $n = 5$, G, $n = 6$, S_{rec}, $n = 5$, G_{rec}, $n = 6$.

provided by the manufacturer. Objects between 100 and 2,000 μm³ volume were assumed to be osteocyte lacunae, while objects greater than 2,000 μm³ were regarded as vascular canals and objects below 100 μm³ were excluded as noise.

Osteocyte canaliculi evaluation

A modified Ploton's silver nitrate precipitation with thionine contrast was performed on PMMA sections of the distal tibia as previously described (Derkx and Birkenhäger-Frenkel 1995; Jáuregui *et al.*, 2016). Osteocyte canaliculi in Tb- and Ct-bone 1 mm proximal of the growth plate were determined using ImageJ software (NIH, University of Wisconsin, USA), considering canaliculi connected with the lacuna in the plane of view in both the trabecular (Tb-N-Ca/Lc) and cortical (Ct-N-Ca/Lc) regions 10 to 20 osteocyte lacunae were evaluated.

Cellular histomorphometry

To measure osteoclast activity, the number of TRAP-positive cells on the Tb-bone surface, based on TRAP activity, was determined by staining PMMA sections with naphthol AS-MX phosphate (Sigma-Aldrich) and Fast Red Violet LB salt (Sigma-Aldrich) in 40 mmol/L acetate buffer (pH 5.0). The numbers of TRAP-positive cells per bone perimeter ($TRAP^+$Cells/BPm, 1/mm) and TRAP-positive cellular surface per bone surface ($TRAP^+$Cell-S/BS, %) were determined 0.5-1.5 mm below the growth plate in the Tb-bone compartment.

Toluidine blue staining was performed on PMMA-embedded bone sections. Viable osteocytes in the Tb and Ct compartment of the tibia, defined as osteocyte lacunae with stained cell nuclei and osteocyte lacunae without stained cell nuclei, were assessed and presented as the ratio of empty lacunae in relation to total osteocyte lacunae (eLc, %) using the OsteoMeasure system (OsteoMetrics, USA).

Osteocyte apoptosis was assessed by performing a TUNEL assay on the bone sections using a In Situ CellDeathDetection Kit (Roche) according to the manufacturer's instructions. The percentage of TUNEL-positive osteocytes over TUNEL-negative osteocytes (TUNEL+ Ot) in the Ct-bone and Tb-bone was determined

Statistical analysis

All analyses were done in a blinded manner. Collected data are presented as the median ± IQR. Significant differences were determined with the Kruskal-Wallis test and subsequent Dunn's multiple comparison testing (α = 0.05) for the comparisons ground-space, ground-ground recovery, space-space

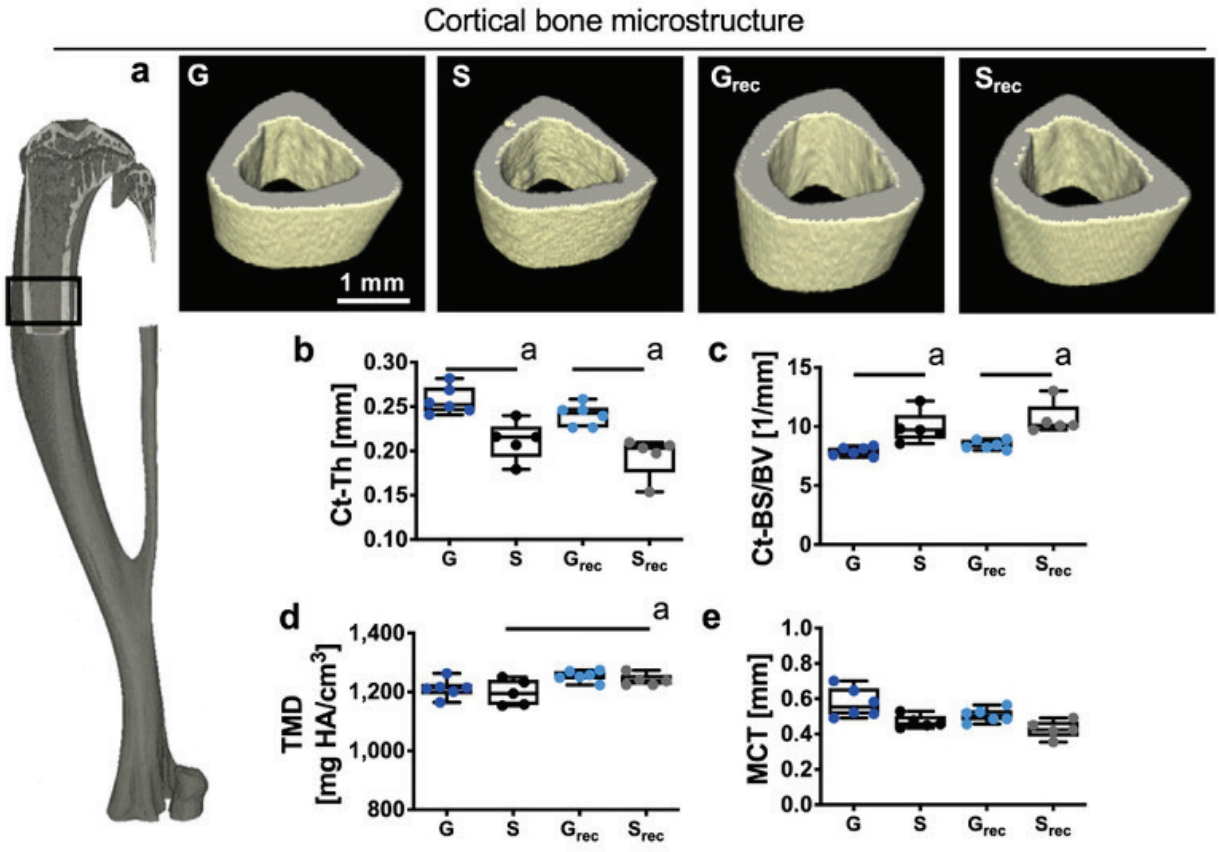

Fig. 2. Lower Ct-Th in space is persistent after subsequent recovery. (**a**) μCT image analysis indicated (**b**) a significantly lower Ct-Th in S than in G, and in S_{rec} than in G_{rec}, along with (**c**) a significantly lower Ct-BS/BV in S than in G, and in S_{rec} than in G_{rec}. (**d**) Ct-TMD was higher in S_{rec} than in S, while (**e**) μCT showed no differences among groups. Groups: S, n = 5, G, n = 6, S_{rec} n = 5, G_{rec} n = 6.

recovery, and ground recovery-space recovery with GraphPad Prism 7 software (GraphPad Software, San Diego, CA).

Results

Tb-bone compartment recovered 7 d post space flight

In this study, bone quality of tibiae from mice that were part of the BION-M1 mission was analysed to specify the effects of a one-month space flight and a 7-day recovery on Earth (Fig. 1**a**). To determine the changes in Tb microstructure following the space mission and recovery time, 3D-reconstructed *μCT* images were analysed (Fig. 1**b**). A significantly lower Tb-BV was determined in the S compared to the G group (Fig. 1**c**, BV/TV, $p = 0.0029$). The bone surface was larger in the S group, suggesting Tb-bone loss by surface removal (Fig. 1**d**, BS/BV, $p = 0.0181$). A loss of Tb-TMD could not be detected in the S group or as a result of recovery on Earth (Fig. 1**e**; TMD). The observed lower Tb-BV/TV in the S group was supported by a significantly lower Tb-N and a lower Tb-Th in S than on the G group (Fig. 1**f**, Tb-N, $p = 0.005$; and Fig. 1**g**, Tb-Th, $p = 0.018$). The resultant Tb separation was significantly higher in the S compared to the G group (Fig. 1**h**, Tb-Sp, $p = 0.004$).

Ct-bone loss did not fully recover post space flight

The 3D reconstruction of the μCT images showed changes in Ct-bone microstructure (Fig. 2**a**). Significantly lower Ct-Th was present in the S group compared to ground (Fig. 2**b**, Ct-Th, $p = 0.025$) and persisted in the S_{rec} group compared to G_{rec} ($p = 0.038$). The loss of bone matrix was further confirmed by larger Ct surface values in the S group compared to the G group (Fig. 2**c**, $p = 0.014$) and in the S_{rec} group compared to the G_{rec} group ($p = 0.050$). Ct material quality in terms of Ct-TMD was significantly elevated upon S_{rec} compared to the S group (Fig. 2**d**, $p = 0.034$). Shape adaptations contributing to Ct-bone loss could not be detected by the calculation of the MCT (Fig. 2**e**, MCT).

Similar Ca contents determined by qBEI

Hydroxyapatite minerals influence the intrinsic toughness and biomechanical properties of the bone matrix. To further investigate the mineralisation degree, qBEI was performed to determine the Ca content of the bone matrix. Representative images of Ct-bone presented with comparable mineralisation (Fig. 3**a**). qBEI confirmed the lack of significant alterations in mean calcium content in Tb- and Ct-bone (Fig. 3**b**,**c**, CaMean).

Higher TRAP-positive cell activity following space flight

The cause of the lower BV/TV in the Tb-bone compartment due to space flight was further analysed by determining TRAP-positive bone-surface cells as a measurement of osteoclast activity (Fig. 4**a**). A significant elevation of TRAP-positive cells per Bpm as well as a larger TRAP-positive cellular surface per BS in space compared to ground were determined (Fig. 4**b**, $p = 0.055$; and Fig. 4**c**, $p = 0.037$). In addition, TRAP-positive osteocytes were seen within the bone matrix (Fig. 4**d**).

Impaired LCN in Ct-bone following space flight

The LCN as a functional entity within the bone matrix is highly dependent on the number individual network characteristics. 3D reconstruction of the Ct shell, including the separation of blood vessel canals and osteocyte lacunae, was performed (Fig. 5**a**).

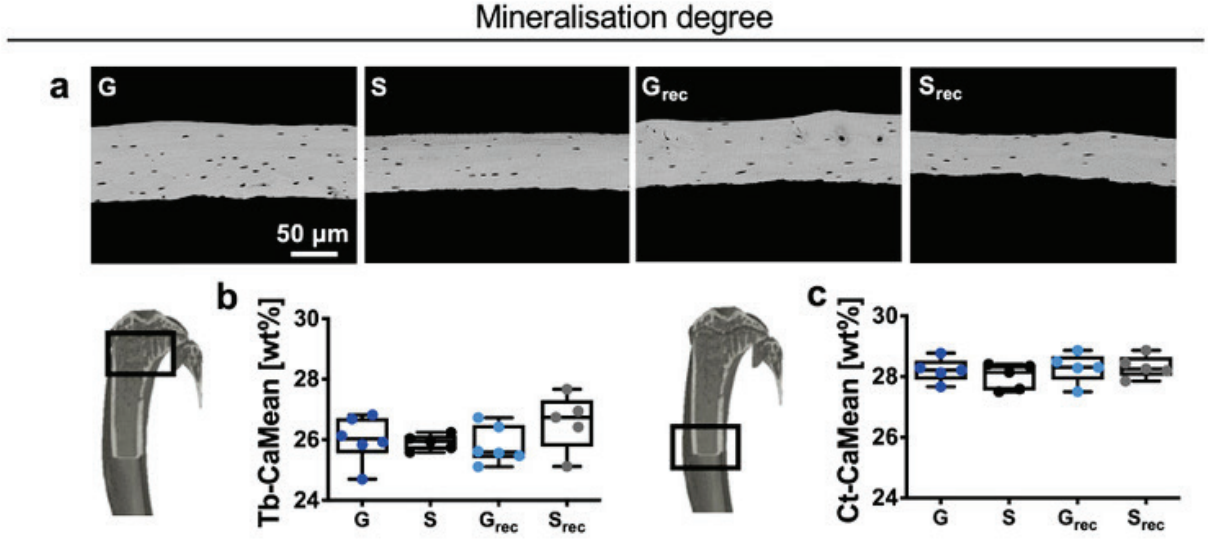

Fig. 3. Bone matrix calcium content was not affected during space flight. (**a**) Representative images of the Ct compartment detected by qBEI. Evaluation showed similar (**b**) CaMean and (**c**) BMDD in Tb- and (**d**,**e**) Ct-bone. Groups: S, $n = 5$, G, $n = 6$, S_{rec}, $n = 5$, G_{rec}, $n = 6$.

In both compartments, the lacunar characteristics were comparable between the groups. Osteocyte lacuna number and mean osteocyte Lc-V were not significantly affected by space flight in Tb- or Ct-bone (Fig. 5**b**,**c**, N-Lc/TV; and Fig. 5**d**,**e**, Lc-V), but also sphericity and orientation of the lacunae was unchanged (data not shown). Therefore, a measurable 3D adaptation of the lacunar characteristics could not be provided, suggesting a small contribution of lacunar remodelling during space flight and recovery. As determinants for mechanotransfer, but also for provision of nutrition and waste product removal, the canaliculi contribute to the LCN connectivity. Canaliculi number in 2D were determined using the Ploton's silver nitrate staining (Fig. 5**f**). While significant changes following the space mission were absent in both Ct- and Tb-bone, a non-significant tendency towards fewer canaliculi per Lc was seen in Ct-bone in the S_{rec} group compared to the G_{rec} group (Fig. 5**g**,**h**, N-Ca/Ot-Lc, Ct: $p = 0.077$).

Cell viability is the primary element to ensure function. Osteocyte viability was determined on histological sections stained with toluidine blue. The percentage of empty osteocyte lacunae in Tb-bone remained unchanged with space flight, while a significantly higher percentage of empty lacunae was determined in the S group compared to the G group (Fig. 5**i**,**j**,**k**, eLc, for Tb: $p = 0.037$). Osteocyte apoptosis determined by a TUNEL assay showed TUNEL+ osteocytes (Fig. 5**l**) with similar results for Ct- and Tb-bone (Fig. 5**m**,**n**).

Discussion

The absence of mechanical loading on bone, as experienced during microgravity in space flight or long-term immobilisation, provokes rapid Ca release, leading to compositional and mechanical changes within the bone matrix. For treatment and prevention techniques as well as a further understanding of load-induced mechanisms in bone, it is essential to characterise these changes. In this study, both bone compartments, Tb and Ct, showed a compromised microstructure immediately following a space mission, while only Tb-bone volume fully recovered by 7 d post space flight. Whereas a reduction in Tb-bone was also observed in previous studies (Gerbaix *et al.*, 2017; Maupin *et al.*, 2019), evidence for its recovery has not yet been presented. Moreover, Ct microstructure is even further compromised following recovery (Gerbaix *et al.*, 2017). A recovery period following a space mission was associated with denser Ct-TMD than when in space, showing the recovery of mineralisation in the Ct compartment, whereas changes in Tb-TMD were absent. Based on the structural recovery solely in the Tb compartment, 2 differential processes are suggested. The results

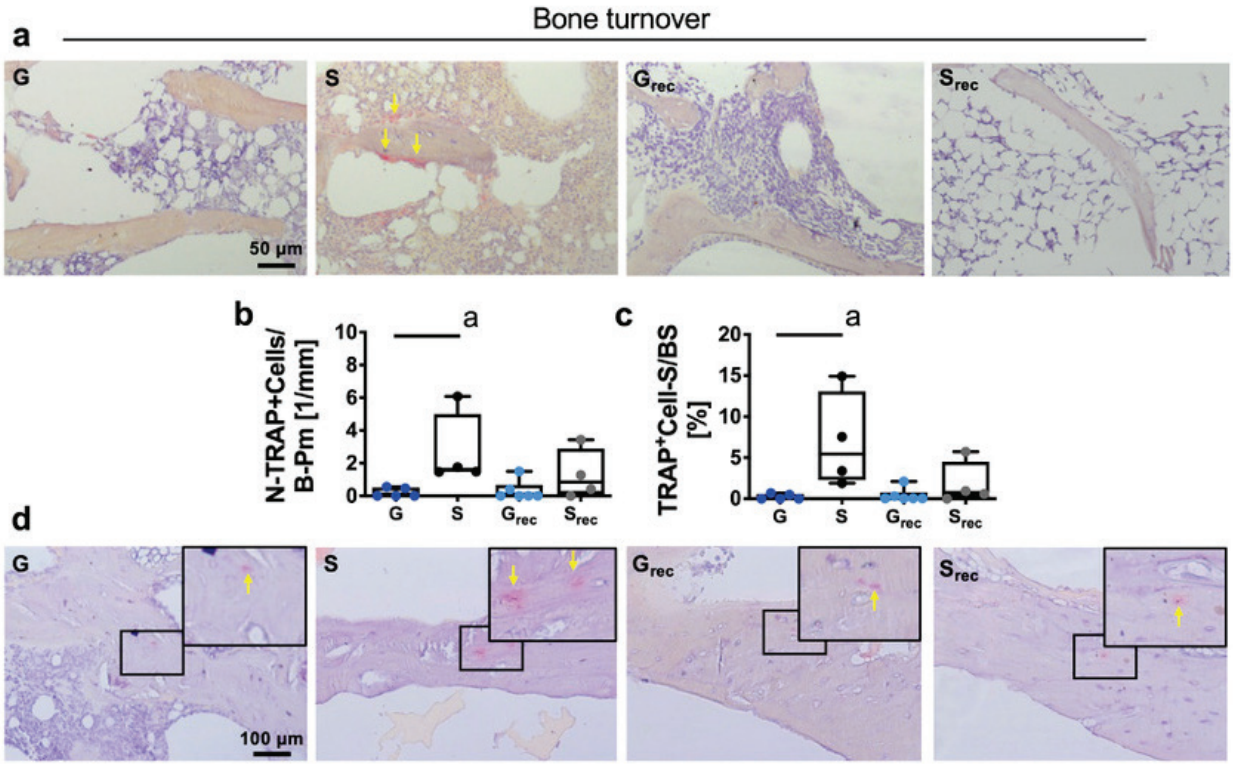

Fig. 4. TRAP-positive cell activity was higher following space flight. (**a**) Analysis of TRAP-stained sections in Tb-bone revealed (**b**) a higher number of TRAP-positive cells per bone perimeter (highlighted by yellow arrows) in S than in G, along with (**c**) a higher TRAP-positive cellular surface over the bone surface in S than in G. (**d**) TRAP staining showed the presence of TRAP-positive osteocytes. Groups: S, $n = 4$, G, $n = 5$, S_{rec}, $n = 4$, G_{rec}, $n = 5$.

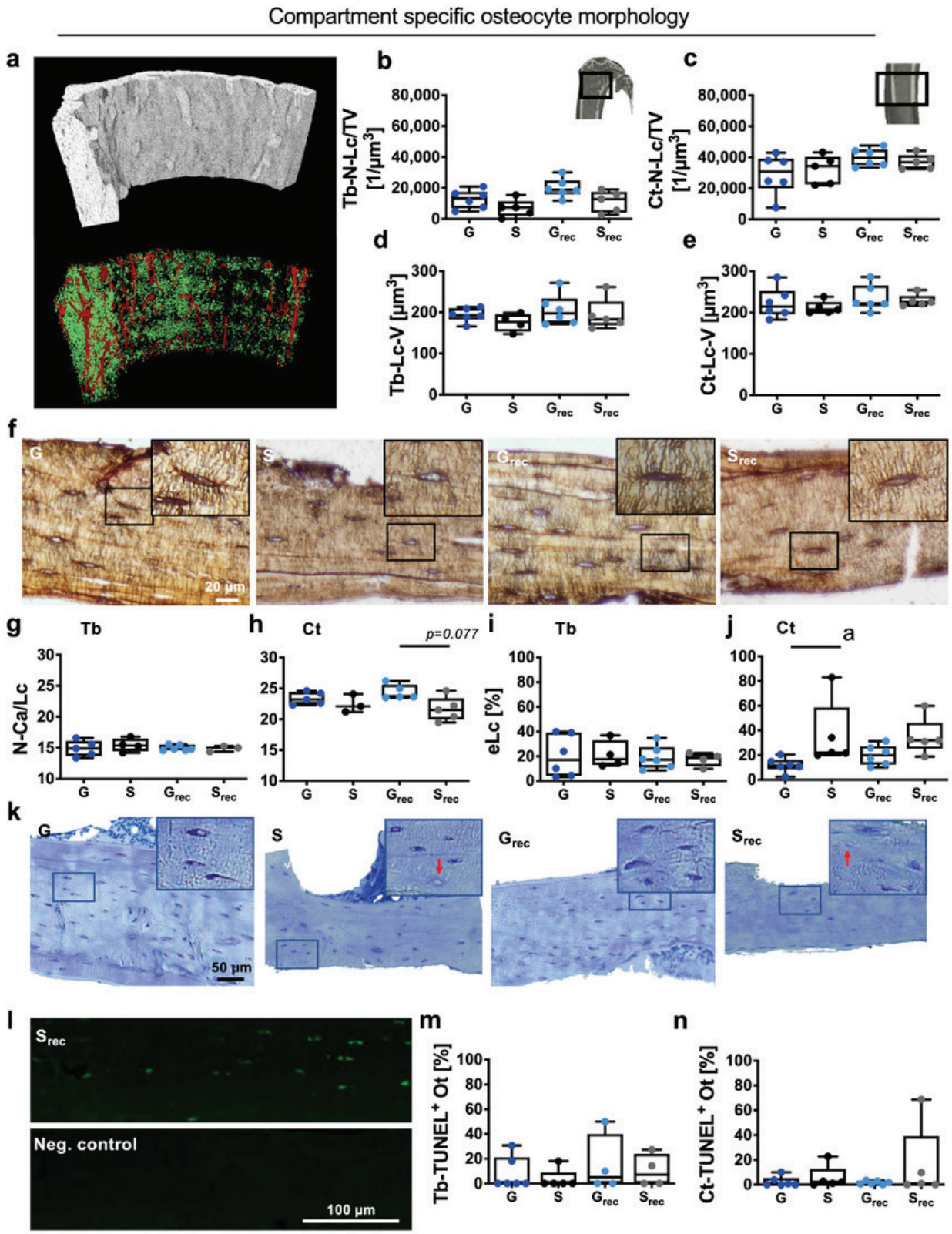

Fig. 5. Osteocyte morphology was similar in all groups for both compartments, along with changes in the number of empty osteocyte lacunae in Ct-bone. (**a**) 3D reconstruction of the Ct-bone with embedded blood vessel channels and osteocyte lacunae (lower panel) showing (**b**) a similar N-Lc/TV in Tb- and (**c**) Ct-bone along with (**d**,**e**) the same Lc-V in both compartments. (**f**) Silver precipitation visualising the LCN revealing (**g**) an unchanged N-Ca/Lc in Tb-bone and (**h**) a tendency towards fewer N-Ca/Lc in Ct-bone of S_{rec} compared to G_{rec}. (**i**) The percentage of eLc was similar in Tb-bone, while in (**j**) Ct-bone, a higher percentage was observed in the S than in the G. (**l**) TUNEL assay to determine local rate of osteocyte apoptosis. TUNEL+ Ot are visible in the Ct bone of one representative section from the Srec group. Negative staining control did not show TUNEL+ cells. (**m**) In Ct and (**n**) Tb bone no significant differences of TUNEL+ Ot were determined. Groups: S, $n = 4$, G, $n = 5$, S_{rec}, $n = 4$, G_{rec}, $n = 5$.

point to a larger bone surface area due to resorption immediately following the space mission, which might contribute to the rebuilding of Tb-bone on ground. The metabolically less-active Ct-bone structure had not yet recovered, pointing towards a recovery of TMD in Ct-bone that was independent of bone volume, reflecting compositional changes in the bone matrix. Others do not find TMD differences in Tb- or Ct-bone when subjected to space flight (Gerbaix *et al.*, 2017), whereas the current study's results confirmed the observed microgravity-induced bone loss of individuals travelling to space (Orwoll *et al.*, 2013). Additionally, further qBEI analysis of regional BMDD showed no significant differences in either compartment. Bone mineral density analyses obtained using μCT were performed on a volumetric and larger region of interest with calibration based on hydroxyapatite phantoms, whereas areal qBEI data were obtained at a higher resolution based on Ca and Al standard calibration.

Bone loss is initiated by imbalanced bone turnover, which was seen in Tb-bone as a higher TRAP-positive cell number, an indication of greater osteoclast activity in the S group than in the G group. Osteocytic osteolysis has been reported by Blaber *et al.* (2013). Enlarged osteocyte lacunae are reported to respond more strongly to mechanical loading (Hemmatian *et al.*, 2018). However, when TRAP-positive osteocytes were examined, no differences in 3D osteocyte lacunar morphology were found within the groups and compartments, indicating the absence of significantly higher osteocytic osteolysis activity following space flight.

Bone remodelling is driven by the regulating action of the mechanosensitive osteocyte network (Prideaux *et al.*, 2016), a well-connected LCN, which is essential for osteocyte-mediated bone turnover. It was found that osteocytes in Ct-bone alone present with a non-significant trend towards fewer canaliculi in the S_{rec} group than in the G_{rec} group, implicating an impairment of the LCN with potential effects on Ct-bone recovery. A well-connected LCN is essential for osteocyte-mediated signalling and is reduced in Ct-bone following unloading (Rolvien *et al.*, 2020). An age-related decrease in osteocyte canaliculi independent of mechanical loading has been shown and linked to the mechanosensitivity of osteocytes (Milovanovic *et al.*, 2013). One fate of osteocytes is cell death, *e.g.* by apoptosis or necrosis, which might occur due to inadequate mechanical loading resulting in an empty osteocyte lacuna within the bone matrix. While in Tb-bone an unchanged percentage of empty lacunae was apparent, Ct-bone presented with a significantly higher proportion of empty lacunae in the S group. In the past, no signs of osteocyte cell death have been detected during a 15 d mission (Blaber *et al.*, 2013), which raises the assumption that osteocyte cell death due to microgravity depends on the exposure time, which is supported by a study analysing bone after a 30 d space mission and 8 d recovery (Gerbaix *et al.*, 2017). Since Ct-bone remodelling following space flight is insufficient, the physiological process of dying osteocytes is not counterbalanced, leading to an accumulation of empty osteocyte lacunae without replacement by newly embedded osteoblasts.

While animals traveling to space are rather unusual and experiments are seldom performed, this study had certain limitations. The material obtained from mice following space flight was limited in number, and focused on only one skeletal element, *i.e.* the tibia. In some samples, the evaluation of the Tb-bone was challenging, reducing the group size for some methods to guarantee high-quality analysis. Furthermore, the absence of Ct-bone recovery might

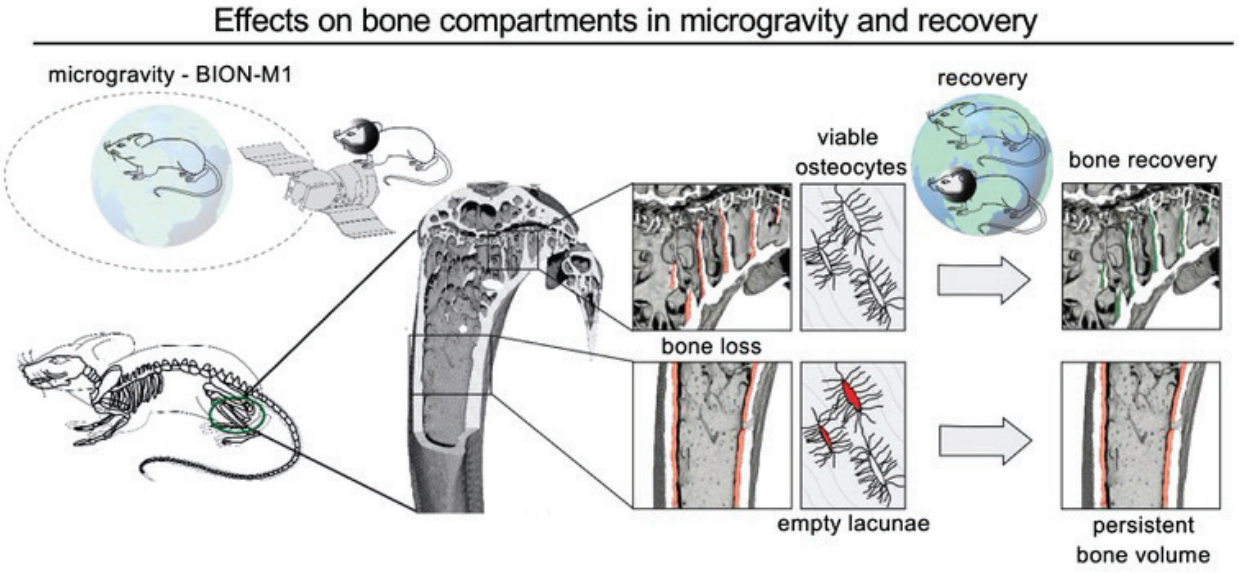

Fig. 6. Differential effects were observed in bone compartments following microgravity and recovery. Overall, the analysis revealed Tb- and Ct-bone loss, which was accompanied by more empty osteocyte lacunae in Ct-bone. While Tb-bone recovered during a recovery period of 7 d, in Ct-bone, the lower bone volume persisted.

be an effect of a compartment-specific prolonged recovery period, which has been postulated for human bone following space flight (Smith *et al*., 2005). While only a relatively short recovery period of 7 d was analysed, the aim was to determine skeletal-specific changes directly following space flight in order to provide further data for possible treatment options to recover bone loss in astronauts.

Conclusions

In summary, the supposition that microgravity induces bone loss in Tb- and Ct-bone was confirmed while providing new information demonstrating that Tb-bone loss recovers, whereas Ct-bone loss persists. Importantly, the assessment of osteocyte viability and morphology identified reduced osteocyte viability solely in Ct-bone without compensatory mechanisms being initiated, while osteocyte canaliculi showed no significant difference, a tendency was noted towards slightly fewer osteocyte canaliculi in the S_{rec} compared to the S group, suggesting an impaired LCN, endangering mechano-dependent bone remodelling and thereby changing the properties of the bone matrix. The observations of differential responses to microgravity and recovery in Tb- and Ct-bone could be linked to different dimensions of osteocyte conservation in both compartments (Fig. 6).

Tb-bone is more accessible to new bone formation upon loading due to a more preserved osteocyte quality. Further studies are needed to validate the contribution of unloading-induced osteocyte pathology to bone material quality following space flight. The observed changes to Ct-bone osteocytes did not recover within 7 d, potentially leading to a manifested change in Ct-bone quality due to microgravity. However, the validation of the contribution of the unloading-induced osteocyte pathology to bone material quality following space flight requires further investigation (i) to identify the underlying mechanisms leading to differential responses of Tb- and Ct-bone under unloading, (ii) to determine whether Ct-bone recovery is prolonged or absent, and (iii) to identify osteocyte apoptosis prevention strategies to obviate irreversible bone loss during space flight and as an approach to treat age-related osteocyte death. These investigations should aim to better understand unloading-induced bone loss to optimise prevention and treatment options for astronauts and patients with immobilisation and other bone diseases.

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Author contributions

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Discussion with Reviewer

Wing Hoi Cheung: The study revealed that microgravity induced changes in cortical bone was in comparison to trabecular bone not reversible within a one-week recovery period. Would these changes be reversible in long term and which measures could be taken to prevent and/or rescue reduced osteocyte viability?

Authors: Within the proposed murine space flight model we determined a lack of cortical bone mass recovery in the tibial midshaft region, while the cortical tissue mineral density recovered 1 week following a 4 week mission in microgravity. In addition, the trabecular compartment of the load-bearing tibia presented overall with a structural recovery; however, one can note the high variability among individual values *i.e.* for BV/TV. Similarly, reports on astronauts and cosmonauts determined remarkably individual differences in bone responses (Sibonga *et al*., 2007). Here, the aBMD measurements at the lumbar spine, mostly entailing trabecular bone, showed in general a recovery with a half life of 151 d, while the change in aBMD values in the femoral neck, entailing cortical bone, presented with a half life of 211 d and presented with individuals not regaining their initial bone mass even 2 years post long-duration space missions. While, the DXA measurements on aBMD might not be optimal to evaluate bone quality and skeletal risks related to microgravity missions (Orwoll *et al*., 2013), the data indicate that i) the cortical bone compartment might need a prolonged period of recovery compared to the trabecular bone compartment and ii) there are quite some individual differences in skeletal response to reambulation.

Data from the current study suggested a role for osteocytes, especially their viability, in the process of skeletal recovery from microgravity. Osteocytes are central regulators of bone remodelling (Prideaux *et al*., 2016). Osteocyte apoptosis and necrosis have previously been reported to coincide with skeletal pathologies (Noble *et al*., 1997 – additional reference). While the RANKL-containing apoptotic bodies from osteocytes are seen to induce bone resorption events *e.g*. upon microcracking (Kennedy *et al*., 2017 – additional reference), the impaired network connectivity might interfere with the bone formation reaction. To determine bone quality, including osteocyte characteristics with microgravity investigations and recovery from it would be of upmost importance. That optimised exercise regimens, as specified for astronauts during space missions, could be a possible intervention to sufficiently reduce bone loss is a current research focus (Orwoll *et al*., 2013). The pharmaceutical application of bisphosphonates administered in combination with exercise prescriptions has also been shown to reduce compartmental loss in trabecular and cortical bone during spaceflight (LeBlanc *et al*., 2013 – additional reference). Bisphosphonates preserve osteocyte viability (Bellido and Plotkin, 2011 – additional reference, in addition to their anti-resorptive activity. Also, drug treatment with sclerostin antibodies are a possible alternative, while potential effects on osteocytes are still under investigation urging for further research.

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Editor's note: The Scientific Editor responsible for this paper was Martin Stoddart.