Received: 1 September 2023 | Revised: 1 September 2023 | Accepted: 20 December 2023

DOI: 10.1002/pmic.202300328

Proteomics
Proteomics and Systems Biology

RESEARCH ARTICLE

Changes in the astronaut serum proteome during prolonged spaceflight

Yayoi Kimura[1] | Yusuke Nakai[1] | Yoko Ino[1] | Tomoko Akiyama[1] | Kayano Moriyama[1] | Tatsuya Aiba[2] | Takashi Ohira[1,3] | Kenji Egashira[1,4] | Yu Yamamoto[1,4] | Yuriko Takeda[5] | Yutaka Inaba[6] | Akihide Ryo[1] | Tomoyuki Saito[7] | Ken Kumagai[6] | Hisashi Hirano[1]

[1]Advanced Medical Research Center, Yokohama City University, Yokohama, Japan

[2]Japan Aerospace Exploration Agency, Tsukuba, Japan

[3]Department of Physiology and Regenerative Medicine, Kindai University Faculty of Medicine, Osaka, Japan

[4]R&D Headquarters, LION Corporation, Tokyo, Japan

[5]Department of Biostatistics, Yokohama City University School of Medicine, Yokohama, Japan

[6]Department of Orthopaedic Surgery, Yokohama City University School of Medicine, Yokohama, Japan

[7]Yokohama Brain and Spine Center, Yokohama, Japan

Correspondence
Yayoi Kimura, Advanced Medical Research Center, Yokohama City University, Fukuura 3-9, Kanazawa-ku, Yokohama 236-0004, Japan.
Email: ykimura@yokohama-cu.ac.jp

Funding information
Japan Aerospace Exploration Agency

Abstract

The molecular mechanisms associated with spaceflight-induced biological adaptations that may affect many healthy tissue functions remain poorly understood. In this study, we analyzed temporal changes in the serum proteome of six astronauts during prolonged spaceflight missions using quantitative comprehensive proteome analysis performed with the data-independent acquisition method of mass spectrometry (DIA-MS). All six astronauts participated in a spaceflight mission for approximately 6 months and showed a decreasing trend in *T*-scores at almost all sites where dual-energy X-ray absorptiometry scans were performed. DIA-MS successfully identified 624 nonredundant proteins in sera and further quantitative analysis for each sampling point provided information on serum protein profiles closely related to several time points before (pre-), during (in-), and after (post-) spaceflight. Changes in serum protein levels between spaceflight and on the ground suggest that abnormalities in bone metabolism are induced in astronauts during spaceflight. Furthermore, changes in the proteomic profile occurring during spaceflight suggest that serum levels of bone metabolism-related proteins, namely ALPL, COL1A1, SPP1, and POSTN, could serve as highly responsive indicators of bone metabolism status in spaceflight missions. This study will allow us to accelerate research to improve our understanding of the molecular mechanisms of biological adaptations associated with prolonged spaceflight.

KEYWORDS
astronaut, human serum, mass spectrometry, proteomics, spaceflight

Abbreviations: BMD, bone mineral density; DIA, data-independent acquisition; DXA, dual-energy X-ray absorptiometry; ISS, International Space Station; JAXA, Japan Aerospace Exploration Agency; RBC, red blood cell.

1 | INTRODUCTION

In space, various factors, including microgravity, space radiation, high carbon dioxide concentrations, the stresses associated with an enclosed environment, and gravitational reloading associated with atmospheric re-entry and landing on Earth, may lead to the deterioration of healthy tissue function [1–4]. In fact, many astronauts who stay on the International Space Station (ISS) face various risks caused by gravity unloading during the transition to a microgravity field, microgravity during prolonged spaceflight, and gravity loading after returning to Earth [5–10]. However, the mechanisms associated with spaceflight-induced biological adaptations in the healthy physical systems of astronauts, including muscular atrophy (loss of muscle mass) and reduced bone mineral density (BMD) (loss of bone mass), remain largely unknown. In space, a 10%–12% decrease in red blood cell (RBC) volume during the first 10 days in spaceflight, called spaceflight anemia, has also been reported [11, 12]. Various causes have been proposed for space anemia, including RBC dysfunction, as well as either decreased production or increased destruction of RBCs; however, the physiological mechanisms are still not fully understood [13]. A better understanding of spaceflight-induced effects on humans at the molecular level and taking measures to inhibit adaptive responses to microgravity will, therefore, be vital to the successful completion of missions that involve landing on extraterrestrial worlds after prolonged spaceflights.

Proteomic approaches employing mass spectrometry (MS) have reached a certain level of maturity and have attracted attention as powerful tools for obtaining information from clinical samples [14, 15]. The proteomes of blood-derived serum and plasma, samples of which can be collected in a minimally invasive manner, accurately reflect the human biological and physiological state, and has, therefore, used in multiple studies for the collection of large amounts of information on health and disease. Therefore, changes in serum protein levels may provide information about mechanisms of adaptation in response to gravity loading. Recently, a targeted MS approach involving multiple reaction monitoring (MRM) in conjunction with stable isotope-labeled standards revealed the behavior of 125 major proteins in plasma samples collected from 18 cosmonauts aboard the ISS [4]. The results have shown that proteins are classified into three groups: proteins whose concentrations stabilize after flight, recover slowly, and recover rapidly to preflight levels. Furthermore, proteins that are differentially expressed in plasma during prolonged spaceflight are associated with pathways that regulate innate immunity, coagulation cascade, lipid metabolism, and extracellular matrix metabolism [4]. Similarly, a targeted MS approach for 20 proteins, including cytokines, from identical twin astronauts (in-flight and on the ground) suggested that the response of these molecules reflects muscle regeneration after atrophy, not a detrimental inflammatory response [16]. In another recent study, the proteome in plasma samples collected at 6 and 1.5 months before launch, mid-duration (~2—4 months in the ISS), and long-term (~5—6 months in the ISS), on the day of return to Earth, and a month after returning, from the same four astronauts has been analyzed using a high-resolution MS-based multidimensional protein identification technology (MudPIT) to enable the detection of several low-abundance plasma proteins [17]. Consequently, they identified a total of 453 unique and nonredundant proteins, 19 of which are involved in inflammatory responses, the cytoskeleton system, and metabolism, which were significantly altered expression by spaceflight. Taken together, these results indicate that proteomic techniques can measure multiple proteins from a sufficient number of biological samples and identify specific proteins within the blood to help elucidate adaptative mechanisms that respond to multiple stresses encountered during spaceflight.

Statement of significance of the study

We aimed to identify serum proteins that can help elucidate the molecular mechanisms of spaceflight-induced biological adaptations associated with prolonged missions aboard the International Space Station. Using blood-derived serum samples collected from six astronauts at different stages before (pre-), during (in-), and after (post-) spaceflight, comprehensive human serum proteomic analysis using the data-independent acquisition method of mass spectrometry may provide novel findings into changes to the astronaut serum proteome profile that occur during spaceflight and after return to Earth. This study promises to accelerate research to determine biological and physiological effects during prolonged spaceflight missions and to detect objective indicators that can predict increased risks of adverse health effects for astronauts.

In this study, we attempted to provide comprehensive information on protein profile changes closely associated with various time points before (pre-), during (in-), and after (post-) prolonged spaceflight by a quantitative proteomic analysis utilizing the data-independent acquisition (DIA) approach of MS technology (DIA-MS) [18]. For this purpose, 72 serum samples were collected from six astronauts before (three time points), during (four time points), and after (five time points) prolonged spaceflight. This study will improve our understanding of spaceflight-induced changes in serum protein levels in astronauts and will greatly improve early detection and management of spaceflight-induced health dysfunction, including bone metabolism abnormalities.

2 | MATERIAL AND METHODS

2.1 | Serum sample collection from astronauts

This is an integrated project by the Japan Aerospace Exploration Agency (JAXA). This research protocol was approved by the Clinical Ethics Committee of Yokohama City University Hospital (#B160401019), the JAXA (#JX-IRBA-20-033), the National

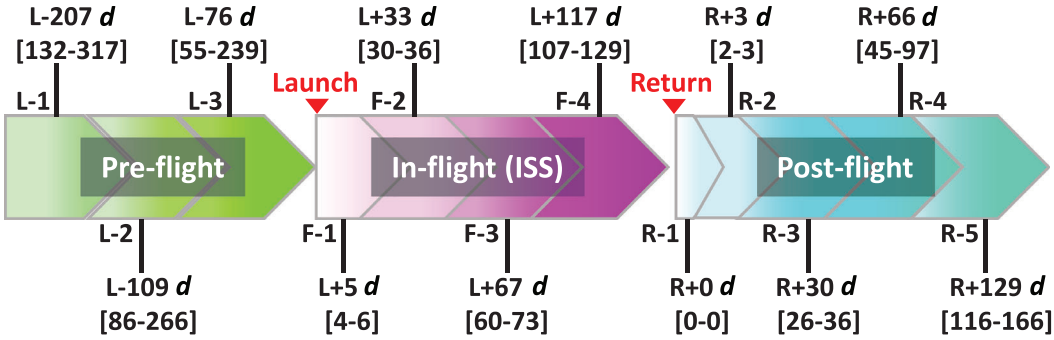

FIGURE 1 Serum sample collection schedule. Serum samples were collected from six participating astronauts at three stages of the spaceflight mission: before launch, while on the ISS, and after returning to Earth. To prevent the identification of specific individuals, data are not indicated by specific date or flight time but by the number of days counted from the launch date for pre- and in-flights and by the number of days counted from the return date to Earth for post-flights. Each day (d) represents the median (minimum day–maximum day) of the serum collection time for the astronauts. However, due to changes in the launch schedule and the spread of COVID-19 infection, blood sampling times occurred over a wide range in the preflight (L-1, L-2, and L-3) and the post-flight (R-4 and R-5) periods. Regardless, this range was within acceptable limits.

Aeronautics and Space Administration (NASA, #Pro2516), EU and European Space Agency Medical Board (ESA MB, #Pro2516), and Human Research Multilateral Review Board (HRMRB, #Pro2516). A total of six astronauts, who participated in a spaceflight mission for approximately 6 months on board the ISS were recruited for this study. The participants included both males and females ranging in age from late 30s to early 50s. All participants signed an informed consent form. This study was conducted in accordance with the Declaration of Helsinki. All data were anonymized before the analyses. Dual-energy X-ray absorptiometry (DXA) scans of the lumbar spine, femoral neck, and trochanter were performed by the space agency to which the individual astronauts belonged both preflight (before launch) and post-flight (after return to Earth). The *T*-score was subsequently calculated from the DXA scan data and used as an indicator of BMD. For each astronaut, serum samples were collected at different stages pre-, in-, and post-flight in space. There were also three sampling times preflight (L-1, L-2, and L-3), four sampling times in-flight (F-1, F-2, F-3, and F-4), and five sampling times post-flight (R-1, R-2, R-3, R-4, and R-5) according to a prespecified schedule. However, due to changes in the launch schedule and the spread of coronavirus disease 2019 (COVID-19) infection, blood sampling occurred over a wide range of times during the preflight and post-flight periods, but these were within acceptable limits. The collection schedule for serum samples is shown in Figure 1. In-flight blood samples were collected and centrifuged, and blood collection tubes were subsequently stored at −95°C on the ISS until they were returned to Earth. Pre- and post-flight blood samples were collected at the Johnson Space Center and the European Astronaut Center, and stored at −80°C after centrifugation and serum collection. Both blood samples collected in-flight and sera collected on the ground (pre- and post-flight) were stored frozen together temporarily at the JSC Clinical Lab. After all samples were collected, they were transported to Yokohama City University and stored at −80°C until they were subjected to further analysis.

2.2 | Proteomic analysis

To prepare samples for MS analysis, 14 human proteins, namely albumin, IgG, antitrypsin, IgA, transferrin, haptoglobin, fibrinogen, alpha2-macroglobulin, alpha1-acid glycoprotein, IgM, apolipoprotein AI, apolipoprotein AII, complement C3, and transthyretin were first removed from serum samples using a Human 14 Multiple Affinity Removal System column (Agilent Technologies). Proteins in 2 μL serum were reduced with 10 mM DTT and alkylated with 25 mM 2-iodoacetamide. After dilution from 8 to 2 M urea in 50 mM NH_4HCO_3, proteins were incubated with 15 ng/μL trypsin at 37°C for 16 h. The resultant peptides were desalted using a StageTip [19] for MS analysis. Subsequently, eluted peptides were completely lyophilized and kept at −80°C until use.

DIA-MS analysis was performed according to a previous study [20]. To identify and quantify peptides and proteins, serum peptide samples were analyzed twice each in DIA mode. DIA-MS data were analyzed using Spectronaut Pulsar X (ver.12.0.2, Biognosys) against a customized spectral library containing information on 1534 proteins. The retention time among different samples was calibrated using iRT peptides (Biognosys). The Biognosys default settings were applied for identification; duplicate assays were excluded, and FDRs were estimated using a *q*-value of 0.01 for both precursors and proteins. Interference correction was activated and a minimum of three fragment ions and two precursor ions were retained for quantification. The area of the extracted ion chromatogram at the MS/MS level was used for quantification. Peptide quantity was measured by the mean of the peak area of each peptide's respective precursor ions, and protein quantity was calculated accordingly by summing the intensity of their respective peptides. The global normalization strategy and *q*-value sparse selection were used for cross-run normalization. All other settings were set to their defaults. To perform downstream quantitative analysis, we used the Perseus software platform (ver. 1.6.2.2, Max-Planck-Institute of Biochemistry) [21]. For quantitative analysis,

samples were categorized into each group, the intensity values were log_2-transformed, and only proteins detected in 70% of samples in at least one group were subjected to quantitative analysis [20]. Missing values were replaced by random numbers extracted from a normal distribution with a width parameter of 0.3 and a downshift parameter of 1.8. Statistical analysis of proteomic data was performed with the two-tailed Welch's *t*-test and all other settings were set to default. The threshold criteria for increased or decreased proteins was set to *p*-value < 0.01 when considering multiple comparisons and a change of 1.5 fold or more compared to other groups in quantitative analyses on the Perseus software. GO term enrichment analysis for biological function interpretation was performed by DAVID Bioinformatics Resources 6.8 (https://david.ncifcrf.gov/) [22]. For biological function and upstream analysis, Ingenuity Pathway Analysis (IPA) (Content ver. 90348151, Release Date: 2023-2-16, QIAGEN) was also used. All settings were also set to default.

3 | RESULTS AND DISCUSSION

3.1 | Bone mineral density reactions associated with spaceflight

The locomotor system, including skeletal muscles and bones, maintains homeostasis by responding to various stresses derived from gravity and other factors. Therefore, bone loss in astronauts induced by microgravity during spaceflight may be a risk factor for osteoporosis and fractures. To determine whether the astronauts recruited for the present study experienced bone loss from preflight (before launch) to post-flight (after return to Earth), a *T*-score, a measure of BMD, was determined. Although no new cases of osteoporosis (*T*-score < −2.5 SD) were observed, all astronauts showed a decreasing trend in *T*-scores at almost all sites where DXA scans were performed in the present study (Figure S1). In addition, biological sex and age had no noticeable effect on *T*-score changes.

3.2 | Serum proteomic analysis with DIA-MS

Systemic circulating blood contains many proteins secreted by various tissues, including locomotor and cardiovascular organs. Moreover, blood proteins are thought to accurately reflect human biological and physiological responses to the stresses caused by spaceflight. By using a comprehensive analysis system for the serum proteome with simple sample preparation and DIA-MS [20], we attempted to uncover temporal changes in the serum proteome of astronauts that characterize the physiological responses that occur over time during prolonged spaceflight missions. Protein profile information was obtained from blood-derived serum samples collected from six astronauts before (three points in preflight), during (four points in in-flight), and after (five points in post-flight) prolonged spaceflight on the ISS by proteomic analysis based on the previous study (Figure 1). A total of 72 serum samples were analyzed but unfortunately, two serum samples were

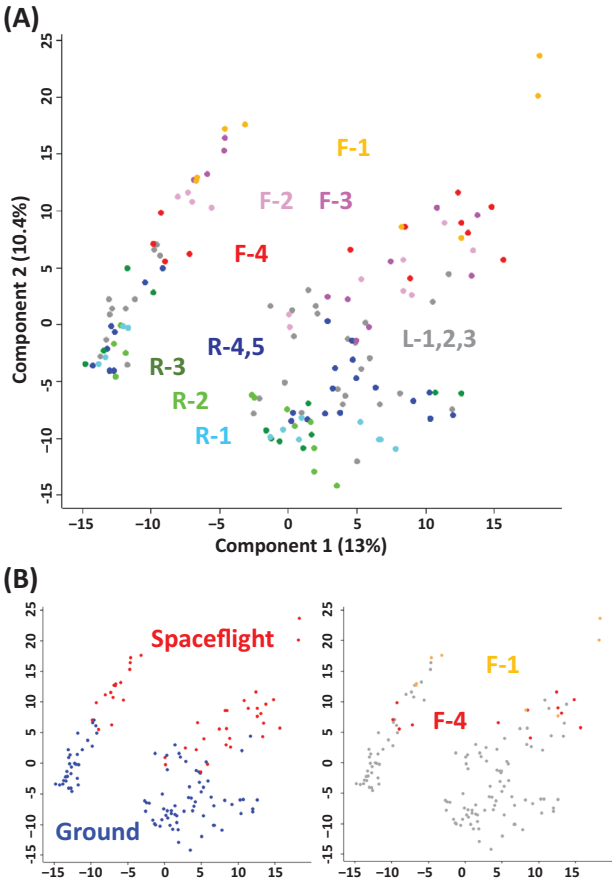

FIGURE 2 A PCA score plot for proteins in each group was generated using Perseus software. (A) All collection points are indicated with different colors. (B) (Left) Serum samples in spaceflight or on the ground are represented by red and blue, respectively. (C) (Right) Serum samples in F-1 or F-4 are represented by orange and red, respectively.

unavailable for quantitative analysis due to severe hemolysis and its consequent effects (Figure S2). However, a search using our spectral DIA library customized for human serum proteome analysis [20] resulted in the successful identification of 624 nonredundant proteins from 140 MS data sets derived from 70 serum samples measured twice. Of these, 566 nonredundant proteins were detected in 70% of samples in at least one group and were, therefore, selected for the sampling point-to-point quantitative analysis. Principal component analysis (PCA), which can visualize the distribution of samples, was conducted using the Perseus software platform. PCA revealed a separation trend between each sampling point (Figure 2A). Additionally, this comparison revealed that the samples could be divided into several subgroups with common characteristics. In particular, the serum proteome profiles differed between immediately after launch (F-1) and immediately before returning to Earth from a prolonged spaceflight (F-4) and also between the spaceflight and ground environments, suggesting that they were affected by various prolonged spaceflight-based stresses, such as physical inactivity associated with gravity unloading (Figure 2B). Using these data, we performed additional

TABLE 1 Proteins expressed differentially in serum collected from astronauts immediately before returning to Earth from a prolonged spaceflight (F-4), compared with those collected immediately after launch (F-1).

Protein accessions	Genes	Protein descriptions	Number of detected peptides	*p*-value (−log)	Difference [F-1/F-4] (log_2)
Increased expression in F-4					
P02452	COL1A1	Collagen alpha-1(I) chain	2	6.93	1.27
Q15063	POSTN	Periostin	14	2.47	1.07
Q96S96	PEBP4	Phosphatidylethanolamine-binding protein 4	3	2.07	0.77
Q9BY67	CADM1	Cell adhesion molecule 1	1	2.31	0.62
P05186	ALPL	Alkaline phosphatase, tissue-nonspecific isozyme	2	3.19	0.59
Decreased expression in F-4					
P12814	ACTN1	Alpha-actinin-1	5	3.38	−2.86
Q01518	CAP1	Adenylyl cyclase-associated protein 1	2	5.65	−1.14
P02788	LTF	Lactotransferrin	16	2.19	−0.93
P31146	CORO1A	Coronin-1A	2	2.38	−0.69
O15144	ARPC2	Actin-related protein 2/3 complex subunit 2	1	3.47	−0.64
Q5SQ64	LY6G6F	Lymphocyte antigen 6 complex locus protein G6f	1	2.03	−0.62

analyses to understand spaceflight-induced health dysfunctions in astronauts.

3.3 | Comparative analysis of serum samples collected immediately after launch (F-1) and immediately before returning to Earth from prolonged spaceflight (F-4)

To determine the proteomic changes in astronaut serum that occur during spaceflight, we compared protein levels at two time points, namely immediately after launch (F-1) and immediately before returning to Earth from a prolonged spaceflight (F-4). The quantitative data for 541 nonredundant proteins detected in 70% of samples in at least one group were subjected to analysis by Perseus (Table S1). Using a statistical method to compare these two sets, we found that the serum levels of only 11 of the 541 proteins were influenced by associated spaceflight time. Most proteins showed no significant change during spaceflight (Table 1). GO term enrichment analysis next indicated that 11 proteins are involved in biological processes such as actin cytoskeleton organization, skeletal system development, endochondral ossification, and osteoblast differentiation. The serum profiles of proteins related to actin cytoskeleton organization, such as CAP1 and CORO1A, revealed distinct features in response to either launch or microgravity (F-1) (Figure 3), gradually decreasing during spaceflight, and returning to preflight levels immediately after returning to Earth (R-1). This response may, therefore, represent a physiological process caused by gravity unloading during the transition to a microgravity field. By contrast, changes in the serum levels of COL1A1 and ALPL, which are involved in biological processes of skeletal system development, endochondral ossification, and osteoblast differentiation, were low immediately after launch (F-1) and continued to increase above preflight levels during the subsequent 6 months on the ISS (Figure 3). Interestingly, COL1A1 and ALPL remained elevated for at least 1 month after return to Earth and began to decline thereafter. Our results suggest that the stressful effects of spaceflight continue for about a month after returning to Earth. Thus, the profile of each serum protein was shown to change in response to the spaceflight stress, but it remains unclear how changes in serum levels of these proteins during spaceflight affect the health of astronauts.

3.4 | Comparative analysis of serum samples collected during spaceflight and on the ground environment

When 70 samples were categorized by spaceflight or ground environment, 530 nonredundant proteins were detected in 70% of samples in at least one group and were subsequently subjected to quantitative analysis using all data obtained from Perseus (Table S2). Consequently, we found 74 proteins whose expression levels in serum samples collected from astronauts during spaceflight were altered compared with those in samples collected on the ground (Table 2). Subsequent functional enrichment analysis within the framework of the IPA showed that proteins whose serum levels were altered by spaceflight are involved in activating processes including phagocytosis of RBCs, proliferation of blood cells, and stimulation of myeloid cells (Table S3).

TABLE 2 List of proteins with altered expression levels in serum samples collected from astronauts during spaceflight, compared with samples collected on the ground.

Protein accessions	Genes	Protein descriptions	Number of detected peptides	*p*-value (−log)	Difference [Flight/Ground] (log_2)
Increased expression in spaceflight (Decreased expression in the ground environment)					
P68363	TUBA1B	Tubulin alpha-1B chain	8	21.34	2.99
P68871	HBB	Hemoglobin subunit beta	8	8.47	2.92
P69905	HBA1	Hemoglobin subunit alpha	19	8.63	2.91
P02042	HBD	Hemoglobin subunit delta	13	7.78	2.87
P00568	AK1	Adenylate kinase isoenzyme 1	1	11.06	2.69
O75083	WDR1	WD repeat-containing protein 1	4	15.36	2.66
Q86UX7	FERMT3	Fermitin family homolog 3	3	19.33	2.48
P00918	CA2	Carbonic anhydrase 2	7	19.03	2.40
P00491	PNP	Purine nucleoside phosphorylase	2	14.53	2.40
P00915	CA1	Carbonic anhydrase 1	5	18.45	2.19
P32119	PRDX2	Peroxiredoxin-2	4	17.23	2.19
P08567	PLEK	Pleckstrin	2	11.11	2.12
Q99497	PARK7	Protein/nucleic acid deglycase DJ-1	1	4.80	2.06
P23528	CFL1	Cofilin-1	5	16.92	2.05
P04406	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	2	20.25	1.89
P60174	TPI1	Triosephosphate isomerase	3	15.18	1.87
Q9Y490	TLN1	Talin-1	19	22.18	1.86
Q01518	CAP1	Adenylyl cyclase-associated protein 1	2	18.96	1.84
P02792	FTL	Ferritin light chain	3	4.50	1.60
Q13228	SELENBP1	Methanethiol oxidase	2	12.90	1.58
P14174	MIF	Macrophage migration inhibitory factor	1	16.10	1.53
P00558	PGK1	Phosphoglycerate kinase 1	3	20.00	1.49
P68366	TUBA4A	Tubulin alpha-4A chain	1	13.74	1.45
O75347	TBCA	Tubulin-specific chaperone A	2	6.08	1.40
P06733	ENO1	Alpha-enolase	3	5.68	1.40
P04040	CAT	Catalase	6	12.74	1.38
P11142	HSPA8	Heat shock cognate 71-kDa protein	3	17.20	1.33
P60660	MYL6	Myosin light polypeptide 6	2	4.53	1.21
P10599	TXN	Thioredoxin	3	5.08	1.20
P05109	S100A8	Protein S100-A8	6	2.10	1.15
P50395	GDI2	Rab GDP dissociation inhibitor beta	2	10.40	1.13
P31146	CORO1A	Coronin-1A	2	10.28	1.10
P62258	YWHAE	14-3-3 protein epsilon	4	15.50	1.08
P52209	PGD	6-phosphogluconate dehydrogenase, decarboxylating	1	8.74	1.07
P78417	GSTO1	Glutathione S-transferase omega-1	3	2.38	1.07
P62937	PPIA	Peptidyl-prolyl cis-trans isomerase A	5	13.65	1.06
P01236	PRL	Prolactin	1	4.75	1.06
P07737	PFN1	Profilin-1	4	11.19	1.04

(Continues)

TABLE 2 (Continued)

Protein accessions	Genes	Protein descriptions	Number of detected peptides	*p*-value (−log)	Difference [Flight/Ground] (log_2)
Q9HBI1	PARVB	Beta-parvin	5	2.86	0.99
P62328	TMSB4X	Thymosin beta-4	2	5.40	0.99
O15144	ARPC2	Actin-related protein 2/3 complex subunit 2	1	12.40	0.97
P67936	TPM4	Tropomyosin alpha-4 chain	5	2.00	0.94
P02647	APOA1	Apolipoprotein A-I	38	4.18	0.91
Q9HDC9	APMAP	Adipocyte plasma membrane-associated protein	5	3.11	0.90
P05164	MPO	Myeloperoxidase	6	10.63	0.89
P37837	TALDO1	Transaldolase	2	12.24	0.88
P80511	S100A12	Protein S100-A12	1	3.50	0.84
Q14019	COTL1	Coactosin-like protein	1	5.29	0.81
P20851	C4BPB	C4b-binding protein beta chain	4	8.26	0.81
Q16851	UGP2	UTP–glucose-1-phosphate uridylyltransferase	1	3.74	0.74
P02671	FGA	Fibrinogen alpha chain	28	15.12	0.66
P02788	LTF	Lactotransferrin	16	3.78	0.62
P14618	PKM	Pyruvate kinase PKM	7	4.80	0.61
Decreased expression in spaceflight (Increased expression in the ground environment)					
O75339	CILP	Cartilage intermediate layer protein 1	1	11.46	−1.63
Q4LDE5	SVEP1	Sushi, von Willebrand factor type A, EGF, and pentraxin domain-containing protein 1	3	9.63	−1.37
Q12884	FAP	Prolyl endopeptidase FAP	7	7.03	−1.21
Q9UBQ6	EXTL2	Exostosin-like 2	3	4.45	−1.01
Q9HCU0	CD248	Endosialin	2	6.33	−0.96
P24043	LAMA2	Laminin subunit alpha-2	6	2.94	−0.92
P02741	CRP	C-reactive protein	6	4.36	−0.87
P08833	IGFBP1	Insulin-like growth factor-binding protein 1	1	2.70	−0.85
O95897	OLFM2	Noelin-2	1	2.09	−0.84
P40967	PMEL	Melanocyte protein PMEL	1	2.65	−0.79
Q6UVK1	CSPG4	Chondroitin sulfate proteoglycan 4	3	3.21	−0.78
P49747	COMP	Cartilage oligomeric matrix protein	6	16.52	−0.72
Q9NPY3	CD93	Complement component C1q receptor	2	3.85	−0.72
P02786	TFRC	Transferrin receptor protein 1	8	3.46	−0.69
P30530	AXL	Tyrosine-protein kinase receptor UFO	1	3.08	−0.68
Q15063	POSTN	Periostin	14	6.59	−0.67
P08253	MMP2	72-kDa type IV collagenase	8	22.38	−0.67
P43121	MCAM	Cell surface glycoprotein MUC18	4	4.36	−0.65
O75023	LILRB5	Leukocyte immunoglobulin-like receptor subfamily B member 5	3	6.02	−0.60
Q6UXB8	PI16	Peptidase inhibitor 16	6	9.90	−0.60
P51884	LUM	Lumican	21	16.36	−0.59

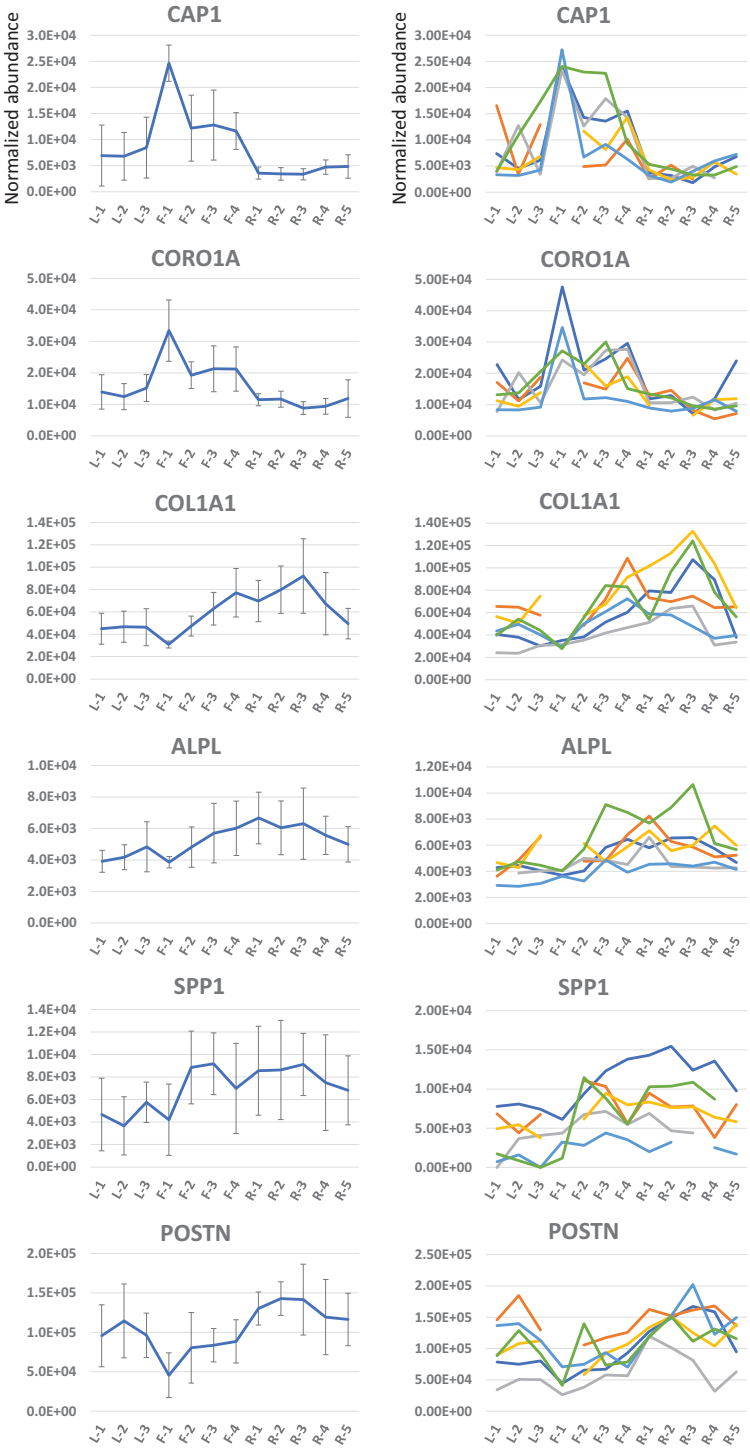

FIGURE 3 Protein profiles in astronaut serum before, during, and after spaceflight. Left, the data are expressed as mean ± SD of normalized abundance. Right, the data represented the proteome profile for each sample.

Hematological changes resulting from spaceflight have been observed since the early days of space exploration. The pattern of hemolysis observed during and after spaceflight confirmed that increased hemolysis is closely related to the space environment [23], and spaceflight anemia is considered a normal adaptation to microgravity [11, 12]. A recent study also suggests that astronauts increase their compensatory production of RBCs and partially restore RBC volume throughout prolonged spaceflight missions due to the body's adaptation to the microgravity environment [24]. Although several theories have been proposed over the years regarding this process, hematological system regulatory mechanisms during prolonged spaceflight remain poorly understood. Our observations concerning proteome profile changes in the serum of astronauts at various time points may help elucidate the physiological mechanisms that regulate hematological systems during spaceflight.

Furthermore, upstream regulator analysis using IPA suggested that increased or decreased levels of several serum proteins during spaceflight may be regulated by the inhibition of EDN1 or the activation

of IL-15, if not both, as upstream cytokines (Figure S3 and Table S4). A previous study has reported that gene expression of EDN1 is significantly suppressed and their release in the supernatant is significantly reduced in endothelial cells grown under simulated microgravity conditions [25]. Another study has reported significant increases in plasma IL-15 concentration in In-flight compared with the preflight baseline [26]. Our estimates of EDN1 inhibition, IL-15 activation, or both, based on serum proteome analysis during spaceflight are consistent with these results. Blood pressure regulation is an integrative process that involves complex factors, such as the nervous system, cardiovascular system, hormones, and renal balance of fluids [27]. EDN1 plays an important role in maintaining these regulatory mechanisms and excessive activation leads to elevation of blood pressure [28, 29]. Regulation by EDN1 inhibition may, therefore, contribute to the phenomenon of lower blood pressure in astronauts during prolonged spaceflights [30, 31]. The role of EDN1 in maintaining bone homeostasis and regulating osteoblast function has also been widely discussed [32]. Mice deficient in EDN1 have craniofacial abnormalities with poor mandibular development and low bone mass, and EDN1 is suggested to affect the regulation of osteogenic cell proliferation and migration [33, 34]. In addition, IL-15, a widely expressed pro-inflammatory cytokine, functions as a myokine secreted from muscles in response to exercise [35], promotes differentiation of osteoclast progenitors into preosteoclasts [36], and stimulates osteoblast apoptosis via natural killer cells [37]. Furthermore, in IL-15 deficient mice with osteonecrosis, femoral BMD and bone mineral content are partially restored and serum osteocalcin, BAP, and BGP, which are used as evaluation markers of osteoblast activity, increase significantly when compared with WT mice with osteonecrosis [38]. Taken together, EDN1 inhibition and/or IL-15 activation, as deduced by changes in the serum proteome profile of astronauts, may be relevant for inducing abnormal bone metabolism in astronauts during spaceflight. In fact, we have observed a decrease in *T*-score during spaceflight in this study (Figure S1). Thus, although the direct relationship between the regulation of these factors and physical changes during spaceflight remains unclear, changes in serum protein levels may allow us to predict increased risks of bone metabolism abnormalities in astronauts.

3.5 | Changes in serum proteome profiles before, during, and after prolonged spaceflight

To identify proteins whose expression changes with spaceflight, serum samples collected from astronauts were used to evaluate changes in the proteome profile over time between each time point before (pre-), during (in-), and after (post-) spaceflight (Tables S5–S10). A list of 124 proteins whose expression levels changed in astronaut serum samples based on sample-to-sample comparisons collected at each time point is shown in Table 3. These were largely inconsistent with the differentially expressed proteins in space detected in a previous study involving comprehensive plasma proteome analysis using MS-based MudPIT, although the only protein common to both studies, LUM, showed a similar decreasing trend during spaceflight compared to pre- and post-flight [17]. Hence, this study may allow detection of numerous novel spaceflight-induced protein changes in astronaut sera, and contribute to a better understanding of the mechanisms of biological adaptations that occur during spaceflight missions.

In our study, we found that many serum proteins are particularly responsive immediately after launch (F-1) compared with preflight (L1, L2, and L3) (Table 3). Functional enrichment analysis within the framework of the IPA predicted that proteome profile changes of these proteins were involved in the increased activation of cell viability, blood cell proliferation, cellular homeostasis, and joint inflammation (Table S3). Furthermore, most of these proteins, that showed significantly lower serum levels immediately after launch (F-1) compared with preflight (L-1, L-2, and L-3), were transient responses (Table 3). GO term enrichment analysis using DAVID, a popular bioinformatics resource, indicated that this group includes proteins related to cell adhesion and extracellular matrix organization. In contrast, proteins that increased in serum level immediately after launch (F-1) continued to increase until late in the prolonged spaceflight missions (F-4), and subsequently most proteins decreased immediately after return to Earth (R-1). Based on GO term enrichment analysis in DAVID, this group was found to contain more proteins related to the innate immune response. Many astronauts experience a variety of adaptations during either launch or immediate post-launch microgravity exposures. Although their respective causal relationships are unknown, the serum proteome profiles of these proteins may, thus, reflect biological adaptations in astronauts resulting from the transition from a gravity-loaded environment to a microgravity field.

Next, we focused on 13 proteins that showed no significant changes in F-1 but were altered in F-4, compared with preflight. Among them, COL1A1 and SPP1 were contained as proteins that increase during spaceflight. COL1A1 are early markers of osteoblast differentiation, while SPP1 (osteopontin) appears later, concomitantly with mineralization [39]. In this study, SPP1 serum levels increased about 1 month after launch (F-2) and remained high during spaceflight (F-3 and F-4) and for about 1 month after return to Earth (R-3), although there were large intersample differences (Figure 3). Analysis of upstream regulation by IPA using a dataset containing proteins with different serum levels compared with preflight (L1, L2, and L3) also suggests that SPP1 is more active immediately before returning from a prolonged spaceflight to Earth (F-4). In addition, another group has revealed that SPP1 expression in cells is slightly but significantly upregulated after being cultured in microgravity compared with 1-*g* controls [25]. Thus, SPP1 may be a protein whose serum levels change in response to microgravity in space. Furthermore, serum levels of POSTN, an osteogenic marker expressed from early to mid-life [39], decreased significantly immediately after launch (F-1) but its profile thereafter resembled that of COL1A1 (Figure 3). Namely, the proteomic profiles of these bone metabolism related-proteins, including ALPL, revealed that the serum levels of each protein after return to Earth from prolonged spaceflight were more variable than preflight, peaking at approximately 1 month after the flight (R-3). Previous studies using serum samples from astronauts on prolonged spaceflights have shown that serum levels of bone formation biomarkers, such as BSAP (bone-specific alkaline phos-

TABLE 3 List of proteins whose expression levels changed in astronaut serum samples based on sample-to-sample comparisons collected at each time point.

				F-1 /Preflight		F-4 /Preflight		/F-1		/R-1		/R-2		/R-3		/R-4, R-5	
Protein accessions	Protein descriptions	Number of detected peptides	Genes	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)
O00151	PDZ and LIM domain protein 1	6	PDLIM1							4.30	1.64	2.63	1.44				
O14498	Immunoglobulin superfamily containing leucine-rich repeat protein	2	ISLR	2.47	−0.75							4.37	−0.63	4.89	−0.78		
O15144	Actin-related protein 2/3 complex subunit 2	1	ARPC2	7.40	1.68	4.33	0.86	3.47	−0.64	2.26	1.07	3.14	1.09	4.03	1.21	6.54	1.12
O75023	Leukocyte immunoglobulin-like receptor subfamily B member 5	3	LILRB5			2.69	−0.59							2.99	−0.88	3.07	−0.68
O75083	WD repeat-containing protein 1	4	WDR1	10.24	3.28							2.49	2.37	2.50	2.41		
O75339	Cartilage intermediate layer protein 1	1	CILP	2.76	−1.55	2.58	−1.52			3.30	−1.87	3.47	−1.77	3.85	−2.30	3.23	−1.71
O75347	Tubulin-specific chaperone A	2	TBCA	3.77	2.80	5.24	2.30			2.59	1.87	4.46	2.67	3.52	1.93	3.11	1.93
P00488	Coagulation factor XIII A chain	13	F13A1											4.07	−0.70		
P00491	Purine nucleoside phosphorylase	2	PNP	3.57	2.88	2.17	1.94			4.89	3.15	5.06	3.70	6.91	3.34	2.82	1.80
P00558	Phosphoglycerate kinase 1	3	PGK1	3.54	1.95	5.06	1.35			5.19	1.52	5.57	1.70	6.59	1.81	5.47	1.53
P00568	Adenylate kinase isoenzyme 1	1	AK1	5.73	3.69	7.99	3.23			5.20	3.66	7.43	3.69	3.49	2.89	4.82	2.53
P00915	Carbonic anhydrase 1	5	CA1	2.98	2.62	4.89	1.94			6.21	2.63	7.18	3.02	7.39	3.01	4.49	1.78
P00918	Carbonic anhydrase 2	7	CA2	2.80	2.73	5.26	2.11			7.26	3.07	7.99	3.70	7.46	3.04	4.50	2.07

(Continues)

TABLE 3 (Continued)

Protein accessions	Protein descriptions	Number of detected peptides	Genes	F-1 /Preflight		F-4 /Preflight		/F-1		/R-1		/R-2		/R-3		/R-4, R-5	
				p-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)
P01024	Complement C3	121	C3							2.67	0.63						
P01236	Prolactin	1	PRL			5.43	1.62			2.19	1.59	3.56	1.55			4.67	1.62
P02042	Hemoglobin subunit delta	13	HBD									2.18	3.27	2.36	3.50		
P02452	Collagen alpha-1(I) chain	2	COL1A1			4.46	0.76	6.93	1.27								
P02671	Fibrinogen alpha chain	28	FGA	5.00	1.05	6.33	0.83							3.04	0.77	4.12	0.61
P02675	Fibrinogen beta chain	13	FGB											2.44	−1.55		
P02741	C-reactive protein	6	CRP									2.81	−1.99				
P02743	Serum amyloid P-component	19	APCS							2.39	0.80	2.91	0.62	2.95	1.47	3.08	0.74
P02750	Leucine-rich alpha-2-glycoprotein	23	LRG1									4.71	−0.61				
P02787	Serotransferrin	12	TF	2.01	0.90												
P02788	Lactotransferrin	16	LTF	4.44	1.24			2.19	−0.93			2.04	0.85				
P02792	Ferritin light chain	3	FTL			2.57	2.04									2.30	1.88
P04003	C4b-binding protein alpha chain	31	C4BPA	2.70	0.64							5.32	0.61	4.81	0.79		
P04040	Catalase	6	CAT	2.19	1.73	3.07	1.20			4.32	1.64	4.54	1.75	4.92	1.90	2.98	1.17
P04075	Fructose-bisphosphate aldolase A	8	ALDOA	2.09	0.70									4.05	0.79	3.62	0.72
P04083	Annexin A1	2	ANXA1	2.19	1.41												
P04406	Glyceraldehyde-3-phosphate dehydrogenase	2	GAPDH	3.80	2.41	4.40	1.56			5.91	2.06	4.70	2.65	6.45	2.31	4.93	1.72
P05164	Myeloperoxidase	6	MPO	2.98	1.35					4.69	0.98			2.63	0.97	2.62	0.70
P05186	Alkaline phosphatase, tissue-nonspecific isozyme	2	ALPL					3.19	0.59								
P06732	Creatine kinase M-type	6	CKM	2.55	−1.26												
P06733	Alpha-enolase	3	ENO1	3.44	2.18												
P06744	Glucose-6-phosphate isomerase	2	GPI	3.50	1.33												

(Continues)

TABLE 3 (Continued)

Protein accessions	Protein descriptions	Number of detected peptides	Genes	F-1 /Preflight		F-4 /Preflight		/F-1		/R-1		/R-2		/R-3		/R-4, R-5	
				p-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)
P07737	Profilin-1	4	PFN1	6.84	1.30					4.94	1.52	4.33	1.32	2.03	0.85	3.53	1.14
P07858	Cathepsin B	4	CTSB									2.88	−0.64				
P07996	Thrombospondin-1	32	THBS1									4.26	0.83				
P08174	Complement decay-accelerating factor	2	CD55	5.69	−0.75												
P08253	72 kDa type-IV collagenase	8	MMP2	5.94	−0.61					6.14	−0.73	4.45	−0.69	7.50	−0.93	6.13	−0.70
P08294	Extracellular superoxide dismutase [Cu–Zn]	6	SOD3	3.01	−0.60							4.35	−0.61	4.32	−0.63		
P08519	Apolipoprotein(a)	15	LPA	2.39	−1.53												
P08567	Pleckstrin	2	PLEK	2.25	1.78	3.75	1.71			7.91	3.55	6.16	3.20	2.29	2.42	5.99	2.49
P08833	Insulin-like growth factor-binding protein 1	1	IGFBP1									2.22	−0.69				
P10153	Non-secretory ribonuclease	2	RNASE2							2.27	0.60						
P10451	Osteopontin	4	SPP1			3.03	1.42										
P10599	Thioredoxin	3	TXN							2.91	1.77						
P11142	Heat shock cognate 71-kDa protein	3	HSPA8	3.38	1.79	4.59	1.26			6.42	1.83	5.44	1.51	4.93	1.37	4.12	1.15
P11597	Cholesteryl ester transfer protein	8	CETP									2.21	1.68				
P12109	Collagen alpha-1(VI) chain	3	COL6A1											5.01	−0.64		
P12111	Collagen alpha-3(VI) chain	25	COL6A3	5.11	−0.62									5.39	−0.61		
P12814	Alpha-actinin-1	5	ACTN1	6.15	2.85			3.38	−2.86								
P12821	Angiotensin-converting enzyme	11	ACE			3.48	−0.94							3.32	−0.98	2.45	−0.74
P13797	Plastin-3	1	PLS3											2.38	−1.20		
P14174	Macrophage migration inhibitory factor	1	MIF	2.89	1.97	4.23	1.38			5.40	1.77	5.69	1.91	5.40	1.73	4.18	1.34
P14618	Pyruvate kinase PKM	7	PKM	2.27	0.91											2.33	0.87

(Continues)

TABLE 3 (Continued)

Protein accessions	Protein descriptions	Number of detected peptides	Genes	F-1 /Preflight		F-4 /Preflight		/F-1		/R-1		/R-2		/R-3		/R-4, R-5	
				p-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)
P16035	Metalloproteinase inhibitor 2	2	TIMP2	5.05	−0.59									4.08	−0.70		
P17174	Aspartate aminotransferase, cytoplasmic	3	GOT1													2.05	0.72
P20023	Complement receptor type 2	6	CR2	4.32	1.72												
P20851	C4b-binding protein beta chain	4	C4BPB	3.52	0.96	2.88	0.75										
P21333	Filamin-A	14	FLNA							4.54	1.28	2.29	0.88				
P22105	Tenascin-X	38	TNXB											6.39	−0.71		
P23142	Fibulin-1	22	FBLN1									3.67	−0.73	2.87	−0.64		
P23381	Tryptophan–tRNA ligase, cytoplasmic	1	WARS									3.91	−0.64				
P23528	Cofilin-1	5	CFL1	6.74	2.28	4.53	1.54			5.53	3.08	4.28	3.22	4.65	1.83	5.57	2.11
P27169	Serum paraoxonase/arylesterase 1	15	PON1											2.95	0.93		
P29401	Transketolase	3	TKT	2.77	0.86												
P31146	Coronin-1A	2	CORO1A	6.72	1.47	3.14	0.79	2.38	−0.69	3.06	1.16	2.57	1.21	5.66	1.22	4.94	1.10
P32119	Peroxiredoxin-2	4	PRDX2	2.69	2.58	4.04	1.93			5.40	2.68	5.97	3.02	6.35	3.00	3.41	1.69
P35443	Thrombospondin-4	11	THBS4	2.36	−0.61									4.91	−0.95	3.85	−0.63
P37802	Transgelin-2	7	TAGLN2							6.96	1.47	3.35	1.23				
P37837	Transaldolase	2	TALDO1	3.50	1.37	3.28	0.80			2.06	0.67	2.09	0.99	4.39	1.04	3.21	1.03
P43121	Cell surface glycoprotein MUC18	4	MCAM			2.01	−0.96			2.14	−1.00			3.07	−1.36		
P49747	Cartilage oligomeric matrix protein	6	COMP	4.69	−0.72	4.25	−0.65			3.74	−0.59	5.40	−0.84	6.17	−1.17	4.97	−0.84
P50395	Rab GDP dissociation inhibitor beta	2	GDI2	2.74	1.85	3.05	1.03			3.29	1.14	2.82	1.16	3.38	1.13	2.58	0.89
P50552	Vasodilator-stimulated phosphoprotein	3	VASP							3.95	1.64						
P51884	Lumican	21	LUM	5.18	−0.68									6.21	−0.87	4.93	−0.61

(Continues)

TABLE 3 (Continued)

Protein accessions	Protein descriptions	Number of detected peptides	Genes	F-1 /Preflight		F-4 /Preflight		/F-1		/R-1		/R-2		/R-3		/R-4, R-5	
				p-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)
P52209	6-phosphogluconate dehydrogenase, decarboxylating	1	PGD	4.92	1.76	4.40	1.02			3.37	1.11	2.53	1.15	7.00	1.73	5.04	1.20
P54289	Voltage-dependent calcium channel subunit alpha-2/delta-1	9	CACNA2D1			2.61	−0.83									2.24	−0.75
P60174	Triosephosphate isomerase	3	TPI1			2.40	1.30			4.15	2.26	4.24	2.28	4.93	2.31	2.75	1.68
P60660	Myosin light polypeptide 6	2	MYL6	4.64	2.20	2.43	1.36										
P62258	14-3-3 protein epsilon	4	YWHAE	3.48	1.52	4.39	1.03			4.48	1.06	4.75	1.15	5.46	1.30	4.98	1.18
P62328	Thymosin beta-4	2	TMSB4X							2.64	1.76	2.21	1.50				
P62805	Histone H4	3	HIST1H4A			2.81	−2.02										
P62937	Peptidyl-prolyl cis-trans isomerase A	5	PPIA	3.54	1.41	3.30	0.71			7.09	1.40	4.99	1.71	4.14	1.07	5.17	0.98
P62942	Peptidyl-prolyl cis-trans isomerase FKBP1A	1	FKBP1A	2.47	1.12												
P67936	Tropomyosin alpha-4 chain	5	TPM4							2.97	2.36	3.25	2.35				
P68363	Tubulin alpha-1B chain	8	TUBA1B	8.10	2.81	5.83	2.69			5.57	4.15	4.56	4.03	3.27	2.86	6.36	3.56
P68366	Tubulin alpha-4A chain	1	TUBA4A	6.33	1.71	4.04	1.53			4.55	2.20	3.87	1.65			2.66	1.52
P68871	Hemoglobin subunit beta	8	HBB									2.41	3.34	2.51	3.45		
P69905	Hemoglobin subunit alpha	19	HBA1									2.15	3.10	2.37	3.36		
P78417	Glutathione S-transferase omega-1	3	GSTO1			2.25	1.75										
P80108	Phosphatidylinositol-glycan-specific phospholipase D	18	GPLD1											4.65	0.65		

(Continues)

TABLE 3 (Continued)

Protein accessions	Protein descriptions	Number of detected peptides	Genes	F-1 /Preflight		F-4 /Preflight		/F-1		/R-1		/R-2		/R-3		/R-4, R-5	
				p-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)
Q01518	Adenylyl cyclase-associated protein 1	2	CAP1	10.40	2.27	4.15	1.05	5.65	−1.14	5.86	1.89	6.96	1.91	5.54	2.02	6.16	2.12
Q03167	Transforming growth factor beta receptor type 3	4	TGFBR3											2.33	−0.65		
Q12884	Prolyl endopeptidase FAP	7	FAP											4.42	−1.36	2.97	−0.93
Q13201	Multimerin-1	6	MMRN1									5.09	1.17				
Q13228	Methanethiol oxidase	2	SELENBP1	2.05	1.90	5.72	1.84			3.26	2.06	6.00	1.91	5.12	2.22	4.62	1.54
Q14019	Coactosin-like protein	1	COTL1							2.60	1.43						
Q14766	Latent-transforming growth factor beta-binding protein 1	6	LTBP1									3.26	0.93				
Q14956	Transmembrane glycoprotein NMB	2	GPNMB	2.02	−0.61												
Q15063	Periostin	14	POSTN	2.85	−1.22			2.47	1.07	3.07	−0.61	3.87	−0.74	2.43	−0.66		
Q16851	UTP–glucose-1-phosphate uridylyltransferase	1	UGP2	3.72	1.32												
Q4LDE5	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1	3	SVEP1	2.41	−1.31	2.72	−1.25					3.12	−1.36	3.29	−1.75	3.36	−1.53
Q5SQ64	Lymphocyte antigen 6 complex locus protein G6f	1	LY6G6F			3.17	−1.37	2.03	−0.62								
Q6UXB8	Peptidase inhibitor 16	6	PI16	3.27	−0.86									3.67	−0.90		
Q6UY14	ADAMTS-like protein 4	9	ADAMTSL4	3.57	−0.77												
Q86UX7	Fermitin family homolog 3	3	FERMT3	5.95	2.52	5.69	1.93			6.17	3.38	4.11	3.14	2.73	2.54	8.96	2.47

(Continues)

TABLE 3 (Continued)

Protein accessions	Protein descriptions	Number of detected peptides	Genes	F-1 /Preflight		F-4 /Preflight		/F-1		/R-1		/R-2		/R-3		/R-4, R-5	
				p-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)	*p*-value (-log)	Difference (log_2)
Q86YW5	Trem-like transcript 1 protein	2	TREML1	2.14	−3.00												
Q8IUL8	Cartilage intermediate layer protein 2	3	CILP2											2.93	−0.91	3.00	−0.80
Q92743	Serine protease HTRA1	2	HTRA1			4.55	0.99										
Q96RD9	Fc receptor-like protein 5	3	FCRL5	3.49	0.96												
Q96S96	Phosphatidylethanolamine-binding protein 4	3	PEBP4	3.01	−0.79			2.07	0.77								
Q99497	Protein/nucleic acid deglycase DJ-1	1	PARK7									2.74	3.74				
Q9BY67	Cell adhesion molecule 1	1	CADM1					2.31	0.62								
Q9H4B7	Tubulin beta-1 chain	2	TUBB1	2.20	2.28	3.51	2.24			2.89	2.47	3.64	2.84			3.57	2.33
Q9HCU0	Endosialin	2	CD248											3.78	−1.30		
Q9HDC9	Adipocyte plasma membrane-associated protein	5	APMAP									3.52	2.21				
Q9NPY3	Complement component C1q receptor	2	CD93											3.56	−1.02		
Q9NQ38	Serine protease inhibitor Kazal-type 5	3	SPINK5	3.22	−0.71												
Q9UBQ6	Exostosin-like 2	3	EXTL2	2.23	−1.75												
Q9Y279	V-set and immunoglobulin domain-containing protein 4	1	VSIG4									2.23	−0.63				
Q9Y490	Talin-1	19	TLN1	6.12	1.75	5.60	1.44			10.60	2.97	6.17	2.65	4.10	1.61	7.32	1.98
Q9Y5Y7	Lymphatic vessel endothelial hyaluronic acid receptor 1	4	LYVE1											2.09	−0.74		
	Total number of proteins with altered expression levels;			**68**		**46**		**11**		**47**		**62**		**65**		**48**	

phatase) and P1NP (procollagen type 1 amino-terminal propeptide), also gradually increased immediately after launch and during spaceflight, and remained high for approximately 1 month after returning to Earth before decreasing [40, 41]. The altered patterns of these serum biomarkers of bone formation were consistent with changes in bone metabolism-related proteins observed during spaceflight in this study. Spaceflight, especially exposure to microgravity, has a wide range of effects on human musculoskeletal health, including muscle atrophy and abnormal bone metabolism. For this reason, astronauts who have spent extended periods of time in space undergo reconditioning after returning to Earth, focusing on the neuro-musculoskeletal system [42]. Based on functional enrichment analysis within the framework of the IPA, serum proteome profiles showed that 1 month after return, quantities of connective tissue cells may be increased and cell death of blood cells and damage to the vascular system may have recovered (Table S3). Therefore, serum proteome profiles, including proteins associated with bone metabolism, may also indicate that astronauts begin recovering physical and physiological status after the first month Post-flight. Although how spaceflight-induced changes in serum levels of these proteins relate to bone metabolism needs to be investigated further, serum levels of ALPL, COL1A1, SPP1, and POSTN may also function as objective indicators of bone metabolism in astronauts caused by spaceflight missions.

In this study, proteomic analysis with DIA-MS provided comprehensive, longitudinal astronaut serum proteome profile data before, during, and after prolonged spaceflight missions, providing scientific information for future studies and promising to accelerate research on elucidating the biological and physiological status associated with prolonged spaceflight-based stresses. However, the causal relationship between spaceflight and chance cannot be determined, since only six individuals were studied. Additional studies are needed to determine the relationship between changes in proteome profiles and the individual's adaptation to spaceflight conditions. Therefore, this study should be considered as providing information for hypothesis generation and should be complemented by future studies involving more astronauts.

4 | CONCLUDING REMARKS

In summary, we conducted comprehensive proteomic analysis on serum samples collected from six astronauts pre-, in-, and post-flight with DIA-MS, and generated proteome profiles. All six astronauts showed a decreasing trend in *T*-scores at almost all sites where DXA scans were performed. We successfully identified 624 nonredundant proteins using 70 serum samples out of a total of 72 serum samples. Subsequent quantitative analysis detected proteins with significantly altered levels in serum before, during, and after spaceflight. Changes in serum protein levels between the spaceflight and ground environments predicted a spaceflight-induced decrease in serum levels of EDN1 and an increase in IL-15. This finding may be relevant to the induction of abnormal bone metabolism in astronauts during spaceflight. Furthermore, the proteomic profile alteration suggested that serum levels of bone metabolism-related proteins, namely ALPL, COL1A1, SPP1, and POSTN, may act as highly responsive indicators of bone metabolism status in spaceflight missions. We expect this study to lead to novel insights into mechanisms of adaptation to spaceflight and contribute to the discovery of objective indicators that can predict increased risks of adverse health effects for astronauts.

ACKNOWLEDGMENTS

This work was supported in part by funding provided by the JAXA for the Japan Experiment Module (JEM) utilization program. Special thanks are due to the astronauts who participated in this study. The authors wish to thank Dr Satoshi Furukawa (JAXA) and the Japan Space Forum for supporting this work.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

DATA AVAILABILITY STATEMENT

All mass spectrometry proteomics data have been deposited in the ProteomeXchange Consortium (http://www.proteomexchange.org) via the jPOST (https://jpostdb.org) partner repository with the dataset identifier PXD027635 (Spectral library data) or PXD044609 (DIA-MS analysis data). All data are available in full without restriction.

ORCID

Yayoi Kimura https://orcid.org/0000-0002-4612-4602
Akihide Ryo https://orcid.org/0000-0001-6028-8091

REFERENCES

1. Smith, S. M., Krauhs, J. M., & Leach, C. S. (1997). Regulation of body fluid volume and electrolyte concentrations in spaceflight. *Advances in Space Biology and Medicine*, *6*, 123–165. https://doi.org/10.1016/s1569-2574(08)60081-7
2. Stein, T. P. (2013). Weight, muscle and bone loss during space flight: Another perspective. *European Journal of Applied Physiology*, *113*(9), 2171–2181. https://doi.org/10.1007/s00421-012-2548-9
3. Moreno-Villanueva, M., Wong, M., Lu, T., Zhang, Y., & Wu, H. (2017). Interplay of space radiation and microgravity in DNA damage and DNA damage response. *NPJ Microgravity*, *3*, 14. https://doi.org/10.1038/s41526-017-0019-7
4. Larina, I. M., Percy, A. J., Yang, J., Borchers, C. H., Nosovsky, A. M., Grigoriev, A. I., & Nikolaev, E. N. (2017). Protein expression changes caused by spaceflight as measured for 18 Russian cosmonauts. *Scientific Reports*, *7*(1), 8142. https://doi.org/10.1038/s41598-017-08432-w
5. Droppert, P. M. (1993). A review of muscle atrophy in microgravity and during prolonged bed rest. *Journal of the British Interplanetary Society*, *46*(3), 83–86.
6. Green, D. A., & Scott, J. P. R. (2017). Spinal health during unloading and reloading associated with spaceflight. *Frontiers in Physiology*, *8*, 1126. https://doi.org/10.3389/fphys.2017.01126
7. Keyak, J. H., Koyama, A. K., LeBlanc, A., Lu, Y., & Lang, T. F. (2009). Reduction in proximal femoral strength due to long-duration spaceflight. *Bone*, *44*(3), 449–453. https://doi.org/10.1016/j.bone.2008.11.014
8. LeBlanc, A., Schneider, V., Shackelford, L., West, S., Oganov, V., Bakulin, A., & Voronin, L. (2000). Bone mineral and lean tissue loss after long duration space flight. *Journal of Musculoskeletal & Neuronal Interactions*, *1*(2), 157–160.

9. Roffino, S., Camy, C., Foucault-Bertaud, A., Lamy, E., Pithioux, M., & Chopard, A. (2021). Negative impact of disuse and unloading on tendon enthesis structure and function. *Life Sciences in Space Research*, *29*, 46–52. https://doi.org/10.1016/j.lssr.2021.03.001
10. Vandenburgh, H., Chromiak, J., Shansky, J., Del Tatto, M., & Lemaire, J. (1999). Space travel directly induces skeletal muscle atrophy. *FASEB Journal*, *13*(9), 1031–1038. https://doi.org/10.1096/fasebj.13.9.1031
11. Leach, C. S., & Johnson, P. C. (1984). Influence of spaceflight on erythrokinetics in man. *Science*, *225*(4658), 216–218. https://doi.org/10.1126/science.6729477
12. Smith, S. M. (2002). Red blood cell and iron metabolism during space flight. *Nutrition (Burbank, Los Angeles County, Calif.)*, *18*(10), 864–866. https://doi.org/10.1016/s0899-9007(02)00912-7
13. Alfrey, C. P., Rice, L., Udden, M. M., & Driscoll, T. B. (1997). Neocytolysis: Physiological down-regulator of red-cell mass. *Lancet*, *349*(9062), 1389–1390. https://doi.org/10.1016/S0140-6736(96)09208-2
14. Dayon, L., Cominetti, O., & Affolter, M. (2022). Proteomics of human biological fluids for biomarker discoveries: Technical advances and recent applications. *Expert Review of Proteomics*, *19*(2), 131–151. https://doi.org/10.1080/14789450.2022.2070477
15. Deutsch, E. W., Omenn, G. S., Sun, Z., Maes, M., Pernemalm, M., Palaniappan, K. K., Letunica, N., Vandenbrouck, Y., Brun, V., Tao, S. C., Yu, X., Geyer, P. E., Ignjatovic, V., Moritz, R. L., & Schwenk, J. M. (2021). Advances and utility of the human plasma proteome. *Journal of Proteome Research*, *20*(12), 5241–5263. https://doi.org/10.1021/acs.jproteome.1c00657
16. Gertz, M. L., Chin, C. R., Tomoiaga, D., MacKay, M., Chang, C., Butler, D., Afshinnekoo, E., Bezdan, D., Schmidt, M. A., Mozsary, C., Melnick, A., Garrett-Bakelman, F., Crucian, B., Lee, S. M. C., Zwart, S. R., Smith, S. M., Meydan, C., & Mason, C. E. (2020). Multi-omic, single-cell, and biochemical profiles of astronauts guide pharmacological strategies for returning to gravity. *Cell Reports*, *33*(10), 108429. https://doi.org/10.1016/j.celrep.2020.108429
17. Martin, D., Makedonas, G., Crucian, B., Peanlikhit, T., & Rithidech, K. (2022). The use of the multidimensional protein identification technology (MudPIT) to analyze plasma proteome of astronauts collected before, during, and after spaceflights. *Acta Astronautica*, *193*, 9–19. https://doi.org/10.1016/j.actaastro.2021.12.054
18. Gillet, L. C., Navarro, P., Tate, S., Rost, H., Selevsek, N., Reiter, L., Bonner, R., & Aebersold, R. (2012). Targeted data extraction of the MS/MS spectra generated by data-independent acquisition: A new concept for consistent and accurate proteome analysis. *Molecular & Cellular Proteomics*, *11*(6), O111.016717. https://doi.org/10.1074/mcp.O111.016717
19. Rappsilber, J., Mann, M., & Ishihama, Y. (2007). Protocol for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics using StageTips. *Nature Protocols*, *2*(8), 1896–1906. https://doi.org/10.1038/nprot.2007.261
20. Kimura, Y., Nakai, Y., Shin, J., Hara, M., Takeda, Y., Kubo, S., Jeremiah, S. S., Ino, Y., Akiyama, T., Moriyama, K., Sakai, K., Saji, R., Nishii, M., Kitamura, H., Murohashi, K., Yamamoto, K., Kaneko, T., Takeuchi, I., Hagiwara, E., … Ryo, A. (2021). Identification of serum prognostic biomarkers of severe COVID-19 using a quantitative proteomic approach. *Scientific Reports*, *11*(1), 20638. https://doi.org/10.1038/s41598-021-98253-9
21. Tyanova, S., Temu, T., Sinitcyn, P., Carlson, A., Hein, M. Y., Geiger, T., Mann, M., & Cox, J. (2016). The Perseus computational platform for comprehensive analysis of (prote)omics data. *Nature Methods*, *13*(9), 731–740. https://doi.org/10.1038/nmeth.3901
22. Huang, D. W., Sherman, B. T., & Lempicki, R. A. (2009). Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nature Protocols*, *4*(1), 44–57. https://doi.org/10.1038/nprot.2008.211
23. Trudel, G., Shahin, N., Ramsay, T., Laneuville, O., & Louati, H. (2022). Hemolysis contributes to anemia during long-duration space flight. *Nature Medicine*, *28*(1), 59–62. https://doi.org/10.1038/s41591-021-01637-7
24. Kunz, H., Quiriarte, H., Simpson, R. J., Ploutz-Snyder, R., McMonigal, K., Sams, C., & Crucian, B. (2017). Alterations in hematologic indices during long-duration spaceflight. *BMC Hematol*, *17*, 12. https://doi.org/10.1186/s12878-017-0083-y
25. Infanger, M., Ulbrich, C., Baatout, S., Wehland, M., Kreutz, R., Bauer, J., Grosse, J., Vadrucci, S., Cogoli, A., Derradji, H., Neefs, M., Küsters, S., Spain, M., Paul, M., & Grimm, D. (2007). Modeled gravitational unloading induced downregulation of endothelin-1 in human endothelial cells. *Journal of Cellular Biochemistry*, *101*(6), 1439–1455. https://doi.org/10.1002/jcb.21261
26. Krieger, S. S., Zwart, S. R., Mehta, S., Wu, H., Simpson, R. J., Smith, S. M., & Crucian, B. (2021). Alterations in saliva and plasma cytokine concentrations during long-duration spaceflight. *Frontiers in Immunology*, *12*, 725748. https://doi.org/10.3389/fimmu.2021.725748
27. Nishida, Y., Tandai-Hiruma, M., Kemuriyama, T., & Hagisawa, K. (2012). Long-term blood pressure control: Is there a set-point in the brain? *The Journal of Physiological Sciences*, *62*(3), 147–161. https://doi.org/10.1007/s12576-012-0192-0
28. Kostov, K. (2021). The causal relationship between endothelin-1 and hypertension: Focusing on endothelial dysfunction, arterial stiffness, vascular remodeling, and blood pressure regulation. *Life (Basel)*, *11*(9), 986. https://doi.org/10.3390/life11090986
29. Schiffrin, E. L. (2001). Role of endothelin-1 in hypertension and vascular disease. *American Journal of Hypertension*, *14*(6 Pt 2), S83–S89. https://doi.org/10.1016/s0895-7061(01)02074-x
30. Baevsky, R. M., Baranov, V. M., Funtova, I. I., Diedrich, A., Pashenko, A. V., Chernikova, A. G., Drescher, J., Jordan, J., & Tank, J. (2007). Autonomic cardiovascular and respiratory control during prolonged spaceflights aboard the International Space Station. *Journal of Applied Physiology(1985)*, *103*(1), 156–161. https://doi.org/10.1152/japplphysiol.00137.2007
31. Norsk, P., Asmar, A., Damgaard, M., & Christensen, N. J. (2015). Fluid shifts, vasodilatation and ambulatory blood pressure reduction during long duration spaceflight. *The Journal of Physiology*, *593*(3), 573–584. https://doi.org/10.1113/jphysiol.2014.284869
32. Sin, A., Tang, W., Wen, C. Y., Chung, S. K., & Chiu, K. Y. (2015). The emerging role of endothelin-1 in the pathogenesis of subchondral bone disturbance and osteoarthritis. *Osteoarthritis and Cartilage*, *23*(4), 516–524. https://doi.org/10.1016/j.joca.2014.11.002
33. Kitano, Y., Kurihara, H., Kurihara, Y., Maemura, K., Ryo, Y., Yazaki, Y., & Harii, K. (1998). Gene expression of bone matrix proteins and endothelin receptors in endothelin-1-deficient mice revealed by in situ hybridization. *Journal of Bone and Mineral Research*, *13*(2), 237–244. https://doi.org/10.1359/jbmr.1998.13.2.237
34. Kurihara, Y., Kurihara, H., Suzuki, H., Kodama, T., Maemura, K., Nagai, R., Oda, H., Kuwaki, T., Cao, W. H., & Kamada, N. (1994). Elevated blood pressure and craniofacial abnormalities in mice deficient in endothelin-1. *Nature*, *368*(6473), 703–710. https://doi.org/10.1038/368703a0
35. Hamrick, M. W. (2012). The skeletal muscle secretome: An emerging player in muscle–bone crosstalk. *BoneKEy Reports*, *1*, 60. https://doi.org/10.1038/bonekey.2012.60
36. Ogata, Y., Kukita, A., Kukita, T., Komine, M., Miyahara, A., Miyazaki, S., & Kohashi, O. (1999). A novel role of IL-15 in the development of osteoclasts: Inability to replace its activity with IL-2. *Journal of Immunology*, *162*(5), 2754–2760.
37. Takeda, H., Kikuchi, T., Soboku, K., Okabe, I., Mizutani, H., Mitani, A., Ishihara, Y., & Noguchi, T. (2014). Effect of IL-15 and natural killer cells on osteoclasts and osteoblasts in a mouse coculture. *Inflammation*, *37*(3), 657–669. https://doi.org/10.1007/s10753-013-9782-0
38. Zhou, Z., Lin, Y., Pan, C., Wang, N., Zhou, L., Shan, H., Gao, Y., & Yu, X. (2020). IL-15 deficiency alleviates steroid-induced osteonecrosis of the femoral head by impact osteoclasts via RANKL-RANK-OPG

system. *Immunity & Ageing*, *17*, 19. https://doi.org/10.1186/s12979-020-00190-0

39. Paolella, F., Gabusi, E., Manferdini, C., Schiavinato, A., & Lisignoli, G. (2019). Specific concentration of hyaluronan amide derivative induces osteogenic mineralization of human mesenchymal stromal cells: Evidence of RUNX2 and COL1A1 genes modulation. *Journal of Biomedical Materials Research. Part A*, *107*(12), 2774–2783. https://doi.org/10.1002/jbm.a.36780
40. Gabel, L., Liphardt, A. M., Hulme, P. A., Heer, M., Zwart, S. R., Sibonga, J. D., Smith, S. M., & Boyd, S. K. (2022). Incomplete recovery of bone strength and trabecular microarchitecture at the distal tibia 1 year after return from long duration spaceflight. *Scientific Reports*, *12*(1), 9446. https://doi.org/10.1038/s41598-022-13461-1
41. Sibonga, J., Matsumoto, T., Jones, J., Shapiro, J., Lang, T., Shackelford, L., Smith, S. M., Young, M., Keyak, J., Kohri, K., Ohshima, H., Spector, E., & LeBlanc, A. (2019). Resistive exercise in astronauts on prolonged spaceflights provides partial protection against spaceflight-induced bone loss. *Bone*, *128*, 112037. https://doi.org/10.1016/j.bone.2019.07.013
42. Lambrecht, G., Petersen, N., Weerts, G., Pruett, C., Evetts, S., Stokes, M., & Hides, J. (2017). The role of physiotherapy in the European Space Agency strategy for preparation and reconditioning of astronauts before and after long duration space flight. *Musculoskeletal Science & Practice*, *27*(Suppl 1), S15–S22. https://doi.org/10.1016/j.math.2016.10.009

SUPPORTING INFORMATION

Additional supporting information may be found online https://doi.org/10.1002/pmic.202300328 in the Supporting Information section at the end of the article.

How to cite this article: Kimura, Y., Nakai, Y., Ino, Y., Akiyama, T., Moriyama, K., Aiba, T., Ohira, T., Egashira, K., Yamamoto, Yu., Takeda, Y., Inaba, Y., Ryo, A., Saito, T., Kumagai, K., & Hirano, H. (2024). Changes in the astronaut serum proteome during prolonged spaceflight. *Proteomics*, e2300328. https://doi.org/10.1002/pmic.202300328