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BRIEF COMMUNICATION

Cell-Free Mitochondrial DNA as a Potential Biomarker for Astronauts' Health

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BACKGROUND: Space travel–associated stressors such as microgravity or radiation exposure have been reported in astronauts after short- and long-duration missions aboard the International Space Station. Despite risk mitigation strategies, adverse health effects remain a concern. Thus, there is a need to develop new diagnostic tools to facilitate early detection of physiological stress.

METHODS AND RESULTS: We measured the levels of circulating cell-free mitochondrial DNA in blood plasma of 14 astronauts 10 days before launch, the day of landing, and 3 days after return. Our results revealed a significant increase of cell-free mitochondrial DNA in the plasma on the day of landing and 3 days after return with vast ~2 to 355-fold interastronaut variability. In addition, gene expression analysis of peripheral blood mononuclear cells revealed a significant increase in markers of inflammation, oxidative stress, and DNA damage.

CONCLUSIONS: Our study suggests that cell-free mitochondrial DNA abundance might be a biomarker of stress or immune response related to microgravity, radiation, and other environmental factors during space flight.

Key Words: astronaut ■ biomarker ■ cell-free DNA ■ space medicine

From the first crewed spaceflights to low Earth orbit in the early 1960s to the current long-duration missions aboard the International Space Station, various adverse health effects have been reported.[1] Some of these adverse effects include but are not limited to body fluid redistribution, muscle atrophy, impaired immune responses, microbiome alterations, neurovestibular impairment, and adverse cardiovascular events.[2] These effects may be significantly increased during future exploration-type space missions, as Apollo astronauts exhibited a higher morbidity/mortality associated with cardiovascular dysfunction compared with nonflight and low Earth orbit astronauts.[3] The frequency and severity of adverse health effects may increase during Moon and Mars missions, where astronauts will be reentering ~16% and ~37% of Earth's gravity during take-off and landing, respectively. Therefore, the risk of adverse health effects is more likely during deep-space exploration, and it is necessary to develop effective and timely diagnostic strategies, including new predictive biomarkers.

Deep-space travel–associated stressors (radiation, microgravity, etc) can damage cell integrity and release the intracellular content into the extracellular milieu. This content can act as a stress signal named damage-associated molecular patterns or alarmins.[4] Damage-associated molecular patterns include DNA, high mobility group box-1, and heat shock proteins implicated in various diseases.[4] While sources of damage-associated molecular patterns include components of the nucleus, plasma membrane, and intracellular proteins, mitochondria can also become dysfunctional and

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promote the production of reactive oxygen species.[5] Of various mitochondrial damage-associated molecular patterns, mitochondrial DNA (mtDNA) can be found in the extracellular space, circulating as short DNA fragments or encapsulated in vesicles.[6] Increasing evidence suggests that differential levels of circulating cell-free mtDNA (cf-mtDNA) play a significant role in various human diseases.[7–9] Indeed, the National Aeronautics and Space Administration's Twin Study reported that cf-mtDNA correlated with prolonged space flight.[10] Compared with the ground-based twin, increased mitochondrial gene expression was detected during the flight, which was coupled with increase in 2 urinary markers of oxidative stress (8-hydroxy-2′-deoxyguanosine [8-OHdG] and platelet-derived growth factor 2-α).[11]

In this report, we extend this analysis to 14 astronauts who flew relatively short ~5- to 13-day missions and found that (1) there is a significant increase of cf-mtDNA in the plasma of all astronauts on the day of return (R-0), which continues to rise 3 days after return (R+3); (2) there is vast interastronaut variability of ~2- to 355-fold increases in cf-mtDNA, signifying the need for retrospective and prospective studies of individual susceptibility to cellular stress in astronauts; (3) the release of cf-mtDNA from mitochondria is associated with the activation of multiple pathways related to inflammation, oxidative stress, and DNA damage; and (4) we demonstrate the utility of using a large number of retrospectively (>20 years) collected blood samples (plasma and cells) from astronauts for further analysis.

METHODS

The data that support the findings of this study are available from the corresponding author upon reasonable request. This study was approved by the National Aeronautics and Space Administration and the Icahn School of Medicine at Mount Sinai's Institutional Review Board (MOD00001074 and HSM19-00367, respectively). All study participants provided written informed consent at time of sample collection.

We measured cf-mtDNA levels in the blood plasma of 14 astronauts who flew short (~5- to 13-day) International Space Station missions between 1998 and 2001. Blood was sampled at 3 different time points: 10 days before launch (L-10), R-0, and R+3. mtDNA and nuclear DNA abundance was measured by real-time quantitative polymerase chain reaction (qPCR). Human β-globin primers were used for normalizing the nuclear DNA.

We also isolated the extracellular vesicles (EVs) from the blood plasma of 3 astronauts at each time point using the ExoQuick method. EVs were characterized by nanoparticle tracking analysis (Nanosight). EV population was further validated using Exo-Check Exosome Antibody Array, and EV-mtDNA was assessed by qPCR using specific primers for mitochondrially encoded cytochrome C oxidase I (MT-CO1) and mitochondrially encoded cytochrome C oxidase III (MT-CO3). Purified exosomal RNA was analyzed by small RNA sequencing.

Further, total RNA was isolated from peripheral blood mononuclear cells (PBMCs) from 6 astronauts at L-10, R-0, and R+3 to measure the expression of genes encoding inflammation, oxidative stress, and DNA damage markers. Additionally, we analyzed DNA/RNA oxidative damage by measuring 8-OHdG levels using the high-sensitivity DNA/RNA Oxidation Damage ELISA kit (Cayman Chemical), which allows the detection of 3 oxidized guanine species as a marker for DNA/RNA oxidative damage—8-hydroxy-2′-deoxyguanosine (8-OHdG) from DNA, 8-hydroxyguanosine from RNA, and 8-hydroxyguanine from either DNA or RNA. The assay has a high sensitivity of ~30 pg/mL. Analyses were performed using PBMCs from astronauts at L-10, R-0, and R+3. See extended methodology in Data S1.

RESULTS AND DISCUSSION

Analysis of cf-mtDNA fractions relative to cell-free nuclear DNA revealed a significant increase of cf-mtDNA at R+3, with significant variability in both postflight samples (Figure 1A and 1D). Given this interastronaut variability, we calculated the individual fold increase of cf-mtDNA for each astronaut for R-0 and R+3 relative to their value at L-10. The increases in cf-mtDNA in the postflight samples of individual astronauts were found to be between 2- and 355-fold compared with preflight levels (Figure 1B, 1C, 1E, and 1F).

Most cells secrete a range of EVs that act as communication vehicles by carrying proteins and nucleic acids between cells. Additionally, EV content may reveal the presence of mtDNA enrichment under stress. Therefore, we isolated EVs from the blood plasma of 3 astronauts at 3 different time points (L-10, R-0, and R+3) and quantified mtDNA abundance. Nanosight analysis of particle size and concentration confirmed the isolation of EVs (Figure 2A). We further characterized the EVs by detecting the presence of protein markers using an exosome-specific antibody array (Figure 2B and 2C). The GM130 control showed only a signal above the background with limited cellular contamination in the exosome preparations. Next, we isolated the mt-DNA from EVs and quantified the MT-CO1 and MT-CO3 abundance by qPCR.

While MT-CO1 was undetectable, our results showed that MT-CO3 levels were not significantly changed at both postflight time points in these 3 astronauts (Figure 2D). Altogether, our results suggested that mt-DNA (MT-CO1 and MT-CO3) levels were increased

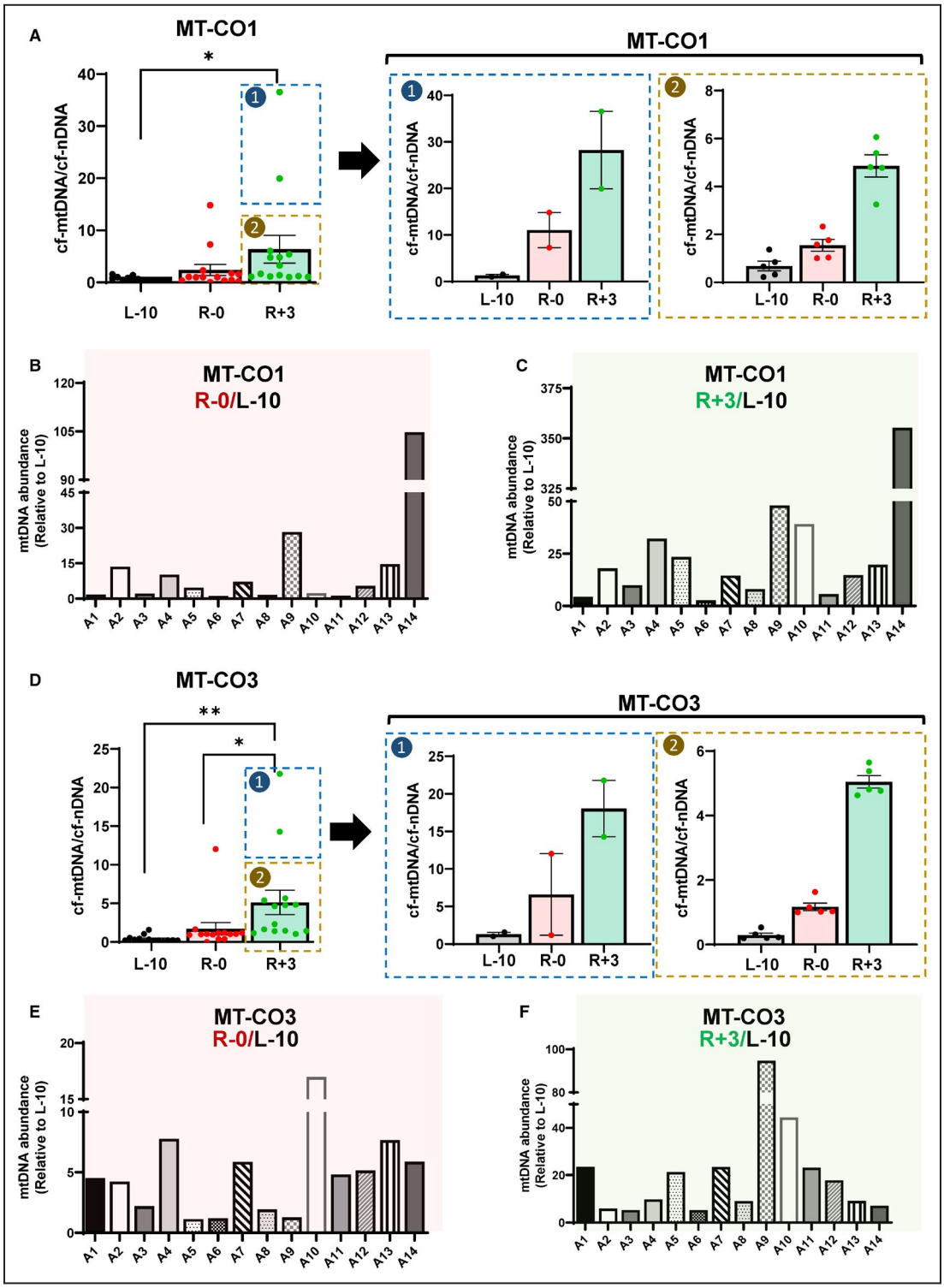
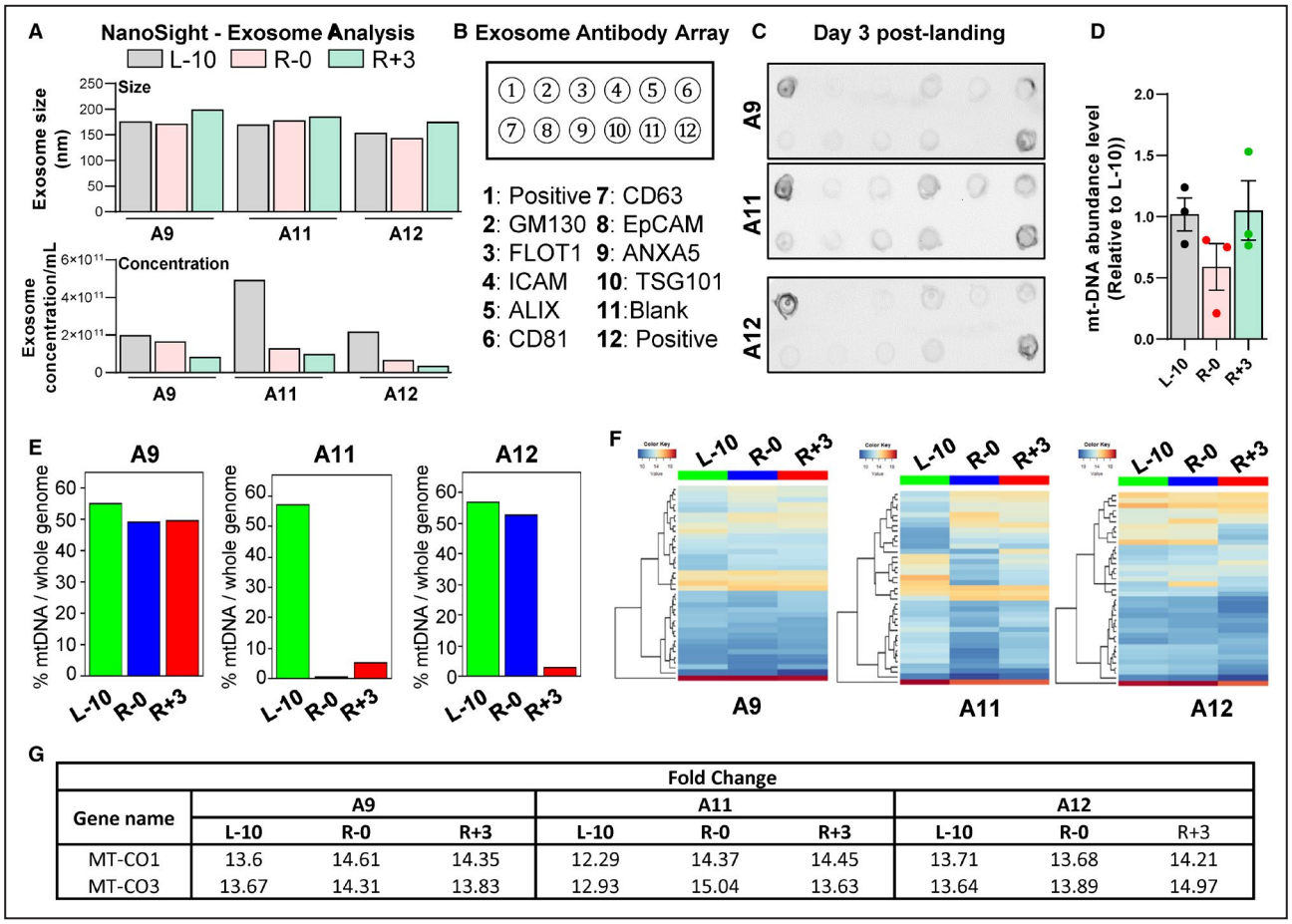


Figure 1. Increased cf-mtDNA levels in the plasma of 14 astronauts after space flight.
A, Quantification of mtDNA content using MT-CO1–specific primers; insets 1 and 2 show mtDNA levels in 2 and 5 astronauts, respectively. **B** and **C**, Each bar represents fold increases of MT-CO1 at R-0 and R+3 relative to L-10 for each astronaut. **D**, Quantification of mtDNA content using MT-CO3–specific primers; insets 1 and 2 show mtDNA levels in 2 and 5 astronauts, respectively. **E** and **F**, Each bar represents fold increases of MT-CO3 at R-0 and R+3 relative to L-10 for each astronaut. cf-mtDNA indicates cell-free mitochondrial DNA; cf-nDNA, cell-free nuclear DNA; L-10, 10 days before launch; MT-CO1, mitochondrially encoded cytochrome C oxidase I; MT-CO3, mitochondrially encoded cytochrome C oxidase II; mtDNA, mitochondrial DNA; R+3, 3 days after return; and R-0, day of landing. Data are presented as ±SEM; **P*<0.05, ***P*<0.01.

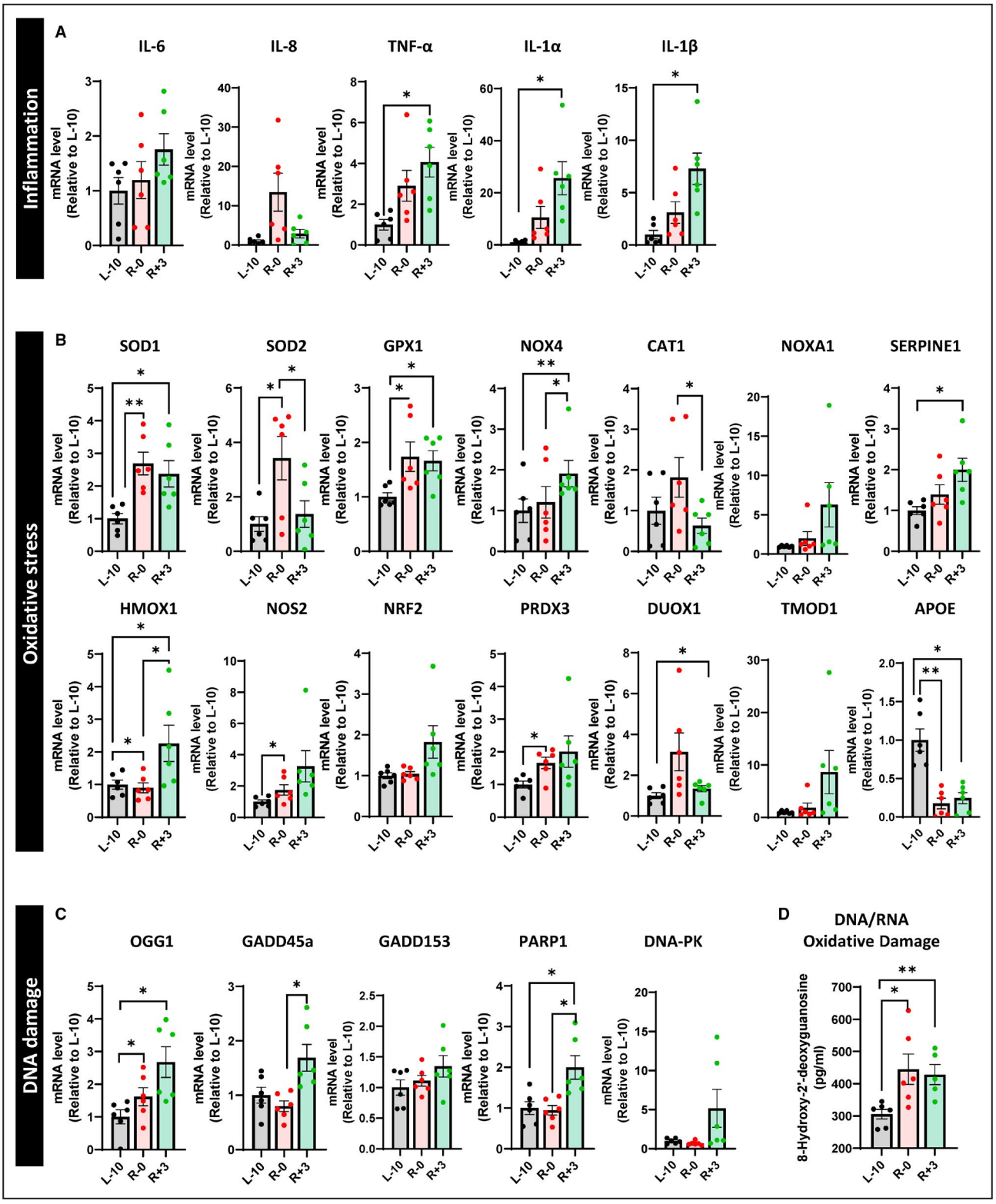


Gene name	Fold Change								
	A9			A11			A12		
	L-10	R-0	R+3	L-10	R-0	R+3	L-10	R-0	R+3
MT-CO1	13.6	14.61	14.35	12.29	14.37	14.45	13.71	13.68	14.21
MT-CO3	13.67	14.31	13.83	12.93	15.04	13.63	13.64	13.89	14.97

Figure 2. Analysis of mt-DNA and mt-RNA abundance in EVs.
A, EVs were isolated from the blood plasma of 3 astronauts (A9, A11, and A12) at L-10, R-0, and R+3, and EV size was characterized by Nanosight analysis. **B**, Exosome isolation was further validated by evaluating the presence of protein markers using an exosome-specific antibody array. Spot positions are provided for the following exosome markers: CD63, CD81, ALIX, FLOT1, ICAM1, EpCam, ANXA5, and TSG101. A GM130 cis-Golgi marker was also included to monitor any cellular contamination in exosome isolation and a positive control spot derived from human serum exosomes. **C**, Data for array spots obtained using 50 μg of exosome lysate isolated from astronauts A9, A11, and A12. **D**, MT-CO3 abundance was measured by qPCR in mt-DNA isolated from EVs from 3 astronauts. **E**, The graphs represent the fraction of mt-DNA normalized by the whole genome in 3 astronauts (A9, A11, and A12) at 3 time points (L-10, R-0, R+3). **F**, Heatmap, displaying gene expression for each sample in the RNA sequencing data set, is shown. Each row of the heatmap represents an mtRNA gene, and each column represents a sample. The color key is log2 transformed counts. **G**, Fold changes in gene expression for MT-CO1 and MT-CO3 are shown in EVs isolated from blood plasma from 3 astronauts (A9, A11, and A12) at L-10, R-0, and R+3. L-10 indicates 10 days before launch; MT-CO1, mitochondrially encoded cytochrome C oxidase I; MT-CO3, mitochondrially encoded cytochrome C oxidase III; mt-DNA, mitochondrial DNA; mt-RNA, mitochondrial RNA; qPCR, quantitative polymerase chain reaction; R+3, 3 days after return; and R-0, day of landing.

only in plasma as cf-mtDNA rather than EV-mtDNA. We further assessed the transcriptome changes associated with space flight by small RNA sequencing in the same astronauts at each time point. Analysis of the RNA sequencing data sets suggests that mtDNA content was decreased at R-0 and R+3 (Figure 2E) and

Figure 3. Transcript levels of stress markers in PBMCs from 6 astronauts.
Relative mRNA expression of (**A**) inflammatory (IL-6, IL-8, TNF-α, IL-1α, IL-1β); (**B**) oxidative stress (SOD1, SOD2, GPX1, NOX4, CAT1, NOXA1, SERPINE1, HMOX1, NOS2, NRF2, PRDX3, DUOX1, TMOD1, APOE); (**C**) oxidative DNA damage stress markers (OGG1, GADD153, GADD45a, PARP1, and DNAPK) was measured by qPCR; (**D**) DNA/RNA oxidative damage (8-hydroxy-2′-deoxyguanosine, 8-hydroxyguanosine, and 8-hydroxyguanine) was measured by direct ELISA. Each dot represents an individual astronaut. APOE indicates apolipoprotein E; CAT1, catalase; DNAPK, DNA-dependent protein kinase; DUOX1, dual oxidase 1; GADD153, DNA damage inducible transcript 3; GADD45a, growth arrest and DNA damage inducible alpha; GPX1, glutathione peroxidase 1; HMOX1, heme oxygenase 1; IL-1α, interleukin-1 alpha; IL-1β, interleukin-1 beta; IL-6, interleukin 6; IL-8, interleukin-8; NOS2, nitric oxide synthase 2; NOX4, NADPH oxidase 4; NOXA1, NADPH oxidase activator 1; NRF2, nuclear factor, erythroid 2 like 2; OGG1, 8-oxoguanine DNA glycosylase; PARP1, poly(ADP-ribose) polymerase 1; PRDX3, peroxiredoxin 3; qPCR, quantitative polymerase chain reaction; SERPINE1, serpin family E member 1; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; TMOD1, tropomodulin 1; and TNF-α, tumor necrosis factor. Data are presented as ±SEM; **P*<0.05, ***P*<0.01.



further confirmed the changes in the pattern of gene expression across the astronauts (Figure 2F). The transcript levels of MT-CO1 and MT-CO3 mtRNA expression were not significantly changed (Figure 2G). The expression of all detectable mtDNA–encoded genes are provided in Table S1.

Previous studies have shown that mtDNA release from mitochondria triggers multiple pathways related to inflammation, oxidative stress, and DNA damage.[12] Thus, we analyzed the transcript levels of various markers related to these pathways (Figure 3). Our results revealed a significant increase in *TNF-α, IL-1α, IL-1β* gene expression

levels at R+3 days (Figure 3A). Although not statistically significant, *IL-6* mRNA expression showed an upward trend on R+3 days, whereas *IL-8* was significantly upregulated on R-0 but not on R+3 days (Figure 3A). Oxidative stress genes *SOD1* and *GPX1* were significantly upregulated at both postflight time points, while *APOE* was significantly downregulated at R-0 and R+3. Of note, *APOE* deficiency has been reported to promote oxidative stress and lead to a compensatory increase in antioxidant enzymes in brain tissue.[13] *HMOX1* transcript levels were significantly decreased at R-0 and significantly increased at R+3 (Figure 3B). *SOD2, NOS2, PRDX3* were significantly elevated only at R-0, while levels of *NOX4, SERPINE1,* and *DUOX1* were significantly increased only at R+3 (Figure 3B). *CAT1* mRNA expression was significantly increased at R-0 compared with R+3. DNA damage gene *OGG1* was significantly upregulated at both postflight time points, while *GADD45a* and *PARP1* were significantly upregulated only at R+3 days (Figure 3C). However, *GADD153* and *DNA-PK* mRNA levels showed only an elevated trend at R+3.

Direct measurement of DNA/RNA oxidative damage in PBMCs revealed a significant increase in 8-OHdG levels at R-0 and R+3 (Figure 3D), suggesting increases in DNA/RNA oxidative damage after flight. These results are consistent with the gene expression profile of oxidative stress enzymes and further confirm increased oxidative stress in PBMCs from astronauts after space flight. Considering interastronaut variability, we also looked at the association between cf-mtDNA and mRNA transcripts levels in 3 astronauts for whom PBMCs were available (Figure S1). We found postflight levels of *IL-1α, IL-1β, TNFα*, and *OGG1* followed a similar elevated trend to cf-mtDNA in all 3 astronauts (Figure S1, shaded light green). Additionally, 2 of these 3 astronauts had elevated levels of *IL-6, IL-8, SOD1, SOD2, GPX1, NOX4, GADD45, CAT1, DNA-PK*, and *PARP1* in at least 1 of the postflight time points (Figure S1, shaded light blue). *GADD153* remained unchanged for all 3 time points.

Increased cf-mtDNA levels have shown prognostic value in chronic diseases such as diabetes, rheumatoid arthritis, and neurodegenerative and cardiovascular diseases.[7–9] Furthermore, mitochondrial dysfunction has been strongly associated with the onset and progression of dilated cardiomyopathies and heart failure.[14] Here, we report elevation in plasma cf-mtDNA in 14 astronauts who flew relatively short missions. Not only were cf-mtDNA levels increased at the time of landing, but levels also continued to increase 3 days after landing. Our data suggest that cf-mtDNA may serve as a prognostic biomarker for the detection and follow-up of space travel–associated mitochondrial dysfunction. The extent to which this elevation continues will require additional evaluation. Consistent with previous reports, our results also showed that space travel was associated with elevated expression of antioxidant enzymes, DNA damage markers, and inflammatory responses. Thus, the release of mtDNA may potentiate stress responses, but defining causal relationships will require further studies. Notably, these measurements may indicate DNA damage, immune response, and RNA-regulatory changes, with an innate capacity to reveal the origin of cells undergoing apoptosis or necrosis.[15] Though cf-mtDNA was not significantly correlated with space flight duration, our study revealed that cf-mtDNA abundance might serve as a potential new biomarker of stress or immune response related to microgravity, radiation exposure, and other environmental factors associated with space flight.

In this study, limitations include some variation in the gene-expression profile across individual crew members. This can be explained, in part, by the smaller study size because of the exceptionally rare availability of such samples. In addition, while the approximate age of the crew members was similar, heterogeneity in space flight duration was noted. However, further analysis of our data showed no association between flight duration and cf-mtDNA abundance in these astronauts, probably attributable to the small sample size and lack of appropriate controls because of the inability to access control-age matched astronauts who did not undergo space flight. Additionally, given the low amounts of blood plasma available, EVs could only be isolated using the ExoQuick method. However, EV isolation methods have different yields and purities. For example, ultracentrifugation-based methods are the most widely used for exosome isolation with higher degrees of purity; however, much more sample input is needed.[10] Therefore, future studies using high-purity isolation methods should be considered to further evaluate mtDNA/RNA abundance in EVs. Finally, it is worth mentioning that cf-mtDNA was isolated from the blood plasma of astronauts who flew short (~5- to 13-day) International Space Station missions between 1998 and 2001, and these samples were deidentified. As such, we do not have access to clinical data related to cardiovascular function from these astronauts to extrapolate possible short-term postflight correlations and, more importantly, long-term follow-up studies. Therefore, additional functional studies may help better understand the role of cf-mtDNA on adverse health risks using a controlled environment that experimentally replicates the multiple stressors of the space environment, such as space radiation or microgravity.

ARTICLE INFORMATION

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Author contributions: Drs Bisserier, Garikipati, Shanmughapriya, Mills, and Goukassian designed research; Drs Bisserier, Shanmughapriya, Rai, Garikipati, Mills, Brojakowska and Gonzalez conducted experiments; Drs Garikipati, Shanmughapriya, Bisserier, Rai, Arakelyan, Goukassian, and Brojakowska analyzed data; Drs Garikipati, Shanmughapriya, Bisserier, Mills, Madesh, Walsh, Kishore, Hadri, Goukassian, and Brojakowska wrote the article; and Dr Goukassian provided project administration and funding acquisition. All authors have read and agreed to the published version of the article.

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Disclosures

None.

Supplementary Material

Data S1
Table S1
Figure S1

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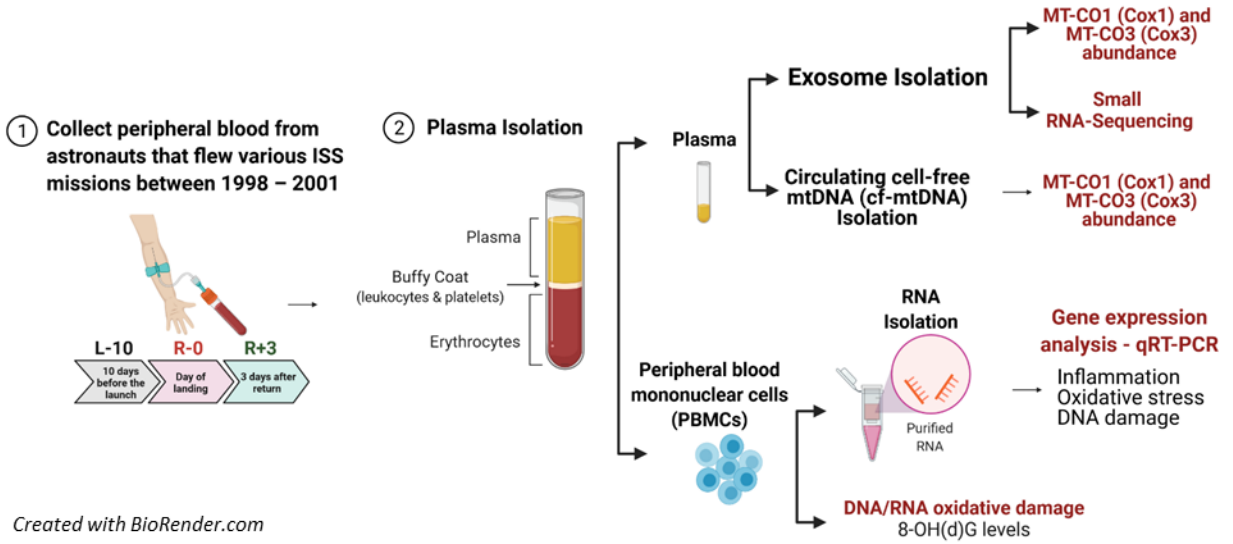
SUPPLEMENTAL MATERIAL

Data S1.

Supplemental Methods

Astronaut samples

We studied the levels of cf-mtDNA in the blood plasma of 14 astronauts who flew short (~5-13-day) ISS missions between 1998 and 2001. Information regarding de-identified blood samples, including Shuttle Space mission code, the approximate average age of the crew members, and time spent in space, along with experimental strategy, are depicted graphically and in the table below.



Sample ID #	Shuttle Mission Code	Approximate Age of The Crew	Time in Space
A1	STS99	45.2 ± 3.9	11d 5hr
A2	STS99	45.2 ± 3.1	11d 5hr
A3	STS106	42.0 ± 4.9	11d 19hr
A4	STS100	42.0 ± 4.1	11d 21hr
A5	STS102	44.8 ± 6.3	12d 21hr
A6	STS92	42.3 ± 4.0	12d 19hr
A7	STS92	42.3 ± 4.0	12d 19hr
A8	STS103	42.6 ± 5.5	7d 23hr
A9	STS100	42.0 ± 4.1	11d 21hr
A10	STS104	42.4 ± 3.1	12d 18hr
A11	STS104	42.4 ± 3.1	12d 18hr
A12	STS104	42.4 ± 3.1	12d 18hr
A13	STS93	44.2 ± 3.9	4d 22hr
A14	STS88	41.3 ± 4.1	11d 16hr

Blood was sampled at three different time points: 10 days before launch (L-10), the day of landing (R-0), and 3 days after return (R+3). Pre- and post-flight samples were stored at -80°C until use.

Nuclear and MT-DNA Measurements

cf-mtDNA was isolated using the DNeasy Blood and Tissue kit from Qiagen according to the manufacturer's protocol (Qiagen, USA). Mitochondrial DNA (mtDNA) and nuclear DNA (nDNA) abundance was measured by real-time quantitative PCR (qPCR) using an Applied Biosystems 7900 Sequence Detection System (Applied Biosystems, USA). Primer pairs specific for mtDNA (MT-CO1 and MT-CO3) were designed to quantify mtDNA abundance, and human beta-globin primers were used for normalizing nDNA. Forward and reverse primer sequences are depicted below.

	Gene symbol	Species	Forward primer (5′-3′)	Reverse primer (5′-3′)
Mt-DNA abundance	MT-CO1	Human	GCCTCCGTAGACCTAACCATCTTC	GTAAGTTACAATATGGGAGATTATTCC
	MT-CO3	Human	ATGACCCACCAATCACATGC	ATCACATGGCTAGGCCGGAG
	GV1	Human	TTCACTAGCAACCTCAAACAG ACA	TGTCTCCACATGCCCAGTTTCT
	ACTB	Human	CTGGAACGGTGAAGGTGACA	AAGGGACTTCCTGTAACAATGCA

Thrombin plasma preparation for exosome precipitation

Exosomes were isolated from blood plasma samples of 3 astronauts (A9, A11, and A12) at L-10, R-0, R+3 using the ExoQuick Plasma prep and Exosome precipitation kit (Cat # EXOQ5TM, System Biosciences, CA, USA). In brief, 400 µl of plasma was mixed with thrombin and kept at room temperature (RT) for 5 minutes. Subsequently, samples were centrifuged at 10,000 rpm for 5 minutes. According to the manufacturer's protocol, the supernatant was collected into a new sterile microcentrifuge tube. Samples were then incubated with the exosome precipitation solution and refrigerated at 4°C for 30 minutes. After centrifugation at 1,500 x g for 30 minutes at 4°C, a beige-colored pellet was observed. Finally, the supernatant was aspirated, and the pellet was dissolved in 100 µl of sterile 1x PBS.

Exosomal DNA isolation

Exosomal DNA isolation was performed using the XCF Exosomal DNA isolation kit (Cat # XCF200S-1, System Biosciences, CA, USA). Isolated exosomes were dissolved in 1x PBS, and the final volume was adjusted to 500 µl. Next, the binding buffer was added to the PBS-dissolved

exosomes. Exosomes were centrifuged using a column provided by the manufacturer and further washed using the washing buffer. Exosomal DNA was eluted using the elution buffer, according to the manufacturer's protocol.

Exosome antibody array

The exosome antibody array was performed using the Exo-check exosome antibody arrays (Cat # EXORAY210A-8, System Biosciences, CA, USA). Briefly, isolated exosomes were quantified for protein using the BCA assay kit (Cat # 23225, Thermo scientific, Il, USA.). We used 50 µg of protein to incubate with the labeling reagent at RT for 30 minutes. Excess labeling reagent was removed according to the manufacturer's protocol. Labeled exosomes were blocked using a blocking buffer, and the membrane was exposed with exosomes facing up at 4º C overnight. The next day, the membrane was washed for 5 minutes at RT. The membrane was then incubated with the detection buffer at RT for 30 minutes. Subsequently, washing was done with wash buffer three times for five minutes at RT and developed using the chemiluminescence detection system (Clarity Western ECL substrate, cat # 170-5060S, Bio-Rad, USA).

Library preparation and small RNA sequencing

RNA quality was assessed using an Agilent TapeStation (Agilent, Palo Alto, CA, USA), and RNA concentration was quantified by Qubit 4.0 spectrophotometer. The library for small RNA sequencing was prepared using the Smarter smRNA-seq kit for Illumina (Takara Bio Inc., USA). The quantity and quality of amplified libraries were evaluated using Qubit (Invitrogen, Carlsbad, CA, USA) and Agilent TapeStation high sensitivity D1000 Screen Tape. Small RNA-seq libraries were sequenced using single-end 75 base pairs (PE75) sequencing chemistry on NextSeq 500 instruments following the manufacturer's protocols (Illumina).

Sequencing data analysis

Raw Fastq files were trimmed using cutadapt and built-in Illumina adapters. The quality of trimmed reads was assessed with FastQC, which is freely available at https://www.bioinformatics.babraham.ac.uk/projects/fastqc. Reads were aligned to human genome reference build GRCh38/hg38 with STAR aligner allowing for reads multi-mapping to different parts of the genome. GENCODE release 38 was used for gene and transcript annotations.

Raw counts were normalized by library size and transformed to log2 with edgeR package. The proportion of cf-mt-DNA vs. cf-nDNA gene expression was calculated as a ratio of total mt-DNA read counts to nDNA read counts. Differential expression of mt-DNA genes was assessed using limma.

Real-time quantitative reverse transcription PCR

We also isolated total RNA from peripheral blood mononuclear cells (PBMCs) of six astronauts pre-flight (L-10) and at two time points post-flight (R-0 and R+3). The real-time polymerase chain reaction was performed using SYBR green (Power up SYBR green master mix, cat # A25742, Applied Biosystem, USA) and QuantStudio™ 3 real-time PCR systems as recommended by the manufacturer. We measured the expression of genes encoding inflammation (*IL-6*, *IL-8*, *TNF-α, IL-1α, IL-β*), oxidative stress (*SOD1*, *SOD2*, *GPX1*, *NOX4*, *CAT1*, *NOXA1*, *SERPINE1*, *HMOX1*, *NOS2*, *NRF2*, *PRDX3*, *DUOX1*, *TMOD1*, *APOE*) and DNA damage markers (*OGG1*, *GADD153*, *GADD45a*, *PARP1*, and *DNAPK*). Please note, out of 14 astronauts, buffy coats were available only for individuals A7, A8, and A12. Forward and reverse primer sequences are depicted below.

Application	Gene symbol	Species	Forward primer (5′-3′)	Reverse primer (5′-3′)
RT-qPCR	APOE	Human	TGGGTCGCTTTTGGGATTACCT	AGGCCTTCAACTCCTTCATGGT
	CAT1	Human	CTTCTTGTTCAGGATGTGGTTTTCA	TACCTTTGCCTTGGAGTATTTGGTA
	DNAPK	Human	CAGGAGACCTTGTCCGCTG	AATACAAGCAAACCGAAATCTCTGG
	DUOX1	Human	AGAAATGCCAGCTGCCACTT	CCGCACATCTTCAACCAACACA
	GADD145A	Human	CGAAAGGATGGATAAGGTGGGG	GGATCAGGGTGAAGTGGATCTG
	GADD153	Human	CAGATGTGCTTTTCCAGACTGATC	TGATTCTTCCTCTTCATTTCCAGGA
	GAPDH	Human	CGACCACTTTGTCAAGCTCA	AGGGGAGATTCAGTGTGGTG
	GPX1	Human	AGGTACTACTTATCGAGAATGTGGC	TGAGGGAATTCAGAATCTCTTCGTT
	HMOX1	Human	GGTGATGGCCTCCCTGTACC	CTTGCGGTGCAGCTCTTCTG
	HSP60	Human	AGATGTAAAATTTGGTGCAGATGCC	CACACCATCTTTTGTTACTTTGGGA
	HSP70	Human	GTCCTAAGAATCGTTCAATTGGAGC	GCAACTGCACAATATCATATGCAAG
	HSP90	Human	CCCAGAGTGCTGAATACCCG	CTGTTTCCAGAGACAGAGTAGAGTG
	IL1A	Human	AAGAAGACAGTTCCTCCATTGATCA	CCTTGAAGGTAAGCTTGGATGTTTT
	IL1B	Human	ATGATGGCTTATTACAGTGGCAATG	ATCTTCCTCAGCTTGTCCATGG
	IL6	Human	ACAAGAGTAACATGTGTGAAAGCAG	ACTCTCAAATCTGTTCTGGAGGTAC
	IL8	Human	TTGCCAAGGAGTGCTAAAGAACTTA	AGCTCTCTTCCATCAGAAAGCTTTA
	NOXA1	Human	AACCATGATGCCAGGTCCCTAA	AGAGGAGCCTGTTTGCCAACTT
	NOS2	Human	ACACGTGCGTTACTCCACCA	GTCCCCTCTGATGCTGCCAT
	Nox4	Human	CTGTATAACCAAGGGCCAGAGTATC	TTATCCAACAATCTCCTGGTTCTCC

	NRF2	Human	CACTCACGTGCATGATGCCC	TGAGATGAGCCTCCAAGCGG
	OGG1	Human	ATCGTACTCTAGCCTCCACTCC	GTCTGAGTCAGTGTCCATACTTGAT
	PARP1	Human	GGATAAGCTCTATCGAGTCGAGTAC	CTTCCAGAAGCAGGAGAAGTGGTAC
	PRDX3	Human	TCAACGATCTCCCAGTGGGC	AGCAGCTGGACTTGGCTTGA
	SERPINE1	Human	ACATTCTGAGTGCCCAGCTCAT	ACATGTCGGTCATTCCCAGGTT
	SOD1	Human	CATCATCAATTTCGAGCAGAAGGAA	ATAGAGGATTAAAGTGAGGACCTGC
	SOD2	Human	CACATCAACGCGCAGATCATG	GATATGACCACCACCATTGAACTTC
	TMOD1	Human	GAGGAAGCCTTGGCAAATGCTT	AATTTGGTTCTTCGTCGGGCAC

DNA/RNA Oxidative Damage

DNA/RNA oxidative damage was measured in PBMCs isolated from 6 astronauts (L-10, R-0) and 5 astronauts (R+3) using the DNA/RNA Oxidative Damage (High Sensitivity) ELISA Kit (Cayman Chemical, USA). This competitive assay can be used to measure all three OxGua species: 8-hydroxyguanosine (8-OHG), 8-hydroxy-2’-deoxyguanosine (8-OHdG), and 8-hydroxyguanine. The antibody recognizes damaged nucleic acid species and binds to the goat polyclonal anti-mouse IgG previously attached to the well. The plate was washed to remove any unbound reagents, after which Ellman's Reagent was added to the well. The product of this enzymatic reaction has a distinct yellow color and absorbs strongly at 412 nm. The intensity of this color, determined spectrophotometrically, is inversely proportional to the amount of free 8-OHdG present in the well during the incubation.

Statistical analysis

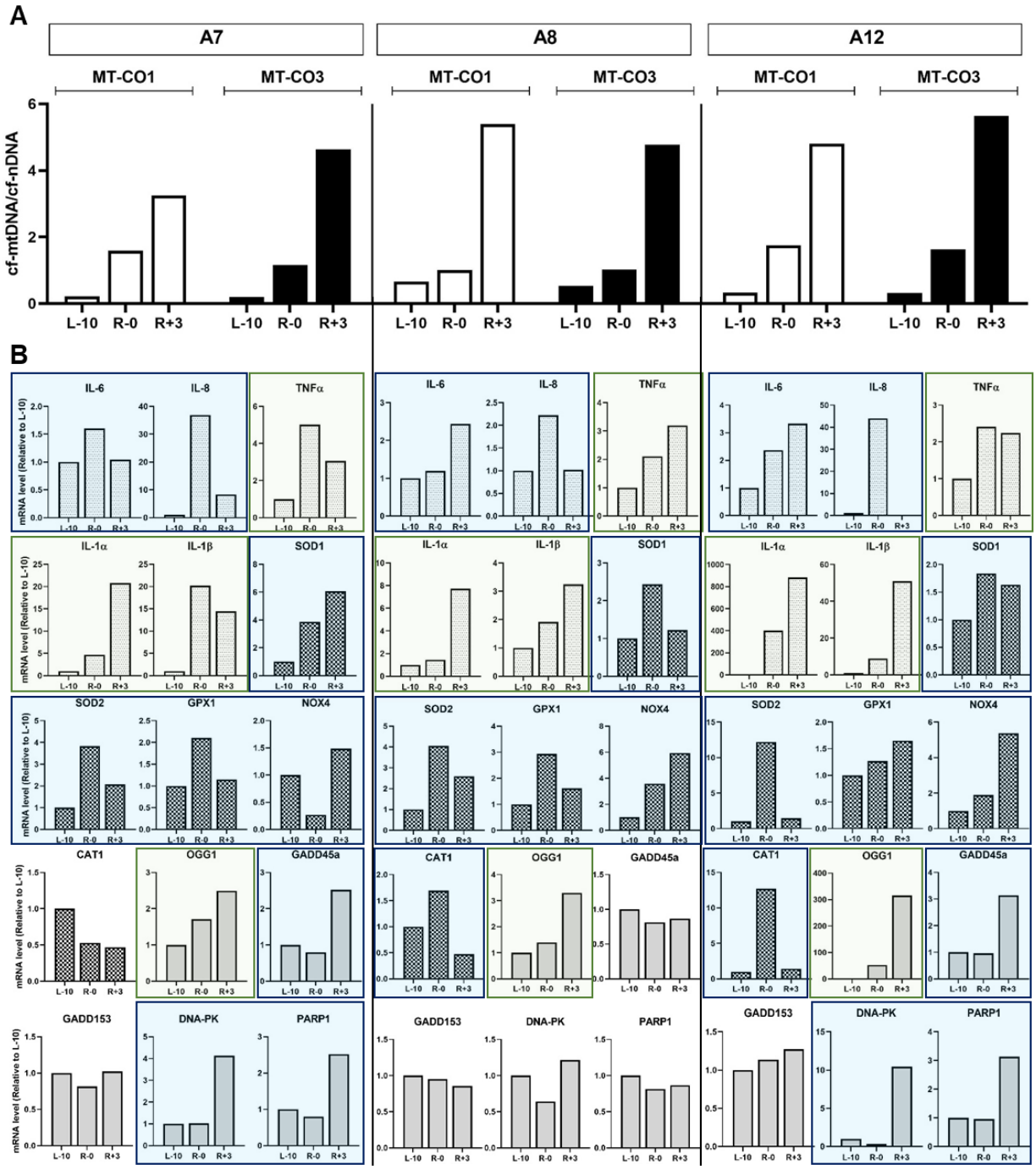
Results are presented as mean ± standard error of the mean (SEM) and were analyzed using a paired t-test for comparisons between means, or one-way ANOVA for repeated measures using the mixed-effects model followed by a Tukey post hoc test for multiple comparisons. Statistical analysis was performed using GraphPad Prism 6, version 6.07 (GraphPad Software, Inc., La Jolla, CA, USA). Differences were considered statistically significant at $p < 0.05$.

Table S1. List of differentially regulated genes.

Gene name	Fold Change								
	A9			A11			A12		
	L-10	R-0	R+3	L-10	R-0	R+3	L-10	R-0	R+3
MT-TF	11.91	11.72	11.18	12.58	9.99	11.24	11.27	10.78	10.45
MT-RNR1	14.77	14.59	14.76	14.59	14.94	14.63	14.41	14.82	14.9
MT-TV	14.24	13.87	14.23	14.42	10.92	13.48	14.22	13.98	12.01
MT-RNR2	19.57	19.61	19.45	19.42	17.79	17.77	19.46	19.7	18.02
MT-TL1	13.18	12.98	12.93	13.52	11.22	12.43	13	12.77	11.19
MT-ND1	12.99	13.23	13.28	12.07	13.47	13.64	12.76	13.6	13.34
MT-TI	9.91	9.89	8.84	9.47	8.12	8.96	10.03	9.79	8.53
MT-TQ	12.04	11.27	11.29	13.08	11.67	10.82	11.96	11.72	11.59
MT-TM	10.59	11.18	11.23	11.9	10.78	11.22	10.8	11.13	11.01
MT-ND2	14.8	15.23	14.89	14.89	15.33	15.21	16.32	15.57	15.37
MT-TW	11	10.66	11.09	11.49	11.13	12.03	10.44	11.05	11.45
MT-TA	11.96	10.21	11.19	11.75	10.27	11.55	10.85	11.22	9.74
MT-TN	10.66	10.21	10.49	11.28	10.59	14.27	10.63	10.76	10.36
MT-TC	11.44	11.01	11.22	11.12	10.08	11.1	11.18	10.97	10
MT-TY	10.65	10.14	10.19	11.4	9.43	9.49	11.37	10.93	9.74
MT-CO1	13.6	14.61	14.35	12.29	14.37	14.45	13.71	13.68	14.21
MT-TS1	13.62	13.04	12.98	14.42	12.25	13.02	14.45	14.11	12.39
MT-TD	12.12	11.42	12.3	12.36	9.45	11.56	12.06	11.58	10.11
MT-CO2	11.94	12.76	12.64	10.44	13.4	13.75	11.91	12.5	13.16
MT-TK	12.78	12.6	13.08	13.79	11.64	12.91	13.54	13.2	12.52
MT-ATP8	12.75	13.64	14.36	11.74	14.62	13.36	12.64	12.74	14.27
MT-ATP6	12.43	13.34	14.13	11.29	14.28	14.59	12.03	12.34	13.5
MT-CO3	13.67	14.31	13.83	12.93	15.04	13.63	13.64	13.89	14.97
MT-TG	15.62	15.23	15.01	15.93	11.37	12.41	15.77	15.42	12.21
MT-ND3	12.03	12.99	13	10.39	12.27	12.49	12.35	12.6	12.17
MT-TR	12.04	11.58	12.28	12.71	9.79	11.83	11.3	10.8	10.79
MT-ND4L	13.25	14	13.08	10.22	13.12	11.62	12.3	12.93	13.1
MT-ND4	13.83	14.68	14.54	12.85	14	14.18	13.71	15.36	13.94
MT-TH	11.46	11.17	12.18	12.16	10.74	12.38	11.64	11.49	10.98
MT-TS2	10.68	10.55	10.91	12.64	9.35	11.52	11.61	11.21	10.38
MT-TL2	14.7	13.76	13.66	15.15	10.37	12.21	14.09	13.94	11.5
MT-ND5	14.56	15.61	15.55	13.03	15.18	15.13	15.47	14.81	15.18
MT-ND6	11.94	13.12	13.08	10.28	12.98	12.83	12.04	15.26	12.67
MT-TE	11.37	11.48	11.15	12.56	10.48	12	12.06	11.91	11.08
MT-CYB	13.31	14.05	14.01	11.87	13.62	13.59	13.42	13.42	13.5
MT-TT	11.8	12.06	12.17	12.13	10.21	11.33	12.24	11.8	10.39
MT-TP	11.96	11.72	11.72	12.34	10.24	11.71	12.24	12.14	11.27

Gene expression of mt-DNA-encoded genes between control (L-10) and post-spaceflight (R0, R+3). Data are shown as fold change.

Figure S1. Comparison of cf-mtDNA and transcript levels of stress markers in PBMCs from three astronauts.



(A) Levels of cf-mtDNA at R0 and R+3 days in A7, A8, and A12 individuals. (B) Transcript levels of inflammatory markers (IL-1α, IL-1β, TNFα), and DNA damage markers (OGG1) follow similar trends to cf-mtDNA in 3 individual astronauts (light green background), whereas IL-6, IL8, SOD1, SOD2, GPX1, NOX4, GADD45, CAT1, DNA-PK, and PARP1 in at least one of the post-flight time points (light blue background).