

## 1 **Cell-free DNA (cfDNA) and exosome profiling from a year-long human** 2 **spaceflight reveals circulating biomarkers**

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48 Station, NASA Twins Study

49

## 50 **Abstract**

51 The health impact of prolonged space flight on the human body is not well understood. Liquid  
52 biopsies based on cell-free DNA (cfDNA) or exosome analysis provide a noninvasive approach to  
53 monitor the dynamics of genomic, epigenomic and proteomic biomarkers, and the occurrence of  
54 DNA damage, physiological stress, and immune responses. To study the molecular consequences  
55 of spaceflight we profiled cfDNA isolated from plasma of an astronaut (TW) during a year-long  
56 mission on the International Space Station (ISS), sampling before, during, and after spaceflight,  
57 and compared the results to cfDNA profiling of the subject's identical twin (HR) who remained  
58 on Earth, as well as healthy donors. We characterized cfDNA concentration and fragment size,  
59 and the positioning of nucleosomes on cfDNA, observing a significant increase in the proportion  
60 of cell-free mitochondrial DNA inflight, suggesting that cf-mtDNA is a potential biomarker for  
61 space flight-associated stress, and that this result was robust to ambient transit from the  
62 International Space Station (ISS). Analysis of exosomes isolated from post-flight plasma revealed  
63 a 30-fold increase in circulating exosomes and distinct exosomal protein cargo, including brain-  
64 derived peptides, in TW compared to HR and all known controls. This study provides the first  
65 longitudinal analysis of astronaut cfDNA during spaceflight, as well as the first exosome profiles,  
66 and highlights cf-mtDNA levels as a potential biomarker for physiological stress or immune  
67 system responses related to microgravity, radiation exposure, and other unique environmental  
68 conditions on the ISS.

69

## 70 **Introduction**

71 A wide range of physiological effects impact the human body during a prolonged stay in  
72 microgravity, such as headward fluid shift, atrophy of muscles, and decreases in bone density,  
73 which have been described for astronauts on the international space station (ISS)(Williams et al.,  
74 2009). In recent years, an increasing number of government and private space agencies have  
75 formed, and missions to the Moon and Mars are now planned for the late 2020s and 2030s (Iosim  
76 et al, 2020). These pending missions may span 30 months and require landing on a planet with  
77 almost no clinical infrastructure for medical monitoring or treatments. Yet, data on physiological  
78 changes of long-term missions (>6 months) is almost non-existent. These long-duration missions  
79 and the increasing exposure of humans to spaceflight-specific conditions necessitates the study of  
80 molecular changes in the human body induced by exposure to spaceflight stressors such as  
81 microgravity, radiation, noise, restricted diet, and reduced physical work opportunities. The NASA  
82 Twins study (Garrett-Bakelman et al., 2019) enabled interrogation of the impact of prolonged  
83 spaceflight on the human biology and cell-to-cell variations in the immune system (Gertz et al.,  
84 2020); however, there has never been a study on the impact of spaceflight on cell-free DNA  
85 (cfDNA).

86

87 Molecular signatures informative of human health and disease can be found in cfDNA and nucleic  
88 acids isolated from plasma, saliva, or urine (Heitzer et al., 2018; Hummel et al., 2018; Siravegna  
89 et al., 2017; Verhoeven et al., 2018; Volik et al., 2016). Non-invasive methods for monitoring  
90 health-related biomarkers in liquids such as plasma ('liquid biopsy') have already been  
91 successfully introduced in a wide range of contexts, including: prenatal testing for detection of  
92 trisomy and micro-deletions (Bianchi et al., 2014; Zhang et al., 2019), cancer diagnostics  
93 (Bettegowda et al., 2014; Diehl et al., 2008; Wang et al., 2017), monitoring of cancer therapies

94 (Birkenkamp-Demtröder et al., 2016; Wan et al., 2020), monitoring of the health of solid-organ  
95 transplants (Verhoeven et al., 2018; De Vlaminck et al., 2014), and screening for infections  
96 (Blauwkamp et al., 2019; Burnham et al., 2018; De Vlaminck et al., 2013). Hence, liquid biopsy  
97 is a potentially useful method for monitoring physiologic conditions of astronauts before, during  
98 and after spaceflight.

99

100 Indeed, cfDNA is extremely dynamic and responsive, providing strong indicators of DNA damage  
101 and tumor growth in distal tissues (Newman et al., 2016), immune response or infection (Zwirner  
102 et al., 2018), and RNA regulatory changes, with an innate capacity to reveal the cells of origin  
103 undergoing apoptosis or necrosis (Thierry et al., 2016). Various studies have reported changes in  
104 cfDNA concentration (Zwirner et al., 2018), cfDNA fragment length distribution (Mouliere et al.,  
105 2011; Underhill et al., 2016), mutation profiles and signatures (Newman et al., 2016), and cfDNA  
106 methylation (Shen et al., 2018) indicative of physiological conditions such as cancer.  
107 Mitochondrial DNA (mtDNA) can also be found in the extracellular space, circulating as short  
108 DNA fragments, encapsulated in vesicles and even as whole functional mitochondria (Amir Dache  
109 et al., 2020; Song et al., 2020). Several recent studies observed increased levels of cell-free  
110 mitochondrial DNA (cf-mtDNA) in psychological conditions (Lindqvist et al., 2016, 2018) and  
111 reduced cf-mtDNA levels in Hepatitis B infected patients associated with a higher risk of  
112 developing hepatocellular carcinoma (Li et al., 2016). However, since no such information exists  
113 for using these metrics for astronauts, we investigated the utility of cfDNA for the monitoring of  
114 the physiologic conditions of astronauts to spaceflight.

115

116 Of note, cfDNA comprises the footprints of nucleosomes, and these nucleosome features enable  
117 tracing of the tissue-of-origin for cfDNA in normal and disease states, through analysis of nuclear  
118 architecture, gene structure and expression (Murtaza and Caldas, 2016; Snyder et al., 2016). In  
119 particular, nucleosome positioning and depletion of short cfDNA sequences reveal footprints of  
120 transcription factor binding, promoter activity, and splicing, ultimately informing gene regulatory  
121 processes in the tissue/cell of origin (Snyder et al., 2016). Similar information can be revealed  
122 from exosomes, which are nano-sized vesicles (size 30–150nm) derived from perinuclear luminal  
123 membranes of late endosomes/multivesicular bodies and released into extracellular environment  
124 via multivesicular body fusion within the cell membrane ((Kalluri and LeBleu, 2020; Mathieu et  
125 al., 2019)) that can mediate long-range physiological crosstalk (Hoshino et al., 2015; Mathieu et  
126 al., 2019). Exosomes act as vehicles for horizontal transfer of information through their cargo:  
127 proteins, lipids, metabolites and DNA, as well as coding and non-coding RNAs (Valadi et al.,  
128 2007; Wortzel et al., 2019). Moreover, exosomes can be powerful mediators of responses to  
129 environmental stimuli as external and physiological stress impact their release, cargo and function,  
130 contributing to pathogenesis (Harmati et al., 2019; O'Neill et al., 2019; Qin et al., 2020). Since  
131 exosomes are abundant in plasma, they are critical components of liquid biopsies (Colombo et al.,  
132 2014; Hoshino et al., 2020) and analysis of their content can complement the information obtained  
133 from cfDNA, but there is no information about exosomes in astronauts.

134

135 To address this gap in knowledge, we profiled cfDNA isolated from plasma samples before,  
136 during, and after the one-year mission on the International Space Station (ISS) to evaluate the  
137 utility of cfDNA as a means to monitor physiological problems during extended missions in space.  
138 We also profiled the exosomes of both astronauts after the mission completion. While bulk RNA  
139 sequencing data have shown widespread gene expression changes in astronauts, including

140 mitochondrial RNA (mtRNA) spikes in flight samples from the One-Year Mission (Garrett-  
141 Bakelman et al., 2019), there has not yet been a study of astronauts that has leveraged cfDNA and  
142 exosomes. We focused on quantitative measures such as the levels of mitochondrial DNA, cfDNA  
143 fragment length, and the depletion of nucleosome signatures at transcription start sites. Together,  
144 our NGS results provide a “whole-body molecular scan”, which can provide a novel measurement  
145 of the impact of spaceflight on the human body, as well as serve as a continued metric of  
146 physiology and cellular stress for future long-duration missions.

147

## 148 **Results**

### 149 *Study design and sample collection*

150 We analyzed circulating cfDNA of a pair of male monozygotic twins over two years, starting when  
151 they were both 50 years old. During the NASA Twin Study, the flight subject (TW) was aboard  
152 the International Space Station (ISS) for 340 days, while his identical twin, the ground subject  
153 (HR), remained on Earth. We collected cfDNA at 12 time points from HR and 11 time points from  
154 TW. Of the latter, four samples were collected in-flight on board of the ISS or space shuttle. In  
155 addition, we profiled the cfDNA of an unrelated control subject (MS) to simulate the ambient  
156 return from the ISS. To control for ambient return (AR) effects (return of samples in the Soyuz  
157 capsule) on the molecular signatures of cfDNA, we subjected two MS samples and one HR sample  
158 to an extended shipping procedure (see Methods). Plasma and cfDNA were extracted using the  
159 same protocol for all samples (Methods). We observed a broad range of cfDNA concentration  
160 between 6.7 ng/ml and 79.9 ng/ml plasma (mean = 27.9 ng/ml, median = 23 ng/ml) across samples  
161 (**Table 1**). However, we found no significant difference in cfDNA concentrations between flight,  
162 ground or control subjects (ANOVA  $p = 0.49$ , **Supp. Fig. 1A**), TW and HR (Wilcoxon rank test  $p$   
163 = 0.65), and flight and ground samples (Wilcoxon rank test  $p = 0.352$ ). TW showed borderline  
164 significantly higher cfDNA concentration pre- and post-flight compared to in-flight (Wilcoxon rank  
165 test  $p = 0.043$ ), however, this is not significant when comparing TW in-flight, TW ground, and  
166 HR/MS ground (ANOVA  $p = 0.4$ , **Supp. Fig 1B**). Complementary metadata on the health status  
167 of TW and HW during the mission has been previously published (Garret-Bakelman et al., 2019),  
168 and no deviations in medication or exercise regimen were noted in the medical records.

169

### 170 *Cf-DNA fragment length distribution is influenced by the ambient return*

171 It has previously been shown that cfDNA derived from tumor cells is shorter than cfDNA derived  
172 from healthy cells (Jiang et al., 2015; Mouliere et al., 2011). This effect can be explained by a  
173 change in nucleosome binding or by a degradation of nucleotides at the end of nucleosome loops.  
174 We therefore hypothesized that environmental stressors such as microgravity or radiation could  
175 also impact the length distribution of cfDNA. Indeed, we found a slight shift to longer cfDNA  
176 fragment lengths in TW in-flight samples (**Fig. 1A**). However, a similar shift was observed in  
177 ground samples subjected to ambient return simulation (**Fig. 1A**, boxplots with yellow border).  
178 Ambient return samples show a similar peak at the 300 to 400bp fragment length, which is only  
179 marginally visible for fresh samples (**Fig. 1B**). Thus, some proportion of long cfDNA fragments  
180 likely originate from blood cells damaged during return flight or transport from the ISS.

181

182 To examine how this might affect other cfDNA fractions, we next examined cell-free  
183 mitochondrial DNA (cf-mtDNA). Recent studies indicate that a prominent fraction of cf-mtDNA  
184 in the plasma is contained within intact, circulating mitochondria (Al Amir Dache, 2020) and that  
185 larger mtDNA fragments can also arise from blood cell degradation. However, our centrifugation

186 step largely removed intact mitochondria and our library preparation comprised mostly smaller  
187 DNA fragments (at least 75% are <350bp )(Supplemental Fig. 2), including an even smaller  
188 fraction (<10%) of the aligned reads (Fig. 2). Thus, the observed fractions of cf-mtDNA are mostly  
189 derived from shorter cf-mtDNA molecules and should represent cf-mtDNA that is randomly  
190 fragmented and sequenced across the entire mitochondria.

191

192 As further evidence of this, the mitochondrial genome showed continuous read coverage in all  
193 samples, ranging from 50x-200x coverage (Supplemental Fig. 3), regardless of the collection  
194 method. Indeed, the length distribution of cf-mtDNA is not affected by ambient return as observed  
195 for chromosomal cfDNA (Fig. 2B), and the average length does not change significantly in inflight  
196 samples or AR simulation samples. Even though cf-mtDNA amounts can significantly vary based  
197 on the donor profiles (Lindqvist et al, 2016) and degree intact vs. fragmented mitochondria, these  
198 NGS data showed that the total cf-mtDNA profiles show relative uniformity in both length and  
199 proportion of reads (Fig. 2).

200

#### 201 *Levels of cell-free mitochondrial DNA are increased during space flight*

202 Next we investigated the fraction of cf-mtDNA relative to chromosomal cfDNA in plasma of  
203 TW, HR, and MS. In order to characterize the cfDNA originating from mitochondria during  
204 spaceflight, we normalized the count of NGS reads mapping to the mitochondrial chromosome  
205 (chrM) by chromosome length and the total number of reads in the library, generating a RPKM  
206 measurement. For comparison, we performed the same procedure with reads mapping to  
207 chromosome 21. We found a sharp increase of cf-mtDNA for subject TW for inflight samples  
208 (Fig. 3A) compared to TW ground samples (Wilcoxon rank test  $p=0.012$ ), compared to HR  
209 ground samples (Wilcoxon rank test  $p=0.018$ ), and compared to all ground samples of HR and  
210 TW (Wilcoxon rank test  $p=0.0045$ , ANOVA  $p=0.00049$ ). In contrast, we found no significant  
211 increase in cfDNA mapping to chromosome 21 (Fig. 3B) in TW-inflight compared to ground  
212 samples of TW and HR.

213

214 Notably, the mtDNA levels in whole blood increased steadily inflight while on the ISS. Indeed,  
215 TW had the highest fraction of cf-mtDNA within the first inflight timepoint (T4), including more  
216 than a 24-fold increase, when compared to ground samples (Fig. 3C, 3D). In the two later  
217 inflight time points, he had 4- and 8-fold increases compared to pre-flight levels. The normalized  
218 levels of chromosome 21 cfDNA were stable for both TW and HR for the duration of the  
219 mission (0.25-0.26 RPKM), revealing no obvious bias due to sample handling (Fig. 3D).  
220 Interestingly, a positive correlation between mtDNA copy number and telomere length in healthy  
221 adults has been previously reported, and telomere elongation in blood and urine was also  
222 observed during spaceflight for TW (Garrett-Bakelman et al., 2019, Luxton et al, 2020).

223

224 Given the previously discussed effects of AR on cfDNA lengths, we tested for potential bias in  
225 cf-mtDNA levels due to AR. To do this, we compared the cf-mtDNA fraction observed in the  
226 MS simulated-AR samples (2 samples) to the MS control sample. We found that cf-mtDNA  
227 levels were actually lower in AR than in FR samples (Fig. 3E), suggesting that the shipping  
228 procedure from the ISS is likely not causing the observed increase in cf-mtDNA levels seen in  
229 the inflight samples. In addition, the AR simulation of the ground subject (HR) did not show a  
230 significant increase of cf-mtDNA levels compared to other HR samples (Fig. 3F). Thus, these

231 data suggest that the cf-mtDNA fraction was significantly increased during space flight, and not  
232 due to the AR blood-from-ISS transport process.

233

234 *Nucleosome positioning suggests a shift in cell of origin of cfDNA due to transport conditions*

235 Given that nucleosome positions are associated with both cfDNA and gene expression (Jiang and  
236 Pugh, 2009), we computed the nucleosome depletion around nucleosomes at transcription start  
237 sites (TSS) to infer gene expression (**Fig. 4A**), as previously demonstrated by Ulz and colleagues  
238 (Ulz et al., 2016). Indeed, these data indicated that the strength of nucleosome depletion is  
239 correlated to bulk gene expression from RNA-seq of the same subjects (Garrett-Bakelman et al.,  
240 2019) (**Fig. 4A**), with a decreased coverage at the site of the transcriptional start site (TSS) for  
241 highly expressed genes. Second, we identified the nucleosome footprint of CTCF in gene bodies,  
242 hypothesizing that nucleosome positioning patterns could reveal broad changes in gene  
243 regulation during spaceflight. A t-SNE analysis of TW and HR samples showed no flight-  
244 specific clustering (**Fig 4B**), indicating that nucleosome positioning identified through cfDNA  
245 may not be sensitive enough to identify spaceflight-related gene expression changes.

246

247 However, based on the correlations between per-tissue gene expression values (Kim et al., 2014)  
248 and nucleosome positioning observed on cfDNA, clear tissue signals in cfDNA were inferred for  
249 all plasma samples. Higher values (Pearson's correlation coefficient) suggested higher gene  
250 expression and stronger tissue signal (**Fig. 5A**) for hematopoietic lineages (up to  $\rho = 0.156$ ,  $n =$   
251 1087411335), mid-range for liver, adrenal gland, and the retina (0.04-0.07) and less so for other  
252 peripheral tissues (e.g. lung, esophagus, 0.00-0.01). These results are consistent with the  
253 expected cfDNA prevalence in blood and with previous findings (Snyder et al., 2016). Despite  
254 such clear signals on tissue of origin, strong clustering of samples was observed, due to the  
255 confounding effect of ambient return. This was seen in both the tissue-of-origin analysis (**Fig.**  
256 **5A**) as well as TSS protection (**Fig. 5B**), highlighting the need for controls and correction for any  
257 degradation. Further, this analysis does not take into account the cf-mtDNA reads, and therefore  
258 may not reflect the tissue of origin for mitochondrial reads or heteroplasmy.

259

260 *Analysis of plasma-circulating exosomes post-flight*

261 To determine how prolonged space missions and Earth re-entry impact circulating exosomes, we  
262 analyzed exosomes from the plasma of TW three years post-return to Earth, and compared their  
263 size, number and proteomes to plasma-derived exosomes isolated from HR and 6 age-matched,  
264 healthy controls. Exosomes were isolated by differential ultracentrifugation and both the size and  
265 number of exosomes were characterized by nanoparticle tracking analysis (NTA) (**Fig. 6A-E**).  
266 While the median size of exosomes was similar between HR, TW and healthy controls (**Fig. 6 A-**  
267 **D**), the number of particles was ~30 times higher in TW compared to HR and healthy controls  
268 (**Fig. 6 E**). Proteomic mass spectrometry analysis revealed that TW, HR and control exosomes  
269 packaged similar numbers of proteins, including a total of 191 exosomal proteins shared among  
270 all samples. HR's exosome catalog contained 26 unique proteins, TW exosomes contained 61  
271 unique proteins, and healthy controls contained 105 unique proteins (**Fig. 6F**).

272

273 Hierarchical clustering of the exosomal proteins revealed distinct signatures of HR and TW, which  
274 clustered apart from the six controls. Interestingly, classification of the pathways using  
275 Metascape (GO processes, KEGG pathways, Reactome gene sets, canonical pathways, and  
276 CORUM complexes)(Zhou et al., 2019) revealed that TW exosomes were enriched in proteins

277 involved in proteasome pathways (**Fig. 6H**). TW exosomes also packaged CD14, a pro-  
278 inflammatory monocyte marker, consistent with the increase in CD14<sup>+</sup> monocytes observed post-  
279 return to gravity in immune markers studied upon return to Earth (Gertz et al, 2020). Notably,  
280 basigin and integrin  $\beta$ 1 proteins, which are correlated with cancer progression and inflammation  
281 (Hoshino et al., 2015, 2020; Keller et al., 2009; Yoshioka et al., 2014), were also detected in TW  
282 exosomes, but not in HR or healthy control exosomes.

283

284 Consistent with previous findings demonstrating microgravity downregulating adaptive  
285 immunity, particularly B cells (Cao et al., 2019), both TW and HR exosomes contained fewer  
286 immunoglobulins compared to healthy controls (**Fig. 6G**). Surprisingly, two brain-specific  
287 proteins, Brain-specific angiogenesis inhibitor 1-associated protein 2 (BAIAP2) and Brain-  
288 specific angiogenesis inhibitor 1-associated protein 2-like protein 1 (BAIAP2L1), were found in  
289 TW plasma-derived exosomes (**Supplemental Table 1, Supplemental Fig. 4A**), yet were not  
290 detected in the plasma of HR or healthy controls. In contrast, HR exosomal cargo was enriched  
291 in proteins associated with regulation of apoptotic pathways (Théry et al., 2001) and ATP  
292 biosynthesis (**Fig. 6I**). Moreover, we observed that the 20S proteasome, but not the regulatory  
293 19S proteasome, is found uniquely associated with the plasma-circulating exosomes in the flight  
294 subject (TW) 3 years after his return to Earth (**Fig. 6G**). Finally, both TW and HR exosomes, but  
295 not controls, were enriched in specific components of the humoral immune response and  
296 leukocyte migration, including the CD53 tetraspanin (**Supplemental Table 2, Supplemental**  
297 **Fig. 4B**) which could reflect either biology shared by the twins or changes associated with travel  
298 to space; however, analysis of plasma exosome samples from genetically unrelated astronauts  
299 would be required to distinguish between these possibilities.

300

## 301 **Discussion**

302 Our study focused on cfDNA and exosomes collected during the NASA Twins study, a  
303 longitudinal, multi-omic experiment examining the effects of long-term spaceflight on the human  
304 body. In particular, we revealed cf-mtDNA fraction to be a potential new biomarker of  
305 physiological stress during prolonged spaceflight, though the total cfDNA concentration is not  
306 significantly correlated with spaceflight. We further observed unique exosome and exosomal  
307 protein signatures within TW several years after the year-long mission, including an increased  
308 amount of exosomes and brain-specific proteins (BAIAP2 and BAIAP2L1). Of note, we identified  
309 multiple biases likely caused by ambient return (AR) blood draws from the ISS, including results  
310 of tissue of origin deconvolution through nucleosome positioning as well as cfDNA fragment  
311 length. As such, future studies will need to control for AR affects if they wish to examine these  
312 molecular dynamics. As an example, DNA could be extracted in space (Castro-Wallace *et al.*,  
313 2017) and either cryopreserved to increase its stable during transport or directly sequencing inflight  
314 to minimize biases and obtain results faster (McIntyre *et al.*, 2016, McIntyre *et al.*, 2019).

315

316 Interestingly, analysis of plasma exosomes isolated post-return to Earth revealed unique  
317 alterations in TW relative to HR and healthy controls, such as a dramatic increase in the number  
318 of circulating particles as well as changes in the types of protein cargo. Since the majority of  
319 plasma circulating exosomes are derived from immune cells, it is likely that these alterations  
320 reflect immune dysfunction associated with space travel and return to gravity. Specifically, the  
321 reduction in TW exosomal immunoglobulin levels and the presence of CD14, a macrophage  
322 marker, may signal a shift towards innate immunity, as even short-term chronic exposure to

323 cosmic radiation and microgravity leads to a decrease in adaptive immune cells (Cao et al., 2019;  
324 Fernandez-Gonzalo et al., 2017). However, circulating exosomes also reflect systemic changes in  
325 homeostasis and physiology, as demonstrated by the packaging of brain-specific proteins in TW  
326 which were not seen in control exosomes, which may indicate long-term altered expression of  
327 exosomes from the brain after spaceflight. Previous studies had shown that microgravity affects  
328 tight junction protein localization within intestinal epithelial cells (Alvarez et al., 2019). It is  
329 conceivable that prolonged space travel could exert similar effects on tight junctions within the  
330 blood-brain barrier, allowing for more exosomes to enter the peripheral blood.

331  
332 One remarkable finding of our study is that the 20S proteasome, but not the regulatory 19S  
333 proteasome, is found uniquely associated with the plasma-circulating exosomes in the flight  
334 subject 3 years post his return to Earth. Recent research has discovered the ubiquitin-independent  
335 proteolytic activity of the 20S proteasome and its role as the major degradation machinery under  
336 oxidizing conditions (Aiken et al., 2011; Deshmukh et al., 2019; Pickering and Davies, 2012).  
337 Elevated levels of 20S proteasome have been detected in the blood plasma from patients with  
338 various blood cancers, solid tumors, autoimmune diseases and other non-malignant diseases  
339 (Deshmukh et al., 2019; Sixt and Dahlmann, 2008). It is also reported that active 20S  
340 proteasomes within apoptotic exosome-like vesicles can induce autoantibody production and  
341 accelerate organ rejection after transplantation (Dieudé et al., 2015), reduce the amount of  
342 oligomerized proteins (Schmidt et al., 2020).and reduce tissue damage after myocardial injury  
343 (Lai et al., 2012), and are correlated with cancer and other pathological status such as viral  
344 infection and vascular injury (Dieudé et al., 2015; Gunasekaran et al., 2020; Tugutova et al.,  
345 2019). The elevated circulating exosomal 20S proteasome in the flight subject may reflect the  
346 increased physiological need to clear these proteins resulting from long-term blood, immune or  
347 other physiological disorders caused by various stress factors during the flight or return to  
348 gravity (Ben-Nissan and Sharon, 2014, Vernice et al, 2020). Study of plasma exosomes obtained  
349 from flight subjects at other time points including pre- and inflight will be necessary to further  
350 examine whether plasma exosomal proteasome can serve as biomarker for pathological  
351 processes associated with space flight.

352  
353 There are limitations in the study design that prevent broad biological conclusions. First, the  
354 sample number is too small to control for all types of potential biases and results may be  
355 somewhat driven by individual health issues. Second, there is no comparable experimental data  
356 to date and the effect of return to gravity in the Soyuz capsule on the integrity of the sampled  
357 material is unknown. Third, the exosome samples have been taken post-flight and can only  
358 inform about long-term effects of extended spaceflight. However, this study stands as a  
359 demonstration of the applications and possibilities of utilizing cfDNA and exosome profiling to  
360 monitor astronaut health and can improve the study design of future missions and research  
361 (Iosim et al, 2019, Nangle et al, 2020).

362  
363 In summary, we identified cell-free mitochondrial DNA (cf-mtDNA) as a novel biomarker of  
364 physiological stress during prolonged spaceflight, which is stable even during transport from the  
365 ISS. However, we demonstrated that transport-induced biases for cell-type deconvolution from  
366 cfDNA needs to be improved in order to be used as a “molecular whole body scan”, and/or  
367 deployment of more real-time methods (e.g. inflight sequencing). Also, we observed that  
368 exosome concentration in plasma and unique exosomal proteins such as 20S proteasomes, CD14,



369 and BAIAP2 demonstrate characteristic changes in the flight subject (TW), potentially caused by  
370 physiological stress during prolonged spaceflight. Overall, these data and methods provide novel  
371 metrics and data types that can be used in planning for future types of astronaut health  
372 monitoring, as well as help establish non-invasive molecular tools for tracking the impact of  
373 stress and spaceflight during future missions.

374

375

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387

### 388 **Disclosure Statement**

389 S.M.B. is a cofounder and Scientific Advisory Board member of KromaTiD, Inc., CEM is a  
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393

### 394 **Author Contributions**

395 CEM, DB and DCL conceived the study, DB, CM, EA, SO, FAV, HZ, IM, KG and PB and, wrote  
396 the manuscript DB,BS, FEG, DBU, DPK, KHY, KN, TL, VR, FAV sample collection and/or  
397 processing DB, CM, SO, HZ, IM, JF, KG and PB Bioinformatic and Analytics, AM, BS, CEM,  
398 CM, CW, EA, FEG, IV, MC, MPS, RKB, SL, SO and TM review manuscript and guided  
399 interpretation. All authors read and approved the manuscript.

400

401

### 402 **Methods**

403

#### 404 *Sample collection*

405 In the NASA Twin Study spanning 24 months we collected blood samples at 12 time points from  
406 the twin on earth (HR) and 11 time points from the twin in space (TW), as previously  
407 described(Garrett-Bakelman et al., 2019). From TW, samples were collected before the flight  
408 (PRE-FLIGHT), during the flight (FLIGHT) and after the flight (POST-FLIGHT). Specimens  
409 were processed as previously described(Garrett-Bakelman et al., 2019). Briefly, whole blood was  
410 collected in 4mL CPT vacutainers (BD Biosciences Cat # 362760,) per manufacturer's  
411 recommendations, which contained 0.1M sodium citrate, a thixotropic polyester gel and a FICOLL  
412 Hypaque solution. Hence our specimens were not exposed to heparin. Samples were mixed by  
413 inversion. Samples collected on ISS were stored at 4°C after processing and returned by the Soyuz  
414 capsule. There was an average of 35-37 hours from collection to processing, including repatriation

415 time. Plasma was obtained by centrifugation of the CPT vacutainers at 1800 X g for 20 minutes at  
416 room temperature, both for the ISS and for the ground-based samples. Finally, plasma was  
417 collected from the top layer in the CPT vacutainer and flash frozen prior to long term storage at -  
418 80°C.

419

420 To simulate batch effects between fresh material (samples collected on earth) and ambient return  
421 material (samples collected during flight and returned via Soyuz capsule at 4°C), we generated 3  
422 control samples (MS) representing fresh (FR) and ambient return (AR) material as described  
423 before (Garrett-Bakelman et al., 2019). Whole blood of a male volunteer of similar age and  
424 ethnicity as HR/TW was collected in three CPT tubes. Plasma was collected and stored as  
425 described for the TW and HR specimens. To generate the ambient return control (AR) two CPT  
426 vacutainers were shipped at ambient temperature (4°C) from Stanford University to Weill Cornell  
427 Medicine and back as air cargo. The returned CPT vacutainers were spun at 300 X g for 3 minutes  
428 and aliquoted. One aliquot from each tube was spun once more at 1800 X g for 3 minutes to  
429 completely clear the plasma of cell debris, resulting in the final AR controls. The aliquoted plasma  
430 was stored at -80C.

431

#### 432 *cfDNA extraction and comparison of cfDNA concentrations in ground and flight subjects*

433 Between 250ul and 1 ml plasma was retrieved from HR, TW and MS samples. The frozen plasma  
434 was thawed at 37C for 5min and spun at 16000g for 10 minutes at 4C to remove cryo-precipitates.  
435 The volume of each plasma sample was brought up to 1ml using sterile, nuclease-free 1X  
436 phosphate buffered saline pH 7.4. Circulating cell-free nucleic acid (ccfNA) was extracted using  
437 the Qiamp Circulating Nucleic Acid kit (Qiagen, USA) following the manufacturer's protocol.  
438 ccfNA was extracted in 50ul AE buffer. Concentration and size distribution information was  
439 obtained by running 1ul of ccfNA on the Agilent Bioanalyzer using the High Sensitivity DNA chip  
440 (Agilent technologies, CA, USA). ~15ul aliquots were set aside for cell-free DNA or DNA  
441 methylation analyses and stored at -80C. A range of extracted cfDNA of 1ng-38ng/mL plasma has  
442 been reported for healthy donors, while cancer patients often show higher levels of 30-50ng/ml  
443 (Table 1). In the HR, TW and control samples we extracted between 6.7 ng/ml and 79.9 ng/ml  
444 plasma (mean = 27.9 ng/ml, median = 23 ng/ml)(Table 1). We tested if there is a significant  
445 difference between HR, TW or MS as well as FR and AR samples using Wilcoxon rank test (R  
446 function wilcox) for pairwise comparisons and ANOVA (R function anova) for multi-group  
447 comparisons. We furthermore visualized the distributions of the groups HR, TW and MS as  
448 boxplots using the R package ggplot2.

449

#### 450 *Q-PCR Analysis of cfDNA*

451 The frozen plasma was thawed at 37C for 5min and spun at 16000g for 10 minutes at 4C to remove  
452 cryo-precipitates. DNA level in samples was measured by SYBR Green dye-based qPCR assay  
453 using a PRISM 7300 sequence detection system (Applied Biosystems) as described previously  
454 (Nakahira PLoS Med. 2013, Garrett-Bakelman Science 2019, PMIDs: 24391478 and 30975860).  
455 The primer sequences were as follows: human NADH dehydrogenase 1 gene (hu mtNd1): forward  
456 5'-ATACCCATGGCCAACCTCCT-3', reverse 5'-GGGCCTTTGCGTAGTTGTAT-3'. Plasmid  
457 DNA with complementary DNA sequences for human mtDNA was obtained from ORIGENE  
458 (SC101172). Concentrations were converted to copy number using the formula;  
459 mol/gram×molecules/mol = molecules/gram, via a DNA copy number calculator  
460 (<http://cels.uri.edu/gsc/cndna.html>; University of Rhode Island Genomics and Sequencing Center).

461 The thermal profile for detecting mtDNA was carried out as follows: an initiation step for 2 min  
462 at 50°C is followed by a first denaturation step for 10 min at 95°C and a further step consisting of  
463 40 cycles for 15 s at 95°C and for 1 min at 60°C. MtDNA levels in all of the plasma analyses were  
464 expressed in copies per microliter of plasma based on the following calculation:  $c=Q \times$   
465  $VDNA/VPCR \times 1/Vext$ ; where  $c$  is the concentration of DNA in plasma (copies/microliter  
466 plasma);  $Q$  is the quantity (copies) of DNA determined by the sequence detector in a PCR;  $VDNA$   
467 is the total volume of plasma DNA solution obtained after extraction;  $VPCR$  is the volume of  
468 plasma DNA solution used for PCR; and  $Vext$  is the volume of plasma extracted.

469

#### 470 *Library generation and sequencing*

471 DNA libraries were generated using the NEBNext DNA Library Preparation Kit Ultra II (New  
472 England Biolabs, USA). Libraries were generated using 15ul of the ccfNA according to the  
473 manufacturer's instruction. Following end-repair and dA-tailing, adaptor ligation was performed  
474 using 15-fold diluted adaptors. After removal of free adaptors using Agencourt magnetic beads  
475 (Beckman Coulter, USA), the libraries were PCR-amplified for 12 cycles using primers  
476 compatible Illumina dual-index sequences. Following bead cleanup for primer removal, the  
477 libraries were run on the Agilent Bioanalyzer to estimate size and concentration. All libraries were  
478 pooled at equal concentration and sent to New England Biolabs, Ipswich MA for sequencing.  
479 Preliminary sequencing on Illumina Miseq indicated the presence of adaptor dimers in some of the  
480 libraries. Therefore, the individual libraries were subjected to an additional round of bead  
481 purification and size and concentration estimation. Subsequently, all libraries were pooled again  
482 and sequenced on the NovaSeq 6000 using an S2 flow cell and 200-cycle kits (2x100). We finally  
483 obtained 4.9 and 4.1 billion reads passing quality filters.

484

#### 485 *cfDNA sequence analysis*

486 Samples were de-multiplexed using the standard Illumina tools. Low quality bases were trimmed  
487 and Illumina-specific sequences and low quality sequences were removed from the sequencing  
488 data using Trimmomatic-0.32. Filtered, paired-end reads were aligned using BWA-mem to the  
489 hg38 human reference genome with the bwa-postalt option to handle alternative alignments. The  
490 resulting BAM files were post-processed (e.g. sorted) using samtools. Duplicate sequences were  
491 removed and only reads aligning in concordant pairs were used for further analysis. The fragment  
492 length distribution was generated by plotting the distance between read 1 and 2 obtained from the  
493 BAM file of each sample. Histograms and boxplots of the fragment length distribution for the  
494 autosomes (Figure 1) and for the mitochondrial genome (Figure 2) for all samples were generated  
495 using the R package ggplot2.

496

#### 497 *Analysis of cell free mitochondrial DNA*

498 cfDNA read counts by chromosome (including the mitochondrial genome labeled ChrMT) were  
499 extracted using a 'edtools coverage -a feature\_file -b sample.bam -counts', where feature\_file  
500 contains the definition (name, start, end) of all chromosomes. Read counts per feature were length-  
501 normalized using the well-established reads-per-kilobase per million formula frequently applied  
502 to normalize RNA-seq data (Mortazavi et al., 2008). We used the R package ggplot 2 to visualize  
503 differences in the normalized cfDNA fraction (RPKM) originating from the mitochondrial genome  
504 between HR, TW and MS and over time during the mission (longitudinal analysis). We used  
505 Wilcoxon rank test (R function Wilcox) to test if the measurements of two conditions (e.g. TW on

506 ground vs. TW in flight) are significantly different. To analyze the differences among multiple  
507 groups we applied ANOVA (R function anova).

508

#### 509 *Nucleosome positioning analysis*

510 Filtered, paired-end reads were aligned using BWA-mem to the hg37 human reference genome  
511 and post-processed using samtools: duplicate sequences were removed, and only reads aligning in  
512 concordant pairs were used for final analysis. The sequence read coverage in 10-kbp windows (-5  
513 kbp to 5kbp) around the transcription start sites of all genes was determined using the samtools  
514 depth function. From the positions of the reads, nucleosome occupancy was inferred, and its  
515 periodograms calculated. A list of transcription start sites organized by transcriptional activity  
516 (measured in FPKM) was used to assign activity (Ulz et al., 2016). The depth of coverage was  
517 summed across genes according to transcriptional activity category (as depicted in Figure 4A).  
518 The coverage was normalized by subtracting the mean value from the intervals [TSS-3 kbp, TSS-  
519 1 kbp] and [TSS+1 kbp, TSS+3 kbp]. According to the method in Snyder et al., 2015, FFT values  
520 for the periods of 193-199bp were correlated with the gene level expression matrix, and the  
521 resulting tissue-periodicity correlations ranked by the value of Pearson's correlation coefficient  
522 and clustered (Ward method with Euclidean distances) to investigate characteristics of tissue-of-  
523 origin dependent on sample type.

524

#### 525 *Purification and Mass spectrometry analysis of plasma-circulating exosomes*

526 Blood plasma was collected from TW 3 years post return of TW to Earth. Blood was also collected  
527 from HR (within one day of blood collection from TW) and from six age-matched healthy controls.  
528 Exosomes were purified by sequential ultracentrifugation, as previously described (Hoshino et al.,  
529 2015). Plasma samples were centrifuged for 10 minutes at 500xg, 20 minutes at 3,000xg, 20  
530 minutes at 12,000xg, and the supernatant was collected and stored at -80°C for exosome isolation  
531 and characterization by NTA (NanoSight NS500, Malvern Instruments, equipped with a violet  
532 laser (405 nm). Samples were thawed on ice and centrifuged at 12,000xg for 20 min to remove  
533 large microvesicles. Exosomes were collected by spinning at 100,000xg for 70min, washed in PBS  
534 and pelleted again by ultracentrifugation in a 50.2 Ti rotor, Beckman Coulter Optima XE or XPE  
535 ultracentrifuge. The final exosome pellet was resuspended in PBS, and protein concentration was  
536 measured by BCA (Pierce, Thermo Fisher Scientific). Mass spectrometry analyses of exosomes  
537 were performed at the Rockefeller University Proteomics Resource Center using 10 µg of  
538 exosomal protein as described previously (Hoshino et al., 2015; Zhang et al., 2018). Heatmap and  
539 complete Euclidean clustering was performed with Morpheus,  
540 (<https://software.broadinstitute.org/morpheus>). Pathway analysis was performed with  
541 Metascape (Zhou et al., 2019).

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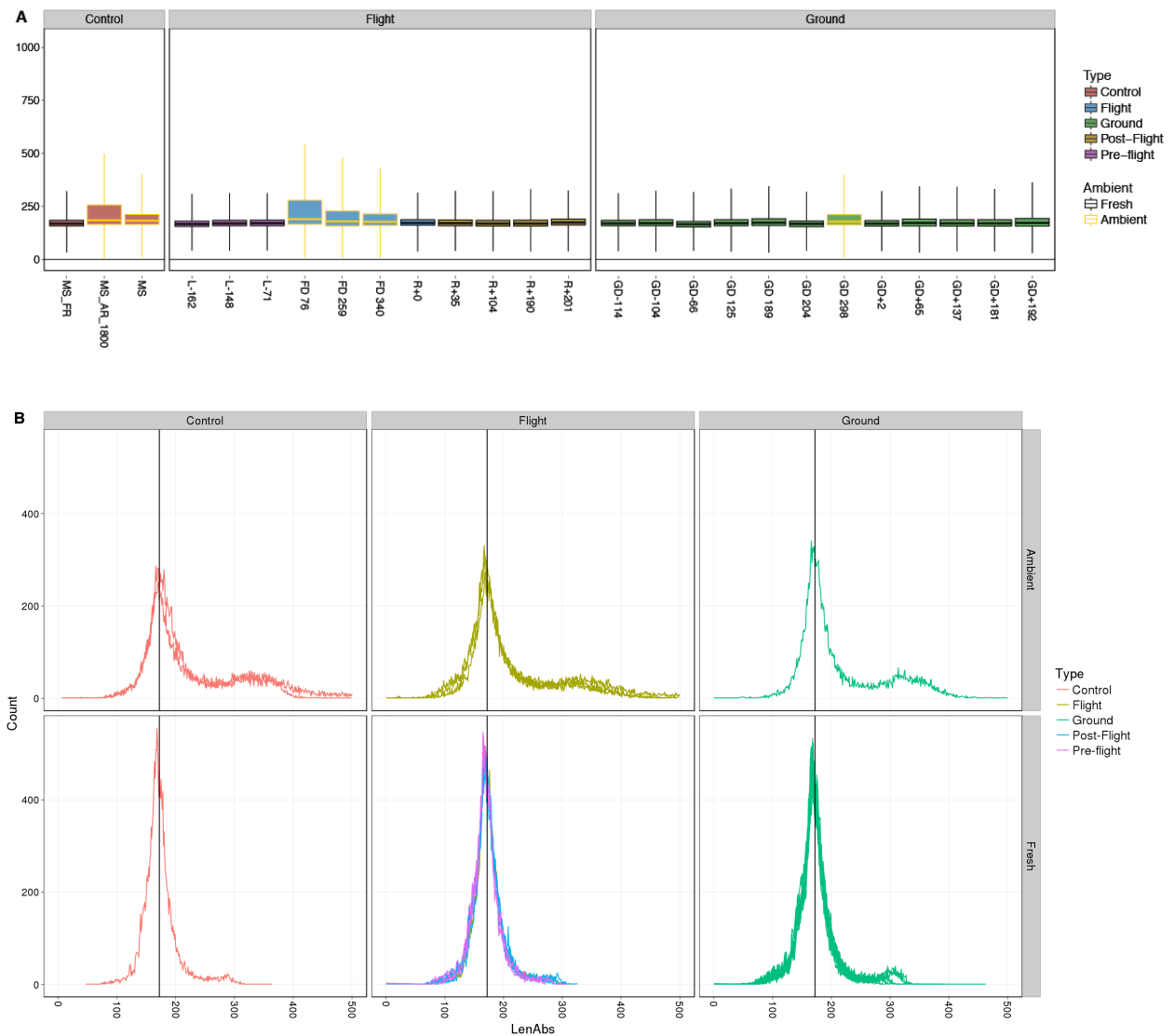


## Figures, Tables, and Supplementary Tables/Figures for:

### Cell-free DNA (cfDNA) and exosome profiling from a year-long human spaceflight reveals circulating biomarkers

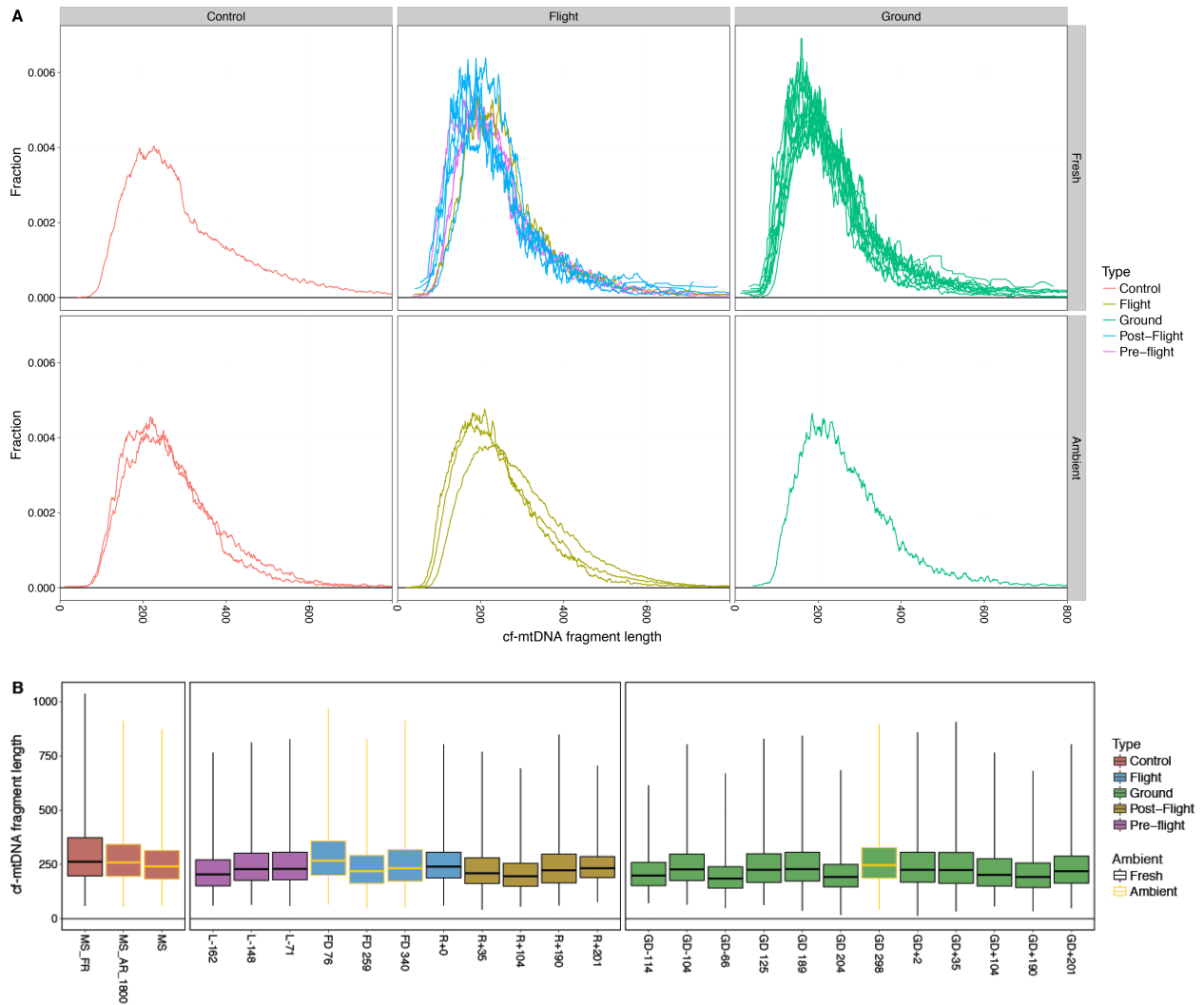
*Daniela Bezdan<sup>1</sup>, Kirill Grigorev<sup>1</sup>, Cem Meydan<sup>1</sup>, Fanny A. Pelissier Vatter<sup>2</sup>, Michele Cioffi<sup>2</sup>, Varsha Rao<sup>3</sup>, Kiichi Nakahira<sup>4</sup>, Philip Burnham<sup>5</sup>, Ebrahim Afshinnekoo<sup>1,6,7</sup>, Craig Westover<sup>1</sup>, Daniel Butler<sup>1</sup>, Chris Moszary<sup>1</sup>, Matthew MacKay<sup>1</sup>, Jonathan Foon<sup>1</sup>, Tejaswini Mishra<sup>3</sup>, Serena Lucotti<sup>2</sup>, Brinda K. Rana<sup>8</sup>, Ari M. Melnick<sup>9</sup>, Haiying Zhang<sup>10</sup>, Irina Matei<sup>2</sup>, David Kelsen<sup>10</sup>, Kenneth Yu<sup>10</sup>, David C Lyden<sup>2</sup>, Lynn Taylor<sup>11</sup>, Susan M Bailey<sup>11</sup>, Michael P. Snyder<sup>3</sup>, Francine E. Garrett-Bakelman<sup>12,13,14</sup>, Stephan Ossowski<sup>15</sup>, Iwijn De Vlaminck<sup>16</sup>, Christopher E. Mason<sup>1,6,7,17\*</sup>*

## Figures

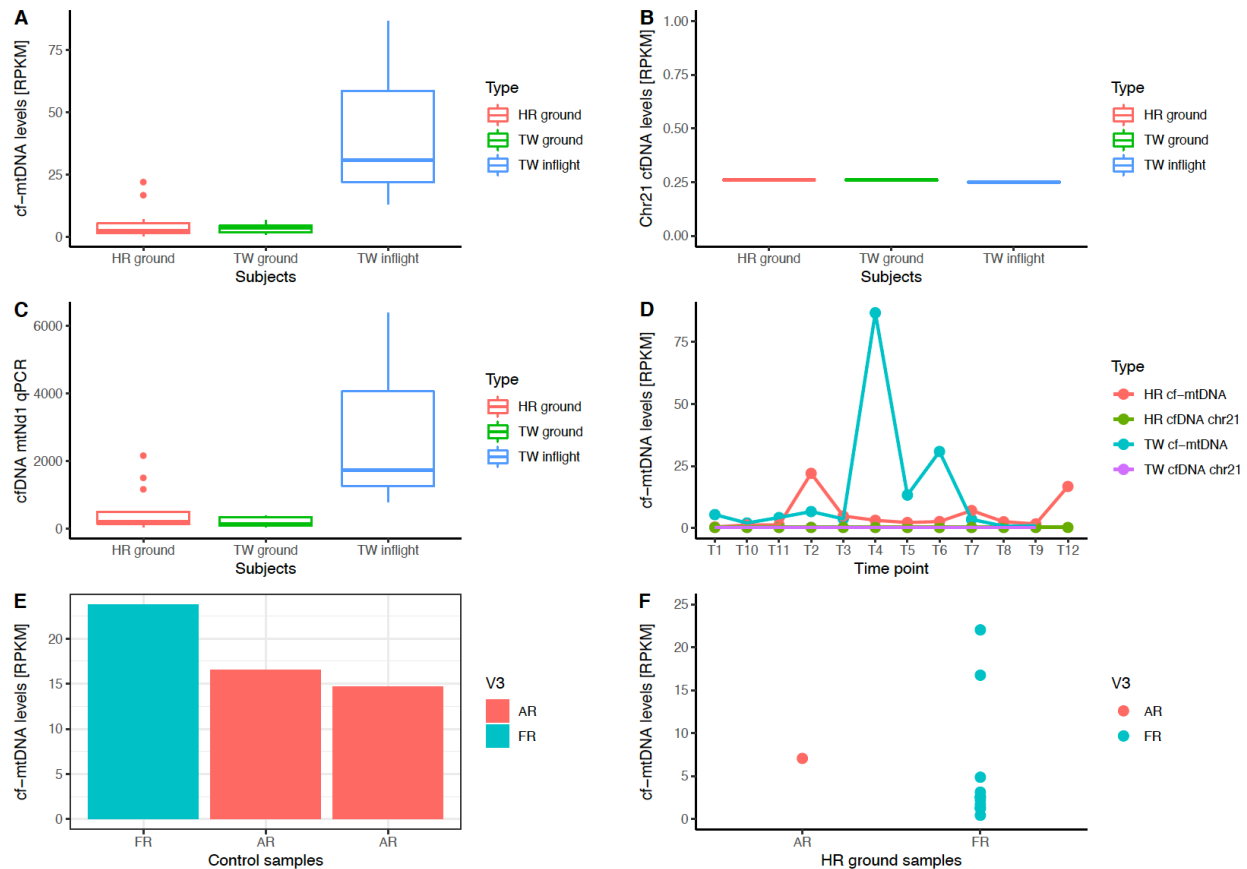


**Figure 1. Size distribution of cfDNAs in ambient return, ambient return simulation and fresh samples.** (A). Ambient return simulation samples (control and ground samples with yellow border) show a highly similar pattern as observed for inflight samples (blue box with yellow border). Long cfDNA fragments likely originate from blood cells damaged during transport. (B) Ambient return samples show an increased fraction of cfDNA with fragment length > 300bp compared to fresh samples. Our experimental procedure does only allow interrogation of DNA fragments up to a

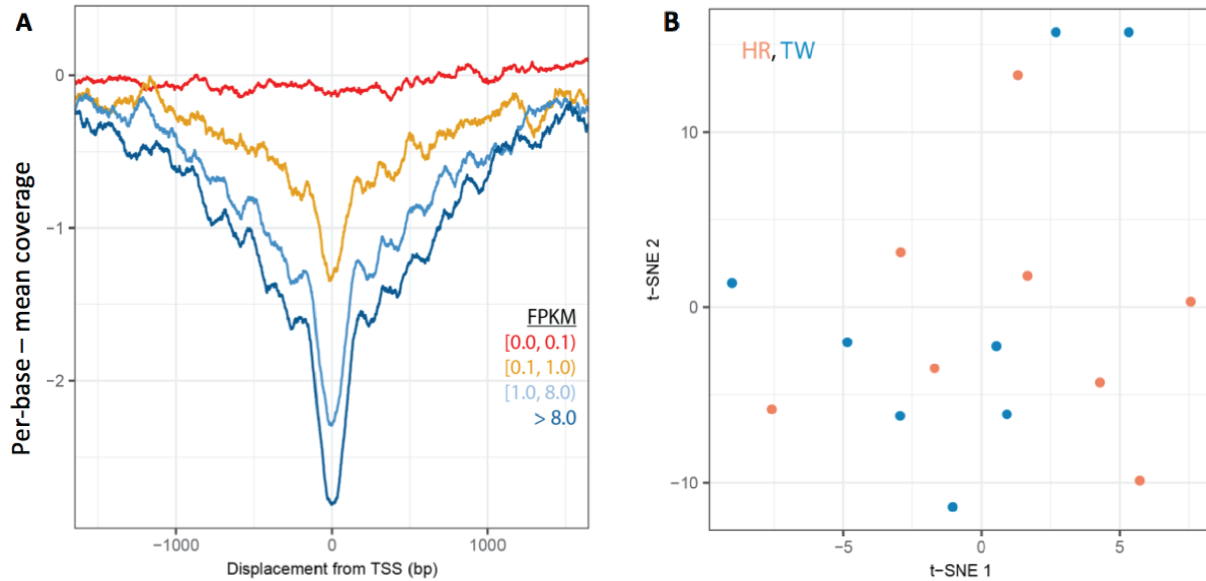
length of 500bp, thus the content of long mtDNA fragments contained in intact circulating mitochondria is not reflected in this analysis.



**Figure 2. Size distribution of cf-mtDNAs.** We observed a wider range of cf-mtDNA lengths compared to total cfDNA (from 100 to 600bp). (A) cf-mtDNA size distributions are similar in ground, flight and control samples, and are not affected by ambient return (AR) or AR simulation. (B) Average length of cf-mtDNA is significantly longer than the average length reported for chromosomal cfDNA (~250bp vs. ~160bp). The average length of cf-mtDNA is not affected by sample type (control, flight, ground) or sample handling (fresh, AR, AR simulation).

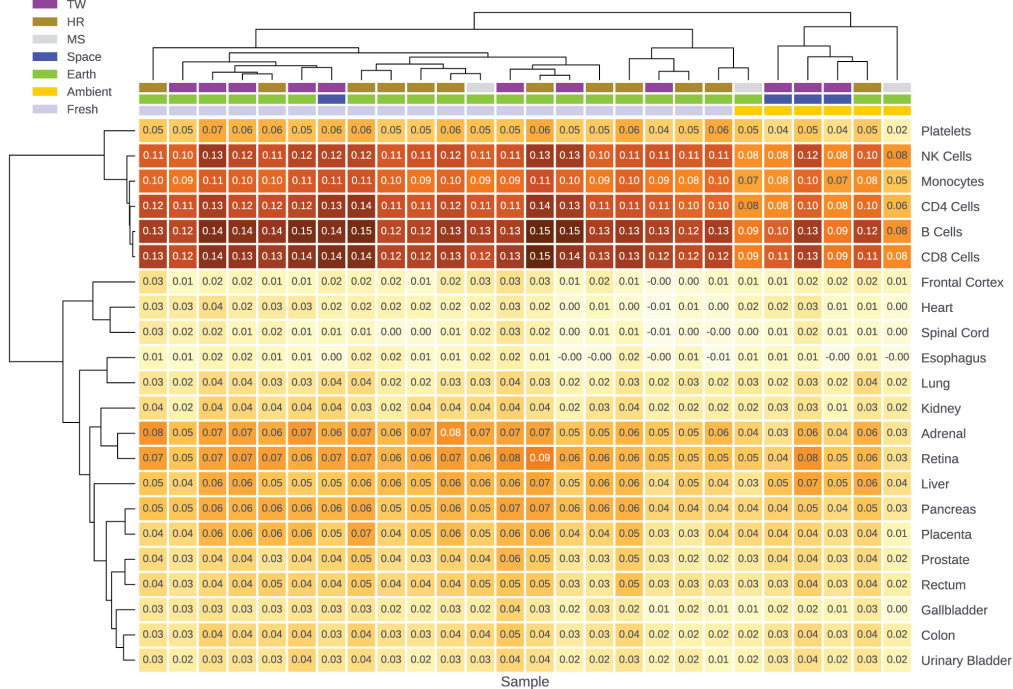


**Figure 3. Analysis of normalized cfDNA read counts by chromosome, including the mitochondrial genome.** (A) TW exhibits a significant increase in cell-free mtDNA during space flight compared to TW and HR ground samples. Counts are reads per kilobase per Million reads, or RPKM (B) Chromosomes do not show any change in RPKM during space flight, as exemplified using chr21. (C) Q-PCR based validation of increased cf-mtDNA fraction in plasma during space flight. (D) Normalized cf-mtDNA fraction and fraction of reads mapping to chr21 for 12 time point during the mission (T4-T6 = space flight). The highest increase in cf-mtDNA fraction is observed during the first months on ISS. (E) Ambient return simulation using two control samples showed no increase in cf-mtDNA compared to fresh samples, but a slight reduction. (F) Ambient return simulation (AR) using one HR ground sample did not show a significant increase in cf-mtDNA fraction. Two outliers within the fresh samples (FR) indicate that other conditions (e.g. stress, disease, immune reaction) could have influenced cf-mtDNA levels of HR on the ground.

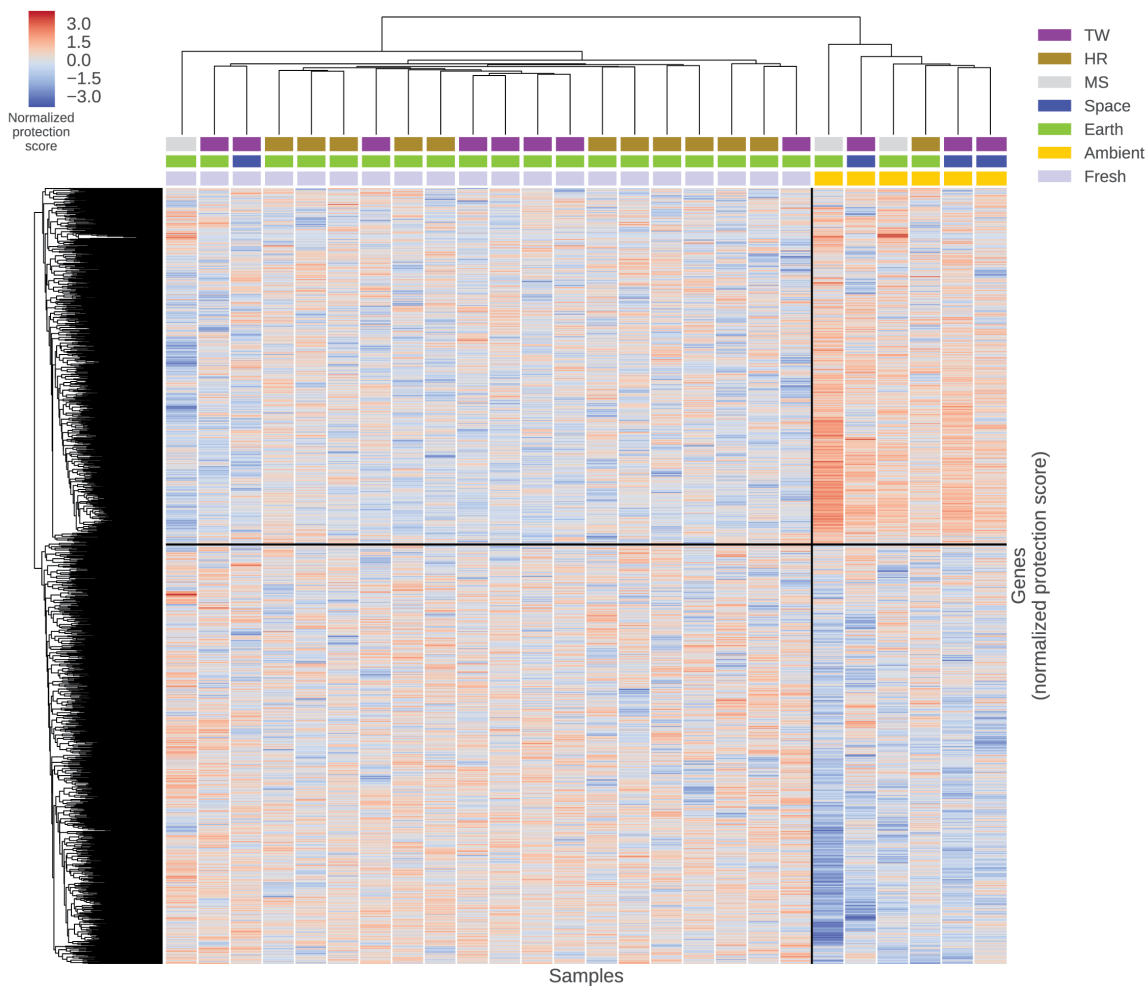


**Figure 4. cfDNA nucleosome footprinting.** (A) Nucleosome depletion in cfDNA around transcription start sites (TSS) is highly correlated with the expression of the respective genes and can therefore be used to estimate promoter activity and gene expression. (B) t-SNE based on genome-wide promoter nucleosome footprint of cfDNA samples reveals no clustering of flight subject and ground subject samples.

A

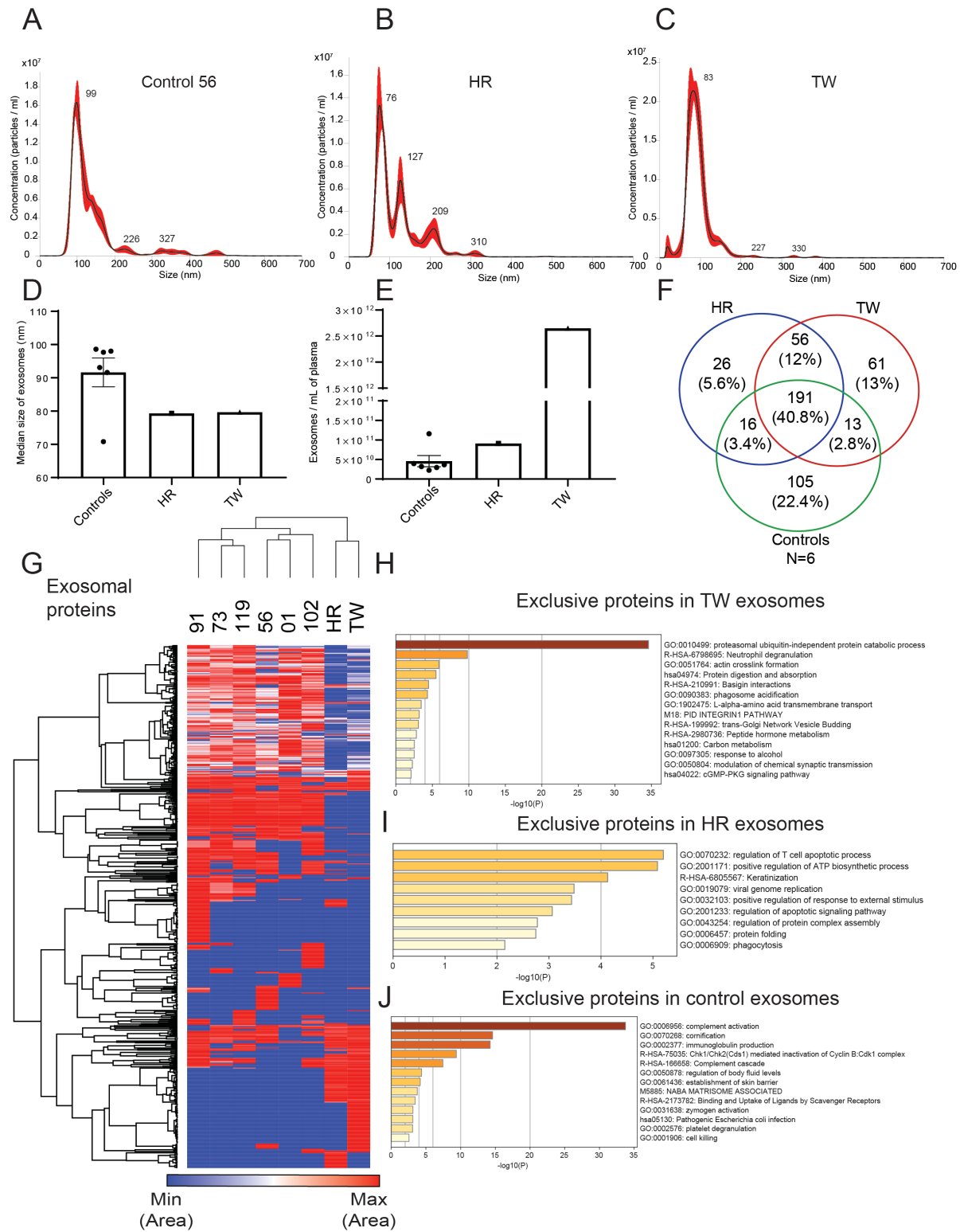


B




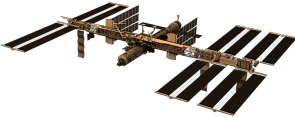
**Figure 5. Tissue of origin deconvolution.** (A) Correlation coefficients (multiplied by -1) for each tissue in each sample, clustered by sample and by tissue. The highest signals are, expectedly, from cells of hematopoietic origin. Spaceflight-dependent dynamics of tissue signal are confounded by the effect of ambient return, as suggested by ambient return samples tending to cluster together regardless of other features. (B) Clustering of samples using TSS protection in cfDNA as a measure of gene expression (lower protection correlates to higher expression). Ambient return samples cluster tightly together and uncover two major clusters of genes whose expression differs significantly from other samples, suggesting transport-related degradation processes or nucleosome detachment. Distribution of mean TSS protection per gene in ambient return and fresh samples is significantly different (t-test  $p < 1e-3$ ).

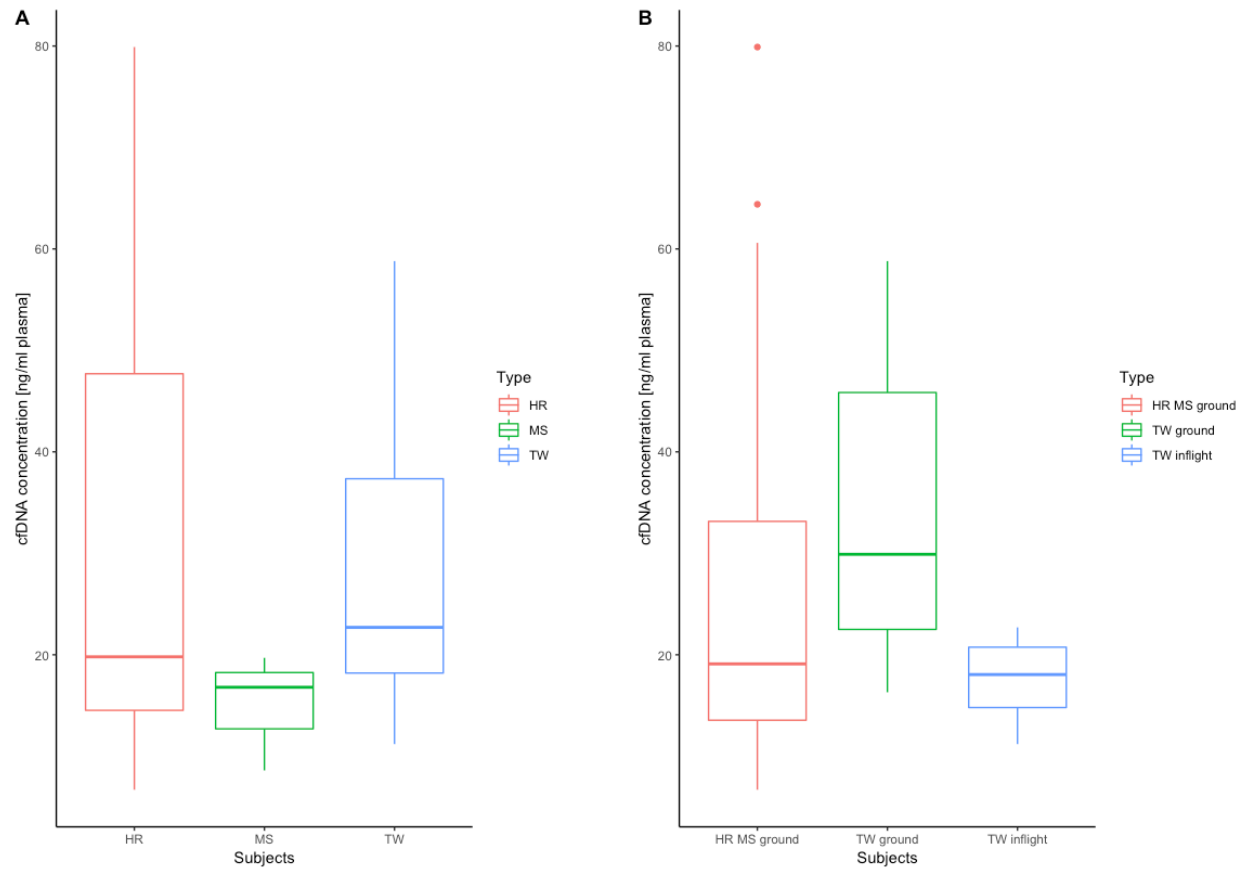




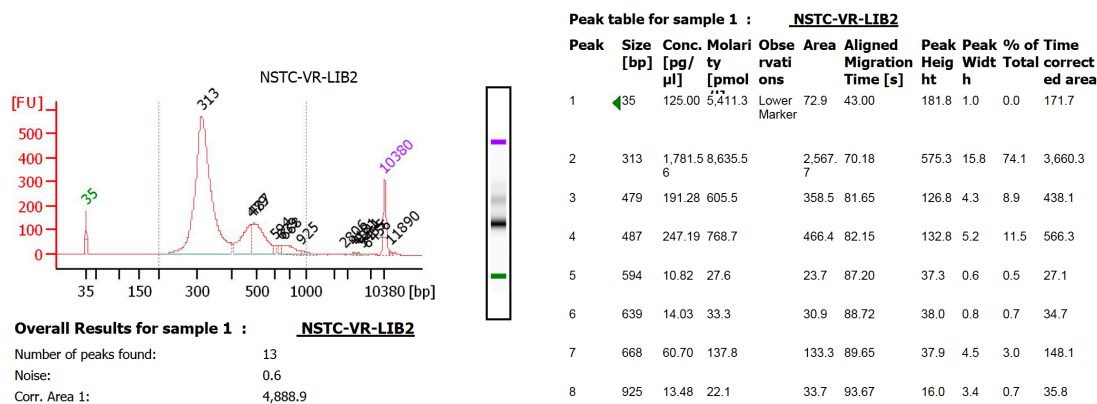
**Figure 6. Characterization of plasma-derived exosomes isolated from HR and TW.** Plasma samples were collected 3 years (TW) and 9 years (HR) post-flight. Nanosight profiles showing size distribution for exosomes isolated from the plasma of (A) Control, (B) HR, and (C) TW. Median size of exosomes (D) and exosome concentration (E) in TW (n=1), HR (n=1), and controls (n=6). (F) Venn diagram of exosomal proteins identified by mass spectrometry in plasma isolated from HR, TW and age-matched healthy controls. (G) Heatmap of plasma-derived exosomal proteins for HR, TW, and age-matched healthy controls. Pathway analysis of exclusive plasma-derived exosomal proteins from (H) TW, (I) HR, and (J) age-matched healthy controls.

**Table 1. Overview of all plasma samples obtained during the 1-year mission.** Subjects for this mission included the ground subject HR (blue), flight subject TW (green) and control subject MS (yellow). Samples taken on the ISS are highlighted in red. The last two columns show the concentration of cfDNA per ml plasma and the Q-PCR results for the mitochondrial transcript mtNd1 in copy/ $\mu$ l plasma.

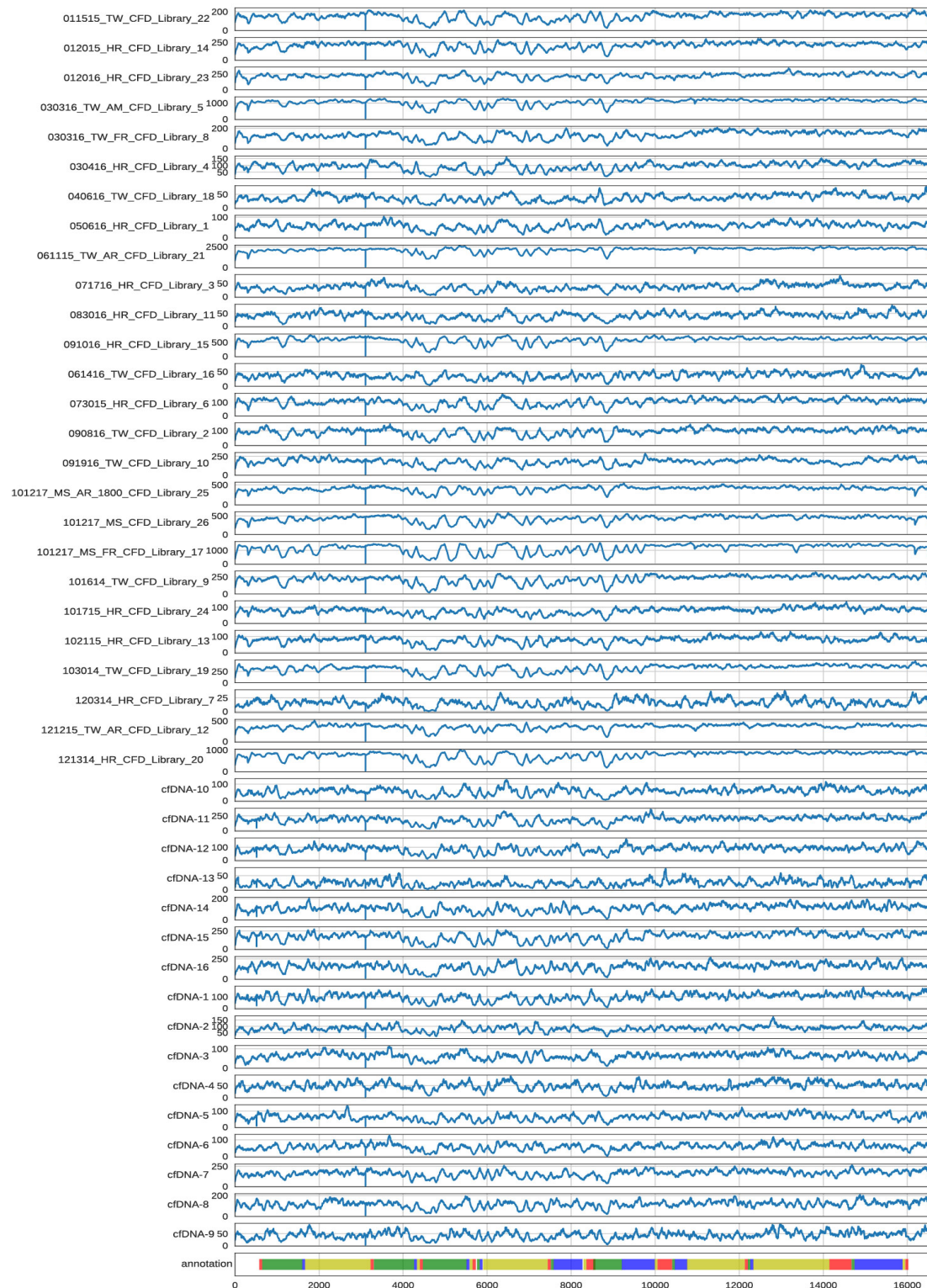
Time	Subject	Sample name	Total per plasma [ng/ml plasma]	mtNd1 Q-PCR [cp/ $\mu$ l plasma]	
PRE-FLIGHT		GD-114	15.5	44	
		GD-104	79.9	2159.6	
		GD-66	11.6	251.7	
FLIGHT		GD 125	6.7	277	
		GD 189	20.5	215.1	
		GD 204	64.4	193.7	
		GD 298	43.4	1502.8	
POST-FLIGHT		GD+2	60.6	157.7	
		GD+65	16.1	136.3	
		GD+137	19.1	68.7	
		GD+181	7.1	147.3	
		GD+192	22.9	1165.2	
AMBIENT			MS	8.6	3080.6
RETURN			MS_AR	19.7	3590.8
CONTROL			MS_AR_1800	16.8	1330.4
PRE-FLIGHT		L-162	44.8	543.1	
		L-148	16.3	737.6	
		L-71	46.9	466	
FLIGHT		FD 76	20.1	6379.7	
		FD 259	11.2	786.9	
		FD 340	22.7	1735.3	
POST-FLIGHT		R+0	16	374.7	
		R+35	23.5	86.4	
		R+104	21.5	37.7	
		R+190	58.8	138.5	
		R+201	29.9	349.1	



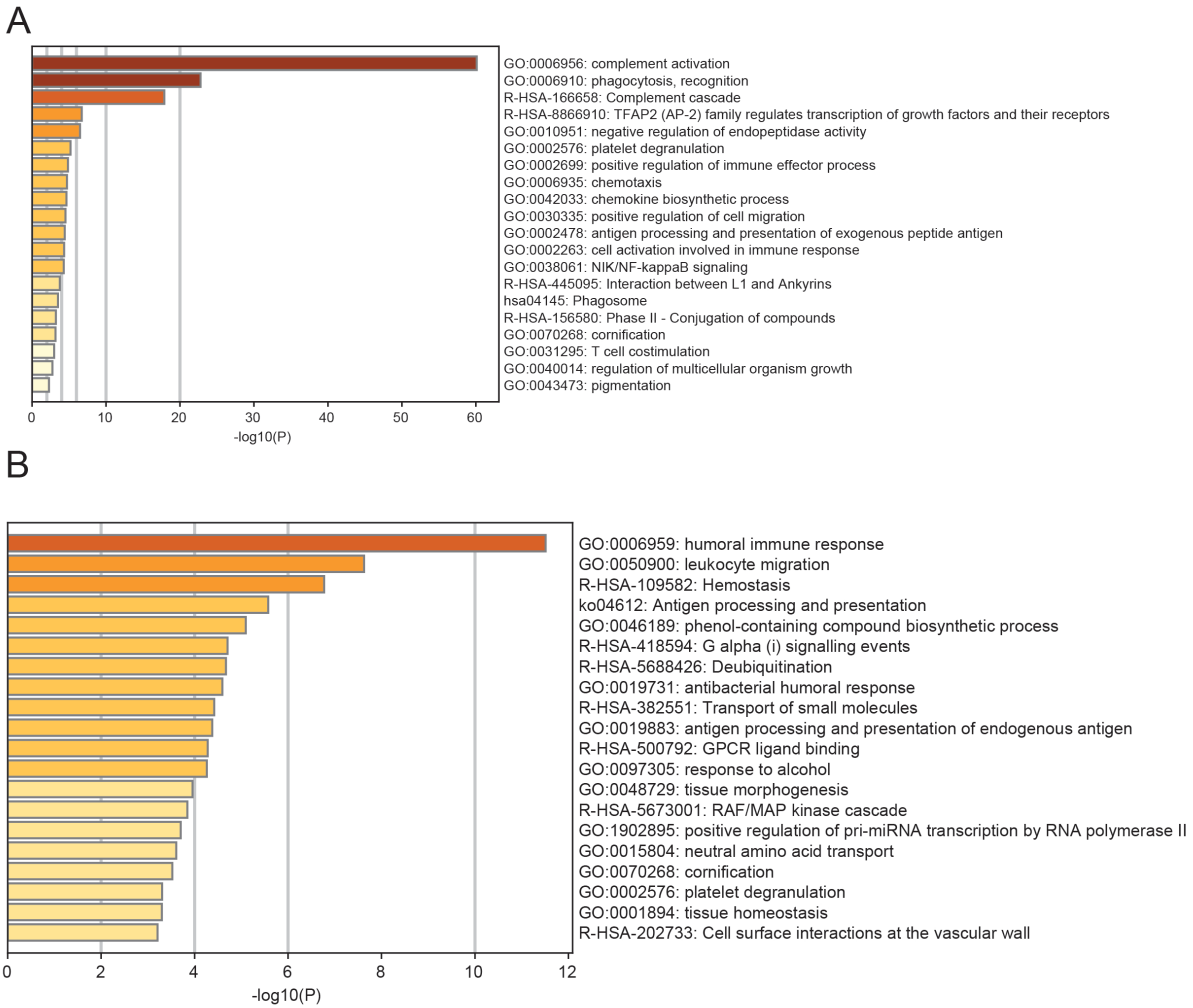
**Supplemental Figure 1:** cfDNA concentration in plasma of ground subject (HR), flight subject (TW) and ground controls (MS). (A) Comparison of cfDNA concentrations between HR, MS and TW samples. (B) Comparison of TW pre- and -post-flight to TW inflight and to the combined ground samples of HR and MS. No significant differences were observed.



**Supplemental Figure 2. Library Fragment distribution for the cell-free DNA libraries.** Pooled libraries were run on the Agilent Bioanalyzer 2100, with the entire fragment range area estimated to be 4,888.9. The first fraction peak was estimated to be at 313bp and the second peak was at 466bp. Given that the Illumina adapters add 120bp to each fragment size, this means that the estimated size of the first fragment set is 193bp and the second set is 346bp. The total area of the first peak represents 74.8% (3,660.3/4,888.9) of the signal.



**Supplemental Figure 3:** Read coverage distribution across the mitochondrial genome for all samples analyzed in this study. We observed continuous coverage of the complete mitochondrial genome in all samples.



**Supplemental Figure 4:** (A) Pathway analysis of inclusive plasma-derived exosomal proteins from TW (n=1), HR (n=2), and (J) age-matched healthy controls (n=6). (B) Pathway analysis of inclusive plasma-derived exosomal proteins from TW and HR excluding age-matched healthy controls

## Supplementary Tables

**Supplemental Table 1:** List of inclusive proteins in exosomes isolated from the plasma of TW, HR and age-matched healthy controls.

Inclusive in TW, HR and control exosomes		
35 kDa inter-alpha-trypsin inhibitor heavy chain H4	Fibrinogen gamma chain	Platelet glycoprotein 4
Actin, cytoplasmic 1	Fibulin 1	Polymeric immunoglobulin receptor
Adiponectin	Ficolin-2	Protein AMBP
Afamin	Ficolin-3	Protein IGHV1-46
Alpha-1-acid glycoprotein 1	Galectin-3-binding protein	Protein IGHV1OR15-1
Alpha-1-acid glycoprotein 2	Gelsolin	Protein IGHV2-26
Alpha-1-antichymotrypsin	Glutathione peroxidase	Protein IGHV3-13
Alpha-1-antitrypsin	Glyceraldehyde-3-phosphate dehydrogenase	Protein IGHV3-15
Alpha-1B-glycoprotein	Haptoglobin	Protein IGHV3-21
Alpha-2-antiplasmin	Haptoglobin-related protein	Protein IGHV3-35
Alpha-2-HS-glycoprotein	HCG2043239	Protein IGHV3-38
Alpha-2-macroglobulin	Heat shock cognate 71 kDa protein	Protein IGHV3-43
Angiotensinogen	Hemoglobin subunit alpha	Protein IGHV3-49
Antithrombin-III	Hemoglobin subunit beta	Protein IGHV3-64
Apolipoprotein A-I	Hemoglobin subunit delta	Protein IGHV3-73
Apolipoprotein A-II	Hemopexin	Protein IGHV3OR15-7
Apolipoprotein A-IV	Heparin cofactor 2	Protein IGHV3OR16-12
Apolipoprotein B-100	Histidine-rich glycoprotein	Protein IGHV3OR16-13
Apolipoprotein C-III	Ig alpha-1 chain C region	Protein IGHV3OR16-9
Apolipoprotein C-IV	Ig alpha-2 chain C region	Protein IGHV4-28
Apolipoprotein D	Ig delta chain C region	Protein IGHV4-34
Apolipoprotein E	Ig gamma-1 chain C region	Protein IGHV4-4
Apolipoprotein L1	Ig gamma-2 chain C region	Protein IGHV5-51
Apolipoprotein M	Ig gamma-3 chain C region	Protein IGHV6-1
Apolipoprotein(a)	Ig gamma-4 chain C region	Protein IGKV1-16
Band 3 anion transport protein	Ig heavy chain V-I region 5	Protein IGKV1-17
Beta-2-glycoprotein 1	Ig kappa chain V-IV region	Protein IGKV1-33
C4b-B	Ig lambda-2 chain C regions	Protein IGKV2-30
C4b-binding protein alpha chain	Ig mu chain C region	Protein IGKV2-40
C4b-binding protein beta chain	IgGfC-binding protein	Protein IGKV2D-24
Carboxypeptidase N catalytic chain	Immunoglobulin heavy variable 3-43D	Protein IGKV3D-20
Carboxypeptidase N subunit 2	Immunoglobulin heavy variable 4-38-2	Protein IGLV1-47
CD5 antigen-like	Immunoglobulin kappa constant	Protein IGLV2-11
CD81 antigen	Immunoglobulin lambda variable 1-51	Protein IGLV2-14
Ceruloplasmin	Immunoglobulin lambda variable 3-10	Protein IGLV3-19
Clusterin	Immunoglobulin lambda variable 8-61	Protein IGLV3-27
Coagulation factor V	Immunoglobulin lambda-like polypeptide 5	Protein IGLV7-43
Coagulation factor XII	Integrin alpha-IIb	Protein IGLV7-46
Coagulation factor XIII A chain	Inter-alpha-trypsin inhibitor heavy chain H1	Protein IGLV9-49
Coagulation factor XIII B chain	Inter-alpha-trypsin inhibitor heavy chain H2	Protein S100-A8
Collectin-11	Keratin, type I cuticular Ha1	Protein S100-A9
Complement C1q subcomponent subunit A	Keratin, type I cuticular Ha3-II	Proteoglycan 4
Complement C1q subcomponent subunit B	Keratin, type I cuticular Ha5	Prothrombin
Complement C1q subcomponent subunit C	Keratin, type I cuticular Ha6	Ras-related protein Rap-1b
Complement C1q tumor necrosis factor-related protein 3	Keratin, type I cytkeletal 10	Reelin
Complement C1r subcomponent	Keratin, type I cytkeletal 14	Retinol binding protein 4, plasma, isoform CRA_b
Complement C1s subcomponent	Keratin, type I cytkeletal 9	Serotransferrin
Complement C3	Keratin, type II cytkeletal 1	Serum albumin
Complement C4 beta chain	Keratin, type II cytkeletal 2 epidermal	Serum amyloid P-component
Complement C5	Keratin, type II cytkeletal 4	Serum paraoxonase/arylesterase 1
Complement component C6	Keratin, type II cytkeletal 5	Solute carrier family 2, facilitated glucose transporter member 1
Complement component C7	Keratin, type II cytkeletal 6B	Thrombospondin-1
Complement component C8 alpha chain	Kininogen-1	Transferrin receptor protein 1
Complement component C8 beta chain	Lipopolysaccharide-binding protein	Transthyretin
Complement component C8 gamma chain	Lysozyme C	Truncated apolipoprotein C-I
Complement component C9	Mannan-binding lectin serine protease 1	Vitamin D-binding protein
Complement factor B	Mannan-binding lectin serine protease 2	Vitamin K-dependent protein S
Complement factor H	Oncoprotein-induced transcript 3 protein	Vitronectin
Complement factor H-related protein 1	Phosphatidylinositol-glycan-specific phospholipase D	von Willebrand factor
Complement factor H-related protein 5	Pigment epithelium-derived factor	Zinc-alpha-2-glycoprotein
Complement factor I light chain	Plasma kallikrein heavy chain	TTR
Corticosteroid-binding globulin	Plasma protease C1 inhibitor	VTN
Erythrocyte band 7 integral membrane protein	Plasminogen	VWF
Extracellular matrix protein 1	Platelet factor 4	



**Supplemental Table 2:** List of unique proteins in exosomes isolated from the plasma of TW, HR and age-matched healthy controls.

Unique in TW exosomes		Unique in HR exosomes
Acetyl-CoA carboxylase 1	Nephrilysin	Alpha-enolase
Alpha-soluble NSF attachment protein	Preylcysteine oxidase 1	Cell division control protein 42 homolog
Aminopeptidase N	Proteasome subunit alpha type	Clathrin heavy chain 1
Angiotensin-related protein 6	Proteasome subunit alpha type-2	Coagulation factor VIII
Atypical chemokine receptor 1	Proteasome subunit alpha type-3	Creatine kinase M-type
Basement membrane-specific heparan sulfate proteoglycan core protein	Proteasome subunit alpha type-5	EMILIN-1
Basigin	Proteasome subunit alpha type-6	Galectin-3
Beta-Ala-His dipeptidase	Proteasome subunit alpha type-7	Galectin-9
Brain-specific angiogenesis inhibitor 1-associated protein 2	Proteasome subunit beta type	Heat shock protein 75 kDa, mitochondrial
Brain-specific angiogenesis inhibitor 1-associated protein 2-like protein 1	Proteasome subunit beta type-1	Immunoglobulin heavy variable 2-70D
Calmodulin-like protein 5	Proteasome subunit beta type-2	Kallicrein-1
Catalase	Proteasome subunit beta type-3	Keratin, type I cuticular Ha3-I
Cation-dependent mannose-6-phosphate receptor O	Proteasome subunit beta type-4	Keratin, type II cuticular Hb2
CD82 antigen	Proteasome subunit beta type-5	Keratin-associated protein 11-1
Collagen alpha-1(VI) chain	Proteasome subunit beta type-6	Keratin-associated protein 2-2
Complement factor H-related protein 4	Proteasome subunit beta type-7	Peptidyl-prolyl cis-trans isomerase A-like 4H
Dipeptidyl peptidase 4	Proteasome subunit beta type-8	Plectin
Epidermal growth factor receptor kinase substrate 8	Protein IGKV1-8	Prolactin-inducible protein
Erythroid membrane-associated protein	Ras-related protein Rab-5C	Protein-glutamine gamma-glutamyltransferase 2
Gamma-glutamyltranspeptidase 1	Ras-related protein Rab-7a	Putative keratin-87 protein
G-protein coupled receptor family C group 5 member C	Rib e-5-ph phate isomerase	Sialic acid-binding Ig-like lectin 16
Heat shock protein beta-1	Septin-5	Tandem C2 domains nuclear protein
Histone H2A	Sodium/potassium-transporting ATPase subunit alpha	Thym in beta-4
Immunoglobulin kappa variable 1-6	Suprabasin	Transitional endoplasmic reticulum ATPase
Immunoglobulin lambda variable 5-39	Syntenin-1	Transmembrane protein 198
Integrin beta-1	Tri eph phate isomerase	Tripeptidyl-peptidase 2
Lactadherin	Trypsin-1	
Large neutral amino acids transporter small subunit 1	Vasodilator-stimulated ph phoprotein	
Large neutral amino acids transporter small subunit 3	Vesicle transport protein	
Monocyte differentiation antigen CD14	V-type proton ATPase 16 kDa proteolipid subunit	
	V-type proton ATPase catalytic subunit A	

	Unique in Control exosomes	
14-3-3 protein epsilon	HLA class I histocompatibility antigen, B-73 alpha chain	Protein IGHV2-70
14-3-3 protein eta	HLA class I histocompatibility antigen, B-8 alpha chain	Protein IGHV3-30
14-3-3 protein gamma	HLA class I histocompatibility antigen, B-82 alpha chain	Protein IGHV3-33
14-3-3 protein sigma	HLA class I histocompatibility antigen, Cw-18 alpha chain	Protein IGHV3-48
14-3-3 protein theta	HLA class I histocompatibility antigen, Cw-4 alpha chain	Protein IGHV3-53
Actin, alpha cardiac muscle 1	Homerin	Protein IGHV3-74
Actin, cytoplasmic 2	Hyaluronan-binding protein 2	Protein IGHV3OR16-10
Beta-actin-like protein 2	Ig heavy chain V-III region 23	Protein IGHV3OR16-8
Carboxypeptidase B2	Ig kappa chain V-III region VG	Protein IGHV4-31
Cholinesterase	Immunoglobulin heavy variable 1-8	Protein IGHV4-59
Collectin-10	Immunoglobulin heavy variable 3-20	Protein IGKV1-27
Complement C1r subcomponent-like protein	Immunoglobulin J chain	Protein IGKV1-39
Complement C2	Immunoglobulin kappa joining 1	Protein IGKV2-24
Complement factor H-related protein 2	Immunoglobulin kappa variable 2-29	Protein IGKV2D-30
Complement factor H-related protein 3	Insulin-like growth factor-binding protein complex acid labile subunit	Protein IGKV3-7
HCG1745306, isoform CRA_a	Integrin beta-3	Protein IGKV6D-21
Hemoglobin subunit gamma-1	Inter-alpha-trypsin inhibitor heavy chain H3	Protein IGLV10-54
Hepatocyte growth factor activator	Keratin, type I cuticular Ha2	Protein IGLV1-44
Hepatocyte growth factor-like protein	Keratin, type I cuticular Ha7	Protein IGLV3-1
HLA class I histocompatibility antigen, A-11 alpha chain	Keratin, type I cuticular Ha8	Protein IGLV3-21
HLA class I histocompatibility antigen, A-2 alpha chain	Keratin, type I cytkeletal 15	Protein IGLV3-25
HLA class I histocompatibility antigen, A-23 alpha chain	Keratin, type I cytkeletal 16	Protein IGLV4-60
HLA class I histocompatibility antigen, A-25 alpha chain	Keratin, type I cytkeletal 17	Protein SAA2-SAA4
HLA class I histocompatibility antigen, A-30 alpha chain	Keratin, type I cytkeletal 18	Protein tyr ine phatase, receptor type, C
HLA class I histocompatibility antigen, A-31 alpha chain	Keratin, type II cytkeletal 1b	Putative HLA class I histocompatibility antigen, alpha chain H
HLA class I histocompatibility antigen, A-34 alpha chain	Keratin, type II cytkeletal 6A	Putative V-set and immunoglobulin domain-containing-like protein IGHV4OR15-8
HLA class I histocompatibility antigen, A-41 alpha chain	Keratin, type II cytkeletal 75	Pyruvate kinase isozymes M1/M2
HLA class I histocompatibility antigen, A-68 alpha chain	Keratin, type II cytkeletal 79	Radixin
HLA class I histocompatibility antigen, A-74 alpha chain	Keratin, type II cytkeletal 80	Selenoprotein P
HLA class I histocompatibility antigen, B-39 alpha chain	Latent-transforming growth factor beta-binding protein 1	Serum amyloid A-1 protein
HLA class I histocompatibility antigen, B-41 alpha chain	Platelet factor 4 variant	SWISS-PROT:P15636 Protease I precursor Lysyl endopeptidase Achromobacter lyticus.
HLA class I histocompatibility antigen, B-42 alpha chain	Pregnancy zone protein	Tetranectin
HLA class I histocompatibility antigen, B-44 alpha chain	Properdin	THBS3 protein
HLA class I histocompatibility antigen, B-55 alpha chain	Protein IGHV1-2	Thyroxine-binding globulin
HLA class I histocompatibility antigen, B-59 alpha chain	Protein IGHV1-69	Uncharacterized protein
HLA class I histocompatibility antigen, B-7 alpha chain		

Shared by TW and HR exosomes (not in controls)	
4F2 cell-surface antigen heavy chain	Immunoglobulin heavy variable 5-10-1
60 kDa heat shock protein, mitochondrial	Immunoglobulin J chain
A disintegrin and metalloproteinase with thrombospodin motifs 13	Immunoglobulin kappa variable 3-15
Actin, gamma-enteric smooth muscle	Immunoglobulin kappa variable 3-20
Aldehyde dehydrogenase family 16 member A1	Keratin, type I cytoskeletal 13
APOC4-APOC2 readthrough (NMD candidate)	Keratin, type II cuticular Hb5
Bone morphogenetic protein 1	Keratin, type II cuticular Hb6
Carbonic anhydrase 1	Leukosialin
Cathelicidin antimicrobial peptide	Leukocyte surface antigen CD53
CD99 antigen	Major vault protein
Cholesteryl ester transfer protein	Metalloreductase STEAP3
Dermcidin	Neutral amino acid transporter B(0)
Equilibrative nucle ide transporter 1	Peroxiaredoxin-2
Ferritin heavy chain	Probable sodium-coupled neutral amino acid transporter 6
Ferritin light chain	Protein IGHV3-72
Guanine nucleotide-binding protein G(I)/G(S)/G(T) subunit beta-1	Protein IGKV2D-29
Guanine nucleotide-binding protein G(t) subunit alpha-1	Protein IGLV2-8
HCG2041221	Protein TFG
Heat shock 70 kDa protein 1B	Protein TRAJ61
High affinity cationic amino acid transporter 1	Ras-related protein Rab-10
Histone H2B type 1-K	Ribonuclease 4
Histone H3.3	Secreted ph phoprotein 24
Histone H4	Selenoprotein P
HLA class I histocompatibility antigen, A-3 alpha chain	Serum amyloid A-4 protein
HLA class I histocompatibility antigen, B-73 alpha chain	Sushi, von Willebrand factor type A, EGF and pentraxin domain-containing protein 1
Immunoglobulin heavy variable 1-3	Syntenin-7
Immunoglobulin heavy variable 3-64D	Ubiquitin-60S ribosomal protein L40
Immunoglobulin heavy variable 3-7	V-type proton ATPase subunit d 1