Human blood contains circulating cell-free mitochondria, but are they really functional?

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Running title: Circulating cell-free mitochondrial bioenergetics

Abstract

Dache et al. (2020, *FASEB J.* **15**, e2002338–15) recently reported the presence of *respiratory-competent* cell-free mitochondria in human blood (up to 3.7 x 10^6 per mL of blood), providing exciting perspectives on the potential role of these extra-cellular mitochondria. While their evidence for the presence of cell-free mitochondria in human blood is compelling, their conclusion that these cell-free mitochondria are respiratory-competent or functional has to be re-evaluated. To this end, we evaluated the functionality of cell-free mitochondria in human blood using high-resolution respirometry and mitochondria extracted from platelets of the same blood samples as positive controls. While cell-free mitochondria were present in human plasma (*i.e.* significant MitoTracker Green Fluorescence and complex IV activity), there was no evidence suggesting that their mitochondrial electron transport system (ETS) was functional (*i.e.* respiration rate not significantly different from 0; no significant responses to ADP, uncoupler or mitochondrial inhibitors oligomycin and antimycin A). Yet, *in vitro* complex IV activity was detectable and even slightly higher than levels found in mitochondria extracted from platelets, suggesting that cell-free mitochondria in human blood are likely to only retain a non-functional part of the electron transport system. Despite being unlikely to be fully functional in the narrow-sense (*i.e.* capable of oxidative phosphorylation), circulating cell-free mitochondria may have significant physiological roles that remain to be elucidated.

Keywords: mitochondrial function, blood cells, platelet, extra-cellular mitochondria

34 **Introduction**

35 Mitochondria are the powerhouse of eukaryotic cells using oxidative phosphorylation
36 to convert nutrients into ATP. They also have key roles in cell signalling (1) and ageing (2).
37 Vertebrates possess functional mitochondria in every cell type, with the notable exception of
38 mammalian mature erythrocytes (3). Dache et al. (2020) recently reported the presence of
39 cell-free mitochondria in human blood, providing exciting perspectives on the role of these
40 extra-cellular mitochondria, and their potential to be used as prognosis tool for various
41 diseases (4). While their evidence for the presence of cell-free mitochondria in human blood
42 is compelling and has been supported by other studies (*e.g.* (5, 6)), their conclusion that these
43 cell-free mitochondria are respiratory-competent or functional has to be re-evaluated (7)
44 since this is critical to uncover the physiological role they may or may not play (8).

45 Indeed, the interpretation that circulating cell-free mitochondria are functional is
46 exclusively based on the authors' graphical interpretation of *in vitro* O_2 consumption rates
47 (Fig. 7 in (4)), and not on statistical testing considering their sample size was N = 1 (despite
48 having n = 3 replicates of the same pooled sample; Dache et al. *pers. com.*). Based on the data
49 being presented in (4), it seems impossible to conclude that circulating cell-free mitochondria
50 are functional, as recently highlighted by (7). The *in vitro* respirometry assay used in (4) to
51 assess functionality has several limitations. First, the addition of ascorbate and TMPD to test
52 complex IV maximal capacity indeed elicits a rise in O_2 consumption, but no negative control
53 is shown by the authors, while there is a known chemical O_2 consumption linked to the auto-
54 oxidation of ascorbate and TMPD (9). Second, there is no visible inhibition of O_2 consumption
55 by antimycin A (*i.e.* a complex III inhibitor), suggesting that the minor O_2 consumption being
56 observed is unlikely to be of mitochondrial origin. Third, the role of mitochondria is not to
57 consume O_2 *per se*, but to produce ATP, and no test of oxidative phosphorylation functionality

58 (*e.g.* stimulation of respiration by ADP addition, or inhibition of respiration by oligomycin
59 addition, an inhibitor of ATP synthase) is provided (7).

60 Two alternative explanations could explain the apparent lack of functionality of these
61 circulating cell-free mitochondria. It is possible that circulating cell-free mitochondria are not
62 functional because they are damaged, or that only a small fraction of them (*i.e.* potentially the
63 ones that arrived in the blood stream only very recently) are still somewhat functional.
64 Mitochondrial integrity might be evaluated to some extent by the addition of exogenous
65 cytochrome c during respirometry assay. Cytochrome c does not penetrate intact
66 mitochondria but penetrates within partially damaged ones eliciting a rise in O_2 consumption
67 (10). Alternatively, it is possible that circulating cell-free mitochondria are functional, but that
68 the *in vitro* respirometry assay of (4) was not well suited for these mitochondria (7). Indeed,
69 the mitochondrial uncoupler FCCP has been added directly to the respiration medium at a
70 concentration of 4μM. However, uncouplers like FCCP have a narrow optimal working range
71 and require a progressive titration (*e.g.* 0.5μM steps; (10)). Indeed, when added in excess,
72 uncouplers can partially to fully inhibit mitochondrial respiration, and partially damaged
73 mitochondria are likely to be more sensitive to such phenomenon than intact ones (10).
74 Consequently, it is possible that the cell-free circulating mitochondria observed in (4) were
75 functional, but that they were fully inhibited by an inappropriate amount of mitochondrial
76 uncoupler in the respiratory medium.

77 A proper assessment of the functionality of circulating cell-free mitochondria is
78 therefore required to establish if they are really functional (7), or potentially non-functional
79 remnants of mitochondria that have been extruded by cells in the blood stream. In this study,
80 we aim to provide elements to answer this question by characterizing mitochondrial
81 bioenergetics properties of circulating cell-free mitochondria using high-resolution

82 respirometry and mitochondria extracted from platelets of the same blood samples as positive
83 controls.
84
85 **Methods**
86 Sample collection
87 This study was performed in accordance with the WMA Declaration of Helsinki. Blood
88 samples were collected at the University of Turku by a trained and habilitated lab technician
89 from six healthy volunteers (2 males and 4 females) following signature of an informed
90 consent form. Approximately 80mL of blood was taken from each volunteer using BD
91 vacutainer K_2EDTA tubes, which were progressively cooled and stored on ice before
92 processing (max. 1 hour).
93
94 Sample processing
95 We used a protocol similar to the one used by (6) to isolate circulating cell-free
96 mitochondria. Blood samples were first centrifugated at 150*g* for 20min (4°C) to isolate
97 platelet-rich plasma (PRP) from other blood cells. PRP was subsequently centrifugated at
98 2500*g* for 30min (4°C) to pellet platelets and obtain cell-free plasma. Mitochondria were
99 isolated from platelets pellet following (11), with 4 cycles of 10 passes in glass/glass Potter-
100 Elvehjem pestle homogenizer and 2500*g* centrifugation (4°C, 1min) to re-form platelet pellet
101 and facilitate cellular membrane disruption by homogenization. Platelet samples were then
102 centrifugated at 800*g* (4°C, 10min) and the supernatant containing isolated mitochondria was
103 subsequently processed along with cell-free plasma. Both sample types were centrifugated at
104 10000*g* (4°C, 10min) to pellet mitochondria. Mitochondrial pellets were resuspended in 2mL
105 of Mir05 respiration buffer (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM

106 taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, free fatty acid bovine serum albumin
107 (1 g/L)), and subsequently washed by centrifugation at 10000*g* (4°C, 10min). Mitochondrial
108 pellets were finally resuspended in 100µL of Mir05. Mitochondrial proteins were quantified
109 using the Biuret method, accounting for the fact that Mir05 contains 1mg.mL^{-1} of BSA.
110 Mitochondrial content was measured using MitoTracker Green fluorescent probe
111 (Invitrogen). Mitochondria were incubated in Mir05 supplemented with 200 nM of
112 MitoTracker Green for 30min at 37°C, washed by centrifugation at 10000*g* (4°C, 10min) and
113 re-suspended in 250µL of Mir05 before being transferred in 96-well black microplates
114 (duplicates of 100µL) for fluorescence signal acquisition using an Enspire 2300 multilabel plate
115 reader. Positive (standard dilution of *Saccharomyces cerevisae*) and negative (Mir05 and
116 unstained cells) controls were run on each plate. MitoTracker Green data is expressed as
117 relative fluorescence per mg of mitochondrial protein (see Fig. 1).
118
119 <u>High-resolution respirometry assay</u>
120 Mitochondrial respiration was assessed using the high-resolution respirometry system
121 O2k (Oroboros Instruments, Innsbruck, Austria) at 37°C using *ca.* 100 µg (range: [75 ; 125],
122 cell-free mitochondria = 93.4 ± 7.5 µg, platelet mitochondria = 108.2 ± 4.9 µg, p = 0.12) of
123 mitochondrial protein per assay (for comparison, 64µg of mitochondrial protein was used in
124 (4)). Mitochondria were added to the respirometry chambers containing 2mL of Mir05 pre-
125 equilibrated at 37°C and chambers were closed after 2min of equilibration. A substrate-
126 uncoupler-inhibitor titration (SUIT) protocol was used to assess the functionality of different
127 components of the mitochondrial electron transport system. Substrates of complex I (P:
128 pyruvate 5mM and M: malate 2mM) were first added, followed by a saturating amount of ADP
129 (2mM). Substrate of complex II (S: succinate 10mM) was then added to stimulate

130 mitochondrial respiration fueled by both complexes I and II. Cytochrome c (Cyt c: 10µM) was
131 added to test potential issues linked to mitochondrial inner membrane integrity. ATP synthesis
132 was then inhibited with oligomycin (O: 2.5µM). A progressive titration (0.5µM steps) of the
133 uncoupler FCCP was subsequently conducted until no further stimulation could be observed
134 (maximum stimulation was reached at *ca.* 1.5µM), at which stage mitochondrial respiration
135 was fully inhibited using the complex III inhibitor antimycin A (AA: 2.5µM). Finally, the maximal
136 activity of complex IV was assessed using ascorbate (Asc: 2mM) and N,N,N',N'-tetramethyl-p-
137 phenylenediamine (TMPD: 0.5mM). Since there is a known chemical O_2 consumption linked
138 to the auto-oxidation of ascorbate and TMPD (9), 2 negative control samples (*i.e.* 50µL of
139 Mir05 instead of mitochondria) were analyzed following the full SUIT protocol, so chemical O_2
140 consumption could be subtracted from ascorbate+TMPD values of biological samples (see Fig.
141 2A).
142
143 Data analysis
144 Difference in mitochondrial content (MitoTracker Green relative fluorescence)
145 between cell-free and platelet mitochondria was analyzed using non-parametric Wilcoxon
146 paired test, and difference to 0 for each sample type using non-parametric one-way Wilcoxon
147 test.
148 Oxygen consumption rates ($pmol.s^{-1}.mL^{-1}$) were extracted using DatLab 7.0 (Oroboros
149 Instruments, Innsbruck, Austria) after correction for background noise. Responses to SUIT
150 protocol of both plasma and platelet mitochondria were analyzed using a linear mixed model
151 with sample type (cell-free *vs.* platelet), stage (P+M, ADP, S, Cyt C, O, FCCP, AA, Asc+TMPD)
152 and their interaction as fixed factor; while controlling for pseudo-replication by including
153 stage(ID) as a random factor to account for the fact that plasma and platelet samples came

154 from the same individual (ID), as well as that measurements were repeated over the same
155 samples in the different stages of the SUIT protocol. Since a significant interaction between
156 sample type and stage was detected (see results), data was subsequently analyzed for each
157 sample type individually to assess the differences between stages using Bonferroni-corrected
158 post-hoc tests.

159 Mitochondrial respirations rates were calculated by subtracting non-mitochondrial
160 oxygen consumption (after AA inhibition) to specific stages ($OXPHOS_{CI+II}$ = S - AA; $LEAK_{CI+II}$ = O
161 - AA; ETS_{CI+II} = FCCP - AA), and by subtracting chemical O_2 consumption (*i.e.* obtained from
162 negative controls, see Fig 2A) to Asc+TMPD values (*COX* = Asc+TMPD - AA - chemical O_2
163 consumption). Those values were corrected by the quantity of mitochondrial protein used in
164 each assay and therefore expressed as $pmol.s^{-1}.mg^{-1}$. Differences between cell-free and
165 platelet mitochondria for each parameter were analyzed using non-parametric Wilcoxon
166 paired tests, and differences to 0 of each parameter were tested using non-parametric one-
167 way Wilcoxon tests. All analyses were conducted using SPSS 25.0, p-values < 0.05 were
168 considered significant and values are expressed as mean ± 95% C.I.

169

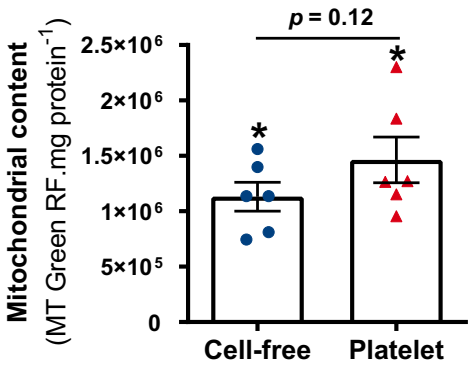
170 **Results**

171

172

173

174



175 **Fig. 1:** ***Mitochondrial content evaluated with MitoTracker Green relative fluorescence between***
176 ***circulating cell-free mitochondria and mitochondria extracted from platelets.*** *Significant difference*
177 *from 0 for each sample type is indicated by an *. Mean values are plotted ± 95% C.I., N = 6.*

178 There was a significant MitoTracker Green signal in both circulating cell-free (p = 0.023)
179 and platelet (p = 0.023) mitochondria samples (Fig. 1). Mitochondrial content was lower (by
180 *ca.* 33%) in circulating cell-free than in platelet mitochondria, but not significantly so (p = 0.12,
181 Fig. 1).

182 Responses to SUIT protocol of both cell-free and platelet mitochondria, as well as
183 negative controls, are presented in Fig. 2A. There was a significant interaction between sample
184 type and stage in the way mitochondria responded to the SUIT protocol ($F_{7,40}$ = 16.99, p <
185 0.001). Platelet mitochondria responded in the expected way to the SUIT protocol ($F_{7,8.1}$ =
186 84.4, p < 0.001; Fig. 2A), with an increase in O_2 consumption following ADP addition (P+M *vs.*
187 ADP: p = 0.012), stimulation by complex II substrate S (ADP *vs.* S: p = 0.005), non-significant
188 effect of Cyt c addition (S *vs.* Cyt c: p = 1.00), a decrease in O_2 consumption following ATP
189 synthase inhibition (Cyt c *vs.* O: p = 0.042), stimulation by FCCP titration (O *vs.* FCCP: p = 0.028),
190 full inhibition by complex III inhibitor AA (FCCP *vs.* AA: p = 0.007) and clear stimulation by
191 fueling directly complex IV with electrons (AA *vs.* Asc+TMPD: p < 0.001). On the contrary,
192 although cell-free mitochondrial O_2 consumption was affected by SUIT stage ($F_{7,7.9}$ = 159.1, p
193 < 0.001; Fig. 2A), they did not significantly respond to the SUIT protocol (P+M *vs.* ADP: p =
194 1.00; ADP *vs.* S: p = 1.00; S *vs.* Cyt c: p = 1.00; Cyt c *vs.* O: p = 1.00; O *vs.* FCCP: p = 1.00; FCCP
195 *vs.* AA: p = 1.00), except for the clear stimulation obtained by fueling directly complex IV with
196 electrons (AA *vs.* Asc+TMPD, p < 0.001).

197 While mitochondrial respiration rates of platelet mitochondria were all significantly
198 different from 0 (all p < 0.03; Fig. 2B), this was not the case for cell-free mitochondria
199 ($OXPHOS_{CI+II}$: p = 0.60; $LEAK_{CI+II}$: p = 0.60; ETS_{CI+II}: p = 0.25), except for complex IV respiration
200 (*COX*: p = 0.028). Mitochondrial respiration rates of platelet mitochondria were significantly
201 higher than cell-free ones ($OXPHOS_{CI+II}$, $LEAK_{CI+II}$ and ETS_{CI+II}: all p < 0.03; Fig 2B), except for

complex IV respiration (*i.e. COX*), for which cell-free mitochondria actually had a slightly higher respiration rate than platelet ones (*COX*: $p = 0.046$; Fig. 2B).

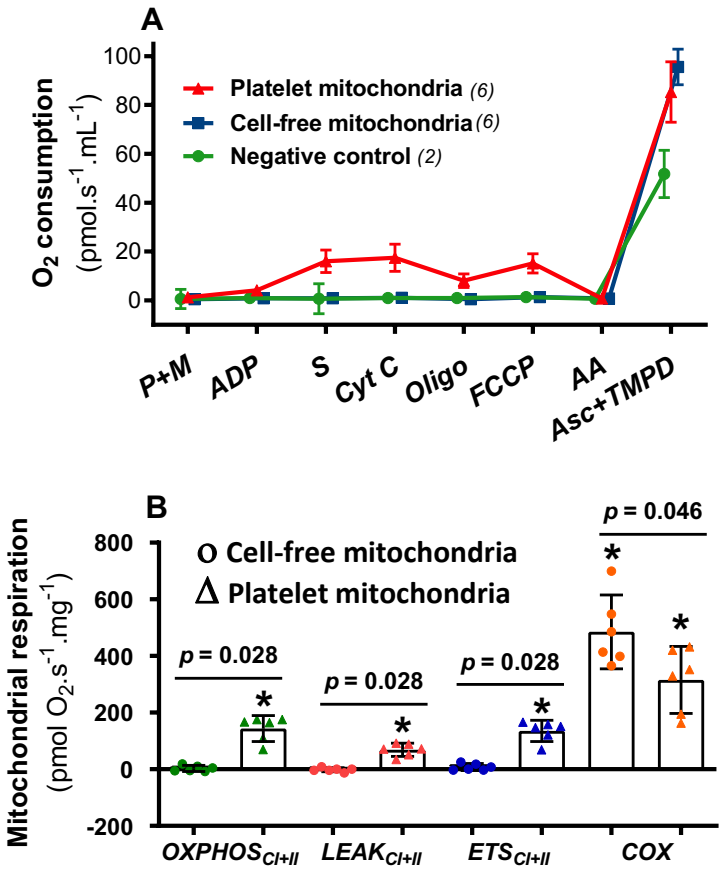


Fig. 2: ***Mitochondrial bioenergetics properties of human circulating cell-free mitochondria and mitochondria isolated from platelets. (A) Responses to a typical substrate-uncoupler-inhibitor protocol evaluated by high-resolution respirometry assay*** *(see methods for full details, and results for information on statistics). Negative control samples consisted of 50µL of respiration buffer instead of mitochondria. While platelets exhibited a typical response to SUIT protocol (all $p < 0.05$), cell-free mitochondria only responded to Asc+TMPD ($p < 0.001$, all other $p = 1.00$).* ***(B) Mitochondrial respiration rates of platelet vs. cell-free mitochondria****. Significant difference from 0 for each parameter is indicated by an *. All mean values are plotted ± 95% C.I., N = 6 in every case, except for negative controls (N = 2).*

232 **Discussion**

233 As noted by (7), the evidence provided by (4) was insufficient to infer the functionality
234 or the respiratory competence of human circulating cell-free mitochondria. By conducting a
235 comprehensive analysis of mitochondrial bioenergetics of human circulating cell-free
236 mitochondria in direct comparison to mitochondria extracted from platelets of the same
237 blood samples, we found no significant support for the functionality or the respiratory
238 competence of those cell-free mitochondria. Indeed, there was no significant evidence that
239 cell-free mitochondria possess a functional electron transport system since they exhibited no
240 responses to mitochondrial substrates, ADP or inhibitors. Yet, when fueling directly complex
241 IV with exogenous electrons (*i.e.* Asc+TMPD), a significant mitochondrial O_2 consumption was
242 detected (similarly to (4), except that no negative control was provided in their study). This
243 shows that cell-free mitochondria retain a small fraction of the ETS, but that they are unlikely
244 to be really functional or respiratory competent *in vivo* since electrons have to enter the ETS
245 through complex I and/or II. In contrast, mitochondria isolated from platelets show the typical
246 response to the SUIT protocol. Mitochondrial quantity per assay is unlikely to be a bias in the
247 data being presented here since cell-free mitochondria only exhibited a slightly lower
248 MitoTracker Green signal compared to mitochondria isolated from platelets, and even had a
249 slightly higher complex IV activity (sometimes used as a proxy of mitochondrial quantity (12)).
250 The absence of narrow-sense functionality (*i.e.* capability of oxidative phosphorylation) of
251 circulating cell-free mitochondria suggested by our results is a key point regarding the
252 identification of their potential physiological role(s). For instance, (8) hypothesized that
253 extracellular mitochondria could help in restoring homeostasis by accumulating at sites of
254 energy deficits, which is unlikely to be true if those mitochondria have no or an extremely low
255 capacity for oxidative phosphorylation.

256 Yet, using flow-cytometry fluorescence assays, (6) recently reported that at least part
257 of the cell-free mitochondria of human and murine blood could remain functional. Indeed,
258 part of the cell-free mitochondria identified by flow cytometry and MitoTracker Green
259 fluorescence appear capable of maintaining a mitochondrial membrane potential since they
260 retained TMRE probe, and such retention was lowered by the addition of the mitochondrial
261 uncoupler FCCP (6). Considering that the isolation protocol for cell-free mitochondria is likely
262 to result in many microparticles not containing 'intact' mitochondria (6), one hypothesis could
263 be that mitochondrial quantity is too low to enable the detection of significant functionality
264 by high-resolution respirometry. Yet, considering the estimated density of functional cell-free
265 mitochondria provided by (6) (*i.e.* 1.4 x 10^6/mL of blood), the samples used in the present
266 study should have contained *ca.* 110 x 10^6 'intact' extracellular mitochondria. Considering that
267 human platelets contain on average 4.9 mitochondria per cell (13), mitochondrial respiration
268 of our cell-free mitochondria samples should be approximately similar to the respiration of 22
269 x 10^6 platelets. Mitochondrial respiration of human platelets has been reported by others to
270 be around 0.35 pmol.s^{-1}.10^6 $cells^{-1}$ ($OXPHOS_{CI+II}$ in (14)). Thereby, if cell-free mitochondria from
271 human blood had respiration rates in the same range as platelet mitochondria, we would
272 expect our cell-free mitochondria to exhibit a respiration of *ca.* 7.6 pmol.s^{-1}.mL^{-1}, which is
273 approximately 8 times above the detection limit of our high-resolution respirometry assay
274 (*e.g.* $LEAK_{CI+II}$ of platelet mitochondria was 7.3 ± 2.4 pmol.s^{-1}.mL^{-1} in our study).
275 Although fully rejecting the hypothesis that human circulating cell-free mitochondria
276 are functional would require a way larger sample size (*e.g.* N > 90 for reaching a statistical
277 power of 0.80 for $OXPHOS_{CI+II}$ based on data presented in Fig. 1B), our results do not support
278 the functionality of circulating cell-free mitochondria. Further studies investigating the

279 functionality or lack of functionality of circulating cell-free mitochondria are now required,
280 since this appears as a critical point to elucidate their potential physiological role(s) (*e.g.* (8)).
281

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287

288 **Conflict of interest**

289 The author declares having no conflict of interest.
290

291 **Author contribution**

292 AS designed the study and conducted all the laboratory work, statistical analysis and writing
293 of the manuscript.
294

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