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 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 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 very similar to the one used by (6). Blood samples were first
96 centrifugated at 150*g* for 20min (4°C) to isolate platelet-rich plasma (PRP) from other blood
97 cells. PRP was subsequently centrifugated at 2500*g* for 30min (4°C) to pellet platelets and
98 obtain cell-free plasma. Mitochondria were isolated from platelets pellet following (11), with
99 4 cycles of 10 passes in glass/glass Potter-Elvehjem pestle homogenizer and 2500*g*
100 centrifugation (4°C, 1min) to re-form platelet pellet and facilitate cellular membrane
101 disruption by homogenization. Platelet samples were then centrifugated at 800*g* (4°C, 10min)
102 and the supernatant containing isolated mitochondria was subsequently processed along with
103 cell-free plasma. Both sample types were centrifugated at 10000*g* (4°C, 10min) to pellet
104 mitochondria. Mitochondrial pellets were resuspended in 2mL of Mir05 respiration buffer (0.5
105 mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes,

106 110 mM sucrose, free fatty acid bovine serum albumin (1 g/L)), and subsequently washed by
107 centrifugation at 10000*g* (4°C, 10min). Mitochondrial pellets were finally resuspended in
108 100µL of Mir05. Mitochondrial proteins were quantified using the Biuret method, accounting
109 for the fact that Mir05 contains 1mg.mL^{-1} of BSA.
110
111 High-resolution respirometry assay
112 Mitochondrial respiration was assessed using the high-resolution respirometry system
113 O2k (Oroboros Instruments, Innsbruck, Austria) at 37°C using *ca.* 100µg (range: [75 ; 125]) of
114 mitochondrial protein per assay (for comparison, 64µg of mitochondrial protein was used in
115 (4)). Mitochondria were added to the respirometry chambers containing 2mL of Mir05 pre-
116 equilibrated at 37°C and chambers were closed after 2min of equilibration. A substrate-
117 uncoupler-inhibitor titration (SUIT) protocol was used to assess the functionality of different
118 components of the mitochondrial electron transport system. Substrates of complex I (P:
119 pyruvate 5mM and M: malate 2mM) were first added, followed by a saturating amount of ADP
120 (2mM). Substrate of complex II (S: succinate 10mM) was then added to stimulate
121 mitochondrial respiration fueled by both complexes I and II. Cytochrome c (Cyt c: 10µM) was
122 added to test potential issues linked to mitochondrial inner membrane integrity. ATP synthesis
123 was then inhibited with oligomycin (O: 2.5µM). A progressive titration (0.5µM steps) of the
124 uncoupler FCCP was subsequently conducted until no further stimulation could be observed
125 (maximum stimulation was reached at *ca.* 1.5µM), at which stage mitochondrial respiration
126 was fully inhibited using the complex III inhibitor antimycin A (AA: 2.5µM). Finally, the maximal
127 activity of complex IV was assessed using ascorbate (Asc: 2mM) and N,N,N',N'-tetramethyl-p-
128 phenylenediamine (TMPD: 0.5mM). Since there is a known chemical O_2 consumption linked
129 to the auto-oxidation of ascorbate and TMPD (9), 2 negative control samples (*i.e.* 50µL of

130 Mir05 instead of mitochondria) were analyzed following the full SUIT protocol, so chemical O_2
131 consumption could be subtracted from ascorbate+TMPD values of biological samples (see Fig.
132 1A).
133
134 Data analysis
135 Oxygen consumption rates (pmol.s^{-1}.mL^{-1}) were extracted using DatLab 7.0 (Oroboros
136 Instruments, Innsbruck, Austria) after correction for background noise. Responses to SUIT
137 protocol of both plasma and platelet mitochondria were analyzed using a linear mixed model
138 with sample type (cell-free *vs.* platelet), stage (P+M, ADP, S, Cyt C, O, FCCP, AA, Asc+TMPD)
139 and their interaction as fixed factor; while controlling for pseudo-replication by including
140 stage(ID) as a random factor to account for the fact that plasma and platelet samples came
141 from the same individual (ID), as well as that measurements were repeated over the same
142 samples in the different stages of the SUIT protocol. Since a significant interaction between
143 sample type and stage was detected (see results), data was subsequently analyzed for each
144 sample type individually to assess the differences between stages using Bonferroni-corrected
145 post-hoc tests.
146 Mitochondrial respirations rates were calculated by subtracting non-mitochondrial
147 oxygen consumption (after AA inhibition) to specific stages ($OXPHOS_{CI+II}$ = S - AA; $LEAK_{CI+II}$ = O
148 - AA; ETS_{CI+II} = FCCP - AA), and by subtracting chemical O_2 consumption (*i.e.* obtained from
149 negative controls, see Fig 1A) to Asc+TMPD values (*COX* = Asc+TMPD - AA - chemical O_2
150 consumption). Those values were corrected by the quantity of mitochondrial protein used in
151 each assay and therefore expressed as pmol.s^{-1}.mg^{-1}. Differences between cell-free and
152 platelet mitochondria for each parameter were analyzed using non-parametric Wilcoxon
153 paired tests, and differences to 0 of each parameter were tested using non-parametric one-

154 way Wilcoxon tests. All analyses were conducted using SPSS 25.0, p-values < 0.05 were
155 considered significant and values are expressed as mean ± 95% C.I.
156
157 **Results**
158 Responses to SUIT protocol of both cell-free and platelet mitochondria, as well as
159 negative controls, are presented in Fig. 1A. There was a significant interaction between sample
160 type and stage in the way mitochondria responded to the SUIT protocol ($F_{7,40}$ = 16.99, p <
161 0.001). Platelet mitochondria responded in the expected way to the SUIT protocol ($F_{7,8.1}$ =
162 84.4, p < 0.001; Fig. 1A), with an increase in O_2 consumption following ADP addition (P+M *vs.*
163 ADP: p = 0.012), stimulation by complex II substrate S (ADP *vs.* S: p = 0.005), non-significant
164 effect of Cyt c addition (S *vs.* Cyt c: p = 1.00), a decrease in O_2 consumption following ATP
165 synthase inhibition (Cyt c *vs.* O: p = 0.042), stimulation by FCCP titration (O *vs.* FCCP: p = 0.028),
166 full inhibition by complex III inhibitor AA (FCCP *vs.* AA: p = 0.007) and massive stimulation by
167 fueling directly complex IV with electrons (AA *vs.* Asc+TMPD: p < 0.001). On the contrary,
168 although cell-free mitochondrial O_2 consumption was affected by SUIT stage ($F_{7,7.9}$ = 159.1, p
169 < 0.001; Fig. 1A), they did not significantly respond to the SUIT protocol (P+M *vs.* ADP: p =
170 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
171 *vs.* AA: p = 1.00), except for the massive stimulation obtained by fueling directly complex IV
172 with electrons (AA *vs.* Asc+TMPD, p < 0.001).

173 While mitochondrial respiration rates of platelet mitochondria were all significantly
174 different from 0 (all p < 0.03; Fig. 1B), this was not the case for cell-free mitochondria
175 ($OXPHOS_{CI+II}$: p = 0.60; $LEAK_{CI+II}$: p = 0.60; ETS_{CI+II}: p = 0.25), except for complex IV respiration
176 (*COX*: p = 0.028). Mitochondrial respiration rates of platelet mitochondria were significantly
177 higher than cell-free ones ($OXPHOS_{CI+II}$, $LEAK_{CI+II}$ and ETS_{CI+II}: all p < 0.03; Fig 1B), except for

178 complex IV respiration (*i.e. COX*), for which cell-free mitochondria actually had a slightly higher

179 respiration rate than platelet ones (*COX*: p = 0.046; Fig. 1B).

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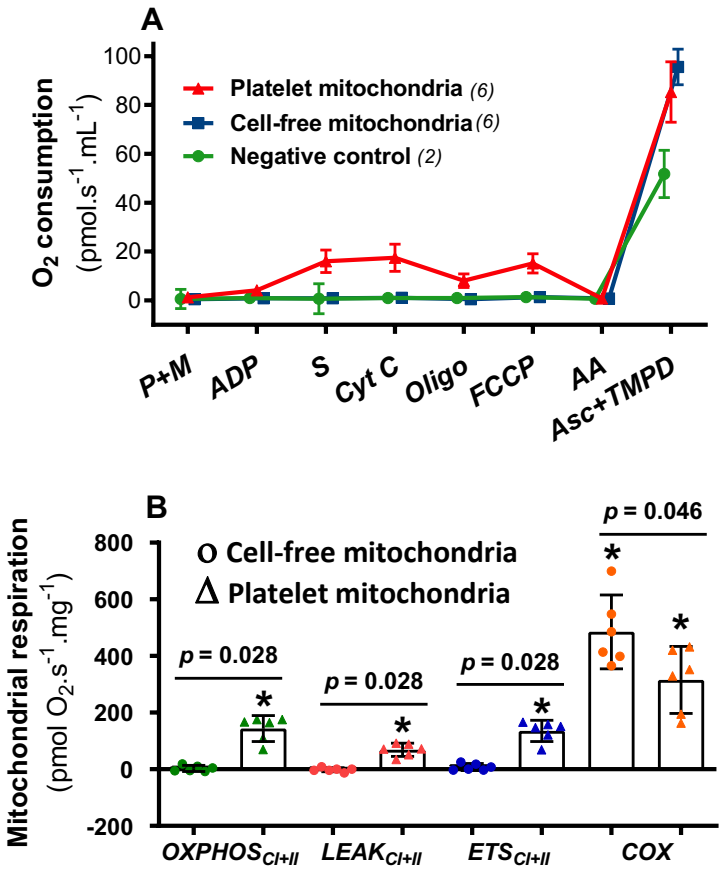

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198 **Fig. 1:** ***Mitochondrial bioenergetics properties of human circulating cell-free mitochondria and***

199 ***mitochondria isolated from platelets. (A) Responses to a typical substrate-uncoupler-inhibitor***

200 ***protocol evaluated by high-resolution respirometry assay*** *(see methods for full details, and results for*

201 *information on statistics). Negative control samples consisted of 50µL of respiration buffer instead of*

202 *mitochondria. While platelets exhibited a typical response to SUIT protocol (all p < 0.05), cell-free*

203 *mitochondria only responded to Asc+TMPD (p < 0.001, all other p = 1.00).* ***(B) Mitochondrial respiration***

204 ***rates of platelet vs. cell-free mitochondria****. Significant difference from 0 for each parameter is*

205 *indicated by an *. All mean values are plotted ± 95% C.I., N = 6 in every case except for negative controls*

206 *(N = 2).*

207

208 **Discussion**

209 As noted by (7), the evidence provided by (4) was insufficient to infer the functionality
210 or the respiratory competence of human circulating cell-free mitochondria. By conducting a
211 comprehensive analysis of mitochondrial bioenergetics of human circulating cell-free
212 mitochondria in direct comparison to mitochondria extracted from platelets of the same
213 blood samples, we found no significant support for the functionality or the respiratory
214 competence of those cell-free mitochondria. Indeed, there was no significant evidence that
215 cell-free mitochondria possess a functional electron transport system since they exhibited no
216 responses to mitochondrial substrates, ADP or inhibitors. Yet, when fueling directly complex
217 IV with exogenous electrons (*i.e.* Asc+TMPD), a significant mitochondrial O_2 consumption was
218 detected (similarly to (4), except that no negative control was provided in their study). This
219 shows that cell-free mitochondria retain a small fraction of the ETS, but that they are unlikely
220 to be functional or respiratory competent *in vivo* since electrons have to enter the ETS through
221 complex I and/or II. In contrast, mitochondria isolated from platelets show the typical
222 response to the SUIT protocol. Mitochondrial quantity per assay is unlikely to be a bias in the
223 data being presented here since cell-free mitochondria even had a slightly higher complex IV
224 activity (sometimes used as a proxy of mitochondrial quantity (12)) than mitochondria isolated
225 from platelets. The absence of narrow-sense functionality (*i.e.* capability of oxidative
226 phosphorylation) of circulating cell-free mitochondria suggested by our results is a key point
227 regarding the identification of their potential physiological role(s). For instance, (8)
228 hypothesized that extracellular mitochondria could help in restoring homeostasis by
229 accumulating at sites of energy deficits, which is unlikely to be true if those mitochondria have
230 no or an extremely low capacity for oxidative phosphorylation.

231 Yet, using flow-cytometry fluorescence assays, (6) recently reported that at least part
232 of the cell-free mitochondria of human and murine blood could remain functional. Indeed,
233 part of the cell-free mitochondria identified by flow cytometry and MitoTracker Green
234 fluorescence appear capable of maintaining a mitochondrial membrane potential since they
235 retained TMRE probe, and such retention was lowered by the addition of the mitochondrial
236 uncoupler FCCP (6). Considering that the isolation protocol for cell-free mitochondria is likely
237 to result in many microparticles not containing 'intact' mitochondria (6), one hypothesis could
238 be that mitochondrial quantity was too low in our study to enable the detection of significant
239 functionality by high-resolution respirometry. Yet, considering the estimated density of
240 functional cell-free mitochondria provided by (6) (*i.e.* 1.4 x 10^6/mL of blood), the samples used
241 in the present study should have contained *ca.* 110 x 10^6 intact extracellular mitochondria.
242 Considering that human platelets contain on average 4.9 mitochondria per cell (13),
243 mitochondrial respiration of our cell-free mitochondria samples should be approximately
244 similar to the respiration of 22 x 10^6 platelets. Mitochondrial respiration of human platelets
245 has been reported by others to be around 0.35 pmol.s^{-1}.10^6 $cells^{-1}$ ($OXPHOS_{CI+II}$ in (14)).
246 Thereby, if cell-free mitochondria from human blood had respiration rates in the same range
247 as platelet mitochondria, we would expect our cell-free mitochondria to exhibit a respiration
248 of *ca.* 7.6 pmol.s^{-1}.mL^{-1}, which is approximately 8 times above the detection limit of our high-
249 resolution respirometry assay (*e.g.* $LEAK_{CI+II}$ of platelets was 7.3 ± 2.4 in this study).
250 Although fully rejecting the hypothesis that human circulating cell-free mitochondria
251 are functional would require a way larger sample size (*e.g.* N > 90 for reaching a statistical
252 power of 0.80 for $OXPHOS_{CI+II}$ based on data presented in Fig. 1B), our results do not support
253 the functionality of circulating cell-free mitochondria. Further studies should be performed to

254 further ascertain the functionality or lack of functionality of circulating cell-free mitochondria
255 since this appears critical as to elucidate their physiological role(s) (*e.g.* (8)).
256

257 **Acknowledgement**
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261 collecting blood, to the six volunteers for giving their blood, as well as to the 'Turku Collegium
262 for Science and Medicine' for funding his research, and to the CafR team of La Piclière La Clusaz
263 for hosting him while writing the initial commentary.
264

265 **Conflict of interest**
266 The author declares having no conflict of interest.
267

268 **Author contribution**
269 AS designed the study and conducted all the laboratory work, statistical analysis and writing
270 of the manuscript.
271

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