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

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4

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10

11

12 Running title: Circulating cell-free mitochondrial bioenergetics

13 **Abstract**

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

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

32 **New & Noteworthy:**

33 The recently reported cell-free mitochondria in human blood have been thought to be
34 respiratory competent, giving rise to speculation about their biological function(s). By
35 characterizing their bioenergetics *in vitro*, we show that circulating cell-free mitochondria

36 are unlikely to be functional in vivo since they display no potential for oxidative
37 phosphorylation.

38 **Introduction**

39 Mitochondria are the powerhouse of eukaryotic cells using oxidative phosphorylation
40 to convert nutrients into ATP. They also have key roles in cell signalling (3) and ageing (14).
41 Vertebrates possess functional mitochondria in every cell type, with the notable exception
42 of mammalian mature erythrocytes (13). Dache et al. (2020) recently reported the presence
43 of cell-free mitochondria in human blood, providing exciting perspectives on the role of
44 these extra-cellular mitochondria, and their potential to be used as prognosis tool for
45 various diseases (1). While their evidence for the presence of cell-free mitochondria in
46 human blood is compelling and has been supported by other studies (*e.g.* (11, 12)), their
47 conclusion that these cell-free mitochondria are respiratory-competent or functional has to
48 be re-evaluated (2) since this is critical to uncover the physiological role they may or may not
49 play (4).

50 Indeed, the interpretation that circulating cell-free mitochondria are functional is
51 based on the graphical interpretation of *in vitro* O₂ consumption rates (Fig. 7 in (1)), and not
52 on statistical testing considering the sample size of N = 1 (n = 3 replicates of the same pooled
53 sample; Dache et al. *pers. com.*). Based on the data being presented in (1), it seems
54 impossible to conclude that circulating cell-free mitochondria are functional, as recently
55 highlighted by (2). The *in vitro* respirometry assay used in (1) to assess functionality has
56 several limitations. First, the addition of ascorbate and TMPD to test complex IV maximal
57 capacity indeed elicits a rise in O₂ consumption, but no negative control is presented, while
58 there is a known chemical O₂ consumption linked to the auto-oxidation of ascorbate and
59 TMPD (6). Second, there is no visible inhibition of O₂ consumption by antimycin A (*i.e.* a
60 complex III inhibitor), suggesting that the minor O₂ consumption being observed is unlikely
61 to be of mitochondrial origin. Third, the role of mitochondria is not to consume O₂ *per se*,

62 but to produce ATP, and no test of oxidative phosphorylation functionality (*e.g.* stimulation
63 of respiration by ADP addition, or inhibition of respiration by oligomycin addition, an
64 inhibitor of ATP synthase) is provided (2).

65 Two alternative explanations could explain the apparent lack of functionality of these
66 circulating cell-free mitochondria. It is possible that circulating cell-free mitochondria are not
67 functional because they are damaged, or that only a small fraction of them (*i.e.* potentially
68 the ones that arrived in the blood stream only very recently) are still somewhat functional.
69 Mitochondrial integrity might be evaluated to some extent by the addition of exogenous
70 cytochrome c during respirometry assay. Cytochrome c does not penetrate intact
71 mitochondria but penetrates within partially damaged ones eliciting a rise in O₂
72 consumption (7). Alternatively, it is possible that circulating cell-free mitochondria are
73 functional, but that the *in vitro* respirometry assay used in (1) was not well suited for these
74 mitochondria (2). Indeed, the mitochondrial uncoupler FCCP has been added directly to the
75 respiration medium at a concentration of 4μM. However, uncouplers like FCCP have a
76 narrow optimal working range and require a progressive titration (*e.g.* 0.5μM steps; (7)).
77 Indeed, when added in excess, uncouplers can partially to fully inhibit mitochondrial
78 respiration, and partially damaged mitochondria are likely to be more sensitive to such
79 phenomenon than intact ones (7). Consequently, it is possible that the cell-free circulating
80 mitochondria observed in (1) were functional, but that they were fully inhibited by an
81 inappropriate amount of mitochondrial uncoupler in the respiratory medium.

82 A proper assessment of the functionality of circulating cell-free mitochondria is
83 therefore required to establish if they are really functional (2), or potentially non-functional
84 remnants of mitochondria that have been extruded by cells in the blood stream. In this
85 study, we aim to provide elements to answer this question by characterizing mitochondrial

86 bioenergetics properties of circulating cell-free mitochondria using high-resolution
87 respirometry and mitochondria extracted from platelets of the same blood samples as
88 matched positive controls.

89

90 **Methods**

91 Sample collection

92 This study was performed in accordance with the WMA Declaration of Helsinki. Blood
93 samples were collected at the University of Turku by a trained and habilitated lab technician
94 from six healthy volunteers (2 males and 4 females) following signature of an informed
95 consent form. Approximately 80mL of blood was taken from each volunteer using BD
96 vacutainer K₂EDTA tubes, which were progressively cooled and stored on ice before
97 processing (max. 1 hour).

98

99 Sample processing

100 We used a protocol similar to the one used by (12) to isolate circulating cell-free
101 mitochondria. This protocol differs slightly from the one used in (1), to enable the separation
102 of platelets to be used as matched positive controls (*i.e.* from the same blood samples).
103 Blood samples were first centrifugated at 150g for 20min (4°C) to isolate platelet-rich
104 plasma (PRP) from other blood cells. PRP was subsequently centrifugated at 2500g for
105 30min (4°C) to pellet platelets and obtain cell-free plasma. Cell-free plasma was kept on ice
106 while mitochondria were isolated from platelets pellet following (5). For platelets, we used 4
107 cycles of 10 passes in glass/glass Potter-Elvehjem pestle homogenizer and 2500g
108 centrifugation (4°C, 1min) to re-form platelet pellet and facilitate cellular membrane
109 disruption by homogenization. Platelet samples were then centrifugated at 800g (4°C,

110 10min) and the supernatant containing isolated mitochondria was subsequently processed
111 along with the cell-free plasma.

112 Both sample types were centrifugated at 10000g (4°C, 10min, similarly to (12)) to
113 pellet mitochondria. Mitochondrial pellets were resuspended in 2mL of Mir05 respiration
114 buffer (0.5 mM EGTA, 3 mM MgCl₂, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH₂PO₄,
115 20 mM Hepes, 110 mM sucrose, free fatty acid bovine serum albumin (1 g/L)), and
116 subsequently washed by centrifugation at 10000g (4°C, 10min). Mitochondrial pellets were
117 finally resuspended in 100µL of Mir05. Mitochondrial proteins were quantified using the
118 Biuret method, accounting for the fact that Mir05 contains 1mg.mL⁻¹ of BSA. Mitochondrial
119 content was measured using MitoTracker Green fluorescent probe (Invitrogen).
120 Mitochondria were incubated in Mir05 supplemented with 200 nM of MitoTracker Green for
121 30min at 37°C, washed by centrifugation at 10000g (4°C, 10min) and re-suspended in 250µL
122 of Mir05 before being transferred in 96-well black microplates (duplicates of 100µL) for
123 fluorescence signal acquisition using an Enspire 2300 multilabel plate reader. Positive
124 (standard dilution of *Saccharomyces cerevisiae*) and negative (Mir05 and unstained cells)
125 controls were run on each plate. MitoTracker Green data is expressed as relative
126 fluorescence per mg of mitochondrial protein (see Fig. 1).

127

128 High-resolution respirometry assay

129 Mitochondrial respiration was assessed using the high-resolution respirometry
130 system O2k (Oroboros Instruments, Innsbruck, Austria) at 37°C using *ca.* 100 µg (range: [75 ;
131 125], cell-free mitochondria = 93.4 ± 7.5 µg, platelet mitochondria = 108.2 ± 4.9 µg, *p* = 0.12)
132 of mitochondrial protein per assay (for comparison, 64µg of mitochondrial protein was used
133 in (1)). Mitochondria were added to the respirometry chambers containing 2mL of Mir05

134 pre-equilibrated at 37°C and chambers were closed after 2min of equilibration. A substrate-
135 uncoupler-inhibitor titration (SUIT) protocol was used to assess the functionality of different
136 components of the mitochondrial electron transport system. Substrates of complex I (P:
137 pyruvate 5mM and M: malate 2mM) were first added, followed by a saturating amount of
138 ADP (2mM). Substrate of complex II (S: succinate 10mM) was then added to stimulate
139 mitochondrial respiration fueled by both complexes I and II. Cytochrome c (Cyt c: 10µM) was
140 added to test potential issues linked to mitochondrial inner membrane integrity. ATP
141 synthesis was then inhibited with oligomycin (O: 2.5µM). A progressive titration (0.5µM
142 steps) of the uncoupler FCCP was subsequently conducted until no further stimulation could
143 be observed (maximum stimulation was reached at *ca.* 1.5µM), at which stage mitochondrial
144 respiration was fully inhibited using the complex III inhibitor antimycin A (AA: 2.5µM).
145 Finally, the maximal activity of complex IV was assessed using ascorbate (Asc: 2mM) and
146 N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD: 0.5mM). Since there is a known
147 chemical O₂ consumption linked to the auto-oxidation of ascorbate and TMPD (6), 2 negative
148 control samples (*i.e.* 50µL of Mir05 instead of mitochondria) were analyzed following the full
149 SUIT protocol, so chemical O₂ consumption could be subtracted from ascorbate+TMPD
150 values of biological samples (see Fig. 2A).

151

152 Data analysis

153 Difference in mitochondrial content (MitoTracker Green relative fluorescence)
154 between cell-free and platelet mitochondria was analyzed using non-parametric Wilcoxon
155 paired test, and difference to 0 for each sample type using non-parametric one-way
156 Wilcoxon test.

157 Oxygen consumption rates ($\text{pmol}\cdot\text{s}^{-1}\cdot\text{mL}^{-1}$) were extracted using DatLab 7.0 (Oroboros
158 Instruments, Innsbruck, Austria) after correction for background noise. Responses to SUI
159 protocol of both cell-free and platelet mitochondria were analyzed using a linear mixed
160 model with sample type (cell-free vs. platelet), stage (P+M, ADP, S, Cyt C, O, FCCP, AA,
161 Asc+TMPD) and their interaction as fixed factor; while controlling for pseudo-replication by
162 including stage(ID) as a random factor to account for the fact that plasma and platelet
163 samples came from the same individual (ID), as well as that measurements were repeated
164 over the same samples in the different stages of the SUI protocol. Since a significant
165 interaction between sample type and stage was detected (see results), data was
166 subsequently analyzed for each sample type individually to assess the differences between
167 stages using Bonferroni-corrected post-hoc tests.

168 Mitochondrial respirations rates were calculated by subtracting non-mitochondrial
169 oxygen consumption (after AA inhibition) to specific stages ($\text{OXPHOS}_{\text{CI+II}} = \text{S} - \text{AA}$; $\text{LEAK}_{\text{CI+II}} = \text{O}$
170 $- \text{AA}$; $\text{ETS}_{\text{CI+II}} = \text{FCCP} - \text{AA}$), and by subtracting chemical O_2 consumption (*i.e.* obtained from
171 negative controls, see Fig 2A) to Asc+TMPD values ($\text{COX} = \text{Asc+TMPD} - \text{AA} - \text{chemical } \text{O}_2$
172 consumption). Those values were corrected by the quantity of mitochondrial protein used in
173 each assay and therefore expressed as $\text{pmol}\cdot\text{s}^{-1}\cdot\text{mg}^{-1}$. Differences between cell-free and
174 platelet mitochondria for each parameter were analyzed using non-parametric Wilcoxon
175 paired tests, and differences to 0 of each parameter were tested using non-parametric one-
176 way Wilcoxon tests. All analyses were conducted using SPSS 25.0, p-values < 0.05 were
177 considered significant and values are expressed as mean \pm 95% C.I.

178

179 **Results**

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

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

199

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

204 higher than cell-free ones ($OXPHOS_{CI+II}$, $LEAK_{CI+II}$ and ETS_{CI+II} : all $p < 0.03$; Fig 2B), except for
205 complex IV respiration (*i.e.* COX), for which cell-free mitochondria actually had a slightly
206 higher respiration rate than platelet ones (COX : $p = 0.046$; Fig. 2B).

207

208 **Discussion**

209 As noted by (2), the evidence provided by (1) was not sufficient to assess the
210 functionality or the respiratory competence of human circulating cell-free mitochondria. By
211 conducting a comprehensive analysis of mitochondrial bioenergetics of human circulating
212 cell-free mitochondria in direct comparison to mitochondria extracted from platelets of the
213 same 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
216 no responses to mitochondrial substrates, ADP or inhibitors. Yet, when fueling directly
217 complex IV with exogenous electrons (*i.e.* $Asc+TMPD$), a significant mitochondrial O_2
218 consumption was detected (similarly to (1), except that no negative control was provided in
219 their study). This shows that cell-free mitochondria retain a small fraction of the ETS, but
220 that they are unlikely to be really functional or respiratory competent *in vivo* since electrons
221 have to enter the ETS through complex I and/or II. In contrast, mitochondria isolated from
222 platelets show the typical response to the SUI protocol. Mitochondrial quantity per assay is
223 unlikely to be a bias in the data being presented here since cell-free mitochondria only
224 exhibited a non-significantly slightly lower MitoTracker Green signal compared to
225 mitochondria isolated from platelets, and even had a slightly higher complex IV activity
226 (sometimes used as a proxy of mitochondrial quantity (8)). The integrity of cell-free
227 mitochondria might have been altered during the *in-vitro* steps, but this hypothesis is not

228 supported by the cytochrome c test (see Fig. 2A), and the matched positive controls (*i.e.*
229 platelet mitochondria) have been submitted to a harsher isolation procedure (*i.e.* due to
230 glass/glass Potter-Elvehjem use) without any visible loss of integrity. The absence of narrow-
231 sense functionality (*i.e.* capability of oxidative phosphorylation) of circulating cell-free
232 mitochondria suggested by our results is a key point regarding the identification of their
233 potential physiological role(s). For instance, (4) hypothesized that extracellular mitochondria
234 could help in restoring homeostasis by accumulating at sites of energy deficits, which is
235 unlikely to be true if those mitochondria have no or an extremely low capacity for oxidative
236 phosphorylation.

237 Yet, using flow-cytometry fluorescence assays, (12) recently reported that at least
238 part of the cell-free mitochondria of human and murine blood could remain functional.
239 Indeed, part of the cell-free mitochondria identified by flow cytometry and MitoTracker
240 Green fluorescence appear capable of maintaining a mitochondrial membrane potential
241 since they retained TMRE probe, and such retention was lowered by the addition of the
242 mitochondrial uncoupler FCCP (12). Considering that the isolation protocol for cell-free
243 mitochondria is likely to result in many microparticles not containing 'intact' mitochondria
244 (12), one hypothesis could be that mitochondrial quantity is too low to enable the detection
245 of significant functionality by high-resolution respirometry. Yet, considering the estimated
246 density of functional cell-free mitochondria provided by (12) (*i.e.* 1.4×10^6 /mL of blood), the
247 samples used in the present study should have contained *ca.* 110×10^6 'intact' extracellular
248 mitochondria. Considering that human platelets contain on average 4.9 mitochondria per
249 cell (9), mitochondrial respiration of our cell-free mitochondria samples should be
250 approximately similar to the respiration of 22×10^6 platelets. Mitochondrial respiration of
251 human platelets has been reported by others to be around $0.35 \text{ pmol}\cdot\text{s}^{-1}\cdot 10^6 \text{ cells}^{-1}$

252 ($OXPHOS_{C+II}$ in (10)). Thereby, if cell-free mitochondria from human blood had respiration
253 rates in the same range as platelet mitochondria, we would expect our cell-free
254 mitochondria to exhibit a respiration of *ca.* $7.6 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{mL}^{-1}$, which is approximately 8 times
255 above the detection limit of our high-resolution respirometry assay (*e.g.* $LEAK_{C+II}$ of platelet
256 mitochondria was $7.3 \pm 2.4 \text{ pmol}\cdot\text{s}^{-1}\cdot\text{mL}^{-1}$ in our study).

257 Although fully rejecting the hypothesis that human circulating cell-free mitochondria
258 are functional would require a way larger sample size (*e.g.* $N > 90$ for reaching a statistical
259 power of 0.80 for $OXPHOS_{C+II}$ based on data presented in Fig. 1B), our results do not support
260 the functionality of circulating cell-free mitochondria. Further studies investigating the
261 functionality or lack of functionality of circulating cell-free mitochondria are now required,
262 since this appears as a critical point to elucidate their potential physiological role(s) (*e.g.* (4)).

263

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270

271 **Conflict of interest**

272 The author declares having no conflict of interest.

273

274 **Author contribution**

275 AS designed the study and conducted all the laboratory work, statistical analysis and writing
276 of the manuscript.

277

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- 313

314 Figure captions

315

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

319

320

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

330

331

332

Mitochondrial content
(MT Green RF.mg protein⁻¹)



