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**Oxygen release from hemoglobin has limited effects on mitochondrial respiration measured from red blood cells.** Reply to the Comment on *“Increased retention of functional mitochondria in mature sickle red blood cells is associated with increased sickling tendency, hemolysis and oxidative stress”*

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## Disclosures

No conflicts of interest to disclose

## Contributions

AS collected and analyzed the data, and wrote the manuscript with input from PC, SE, EN and DR.

## Main text

The measurement of mitochondrial aerobic metabolism from blood cells has gained in popularity over the past decade due to its low invasiveness<sup>1,2</sup>. In non-mammalian vertebrates, red blood cells are nucleated and possess functional mitochondria<sup>3</sup>, which enables the assessment of mitochondrial respiration from small blood samples (as low as 20  $\mu$ L of whole-blood<sup>4</sup>). Recently, we have showed that human mature sickle red blood cells retain some functional mitochondria, which was associated with increased sickling tendency, hemolysis and oxidative stress<sup>5</sup>. Willis et al.<sup>6</sup> recently questioned the methodology used to demonstrate

the functionality of the mitochondria retained in sickle red blood cells, since hemoglobin-O<sub>2</sub> dissociation could influence the oxygen consumption rate (JO<sub>2</sub>) measured with high-resolution respirometry.

As rightly noted by Willis et al.<sup>6</sup>, the PO<sub>2</sub> within the *in-vitro* chamber declines over time due to oxygen consumption by the cells. Such a decline in PO<sub>2</sub> leads to the potential release of O<sub>2</sub> by hemoglobin, to an extent depending mostly on: 1. the change in PO<sub>2</sub>, 2. the HbO<sub>2</sub> binding parameters, and 3. the amount of hemoglobin in the chamber. Willis et al.<sup>6</sup> state that because the PO<sub>2</sub> is progressively falling, the errors will confound not only the absolute rates but also the relative differences between respiratory states (*i.e.* the proof of mitochondrial functionality we used in<sup>5</sup>). Such statement assumes that O<sub>2</sub> dissociation from hemoglobin will be higher at lower PO<sub>2</sub>. However, using the same protocol as in previous study on avian red blood cells<sup>7</sup> Fig. 1 shows that it would not be the case since non-mitochondrial JO<sub>2</sub> (after antimycin A addition) does not vary over a broad range of PO<sub>2</sub> (see also electronic supplementary material (ESM) S1 for raw data). At low PO<sub>2</sub>, O<sub>2</sub> release from hemoglobin could have been anticipated as assumed by Willis et al.<sup>6</sup>, which would have led to decreased or even negative (*i.e.* O<sub>2</sub> release > O<sub>2</sub> consumed) JO<sub>2</sub>.

Actually, the release of O<sub>2</sub> from hemoglobin will not directly depend on the PO<sub>2</sub>, but on the instantaneous rate of change in PO<sub>2</sub> linked to oxygen consumption (*i.e.* JO<sub>2</sub> in a given state), and on the position in terms of absolute PO<sub>2</sub> on the hemoglobin-O<sub>2</sub> dissociation curve. As supposed by Willis et al.<sup>6</sup> based on Stier et al.<sup>1</sup> and Esperti et al.<sup>5</sup>, our measurements on both avian species and human were conducted at high PO<sub>2</sub> (Fig 2A, see raw data in ESM S2), enabling to remain within the linear and almost flat portion of the hemoglobin-O<sub>2</sub> dissociation curve (Fig. 2B). Extracting the data from Abdu et al. (2008)<sup>8</sup> and Powell (2015)<sup>9</sup> for human and birds (Fig. 2B, ESM S3), respectively, enabled to calculate the change in hemoglobin saturation (%) linked to JO<sub>2</sub>-induced changes in PO<sub>2</sub>, and the associated release of O<sub>2</sub> for each respiratory state (see ESM S4 and S5 for calculations). Based on these calculations, we can note that the release of O<sub>2</sub> is relatively minor (difference between JO<sub>2</sub> and JO<sub>2</sub> corrected in Fig. 2C and 2D). While absolute respiration rates are affected to a minor extent (Fig. 2C & 2D), the relative differences between respiratory states are not, as demonstrated for instance by the similarity between OXPHOS coupling efficiencies calculated from raw vs. corrected JO<sub>2</sub> values (*i.e.* for Japanese quail: raw =  $0.731 \pm 0.013$  vs. corrected =  $0.730 \pm 0.013$ ; for human sickle red blood cells: raw =  $0.437 \pm 0.065$  vs. corrected =  $0.424 \pm 0.061$ ). Since O<sub>2</sub> release is proportional to JO<sub>2</sub> and

influenced by hemoglobin content (that does not vary between the different respiratory states), it is not surprising from our perspective that the relative differences between respiratory states are not influenced by O<sub>2</sub> release from hemoglobin, as long as the assay is conducted within the linear and almost flat part of the Hb-O<sub>2</sub> dissociation curve (Fig. 2B).

As rightly pointed out by Willis et al.<sup>6</sup>, issues can arise when two groups have different Hb contents and/or O<sub>2</sub> binding kinetics. This is however unlikely to confound the results presented in Esperti et al.<sup>5</sup> because O<sub>2</sub> binding kinetics does not vary between healthy and sickle red blood cells in normoxic conditions<sup>8</sup> (> 92.5% saturation, PO<sub>2</sub> > 65 mm Hg), and Hb content (and thus potential O<sub>2</sub> release) is lower in sickle cell patients than healthy individuals<sup>10</sup>.

Willis et al.<sup>6</sup> also rightly questioned the choice of Mir05 as a respiratory medium and the lack of exogenous substrate (*i.e.* glucose) when assessing mitochondrial respiration of 'intact' red blood cells. Respiration of intact blood cells can for instance be conducted in PBS or plasma<sup>11</sup>, but our own experience with avian blood cells shows that mitochondria loose functionality along the assay with PBS, as evidenced by a FCCP-induced respiration being lower than the endogenous respiration, which does not happen when using Mir05. Mir05 also enables to first measure the endogenous respiration and then to permeabilize the red blood cells for more detailed investigation<sup>12</sup>. From our perspective, refraining from using exogenous substrates enables the measurement of mitochondrial respiration rates being more closely related to the *in-vivo* physiology, where substrates are usually not at saturating levels. Using the subject's own plasma<sup>11</sup>, whenever possible, is likely the best way to obtain the more meaningful information about *in-vivo* mitochondrial metabolism. Regarding the amount of red blood cells used in respirometry assays, Willis et al.<sup>6</sup> also question the precision of pipetting packed red blood cells, which we have questioned before (see<sup>1</sup>). Counting red blood cells is likely the best approach possible (as done in<sup>4,12</sup>).

Around 75% of the patients included in our study<sup>5</sup> were under hydroxyurea therapy, and all patients from the mitochondrial respiration experiments received this treatment. Hydroxyurea has recently been shown to promote erythroid differentiation by accelerating maturation processes<sup>13</sup>, which may impact the degree of mitochondrial retention into mature red blood cells. To answer this question, a study would be needed to test the degree of mitochondrial retention into mature red blood cells and their functionality before and after hydroxyurea therapy.

In light of the excellent comment by Willis et al.<sup>6</sup>, here are some recommendations that should be useful to ensure the best accuracy when measuring mitochondrial respiration from red blood cells: 1. provide the range of PO<sub>2</sub> at which JO<sub>2</sub> measurements are conducted; 2. ensure that measurements are conducted at a PO<sub>2</sub> being within a linear and almost flat part of the Hb-O<sub>2</sub> dissociation curve; 3. check for potential bias between experimental groups in terms of Hb content or O<sub>2</sub> binding kinetics; 4. measure Hb in individual samples to correct JO<sub>2</sub> if deemed necessary based on theoretical expectations (*e.g.* if Hb content is high and variable between samples) or if a difference exists between groups in terms of Hb content.

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## Figure legends

**Fig. 1: Relationship between non-mitochondrial  $JO_2$  and  $PO_2$  in Japanese quail (*Coturnix japonica*) red blood cells measured *in-vitro* with high-resolution respirometry** (N = 2 biological replicates per  $PO_2$ ; mean  $\pm$  SD). Measurements were conducted according to the methodology described in Stier et al.<sup>7</sup>, and the various  $PO_2$  at which non-mitochondrial  $JO_2$  was measured were achieved by letting intact red blood cells consume more or less  $O_2$  within the chamber before adding antimycin A.

**Fig. 2: Relationship between  $PO_2$  and  $JO_2$  in red blood cells of Japanese quail (blue) and human with sickle cell disease (red): A. Raw  $JO_2$  measurements and the associated  $PO_2$  during measurement; B. Hemoglobin-oxygen dissociation curves, redrawn from Powell<sup>9</sup> and Abdu et al.<sup>8</sup>; C. Comparison of raw  $JO_2$  and  $JO_2$  corrected for  $O_2$  release by hemoglobin for Japanese quails; D. Comparison of raw  $JO_2$  and  $JO_2$  corrected for  $O_2$  release by hemoglobin for human sickle red blood cells.** N = 8 biological replicates for each species, data from Stier et al.<sup>7</sup> and Esperti et al.<sup>5</sup> re-analyzed (mean  $\pm$  SE).