

## RESEARCH ARTICLE

# Mitochondrial respiration variability and simulations in human skeletal muscle: The Gene SMART study

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## Funding information

Nir Eynon, Grant/Award Number:  
 NHMRC CDF#APP1140644; Sarah  
 Voisin, Grant/Award Number: NHMRC  
 CDF#APP115777321

## Abstract

Mitochondrial respiration using the oxygraph-2k respirometer (Oroboros) is widely used to estimate mitochondrial capacity in human skeletal muscle. Here, we measured mitochondrial respiration variability, in a relatively large sample, and for the first time, using statistical simulations, we provide the sample size required to detect meaningful respiration changes following lifestyle intervention. Muscle biopsies were taken from healthy, young men from the Gene SMART cohort, at multiple time points. We utilized samples for each measurement with two technical repeats using two respirometer chambers ( $n = 160$  pairs of same muscle after removal of low-quality samples). We measured the Technical Error of measurement ( $TE_M$ ) and the coefficient of variation (CV) for each mitochondrial complex. There was a high correlation between measurements from the two chambers ( $R > 0.7$   $P < .001$ ) for all complexes, but the  $TE_M$  was large ( $7.9\text{--}27$   $\text{pmol s}^{-1} \text{mg}^{-1}$ ; complex dependent), and the CV was  $>15\%$  for all complexes. We performed statistical simulations of a range of effect sizes at 80% power and found that 75 participants (with duplicate measurements) are required to detect a 6% change in mitochondrial respiration after an intervention, while for interventions with 11% effect size, ~24 participants are sufficient. The high variability in respiration suggests that the typical sample sizes in exercise studies may not be sufficient to capture exercise-induced changes.

## KEYWORDS

exercise, intervention, mitochondria, OXPHOS, oxygen consumption

**Abbreviations:** ATP, adenosine triphosphate; CI, complex I, electron input through CI; CV, coefficient of variation;  $CI_p$ , oxidative phosphorylation (OXPHOS) capacity (P) through CI; ETS, electron transport system; CI+CII, convergent electron input through CI and CII  $CI+CI_E$ , capacity (E) through CI+CII;  $CI+CI_p$ , complex II (CII) linked respiration; E, ETS capacity; ETS, measurement of electron transport system; FCCP, p-trifluoromethoxyphenylhydrazine; Gene SMART, genes and skeletal muscle adaptive response to training; Inv-RCR, inverse of respiratory control ratio ( $CIL/CI+II_p$ ); L, leak respiration; LCR, leak control ratio ( $CIL/CI+II_E$ ); OXPHOS, oxidative phosphorylation; P, oxfos capacity; PCR, phosphorylation control ratio ( $CI+II_p/CI+II_E$ ); ROX, residual oxygen consumption; SCR, substrate control ratio at constant P ( $CIP/CI+II_p$ ); TCA, tricarboxylic acid;  $TE_M$ , technical error of measurement.

Sarah Voisin and Nir Eynon are Co-senior authorship.

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## 1 | INTRODUCTION

The mitochondrion is a membrane-enclosed organelle found in eukaryotic cells. With its five specialized complexes, it produces adenosine triphosphate (ATP) and thus constitutes the powerhouse of the cell.<sup>1</sup> ATP is produced during oxidative phosphorylation (OXPHOS) via the tricarboxylic acid (TCA) cycle inside the mitochondrial matrix, and via the electron transport system (ETS) along the inner mitochondrial membrane.<sup>2</sup> Mitochondrial respiration measured by maximal oxygen consumption in skeletal muscle fibers, is currently the primary way of assessing mitochondrial capacity.<sup>3,4</sup> The most common method in human skeletal muscle uses fibers permeabilized by saponin.<sup>5,6</sup> Using a combination of different substrates, this technique is able to mimic the processes (ie, TCA cycle and ETS) occurring within the mitochondrion.

Regular exercise has a beneficial effect on mitochondrial function.<sup>7</sup> Endurance exercise training improves mitochondrial respiration,<sup>8-13</sup> with high-intensity exercise training leading to larger improvements (up to 40%)<sup>8,10-12</sup> than moderate continuous exercise training (up to 20%).<sup>14,15</sup> However, changes in mitochondrial respiration using permeabilized muscle fibers following exercise training have been assessed in relatively small sample sizes, typically within a range of  $n = 8-15$ ,<sup>16</sup> and without assessing respiration changes in a control group.<sup>17</sup> Mitochondrial respiration values, as well as improvements in mitochondrial respiration following exercise training are also quite variable between studies.<sup>3,10,14,16,18,19</sup> For example, Irving et al<sup>18</sup> observed a ~1.5-fold change in mitochondrial respiration after moderate intensity endurance training ( $n = 34$ ), while Robach et al<sup>20</sup> did not observe any change after a similar intervention ( $n = 17$ ). Mitochondrial respiration capacity in human exercise intervention studies is not exclusive to skeletal muscle samples. Although such investigations are less common, different studies have measured respiration capacity in adipose tissue,<sup>21,22</sup> and blood cells (including lymphocytes and platelet).<sup>23-26</sup>

Tests run in duplicates (or more) enable researchers to capture technical variability, and/or biological day-to-day variability within participants.<sup>17</sup> The typical error of measurement ( $TE_M$ ), also called “within-subject standard deviation,” provides an estimate of such variability.<sup>17</sup> In the only study to date investigating the reliability of mitochondrial respiration measurement,<sup>3</sup> the  $TE_M$  between two fiber bundles from the same biopsy was  $10.5 \text{ pmol s}^{-1} \text{ mg}^{-1}$  in the maximal oxidative phosphorylation (OXPHOS) and the coefficient of variations (CVs) were worryingly high (15.2% between two fiber bundles, 23.9% between legs, and 33.1% at different time points).<sup>3</sup> More studies are required to confirm whether such variability is consistently high, but, more importantly, this technical variability needs to be put into perspective with typical mitochondrial respiration changes

following interventions (eg, exercise training interventions). The qualifiers “high” and “low” variability only make sense when compared with the magnitude of the intervention-induced changes, which will determine how likely we are to detect those changes.

Therefore, the aim of the present study was to investigate the reliability of mitochondrial respiration measurements in human *vastus lateralis* muscle using large number of duplicate fiber bundles (160 pairs) collected from a range of participants at different time points. We correlated mitochondrial respiration values between two chambers containing bundles of same muscle sample for complexes  $CI_P$ ,  $CI + CII_P$  and  $CI + CII_E$ , and calculated the  $TE_M$  and the CV between experiments. Using the estimated  $TE_M$  and CV, we performed simulations to determine the minimum number of participants required to detect meaningful mitochondrial respiration changes of various effect sizes following a hypothetical intervention, at 80% power.

## 2 | MATERIALS AND METHODS

### 2.1 | Participants

Participants were from the Genes and Skeletal Muscle Adaptive Response to Training (Gene SMART) cohort. The full study methodology has been previously described elsewhere.<sup>27</sup> 68 apparently healthy, Caucasian men (age =  $31.4 \pm 8.2$  years old; BMI =  $25.2 \pm 3.2 \text{ kg/m}^2$ ) participated in the study and signed a written informed consent. Participants were excluded if they had any preexisting heart condition, health issues, and/or preexisting injury that could potentially impair exercise capacity. The study was approved by the Human Ethics Research Committee at Victoria University (HRE13-223).

### 2.2 | Muscle biopsies

A controlled diet for 48 hours prior to the muscle biopsies was provided to the participants, according to the guidelines of the Australian National Health & Medical Research Council (NHMRC). Muscle biopsies were taken by an experienced medical doctor from the *vastus lateralis* muscle of the participants' dominant leg, following local anesthesia (2 mL, 1% Lidocaine (Lignocaine)). The needle was inserted in the participant leg and manual suction was applied for muscle collection. Care was taken not to contaminate the muscle samples with local anesthetic during the biopsy. Approximately 2-6 mg of muscle was then immediately placed in ice-cold BIOPS for determination of mitochondrial respiration in two individual chambers (duplicates).

## 2.3 | Mitochondrial respiration

Immediately after each biopsy (within max 30 minutes of collection), 2-6 mg of muscle fibers were mechanically separated using pointed forceps under a binocular microscope in 2-mL ice-cold biopsy preservation solution on ice (BIOPS, 2.77 mM CaK<sub>2</sub>EGTA, 7.23 mM K<sub>2</sub>EGTA, 5.77 mM Na<sub>2</sub>ATP, 6.56 mM MgCl<sub>2</sub>•6H<sub>2</sub>O, 20 mM Taurine, 15 mM Na<sub>2</sub>Phosphocreatine, 20 mM Imidazole, 0.5 mM Dithiothreitol, and 50 mM K<sup>+</sup>-MES at pH 7.1).<sup>6</sup> Permeabilization of the plasma membrane occurred in the same solution with 50 µg/ml of saponin (Sigma-Aldrich, St Louis, USA) for 30 minutes rotating on ice. This was followed by rinsing the muscle fibers for 3 × 7 minutes in mitochondrial respiration medium (MiR05, 0.5 mM EGTA, 3 mM MgCl<sub>2</sub>•6H<sub>2</sub>O, 60 mM K-lactobionate, 20 mM Taurine, 10 mM KH<sub>2</sub>PO<sub>4</sub>, 20 mM Hepes, 110 mM sucrose, and 1 g/L bovine serum albumin at pH 7.1)<sup>6</sup> on ice. Mitochondrial respiration was measured in duplicates in washed muscle fibers (between 1 and 3 mg wet weight of muscle fibers/chamber) in MiR05 at 37°C using the high-resolution Oxygraph-2k (Oroboros, Innsbruck, Austria), with additional substrates. Oxygen concentration (mM) and flux (pmol × s<sup>-1</sup> × mg<sup>-1</sup>) were recorded using DatLab software. Reoxygenation by direct syringe injection of O<sub>2</sub> was necessary to maintain O<sub>2</sub> levels between 275 and 450 mM and to avoid potential oxygen diffusion limitation. A substrate-uncoupler-inhibition titration sequence was used. The following substrates were added (final concentration): malate (2 mM) and pyruvate (5 mM) were added to measure the LEAK respiration (L) through Complex I (CI) (CI<sub>L</sub>), followed by MgCl<sub>2</sub> (3mM) and ADP (5 mM) to measure oxidative phosphorylation (OXPHOS) capacity (P) through CI (CI<sub>P</sub>), followed by succinate (10 mM) to measure P through CI + Complex II (CII) linked respiration (CI + CII<sub>P</sub>).<sup>28</sup> This respiration state represents the maximal respiratory capacity in the respirometer chamber.<sup>28</sup> Cytochrome *c* (10 µM) was used to test the integrity of the outer mitochondrial membrane, in this step if the respiration increased >10% when cytochrome *c* was added, values from that chamber were removed due to a damaged membrane. A series of steps (steps of 0.5 µM) p-trifluoromethoxyphenylhydrazon (FCCP) titrations followed for measurement of electron transport system (ETS) capacity (E) through CI + CII (CI + CII<sub>E</sub>). Antimycin (3.75 µM) was added to block the activity of complex III and to measure the residual oxygen consumption (ROX) indicative of non-mitochondrial oxygen consumption. Substrate and coupling control ratios were calculated from the different titration steps obtained from the protocol used.<sup>7</sup> A background calibration for the Oroboros machine was performed every 3 months, and air calibrations were performed before each experiment. The results were pasted into the excel spreadsheet supplied by the manufacturer (Oroboros). If air calibrations

presented more than 5% deviation in the results, membranes were changed, and new background calibration was done. Instrument backgrounds were performed in MiR05, and oxygen levels were at 450 nmol/mL. Highly variable graphs are indicative of poor quality, as shown on the O2K software, were removed.

## 2.4 | Citrate synthase activity

Intrinsic changes in the mitochondria can be determined by quantitative measurements of specific markers. Such measurements can estimate the content of the mitochondria and are commonly used to normalize global measurements of mitochondrial function. The most commonly used measurement is the citrate synthase (CS) enzyme activity.<sup>29</sup> Thus, we have normalized our results by CS activity.

Complete enzyme extractions, from small pieces of frozen tissues, were performed in an ice-cold buffer (KH<sub>2</sub>PO<sub>4</sub> & K<sub>2</sub>HPO<sub>4</sub>) using a TissueLyser II (Qiagen, Hilden, Germany). Protein concentration was assessed using the bicinchoninic acid assay. Total citrate synthase (CS) activity was measured (30°C, pH 7.5) using standard spectrophotometric assays. CS activity is presented in international units (IU).

## 2.5 | Statistical analyses

We calculated three different metrics to show the reliability of mitochondrial respiration measurements. First, we calculated the correlation between duplicates from the two chambers, for each complex, using non-parametric Spearman's test to downweight the influence of outliers, and a stringent *P* value < .005 for significance. Then, we calculated the within-subject standard deviation, also called typical (or technical) error of measurement (TE<sub>M</sub>)<sup>17</sup>:

$$TE_M = \frac{\sqrt{\sum_1^n (x_{i1} - x_{i2})^2}}{2n},$$

where *n* is the number of pairs of duplicates and *x* is the respiration measurement.

We calculated the coefficient of variability (CV) estimated by:

$CV = 100 \times \frac{TE_M}{\mu}$ , where  $\mu$  is the mean respiration across all duplicates and all samples. While TE<sub>M</sub> is expressed in the units of mitochondrial respiration (pmol s<sup>-1</sup> mg<sup>-1</sup>), CV is a percentage.

Lastly, we performed simulations based on the TE<sub>M</sub> for the CI + CII<sub>P</sub> and CI + CII<sub>E</sub> respiration values. We simulated increases of 1%-50% in mitochondrial respiration in each participant after a hypothetical intervention and estimated the

sample size (number of participants) required to detect this change at 80% power.

All analyses were performed using the R software.

### 3 | RESULTS

#### 3.1 | Large technical error in mitochondrial respiration measurement

Two fiber bundles from the same muscle biopsy were run simultaneously in two chambers totaling 160 duplicate pairs after removal of results that indicated damaged membrane (ie, cyt-c increased >10%). Respiration measurements were correlated between chambers ( $R \geq 0.71-0.75$ ,  $P < .005$  for all) (Figure 1). Yet when compared with correlations obtained for gene expression data (generally  $R > 0.9$ ),<sup>30,31</sup> the correlation values obtained here are rather low.

The poor correlation between chambers was consistent with high  $TE_M$  and CV estimates for all complexes (Table 1), as all complexes showed a  $CV \geq 15\%$ . When reporting the Flux Control Ratios (FCRs), to account for lab-to-lab variability,<sup>7</sup> the  $TE_M$  and CV estimates were also significantly elevated, with some reaching more than 100%.

	Mean $\pm$ SD		$TE_M$	CV (%)
	Chamber 1	Chamber 2		
$CI_p$ (pmol s <sup>-1</sup> mg <sup>-1</sup> )	82.9 $\pm$ 30.6	85.1 $\pm$ 28.7	14.9	17.5
$CI + CII_p$ (pmol s <sup>-1</sup> mg <sup>-1</sup> )	123.1 $\pm$ 39.0	123.0 $\pm$ 36.0	19.0	15.3
$CI + II_E$ (pmol s <sup>-1</sup> mg <sup>-1</sup> )	154.9 $\pm$ 48.8	150.6 $\pm$ 44.1	24.4	15.9
LCR ( $CI_L/CI + II_E$ )	0.08 $\pm$ 0.08	0.09 $\pm$ 0.07	0.04	50.8
PCR ( $CI + II_p/CI + II_E$ )	0.80 $\pm$ 0.12	0.82 $\pm$ 0.11	0.07	9.0
RCR ( $CI + II_p/CI_L$ )	11.1 $\pm$ 31.3	11.6 $\pm$ 16.7	22.8	193.7
Inv_RCR ( $CI_L/CI + II_p$ )	0.11 $\pm$ 0.09	0.11 $\pm$ 0.08	0.05	48.6
SCR ( $CI_p/CI + II_p$ )	0.67 $\pm$ 0.11	0.69 $\pm$ 0.08	0.07	10.8

Abbreviations: CI, complex I; CI, electron input through CI; CI+II, convergent electron input through CI and CII; CI+CII, complex I & II; E, ETS capacity; L, leak respiration; Inv-RCR, inverse of respiratory control ratio ( $CI_L/CI+II_p$ ); LCR, leak control ratio ( $CI_L/CI+II_E$ ); P, oxphos capacity; PCR, phosphorylation control ratio ( $CI+II_p/CI+II_E$ ); RCR, respiratory control ratio ( $CI+II_p/CI_L$ ); SCR, substrate control ratio at constant P ( $CI_p/CI+II_p$ ).

FCR were calculated from mass-specific mitochondrial respiration measurements in permeabilized muscle fibers (vastus lateralis).

	Mean $\pm$ SD		$TE_M$	CV (%)
	Chamber 1	Chamber 2		
$CI_p^*/CS^{**}$	5.38 $\pm$ 1.92	5.60 $\pm$ 1.79	1.02	18.5
$CI+CII_p^*/CS^{**}$	8.13 $\pm$ 2.73	8.24 $\pm$ 2.52	1.25	15.2
$CI+II_E^*/CS^{**}$	10.35 $\pm$ 3.65	10.22 $\pm$ 3.32	1.57	15.3

Note: Results based on 128 muscle samples.

\* (pmol s<sup>-1</sup> mg<sup>-1</sup>); \*\* (mIU  $\times$  mg protein<sup>-1</sup>).

The poor correlation along with high  $TE_M$  and CV (> 15%) estimates for all complexes was still observed when we normalized mitochondrial respiration with CS activity, (Table 2).

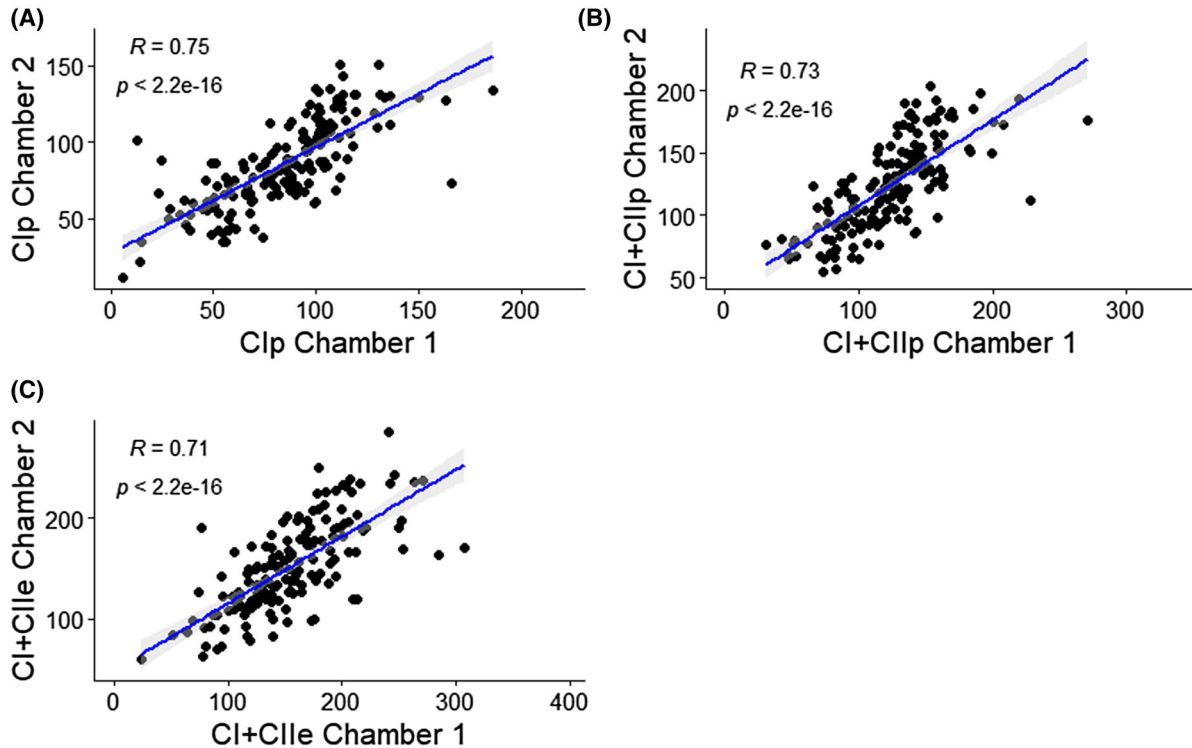
#### 3.2 | Simulations to estimate the sample size required to detect changes in mitochondrial respiration at 80% power

We performed simulations for both the  $CI + CII_p$  and  $CI + II_E$  respiration values since they are the most commonly reported respiration measurements in the literature, as well as the PCR and SCR ratios. We estimated the sample size required to detect true changes in mitochondrial respiration at 80% power. Since  $TE_M$  and CV values for mitochondrial respiration and mitochondrial respiration/CS were similar we have not conducted simulations for the latter. However, we have attached the code for this calculation in the supplementary data.

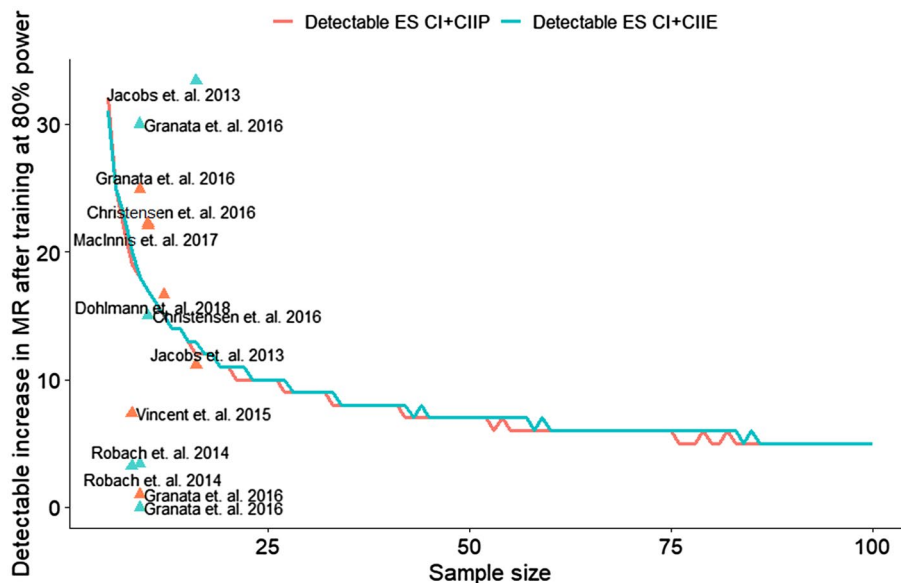
For the coupled and uncoupled respiration, the minimum sample size required to observe a percentage increase at 80% power is shown on Figure 2. An intervention that increases mitochondrial respiration by 10% at the group level requires

**TABLE 1** Chamber-specific respiration values and FCRs, typical error of measurement and coefficient of variation for each substrate

**TABLE 2** Chamber-specific respiration values normalized by CS activity, typical error of measurement, and coefficient of variation for each substrate

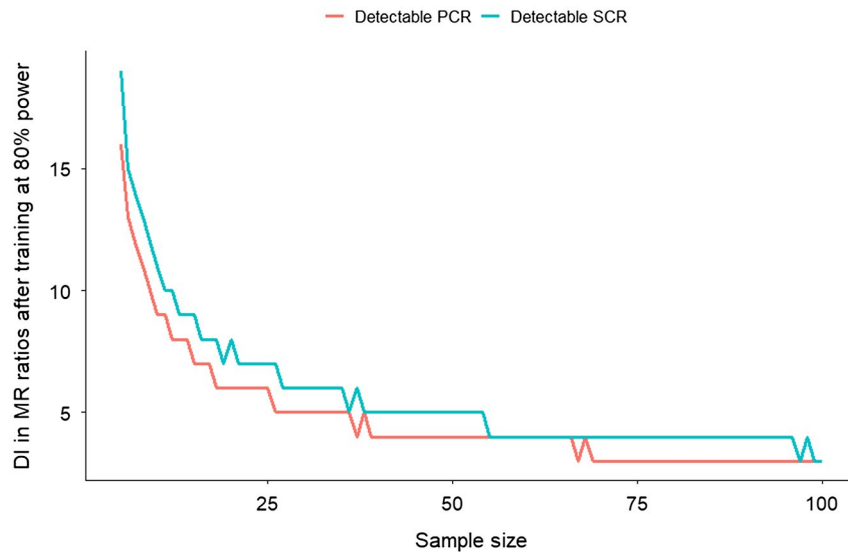


**FIGURE 1** Spearman's correlation between chambers after the addition of (A) oxidative phosphorylation (OXPHOS) capacity (P) through Complex I ( $CI_p$ ), (B), measure P through CI+Complex II (CII) linked respiration (CI+ $CI_{Ip}$ ), (C) electron transport system (ETS) capacity (E) through CI+CII (CI+ $CI_{IE}$ ). All values are in  $\text{pmol s}^{-1} \text{mg}^{-1}$



**FIGURE 2** Minimum sample size required to detect increases in mitochondrial respiration (MR) after training at 80% power. A minimum of  $\sim 55$  (CI+ $CI_{Ip}$ ) and  $\sim 60$  (CI+ $CI_{IE}$ ) pairs of duplicate samples are necessary to detect an increase of 6% in mitochondrial respiration at the group level, at 80% power. An intervention with  $\sim 20$  samples/individuals would require a change of at least 11% in mitochondrial respiration to achieve 80% power for both CI+ $CI_{Ip}$  and CI+ $CI_{IE}$  respiration. Experiments with less than 15 individuals would require a change of at least 13% to achieve 80% power. The triangles represent real effect sizes and sample sizes reported in different studies <sup>8,10-12,14,20,35</sup>





**FIGURE 3** Minimum sample size required to detect increases (DI) in mitochondrial respiration (MR) ratios after training at 80% power. A minimum of ~26 (PCR) and ~38 (SCR) pairs of duplicate samples are necessary to detect an increase of 5% in mitochondrial respiration at the group level, at 80% power. An intervention with ~20 samples/individuals would require a change of at least 6% and 8% in mitochondrial respiration to achieve 80% power for PCR and SCR ratios, respectively. Experiments with less than 20 individuals would require a change of at least 7%-10% to achieve 80% power. Due to inconsistencies in the literature in which ratios each study calculates we have not included data from published studies in our ratios graph

a minimum of 23 participants to detect changes for  $CI + CII_P$  and  $CI + CII_E$  at 80% power. Our results suggest that with the typical sample size in exercise training studies ( $n = 12$ ), only changes of  $>15\%$  in mitochondrial respiration following training would be detectable at 80% power.

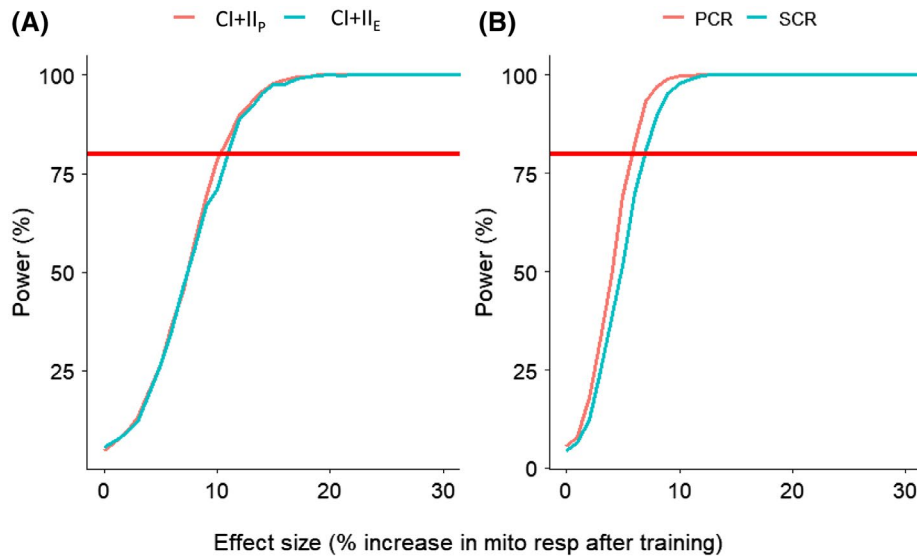
For the PCR and SCR respiration ratios, the minimum sample size required to observe a percentage increase at 80% power is shown on Figure 3. An intervention that increases mitochondrial respiration by 10%, at the group level, requires a minimum of 11 participants to detect changes for PCR ratio and 22 participants for the SCR ratio at 80% power.

We have also simulated percentage increases after hypothetical exercise training intervention in a cohort of 20 individuals (Figure 4A). We observed that an increase of ~11% or more in mitochondrial respiration is necessary for changes to be detected at 80% statistical power if each participant had duplicate respiration measurements. While for ratios (Figure 4B), a minimum of ~6% increase for PCR phosphorylation control ratio ( $CI + II_P/CI + II_E$ ) and ~7% increase for SCR substrate control ratio at constant P ( $CI_P/CI + II_P$ ) is required to be detected at 80% power, if each participant had duplicate respiration measurements.

## 4 | DISCUSSION

In the present study, we reported the  $TE_M$  and CV for measurements of mitochondrial respiration for  $CI_P$ ,  $CI + CII_P$ ,

and  $CI + CII_E$ , using the OROBOROS equipment, and in a large sample ( $n = 160$  pairs of duplicate respiration measurements). We also performed statistical simulations to uncover the minimum number of participants required to detect an intervention-induced change in mitochondrial respiration at 80% power. We found a very large variability in all measurements ( $CV > 15\%$ ), suggesting that this measurement may only be appropriate in studies using large sample sizes ( $n \geq 55$ ) or that detect large effect sizes ( $>15\%$ ). To account for between-lab (lab-by-lab) variability, we have also computed the  $TE_M$  and CV for mitochondrial respiration ratios including: LCR (L/E), PCR (P/E), RCR (P/L), Inv RCR (L/P), and SCR (constant P). Not surprisingly, the  $TE_M$  and CV remained  $>9\%$ , and the statistical simulations suggest a sample size of  $\geq 26$  is required to achieve 80% power. Mitochondrial respirations values were also normalized by CS activity, but no significant changes in  $TE_M$  or CVs were observed. In other words, the type of training should be carefully selected to achieve effect sizes in mitochondrial respiration experiments if sample size is a limitation. For example, participants who did sprint interval training (SIT) ( $n = 9$ ) presented  $>19\%$  change in mitochondrial respiration after 4 weeks of training, while participants who did only moderate intensity ( $n = 9$ ) or high intensity interval training ( $n = 11$ ) did not show any changes in mitochondrial respiration after 4 weeks of training.<sup>10</sup> Higher numbers of technical replicates (ie, number of chambers used for the same muscle—here we used two) could potentially lower the  $TE_M$ , in which case the required sample size would be lower to detect a given effect size.



**FIGURE 4** A, Power to detect percentage change in mitochondrial respiration (effect size) after a training intervention with  $n = 20$  participants. A minimum of  $\sim 11\%$  increase in mitochondrial respiration is needed to be detected at 80% power for both the CI+CI<sub>P</sub> and CI+CI<sub>E</sub>, if each participant had duplicate respiration measurements. B, Power to detect percentage change in mitochondrial respiration ratios (effect size) after a training intervention with  $n = 20$  participants. A minimum of  $\sim 6\%$  increase for PCR phosphorylation control ratio (CI+II<sub>P</sub>/CI+II<sub>E</sub>) and  $\sim 7\%$  increase for SCR substrate control ratio at constant P (CI<sub>P</sub>/CI+II<sub>P</sub>) is required to be detected at 80% power, if each participant had duplicate respiration measurements

$TE_M$  includes variability due to machine calibration and human error, and is potentially specific to each research facility.<sup>17</sup> However, Cardinale et al<sup>3</sup> recently reported a similarly high CV (15.2%), suggesting that the high variability we observed occurs across research facilities and is intrinsic to the OROBOROS equipment. Permeabilization of muscle pieces involves taking a small piece of muscle (typically 2–6 mg) and placing it in a dish with BIOPS solution; then, a technician uses two pairs of sharp forceps to separate individual fiber bundles.<sup>32</sup> Since this process is complicated, it is recommended that the same person performs the procedure in a given study to avoid variability between technicians.<sup>33</sup> The degree of fiber separation determines the amount of mitochondria present after the permeabilization, thus affecting the respiration measurements.<sup>33</sup> It is plausible that technicians vary in their ability to efficiently separate fibers, and this could lead to higher respiration values and potentially higher variability as well. Thus, different technical staff/researchers conducting the experiment can explain why experiments are variable. The largest variability in measurements, recently reported by Cardinale et al<sup>3</sup> was in experiments conducted by two different technicians working on the same piece of muscle (mean  $\pm$  SD =  $31.3 \pm 7.1$  vs  $26.3 \pm 8.1$  pmol s<sup>-1</sup> mg<sup>-1</sup>,  $P = .12$ ). In the present study, some of the experiments were conducted by one technician, and some by another technician (ie, the two technicians never handled the same piece of muscle), which might explain some of the variability we reported. Unfortunately, we are unable to calculate the variability due to technicians in this study as they worked

on different muscle samples. It should also be noted that it is common to use creatine in the respiration chambers when working with permeabilized muscle. However, several papers have not used creatine in their experiments and have reported valid and replicable results.<sup>7,8,10,12</sup>

To the best of our knowledge, the smallest meaningful change in mitochondrial respiration after training or other lifestyle interventions has never been reported, since this is dependent on the overall aim of each study.<sup>10</sup> In the present study, we calculated the minimum number of participants required to detect a change in mitochondrial respiration at 80% power. Our results suggest that with the typical sample size in exercise training studies ( $n = 12$ ), only changes of  $>18\%$  in mitochondrial respiration following training would be detectable at 80% power. This means that it would be a challenge to observe true changes in mitochondrial capacity using the OROBOROS technology, since most studies do not report such large increases following exercise training ( $-9\%$  to  $20\%$ ).<sup>18,19,32,34–38</sup> We acknowledge that some of the studies have observed significant changes in mitochondrial respiration without reaching the effect sizes we presented here. This implies that although such studies were significant, the sample sizes were too low to detect the magnitude of changes they reported at 80% power. The simulations presented in this paper provide important information for planning experiments investigating mitochondrial capacity. Future studies can use this data and the code we provided (see Supplementary File 1) as a guide to determine the number of participants required to

detect changes in mitochondrial respiration in their study. Alternatively, if the number of participants is a limitation, then a careful consideration of the exercise intervention duration is recommended, to trigger changes that are large enough to achieve 80% power.

In conclusion, we found very large variability in mitochondrial respiration measurements, reflected by  $TE_M$  and CV, and calculated the required sample size necessary for studies aimed at detecting changes in mitochondrial respiration. We recommend that future studies utilizing this method in skeletal muscle would follow the guidelines we provided here to detect significant changes in mitochondrial capacity, following lifestyle interventions. Finally, it should be noted that mitochondrial respiration is only one measure of mitochondrial function. The use of integrative approaches,<sup>1,16,32</sup> such as mitochondrial protein expression, mitochondrial content quantification, mitochondrial DNA sequencing, and appropriate statistical methods<sup>17</sup> may allow discoveries in the complex and integrative nature of exercise adaptations<sup>16,39</sup> as well as strengthen results from mitochondrial respiration measurements.

## ACKNOWLEDGMENT

This study was chiefly supported by Nir Eynon's National Health and Medical Research Council, Australia Career Development Fellowship (NHMRC CDF# APP1140644), and Sarah Voisin's NHMRC Early Career Fellowship (APP11577321). The authors would like to acknowledge Dr Lannie O'Keefe for providing the participant's-controlled diet according to the NHMRC guidelines.

## CONFLICT OF INTEREST

The authors have no conflict of interest on the production of this manuscript.

## AUTHOR CONTRIBUTIONS

M Jacques performed the experiments, analyzed the data, and drafted the manuscript; J Kuang performed the experiments and revised the manuscript; DJ Bishop revised the manuscript; X Yan participated in the research design and revised the manuscript; J Alvarez-Romero performed the experiments; F Munson, I Papadimitriou; and A Garnham performed and collected the muscle biopsies and revised the manuscript; S Voisin performed the data analyses and assisted in drafting the manuscript; and N Eynon designed the research and assisted in drafting the manuscript.

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## SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

**How to cite this article:** Jacques M, Kuang J, Bishop DJ, et al. Mitochondrial respiration variability and simulations in human skeletal muscle: The Gene SMART study. *The FASEB Journal.* 2020;34:2978–2986. <https://doi.org/10.1096/fj.201901997RR>