


## RESEARCH PAPER

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# Effects of 30 days bed rest and exercise countermeasures on PBMC bioenergetics

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## Abstract

**Aim:** Altered mitochondrial function across various tissues is a key determinant of spaceflight-induced physical deconditioning. In comparison to tissue biopsies, blood cell bioenergetics holds promise as a systemic and more readily accessible biomarker, which was evaluated during head-down tilt bed rest (HDTBR), an established ground-based analog for spaceflight-induced physiological changes in humans. More specifically, this study explored the effects of HDTBR and an exercise countermeasure on mitochondrial respiration in peripheral blood mononuclear cells (PBMCs).

**Methods:** We subjected 24 healthy participants to a strict 30-day HDTBR protocol. The control group ( $n=12$ ) underwent HDTBR only, while the countermeasure group ( $n=12$ ) engaged in regular supine cycling exercise followed by veno-occlusive thigh cuffs post-exercise for 6 h. We assessed routine blood parameters 14 days before bed rest, the respiratory capacity of PBMCs via high-resolution respirometry, and citrate synthase activity 2 days before and at day 30 of bed rest. We confirmed PBMC composition by flow cytometry.

**Results:** The change of the PBMC maximal oxidative phosphorylation capacity (OXPHOS) amounted to an 11% increase in the countermeasure group, while it decreased by 10% in the control group ( $p=0.04$ ). The limitation of OXPHOS increased in control only while other respiratory states were not affected by either intervention. Correlation analysis revealed positive associations between white blood cells, lymphocytes, and basophils with PBMC bioenergetics in both groups.

**Conclusion:** This study reveals that a regular exercise countermeasure has a positive impact on PBMC mitochondrial function, confirming the potential application of blood cell bioenergetics for human spaceflight.

## KEYWORDS

blood cell bioenergetics, countermeasure, exercise, head-down tilt bed rest, mitochondrial function, PBMC, simulated microgravity, spaceflight

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## 1 | INTRODUCTION

Maintaining crew health during long-duration spaceflights is crucial for mission success. Yet, space conditions compromise health and induce pleiotropic changes including muscular and cardiovascular deconditioning.<sup>1,2</sup> Altered mitochondrial function in different tissues likely contributes to these detrimental health effects.<sup>3</sup> As mitochondria are critical for cellular function and energy production, mitochondrial dysregulation could substantially challenge and exacerbate health risks during spaceflight.<sup>4</sup> In order to facilitate extended missions and maintain astronaut health, adequate countermeasures preserving mitochondrial function are warranted.<sup>5</sup>

Changes in mitochondrial function are also implicated in the pathogenesis of highly prevalent earthbound diseases affecting our society, such as the metabolic syndrome, type 2 diabetes, cardiovascular diseases, certain cancers, and neurodegenerative diseases.<sup>6</sup> Hence, the current emphasis is on comprehending the role of disrupted mitochondrial function in the pathophysiology of those diseases, particularly within compromised tissues.<sup>7</sup>

However, the requirement of an invasive and costly biopsy for assessing tissue-specific mitochondrial function limits application in clinical studies. Thus, a liquid “biopsy,” an analysis of body fluids such as blood, has gained interest in recent years to monitor bioenergetic function of different blood cell types in humans.<sup>8</sup>

One relevant cell population comprises peripheral blood mononuclear cells (PBMCs), a crucial component of the immune system consisting primarily of lymphocytes (T cells, B cells, and natural killer cells) and monocytes.<sup>9,10</sup> Although appealing, the usability of mitochondrial function of PBMCs as a surrogate and systemic marker may be limited due to the heterogeneity and marked differences in the metabolic properties of different immune cell subtypes.<sup>11</sup> Nevertheless, PBMC mitochondrial function appears responsive to various pathologies, including major depression and Alzheimer's and is influenced by physical exercise.<sup>12–15</sup> Per volume of blood, PBMC mitochondrial respiratory function increased following acute exercise in collegiate swimmers<sup>16</sup> and healthy adults.<sup>14</sup> Other studies demonstrate improved bioenergetics in PBMCs after low-intensity exercise,<sup>15</sup> and its modulation by fitness status in females.<sup>17</sup>

Changes in PBMC metabolism may also affect the immune system. Changes in immune system regulation have been found in both short-duration and extended spaceflights, resembling similar findings from studies involving prolonged bed rest.<sup>18–20</sup> Systemic mitochondrial

alterations during spaceflight may further exacerbate immune system dysfunction.<sup>21</sup> Weakening of the immune system of humans in space may thus pose a significant challenge for space exploration. To the best of our knowledge, no study analyzed mitochondrial function in PBMCs during bed rest as an extreme form of inactivity<sup>22</sup> and analog for human spaceflight missions. Therefore, the current study aimed to investigate mitochondrial function in PBMCs during prolonged and strict head-down tilt bed rest (HDTBR). HDTBR is an established ground-based analog for spaceflight<sup>22</sup> and is the gold standard for investigating eligible countermeasures to prevent HDTBR-induced deconditioning<sup>23</sup> such as cardiovascular deconditioning,<sup>24</sup> loss of bone<sup>19</sup> and loss of muscle mass.<sup>25</sup> Organ-specific mitochondrial dysfunction is strongly connected to these responses. Previous bed rest studies demonstrated that muscle mitochondrial respiration decreased after 21, 7 and 3 days of bed rest<sup>26–28</sup> while exercise training has been shown to improve mitochondrial function.<sup>29</sup> Another study observed an increase in intrinsic mitochondrial respiration (normalized to citrate synthase activity) but no changes for non-normalized mitochondrial capacity after 4 days of bed rest,<sup>30</sup> while additional studies showed no significant change in mitochondrial respiration following 10 days of bed rest.<sup>31,32</sup> We reasoned that PBMC mitochondrial function is modulated by bed rest, which could improve our understanding of mitochondrial changes occurring during simulated and real spaceflights.

Considering the limited available evidence, the aims of the present study were to assess (i) the effects of bed rest on changes of mitochondrial function in PBMCs and how these changes associate with clinical immune cell parameters, and (ii) the effect of an exercise countermeasure in combination with veno-occlusive thigh cuffs on PBMC bioenergetics and clinical immune cell parameters during HDTBR.

## 2 | RESULTS

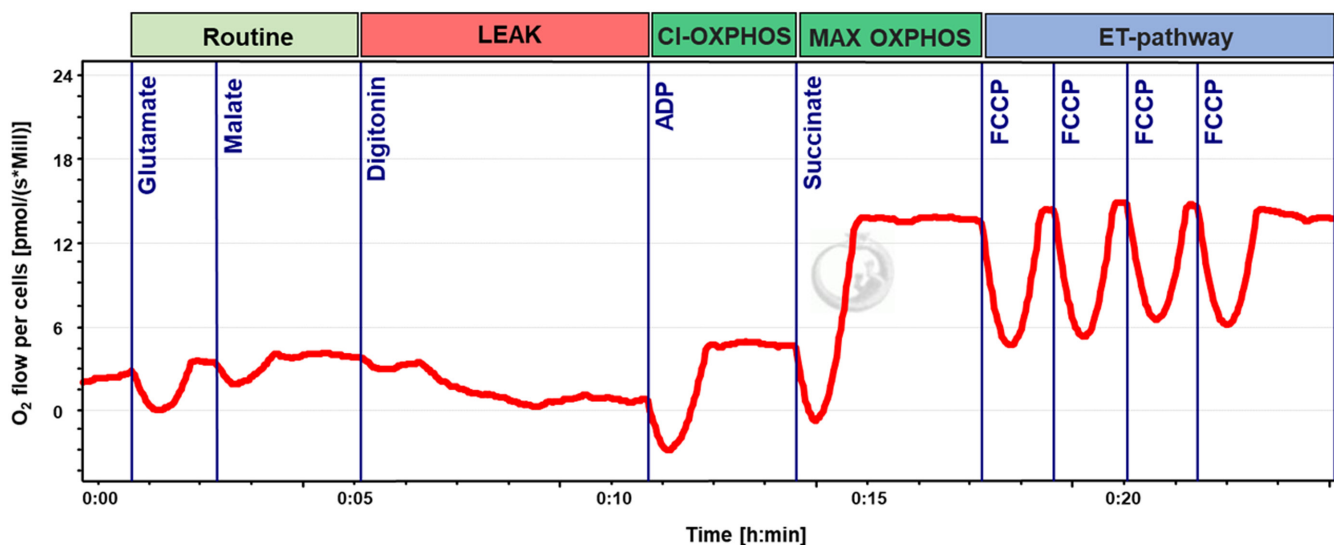
The current study included 24 healthy participants who underwent strict HDTBR (Table 1). Twelve individuals were allocated to the control group and 12 to the countermeasure group. The countermeasure intervention included regular supine cycling exercise in the HDT position followed by veno-occlusive thigh cuffs post-exercise for 6 h. Participant's characteristics are given in Table 1. Both groups were matched for age, BMI and metabolic parameters including fasting blood glucose, blood lipids and transaminases, which were all not significantly different at baseline.

**TABLE 1** Demographics and clinical laboratory parameters of the participants at baseline.

Parameter	Control (n = 12)	Countermeasure (n = 12)
Females (in %)	41.6	33.3
Age (years)	35.2 ± 7.9	34.3 ± 9.3
Height (m)	1.75 ± 0.1	1.74 ± 0.1
Weight (kg)	71.9 ± 13.7	70.0 ± 10.6
BMI (kg/m <sup>2</sup> )	23.3 ± 1.9	23.0 ± 2.8
Fasting blood glucose (mg/dL)	84.4 ± 7.4	82.7 ± 6.7
Triglycerides (mg/dL)	84.4 ± 30.1	83.4 ± 29.3
Cholesterol (mg/dL)	173.5 ± 25.5	189.5 ± 35.7
LDL-Cholesterol (mg/dL)	119.4 ± 25.5	130.2 ± 36.8
HDL-Cholesterol (mg/dL)	53.4 ± 13.1	58.0 ± 12.2
LDH (U/I)	153.5 ± 23.0	152.8 ± 36.2
ALAT (U/I)	24.3 ± 16.1	26.3 ± 16.3
ASAT (U/I)	16.5 ± 5.5	18.1 ± 3.5
Creatinine (mg/dL)	0.87 ± 0.2	0.85 ± 0.1

Note: Blood samples were obtained 14 days before the start of HDTBR. Data are presented as means ± standard deviation (SD).

Abbreviations: ALAT, alanine aminotransferase; ASAT, aspartate aminotransferase; BMI, body mass index; HDL, high-density lipoprotein; LDH, lactate dehydrogenase; LDL, low-density lipoprotein.



**FIGURE 1** Representative high-resolution respirometry trace of PBMC respiration. The protocol comprised of sequential titration of multiple substrates. Malate and glutamate were added to sustain the NADH pathway during routine respiration. Subsequently, digitonin was injected to permeabilize the cell membrane and obtain non-phosphorylating LEAK respiration. Adding adenosine diphosphate (ADP) results in CI-linked OXPPOS state. The addition of succinate provides electron flow through complexes I and II and results in maximal ADP-stimulated mitochondrial respiration (MAX OXPPOS). Maximal electron transfer system capacity (ETC) was measured by stepwise titration of the uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP). The red line represents cell-specific O<sub>2</sub> flow, calculated as the negative time derivative of the O<sub>2</sub> concentration, expressed as pmol/s/10<sup>6</sup> cells.

## 2.1 | The effect of HDTBR with and without an exercise countermeasure on PBMC mitochondrial respiration

We performed high-resolution respirometry of PBMCs 2 days before the start of HDTBR and on day 30 of the HDTBR

period (Figure 1). PBMC composition was confirmed by fluorescence-activated cell sorting (Figure S1). PBMCs were mainly comprised of lymphocytes and monocytes (Figure S2). Mitochondrial respiration in the absence of adenylates (LEAK), complex I-linked oxidative phosphorylation capacity (CI-linked OXPPOS), and coupling efficiency of complex

I, that is, LEAK/CI-linked OXPHOS, were not affected in either group (Figure 2A–C). Maximal ADP-stimulated mitochondrial respiration driven by NADH- and succinate-linked substrates (OXPHOS) was not affected in both groups, regardless of the intervention (Figure 2D). However, maximal OXPHOS increased by 11% in the countermeasure group ( $2.5 \pm 2.9$  pmol/s/ $10^6$  cells) while it decreased by 10% in the control group ( $-2.7 \pm 7.8$  pmol/s/ $10^6$  cells;  $p=0.04$ , Figure 2E). When correcting OXPHOS for the LEAK component (Net-OXPHOS depicting the respiratory capacity relevant for phosphorylation of ADP to ATP), the decrease in the control group was 13%, while the increase due to countermeasure was 4% (Figure 2F,G). The maximal electron transport system capacity (ETC) was also not significantly affected by any intervention (Figure 2H). We found a significant decrease in the P/E flux control ratio (the flux ratio of maximal OXPHOS

over ETC) for the control group ( $p=0.04$ ), while there were no significant changes in L/E flux control ratio (the flux ratio of LEAK respiration over ETC) or L/P flux control ratio (the flux ratio of LEAK respiration over maximal OXPHOS) (Figure 2I). For analysis of sex-specific effects, sex was considered as an independent variable. This analysis revealed no sex-specific differences of PBMC mitochondrial respiration.

## 2.2 | The effect of HDTBR with and without an exercise countermeasure on intrinsic mitochondrial respiration of PBMCs

By quantitatively assessing specific markers, intrinsic mitochondrial alterations can be determined. These

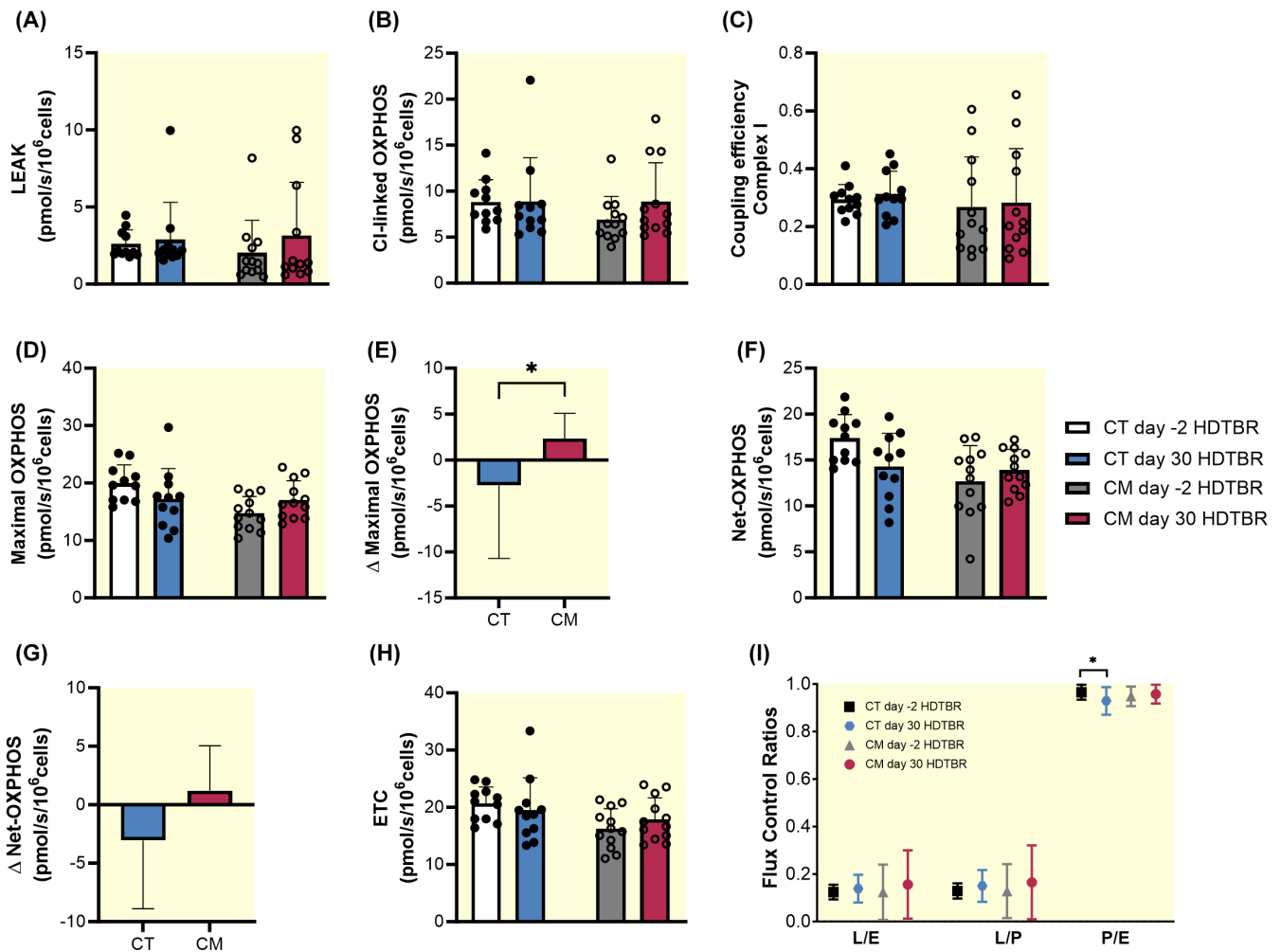
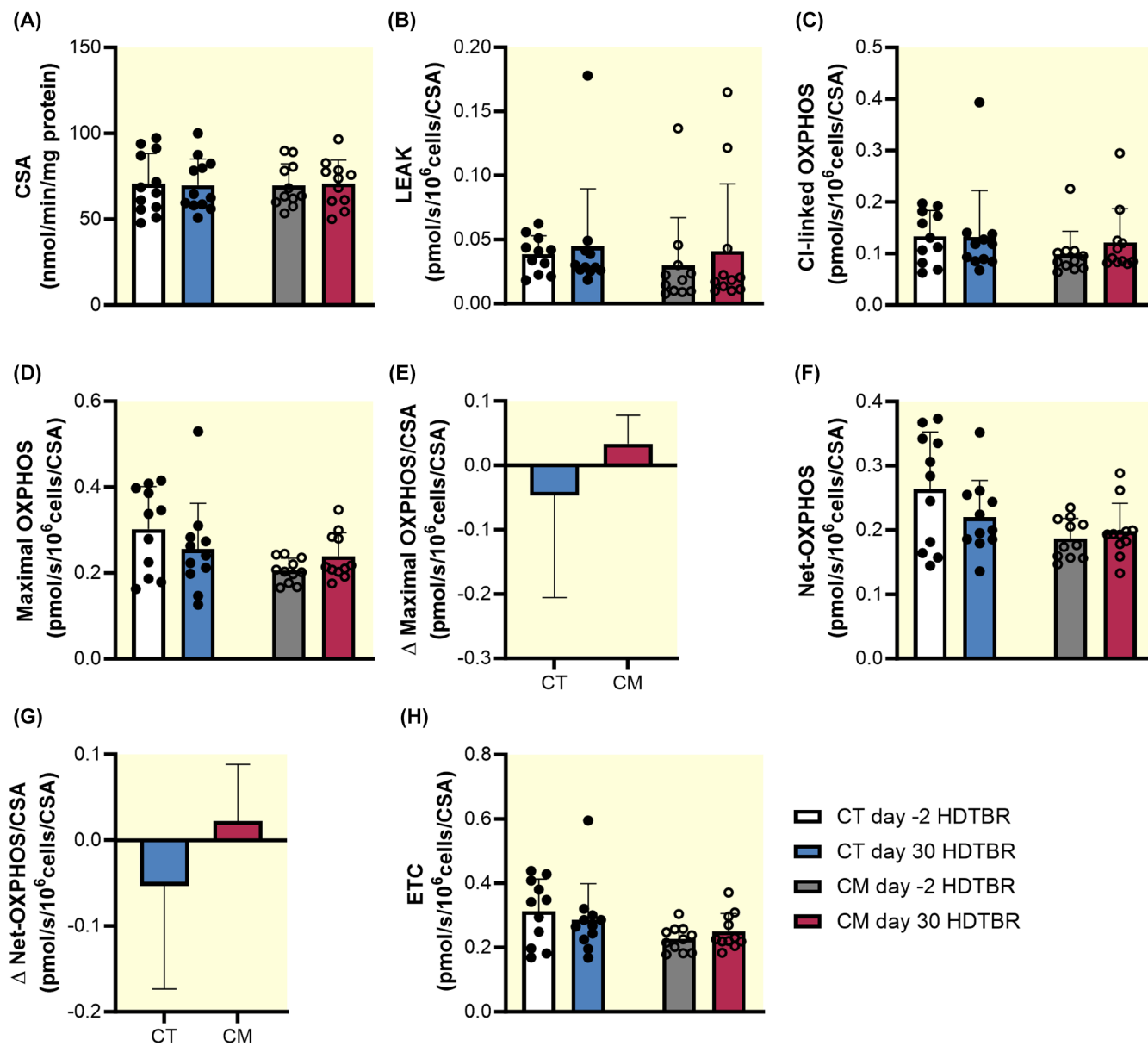


FIGURE 2 Comparison of changes in PBMC mitochondrial respiration through several respiratory states and flux control ratios 2 days prior to HDTBR and on day 30 of HDTBR. (A) LEAK respiration, (B) CI-linked OXPHOS, (C) coupling efficiency of complex I, (D) maximal OXPHOS capacity, (E)  $\Delta$ maximal OXPHOS capacity, (F) Net-OXPHOS capacity, (G)  $\Delta$ Net-OXPHOS capacity, (H) ETC, and (I) different flux control ratios pre and post HDTBR in the control and countermeasure group. L/E flux control ratio given as the flux ratio of LEAK respiration over ETC, L/P flux control ratio given as the flux ratio of LEAK respiration over maximal OXPHOS, and P/E flux control ratio given as the flux ratio of maximal OXPHOS over ETC.<sup>49,50</sup> \*denotes significant changes at  $p < 0.05$ . CT-control-group, CM-countermeasure group, HDTBR-head-down tilt bed rest. Filled circles represent the control group, empty circles represent the countermeasure group.

markers can estimate mitochondrial content and are commonly used to normalize mitochondrial respiration measurements. Citrate synthase activity (CSA) is a widely used surrogate marker of mitochondrial content.<sup>33</sup> The intervention did not significantly impact CSA. On HDTBR day 30, the control group had a mean CSA level of  $69.9 \pm 14.6$  nmol/min/mg protein, while the countermeasure group had a mean CSA level of  $70.9 \pm 13.0$  nmol/min/mg protein. Both groups were similar to their respective baseline mean levels 2 days

prior to HDTBR:  $70.6 \pm 16.8$  for the control group, and  $69.6 \pm 12.1$  for the countermeasure group (see Figure 3A for details).

Consequently, intrinsic mitochondrial respiration, i.e., mitochondrial respiration normalized to CSA, was not altered in either group through all respiratory states (Figure 3B–H). Similarly, to the results for non-normalized respiration, there was only a significant difference before and after HDTBR for the P/E-ratio in the control group ( $p=0.04$ ).



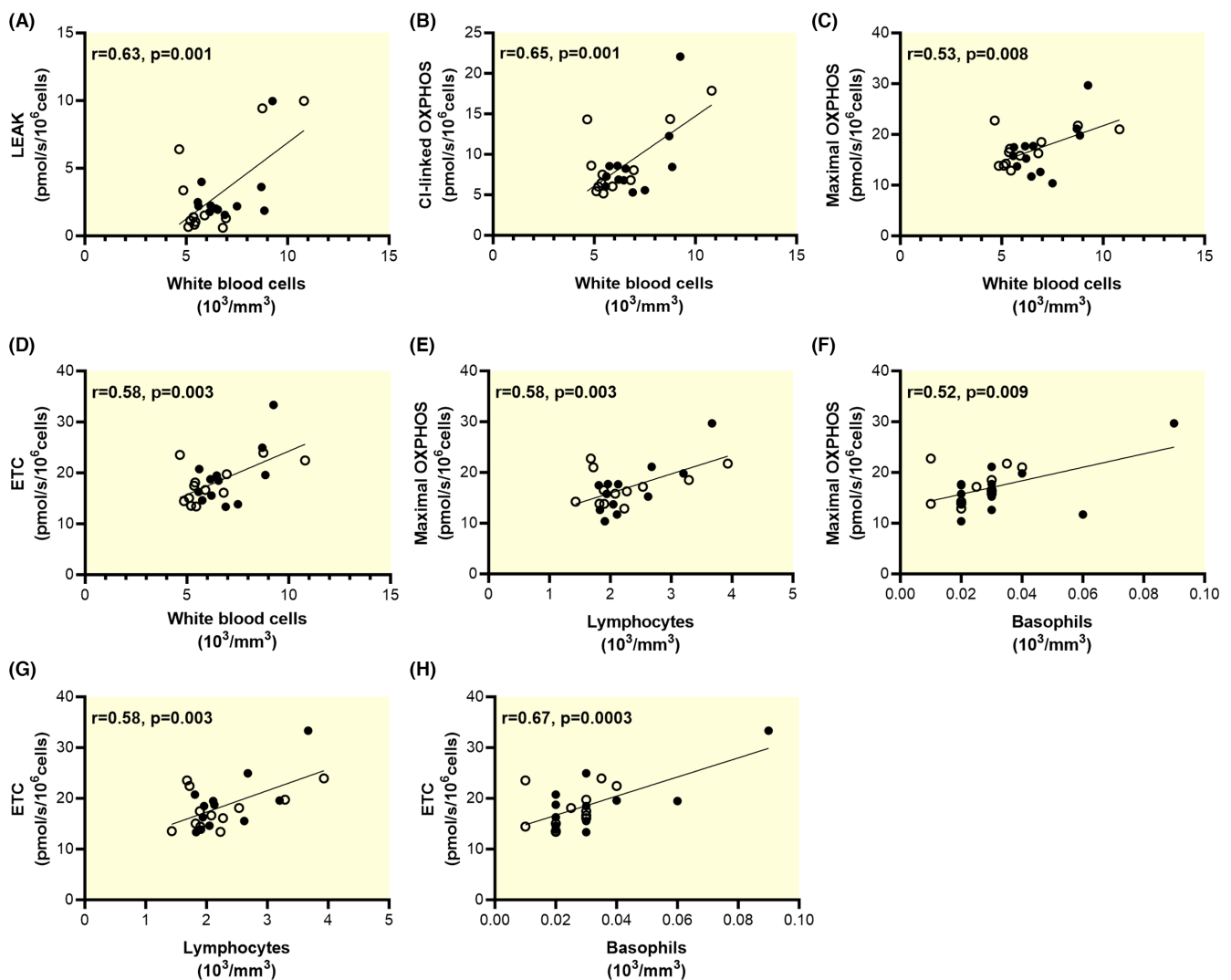
**FIGURE 3** Comparison of citrate synthase activity and different respiratory states normalized for citrate synthase activity 2 days prior to HDTBR and on day 30 of HDTBR. (A) Citrate Synthase Activity (CSA), (B) LEAK respiration per CSA, (C) CI-linked OXPPOS per CSA, (D) maximal OXPPOS capacity per CSA, (E)  $\Delta$ maximal OXPPOS capacity per CSA, (F) Net-OXPPOS capacity per CSA, (G)  $\Delta$ Net-OXPPOS capacity per CSA, and (H) ETC per CSA pre and post HDTBR in the control and countermeasure group. CT-control-group, CM-countermeasure group, HDTBR-head-down tilt bed rest.

### 2.3 | Correlation between PBMC mitochondrial respiration and clinical immune cell parameters and influence of bed rest on clinical laboratory parameters

Next, we performed correlation analyses between clinical immune cell parameters (white blood cell count, lymphocyte count, and basophil count) from blood samples obtained on day 1 after the end of HDTBR and mitochondrial respiration measured on HDTBR day 30. We found a positive correlation between the count of white blood cells per  $10^3/\text{mm}^3$  and several mitochondrial respiratory states (all  $p < 0.01$ ). Specifically, we found a positive correlation with LEAK respiration ( $r = 0.63$ ,  $p = 0.001$ ), CI-linked

OXPHOS ( $r = 0.65$ ,  $p = 0.001$ ), maximal OXPHOS ( $r = 0.53$ ,  $p = 0.008$ ), and ETC ( $r = 0.58$ ,  $p = 0.003$ ) as illustrated in (Figure 4A–D). In addition, we found a positive correlation between the count of lymphocytes per  $10^3/\text{mm}^3$  and maximal OXPHOS ( $r = 0.58$ ,  $p = 0.003$ ) or ETC ( $r = 0.58$ ,  $p = 0.003$ ) as well as basophils per  $10^3/\text{mm}^3$  and maximal OXPHOS ( $r = 0.52$ ,  $p = 0.009$ ) or ETC ( $r = 0.67$ ,  $p = 0.0003$ , Figure 4E–H). We did not find a correlation of CSA with any clinical immune cell parameter.

Some standard clinical laboratory parameters revealed changes from 14 days before and 1 day after the end of HDTBR (Table S1). Immune cell markers, such as the numbers of leucocytes, lymphocytes, and neutrophils, exhibited changes in both groups, whereas only the



**FIGURE 4** Correlation analysis between specific PBMC mitochondrial respiratory states and clinical immune cell parameters. (A) Correlation of LEAK, (B) CI-linked OXPHOS, (C) OXPHOS and (D) ETC with white blood cell count; (E) correlation of OXPHOS with lymphocyte count, (F) OXPHOS with basophil count, (G) ETC with lymphocyte count and (H) ETC with basophil count. LEAK respiration in the absence of adenylates, CI-linked OXPHOS-complex I-linked oxidative phosphorylation, OXPHOS-maximal ADP-stimulated mitochondrial respiration driven by NADH- and succinate-linked substrates (OXPHOS). PBMC mitochondrial respiration was determined on day 30 of bed rest, and clinical immune parameters on day 1 after the bed rest phase. Pearson's correlation analysis was performed.

control group showed a significant change in the number of monocytes, eosinophils, and basophils. Red blood cell-related parameters remained stable in both groups. Alanine aminotransferase decreased in the countermeasure group after 30 days of bed rest. Bilirubin decreased in both groups. Cholesterol numerically decreased in both groups but reached statistical significance only in the countermeasure group. HDL-cholesterol levels were lower after the intervention in both groups. While LDL-cholesterol decreased only in the countermeasure group, triglyceride levels increased only in the control group. Creatinine kinase increased in both groups while urea decreased in both groups.

### 3 | DISCUSSION

This study reveals that strict HDTBR resulted in a 10% reduction in maximal OXPHOS capacity of PBMCs, while an exercise countermeasure combined with veno-occlusive thigh cuffs during HDTBR enhanced this parameter by 11%. Furthermore, we observed that the restriction of maximal OXPHOS capacity by the phosphorylation system, as evidenced by a decrease in the P/E ratio, only occurred in the control group. This was effectively prevented by the exercise countermeasure during HDTBR. The bed rest and countermeasure had no impact on other aspects of PBMC mitochondrial respiratory function or CSA. Additionally, mitochondrial respiratory states of PBMCs exhibited a positive correlation with clinical immune cell parameters, including counts of white blood cells, lymphocytes, and basophils.

One of the benefits to study blood bioenergetics is that samples can be obtained using a minimally invasive approach. Changes in mitochondrial function in PBMCs occur in various infectious, neurological, cardiopulmonary, vascular, and metabolic diseases as well as in cancer.<sup>8</sup> In individuals with Alzheimer's<sup>13</sup> and in geriatric patients with fatigue, mitochondrial respiration of PBMCs is decreased.<sup>34</sup> A study by Karabatsiakos et al. proposed a notable impairment in mitochondrial function among individuals with major depression.<sup>12</sup> The authors also identified a correlation between mitochondrial function and the severity of the depression.<sup>12</sup> A similar correlation was described between disease severity in pulmonary arterial hypertension and PBMC mitochondrial respiration.<sup>35</sup> Also, in patients with heart failure, PBMC respiratory capacity was lower in the group with more severe symptoms according to the New York Heart Association classification.<sup>36</sup>

Growing evidence suggests that PBMC bioenergetics is not only responsive to certain pathologies, but also to exercise training.<sup>14,15</sup> However, after acute exercise,

an increase in mitochondrial respiratory function of PBMCs in response to exercise was observed only when normalized per mL of blood (tissue), but not on a per cell basis.<sup>14</sup> This observation was explained by an egress of circulating immune cells 30–60 min after acute exercise.<sup>37,38</sup> Another study confirmed the impact of acute low-intensity exercise on improved bioenergetics and fatty acid oxidation in PBMCs,<sup>15</sup> suggesting that acute exercise acts as a modulator of immune cell metabolism. In the present study, however, blood sampling was performed in the rested state after an overnight fast in order to assess potential chronic effects of the HDTBR and exercise countermeasure.

Given that blood cell bioenergetics can be modulated by environmental factors, the question thus remains whether PBMCs also respond to bed rest, as a form of extreme inactivity.<sup>22</sup> To our knowledge, no study has assessed this question so far. One study did measure PBMC mitochondrial respiration in persons with spinal cord injury.<sup>39</sup> Regrettably, the absence of a healthy control group in this study makes it impossible to assess the influence of inactivity per se on PBMC bioenergetics. In previous bed rest studies as a simulation of spaceflight, assessments of mitochondrial function have primarily focused on skeletal muscle tissue. Skeletal muscle mitochondrial respiration declined following 3, 7, and 21 days of HDTBR.<sup>26–28</sup> Conversely, when normalized to citrate synthase activity, intrinsic mitochondrial respiration increased after 4 days of HDTBR.<sup>30</sup> Other studies showed no significant changes of mitochondrial respiration following 10 days of HDTBR.<sup>31,32</sup> While mitochondrial function of PBMCs seems to be responsive to particular exercise interventions, the effects of strict HDTBR, in combination with a specific countermeasure, remain understudied. We find, that an exercise countermeasure can prevent certain alterations of OXPHOS, which occur in response to bed rest without any countermeasure. Although PBMC mitochondrial function is modulated by environmental factors, analyzing the entire PBMC population can obscure underlying differences caused by factors other than metabolic processes, particularly changes in PBMC subpopulations. While most of the immune cells behave similarly in the control and countermeasure group, some cell populations including eosinophils, lymphocytes, monocytes, or basophils show a distinct response (Suppl. Table 1). Consequently, bulk PBMC analysis does not allow to assess metabolic properties of different immune cell subtypes and may thus have limited utility in elucidating biological mechanisms due to the intricate heterogeneity of cell subsets within the tissue sample.<sup>11</sup> Future studies elucidating mitochondrial function and content in an immune

cell-specific manner could resolve this biological limitation and further our understanding of the immune system in the spaceflight context. This could eventually pave the way to define biomarkers based on cell-specific mitochondria responsive to bed rest or microgravity.

Mitochondria play a central role in bioenergetics by generating cellular energy in the form of ATP through the process of oxidative phosphorylation. This is also evident in our study, where PBMC maximal OXPHOS capacity increased by 11% due to the countermeasure, while it decreased by 10% in the control group. We also find that bed rest without appropriate countermeasures decreased the P/E ratio. The P/E ratio ranges from the lower limit, representing zero capacity of the phosphorylation system, to the upper threshold of 1.0, under conditions where there is no restriction of OXPHOS, indicating that the capacity of the phosphorylation system matches maximal electron transfer capacity.<sup>40</sup> A decrease in the P/E ratio thus signifies an increased limitation of the phosphorylation system and indicates a potential metabolic consequence of mitochondrial respiratory function during HDTBR at the PBMC level. This study was not designed to assess subpopulations of PBMCs. Thus, quantification of individual variability of circulating immune cell subtypes or the impact of environmental factors on these cell populations warrants further investigations. In general, human immune cells can be activated during isolation or by environmental factors.<sup>41</sup> In order to prevent activation, we carefully maintained a constant temperature during PBMC isolation. As we did not assess the activation of immune cells in this study, we cannot exclude that the intervention may have resulted in phenotypic changes due to activation. However, the correlation of PBMC mitochondrial respiration with specific immune cell subtypes suggests that PBMC bioenergetics can be influenced by the composition of these cells.

Spaceflight induces considerable changes in immune and mitochondrial function.<sup>3,42</sup> Dysregulated immune responses have been consistently observed in short-term and long-term spaceflights, paralleling similar patterns observed in extended periods of bed rest.<sup>18–20</sup> Clinically, this translates to the increased prevalence of allergic reactions, including rhinitis and skin rashes, among 46 International Space Station (ISS) crew members.<sup>43</sup> The spaceflight-associated immune system weakening may pose a significant challenge for human space exploration beyond Earth's orbit. To understand the mechanisms and test countermeasures we use bed rest studies as spaceflight analogs. After a space mission, reduced expression of nuclear-encoded DNA genes responsible for oxidative phosphorylation contribute to impaired

mitochondrial OXPHOS.<sup>44</sup> This environment is also associated with increased production of reactive oxygen species, contributing to oxidative stress.<sup>45</sup> In addition, microgravity exposure likely results in the downregulation of components within the electron transport and OXPHOS system,<sup>3</sup> possibly resulting in reduced production of ATP.<sup>46</sup> Importantly, it has been shown that radiation can prompt severe mitochondrial damage<sup>47</sup> and it is likely that the combination of microgravity with radiation is the main driver inducing mitochondrial stress.<sup>3</sup> For obvious ethical reasons, this element cannot be simulated in spaceflight analogs on earth. Whether or not longer durations of HDTBR or microgravity in combination with radiation during spaceflight impacts PBMC mitochondrial bioenergetics further, remains to be determined.

In our study, we found that the effect of 30 days of HDTBR and countermeasure on clinical blood parameters in healthy participants are minor. The small increase in specific immune cell types is comparable in both the control and exercise countermeasure group and is unlikely to have meaningful clinical implications. Notably, although the exercise countermeasure may not prevent a decline in HDL-cholesterol levels, it appears to have a favorable effect on blood lipids, as indicated by enhancements in LDL-cholesterol and stable triglyceride levels throughout the bed rest phase, considering strictly standardized dietary intake during this period.

In summary, this study reveals that a regular exercise countermeasure performed during HDTBR has a positive effect on PBMC OXPHOS capacity. No significant changes were observed in other respiratory states. Furthermore, specific immune cell parameters exhibited a positive correlation with PBMC mitochondrial respiratory states. These results underscore the impact of HDTBR and an exercise countermeasure on PBMC bioenergetics and confirm their potential application in human spaceflight.

## 4 | MATERIALS AND METHODS

### 4.1 | Participants

This study was a part of the Spaceflight-Associated Neuro-Ocular Syndrome Countermeasure (SANS-CM) study, a joint project between the National Aeronautics and Space Administration (NASA) and the German Aerospace Center (DLR). Campaigns 3 and 4 were carried out between February and July 2023 in the: envihab facility of the Institute of Aerospace Medicine of the DLR in Cologne, Germany. After providing written informed consent, 9 women and 15 men aged between 24 and



54-years ( $35 \pm 8$  years, BMI  $23.2 \pm 2.4$  kg/m<sup>2</sup>) participated in this study. All participants were healthy with no history of cardiovascular disease, were non-smokers for at least 6 months prior to study-start, and with no regular intake of medication. The study was approved by the North Rhine Medical Association (ID 2020211) and was registered at the German Clinical Trials Register (DRKS00030848). The study was conducted in accordance with the declaration of Helsinki and its latest 2013 amendment.

## 4.2 | Study design

The overarching goal of SANS-CM 3 and 4 was to assess the efficacy of an exercise countermeasure for physiological adaptations evoked by 30 days strict 6° HDTBR (Figure 5). All daily activities including personal hygiene were performed in the 6° HDT position. In total, participants spent 58 days at the: envihab research facility at DLR, including the pre- and post-HDTBR phases. All participants ingested strictly standardized diets with controlled fluid intake and were subjected to regulated bed-times. No alcohol or caffeine containing beverages were permitted. Compliance with the protocol was ensured by video-monitoring and around-the-clock staff. After the 14-day baseline data collection, participants were randomly assigned to either HDTBR alone ( $n=12$ ) or HDTBR in combination with an exercise countermeasure following veno-occlusive cuffs for 6 h at 50 mmHg ( $n=12$ ). The HDTBR period of 30 days was followed by 14 days of recovery phase.

## 4.3 | Countermeasure

During HDTBR, participants of the exercise countermeasure group engaged in 60 min of continuous aerobic cycling in the HDT position, followed by 6 h of wearing veno-occlusive thigh cuffs on 6 days per week to sequester blood in the lower limbs. Appropriate work rates for the supine cycling exercises during HDTBR were determined 7 days prior to bed rest by a ramp test on a cycle ergometer in 6°

HDT to assess peak oxygen consumption (VO<sub>2pk</sub>). The VO<sub>2pk</sub> test started with a 3 min warm-up at 50 W, which then increased each minute in increments of 25 W until volitional exhaustion on a recumbent ergometer (Type 917900, Lode B.V., Groningen, The Netherlands) with continuous measurement of oxygen consumption and carbon dioxide production (Quark CPET, Cosmed, Rome, Italy). During the HDTBR, after a warm-up of 5 min, participants cycled on the recumbent ergometer at 45% of peak power achieved during the VO<sub>2pk</sub> test. This was followed by a 5-min cool-down at 50% of the training workload.

Veno-occlusive thigh cuffs were donned within 30 min after cycling and adjusted to 50 mmHg ( $\pm 5$  mmHg) pressure. The rationale for application of the thigh cuffs is to “trap” the blood in the legs, reducing head-ward fluid shift and thereby preventing developing optic disc edema and spaceflight-associated neuro-ocular syndrome. Within the 6 h veno-occlusive thigh cuff wearing period, cuff pressure was monitored every 30 min using a digital gauge (PicoPress, Microlab Elettronica Sas, Ponte San Nicolò, Italy).

## 4.4 | PBMC isolation

To assess mitochondrial respiration in PBMCs, venous blood samples were taken from the cubital vein at BDC-2 and HDT30 after an overnight fast. Prior to bed rest, even after several attempts, the medical personnel was not able to obtain a blood sample from one individual. Therefore, we could not analyze the change in mitochondrial respiration during bed rest for that individual. The blood draw was performed with a Safety-Multifly 21 G (Sarstedt, Nümbrecht, Germany). Two samples of 9 mL were collected in a VACUETTE® K3EDTA (tripotassium ethylenediaminetetraacetic acid). The blood was gently mixed by slowly inverting the tube 6–10 times and transported to the lab at room temperature (RT). For blood cell isolation, 15 mL Ficoll-Paque™ PLUS (GE Healthcare Life Sciences, Solingen, Germany) was prepared in 50 mL falcon tubes (Greiner Bio-One, Kremsmünster, Austria).<sup>48</sup> 9 mL of blood was diluted 1:2

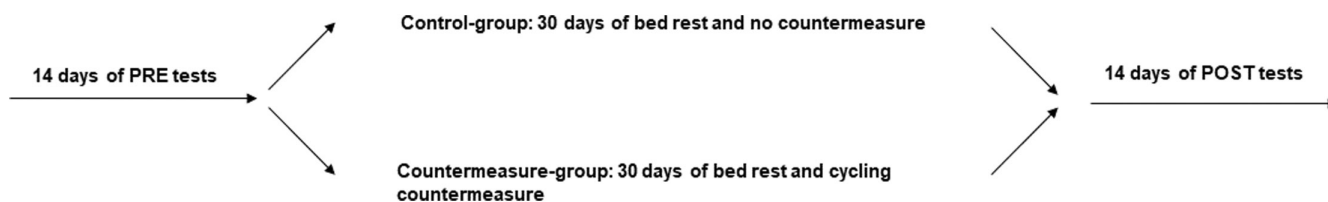


FIGURE 5 Study design. Prior to the bed rest phase, participants spent 14 days in the DLR facility for familiarization and baseline assessment. Participants were randomly assigned to the control group or the exercise countermeasure group. After 30 days of bed rest, participants stayed for an additional 14 days in the: envihab for post-bed rest assessment.

with DPBS (Dulbecco's Phosphate-Buffered Saline), gently poured on the top of the polyethylene barrier and centrifuged at 1000g (10 min, RT, no brakes) in Heraeus Megafuge 1.0R (Thermo Scientific, Waltham, Massachusetts, USA). About 5 mL of the supernatant of the top layer was discarded. The buffy coat was carefully transferred into a new sterile 50 mL Falcon tube. For washing the PBMCs, DPBS was added up to a total volume of 25 mL and centrifuged at 100g for 10 min at RT (intermediate brake 6). The pellet was resuspended in 1 mL DPBS and DPBS was added again up to 25 mL and centrifuged at 100g for 10 min at RT (intermediate brake 6). After discarding the supernatant, the pelleted PBMC fraction was gently resuspended in 1 mL mitochondrial respiration medium [MiR05: ethylene glycol tetraacetic acid (EGTA) (0.5 mM), magnesium dichloride (3 mM), lactobionic acid (60 mM), taurine (20 mM), potassium dihydrogenphosphate (10 mM), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, (HEPES) (20 mM), sucrose (110 mM), and essential fatty acid free bovine serum albumin (1 g/L), pH 7.1 at 37°C].

#### 4.5 | Mitochondrial respiration

PBMCs were counted with the Axiovert 25 (Zeiss, Oberkochen, Germany) and 4 million PBMCs were used per oxygraphy chamber. Before adding the cells to the chamber of the O2k High-Resolution FluoRespirometer (Oroboros Instruments, Innsbruck, Austria), standardized instrumental calibrations and background correction were performed to correct for back-diffusion of O<sub>2</sub> into the chamber. The chambers were filled with MiR05. The required volume to be added from the cell stock was removed and replaced with the adequate volume of cell suspension. Measurements were performed in duplicate.

A substrate-uncoupler-inhibitor-titration protocol was applied to measure mitochondrial respiration during different states.<sup>40</sup> Malate (1 mM) and glutamate (10 mM) were added to the chamber using a Hamilton syringe (Oroboros Instruments) to sustain the NADH-pathway. Subsequently, digitonin (4.05 μM) was injected to permeabilize the plasma membrane and obtain non-phosphorylating LEAK respiration.<sup>9</sup> The addition of ADP (2 mM) leads to the transition from LEAK to CI-linked OXPHOS. Succinate (10 mM) was added to support electron flow through complexes I and II (OXPHOS) and measure maximal ADP-stimulated mitochondrial respiration. Net-OXPHOS capacity i.e. maximal OXPHOS correcting for the dissipative LEAK component, was calculated by subtracting LEAK respiration from maximal OXPHOS capacity. ETC as maximal electron

transfer-pathway capacity was measured by stepwise (0.05 μM/step) titration of uncoupler carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP).

#### 4.6 | Fluorescence-activated cell sorting

PBMCs were isolated as describe above. For the fluorescence-activated cell sorting, Duraclone IM Phenotyping BASIC Tubes (Beckman Coulter, Brea, USA) were used. For sample preparation, 100 μL of PBMC-solution was added to the Duraclone tubes and tubes were vortexed. After 15 min of incubation time in the dark, 500 μL DPBS was added. After vortexing again, PBMCs were characterized by flow cytometry using a CytoFLEX S cytometer (Beckman Coulter Life Sciences, Brea, USA) and flow cytometry analyses was performed using CytExpert Software (Figure S1).

The proportion of singlet cells in all cells was 99.5%, while 94.6% of those cells were CD45-positive (CD45+). Percentages of subtypes B-cells, T-cells, NK-cells, monocytes, and neutrophils are represented in Figure S2.

#### 4.7 | Citrate synthase activity

To determine CSA as a marker of mitochondrial content<sup>33</sup>, a subsample of the remaining cells was centrifuged by 5000 g for 5 min at 4°C using a Microfuge 20R (Beckman Coulter, Brea, USA). The supernatant was discarded, and the pellet was frozen at -80°C. For lysis, the pellet was resuspended with 125 μL Cellytic M C2978 (Sigma Aldrich, St. Louis, USA) and incubated 15 min on ice, thereby vortexed every 5 min. Lysed cells were centrifuged at 12000 g for 15 min, 4°C (Mikrofuge 20R). The protein-containing supernatant was collected and aliquoted in 0.5 mL tubes. The lysate was stored for later use at -80°C.

The amount of protein in the sample with a minimum amount of 8 μg of whole cell extract was determined using a BCA Protein Assay Kit (EMD Millipore Corp, Burlington MA, USA). For the protein determination, the sample was diluted 1:5 with Aqua Bidest distilled water. The sample was incubated at 37°C for 30 min in an incubator (Binder, Tuttlingen, Germany). Protein concentration was assessed spectrophotometrically on a plate reader (Multiscan FC, Thermo Scientific, Waltham, USA) at an absorbance of 570 nm. The microscale assay was performed using a 96-well microplate (Greiner Bio One, Kremsmünster, Austria). Measurements were performed in duplicate, and the absorbance of the blank was subtracted. The standard curve was used to calculate the amount of protein, considering the dilution factor.

CSA was then measured using a commercially available Citrate Synthase Assay Kit CS0720 (Sigma Aldrich, Taufkirchen, Germany). The required components were prepared according to the Sigma Aldrich technical bulletin. During the measurement, the reagents and samples were kept on ice. The lysed sample was diluted 1:10 with molecular biology-grade water. To indicate whether the procedure is optimized and working, a positive control (Catalog Number C4741) was assessed along with the measurement of the samples. The measurement was performed in a 96-well-microplate on a plate reader. The reaction mixture containing 178  $\mu$ L Assay Buffer, 30 mM Acetyl CoA Solution, 10 mM DTNB Solution, and the sample (8  $\mu$ L diluted sample per reaction) for triplicates was prepared. 190  $\mu$ L was placed in each of the 3 wells. Following this initial absorbance reading, 10  $\mu$ L of 10 mM OAA was added to each well using a multipipette (Eppendorf, Hamburg, Germany) to start the enzyme reaction. The absorbance was measured again (10 s shake, wavelength 405 nm, duration 90 s, interval 10 s for obtaining 10 measurements). CSA was calculated by subtracting the endogenous activity from the final reading and expressed as nanomoles per minute per mg protein.

#### 4.8 | Statistical analysis

Mitochondrial respiratory capacity is expressed as respiration rate per million cells and was recorded and quantified using Datlab 7.4.0.4 software (Oroboros Instruments, Innsbruck, Austria). The average of two measurements was determined and used for further analysis. Statistical analysis was performed in GraphPad Prism 8.0.2. The level of significance was set at  $p < 0.05$ . Unless otherwise specified, values are expressed as means  $\pm$  SD. All data were evaluated for normal distribution using Shapiro–Wilk normality tests. Baseline characteristics and changes in mitochondrial respiration ( $\Delta$ ) between groups were compared using an unpaired  $t$ -test. Different mitochondrial ratios (pre and post-bed rest), laboratory parameters and CSA were analyzed by paired student  $t$ -test when normally distributed or Wilcoxon test when data failed the normality or equal variance test. Pearson's correlation analysis was performed for correlations. Due to the exploratory nature, no correction for multiple testing was performed. For analysis of sex-specific effects, a mixed-effects model was fitted in each case and sex was considered as an independent variable in the model in addition to time.

#### AUTHOR CONTRIBUTIONS

DP is responsible for the conception and design of the work. DP and FB contributed to the acquisition and

analysis of the data and drafted the manuscript. TF, JK, LdB, EME, EM, SM, KH, and PFM were involved in data collection, analysis, and manuscript preparation and drafting. JJ and JR provided comments, critical discussions and contributed to manuscript review. All authors have approved the final version of the manuscript and are accountable for all aspects of the work. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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#### CONFLICT OF INTEREST STATEMENT

The authors have no conflict of interest to declare.

#### DATA AVAILABILITY STATEMENT

The datasets generated and analysed during the current study are available from the corresponding author upon reasonable request.

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## SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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