Metabolic and Cellular Differences Between Sedentary and Active Individuals at Rest and During Exercise

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ABSTRACT

Lack of physical activity has been associated with multiple diseases including cardiovascular disease (CVD), cancer, Alzheimer's disease (AD), type 2 diabetes (T2D), Parkinson's disease, depression, dementia and even cancer. Mitochondrial impairment or dysfunction is associated with lack of physical activity and considered to be involved in the pathogenesis of the most prevalent non-communicable diseases (NCDs) afflicting our societies such as T2D, CVD, metabolic syndrome, and even AD.

To our knowledge, there is a scarcity of studies on the metabolic, mitochondrial and cellular characteristics of "healthy sedentary" individuals living without clinical symptoms. Hence, the main aim of our study herein was to characterize multiple metabolic, mitochondrial and cellular bioenergetic signatures in "healthy sedentary" individuals which could already be downregulated compared to moderately active individuals.

Nineteen subjects, 9 sedentary (SED) and 10 moderately active (AC) volunteered for multiple assessments including muscle biopsies, in order to assess muscle metabolism, mitochondrial respiration and bioenergetics both at rest and during exercise.

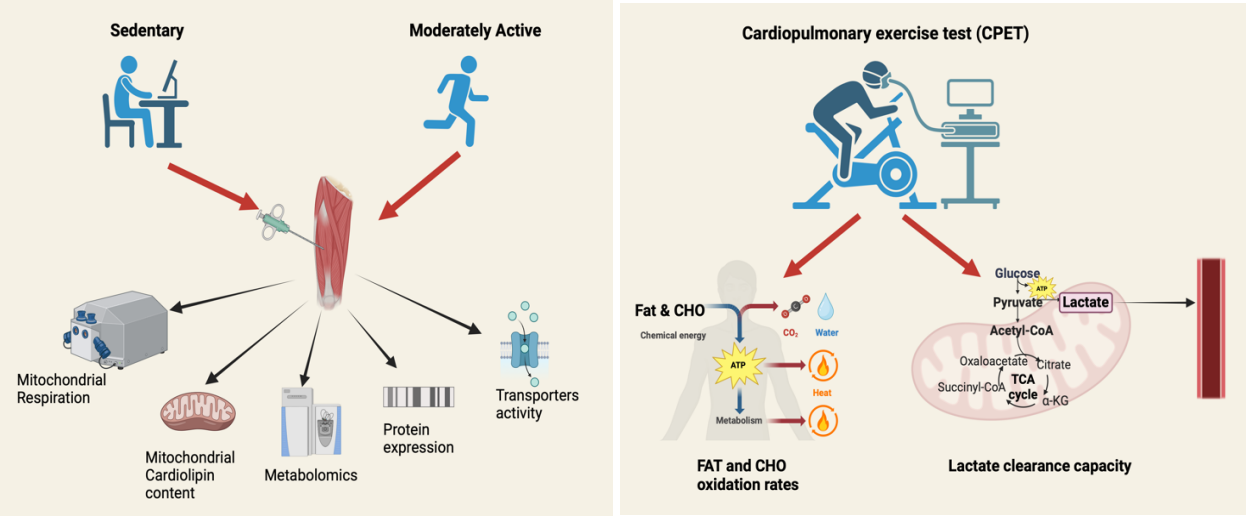
For our exercise studies, we performed graded exercise testing (GXT) to assess carbohydrate and fat oxidation capacity as well as lactate clearance capacity according to our previously developed methodology.

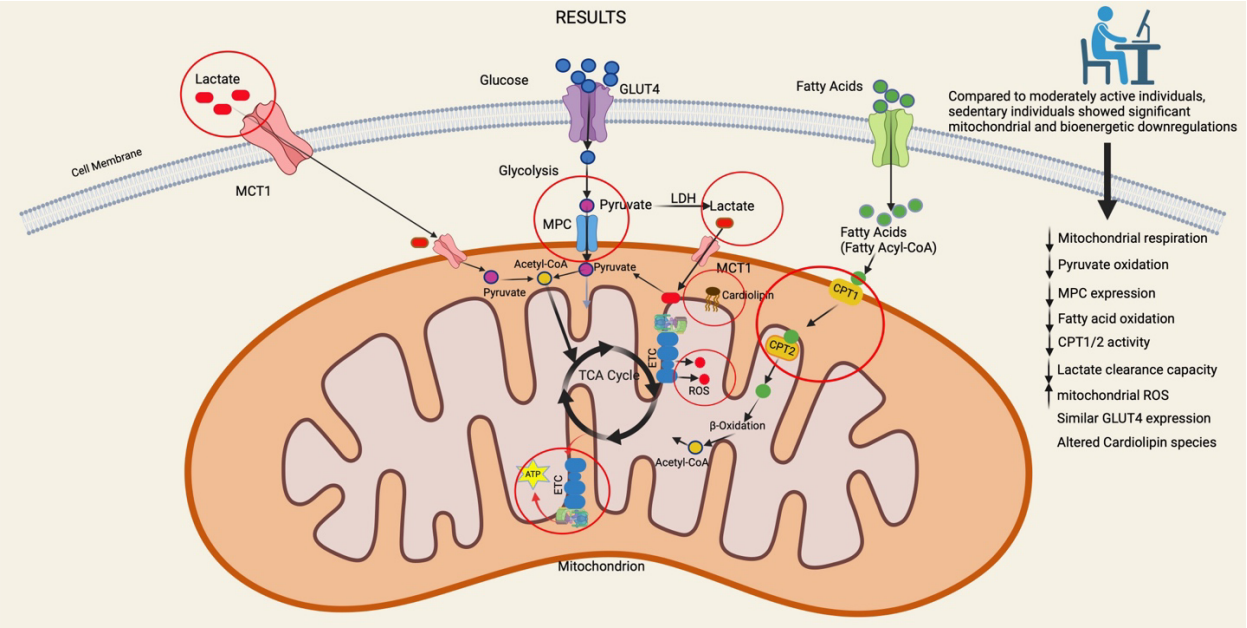
Resting studies showed decreased mitochondrial respiration including decreases in complex I (-36%) and II (-28%) as well as total electron system capacity (-34%) and electron system capacity coupled to ATP production via ATP synthase (-30%). Regarding muscle carbohydrate metabolism, SED individuals showed a decrease in mitochondrial pyruvate oxidation (-37%) as well as reduced expression (-49%) of mitochondrial pyruvate carrier (MPC). Regarding fatty acid metabolism, SED showed decreased activity of carnitine palmitoyltransferase I (CPT1)(-51%) and CPT2 (-44%) as well as decreased mitochondrial fatty acid oxidation (-35%). Metabolomics analysis also confirmed downregulation of carbohydrate and fat metabolism. Partial Least-Squares Discriminant Analysis (PLS-DA) identified distinct metabolic phenotypes through intermediates of glycolysis and fatty acid oxidation. Further, we found significant differences in cardiolipin (CL) species expression between SED and AC groups, which, due to the important role of CL in mitochondrial structure, function, biogenesis and bioenergetics, deserves further attention.

Exercise studies also showed significant differences in substrate utilization between groups where SED possessed a significantly lower fat oxidation capacity as well as lactate clearance capacity. The correlation of different bioenergetic parameters between resting and exercise conditions were robust, suggesting the possibility of performing cardiopulmonary exercise testing (CPET) as a non-invasive methodology to indirectly assess metabolic function in multiple populations.

In summary, in our study herein, we show that “healthy sedentary” individuals already possess a significant decrease in cellular metabolism, mitochondrial respiration and bioenergetics compared to moderately active individuals both during resting and exercising conditions.

Since large numbers of sedentary individuals evolve to develop cardiometabolic disease, a better understanding of decreased cellular bioenergetics and mitochondrial function is needed in order to improve both diagnosis and treatment of multiple metabolic diseases.





CONCLUSIONS:

- Sedentary individuals possess significant decreases in mitochondrial respiration as well as muscle bioenergetics during resting conditions compared to moderately active individuals.
- Despite similar GLUT-4 concentrations, sedentary individuals show significant decreased pyruvate oxidation as well as expression of mitochondrial pyruvate carrier (MPC).
- During exercise studies, sedentary individuals show significantly decreased levels of fat oxidation and lactate clearance capacity which correlate with mitochondrial and bioenergetics parameters from resting muscle biopsies. Hence, cardiopulmonary exercise

testing (CPET) accompanied by measuring blood lactate levels could be a practical manner to assess muscle mitochondrial function and bioenergetics in a non-invasive and ambulatory manner.

- Identifying early signatures of decreased mitochondrial function and bioenergetics capacity could be an important approach to prevent or improve different metabolic diseases through lifestyle changes mainly from exercise and nutrition.

INTRODUCTION

Lack of physical activity has been associated with multiple diseases including cardiovascular disease (CVD), cancer, Alzheimer's disease (AD), type 2 diabetes (T2D), Parkinson's disease, depression and dementia [1-15], accounting for more than 5 million deaths per year worldwide [16]. Moreover, low cardiorespiratory fitness (CRF) is considered responsible for the highest percentage of all attributable fractions for all-cause mortality [17].

According to the World Health Organization (WHO), 81% of adolescents and 27.5% of adults currently do not meet WHO's recommended levels of physical activity [15]. The same recent WHO report states that almost 500 million new cases of preventable non-communicable diseases (NCDs) will occur between 2020–2030, resulting in around US$ 27 billion in treatment costs annually. Moreover, the addition of billions of dollars in productivity losses due to morbidity and mortality will significantly increase the costs associated with lack of physical activity.

Physical activity is a canonical characteristic of humans. Nevertheless, in modern societies, the normalization of the lack of physical activity has led to the perception that physical activity is an intervention even though it remains as the *modus vivendi* engrained in our genes; the reality is that becoming sedentary has been the real intervention and collateral effect of modern societies [18]. This perception of physical activity as an intervention has even resulted in the utilization of "healthy sedentary" individuals as the control cohort in a vast number of medical research studies.

To our knowledge, there is a scarcity of studies on the metabolic and cellular characteristics of "healthy sedentary" individuals without any clinical symptoms, which could already show underlying metabolic and cellular bioenergetic dysregulations and, over the course of time, the

presentation of multiple NCDs. Hence, the main aim of our study herein was to characterize multiple metabolic and cellular bioenergetic signatures in “healthy sedentary” individuals which could already be downregulated compared to moderately active individuals, the true evolutionary control cohort.

Within the multiple metabolic and cellular dysregulations leading to multiple diseases, mitochondrial impairment or dysfunction is considered to be involved in the pathogenesis of the most prevalent NCDs afflicting our societies such as T2D, CVD, metabolic syndrome, and even AD as well as cancer [18]. Hence, our study herein focused on multiple muscle mitochondrial and bioenergetic characteristics and patterns which are clearly identifiable between “healthy sedentary” individuals and moderately active individuals. In our study herein, we chose skeletal muscle as it is the largest and most metabolically active organ in the human body and an ideal organ to study cellular metabolism and bioenergetics. As such, it is suspected that T2D debuts in skeletal muscle as ~80-85% of glucose is disposed by skeletal muscle during resting and postprandial conditions [19]. Under normal metabolic circumstances, most glucose should be successfully oxidized in skeletal muscle mitochondria via oxidative phosphorylation (OXPHOS). However, in the presence of muscle mitochondrial dysfunction or impairment, glucose will face significant challenges in order to be transported into skeletal muscle cells to accomplish its mission to be converted to pyruvate in the cytosol and ultimately mitochondrial OXPHOS instead of cytosolic fermentation to lactate.

To achieve our aim, we performed multiple assessments pertaining to muscle metabolism and bioenergetics both at rest and during exercise. For our resting assessments, we performed muscle biopsies in order to assess mitochondrial respiration via substrate oxidations of carbohydrate derivatives, fatty acids, and amino acids, electron transport chain (ETC) efficiency, production of reactive oxidative species (ROS), fatty acid transporter activity, mitochondrial pyruvate carrier (MPC) quantity and activity and $^{13}C_1$ lactate and $^{13}C_3$ pyruvate fluxomics.

For our exercise studies, we performed graded exercise testing (GXT) to assess carbohydrate and fat oxidation capacity as well as lactate clearance capacity according to our previously developed methodology through the assessment of fatty acid oxidation rates and lactate clearance capacity both mitochondrial substrates, representative of mitochondrial function and metabolic

flexibility[20]. This methodology could be useful to indirectly assess mitochondrial function and metabolic flexibility in an ambulatory and non-invasive manner as it does not require invasive muscle biopsies to measure fat and carbohydrate metabolism. Therefore, we established correlations between the data obtained from muscle biopsies with our proposed methodology through GXT.

In our study herein, we observed statistically significant differences between "healthy sedentary" and moderately active subjects in all parameters studied which are shown and discussed in the sections below. Our results show that "health sedentary" individuals already show significant cellular and bioenergetic downregulations compared to moderately active individuals which could, over time, lead to the development of multiple NCDs. Hence, we believe that the necessity to detect a decay in metabolic and mitochondrial function at early stages is imperative in order to avert multiple NCDs through different lifestyle interventions like exercise, nutrition, and through the development of novel therapeutics.

1. METHODS

*2.*1 *Subject Recruitment*

Nineteen male subjects ((41.9 ± 13.8 years; 82.6 ± 13.9 kg)) participated in this study and were assigned a research arm based upon meeting one of the following criteria related to physical activity:

- Sedentary (SED): n= 10. Does not perform exercise regularly or elevate heart rate outside of daily tasks
- Active (AC): n=10. Performs aerobic exercise for at least 150 minutes per week, and has at least a six-month history of doing so

All subjects were screened via the American College of Sports Medicine Risk Stratification Model for cardiovascular health at the time of enrollment and were approved for study-related exercise; individuals diagnosed with diabetes (type 1 or type 2) were excluded from participating in this study. This research was approved by the Colorado Multiple Institutional Review Board (17-1095).

2.2 Muscle Biopsies

Ethyl Chloride Topical Anesthetic Spray (Gebauer Company, Cleveland OH, USA) followed by local anesthesia injection of 5 mL of 1% lidocaine HCl (Pfizer, New York, NY, USA) on the lateral thigh numbed the site where a 1 cm incision was made. Biopsies of the vastus lateralis muscle were then obtained using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge × 10-cm biopsy needle (Bard Biopsy Systems, Tempe, AZ, U). Three passes of this device were performed under direct ultrasound guidance to ensure blood vessels were avoided; each pass obtained about 25 mg of muscle tissue. The fresh muscle tissue was immediately prepared for respective experimentation or flash frozen in liquid nitrogen and stored at –80°C for future analysis. A Steri-strip (3M Nexcare, St. Paul, MN, USA) was utilized to close the incision and a pressure dressing was placed on the biopsy site using Elastoplast tape (Hamburg, Germany).

2.3. High Resolution Respirometry and Measurement of Reactive Oxygen Species (ROS) Production

Respiration of permeabilized muscle fibers was measured by high resolution respirometry (Oxygraph;, Oroboros Instruments, Innsbruck, Austria) using a stepwise protocol to evaluate various components of the electron transport system and simultaneous measurement of total ROS using amplex red measured with a fluorescence module built into the chamber Approximately 10 mg of muscle tissue was placed in cold biopreservation solution (BIOPS) immediately following biopsy. The relaxing and biopsy preservation solution BIOPS contained 10 mM Ca-EGTA buffer, 0.1 µM free calcium, 20 mM imidazole, 20 mM taurine, 50 mM K-MES, 0.5 mM DTT, 6.56 mM MgCl2, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1 (Veksler et al., 1987, Letellier et al., 1992). Tissue was cut into approximately 2 mg pieces and teased using forceps to separate fibers. The tissue was then placed in a solution of BIOPS containing 30 microg/ml saponin for 30 minutes to permeabilize the plasma membrane and allow substrate delivery to the mitochondria. Fibers were washed for 10 minutes at 4°C in ice-cold mitochondrial respiration medium (Gnaiger et al., 2000). Samples were blotted on filter paper, weighed, and placed in the chambers of the Oroboros O2K apparatus at 37°C containing respiration medium. Standard protocols were followed for calibration of the chambers. Reagents for measurement of ROS were then added: 5 units/ml superoxide dismutase (SOD), 1 unit/ml horseradish peroxidase, and

0.1uM/step H2O2 used to calibrate the machine before starting the addition of substrates. Substrates were added in a stepwise fashion in the following order (final concentrations) 0.2 mM palmitoyl carnitine, 1 mM malate, 4mM ADP (assess long chain fatty acid oxidation), 0.4 mM octanoylcarnitine (assess medium chain fatty acid oxidation), 5 mM pyruvate (assess Complex I through pyruvate dehydrogenase (PDH)), 10 mM glutamate (assess Complex I capacity independent of PDH), 10 mM succinate (assess electron transfer system capacity coupled to ATP production via ATP synthase), 1 microM FCCP (assess maximal respiration/ electron tranfer system capacity), 2 microM rotenone (assess Complex II capacity), and 5 microM Antimycin A (assess respiration due to oxidative side reactions (ROX)). Oxygen flux rates were normalized per milligram of tissue wet weight. Analysis was performed using the Oroboros Datlab software.

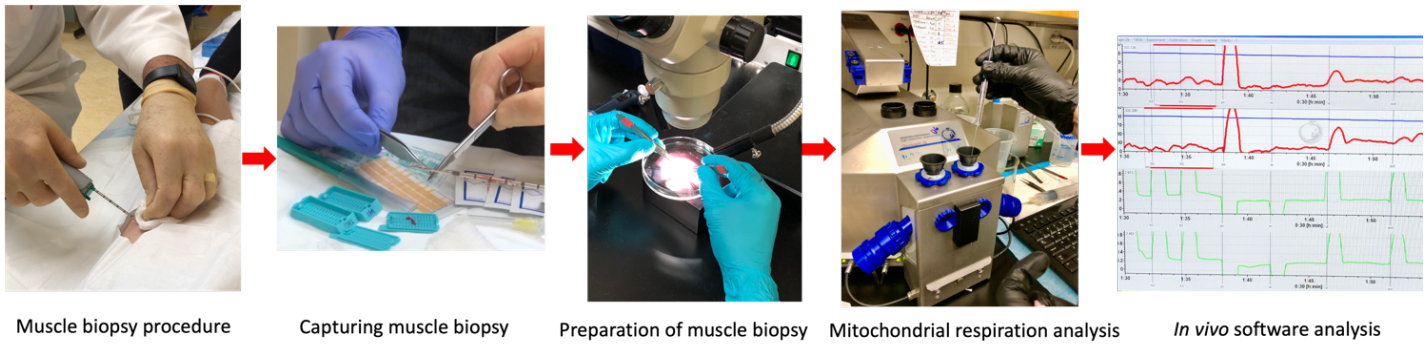


Figure-1. Muscle biopsy procedure included extraction, mitochondrial homogenization, and mitochondrial respiration analysis through Oroboros O2K.

2.4. Protein Expression

Protein expression was evaluated by an automated capillary-based western analysis (Jess, Protein Simple, CA) following the manufacturer's protocol. Briefly, skeletal muscle homogenates with fluorescent master mix (1:4) were heated (95°C, 5 min) to denature the samples. Samples, primary antibodies, biotinylated ladder, HRP-conjugated secondary antibodies, protein normalization reagent, and luminol-peroxide were loaded onto a 12-230 kDa assay plate (Protein Simple, CA) as per the Protein Normalization Module (Protein Simple, CA) layout. Target protein peak area was normalized to total protein content within capillaries using the Compass for Simple Western software (ProteinSimple, CA).

2.5 . Carnitine Palmitoyltransferase I and II activity

Rates of carnitine palmitoyltransterase I and II (CPT1 and CPT2) activity in muscle homogenates were quantified using a 14C carnitine (Perkin Elmer, Waltham, MA, USA) based radioassay [21]. The assay measures the activity of CPT1 by permeabilizing the plasma membrane and measuring the production of palmitoylcarnitine from palmitoyl CoA. By permeabilizing the mitochondrial inner membrane and adding malonyl CoA to inhibit CPT1, the activity of CPT2 was measured.

2.6. Skeletal Muscle Isotope Tracing Analysis

Approximately 10 mg of muscle tissue was placed in Krebs-Ringers bicarbonate solution (pre-warmed to 37°C containing either 21 mM $^{13}C_1$-lactate (CLM-1577-PK, Cambridge Isotope Laboratories, MA, USA) or 2 mM $^{13}C_3$-pyruvate (CLM-2440-PK, Cambridge Isotope Laboratories, MA, USA). Samples were incubated at 37°C for 30 minutes, then centrifuged at 2000g for 10 minutes at room temperature. Supernatants were discarded and remaining pellets were flash frozen on dry ice and stored at -80°C until analysis.

2.7. Mass Spectrometry-based Metabolomics.

Sample Preparation

Skeletal muscle tissue was isolated, flash frozen in liquid nitrogen, and stored at -80°C until analysis. Prior to LC-MS analysis, samples were placed on ice and resuspended with methanol:acetonitrile:water (5:3:2, v:v) at a concentration of 30 mg/ml. Suspensions were vortexed continuously for 30 min at 4°C. Insoluble material was removed by centrifugation at 10,000 g for 10 min at 4°C and supernatants were isolated for metabolomics analysis by UHPLC-MS.

UHPLC-MS analysis

Analyses were performed as previously published [22, 23]. Briefly, the analytical platform employs a Vanquish UHPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled online to a Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA). Samples were resolved over a Kinetex C18 column, 2.1 x 150 mm, 1.7 µm particle size (Phenomenex, Torrance, CA, USA) equipped with a guard column (SecurityGuardTM Ultracartridge – UHPLC C18 for 2.1

mm ID Columns – AJO-8782 – Phenomenex, Torrance, CA, USA) using an aqueous phase (A) of water and 0.1% formic acid and a mobile phase (B) of acetonitrile and 0.1% formic acid for positive ion polarity mode, and an aqueous phase (A) of water:acetonitrile (95:5) with 1 mM ammonium acetate and a mobile phase (B) of acetonitrile:water (95:5) with 1 mM ammonium acetate for negative ion polarity mode. Samples were eluted from the column using either an isocratic elution of 5% B flowed at 250 µl/min and 25ºC or a gradient from 5% to 95% B over 1 minute, followed by an isocratic hold at 95% B for 2 minutes, flowed at 400 µl/min and 45ºC. The Q Exactive mass spectrometer (Thermo Fisher Scientific, San Jose, CA, USA) was operated independently in positive or negative ion mode, scanning in Full MS mode (2 µscans) from 60 to 900 m/z at 70,000 resolution, with 4 kV spray voltage, 45 shealth gas, 15 auxiliary gas. Calibration was performed prior to analysis using the Pierce™ Positive and Negative Ion Calibration Solutions (Thermo Fisher Scientific). Acquired data was then converted from raw to mzXML file format using Mass Matrix (Cleveland, OH, USA). Samples were analyzed in randomized order with a technical mixture injected after every 15 samples to qualify instrument performance. Metabolite assignments, isotopologue distributions, and correction for expected natural abundances of deuterium, 13C, and 15N isotopes were performed using El-MAVEN (Elucidata, CA, USA). Multivariate analyses were performed using MetaboAnalyst 6.0 (Pang et al., 2022). Raw data were normalized by median and ranged scaled (mean-centered and divided by the range of each variable) prior to statistical analysis.

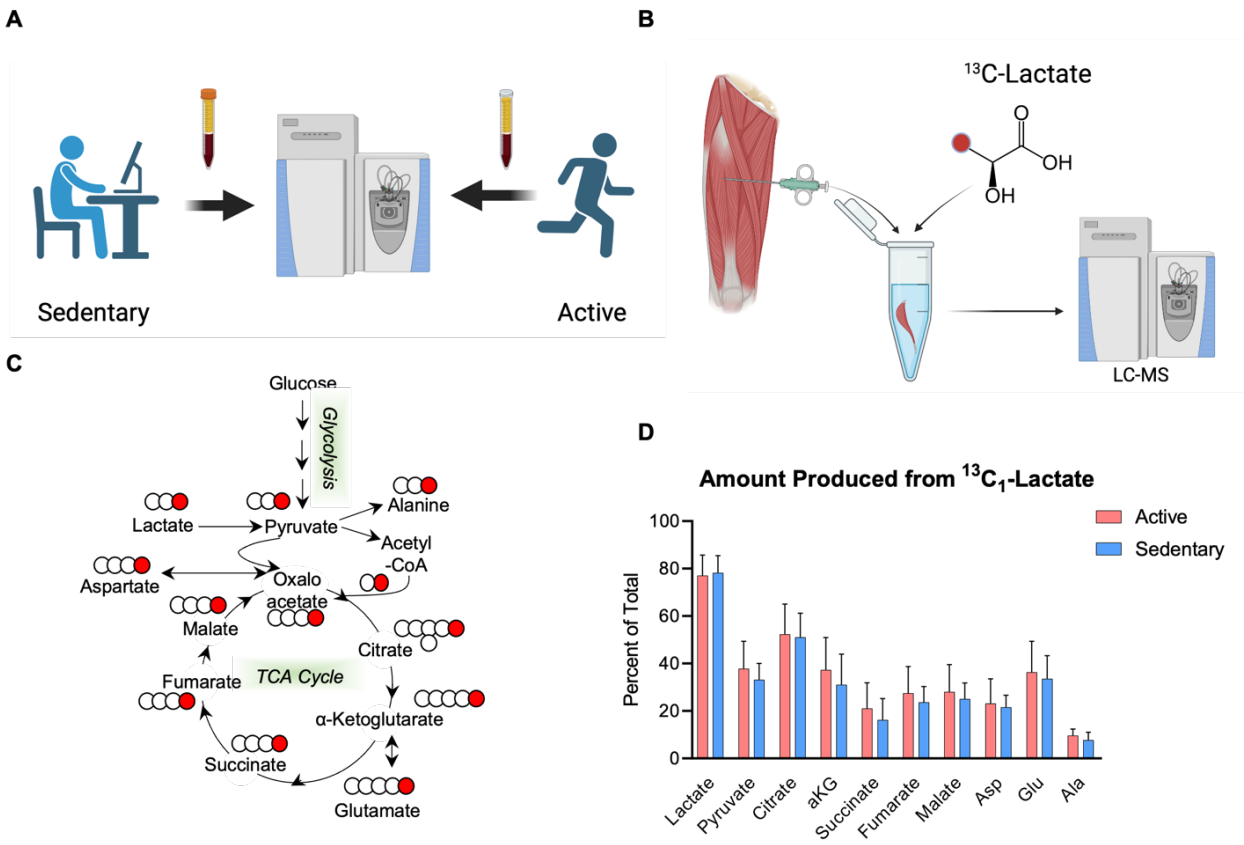


Figure-2 Ex Vivo Skeletal Muscle Isotope Tracing Analysis. (A) Skeletal muscle was isolated from 10 sedentary and 13 active individuals. (B) Isolated tissue was incubated with 13C1-lactate for 30 minutes in a physiological buffer at 37C. (C) Isotope tracing was targeted to metabolites in the TCA Cycle and related transaminase products. (D) Isotope enrichment is reported as a percentage of the total pool in each sample.

2.8. Cardiolipin Quantification

Muscle tissue was homogenized in PBS and lipids extracted. CL was quantified in these lipid extracts using liquid chromatography coupled to electrospray ionization mass spectrometry (LC/MS) in an API 4000 mass spectrometer (Sciex, Framingham, MA, USA) using normal phase solvents as previously published [24]. Total CL was calculated as the sum of the seven dominant species m/z 1422, 1446, 1448, 1450, 1470, 1472 and 1474.

2.9. Graded Exercise Assessment

All subjects were instructed to consume >50% of kcal as carbohydrates the night before and day of testing in order to be properly fueled for exercise. An exercise bicycle (RX 2.0; Raleigh Bicycle Company, Eastwood, United Kingdom) on a leg cycle ergometer (KICKR Smart Trainer; Wahoo Fitness, Atlanta, GA, USA) was fitted to subjects upon arrival. Subjects were asked to warmup freely at an exercise intensity below 65 Watts (W) for 10 minutes. The exercise protocol began at 75 W for all participants and increased by 25 W every 10 minutes until volitional exhaustion.

2.10. Gas Exchange Measurements

Oxygen consumption (VO2), CO2 production (VCO2), and respiratory exchange ratio (RER = VCO2/VO2) were determined using a ParvoMedics TrueOne 2400 Metabolic Measurement System (ParvoMedics, Inc., Sandy, UT, USA). Subjects were required to wear the mouthpiece that collected and analyzed respiratory gases; respiratory gas data were averaged over 15 seconds throughout the entire test.

2.11. Fat and Carbohydrate Oxidation Rate Measurements

For the measurement of total fat oxidation (FATox) and carbohydrate oxidation (CHOox), stoichiometric equations were applied according to the methodology described by Frayn [25] where:

$$\text{FATox } (g \cdot min^{-1}) = 1.67\ VO_2\ (L \cdot min^{-1}) - 1.67\ VCO_2\ (L \cdot min^{-1})$$

$$\text{CHOox } (g \cdot min^{-1}) = 4.55\ VO_2\ (L \cdot min^{-1}) - 3.21\ VCO_2\ (L \cdot min^{-1})$$

2.12. Lactate Concentration Measurement

At the end of every stage throughout the test, a sample of capillary blood was collected from the earlobe to analyze both extracellular levels of L-lactate (Lactate Plus Meter; Nova Biomedical, Waltham, MA, USA). Heart rate was monitored for the duration of the test with a heart monitor (Polar S725x; Polar Electro, Kempele, Finland) as well as rate of perceived exertion.

2.12. Statistical Analysis

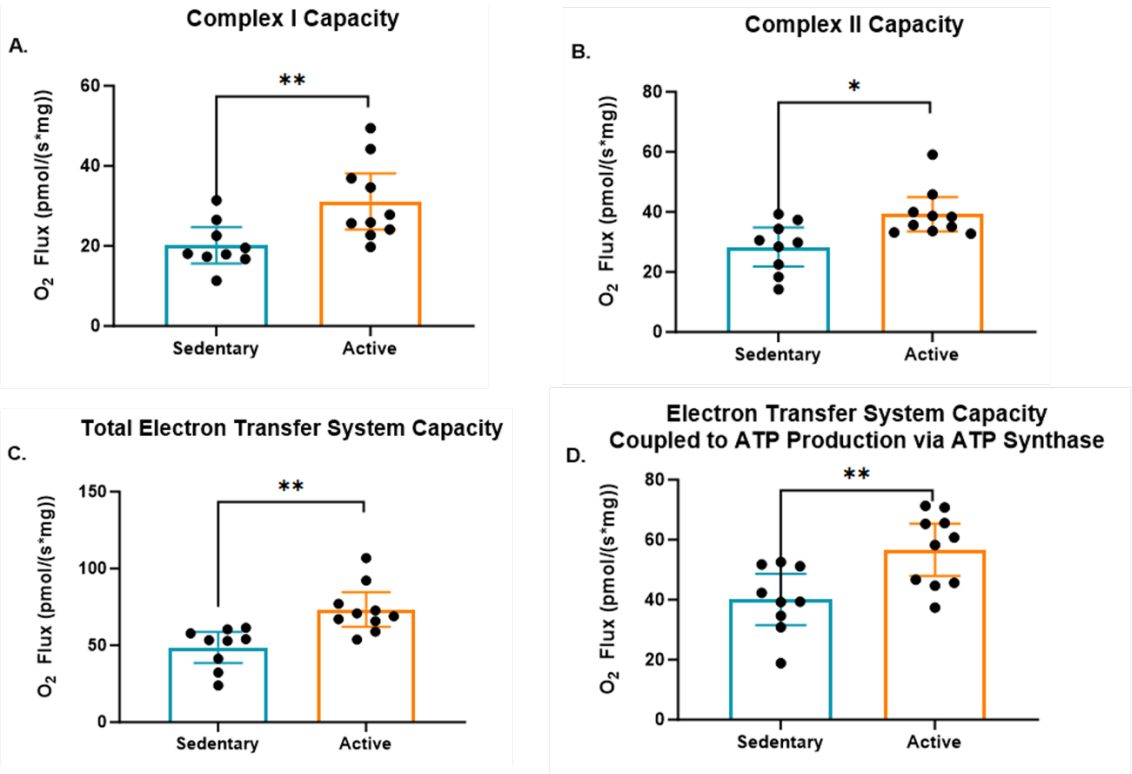
Data was analyzed and visualized via GraphPad Prism (version 9.2.1, GraphPad, San Diego, CA). Independent samples t-test were utilized for group-mean comparisons and employed a Shapiro–Wilk test for normality. Effects were analyzed with $p < 0.05$ being significant and $p < 0.1$ reported as trending toward significance.

3. RESULTS

3.1 Resting Conditions

High Resolution Respirometry – Mitochondrial Complex and Electron Transport System Capacities

Capacity of mitochondrial complex I and II was significantly higher in AC compared to SED ($p<0.01$ and $p<0.05$, respectively) (Fig-3A,B). Total electron transfer system (ETS) capacity as well as total ETS capacity coupled to ATP production via ATP synthase was significantly higher in AC than in SED subjects ($p<0.01$) (Fig-3C,D). Respiration that continues due to oxidative side reactions (residual oxygen consumption-ROX) following complete inhibition of the ET-pathway was also higher in AC individuals compared to SED ($p<0.01$) (Fig-3E).



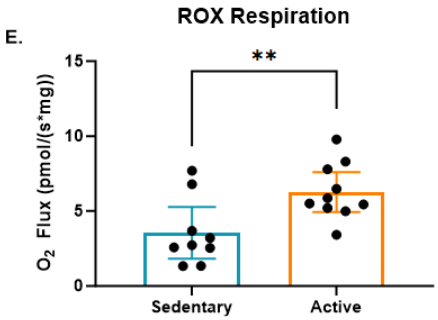


Figure 3. Differences in A) mitochondrial complex I capacity, B) complex II capacity, C) total electron transfer system capacity (maximal respiration), and D) electron transfer system capacity coupled to ATP production via ATP synthase between sedentary and active individuals. E) Differences in residual oxygen consumption (ROX) due to oxidative side reactions that continue after the inhibition of the ET-pathway*p<0.5, **p<0.01.

3.1.1. High Resolution Respirometry - Substrate Oxidation Capacities

Fatty acid oxidation using the substrates palmitoylcarnitine (long-chain fatty acid) or octanoylcarnitine (medium-chain fatty acid) was significantly higher in AC than in SED subjects (p<0.01) (Fig-4A,B). Pyruvate oxidation, the end-product of glycolysis, was also significantly higher in AC than in SED (p<0.01) (Fig-4D). Amino acid oxidation, represented by glutamate, was also significantly higher in AC than in SED (p<0.01) (Fig-4C).

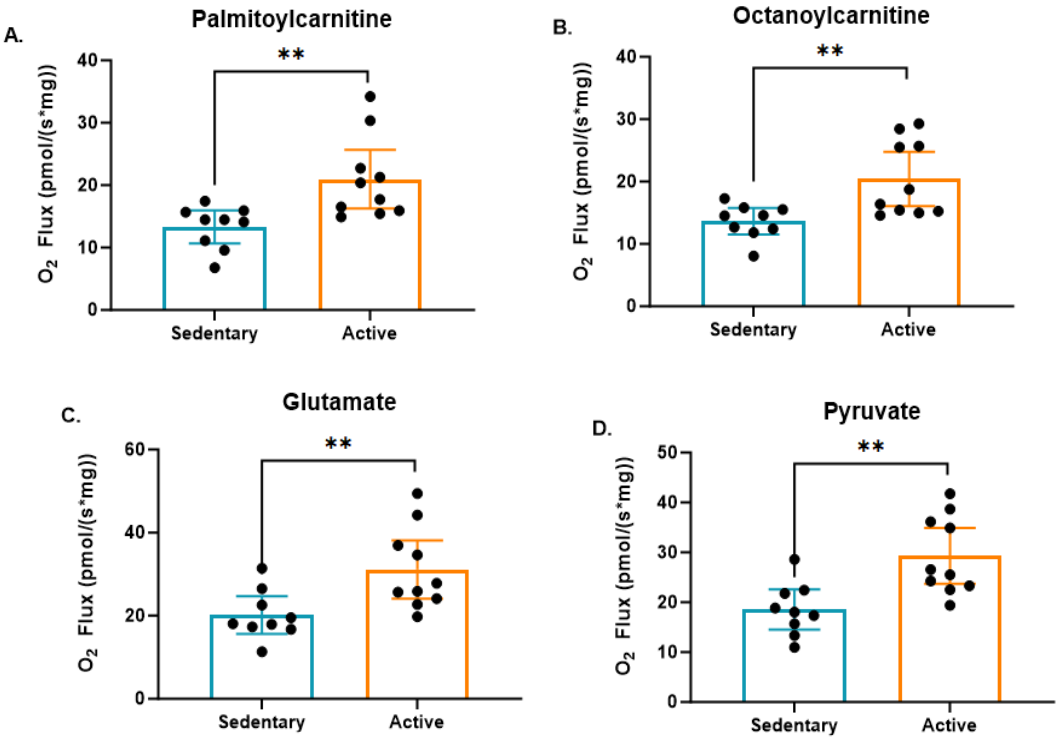


Figure 4. Differences in O_2 flux between sedentary and active individuals following saturating additions of A) palmitoylcarnitine, indicating long-chain fatty acid oxidation, B) octanoylcarnitine, indicating medium-chain fatty acid oxidation C) glutamate through complex I independent of pyruvate dehydrogenase complex and D) pyruvate, indicating carbohydrate-derived oxidation through complex I following transport into mitochondria via mitochondrial pyruvate carrier (MPC).

3.1.2.High Resolution Respirometry - Reactive Oxygen Species (ROS) Production

The amount of reactive oxygen species (ROS) produced normalized to O2 flux trends toward being higher in SED than in AC following the addition of ADP, Octanoylcarnitine, pyruvate, glutamate, and rotenone, with statistically higher ROS production upon addition of antimycin A ($p<0.05$, Fig-5).

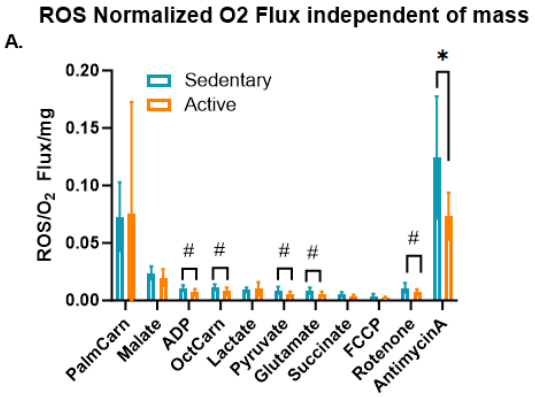


Figure 5. Differences in reactive oxygen species (ROS) production normalized to O_2 flux independent of mass in sedentary and active individuals. #$p<0.1$, *$p<0.05$.

3.1.3. Protein Expression and Activity

The quantity of skeletal muscle glucose transporter (GLUT4) as well as lactate dehydrogenase (LDH) isoforms A and B were similar in both populations studied (Fig-6 AC).

However, mitochondrial pyruvate carrier I (MPC1) content was significantly lower in SED compared to AC individuals ($p<0.01$) (Fig-6D,F). Figure 6E shows the combination of both MPC1 expression and pyruvate oxidation which are reduced in SED compared to AC individuals.

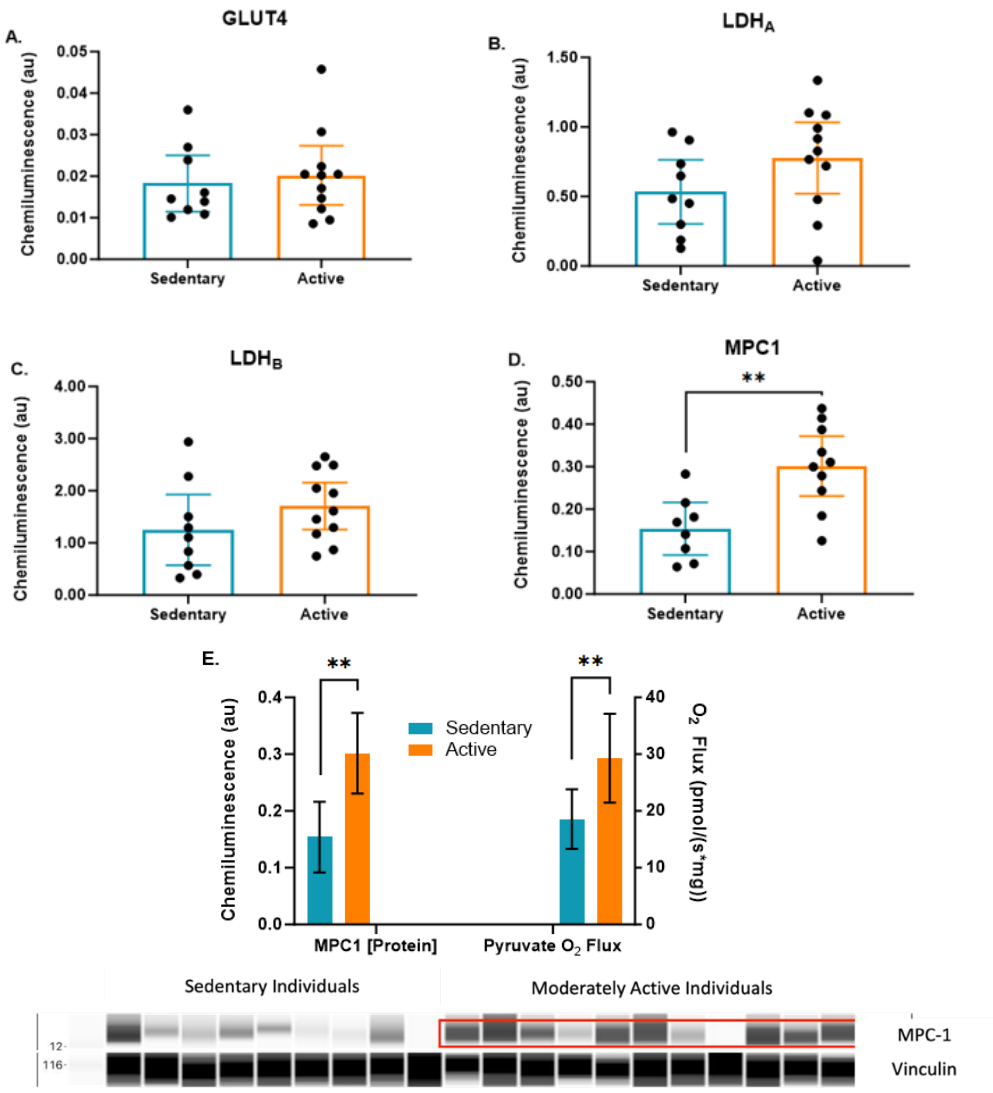


Figure 6. Differences in protein expression of A) GLUT4, B) LDH_A, B) LDH_B, and D&F) MPC1 between sedentary and active individuals. E) The combination of both MPC1 expression and pyruvate oxidation are reduced in SED compared to AC individuals. ** $p<0.01$.

Active individuals possessed higher activity of mitochondrial fatty acid transporter, carnitine palmitoyl transferase I (CPT1), compared to sedentary ones ($p<0.05$) (Fig-7A) whereas CPT2 activity was similar when comparing the two study groups (Fig-7B).

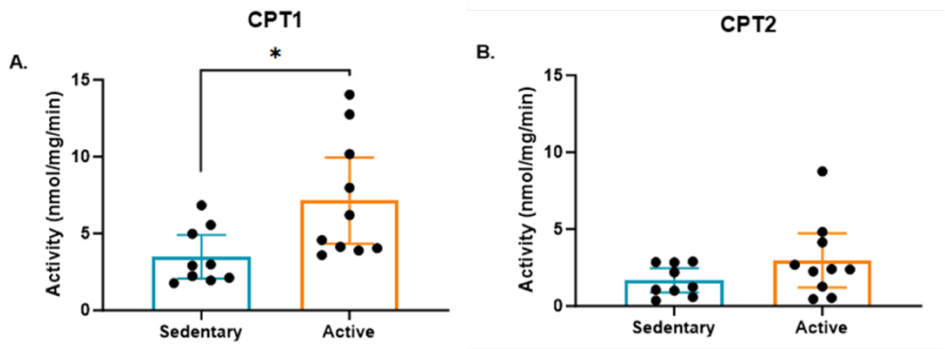


Figure-7. Differences in A) carnitine palmitoyl transferase I (CPT1) and B) carnitine palmitoyl transferase II (CPT2) activity between sedentary and active individuals. *p<0.05.

3.1.4. Cardiolipin Molecular Species

We found significant differences in cardiolipin (CL) content between SED and AC groups, with AC having greater total CL content (p<0.01) (Fig-8A). We also found increased monolysocardiolipin (MLCL; CL missing one fatty acyl chain) content in the AC group (Fig-8B), but the ratio of MLCL to CL was similar between groups (Fig-8C). Moreover, there was a significant increase in tetralineolyl CL (L4CL; CL having four linoleate chains) in the AC group (p<0.01) (Fig-8D), as well as a significant increase in the percent of L4CL, (p<0.01) (Fig-8E).

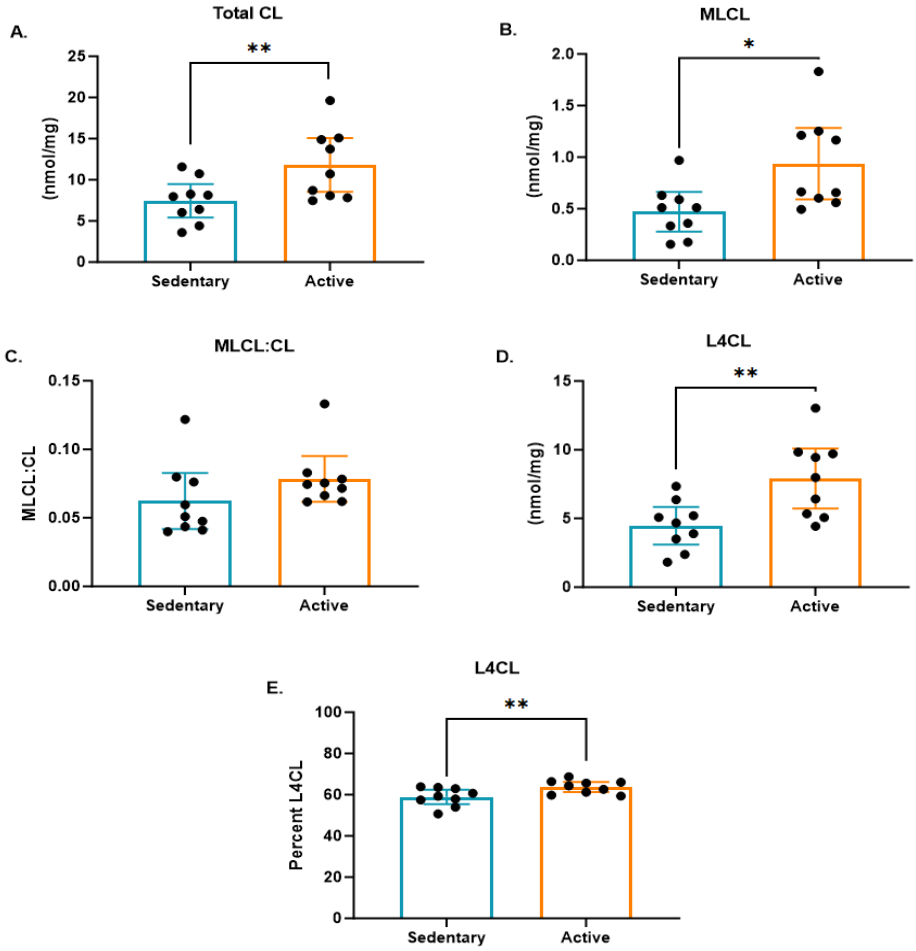


Figure 8. Comparison of A) total cardiolipin (CL), B) monolysocardiolipin (MLCL), C) the ratio of MLCL to total CL, D) tetralineolyl cardiolipin (L4CL), and E) percent L4CL between sedentary and active individuals. *p<0.5, **p<0.01.

3.1.5 Metabolomics

To determine if skeletal muscle tissue isolated from sedentary or active individuals was metabolically different under resting conditions, isolated tissue samples were analyzed by high-throughput mass spectrometry-based metabolomics and quantified 292 named metabolites (Supplementary Table 1). Partially supervised partial least squares discriminant analysis (PLS-DA) identified distinct metabolic phenotypes when comparing these two groups, which separated predominantly by Component 1 that explained 11.1% of the variance (Fig-9A). The top 15 metabolites that contributed to the clustering pattern are listed according to their Variable Importance In Projection (VIP) score (Fig-9B). This list was enriched for intermediates of glycolysis (glucose, glyceraldehyde 3-phosphate) and fatty acid oxidation (acylcarnitine [AC] 12-OH, 14:1-OH, 10, 14:1 and fatty acid [FA]14:1). When selecting for the top 50 significantly different metabolites (students T-test, $p < 0.05$), hierarchical clustering analysis was capable of predominantly distinguishing samples isolated from subjects within the two respective groups, aside from sedentary subjects 1 and 15 (Fig-9C). These clustering patterns were driven largely by higher acylcarnitines and lower glucose, cytidine, pyridoxamine, glyceraldehyde 3-phosphate, ADP-ribose, linolenic acid, and spermine in the active group (Fig-9D).

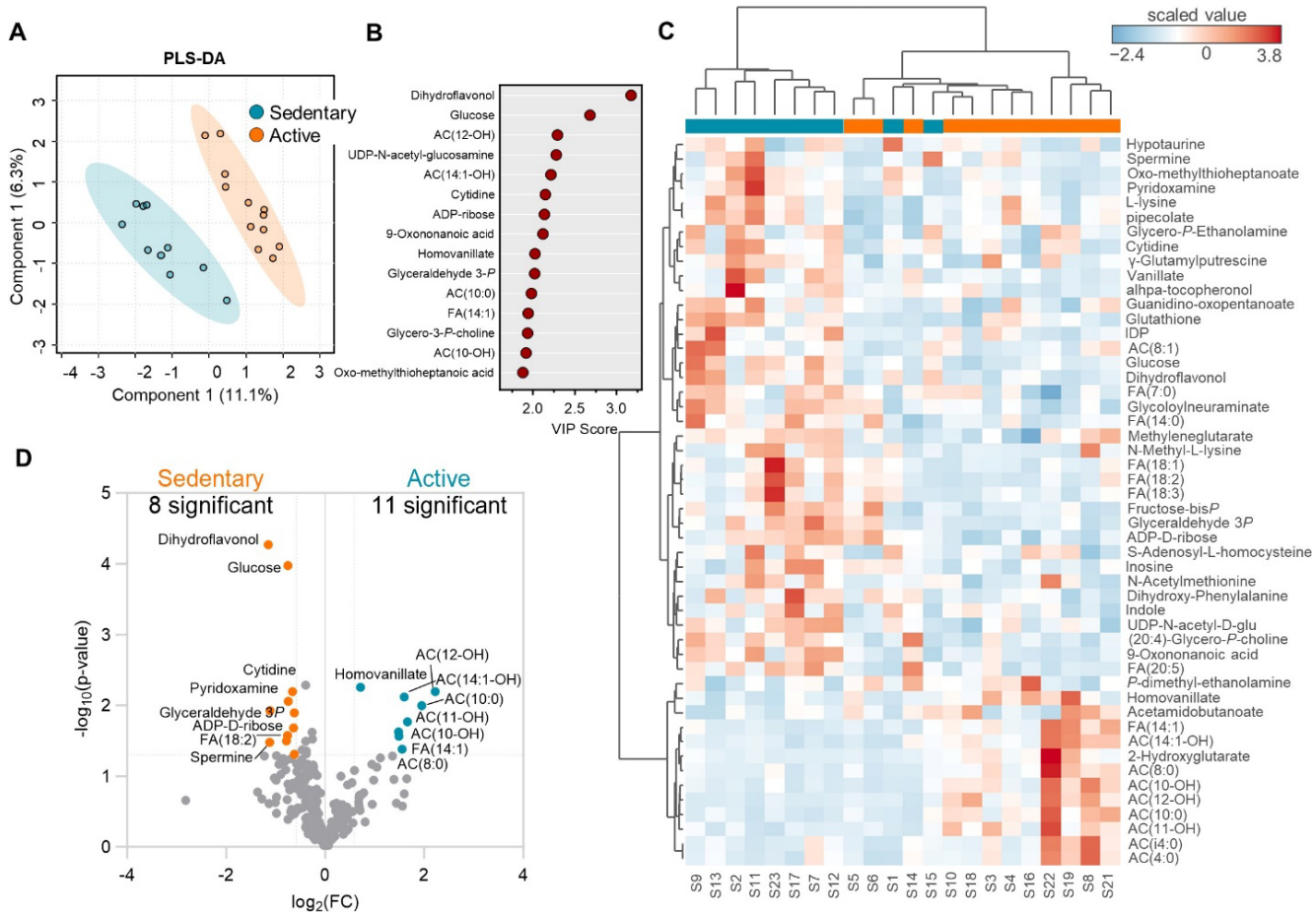


Figure 9. (A) Partial Least Squares Discriminant Analysis (PLS-DA) of metabolomics data. (B) Top 15 metabolites by variable importance in project (VIP) score. (C) Hierarchical clustering analysis of top 50 significant metabolites (Students T-Test, p<0.05). (D) Volcano plot with significantly higher (turquoise) and lower (orange) metabolites highlight for the active group, respectively.

3.2 Exercise Studies

SED individuals possess a significantly lower absolute and relative $VO2_{max}$ compared to AC ($p<0.0001$) (Fig-10A,B). Likewise, both absolute and relative maximal power output during the exercise test was significantly lower in SED compared to AC individuals ($p<0.0001$) (Fig-10C,D).

At both 125 Watts and 150 Watts, lactate clearance capacity was significantly superior in AC compared to SED individuals ($p<0.001$) (Fig-10E,F).

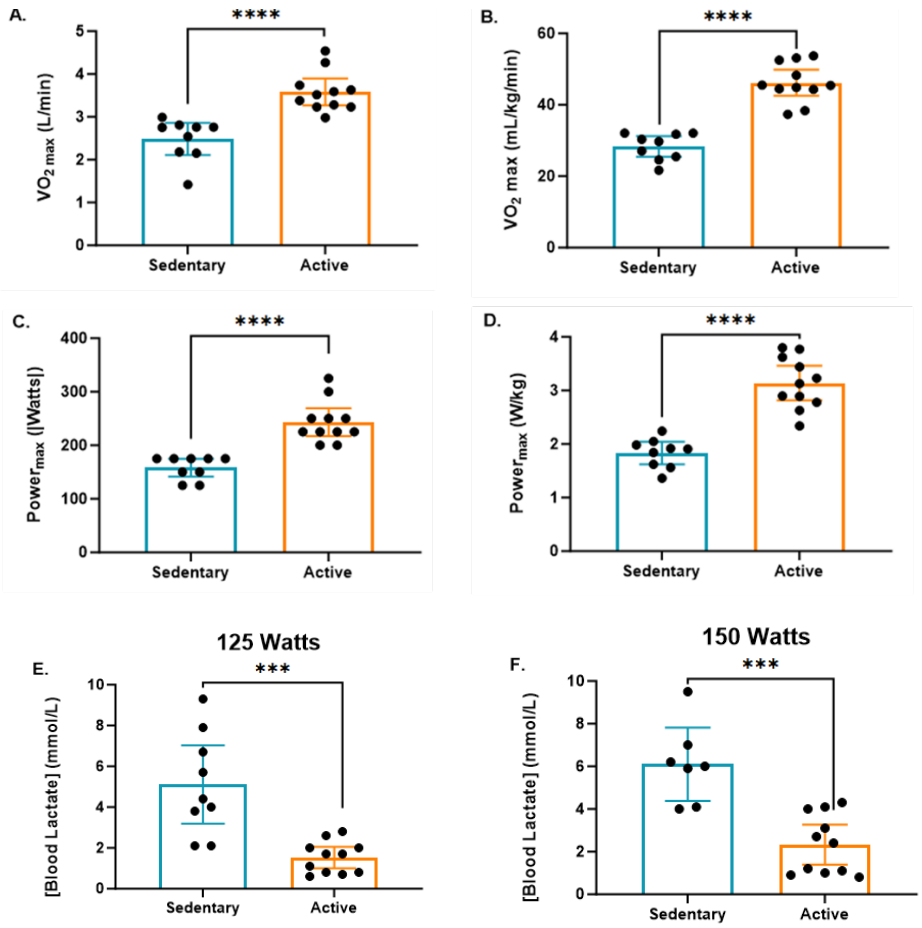


Figure 10. Differences in A) absolute $VO2_{max}$, B) relative $VO2_{max}$, C) absolute $power_{max}$, and D) relative $power_{max}$ between sedentary and active individuals. Differences in blood lactate concentrations at E) 125 Watts and F) 150 Watts between sedentary and active individuals. ***p<0.001, **** p<0.0001

3.2.1.Substrate Oxidations and Correlations During Exercise

During exercise, inverse correlations between blood lactate concentration and global fat oxidation (FATox) were statistically significant for each population studied (AC p<0.001, SED p<0.01) (Fig-11A,B). Likewise, inverse correlations between global carbohydrate oxidation (CHOox) and FATox are statistically significant also for both groups (AC p<0.001, SED p<0.05) (Fig-11 C,D). Finally, correlations between blood lactate concentration and CHOox are also statistically significant for both groups (AC p<0.001, SED p≤0.001) (Fig-11E,F).

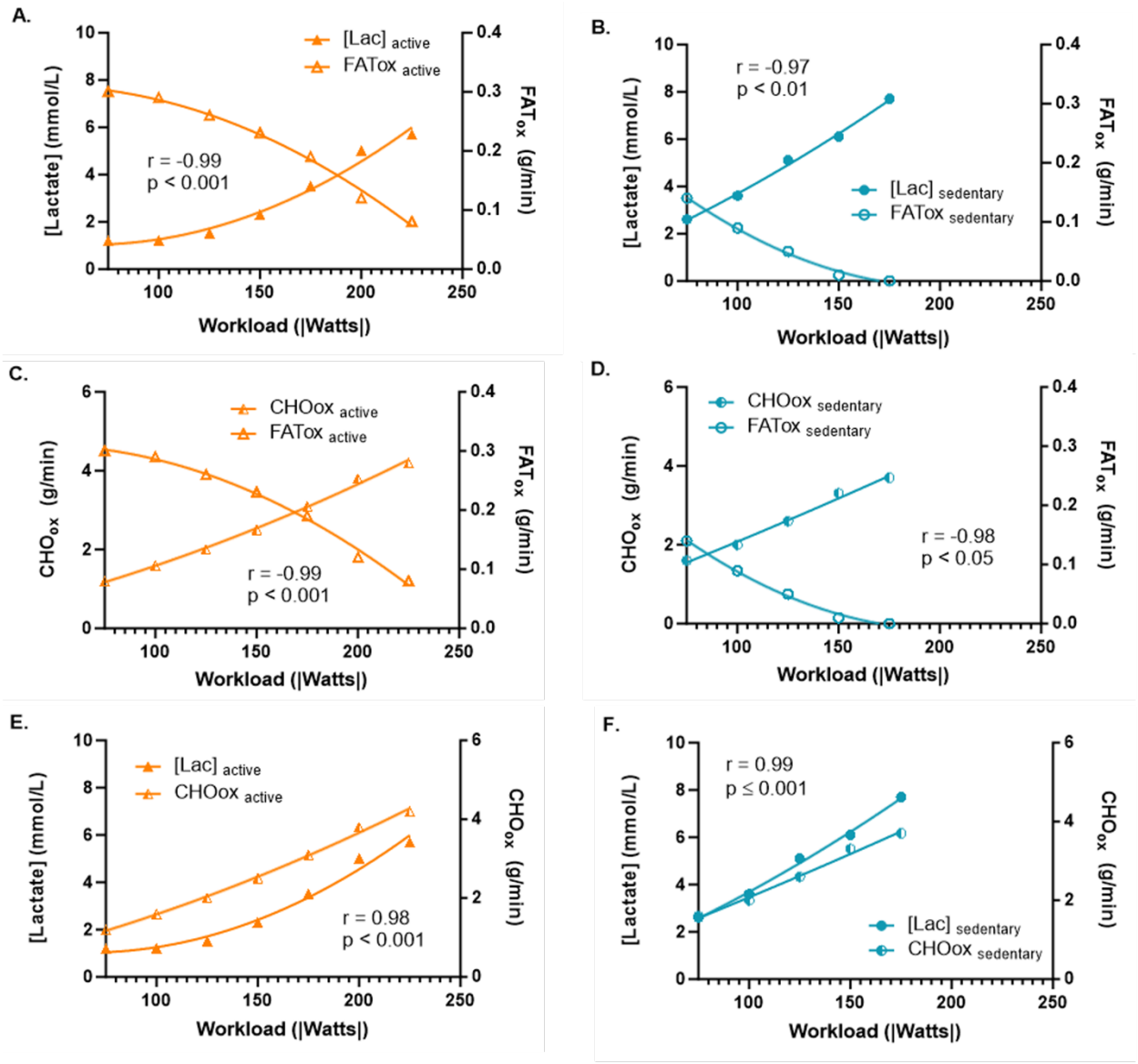


Figure 11. Correlations between blood lactate concentration and global fat oxidation (FATox) in A) active and B) sedentary individuals as workload increases via a graded exercise assessment. Correlations between global carbohydrate oxidation (CHOox) and FATox in C) active and D) sedentary individuals and correlations between blood lactate concentration and CHOox in E) active and F) sedentary individuals as workload increases

3.2.2.Correlations between resting and exercise conditions.

Ratios of blood lactate concentration during exercise at 125 Watts and 150 Watts respective to resting mitochondrial pyruvate oxidation capacity, electron transfer system capacity, and electron transfer system capacity coupled to ATP production via ATP synthase were significantly higher in SED compared to AC (p<0.001-0.0001) (Fig-12).

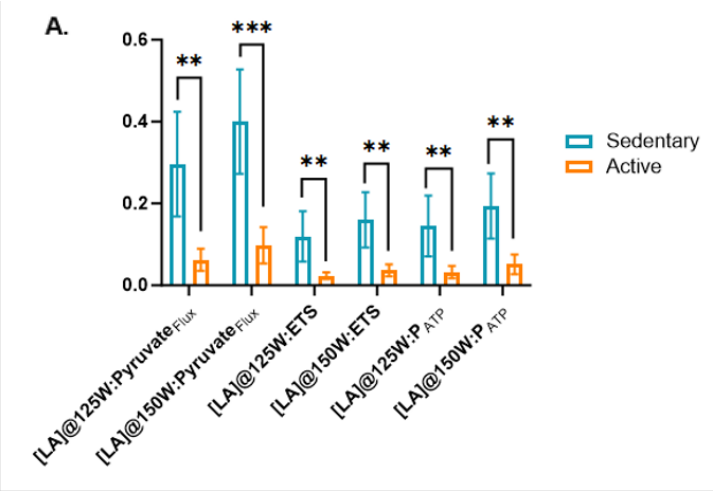


Figure 12. A) Ratios of blood lactate concentration at 125 Watts and 150 Watts to resting mitochondrial pyruvate oxidation capacity, electron transfer system (ETS) capacity, and electron transfer system capacity coupled to ATP production via ATP synthase (P_{ATP}) between sedentary and active individuals. **p<0.01, *** p<0.001

For the study group as a whole, we found robust correlations between FATox during exercise at both 125 Watts and 150 Watts and mitochondrial oxidation of palmitoylcarnitine (LCFA) and octanoylcarnitine (MCFA) at rest between AC and SED groups (p<0.0001) (Fig-13A-D). Furthermore, we also found a strong correlation between FATox during exercise at both 125 Watts and 150 Watts and mitochondrial resting electron transport system capacity (ETS) between AC and SED groups (p<0.01, Fig-13E,F).

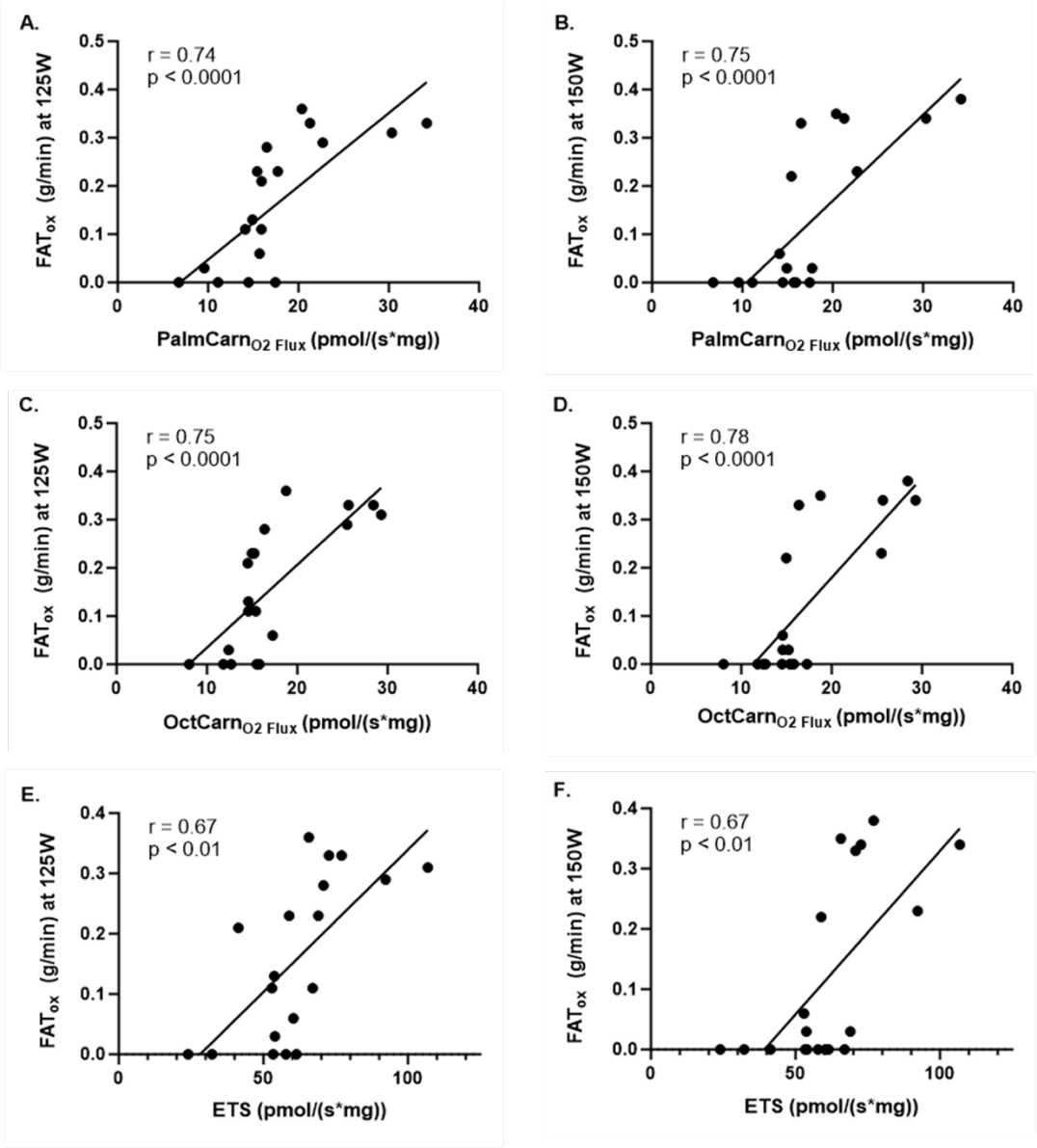


Figure 13. Correlations between mitochondrial oxidation of palmitoylcarnitine (LCFA) at rest and global fat oxidation during exercise at both A) 125 Watts and B) 150 Watts across the entire study population. Correlations between mitochondrial oxidation of octanoylcarnitine (MCFA) at rest and global fat oxidation during exercise at both C) 125 Watts and D) 150 Watts. Correlations between mitochondrial electron transport system capacity and global fat oxidation during exercise at both E) 125 Watts and F) 150 Watts for the entire study population.

Furthermore, there were strong correlations between $VO2_{max}$ and ETS as well as electron transfer system capacity coupled to ATP production via ATP synthase (P_{ATP}) for both groups as (r=0.57, p≤0.01 and r=0.59, p<0.01, respectively) (Fig-14A,B)

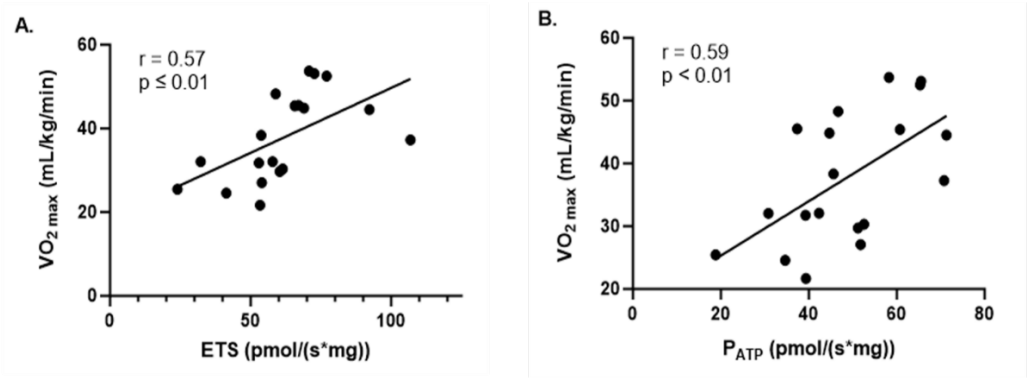


Figure 14. Correlations between A) electron transport system capacity (ETS) and $VO2_{max}$ as well as B) electron transfer system capacity coupled to ATP production via ATP synthase and $VO2_{max}$ across entire study population.

Finally, for both groups combined, there were moderate inverse correlations between exercise blood lactate concentrations at 125 Watts and 150 Watts and resting mitochondrial pyruvate oxidation (r=-0.48, p<0.01 and r=-0.49, p<0.05, respectively) (Fig-15A,B) as well as between blood lactate concentration at 125 Watts and 150 Watts and resting mitochondrial palmitoylcarnitine (LCFA) (r=-0.49, p<0.01 and r=-0.50, p<0.05 respectively) (Fig-15C,D)

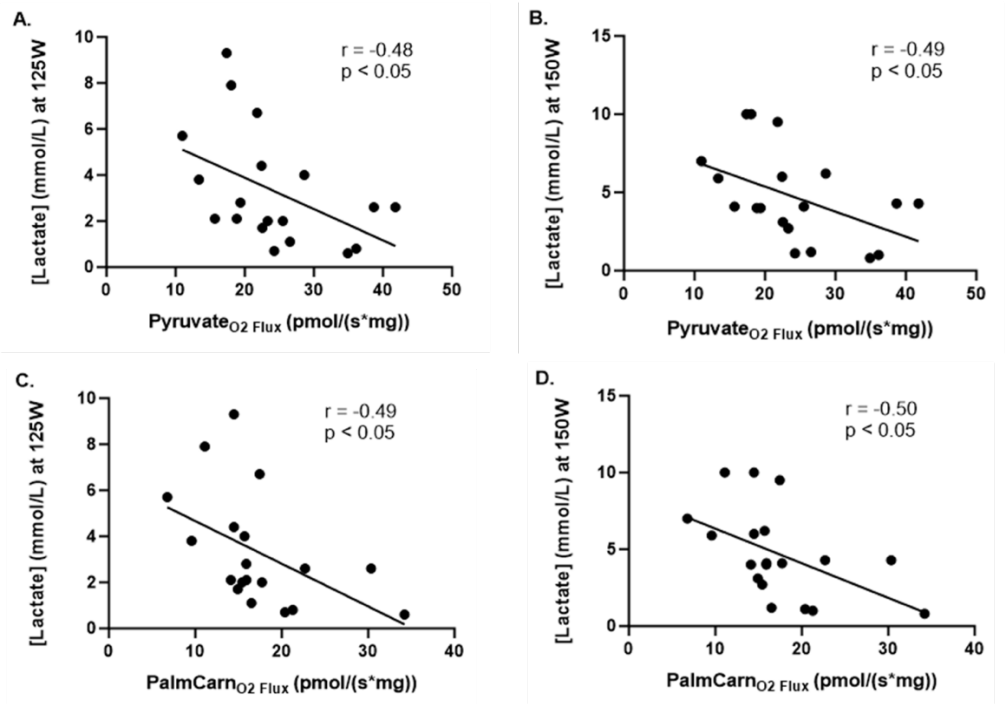


Figure 15. Correlations between mitochondrial oxidation of pyruvate at rest and blood lactate concentrations during exercise at A) 125 Watts and B) 150 Watts across entire study population. Correlations between mitochondrial oxidation of palmitoylcarnitine at rest and blood lactate concentrations during exercise at A) 125 Watts and B) 150 Watts.

DISCUSSION

Historically, sedentary individuals have been portrayed as healthy individuals as they did not manifest clinical symptoms. This population has even been utilized as the control group in a vast number of clinical studies. In the study herein, we show significant metabolic downregulations both at rest and during exercise in "healthy sedentary" individuals (SED) compared to moderately active individuals (AC) across multiple cellular and bioenergetic functions which are involved in the pathogenesis of insulin resistance and type 2 diabetes.

Resting studies

In resting conditions, SED showed significantly reduced mitochondrial respiration and substrate oxidation capacity. SED individuals have a significant decrease in complex I (36%) and II capacities (28%) as well as total electron system capacity (34%) and electron system capacity coupled to ATP production via ATP synthase (30%), compared to AC (Fig-3A-C). Regarding protein content involved in glucose transport and metabolism, SED group showed similar expressions of GLUT4 as well as a similar expression of both isoforms of LDH (A and B) to AC (Fig-6A-C). However, SED individuals possessed a significantly reduced expression (49%) of MPC compared to AC group ($p<0.01$)(Fig-4E and 6D,F). This is a significant finding as although glucose transporters (GLUT4) are equally expressed in both groups, MPC and pyruvate oxidation are already significantly downregulated in SED (Fig-4C,E), showing what could be a signature of internal cellular downregulation in the oxidation of the end-product of glucose, pyruvate.

Historically, the pathogenesis of T2D has clinically focused on hyperglycemia, hyperinsulinemia, IR, as well as the dysregulation of GLUT4 and pancreatic beta cells. However, glucose uptake is just the first part of the journey as full glucose metabolism must endure ten biochemical cytosolic reactions, with pyruvate and lactate as the end products of a "mid point" of the journey. The last part of this journey should be successfully completed by the transportation of pyruvate into mitochondria by MPC for oxidation to Acetyl-CoA and posterior oxidation in mitochondria for ATP synthesis through the TCA cycle and OXPHOS in the ETC.

The discovery of MPC in 2012 by Bricker et al as well as Herzig et al [26, 27] opened a significant new venue for a more complete depiction of the cellular fate of glucose as well as pyruvate

metabolism and therefore novel doors towards a better understanding of the pathogenesis of T2D and IR. Our findings herein show that in fact, pyruvate transport and metabolism are significantly decreased in healthy sedentary individuals (37%) despite the fact that GLUT4 expression is equally expressed in both groups. Through our "inside out" approach, the major finding of our study could mean that the downregulation of glucose metabolism could mechanistically start from within cells, in mitochondria and not at the sarcolemma through a dysregulation of GLUT4 and resistance of insulin receptors. This downregulation of pyruvate metabolism which could develop years before glucose uptake dysregulation, preceded by insulin resistance and GLUT4 dysregulation. Hence, we believe that the role of pyruvate metabolism deserves further attention for a better understanding of the pathogenesis of T2D and IR.

Furthermore, fat metabolism is also significantly dysregulated in SED compared to AC. In our study herein, we show a decrease in both mitochondrial fatty acid oxidation (Fig-4A,B) as well as activity of the CPT1 transporter responsible for getting medium and long-chain fats into mitochondria ($p<0.05$),(Fig-7A) and a trend towards a decreased activity of CPT2 ($p=0.16$) (Fig-7B). The dysregulation of fat oxidation in skeletal muscle and intramuscular/intramyocellular fat has received significant attention in the last two decades as it is known that increase in intramuscular/intramyocellular fat is correlated with IR [28-31]. If SED individuals cannot transport and oxidize fatty acids correctly, it is plausible to posit that SED individuals may accumulate fatty acids overtime right outside muscle mitochondria with possible deleterious effects over time including IR.

Metabolomics analysis also confirms CHO and Fat metabolism downregulations. PLS-DA identified distinct metabolic phenotypes through intermediates of glycolysis (glucose, glyceraldehyde 3-phosphate) and fatty acid oxidation (acylcarnitine [AC] 12-OH, 14:1-OH, 10, 14:1 and fatty acid [FA]14:1) (Fig-9A). Hierarchical clustering analysis was capable of distinguishing samples isolated from subjects within the two respective groups, mainly driven by higher acylcarnitines and lower glucose, cytidine, pyridoxamine, glyceraldehyde 3-phosphate, ADP-ribose, linolenic acid, and spermine in the active group (Fig-9D).

Nevertheless, downregulations in protein content and substrate oxidation are not the only mitochondrial abnormalities we found in SED compared to AC. Cardiolipin (CL) is a dimeric phospholipid residing in the inner mitochondrial membrane with multiple involvements in mitochondria such as structure, function, biogenesis and bioenergetics [18, 32-35] . Alterations in CL acyl chain composition, and/or CL peroxidation have been associated with mitochondrial dysfunction in a variety of pathological conditions, including ischemia, hypothyroidism, aging, and heart failure [32]. In our study herein, we found significant differences in CL between SED and AC groups ($p<0.01$) (Fig-15A). We also found a tendency of increased monolysocardiolipin (MLCL) (Fig-15B). Moreover, there was a significant difference in tetralineolylcardiolipin (L4CL) and the percent of L4CL out of total CL-species between SED and AC groups ($p<0.01$) (Fig-15D,E). The increase in percent L4CL is important because it is a change independent of the number of mitochondria in the muscle and a decrease in this percent has been linked to heart failure in rats and humans and closely tied to the degree to which mitochondria use fatty acids for fuel [24, 36]. The changes in CL we show herein could lead to further exacerbation of mitochondrial dysfunction and substrate oxidation which deserve further attention and studies regarding the role of CL in the pathogenesis of mitochondrial dysfunction pertaining the pathogenesis of TD2.

Moreover, we showed significant differences in reactive oxidative species (ROS) between SED and AC groups. Mitochondria are the main producers of ROS[37-39]. It is well established that ROS can function as pleiotropic physiological signaling molecules [40] necessary for both cellular homeostasis “oxidative eustress” [41, 42] and “mitohormesis” [43, 44]. However, it is also known that excessive ROS production can accumulate in cells and can cause multiple cellular disruptions involved in multiple diseases[45-49] as well as affect mitochondrial function by causing damage to mitochondrial DNA[38, 50]. In our study we found that AC subjects showed increased oxygen flow (Fig-3E) which will cause more total oxygen to escape from the ETC and therefore naturally produce more ROS overall. However, as shown in Figure 5, when ROS were normalized to oxygen flux making this measurement independent of the number of mitochondria in the muscle, higher ROS were detected in muscle mitochondria of SED individuals indicating that ROS are released at a higher rate proportional to flow at many different ETC locations possibly due to a leakier

transport system which could elicit supraphysiological ROS eliciting deleterious effect to mitochondrial and cellular homeostasis.

Exercise studies

It is noteworthy that our exercise studies confirmed what we observed in our resting studies. These findings can have remarkable clinical applications to indirectly assess mitochondrial function in a non-invasive way and in an ambulatory manner through GXT and CPET testing as we previously suggested [20].

We observed significant differences in all parameters we measured between SED and AC groups. Both absolute and relative $VO2_{max}$ were significantly higher (31% and 38%, respectively) in AC compared to SED ($p<0.0001$) (Fig-10A,B). Likewise, both maximal absolute and relative power output were significantly higher (35% and 42%, respectively) in AC compared to SED individuals ($p<0.0001$) (Fig-10C,D).

Blood lactate concentrations were significantly higher in SED vs AC individuals at a same power output (70% at 125 W, 62% at 150 W)($p<0.001$) (Fig-10E,F). This significantly higher blood lactate levels in SED denote a significantly lower pyruvate oxidation capacity (as we shown in our resting studies) leading to a reduction to lactate. As expected, and in agreement with our resting studies, AC individuals showed a significantly higher capacity to oxidize fat compared to SED ones ($p<0.05$-0.001) (Fig-11A-D).

Correlations between blood lactate and FATox, Blood lactate and CHOox and between CHOox and FATox were quite robust across both groups studied (Fig-11). We previously described these correlations as indirect assessments of mitochondrial function and metabolic flexibility between elite athletes, moderately active individuals and patients with metabolic syndrome[20]. In our study herein, we show that these differences in substrate utilization are probably due to decreased mitochondrial transport and oxidation capacities in SED vs AC individuals as we demonstrate through muscle biopsies in our resting studies.

Moreover, our correlations between mitochondrial respiration and capacity between our resting studies and exercise studies are quite robust. As Figure 12 shows, the ratios of blood lactate concentration at 125 Watts and 150 Watts to mitochondrial pyruvate oxidation at rest, electron

transfer system capacity, and electron transfer system capacity coupled to ATP production via ATP synthase at rest were significantly higher in SED compared to AC ($p<0.001$-0.0001)(Fig-12). Furthermore, the correlations between mitochondrial fat oxidation at rest and during exercise were quite robust for both groups observed ($r=0.75$-0.78; $p<0.01$)(Fig-13A-D). Correlations between exercise lactate and resting pyruvate oxidation as well as with fatty acid oxidation at rest were inversely moderate ($r=-0.48$-0.50; $p<0.05$) (Fig-15A-D).

In summary, in our study herein, we demonstrate that "healthy sedentary" individuals already possess significant cellular bioenergetic downregulations compared to moderately active individuals. These differences are profound both during resting and exercising conditions and across multiple key mitochondrial elements such as ETC, substrate transporters and oxidations, cardiolipin species configurations and ROS productions. While these differences were expected during exercising conditions, some of the differences observed at rest were unexpected as SED group are healthy subjects without any known metabolic condition.

Specifically noteworthy is our finding that both MPC and pyruvate oxidation are significantly decreased in SED compared to AC while GLUT4 expressions are similar. We believe that this is an important finding as SED individuals although historically considered healthy, already possess significant downregulation in cellular glucose metabolism despite normal expression of external glucose transporters, GLUT4. This finding could mean that the pathogenesis of the dysregulation of glucose metabolism could already start at the mitochondrial level and proceed a dysregulation of GLUT4 and insulin function by years or decades. We believe that it is important to perform further research studies regarding pyruvate metabolism to better understand the mechanisms behind the pathogenesis of IR and T2D which can lead to the development of novel medications to target MPC and pyruvate oxidation.

Finally, we show that exercise mirrors quite robustly the results obtained from muscle biopsies at rest. Hence, metabolic testing and cardiopulmonary exercise testing (CPET) could be an optimal and practical manner to indirectly assess mitochondrial function in a non-invasive way to millions of people in an ambulatory manner. Although further studies including populations with pre- and T2D are warranted, the fact that MPC and pyruvate metabolism are already significantly

downregulated in sedentary individuals before a possible transitioning to a pre- or T2D status, a fantastic window of opportunity exists in order to intervene through exercise and nutrition to potentially avert T2D and metabolic syndrome in millions of people.

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