# Advances in Cellular Respirometry and its Future in the Clinical Laboratory


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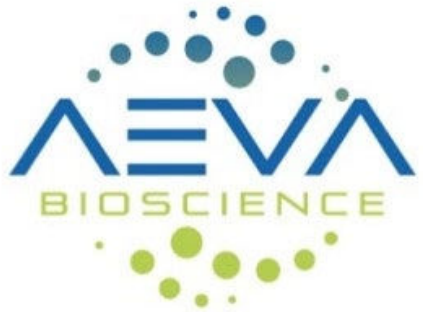
# Disclosure




Chief Science Officer (CSO) of Aeva Bioscience, LLC

*A CRO providing research and consulting services to clinical, translational, and basic science studies investigating mitochondrial function.*

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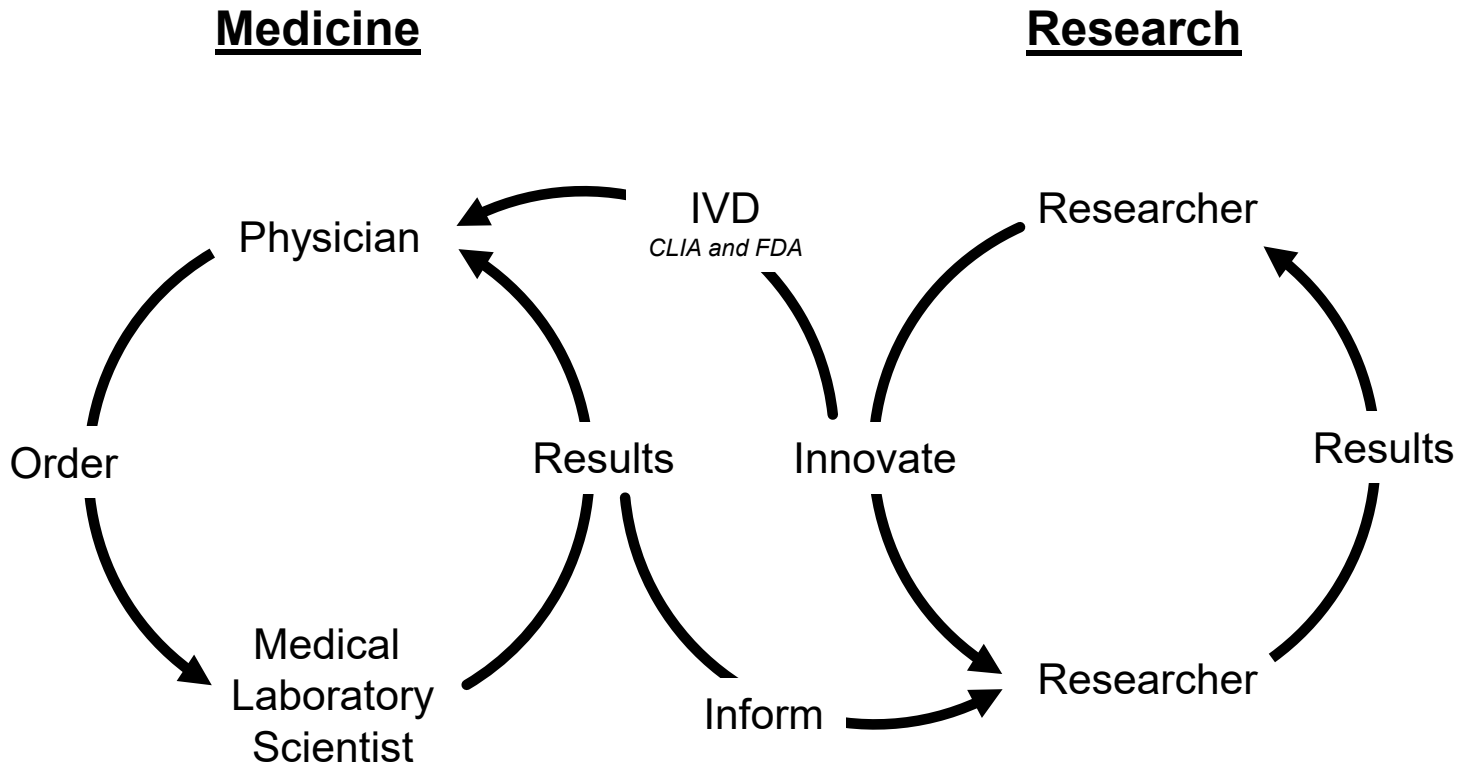
# The Life Cycles of an Assay



# FDA vs. CLIA

- **FDA** determines whether a test can accurately and reliably measure what it claims to measure (analytical validity) and whether the measurement is predictive of a certain state of health (clinical validity).

- **CLIA** verifies certain performance characteristics of a test within the laboratory using it. Analytical validation testing required.

# Outline by FDA and CLIA Requirements

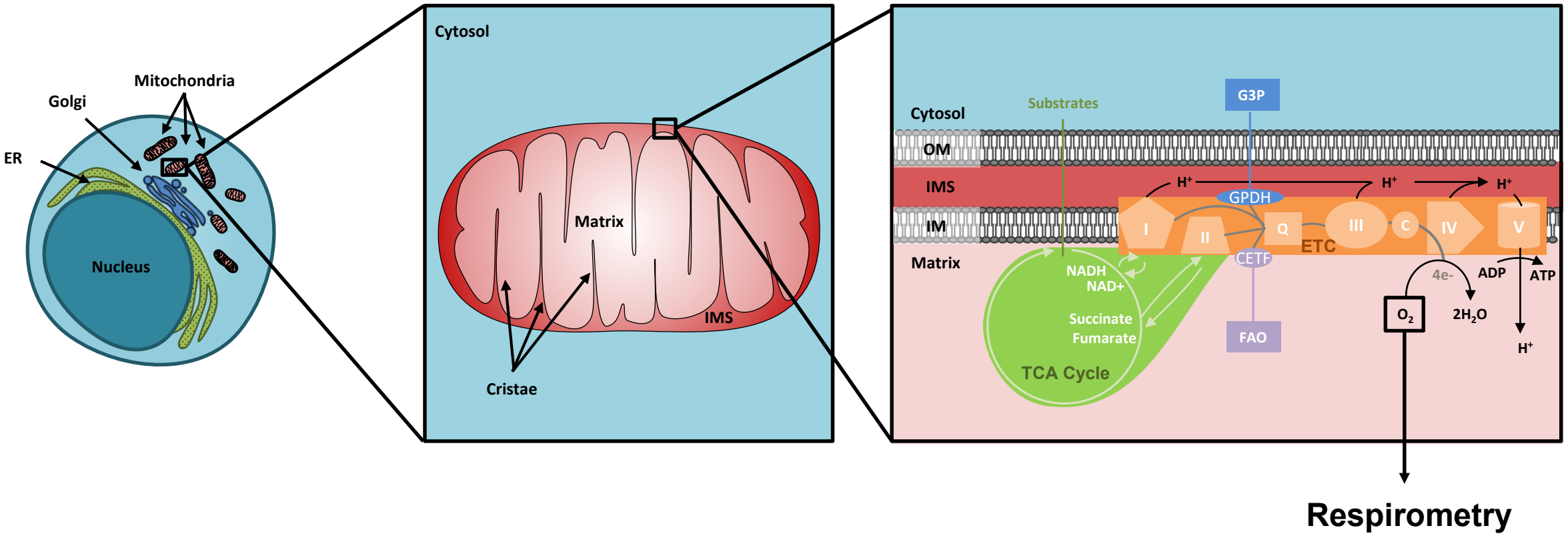
**Clinical Validation:**

- Test purpose
- Specimen type(s)
- Target population(s)
- Clinical Sensitivity and Specificity

**Analytical Validation:**

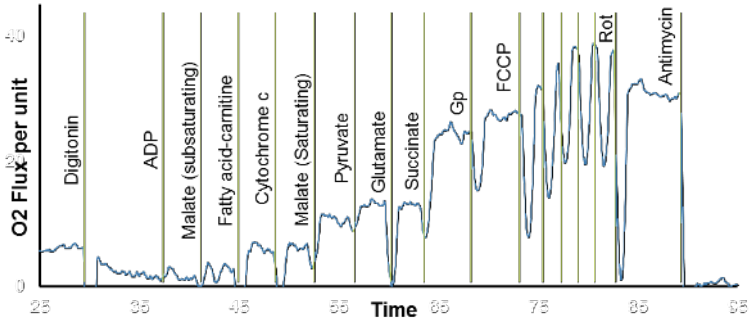
- Precision
- Accuracy
- Analytical sensitivity and specificity
- Reference Range
- Other performance characteristics

# The Fundamentals of Cellular Respirometry



# Technology to Measure Mitochondrial Function

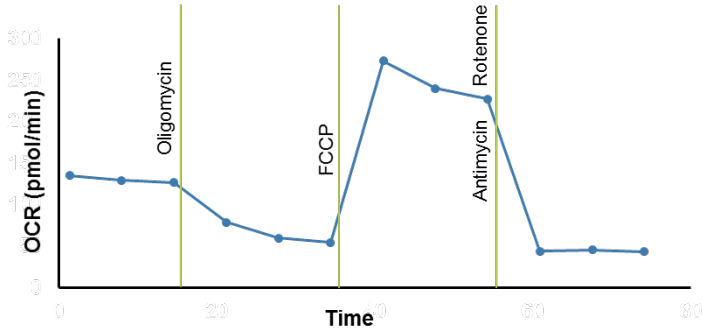
## Polarographic – Oroboros - Austria




**Features:**

- Intact and permeabilized cells and tissues
- Unlimited injections (manual)
- Low Throughput
- High resolution
- Low operating cost

## Fluorometric/optical probes – Seahorse/Agilent - US


**Features:**

- Intact cells
- 4 injection ports (automated)
- High Throughput
- Low resolution
- High operating costs

# Examples of Clinical Indices

## Permeabilized Cell Assay

Routine Respiration
Fatty Acid-Linked Respiration
Cytochrome c Response
Complex I-Linked Respiration
Complex II Respiration
Glycerophosphate Response
Max Uncoupled Respiration
Complex I Uncoupled
Complex II Uncoupled
Complex IV Respiration
Residual O2 Consumption

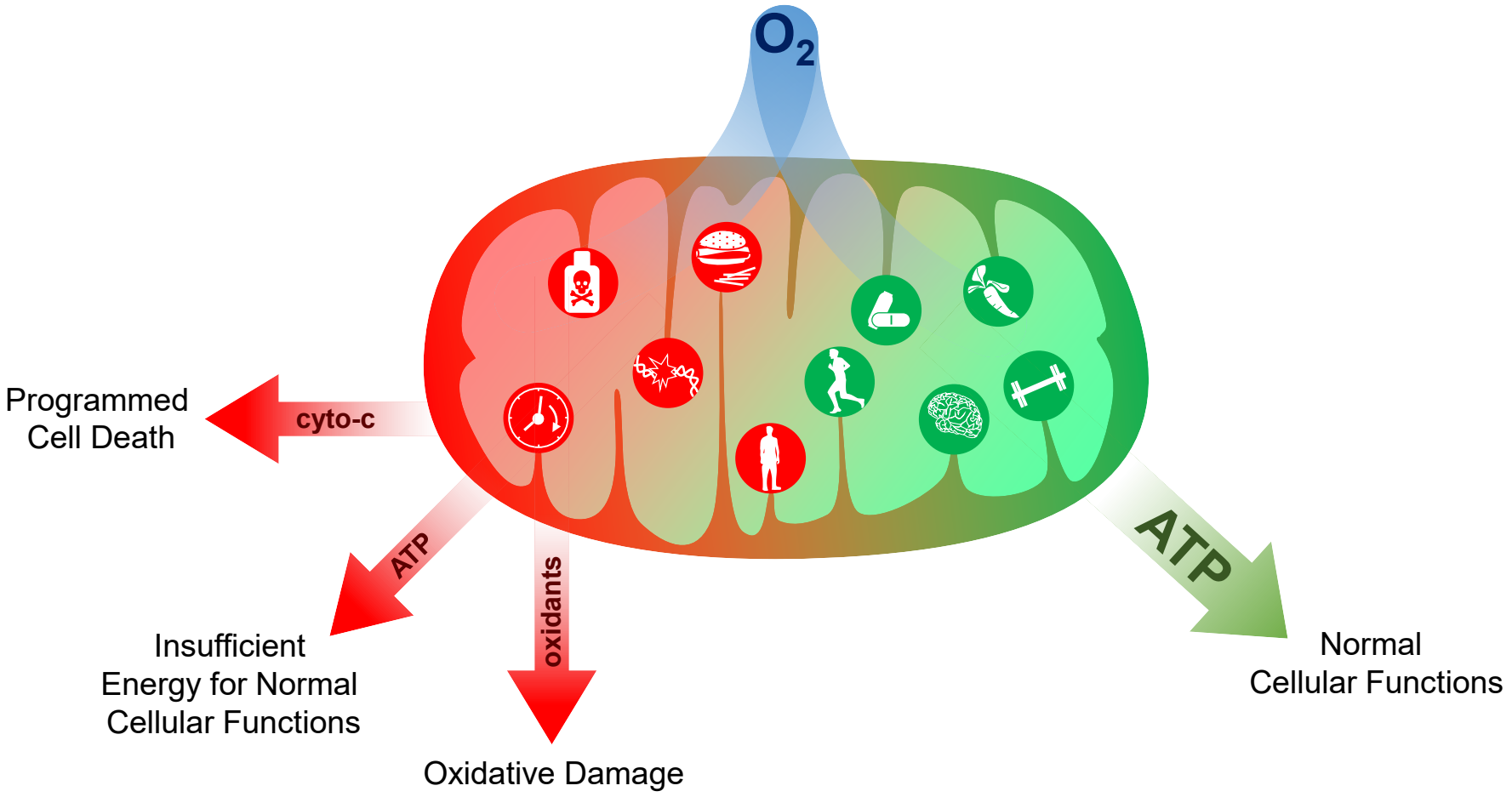
## Intact Cell Assay

Basal Respiration
ATP-Linked Respiration
Proton Leak
Maximal Respiration
Spare Respiratory Capacity
Non-Mitochondrial Oxygen Consumption

Basal glycolytic rate
Glycolytic Flux

Derived Bioenergetic Health Index

# Bioenergetics in Human Health and Disease



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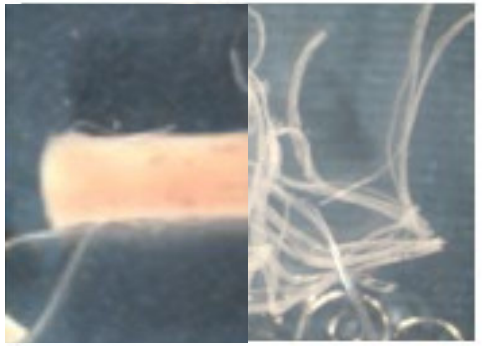
# Historical Clinical Specimen

Vastus Lateralus Biopsy

Sample preservation and transportation

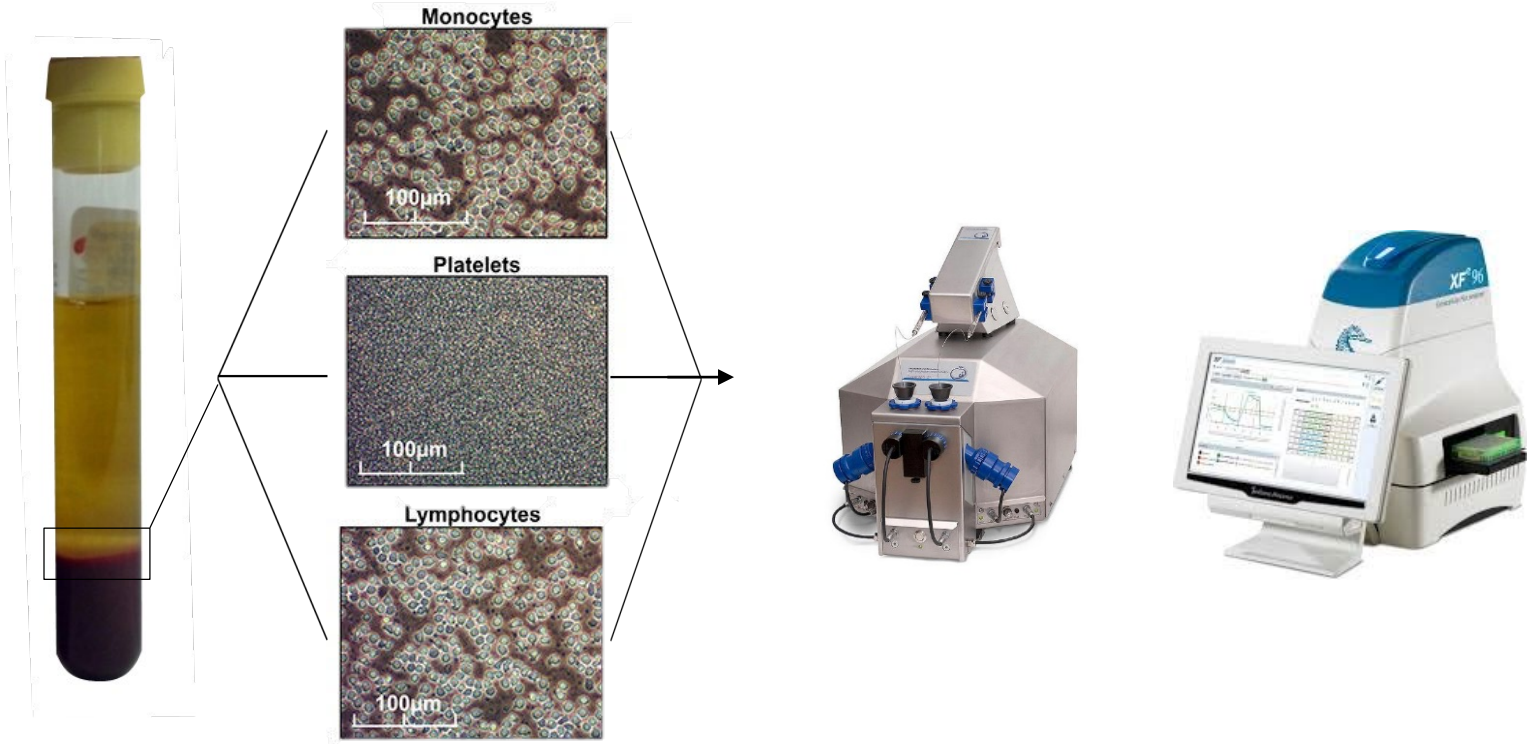
Mechanical preparation and permeabilization of fiber bundle



High-Resolution Respirometry (Oroboros)

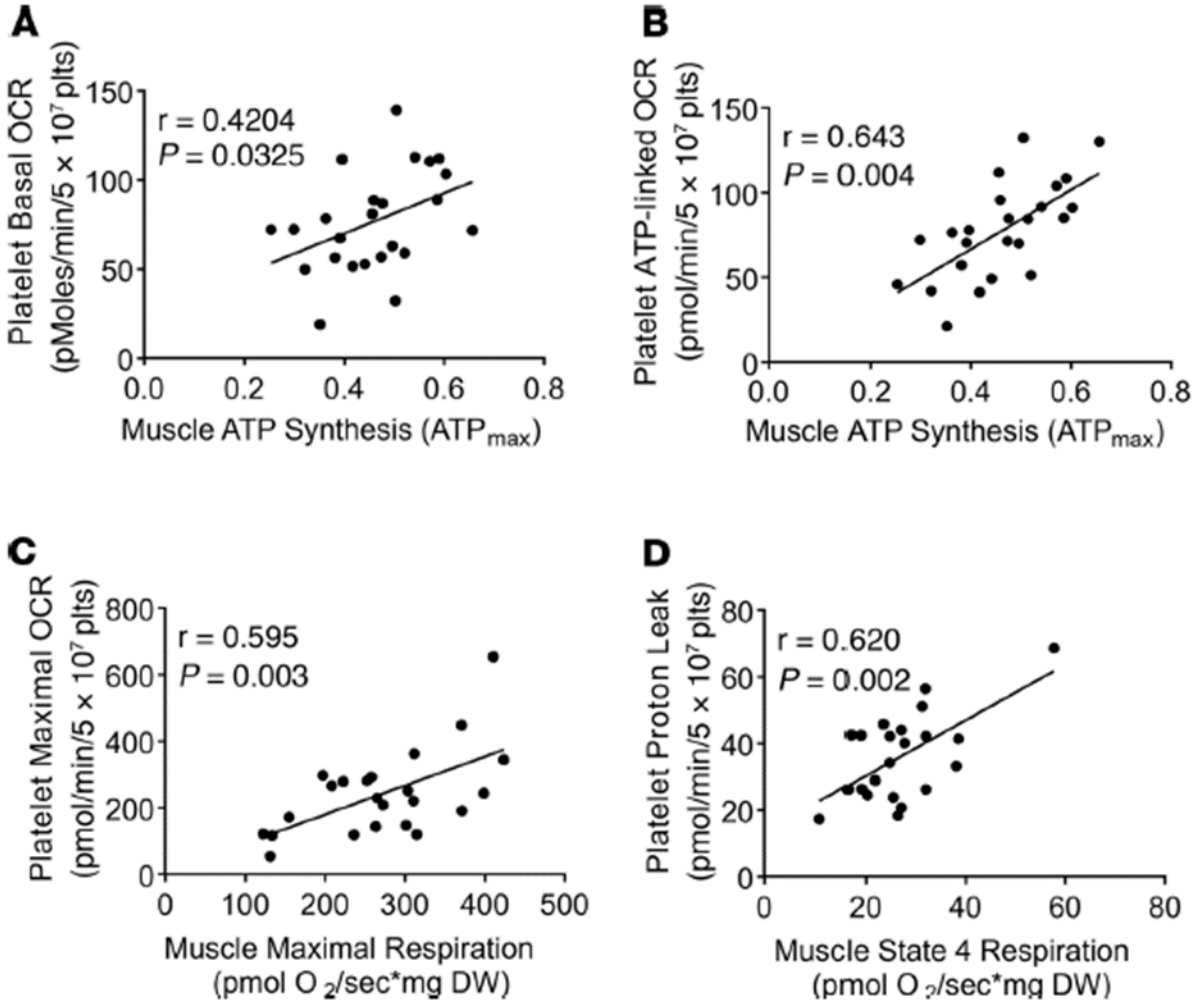
- Painful and Invasive.
- Time-intensive.
- Complex procedure and sample preparation.
- Used to support diagnosis of mitochondrial disease.
- Mostly used in clinical research.

# Blood-Based Bioenergetics



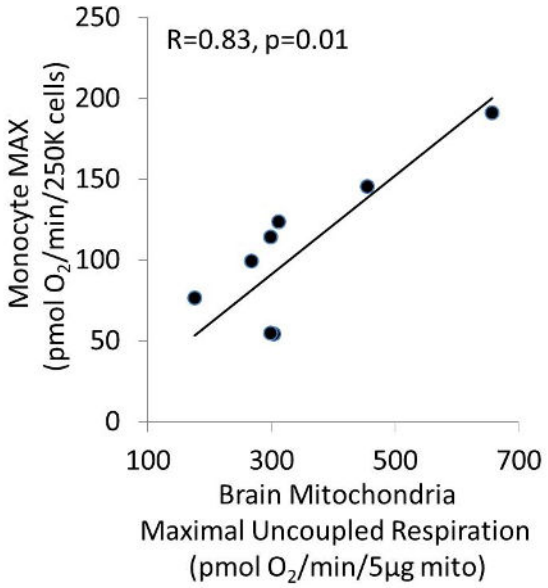

- Minimally invasive. Only takes a single tube of blood.
- Can be sampled routinely.
- Suitable for a wide array of patient populations.
- Reflect physiological and pathological changes throughout the body.
- Monocytes, Platelets, and Lymphocytes contain functional mitochondria.

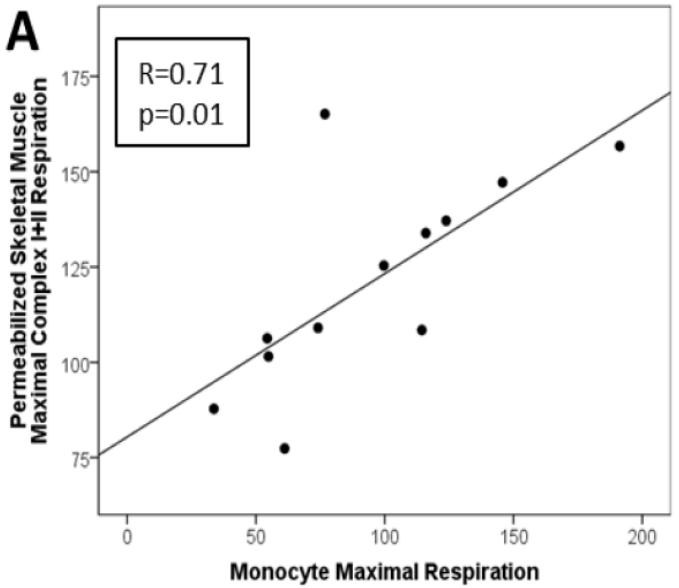
# Blood Cell Respiration Vs. Muscle Respirometry in Humans

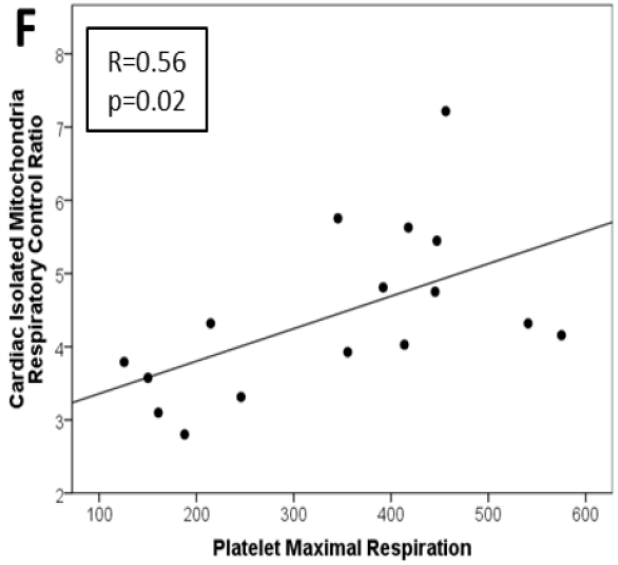



*Braganza et al. Platelet bioenergetics correlate with muscle energetics and are altered in older adults. JCI Insight. 2019 Jul 11; 4(13): e128248.*

# Blood Cell Respiration Recapitulates Brain, Heart, and Skeletal Muscle Respiration in Primates




DJ Tyrrell et al. *Redox Biol* 2016
DJ Tyrrell et al. *Oxid Med Cell Longev* 2017

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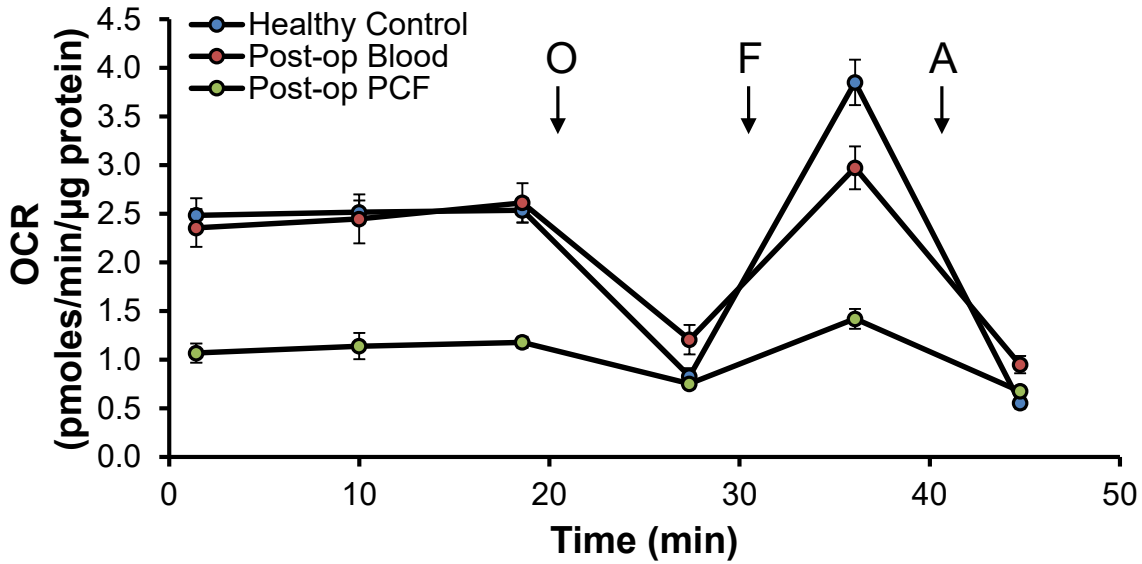
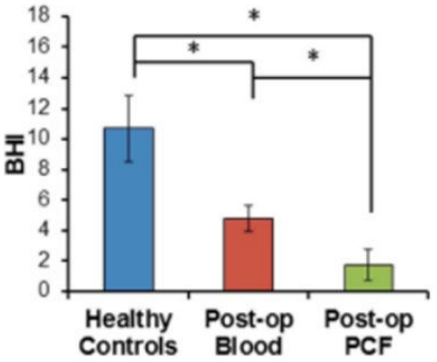
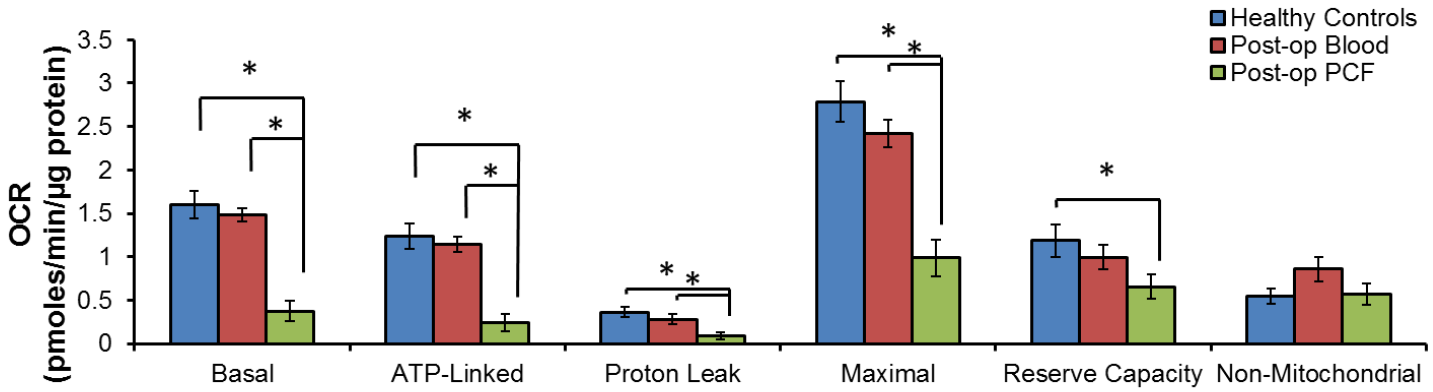
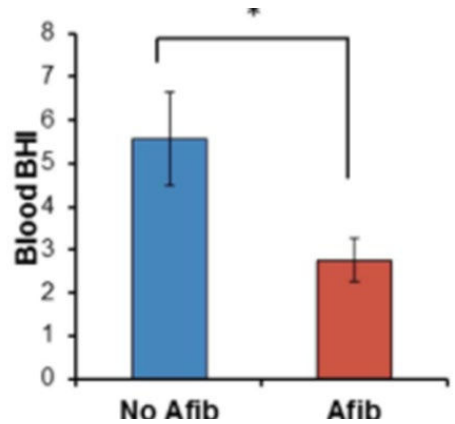
# Blood and Pericardial Fluid Monocyte Bioenergetics After Cardiac Surgery



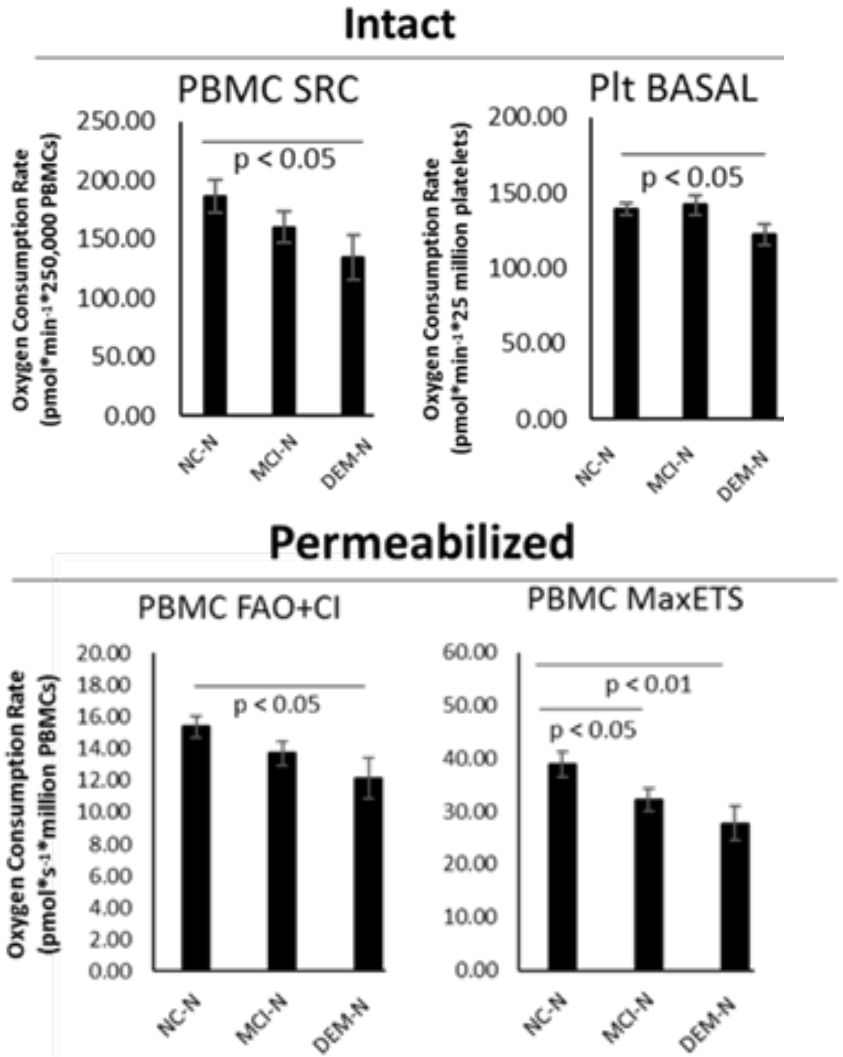




*Kramer et al. Biosci Rep. 2015*

# Brain Morphology and Cognition Reflect PBMC and Platelet Bioenergetic function

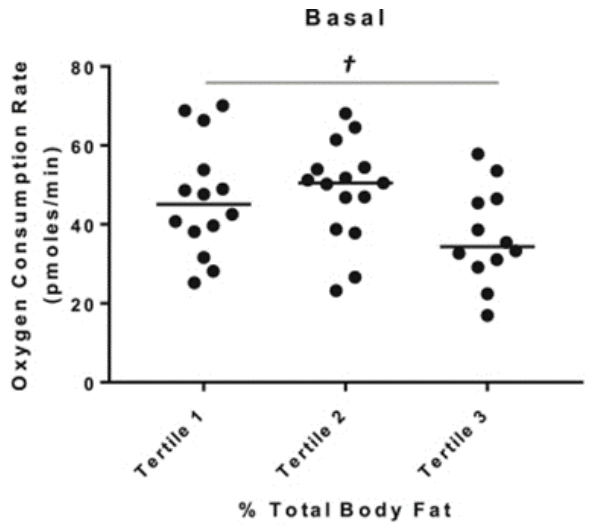
| Respirometry Parameters | TGM | TWM | TICV |
|---|---|---|---|
| Basal Respiration | R = 0.338 | **R = 0.666** | **R = 0.588** |
| | p = 0.218 | **p = 0.007** | **p = 0.021** |
| Maximal Respiration | R = 0.375 | **R = 0.547** | **R = 0.550** |
| | p = 0.169 | **p = 0.035** | **p = 0.034** |
| Spare Respiratory Capacity | R = 0.367 | R = 0.408 | R = 0.477 |
| | p = 0.178 | p = 0.131 | p = 0.072 |
| ATP-linked Respiration | R = 0.253 | **R = 0.563** | R = 0.490 |
| | p = 0.364 | **p = 0.029** | p = 0.064 |
| FAO | R = 0.477 | **R = 0.591** | **R = 0.684** |
| | p = 0.062 | **p = 0.016** | **p = 0.003** |
| FAO+ComplexI | R = 0.467 | **R = 0.519** | **R = 0.564** |
| | p = 0.068 | **p = 0.040** | **p = 0.023** |
| FAO+Compl exI+ComplexII | R = 0.375 | **R = 0.502** | **R = 0.528** |
| | p = 0.152 | **p = 0.047** | **p = 0.035** |
| Max ETS | R = 0.349 | **R = 0.503** | **R = 0.503** |
| | p = 0.199 | **p = 0.047** | **p = 0.047** |

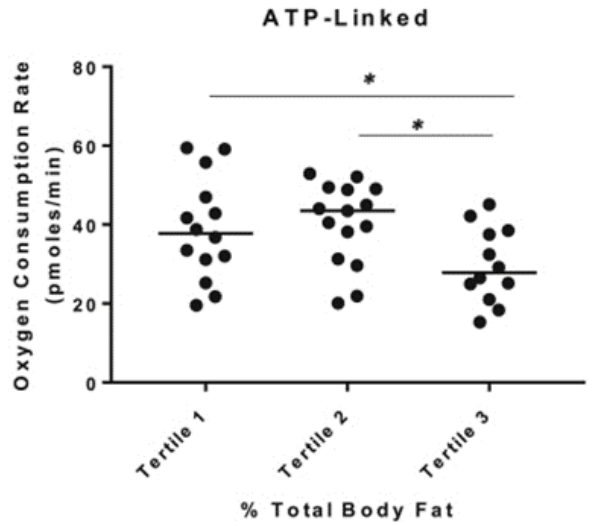


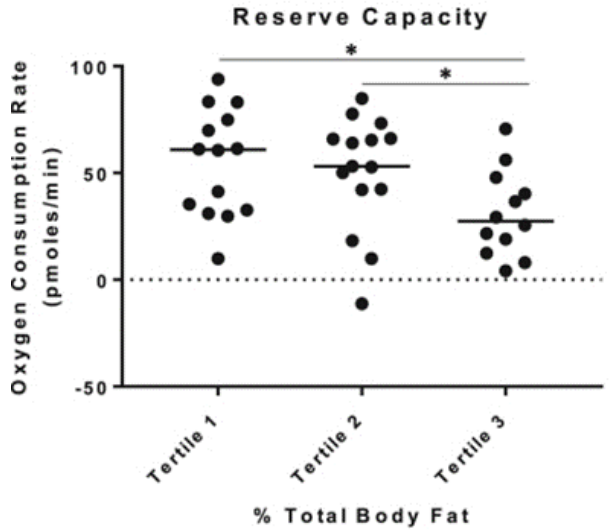

*Mahapatra et al. Clin Sci (Lond). 2018 December 12; 132(23): 2509–2518.*

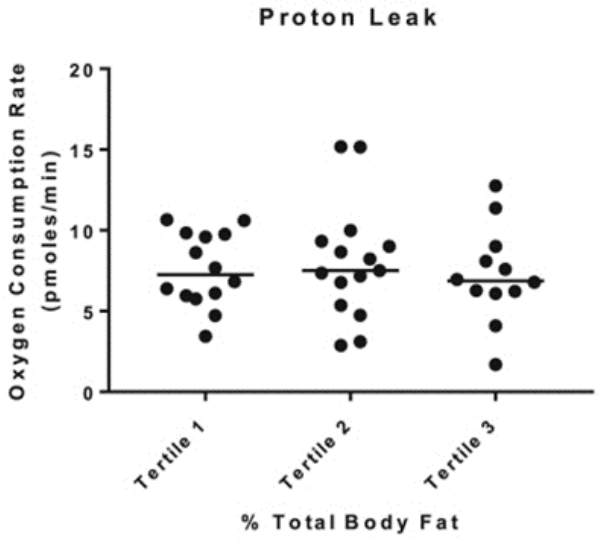
*Mahapatra et al. Unpublished. In Review.*

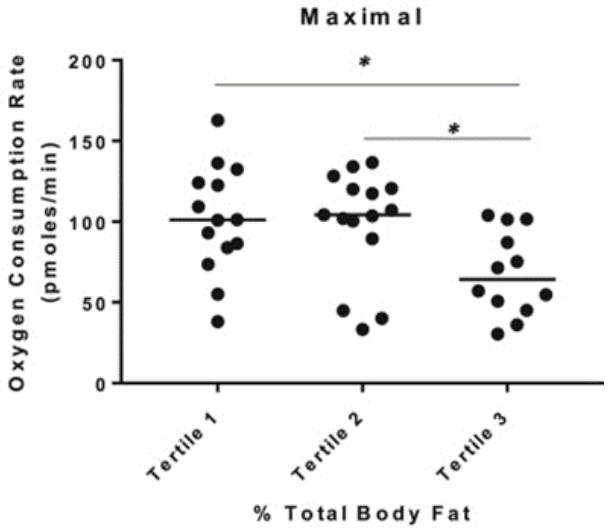
# Blood Bioenergetics Associated with Body Fat Content



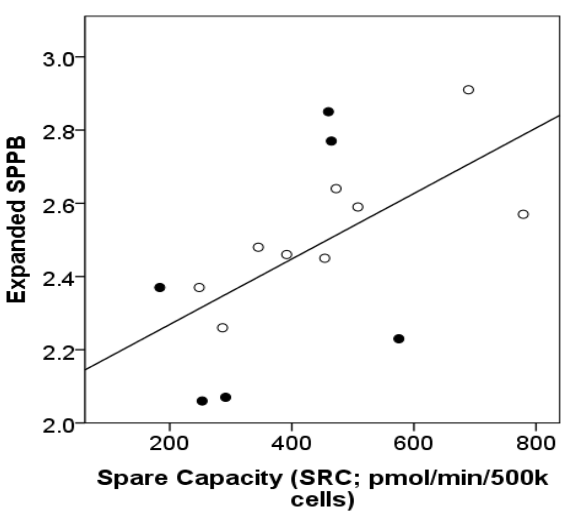



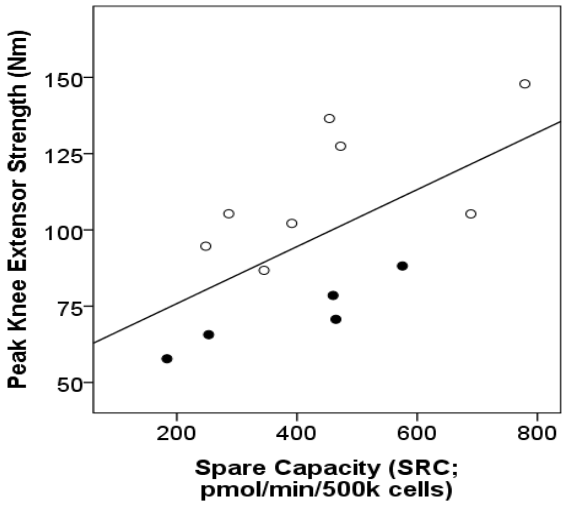



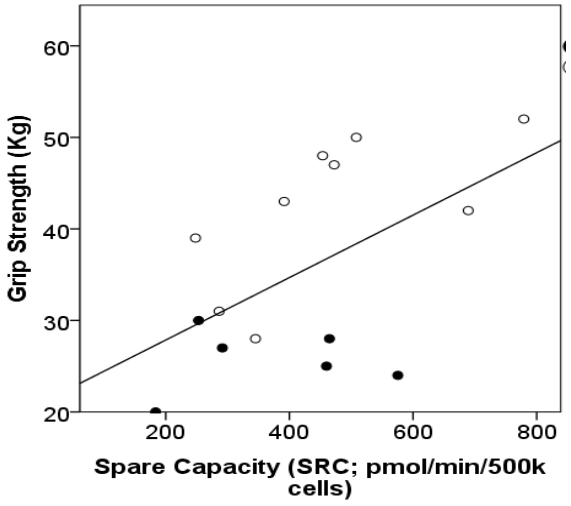



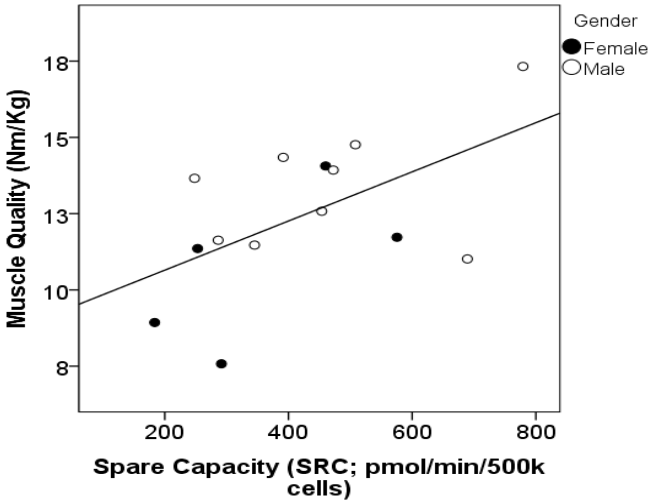

*Willig et al. Redox Biology Volume 12, August 2017, Pages 648-656*

# Muscle Strength and Quality Associated with Better Blood Bioenergetics



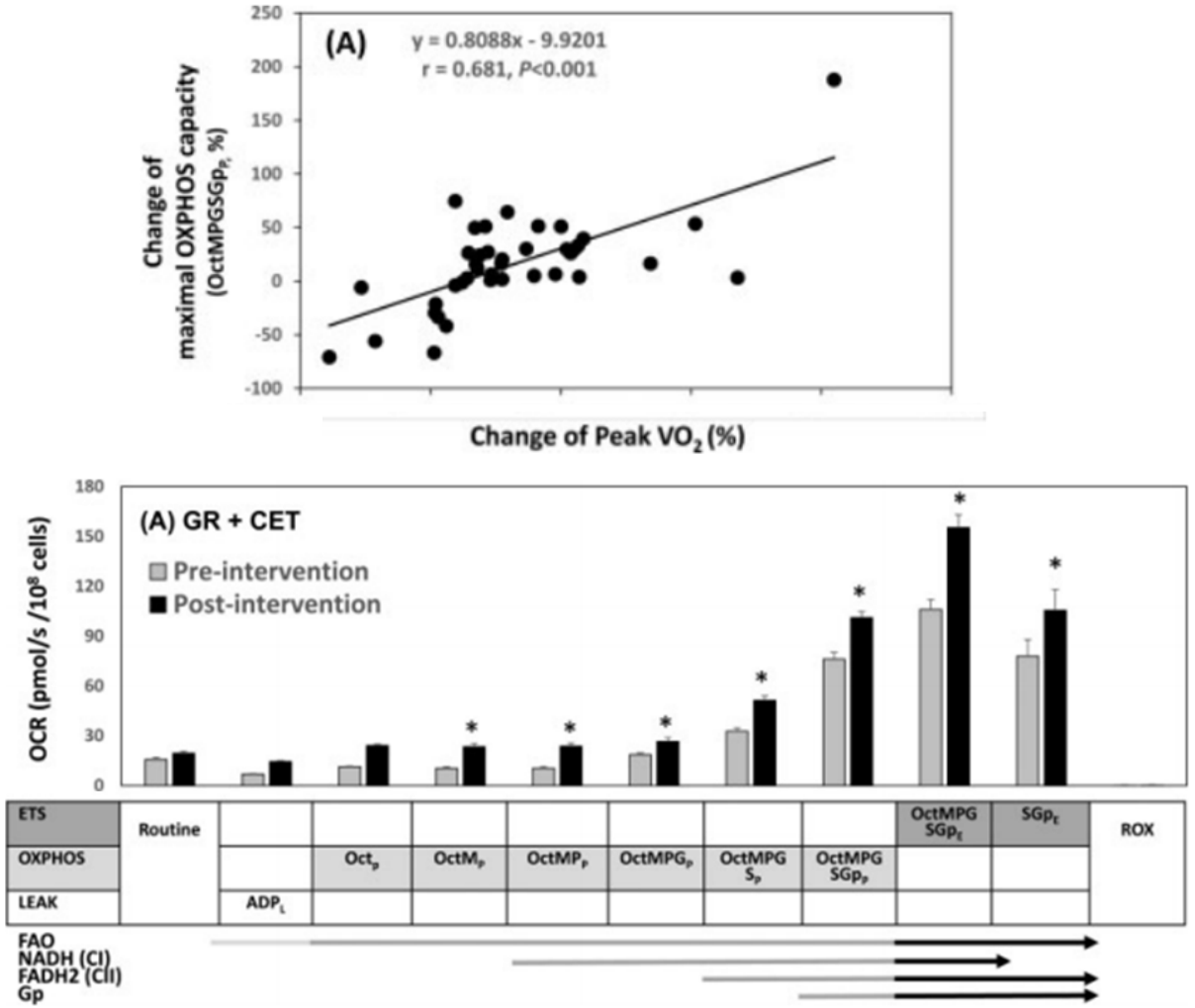



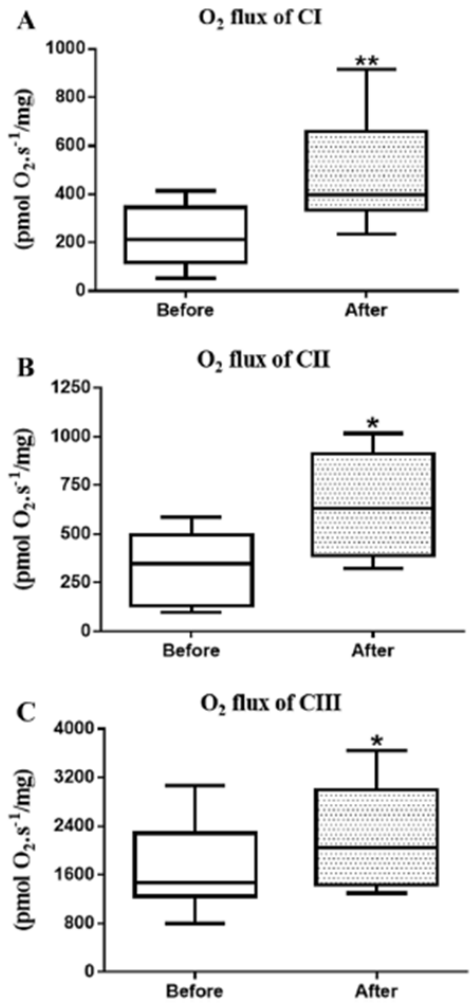




*Tyrrell et al. 2015*

# Exercise Improves Mitochondrial Function of Blood cells

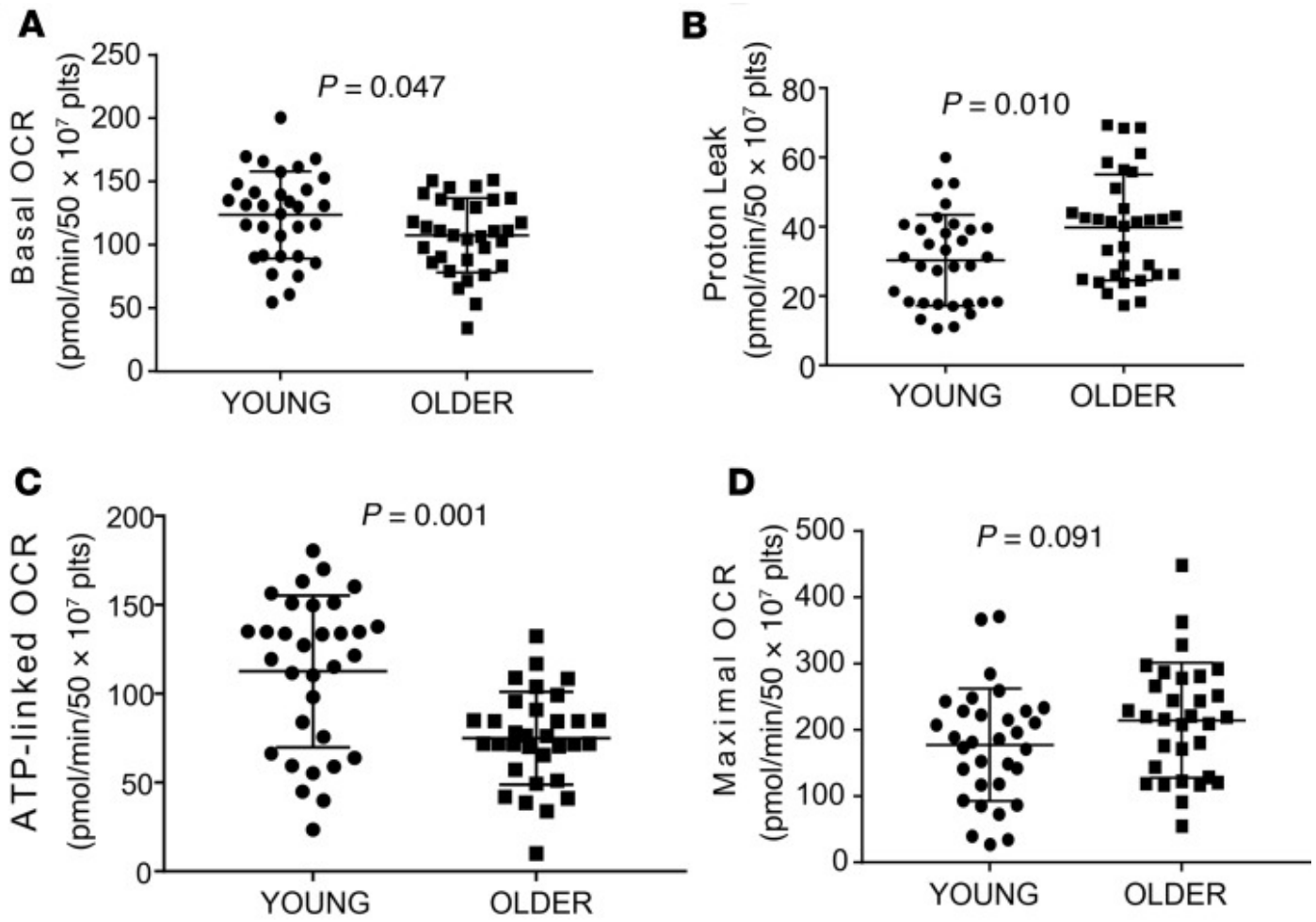



*Exercise Effect on Platelet Mitochondrial Function in PAD Patients. Lin et al. Cellular Haemostasis and Platelets*

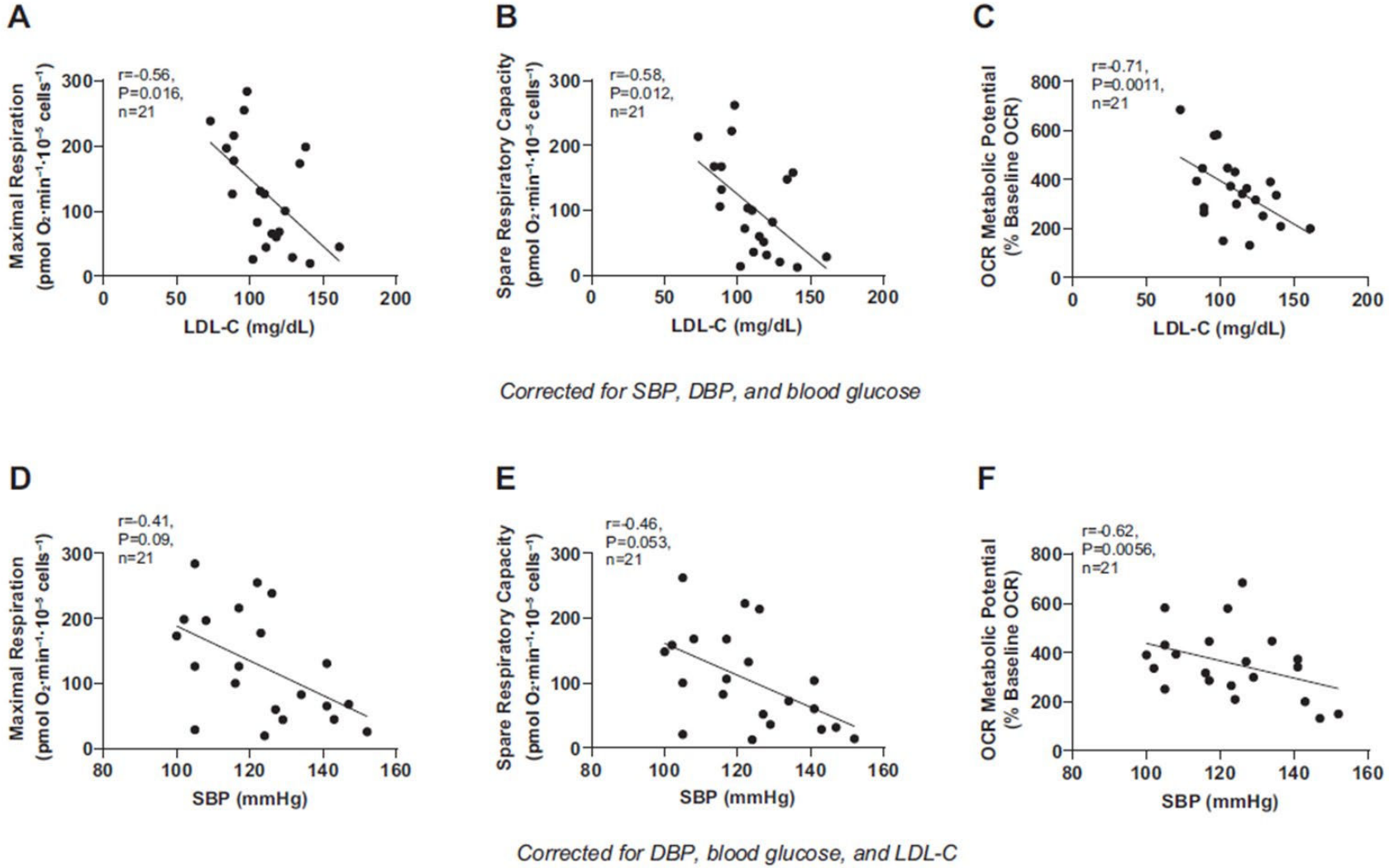
*Rosa et al. Moderate-intensity functional training improves mitochondrial capability and redox state in peripheral blood mononuclear cells of metabolic syndrome women. Sport Sciences for Health*

# Platelet Bioenergetics affected by Age



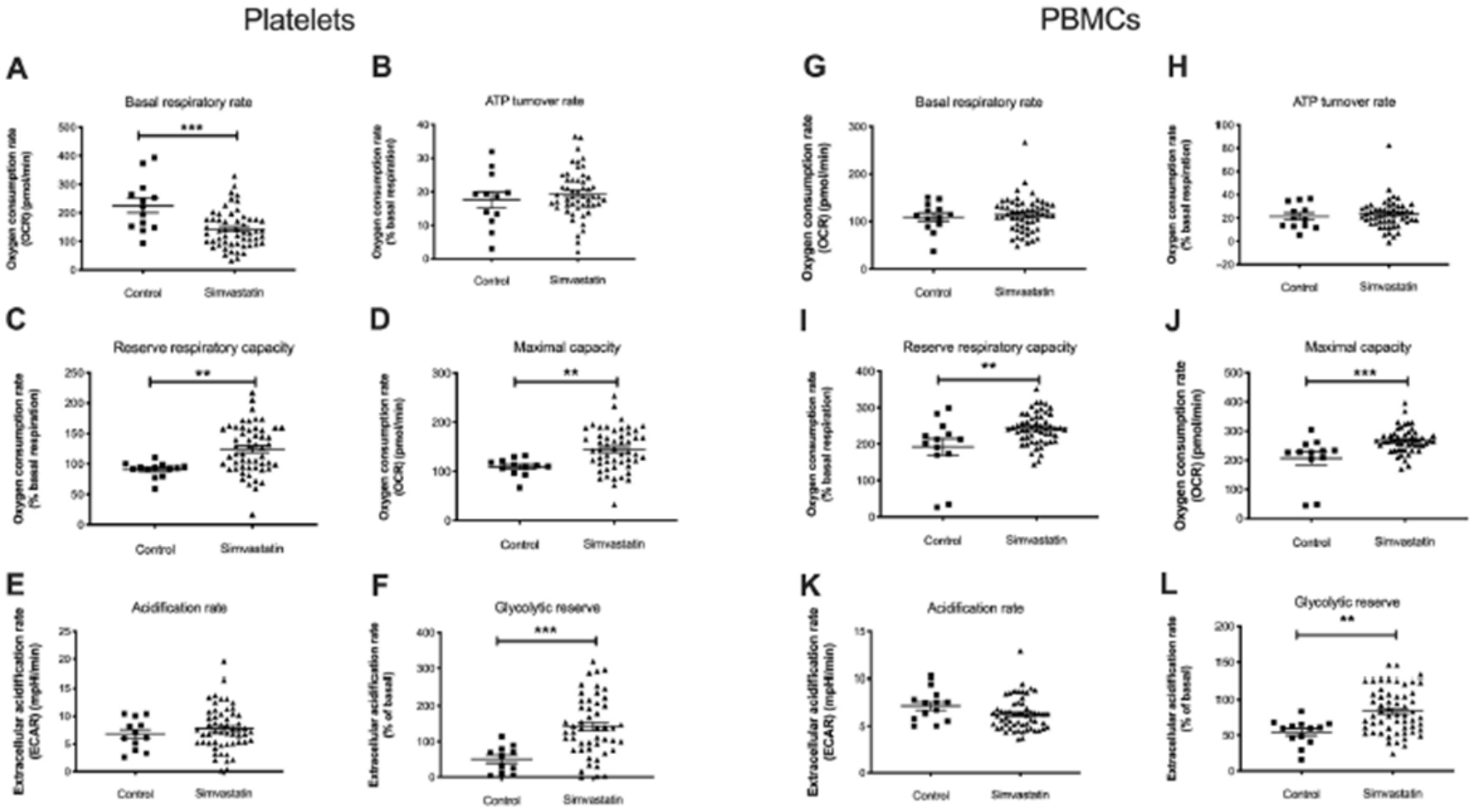

*Braganza et al. Platelet bioenergetics correlate with muscle energetics and are altered in older adults. JCI Insight. 2019 Jul 11; 4(13): e128248.*

# Blood-based Bioenergetics affected by cardiometabolic Factors



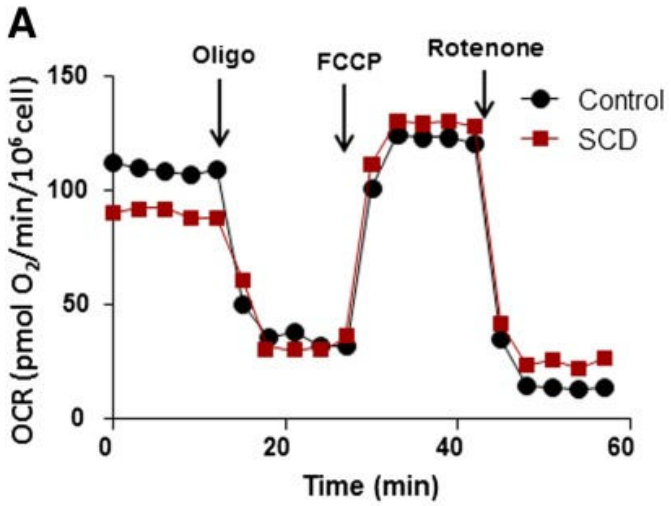

*DeConne et al. Cardiometabolic risk factors are associated with immune cell mitochondrial respiration in humans. Am J Physiol Heart Circ Physiol 319: H481–H487, 2020.*

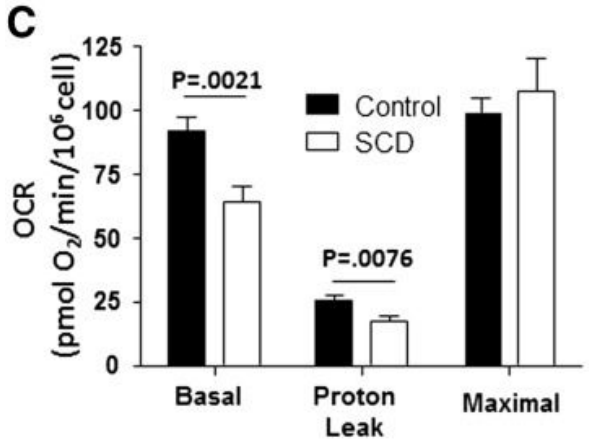
# Blood-based Bioenergetics improved by Statins




*Durhuus et al. Simvastatin improves mitochondrial respiration in peripheral blood cells. Scientifc Reports (2020) 10:17012*

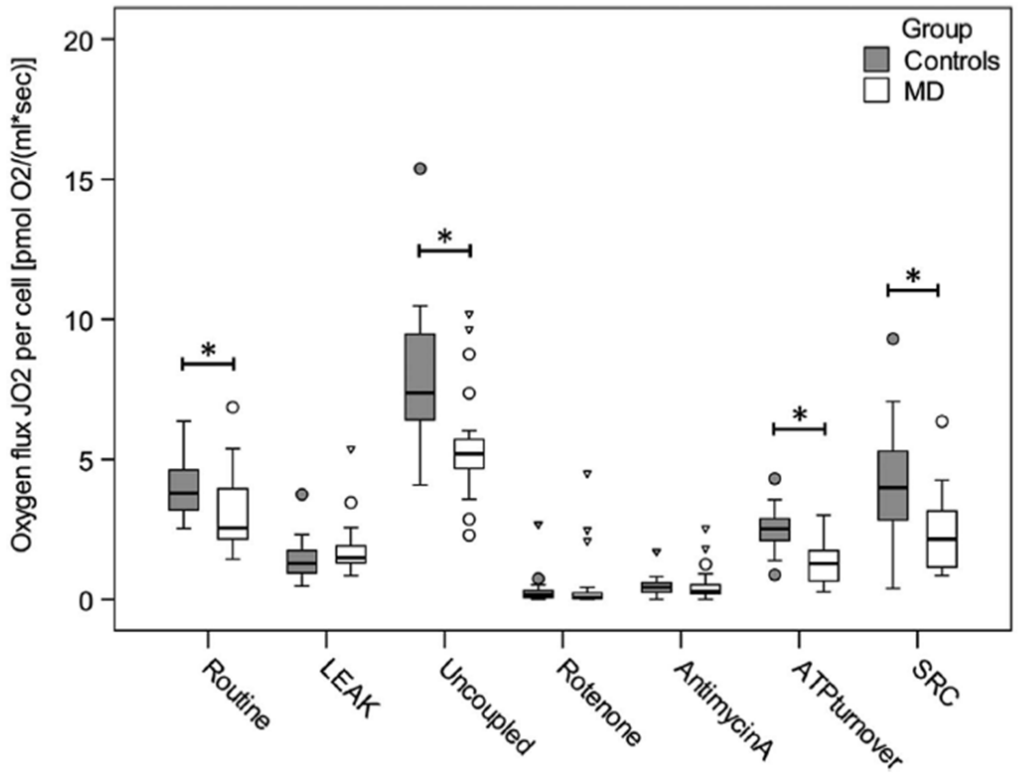
# SCD Increases Platelet Proton Leak and Decreases Basal Respiration through Complex V inhibition



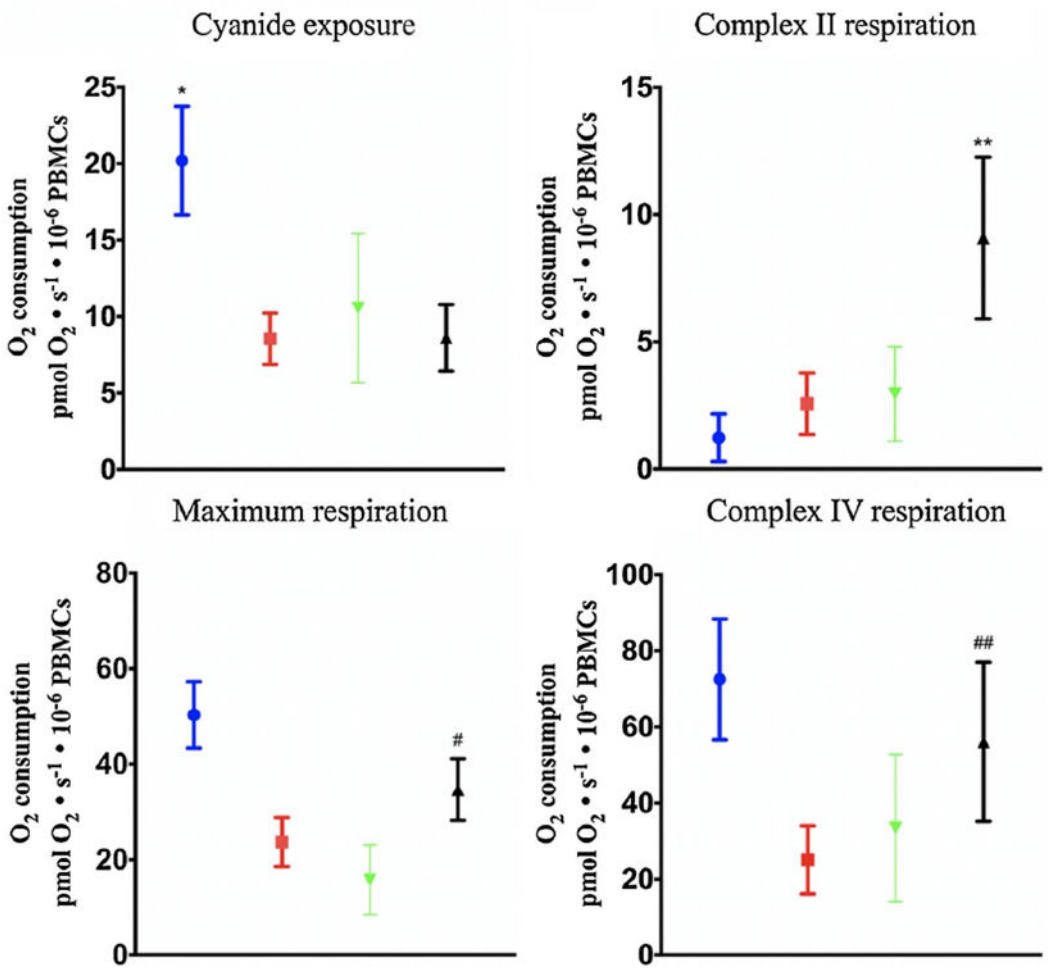

*Cardenes et al. Platelet bioenergetic screen in sickle cell patients reveals mitochondrial complex V inhibition, which contributes to platelet activation. Blood. 2014 May 1; 123(18): 2864–2872.*

# Major Depressive Disorder Associated with Global Decrease in PBMC Mitochondrial Function



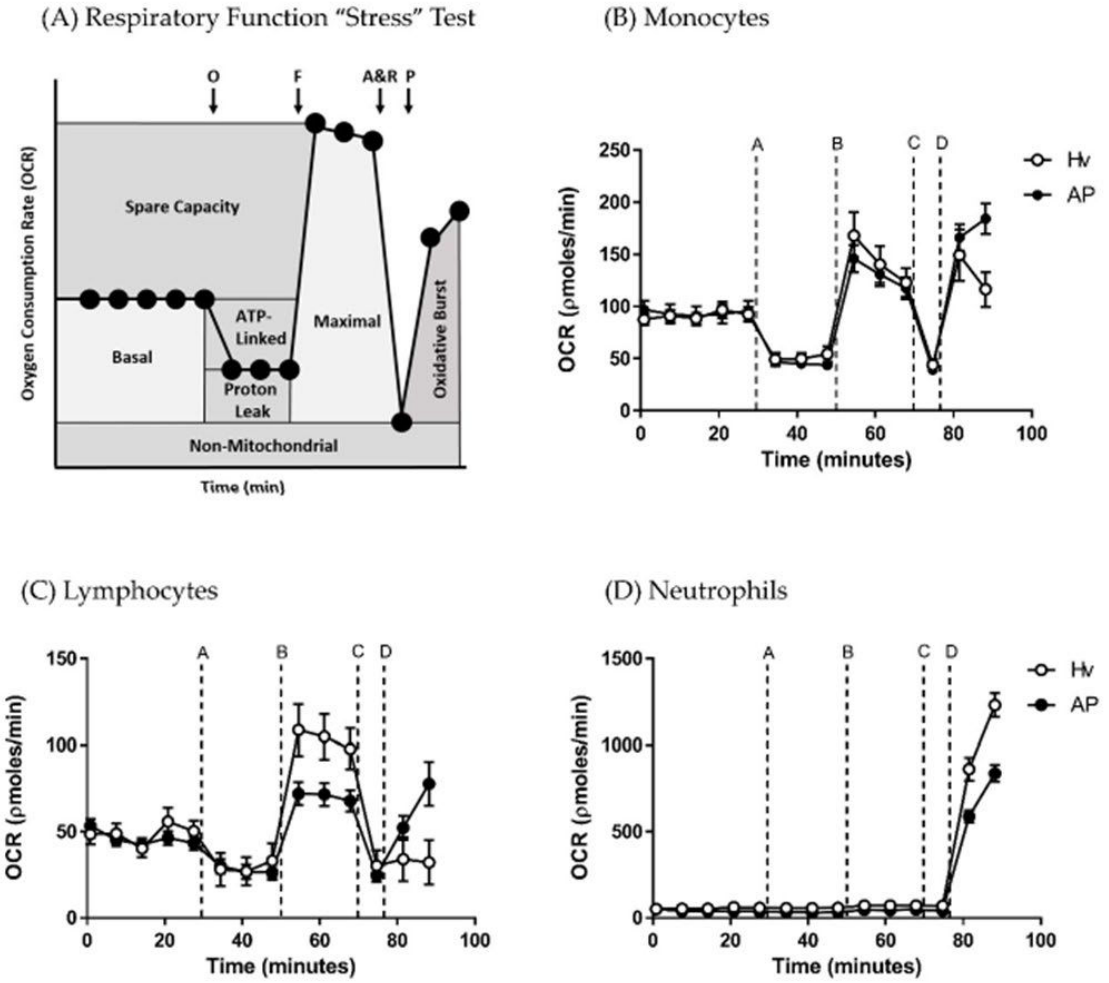

*Karabatsiakis et al. Mitochondrial respiration in peripheral blood mononuclear cells correlates with depressive subsymptoms and severity of major depression Transl Psychiatry (2014) 4, e397*

# Respiration is Impaired in Cyanide poisoning but partly restored with Succinate pro-drug



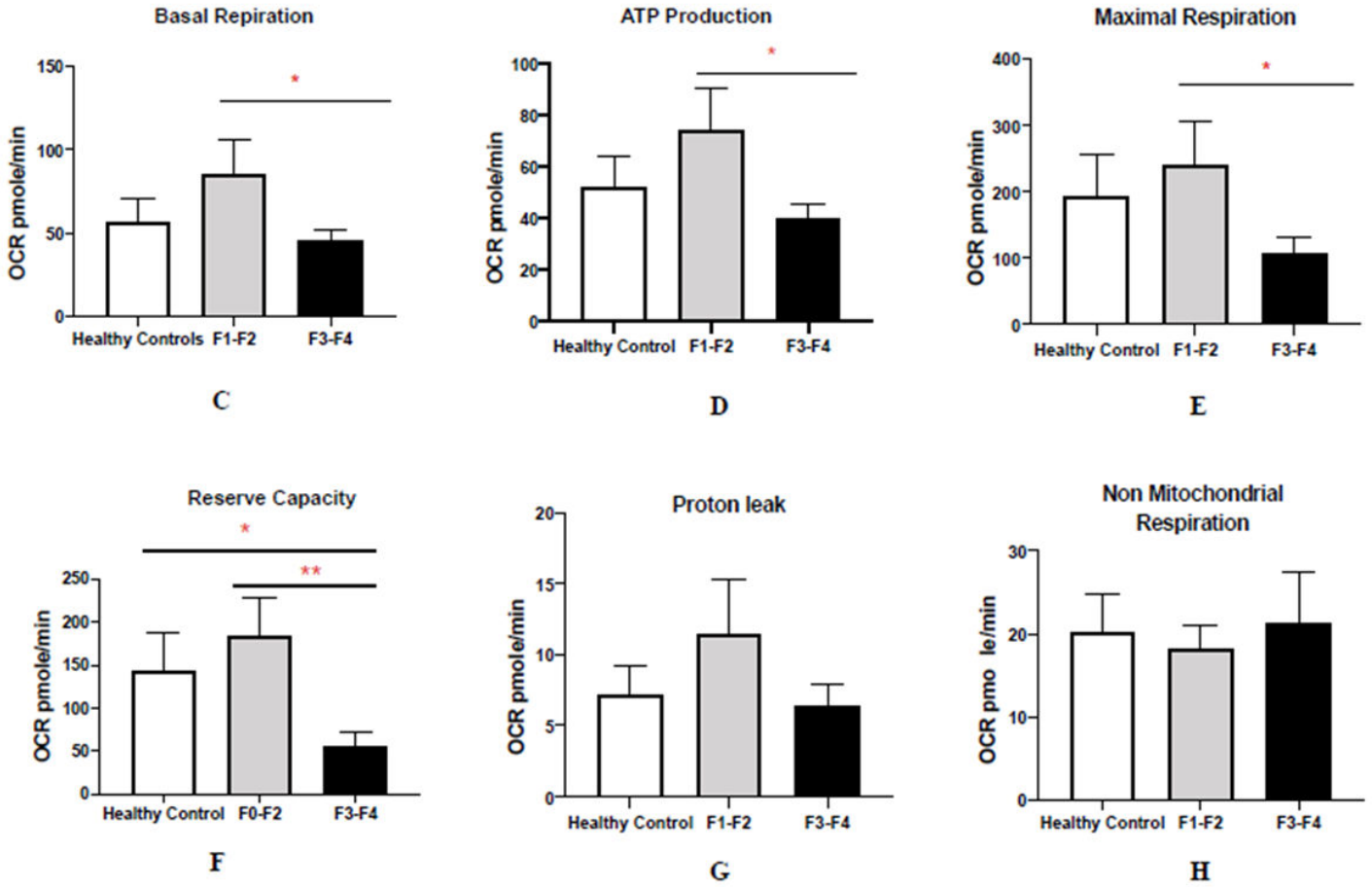

*Owiredu et al. In vitro comparison of hydroxocobalamin (B12a) and the mitochondrial directed therapy by a succinate prodrug in a cellular model of cyanide poisoning. Toxicology Reports 7 (2020) 1263–1271.*

# Leukocyte Mitochondrial and Oxidative Burst Dysfunction in Patients with Acute Pancreatitis



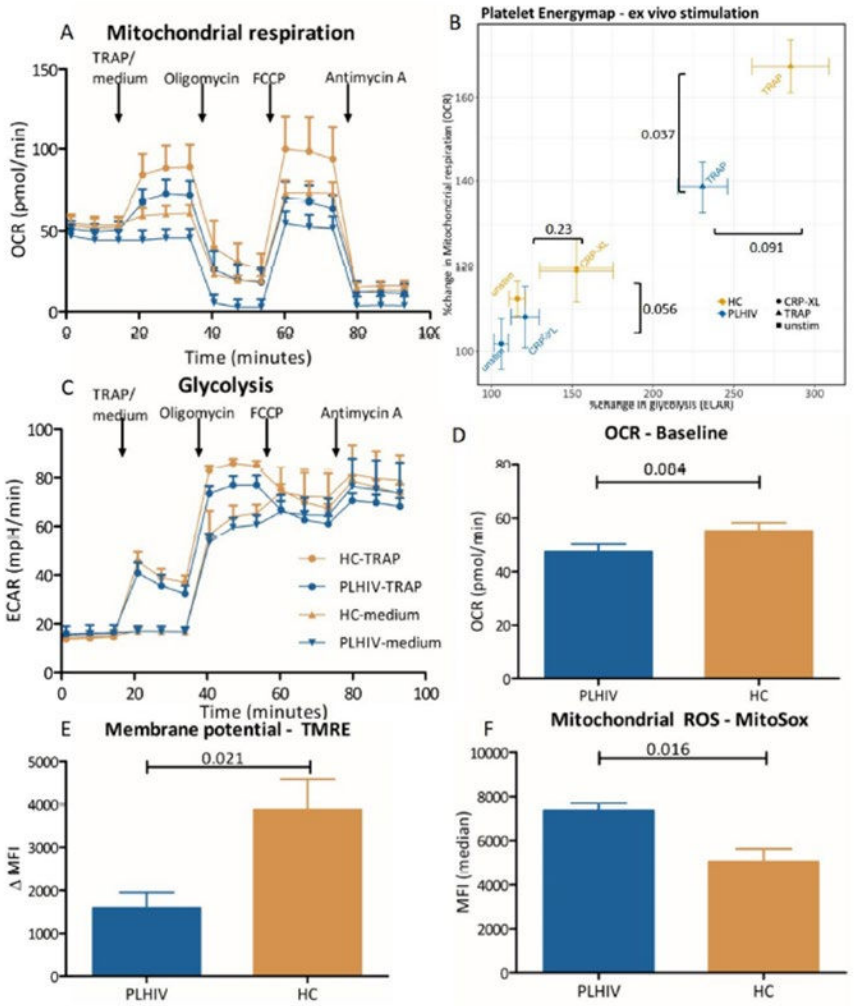

*Morton et al. Altered Bioenergetics of Blood Cell Sub-Populations in Acute Pancreatitis Patients. J Clin Med. 2019 Dec 13;8(12):2201.*

# Severe fibrosis of the Liver in NAFLD patients associated with Mitochondrial dysfunction in PBMCs



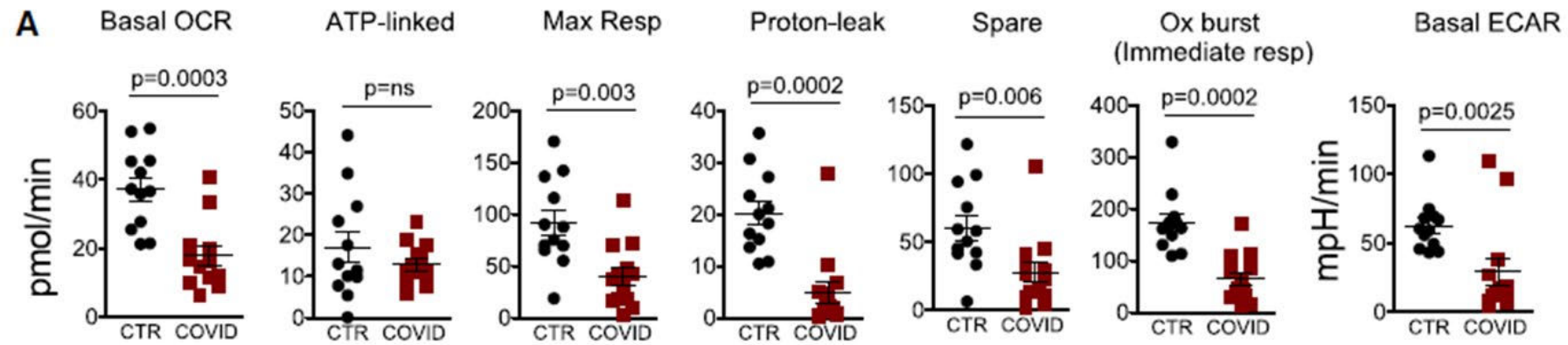

*Ajaz et al. Mitochondrial dysfunction as a mechanistic biomarker in patients with Non-Alcoholic fatty liver disease (NAFLD). Mitochondrion. 2021 Mar;57:119-130120*

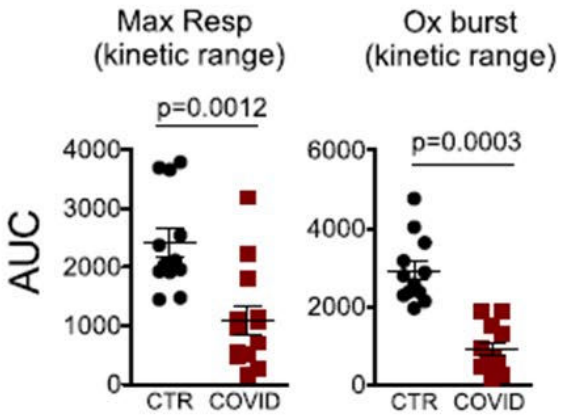
# Long-term treatment of HIV Alters Platelet metabolism Pre and Post Activation

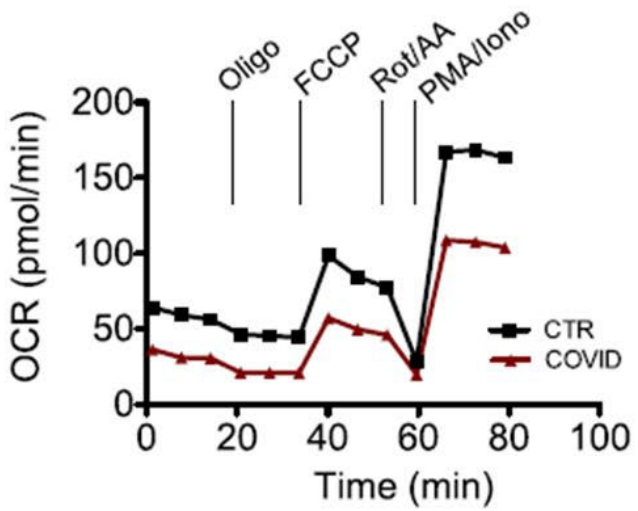


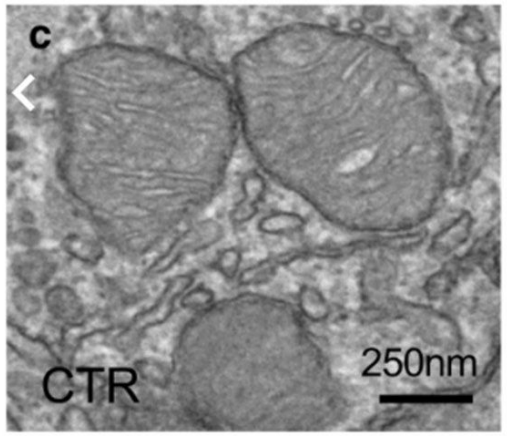

*Heijden et al. Long-term treated HIV infection is associated with platelet mitochondrial dysfunction.. Scientific Reports | (2021) 11:6246*

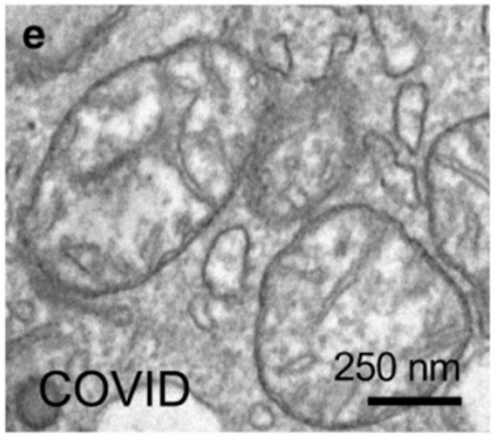
# Monocytes in COVID-19 + Patients have Impaired Oxidative Burst and Significant Mitochondrial Dysfunction




*Gibellini et al. Altered bioenergetics and mitochondrial dysfunction of monocytes in patients with COVID-19 pneumonia. EMBO Mol Med (2020)12:e13001*

# Blood-based Bioenergetics can Aid in the Diagnosis of Genetic Mitochondrial Diseases

| | | | Platelet respiration (pmol $O_2$/s/$10^8$ cells) | | | | | | | | | Routine diagnostics | | | | | | Platelet diagnostics | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Routine respiration | DMP | OXPHOS $CI_{NP}$ | OXPHOS $CI_{NPG}$ | OXPHOS CI+II | LEAK CI+II | ETS CI+CII | ETS CII | CIV activity | Plasma lactate[a] | CSF lactate[a] | OAU[a] | MR[a] | Muscle biopsy[b] | Genetics[b] | OXPHOS | $CI_{NP}$/DMP | Log(OXPHOS CI+II/Routine) | ΔADP/ΔSucc. low | ΔADP/ΔSucc. high |
| Cases with confirmed mitochondrial disease | 1 | Leigh syndrome | 17.4 | 16.2 | 16.4 | 12.0 | 45.5 | 13.2 | 36.2 | 20.2 | - | | | | | | | | | | | |
| | 2 | MELAS | 17.5 | 6.85 | 8.25 | 8.39 | 21.5 | 6.78 | 17.5 | 10.9 | - | | | | | | | | | | | |
| | 3 | Alpers syndrome | 11.1 | 5.53 | 15.9 | 16.6 | 24.3 | 4.21 | 18.5 | 10.9 | - | | | | | | | | | | | |
| | 4 | PDH deficiency | 6.50 | 4.13 | 12.8 | 14.4 | 22.6 | 3.10 | 18.0 | 9.81 | - | | | | | | | | | | | |
| | 5 | Mitochondrial encephalopathy | 25.9 | 19.6 | 30.6 | 31.0 | 41.0 | 6.70 | 42.7 | 16.8 | - | | | | | | | | | | | |
| | 6 | PDH deficiency | 10.3 | 7.76 | 17.0 | 18.7 | 30.3 | 4.91 | 27.2 | 14.9 | - | | | | | | | | | | | |
| | 7 | Alpers-Huttenloher syndrome | 13.2 | 6.67 | 10.7 | 11.4 | 18.7 | 6.37 | 14.1 | 5.89 | - | | | | | | | | | | | |
| | 8 | Mitochondrial encephalopathy | 15.4 | 11.0 | 22.4 | 22.5 | 30.2 | 4.48 | 34.5 | 9.60 | - | | | | | | | | | | | |
| | 9 | CPEO and myopathy | 9.13 | 5.90 | 19.3 | 20.4 | 30.0 | 3.49 | 32.6 | 12.2 | - | | | | | | | | | | | |
| | 10 | Mitochondrial depletion syndrome | 6.92 | 4.10 | 12.9 | 15.3 | 23.7 | 3.60 | 24.5 | 11.1 | - | | | | | | | | | | | |
| | 11 | Mitochondrial hepatopathy | 7.57 | 5.92 | 14.3 | 14.5 | 26.8 | 5.26 | 28.1 | 16.6 | 42.5 | | | | | | | | | | | |
| | 12 | Kearns-Sayre syndrome | 3.89 | 5.07 | 11.3 | 12.6 | 18.8 | 4.02 | 18.3 | 8.85 | 26.6 | | | | | | | | | | | |
| | 13 | LHON plus | 4.92 | 5.87 | 14.6 | 15.6 | 28.0 | 5.36 | 30.8 | 14.9 | 51.6 | | | | | | | | | | | |
| | 14 | Leigh syndrome | 7.65 | 7.26 | 21.2 | 23.6 | 38.3 | 6.64 | 38.7 | 21.3 | 53.4 | | | | | | | | | | | |
| | 15 | PDH deficiency | 6.77 | 3.71 | 8.24 | 7.89 | 22.6 | 4.92 | 20.9 | 16.2 | 37.6 | | | | | | | | | | | |
| | 16 | Leigh syndrome* | 10.1 | 7.42 | 15.7 | 16.3 | 26.0 | 3.89 | 19.7 | 9.06 | 31.8 | | | | | | | | | | | |
| | 17 | Mitochondrial depletion syndrome | 8.05 | 8.73 | 21.8 | 24.2 | 34.4 | 5.41 | 31.4 | 14.3 | 38.3 | | | | | | | | | | | |
| | 18 | Mitochondrial disease** | 6.56 | 4.93 | 13.2 | 14.0 | 35.9 | 8.46 | 40.4 | 26.7 | 60.9 | | | | | | | | | | | |

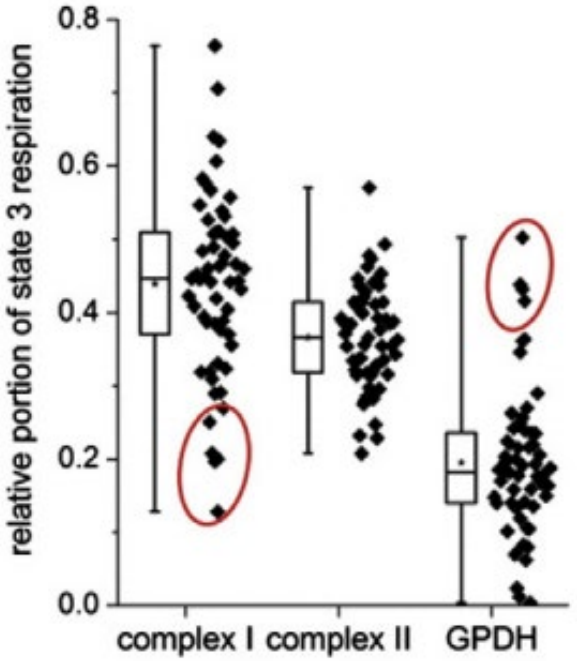
Abbreviations: ADP, adenosine diphosphate; CI, complex I; CPEO, chronic progressive external ophthalmoplegia; CSF, cerebrospinal fluid; DMP, digitonin, malate and pyruvate; ETS, electron transport system; HUPRA, hypertension, renal failure, and alkalosis; LHON, Leber's hereditary optic neuropathy; MD, mitochondrial disease; MELAS, Mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episode; MP, malate and pyruvate; MPG, malate, pyruvate and glutamate; MR, magnetic resonance; OXPHOS, oxidative phosphorylation; PDH, pyruvate dehydrogenase.

(a) green, normal result; red, any pathologic result. (b) green, result not indicating MD; red, pathologic result indicating MD.

*Thiamine transporter-2 deficiency.

**Suspected HUPRA syndrome on genetic basis.

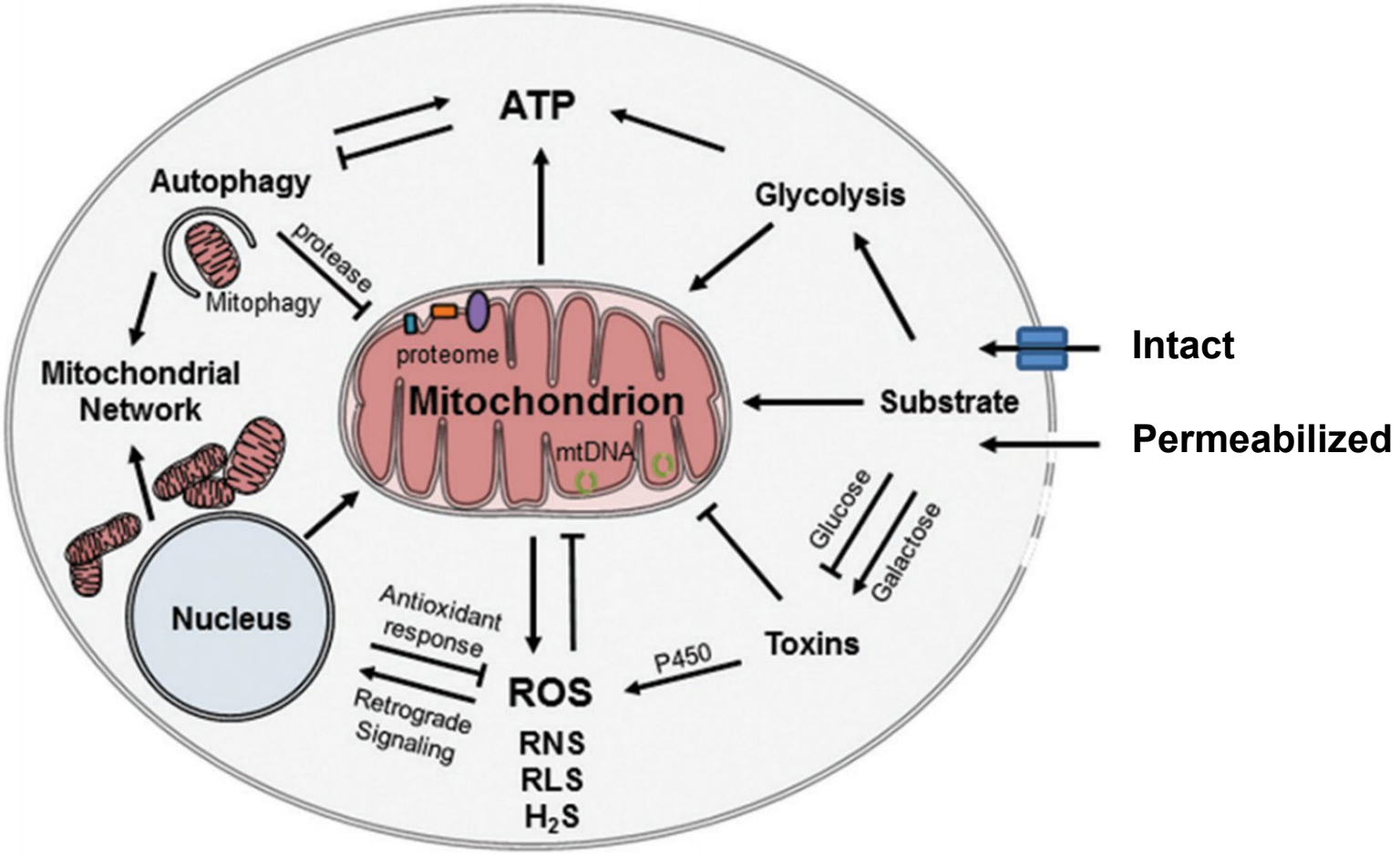
*Westerlund et al. Oxygen consumption in platelets as an adjunct diagnostic method for pediatric mitochondrial disease. Clinical Investigation Volume 83 | Number 2 | February 2018*




*P. Pecina et al. Noninvasive diagnostics of mitochondrial disorders in isolated lymphocytes with high resolution respirometry. BBA Clinical 2 (2014) 62–71*

# The Mitocentric View

*All things affect mitochondria, and mitochondria affect all things.*




*Kramer et al. The emerging theme of redox bioenergetics in health and disease. Biomed J. Jul-Aug 2015;38(4):294-300*

# Outline by FDA and CLIA Requirements

**Clinical Validation:**

- Test purpose
- Specimen type(s)
- Target population(s)
- **Clinical Sensitivity and Specificity**

**Analytical Validation:**

- Precision
- Accuracy
- Analytical sensitivity and specificity
- Reference Range
- Other performance characteristics

# The Problem of Clinical Sensitivity and Specificity

**Definitions:**

- Clinical Sensitivity– The percentage of individuals with the target condition (disease) that will have positive test results.

- Clinical Specificity – The percentage of individuals that do not have the target condition who will have negative test results.

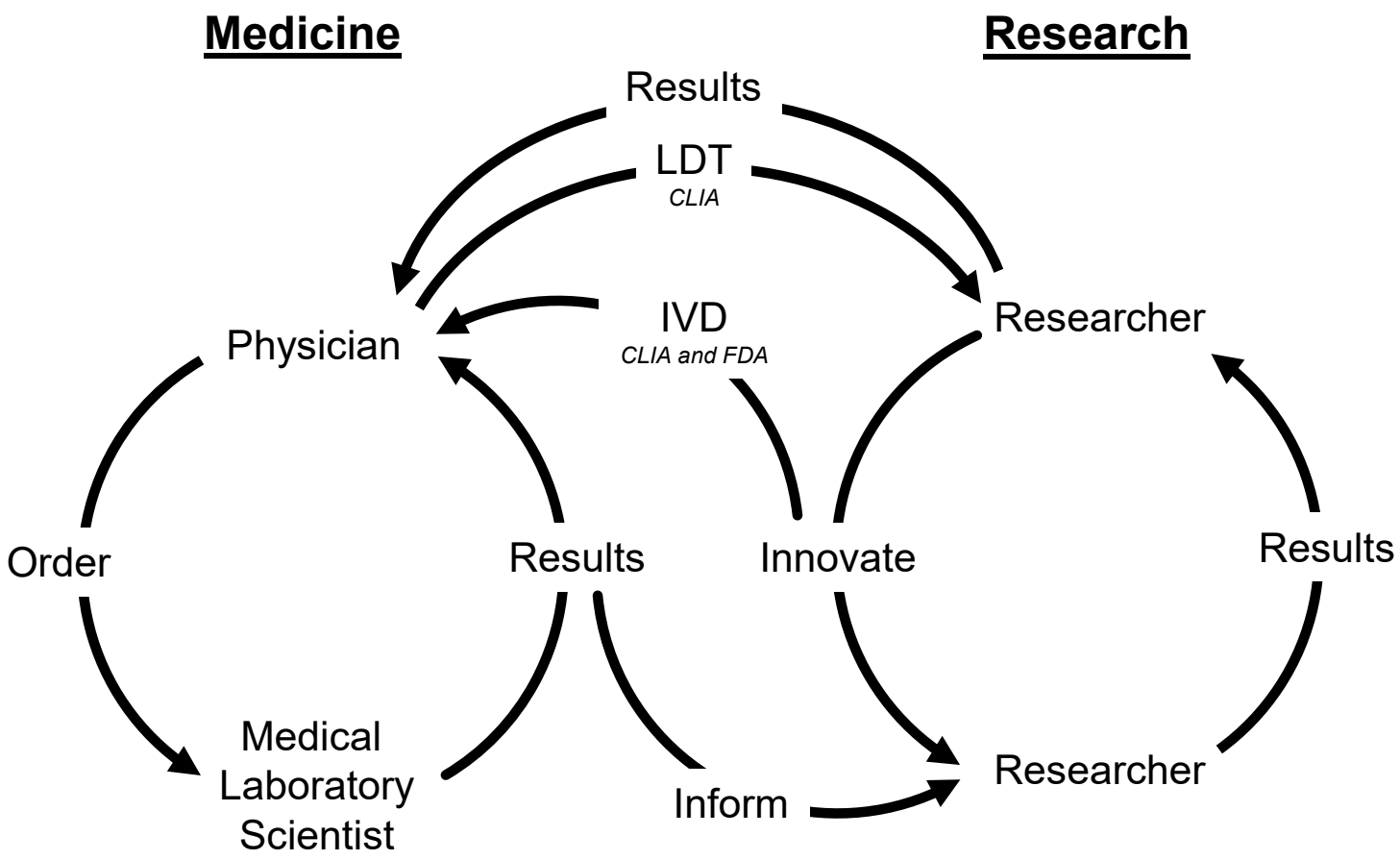
**The Problem:**

- Blood-based bioenergetic assays are sensitive to many genetic and environmental stressors and implicated in many diseases.

- Until distinct patterns of dysfunction can be defined for specific diseases, the test will not be sensitive for any one disease alone.

- May present a problem in FDA's premarket review of an IVD.

# The Laboratory Developed Test



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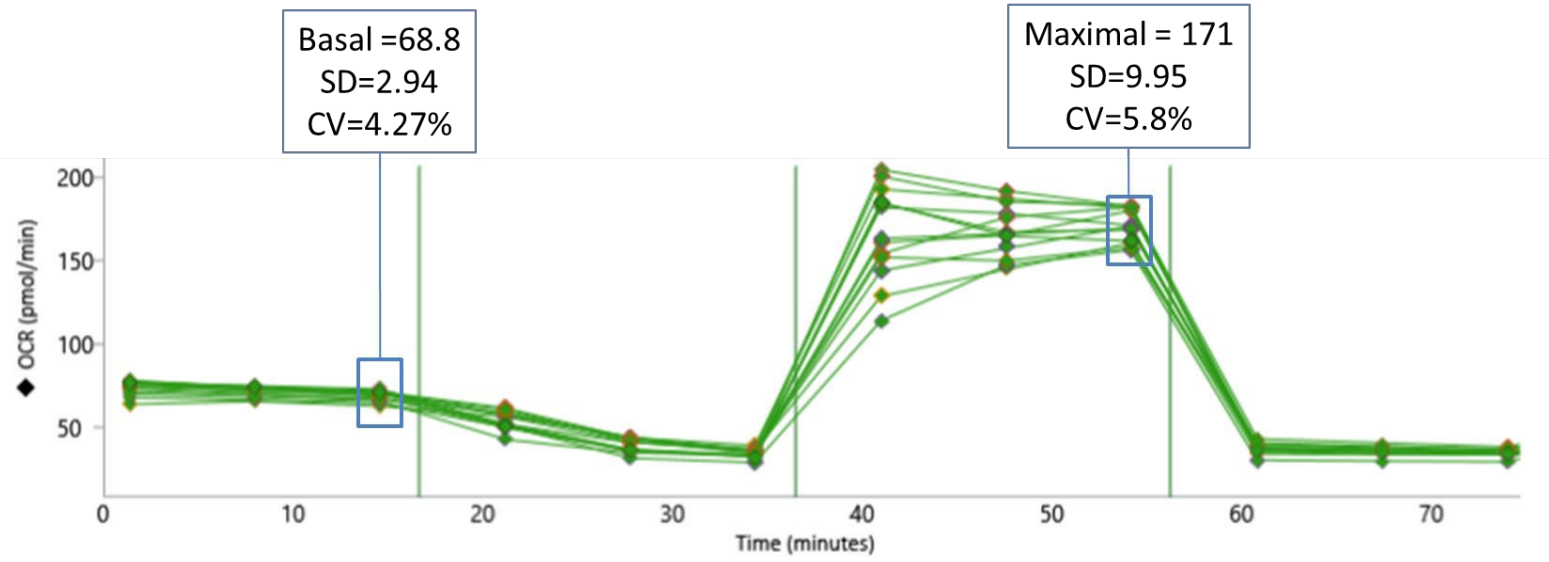
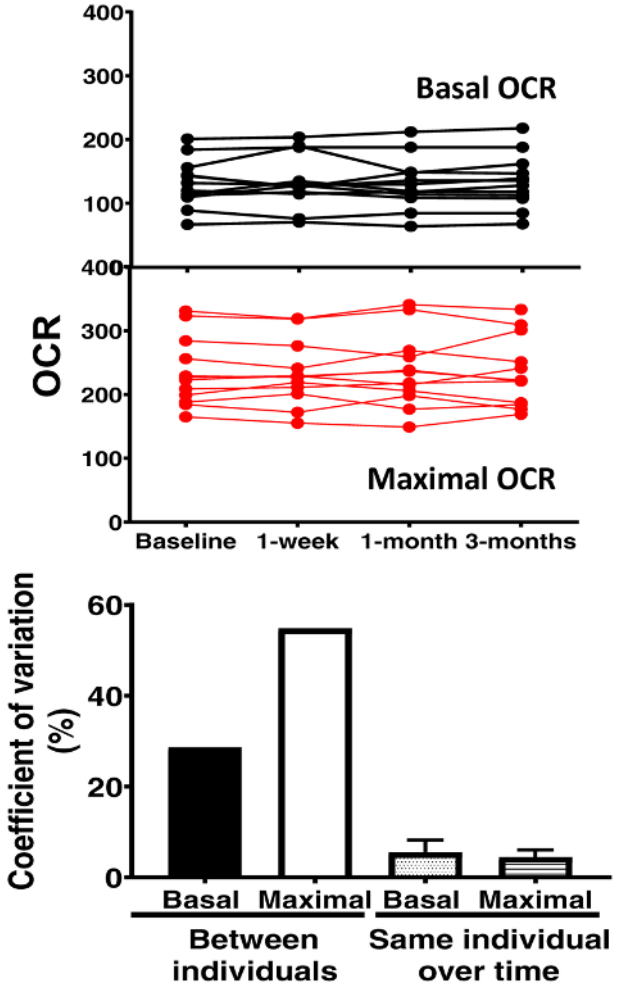
# Precision

- Repeatability among replicates (within-run precision).
- <10% CV will require 3-5 replicates in a Seahorse instrument and duplicate runs in an Oroboros instrument.



# Accuracy

- Conformance to a value, accepted standard, or expected value.
- >20% CV between individuals.
- CV ≈ 7% across 4 time points spanning 3 months for 12 individuals without intervention.
- Ideal for tracking the progression of a disease or condition within an individual or monitoring therapeutic efficacy.




*Braganza et al. Blood-based bioenergetics: An emerging translational and clinical tool. Mol Aspects Med. Epub 2019 Dec 18*

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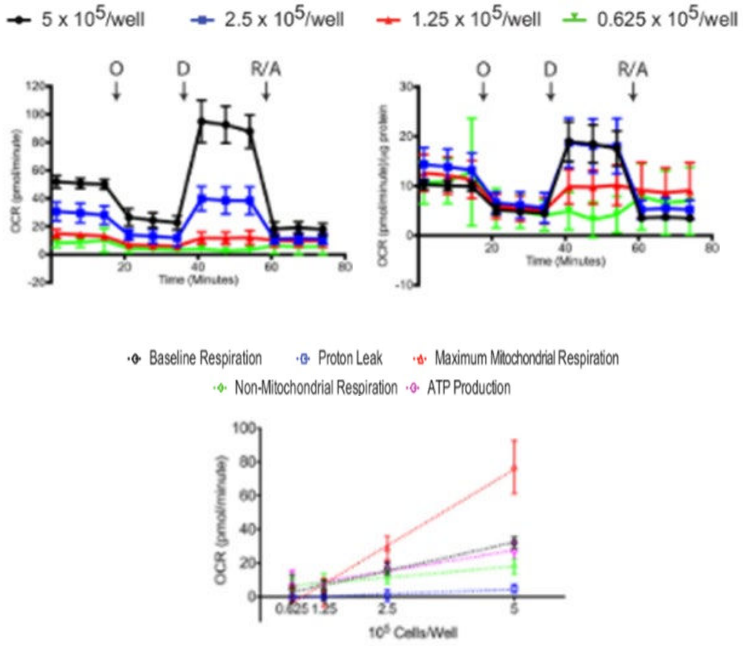
# Analytical Sensitivity

- Also known as the limit of detection, LoD. Analytical sensitivity is the lowest concentration of the analyte which the test can reliably detect above background.
- Helps establish the Reportable Range and target cell number.
- Minimum lymphocyte number per well for reliable readings, taking into account the variation between individuals:

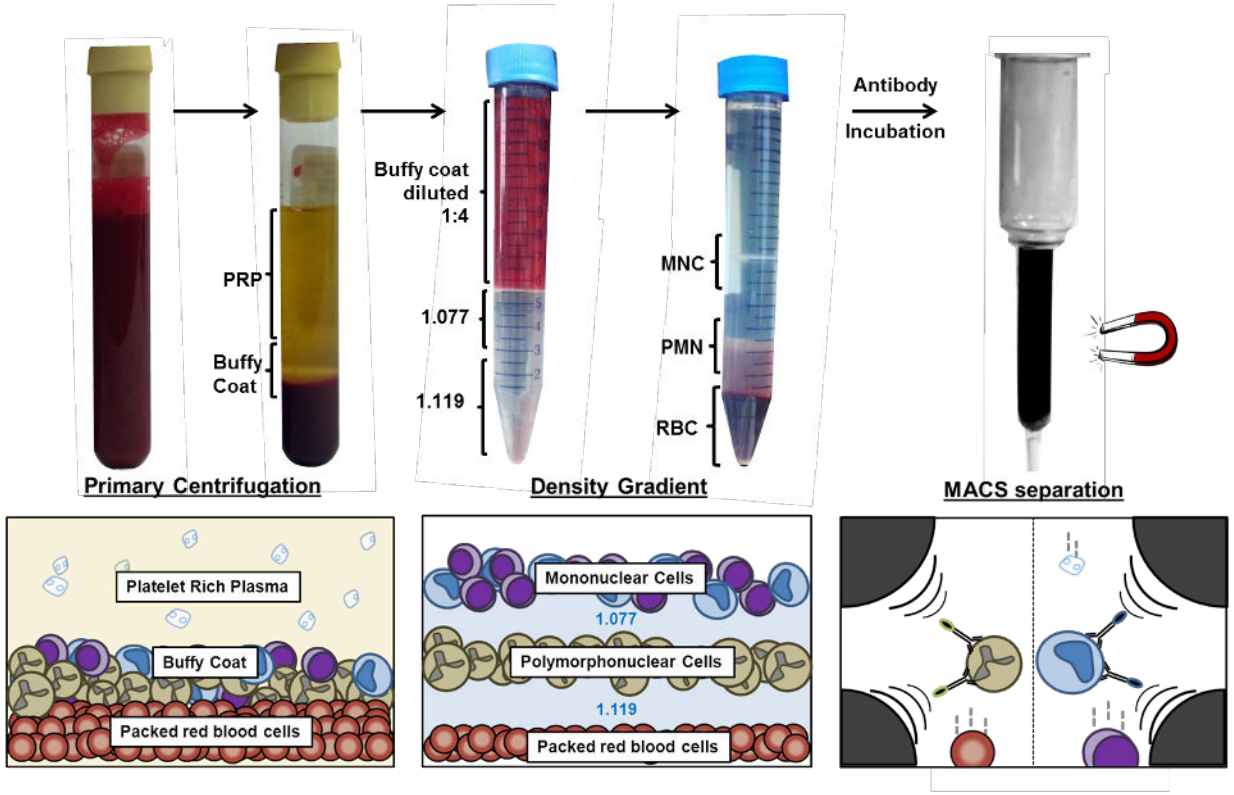
| | |
|---|---|
| XF24 | 2.5x10^5 |
| XF96 | 1.5x10^5 |
| XF HS Mini (8) | 0.5x10^5 |
| O2K | 40x10^5 |
| O2K (1/4) | 10x10^5 |




*Traba et al. An Optimized Protocol to Analyze Glycolysis and Mitochondrial Respiration in Lymphocytes. Immunology and Infection. 2016*

# Analytical Specificity

- This is the ability of the method to detect only the analyte it is designed to detect.
- In blood-based respirometry, other blood cell contaminants may be present.
- Contaminating cells in our standard isolation protocol result in <1% contribution to bioenergetics of platelet and lymphocyte fractions.




| | Selection Purity % total | Contaminants % total | Contribution to bioenergetics after purification % | Contribution to protein after purificaiton % |
|---|---|---|---|---|
| Monocytes (CD14) | 55.80 ± 2.98 | L - 11.62 ± 0.56<br>P - 32.58 ± 3.48 | 84.64 ± 0.41 | 85.64 ± 0.40 |
| Lymphocytes (CD45) | 92.63 ± 1.53 | P - 7.37 ± 1.53 | 99.76 ± 0.05 | 99.78 ± 0.04 |
| Platelets | *99.85 ± 0.03 | R – 0.15 ± 0.03 | 100 | N/A |

# Outline by FDA and CLIA Requirements

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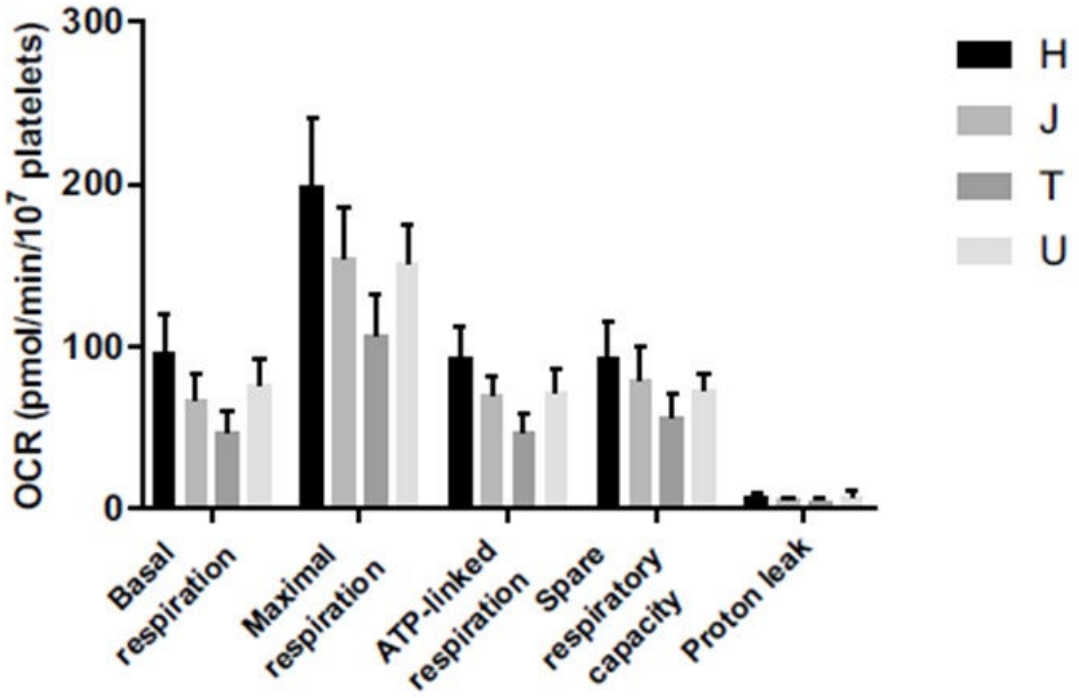
# Reference Range

- The laboratory must establish its own reference range.
- Bioenergetics are influenced by age, genetics, and environment in otherwise healthy individuals.
- “Normal” may need to be based on mitochondrial haplotype.

**Mitochondrial Haplotype**




*Ball et al. Assessment of the impact of mitochondrial genotype upon drug-induced mitochondrial dysfunction in platelets derived from healthy volunteers. Arch Toxicol. 2021*

# Outline by FDA and CLIA Requirements

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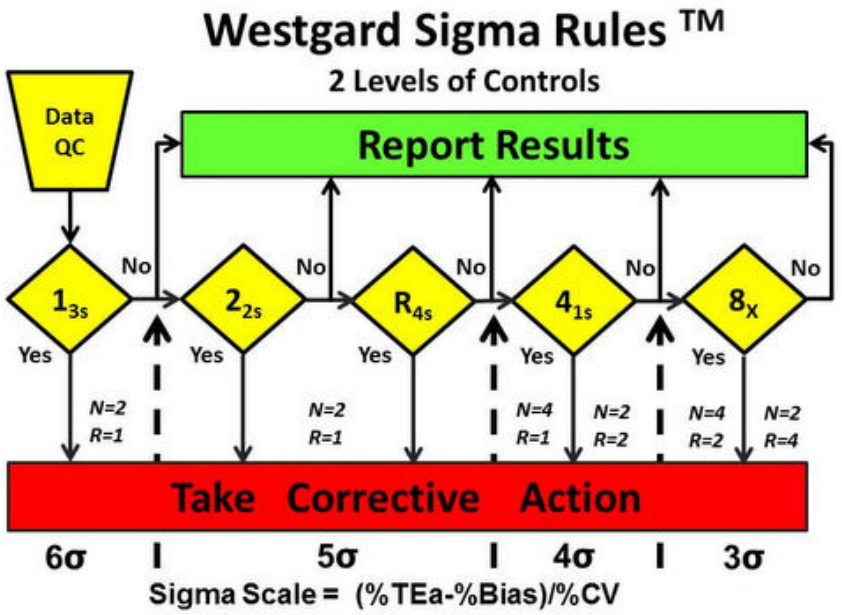
# Quality Controls

- Need a standard for live-cell assay. Challenging because mitochondria are difficult to store and show large variation between individuals.

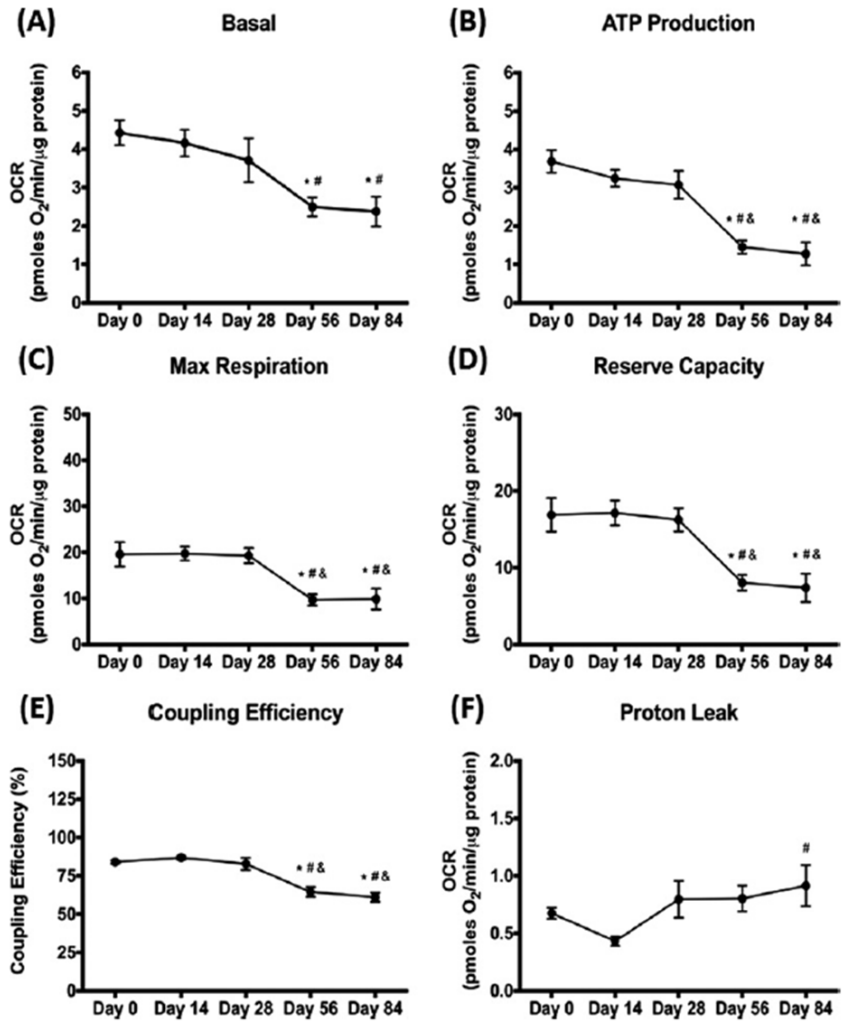
- Bakers Yeast-
  - Dehydrated. Stable for long periods of time.
  - Contain mitochondria which respire.
  - Respond to many mitochondrial substrates and inhibitors.

- Westgard rules to determine when reagents need to be replaced or instrument needs to be calibrated or undergo maintenance.

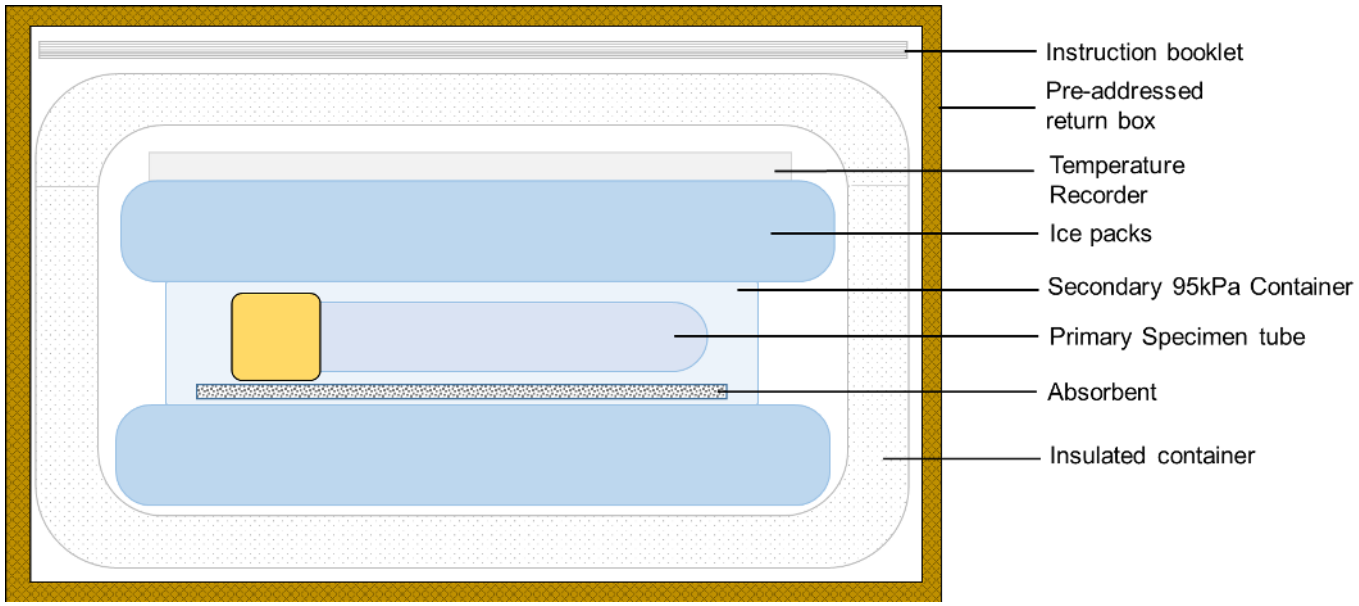
# Specimen Stability

- Usually run within 4 hours of collection in the research setting.
- Live cell assay affected by the many stresses of storage.
- Poses a challenge when specimens need to be shipped from around the country.
- Freeze-thaw damaging to mitochondria. Would require isolation and freezing at the collection site.

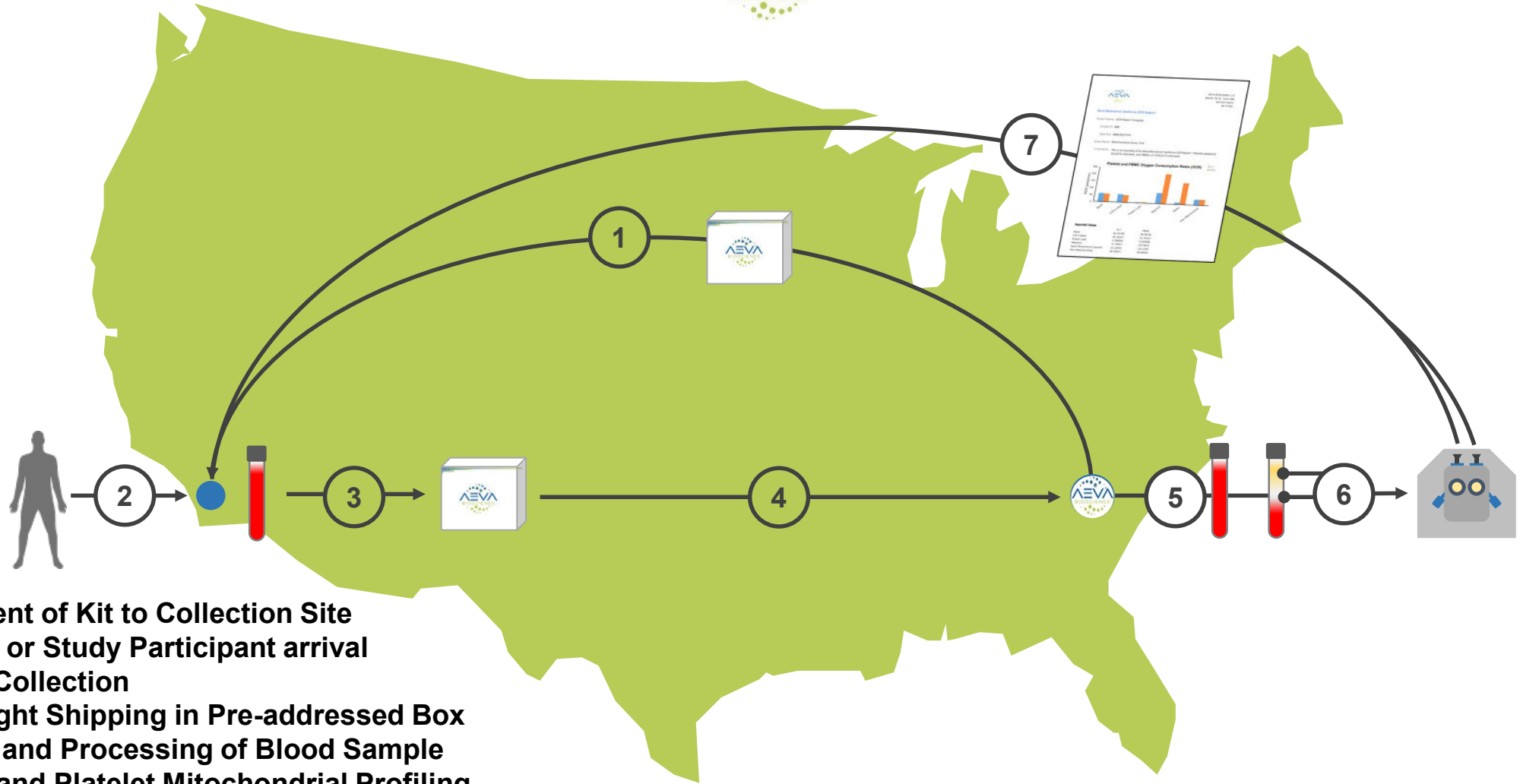



*Keane et al. The impact of cryopreservation on human peripheral blood leucocyte bioenergetics. Clinical Science (2015) 128, 723–733*

# Aeva Bioscience Whole Blood Shipping Kit

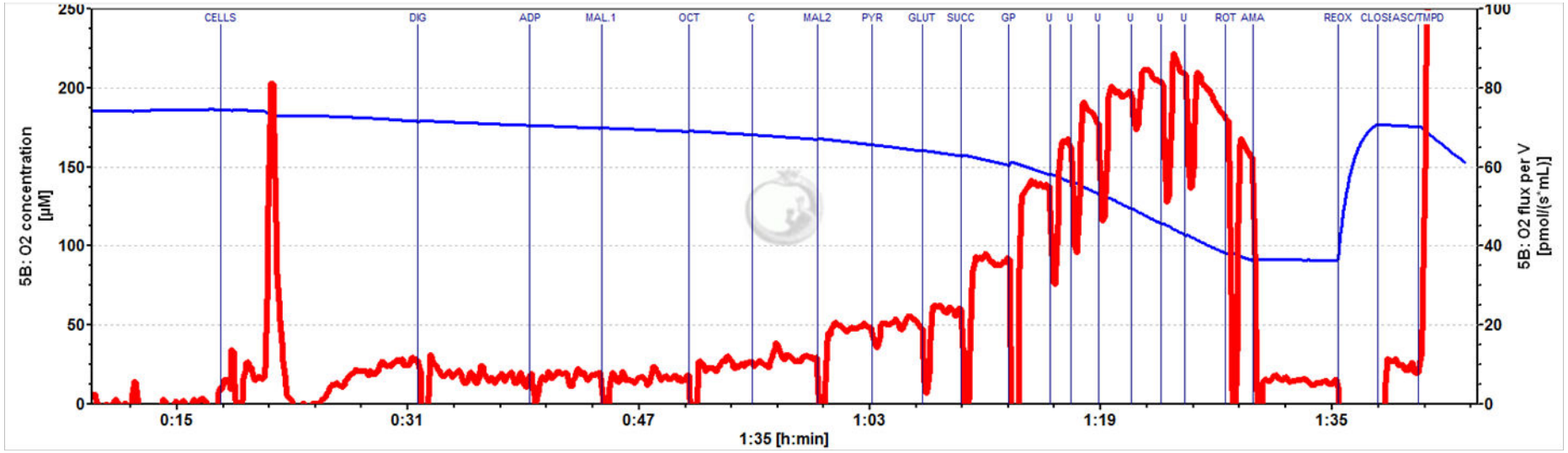
# Aeva Bioscience Whole Blood Shipping Kit



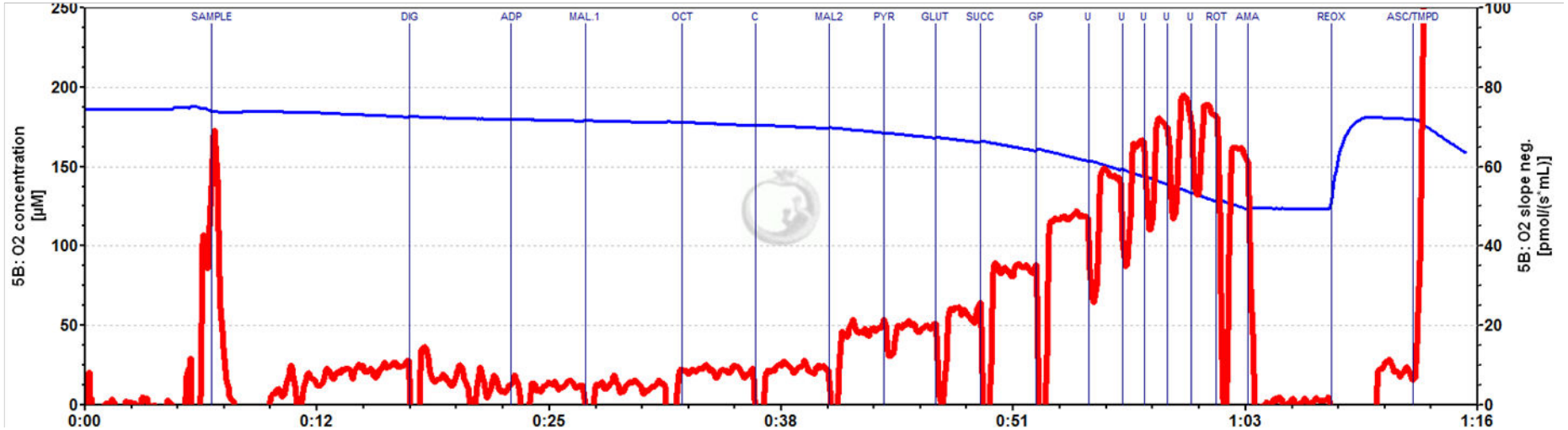

1) Shipment of Kit to Collection Site
2) Patient or Study Participant arrival
3) Blood Collection
4) Overnight Shipping in Pre-addressed Box
5) Arrival and Processing of Blood Sample
6) PBMC and Platelet Mitochondrial Profiling
7) Return Report to Originating Site

# O2k Bioenergetics after 24 hours

0hr




24hr



# Pathway to the Clinical Laboratory

- Blood-based bioenergetics to be established as a high-complexity quantitative LDT (No clinical validation by FDA).
- Immediate applications for general health and wellness, tracking the progression of a disease, or monitoring therapeutic efficacy.
- Use gathered data and machine learning to determine which respirometric indices are unique to their disease or condition for diagnostic and prognostic applications (i.e. 23andMe model).
- Support additional clinical studies/trials in these disease or conditions. (Aeva Bioscience LLC, CRO).
- Seek approval for IVD for use in clinical laboratories.

# Acknowledgments


## The Molina Lab

Anthony Molina PhD
Gargi Mahapatra PhD
Jenny G.A.
Jenny Gao
Amber Dewitt
Kimberly “Allie” Amick
Stephen Dozier
Jemima Piloso

## Darely-Usmar Lab

Victor M. Darley-Usmar
Balu K. Chacko
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Tanecia Mitchell
Michelle Johnson

## CLLS

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Melinda Kramer