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Clinical Tests of Metabolic Dysfunction: With Focus on Blood Respirometry and Lactate

Westerlund, Emil

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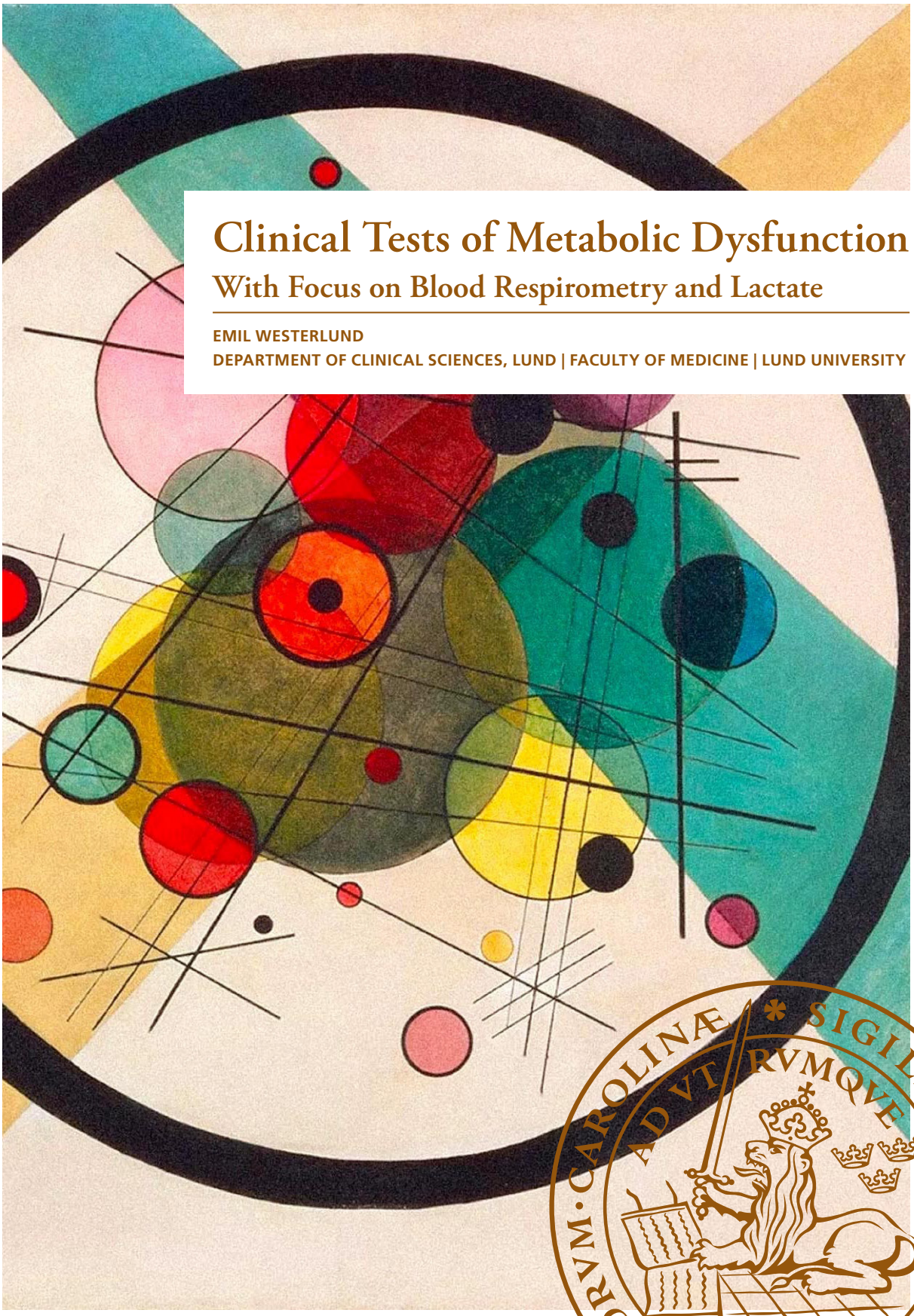

Clinical Tests of Metabolic Dysfunction

With Focus on Blood Respirometry and Lactate

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DOCTORAL DISSERTATION

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Abstract:
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Mitochondrial diseases (MDs) are a group of inborn diseases which affect the function of mitochondria, primarily their ability to supply energy for the cells. The disease presentation is highly variable. It is often a challenge to confirm or rule out the diagnosis, as the assessment is reliant on muscle biopsy or genetic tests. There is a need for better, non-invasive biomarkers that can facilitate the diagnostic process of MD.

High-resolution respirometry in blood cells is a relatively non-invasive and fast way of assessing mitochondrial function. It has the potential to be used in the diagnosis of MD, but it is unclear to what extent, as the disease is not normally manifested in blood cells.

An elevated lactate concentration in blood is a general sign of metabolic dysfunction. It may occur in MD patients, but it is not very specific.

Part of this thesis examines the use of blood cell mitochondria as biomarkers in a general sense (Paper II and III). Mitochondrial function in blood cells did not reflect that of muscles in healthy individuals, nor exhibit typical exercise induced changes, nor age-dependent decline, which is seen in other tissues. However, significant alterations were found in patients with MD, supporting their possible role as biomarker for selected pathological conditions.

In a cohort of paediatric patients with suspected MD, the diagnostic accuracy of tests based on blood cell respirometry was evaluated (Paper I). Blood respi¬rometry was not able to rule out MD with sufficient accuracy. A positive test increased the likelihood of disease and the accuracy increased further in combin¬ation with lactate. Its potential for clinical use is discussed in the thesis.

An elevated blood lactate may have many other causes besides MD. The last part of the thesis examines the use of lactate in the emergency department (ED) in a variety of acute conditions (Paper IV). An elevated lactate is generally known to be associated with mortality. In a large retrospective cohort, the prognostic value of lactate was shown to be highly dependent on its aetiology, and on the presence and degree of concomitant acidaemia. The results could inform the clinical evaluation of an elevated lactate in the ED and be useful for the design of future lactate studies.

Key words: mitochondrial disease, diagnose, lactate, respirometry, PBMCs, platelets, muscle

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Clinical Tests of Metabolic Dysfunction

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"Reality is frequently inaccurate."

– The Hitchhiker's Guide to the Galaxy

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Original articles

The thesis comprises the following articles, referred to in the text by their Roman numerals (I-IV):

I. Westerlund E, Marelsson SE, Ehinger JK, Sjövall F, Morota S, Åsander Frostner E, Oldfors A, Darin N, Lundgren J, Hansson MJ, Fellman V, Elmér E. **Oxygen consumption in platelets as an adjunct diagnostic method for pediatric mitochondrial disease.** Pediatric Research. 2018.

II. Westerlund E, Marelsson SE, Karlsson M, Sjövall F, Chamkha I, Åsander Frostner E, Lundgren J, Fellman V, Eklund EA, Steding-Ehrenborg K, Darin N, Paul G, Hansson MJ, Ehinger JK, Elmér E. **Correlation of mitochondrial respiration in platelets, peripheral blood mononuclear cells and muscle fibers.** Heliyon. 2024.

III. Ehinger JK*, Westerlund E*, Frostner EÅ, Karlsson M, Paul G, Sjövall F, Elmér E. **Mitochondrial function in peripheral blood cells across the human lifespan.** NPJ Aging. 2024.

IV. Westerlund E*, Leffler M*, Ehinger J, Elmér E, Sjövall F. **Elevated lactate in the Emergency Department – prognostic value and role of pH and aetiology.** Manuscript.

*Contributed equally

Abbreviations

AD	Alzheimer's disease
ADP	adenosine diphosphate
ATP	adenosine triphosphate
AUC	area under the curve
AUROC	area under the receiver operating characteristic (curve)
CI	complex I
CII	complex II
CIII	complex III
CIV	complex IV
CV	complex V
CNS	central nervous system
CS	citrate synthase
DMP	digitonin, malate, pyruvate
ED	emergency department
ETS	electron transfer system (also called the 'electron transport chain', ETC)
FAD^+	flavin adenine dinucleotide
FCCP	protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone
FGF-21	fibroblast growth factor-21
GDF-15	growth differentiation factor-15
GTP	guanosine triphosphate
IMM	inner mitochondrial membrane
IMS	intermembrane space
LDH	lactate dehydrogenase
LHON	Leber hereditary optic neuropathy
LR	likelihood ratio
MD	mitochondrial disease
MFRTA	mitochondrial free radical theory of ageing
MMS	Mitochondrial Medicine Society
MRI	magnetic resonance imaging
mtDNA	mitochondrial DNA
NAD^+	nicotinamide adenine dinucleotide

nDNA	nuclear DNA
NEWS	National Early Warning Score
NIH	National Institutes of Health
NLR	negative likelihood ratio
NPV	negative predictive value
OXPHOS	oxidative phosphorylation
PBMC	peripheral blood mononuclear cell
PDC	pyruvate dehydrogenase enzyme complex
PGC1α	peroxisome proliferator-activated receptor gamma coactivator-1α
PLR	positive likelihood ratio
pmf	protonmotive force
PPV	positive predictive value
ROC	receiver operating characteristic
ROS	reactive oxygen species
Rox	residual oxygen consumption
SUIT	substrate uncoupler inhibitor titration

Summary

This thesis concerns clinical applications of high-resolution respirometry in blood cells and lactate concentration in blood.

Mitochondrial diseases (MDs) are a group of inborn diseases which affect the function of mitochondria, primarily their ability to supply energy for the cells. The disease presentation is highly variable. It is often a challenge to confirm or rule out the diagnosis, as the assessment is reliant on muscle biopsy or genetic tests. There is a need for better, non-invasive biomarkers that can facilitate the diagnostic process of MD.

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Part of this thesis examines the use of blood cell mitochondria as biomarkers in a general sense (Paper II and III). Mitochondrial function in blood cells did not reflect that of muscles in healthy individuals, nor exhibit typical exercise-induced changes, nor age-dependent decline, which is seen in other tissues. However, significant alterations were found in patients with MD, supporting their possible role as biomarker for selected pathological conditions.

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Popular summary in Swedish

Denna avhandling handlar om kliniska användningsområden för högupplöst respirometri i blodceller och mätning av laktat i blod.

Mitokondriesjukdomar är en grupp medfödda sjukdomar som beror på fel i mitokondrierna. Mitokondrier finns i nästan alla mänskliga celler och deras viktigaste jobb är att omvandla föda till energi. Detta är avgörande för det mesta som händer i kroppen: för att hjärtat ska slå, för att du ska kunna röra på armar och ben och för att nervsignaler ska kunna skickas i din hjärna.

Patienter med mitokondriesjukdom kan ha varierande symptom från många olika organ. Diagnosen är ibland svår att ställa och utredningen kan ta lång tid. För fullständig diagnostik krävs i regel komplicerade tester, så som ett vävnadsprov från lårmuskeln eller en genetisk utredning. Ett snabbt och enkelt test för mitokondriesjukdom skulle kunna förbättra diagnostiken avsevärt.

Eftersom ett blodprov är ett relativt lätt och skonsamt ingrepp för en patient, har vi undersökt möjligheterna att diagnosticera mitokondriesjukdom genom att analysera mitokondriernas funktion i blodceller. Detta gjordes med högupplöst respirometri för ett antal barn under utredning för mitokondriesjukdom.

Det visade sig att ett negativt test inte var bra nog för att med säkerhet utesluta mitokondriesjukdom. Ett positivt test ökade sannolikheten något för diagnosen och träffsäkerheten förbättrades när testet kombinerade med mätning av laktat i blodet. Sammantaget är det oklart om metoden kan ha en framtida klinisk roll, och i så fall vilken, detta diskuteras närmare i slutet av avhandlingen.

Laktat är ett ämne som ofta är förhöjt i blodet när ämnesomsättningen är rubbad. Detta kan ske vid mitokondriesjukdom men även vid en rad andra tillstånd. Det är sedan tidigare känt att ett förhöjt laktatvärde i allmänhet är förknippat med ökad dödlighet hos patienter på akuten, där det redan idag används för att avgöra prognosen hos akut sjuka patienter. Vi undersökte laktatvärden hos en stor grupp patienter på akuten och såg att sambandet mellan förhöjt laktat och dödlighet var väldigt olika beroende på vilken sjukdom som orsakade laktatstegringen, och även beroende på surhetsgraden i blodet.

Resultaten kan hjälpa läkare att göra en mer nyanserad värdering av ett högt laktatvärde på akuten. Resultaten kan också vara en grund för framtida studier, där man kan välja att titta närmare på situationer där nyttan av laktat är som störst och välja bort dem där nyttan förväntas vara låg.

Introduction

A clinical vignette

A two-year old girl presents to the emergency department with epileptic seizures. The seizures are treated and terminate, but the girl does not return to her normal state. She is pale and ill-looking, she vomits, and her blood lactate is high.

The lactate is no surprise to her physician, as she has just had a generalised seizure. However, the lactate and the symptoms do not subside as expected and she is admitted with the tentative diagnosis of sepsis, or possibly meningitis.

Sepsis is a severe condition with high mortality and her physician recalls that lactate is associated with mortality even in the absence of hypotension, a phenomenon sometimes called "occult shock". The girl is given intravenous antibiotics and fluids.

In the ward, her condition is stabilised. She does not deteriorate but neither does she improve as expected. Her cultures are negative. Her liver tests turn up abnormal, prompting speculation into possible liver conditions. The liver is known to be essential for lactate clearance, which could explain the elevated lactate (which is lower by now, but not yet normalised). A renewed history is taken. This reveals that the girl is under follow-up for suspected developmental delay but the investigation so far has not been conclusive.

At this point, one of her attending physicians makes a new suggestion for the differential diagnosis: mitochondrial disease.

Again, this would explain the elevated lactate but not with certainty, as elevated lactate is known to have multiple causes (at this point, it has already been attributed to seizures, sepsis, and primary liver disease). A quick test to rule in – or preferably rule out – mitochondrial disease would obviously be highly beneficial to both the patient and her doctor but, unfortunately, no such test is available.

The investigation continues…

Thesis disposition

The case above is loosely based on features of authentic cases but altered and fictionalised for reasons of confidentiality. The case introduces the central themes of this thesis and a clinical context for the research questions.

Mitochondrial disease (MD) is difficult to diagnose. The clinical presentation is variable, and the best diagnostic tests are complicated and inaccessible. A quick, informative test would obviously be of much value. Part of the thesis explores whether blood cell respirometry might be such a test (Paper I).

Lactate concentration in blood is an important test in mitochondrial disease and in many other metabolic disorders, as it provides a quick window into the metabolic state of the patient. For the diagnosis of mitochondrial disease, and for many other purposes, it is imprecise. Its interpretation is often fraught with uncertainty and misconceptions but, used properly, the test has the potential to be useful in many clinical situations. Part of the thesis explores lactate as a diagnostic (Paper I) and prognostic test (Paper IV).

The thesis also includes studies of a more preclinical nature, exploring methodological background on blood cell respirometry as a biomarker in a more general sense (Paper II and III). The overarching question of these studies is: To what degree do the mitochondria of blood cells reflect mitochondrial function systemically, and in other, less accessible tissues?

The following parts of the Introduction section reviews the basics of mitochondrial physiology, cellular respiration, mitochondria's role in disease and ageing, and the physiology of lactate production. These subjects are then related to the specific clinical questions and research topics of the included papers.

The Methods section summarises the main methods, but also includes a discussion on limitations and alternative methods.

The Results section summarises the results, but also includes brief comments and discussing remarks adjacent to each finding. The Discussion section features a wider and more general discussion, including future perspectives.

The Results and Discussion sections are organised according to the three main themes of the thesis:

1) Diagnosis of MD by blood respirometry and lactate.
2) Blood respirometry as a biomarker.
3) Lactate as a prognostic test in the emergency department (ED).

An overview of mitochondrial physiology

Cellular metabolism and respiration

All organisms need energy to exist. Autotrophic organisms (mainly plants) harvest energy from the sun and use it to convert simple molecules – water and carbon dioxide – into more complex organic structures. Heterotrophic organisms (including animals) get their energy from consuming autotrophic organisms, breaking down the complex organic structures they contain and harvest the energy released from their chemical bonds. Most animals (including humans) need oxygen for this process.

On a principal level, it is the same thing as wood burning in a campfire. Except that a campfire is very inefficient. A furnace would be a more apt metaphor, but still a far cry from the efficiency human cells aspire to. To illustrate the process, I will describe the classic textbook example of carbohydrate metabolism, starting with glucose (Figure 1).

Glucose is a six-carbon molecule which is taken up into the cell and gradually broken down into two three-carbon molecules of pyruvate. The free energy released in the successive chemical reactions is utilised to phosphorylate two ADP molecules into ATP, and to reduce two NAD^+ molecules into NADH.[1] ATP is the "energy currency" of the cell, used for a wide range of processes that keep cells alive and functioning. The main function of NADH is to move electrons, necessary to keep certain reactions going, but also a means for the cell to move energy around.

The process described so far is called glycolysis and does not require oxygen. Glycolysis is not the most efficient use of glucose, but it was the only option for the first two billion years of life on Earth.[2]

Then came oxygen and mitochondria.

All complex life – and most human cells – contain mitochondria and can continue the breakdown process of glucose with the help of oxygen, greatly increasing efficiency. Pyruvate is the end product of glycolysis, but it can still be further oxidised, and for this purpose it moves into the mitochondrion. There, it is converted into the two-carbon molecule acetyl coenzyme A (acetyl-CoA) with help of the pyruvate dehydrogenase enzyme complex (PDC). Acetyl-CoA is united with the four-carbon molecule oxaloacetate, yielding the six-carbon molecule citrate. Citrate is then further oxidised and broken down in a series of steps called the Krebs cycle, or tricarboxylic acid (TCA) cycle, until it finally becomes oxaloacetate, ready for the next revolution of the cycle.

Like glycolysis, also the Krebs cycle produces ATP – but not many. Instead, the majority of ATP yielded by the Krebs cycle are generated in an indirect

manner, in the electron transfer system (ETS) of the inner mitochondrial membrane (IMM).

The theory of how ATP is created by the ETS was developed by Peter Mitchell and Jennifer Moyle in 1960s.[3,4] It is one of the most remarkable discoveries in biology in the 20th century. At the time, it was believed that all ATP resulting from the Krebs cycle were generated by substrate level phosphorylation, i.e., something resembling glycolysis. But no one knew exactly how. The real answer, presented by Mitchell and Moyle as the "chemiosmotic hypothesis of oxidative phosphorylation", turned out to be much different from glycolysis.[4]

What happens is this: The electrons from the oxidised substrates in the Krebs ultimately react with oxygen (O_2) and a proton (H^+), forming water (H_2O). (Hence, "oxidative".) But they reach there by a highly convoluted route, maximising the energy extracted from the net reaction. The electron carriers

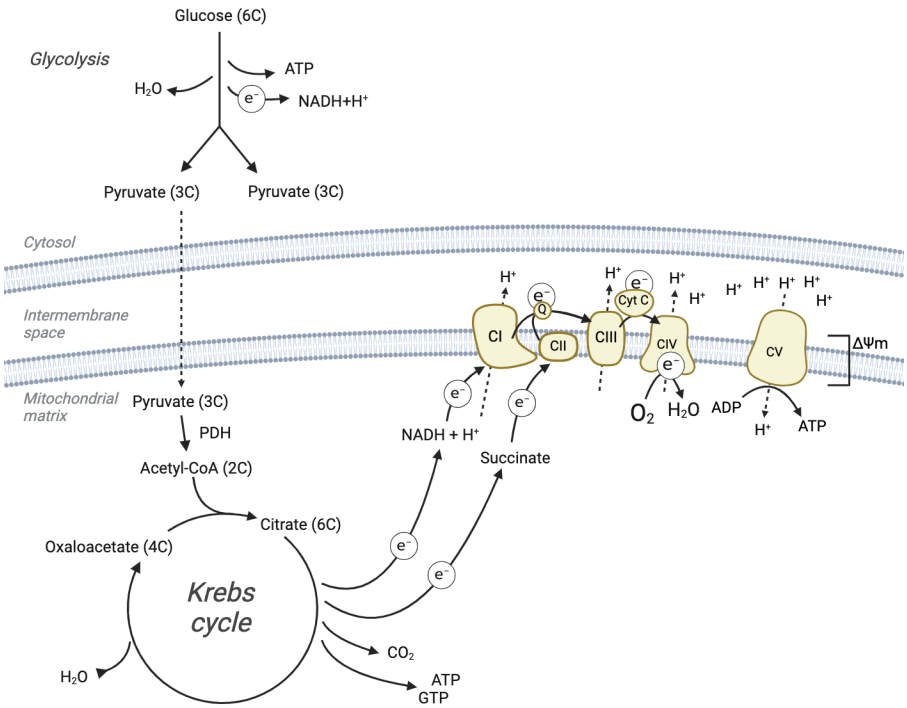


Figure 1 Overview of glycolysis and cellular respiration.
ATP = adenosine triphosphate. ADP = adenosine diphosphate. C = carbon. CI-CV = complex I-V. CoA = Co-ezyme A. Cyt C = cytochrome C. e^- = electron. $FADH_2$ = flavin adenine dinucleotide (reduced). GTP = guanosine triphosphate. $NADH+H^+$ = nicotinamide adenine dinucleotide (reduced). PDC = pyruvate dehydrogenase complex. Q = CoEnzyme Q_{10}. ΔΨm = membrande potential. Please note that the illustration is a simplified summary of metabolic pathways and is not meant to represent balanced biochemical reactions (for example the numbers of electrons or protons). *Created in BioRender.*

NADH and FADH (via succinate) are oxidised by complex I (CI), and inside complex II (CII), respectively, and the electrons continue their way through the complexes of the ETS. The electron paths converge in complex III (CIII) and then pass through complex IV (CIV). Only then do they react with oxygen.

This movement of electrons enables the complexes of the ETS to pump protons (H^+) across the IMM, to the intermembrane space (IMS). As the IMM is otherwise impermeable to protons, this results in the build-up of an electrochemical (or "chemiosmotic") gradient, generating a protonmotive force (pmf). The protons can then be released back into the mitochondrial matrix, a bit like turning on a tap (the flow being driven by the pmf). The "tap" is complex V (CV). CV is also known as ATP synthase, because the flow of protons through the tap is used to phosphorylate ADP into ATP (hence "phosphorylation").

Whereas glycolysis generates two ATP per glucose, the full oxidation of glucose by the Krebs cycle and the ETS generates a final tally of 28–38 ATP per glucose.[1]

Why is the amount of ATP a range rather than a number?

This has to do with various factors that distinguishes oxidative phosphorylation from glycolysis – a chief one being the important concept of "coupling". Under normal conditions, the flow of electrons through the ETS and the consumption of O_2 is proportional – or "coupled" – to the rate of ATP produced by the ATP synthase. This is because the ETS pumps protons across the IMM and the ATP synthase cannot release them back in without simultaneously making ATP.

But respiration is not always coupled. When protons are released back into the matrix by other routes and bypass the ATPase, electrons still flow and oxygen is still consumed, but no ATP is produced. This happens both in certain situations in vivo, and under experimental conditions, and is called "uncoupled" respiration.[5] Coupling is important for efficiency and is also relevant to respirometry studies of mitochondria (more on this later).

Besides carbohydrates, the body can also use lipids and proteins as energy sources. The fatty acids contained in lipids undergo β-oxidation in the mitochondria and enter the Krebs cycle in the form of acetyl-CoA. Protein metabolism is slightly more complex but, in summary, the amino acids that constitute proteins can also enter the Krebs cycle by various pathways. (None of these processes are shown in the figure.) In both cases, oxygen is required to maximise efficiency in terms of ATP production.

Why does efficiency matter in ATP production? For one thing, the demand is staggering. It is estimated that a normal day, a human being turns over roughly their own body weight in ATP. And some of our most important cells need large amounts to be available at an instant. For example, two major ATP consumers

are the electrolyte pumps of nerve cells, that make neuronal signalling possible, and myosin in muscle cells, the protein responsible for muscle contractions.

It is easy to appreciate the importance of mitochondria for sustained life and daily activity, and the potentially devastating effects of mitochondrial dysfunction. (Specific examples of this appear in Paper I and IV, and later in this summary.)

But there is more to mitochondria besides metabolism (in its most narrow definition). Mitochondria also have a pivotal role in apoptosis, calcium homeostasis and cell signalling, with implications that are still not fully mapped out.[6-8] While not directly or obviously related to the mitochondrial capacity to produce ATP, each of these functions is inevitably linked to it in such a way that it is impossible to consider one without the other. Mitochondria might be viewed upon as a sort of indispensable "multi-tool" of eukaryotic cellular life. A fact which may partly be explained by their primeval origins and their crucial role in evolution.

Mitochondrial origins, structure, and genome

A review of the evolution of life on Earth for the last 3.8 billion years is beyond the scope of this thesis.

Nevertheless, a highlight of evolution so far, relevant to the subject, is the appearance of mitochondria as eukaryotic cell organelles sometimes around 1.4 billion years ago.[9] It is believed that mitochondria were once independent organisms, an early form of bacteria with the ability of aerobic metabolism. At one point, these "protobacteria" were engulfed by other prokaryotic cells. Instead of being destroyed (which would normally happen) they lived on inside the host cells as "symbiotes". This is according to the endosymbiotic theory, which was pioneered by Lynn Margulis in the 60s and subsequently corroborated with genetic evidence.[9,10]

The symbiotes – the mitochondria – were able to produce energy with an efficiency that was a great asset to their host cells. It is generally believed that mitochondria had an instrumental role in the evolvement of advanced multicellular life during the Cambrian explosion, starting 539 million years ago, when levels of atmospheric oxygen rose dramatically.[11]

Today, all animals have mitochondria – possibly with some singular exception.[12] Certainly, all humans do, and they exist in all human cell types except for red blood cells. Mitochondria are generally said to be about 0.5–1 μm across and are often depicted as being rod-shaped, but in reality they come in many different shapes and sizes, and these properties are not static.[13] The number of mitochondria per cell varies greatly depending on cell size and function: an average platelet has about 5–7 mitochondria and a liver cell has several thousands.[14,15]

As was mentioned earlier, the mitochondrion is enveloped by a double membrane, which is instrumental to the process of oxidative phosphorylation in the ETS. The inner membrane is highly convoluted, forming so called "cristae" (Figure 2). This increases the area of the inner membrane and the efficiency of the ETS, with high-energy demanding cells tending to have more cristae than others.[16]

Another important feature of mitochondria is that they are encoded by two different genomes. Because mitochondria were once independent organisms, they used to have a complete, self-sufficient genome of their own. Over millions of years of evolution, the mitochondrial genes gradually moved into the DNA of the host cell nucleus (nDNA). But mitochondria still retain a small number of genes in their own genome (mtDNA), circular in shape as a remembrance of their ancient bacterial origins. The human mitochondrial genome consists of approximately 16 569 nucleotide pairs and comprises 37 genes. 13 of these constitute some of the essential parts of the ETS (otherwise mainly coded by nDNA) and the remaining encode ribosomal or transfer RNA.[17] Each mitochondrion usually has several copies of mtDNA within it (Figure 2).

One might say that the mitochondrial structures – much like many modern high-tech products – are assembled domestically (in the mitochondrial matrix) but most of the parts are imported from foreign factories (the cell nucleus).

The dual genome has important clinical implications for primary mitochondrial diseases (which will be described later), and for other conditions where mitochondria play a part. So does the mode of mitochondrial inheritance. Since the fertilised oocyte generally contributes all of the mitochondria to the future individual – and the sperm cell contributes none – mitochondrial inheritance is predominantly maternal.[18]

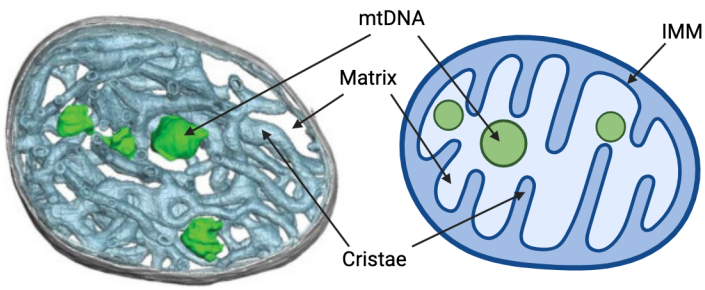


Figure 2 Mitochondrial structure and DNA
The picture shows a cryo-electron tomography image of an actual mitochondrion (left) along with a schematic illustration (right). The left picture is adapted and modified from work by Prole et al. (© 2020, CC Attribution 4.0 by licence).[19] The right picture, and the picture composition, were created with BioRender. IMM = intermembrane space. mtDNA = mitochondrial DNA.

Mitochondria in health and disease

The crossroads of the cell

Since the 1980s there has been a strong genetic paradigm in medical sciences. It arguably peaked with the launch of the Human Genome Project in 1990, a costly enterprise to sequence the entire human genome. (More precisely, *one* of the human genomes.) According to one of its main proponents, the project was expected to lead to the identification and circumvention of a plethora of pathological genes, a revolution in "preventive medicine", and life "without disease".[20]

The project was completed in 2003 but did not succeed in eliminating disease. In fact, many argue that while its impact on medical research was significant, its clinical impact, so far, has been modest at best.[21,22] Improved and cheaper techniques have successively allowed for genome wide association studies (GWAS), sequencing genomes of larger and larger groups of individuals, with the goal to figure out the causes of common diseases. But even as sample sizes have increased, from 146 participates in the first GWAS to over a million nowadays, molecular researchers have frustratingly concluded that only a small fraction of the variance of common diseases can be explained by the studied genes.[23-25]

The lost momentum of nuclear genetics paved the way for a new paradigm: a "metabolic paradigm". At the centre of this paradigm are mitochondria, anticipated by some as a key to explain the major debilitating and degenerative diseases afflicting humanity today.[2,26-28]

Before trading one bursting paradigm bubble for another, it might behove the scientific community to exercise a degree of caution. Perhaps it is best to view mitochondria as an important complement to nuclear genetics, not a competing paradigm. "Life", in the more eloquent words biologist Doug Wallace, "is structure animated by energy".[17] Without touting mitochondrial medicine as the panacea for all human ailments, it is not hard to at least argue that – in addition to nuclear genes, environmental and other factors – they do seem to have a role in many of life's core processes.

One argument for the importance of mitochondria is evolutionary. Nuclear genes were clearly instrumental for eukaryotic cell differentiation and the evolvement of large complex animals. But, as mentioned earlier, the energy production that made that evolution possible (or maybe even caused it) came from mitochondria. One current hypothesis even states that life originated with metabolism, and that metabolism gave rise to the first genetic code, rather than

the other way around.[29] Regardless of whether that specific hypothesis is true, it is evident that without metabolism there would not be much life at all.

Mitochondrial dysfunction has naturally been implicated in the pathogenesis of metabolic conditions such as type 2-diabetes and non-alcoholic fatty liver disease (NAFLD), prevalent diseases with complex aetiologies that are not yet fully understood.[30,31]

There are several possible mechanisms. A signature feature of early type 2-diabetes is peripheral insulin resistance. Skeletal muscle makes up about 40% of the bodyweight and contribute significantly to the basal metabolic rate of the whole body.[32] As such, skeletal muscle is a major insulin target and contributor to glucose homeostasis. Decreased mitochondrial capacity has been shown in skeletal muscle biopsies from diabetes patients.[33] This in turn is linked to obesity, with excessive deposits of lipids in muscle cells. These are believed to be incompletely oxidised and further damage the mitochondria through generation of reactive oxygen species (ROS, see below), instigating a vicious cycle of metabolic dysregulation.[34]

At later stages of type 2-diabetes, not only peripheral insulin sensitivity but also insulin secretion from the pancreatic β-cells will diminish. Interestingly, the physiological mechanism by which β-cells detect glucose and secrete insulin, is mediated by the mitochondria. When the mitochondria of the β-cells metabolise glucose and generate ATP, ATP-sensitive K^+-channels in the plasma membrane close and depolarise the cell membrane. This, in turn, leads to an influx of Ca^{2+} through voltage-sensitive ion channels and the increased calcium ion concentration, finally, triggers the release of insulin-filled vesicles.[35] This signalling pathway explains why β-cells only use glucose (not fatty acids) as energy source. Interestingly, it has been demonstrated that inherited mitochondrial disease can cause a type 1-diabetes-like phenotype, via disruption of this mitochondrial pathway, and recently there has been speculation that the insulin deficiency in type 2-diabetes too may be mitochondrially mediated.[34,36]

There is also growing evidence for the role of mitochondrial dysfunction in other major chronic diseases that have not traditionally been thought of as metabolic.

One example is dementia. The association between diabetes and cognitive impairment was first described in 1922 but has only recently been more widely accepted.[37,38] In fact, there is now a growing research interest in the connection between metabolic disease and dementia, in particular Alzheimer's disease (AD).

Considering that glucose is normally the main energy source of the brain, it is not difficult to imagine that impaired glucose homeostasis might influence brain metabolism through mitochondrial pathways. Interestingly, mitochondrial dysfunction has been shown to precede amyloid plaque deposition (a chief

pathological finding in AD) in animal models.[39] An interesting new hypothesis proposes that a dysfunction in the communication sites between the endoplasmic reticulum (ER) and the mitochondria gives rise to many of the known features of AD: dysregulated lipid metabolism, calcium homeostasis, cognitive symptoms, and eventually – amyloid plaques.[40] The allure of the "MAM hypothesis" (MAM = mitochondria associated membrane) is that it accounts for a multifactorial aetiology, where both glucose homeostasis and the nuclear genome are involved, and where familial and sporadic forms of AD are variants of the same disease.[2,41]

Two other major areas of disease where the role of mitochondria have spurred an increased interest are cancer and immunological disorders. The altered metabolism of cancer cells was originally discovered by Otto Warburg in 1926.[42] He noticed that cancer cells tend to produce energy mainly through glycolysis, without full oxidation in the Krebs cycle and the ETS (Figure 1) – even in the presence of oxygen.[43]

This phenomenon – a propensity of certain cells for aerobic glycolysis – has since been named the "Warburg effect". Its causes and implications have spurred debate for a century but there has been interesting developments in our knowledge surrounding it.[44] For example, emerging evidence suggests that the build-up and release of succinate can activate hypoxia induced factor-1 (HIF-1), a transcription factor that switches the metabolic profile of the cell.[8] This normally happens under hypoxic conditions but certain types of cancer cells may utilise the pathway regardless of oxygen supply, allowing them to promote excessive cell growth and division.[45] The signalling function of succinate and other Krebs cycle metabolites have also been implicated in important immunological processes, both in the body's appropriate defences against infection, and in chronic inflammatory diseases.[8]

The point here is not to suggest that mitochondria are the ultimate cause of inflammatory diseases and cancer. (A singular isolated cause of cancer seems unlikely to ever emerge.) The exposé of mitochondria's role in major age-associated and chronic disease entities such as diabetes, neurodegeneration, and inflammatory diseases, is merely meant to illustrate how central mitochondria are in many seemingly different biological processes. The mitochondria are, in some sense, a "crossroads of the cell", where many vital pathways converge, diverge, and interact.

This account is also a preamble to a brief note on the big unresolved question of ageing. Ageing is a major risk-factor for many of the diseases mentioned above and the role of mitochondria in ageing is an area of continued interest, which may connect the diseases of ageing to the process of ageing itself.

The mitochondrial theory of ageing

To summarise our current knowledge on biological ageing: nobody knows why, and nobody knows how.

That is not to say there has not been a great many *ideas* historically. Today there is certainly an expanding wealth of knowledge on specific aspects of ageing and many good theories on the subject that are being examined.

Those theories may or may not be mutually exclusive. One of the most impactful summaries in the field lists the "nine hallmarks of aging" – important biological changes that have been demonstrated to occur and correlate with cellular senescence and the eventual demise of the organism.[46] This paper has been cited approximately 10 000 times since its publication in 2013 and was inspired by another famous paper, that listed the hallmarks of cancer.[47] It has also been criticised, as the hallmarks provide even less explanatory power than its cancer precedents.[48] This is perhaps unfair to the hallmark authors and more likely a sign of where the field stands in general.

One of the hallmarks of ageing listed in the paper is mitochondrial dysfunction, but, like many of the other hallmarks, its precise role remains to be elucidated. The connection between mitochondria and ageing was first suggested by Denham Harman in the 1950s and is known as the mitochondrial free radical theory of ageing (MFRTA).[49] Free radicals are molecules that are prone to react with, and potentially damage, cellular structures. Most free radicals in the body are generated in the ETS of the mitochondria, in the form of ROS. ROS is produced when the electron transfer through the ETS is hampered, causing electrons to escape and react prematurely with O_2 molecules.[50] This may happen to some degree even under physiological conditions but not extensively (Figure 3A). Important general causes of increased ROS production are diminished oxidative capacity, caused by insufficient oxygen or poor ETS function, or when

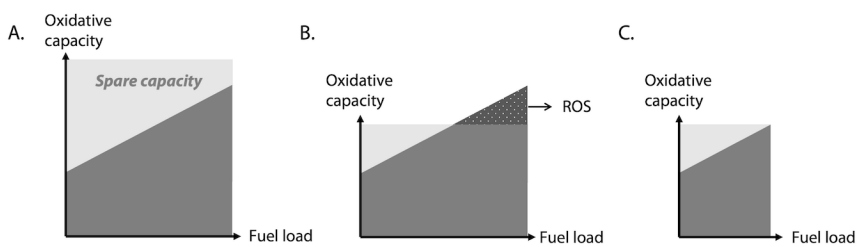


Figure 3 Mitochondrial function and ROS production
An conceptual illustration of the dynamic relationship between oxidative capacity, fuel load and ROS production, which may be related to ageing and chronic diseases. **A.** Oxidative capacity equals or exceeds requirements. **B.** Requirements exceed oxidative capacity, producing ROS. **C.** The effects of diminshed oxidative capacity are mitigated by decreased fuel load. Adapted from Patti and Corvera.[34]

the ETS is overwhelmed by too much substrate (Figure 3B).

According to the MFRTA, mtDNA is a prime target of ROS damage due to its proximity to the ROS production sites. Mutagenic damage to mtDNA will, in turn, cause dysfunction of the ETS and even more ROS in a vicious cycle. The theory states that the exponential decline of mitochondrial function, which must follow, supposedly correlates to the functional decline of organs and tissues characteristic for ageing. Since ROS production is never completely absent, ageing is inevitable.[51]

The theory was supported by studies showing that mtDNA mutations are more abundant than nDNA mutations, that they accumulate with age, and that rate of oxidative mtDNA damage across species is inversely correlated to their lifespan.[52-54] Knock-in mice with a defective mtDNA polymerase have been shown to age prematurely and have a reduced lifespan.[55]

However, after its initial popularity, the MFRTA fell out of grace due to subsequent findings that seemed to be at odds with the theory. If ROS cause ageing, antioxidants, compounds that neutralise ROS, should be expected to reduce signs of ageing and increase lifespan. In contrast, notable animal studies showed that manipulation of antioxidant levels (despite high hopes from the pharmaceutical industry) is useless or even deleterious.[56,57]

Is the MFRTA dead? Perhaps not quite. Experimental calorie restriction has repeatedly been shown to increase lifespan in various mammals.[58-60] There is a clear theoretical connection between reduced food intake and reduced ROS production, as reduced fuel load might mitigate the effects of diminished oxidative capacity (Figure 3C). An allure of the MFRTA is that it accounts for the influence of environmental and nuclear genetic factors on ageing, by modulating ROS production, and that it connects ageing to the pathophysiology of age-associated and chronic diseases.[34]

Does mitochondrial function decrease with age? If so, how does it decline? Is the decline universal or is it confined to certain tissues? These questions are crucial for further evaluating the MFRTA and the mechanisms of ageing in general.

Mitochondria in muscles and exercise

Muscles have a high energy demand when activated and muscle fibres are generally high in mitochondrial content. This has made muscle tissue a long-standing favourite of mitochondrial researchers and, consequently, relatively much is known about muscle mitochondria compared to other tissues.

Even Hans Krebs and Albert Szent-Györgyi, to whom mitochondria's exact function was not clear at the time, found that minced pigeon-breast muscle – which is very rich in mitochondria – was particularly well-suited for the study of

oxidative metabolism.[61] Later, in the 1950's, when breast-muscles of pigeons were compared to those of chickens – who do not use the muscles for flight – it was noticed that they had significantly fewer mitochondria and lower oxidative enzyme activity.[62]

Differences in mitochondrial respiratory capacity do not only account for inter-species variation but also for adaptive changes. John O. Holloszy showed already in 1967 that endurance training induces an increase in mitochondrial content and oxidative capacity in skeletal muscle from rats, and that these changes were associated with increased physical performance.[63] Similar findings followed in several human studies.[64-66]

The molecular mechanisms behind these exercise-induced mitochondrial changes have since been revealed; while not yet fully understood some of the main pathways are well-known. A very important regulator of mitochondrial biogenesis (sometimes called the "master regulator") is the transcriptional coactivator known as peroxisome proliferator-activated receptor gamma coactivator-1α (PGC1α).[67] During bouts of exercise, several acute biochemical alterations such as a changes in the ATP:ADP ratio, calcium influx, and ROS production, promote the activation of PGC1α, which in turn coactivates nuclear transcription factors of genes that cause biogenesis.[68] (This kind of ROS signalling pathway could explain why crude manipulations of antioxidant levels have failed to increase lifespan and health in experimental settings.)

But the role of muscle mitochondria extends beyond athletics. As mentioned earlier, it is well-recognised that muscles contribute a large share of total metabolism, affecting metabolic homeostasis of the whole body. As such, the mitochondrial function of muscles has been implicated in key processes of health, disease, and ageing. For example, some of the beneficial effects of physical exercise on lowering the risk of type 2-diabetes are believed to be mediated partly through alterations of muscle mitochondria, an effect that could be related to increased oxidative capacity and lower chronic ROS production (Figure 3).[34] This fits with epidemiological data, showing that physical activity can counteract the age-dependent association between glucose intolerance and impaired cognition.[69] Conversely, decreased muscle mass has unfavourable associations in old age. Decline in muscle mass and strength (known as sarcopenia) is a well-known phenotype of ageing and correlates with physical disability.[70] Studies have even showed that gait-speed in elderly people has an impressive predictive ability for mortality, exceeding that of many more obvious predictors such as the presence of chronic diseases, smoking, and hospitalisation.[71]

Does physical exercise mainly influence muscle mitochondria or are there also systemic mitochondrial effects of exercise? Are there inherent functional similarities between mitochondria in muscle tissue and other tissues, within the

same individual? These questions have implications for the study of muscle mitochondria both in health and disease, including in ageing.

Mitochondria in inherited and acute diseases

Mitochondrial disease

Mitochondria's role in disease is probably most obvious in a group of diseases normally referred to as "mitochondrial diseases". As is evident from the previous sections, this expression could in some sense be applied to a very wide range of disorders involving mitochondrial dysfunction. What is normally meant by mitochondrial disease (MD) is, however, *primary* or *inherited* MD. This is a heterogenous group of diseases emanating from inborn errors in mtDNA or nDNA genes, that control the proteins of the ETS.

Because mitochondria are abundant in skeletal muscle and crucial to their normal function, muscle symptoms are common and mitochondrial dysfunction in muscle has been well-characterised in MD historically.[72,73]

Mitochondrial dysfunction of blood cells in MD patients has been less studied, since blood is not usually a symptomatic organ. Still, blood cells are easily sampled and could potentially provide a fast and accessible source of mitochondria for diagnostic purposes. (More on this follows later.)

Mitochondrial dysfunction in emergency medicine

The preceding text has described mitochondrial dysfunction in relation to chronic and inherited disease, cancer, ageing and exercise. But mitochondria are also involved in common acute conditions seen in the ED.

For example, cell death due to ischemia is a core pathological process in major, time-sensitive emergencies such as ischemic stroke and myocardial infarction. As mitochondria are the main oxygen consumers of the cell, and the primary mediators of apoptosis, their role is integral to this process and especially to ischaemia-reperfusion injury.[74-76]

As another example, several drug intoxications and medication side-effects, including acetaminophen and metformin, are caused by mitochondrial dysfunction.[77-81] Mitochondrial dysfunction has also been suggested to play an important role in sepsis, which was the rationale behind a clinical trial with thiamine to reduce sepsis-associated kidney injury.[82,83]

A less common but important example is children with mitochondrial disease that present to the ED with acute metabolic decompensation, triggered by fasting, exercise or infection.[84] This is often the initial presentation.

Mitochondrial function is not typically assessed directly in any organ or situation in the ED. However, blood lactate is a well-known by-product of

mitochondrial dysfunction and is measured easily and almost ubiquitously in critically ill patients. Its accessibility combined with its proximity to essential metabolic pathways makes lactate an interesting candidate for prognostic and diagnostic use in the ED.

The search for a mitochondrial biomarker

Blood cell respirometry as a biomarker

What is a biomarker and why do we need it?

A biomarker is a subcategory of medical signs. The word is used frequently in contemporary society but often in rather specific contexts. A layman will probably associate the word with something like taking a quick blood test and finding out whether their diet is healthy, or their ten-year risk of getting a heart attack.

Official definitions of the word biomarker tend to be wider. The National Institutes of Health (NIH) has defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention".[85] In essence, a biomarker is a means to predict something complex or important by a measurement that is obtained with relative ease, often involving a laboratory analysis.

Part of this thesis is concerned with mitochondrial biomarkers in blood cells. There have been high hopes within the research community that blood cell mitochondria can be used as substitutes for less accessible mitochondria, or to reflect systemic mitochondrial dynamics in the body.[86-90]

As was mentioned earlier, muscle tissue has traditionally been of prime interest to mitochondrial researchers. But a muscle biopsy is a substantially more invasive procedure than a blood sample, which is especially a problem when sampling humans.

There are several different techniques to obtain a muscle sample. While generally safe, they all require a trained physician and local anaesthetics, and symptoms such as numbness or soreness may ensue.[91-93] This poses a problem when studying dynamic changes, such as the response to exercise, fasting, or the effects of ageing, where repeated measurements are desired. It is also an impediment for achieving large sample sizes. The biopsy procedure may be even more difficult in clinical medicine, particularly for children, who generally require sedation.[94] Other mitochondrial sites of interest, such as the liver, heart muscle or brain tissue, are rarely sampled in living patients due to the invasiveness, and virtually never in healthy volunteers.

If mitochondria from blood cells could give all or even some of the same information as less accessible tissues, it would obviously be of benefit both to research and clinical practice.

Blood cells as markers of mitochondrial ageing

A premise of the classic MFRTA is that the mitochondrial decline is intrinsic and inevitable at the cellular level. Different people may age biologically at different rates but everyone ages – because their cells "wear out". Consequently, mitochondrial decline should be detectable universally throughout the body.

Is it? Mitochondrial function in human ageing has mainly been studied in muscle tissue, building upon findings from animal studies, where respiratory function was shown to decline with age.[95] This was largely confirmed in ensuing human muscle studies by Trounce (1989), Cooper (1992) and Boffoli (1993), though sample sizes were small compared to the animal studies.[96-98] The mentioned studies agree on a rather pronounced age-dependent decline in respiratory function and enzyme activity, especially for CI and CIV. They do not agree on its cause, nor on the decline of CII function.

A later study by Rasmussen (2003) did not find any clear evidence of declining enzyme activity, nor of mitochondrial content, in sharp contrast to the earlier studies.[99] However, a very large human study soon followed by Short (2005), where decline in all respiratory parameters was found, as well as in mitochondrial content, which also correlated to the degree of oxidative mtDNA damage.[100] Experimental evidence has suggested that methodological errors may account for the variation in effect size between aforementioned studies, but the overall body of evidence does at least support some degree of functional decline with age.[101]

Paradoxically, age-related mitochondrial decline is less well characterised in blood cells even though they are easier to sample. Previous studies have been to the small side, have mainly examined platelets, and the results are more ambiguous than in muscle. Two early studies, by D'Aurelio (2001) and Shi (2008), found increased lactate production and lower membrane potential, respectively, in platelets from older people.[102,103] Also examining platelets, both Braganza (2019) and Jedlička (2021) found lower respiratory function in older individuals, but not for CI (in contrast to the muscle studies).[104,105] As far as we are aware, Alonso (2021) is the only study, preceding ours, that examined age-dependent changes in platelets and peripheral blood mononuclear cells (PBMCs) from the same individual. Several respiratory parameters declined with age in PBMCs but not in platelets.[106] The largest preceding study (n=85) on platelet respiration and age, by Chacko (2019), found no decline in any parameter.[107]

In summary, despite some previous data, the question of mitochondrial age-dependent decline in blood cells is unresolved.

Blood cells as markers of mitochondrial function in muscle

What are the theoretical reasons to believe that mitochondrial respiratory function would correlate between blood cells and muscle tissue?

All mitochondria in the body are descended from the mitochondrial pool of the original zygote and possess maternally inherited mtDNA. This mtDNA, along with many more nuclear genes, influence the ETS and the function of all mitochondria in the body. While there is generally a mixture of mtDNA alleles within the body (a phenomenon called "heteroplasmy"), recent evidence shows that nuclear genes possess some degree of control over both heteroplasmy and mtDNA copy number.[108] On the whole, it is inferable from these facts that respiration *may* correlate to some degree across tissues within the same individual. But if so – how much do they correlate?

And what about dynamic changes? As discussed earlier, much is known about the specific effects of exercise on muscle mitochondria. Assuming that environmental factors such as exercise, food intake, and medications, affect tissues differently, or selectively, this may decrease correlation across tissues from the baseline. For example, if exercise were increasing respiratory capacity in muscle mitochondria but not in blood cells, the correlation between muscle and blood cells would, logically, diminish the more a person exercises. On the other hand, there is some research suggesting that exercise-induced changes may extend beyond muscle mitochondria and have systemic effects. Examples of this has been seen in adipose tissue, the brain, the liver, and the kidney.[109]

Despite the high hopes of blood cell mitochondria as biomarkers for their muscle counterparts, studies making direct comparisons in humans are surprisingly few and small. A preceding study from 2016, in African green monkeys (fellow primates who are biologically similar to humans in many respects), had very promising results.[86]

This was followed by a less convincing body of human studies. The previously mentioned human study by Braganza (2019) examined platelet-muscle correlations, in addition to ageing effects, and found a mixture of strong and absent correlations in a subgroup of the total study population (n=26).[104] The authors summary conclusions were nonetheless favourable. Rose (2019) studied both platelets and PBMCs, but correlations were mainly absent, except for occasional parameters (n=13).[110] Hedges (2019), likewise, failed to find correlation between PBMCs and muscle (n=9). Interestingly, this study also examined the effects of exercise on mitochondrial respiratory function, which occurred in muscle but not in PBMCs.[111]

In conclusion, current evidence is not sufficient to neither support, nor to dismiss, blood cells as biomarkers for muscle mitochondria.

Mitochondria in circulating blood cells

Peripheral blood mononuclear cells (PBMCs)

PBMCs are cells isolated from the circulating blood that carry a single, round nucleus. The category mainly includes lymphocytes and monocytes. It excludes blood cells such as granulocytes, that have irregular or multilobular nuclei, and erythrocytes and platelets, that are anuclear.

Lymphocytes and monocytes are generated in the bone marrow from haematopoietic stem cells (HSCs), via progenitor cells. They differentiate into mature immune cells by a very complex process which will not be reviewed in detail here.[112] Peripheral blood cell mitochondria descend from the progenitor cells, and ultimately from HSCs. Lymphocyte turnover in the peripheral circulation ranges from days to years, depending on cell type, class, and function.[113,114]

Both B and T lymphocytes use oxidative phosphorylation for energy production in their naïve states. When activated, oxidative phosphorylation increases but, in addition, aerobic glycolysis is relatively upregulated to meet the requirements of cytokine production and proliferation.[115] A similar metabolic shift occurs in monocytes when they mature into macrophages.[112]

Apart from metabolism, mitochondria in lymphocytes are also involved in key signalling pathways of innate and adaptive immunity.[115] These are topics of recent discovery and increased research attention.

Platelets

Platelets are very small (~1.5–2.5 μm) components of peripheral blood whose chief known function is to counteract bleeding from blood vessel injury. Platelets are often referred to as "cell fragments" (and originally as the "dust of the blood"), though their status as non-cells has been challenged as we have learned more about them.[116,117] For the purposes of this thesis (mostly out of grammatical convenience) they are referred to as cells.

Platelets are constantly generated by being budded off from megakaryocytes in the bone marrow. It is estimated that each day, progenitor cells in the bone marrow generate around 1×10^8 megakaryocytes, which in turn generate around 1 000 platelets each. The circulatory lifespan of a platelet is 7–10 days.[112]

A non-activated platelet looks like a tiny American pancake: flat, smooth, and slightly ovoid. In their quiescent state, like lymphocytes, platelets use oxidative phosphorylation for their energy production.[118] When platelets are activated by external stimuli, they change dramatically in shape, looking less like pancakes and more like splattered pancake batter. They stick together, aggregate into a thrombus, and initiate a coagulation cascade with the ultimate purpose of stopping a bleed. This process demands a lot of energy rather quickly, which is most likely supplied by an increase in both anaerobic glycolysis and oxidative

phosphorylation.[119] Normally in experimental settings, unless the activation process itself is of interest, measures are taken to keep platelets inactive.

Human platelets lack nuclei and nDNA (which remains in the megakaryocyte). They do, however, contain mtDNA and ample RNA, of both nuclear and mitochondrial origin. This enables them to make new proteins and respond to rapid shifts in energy demand.[120]

Platelets contain about 5–7 mitochondria each, organelles that are allotted to them in a seemingly random fashion as they bud off from the megakaryocyte.[121] Even though each platelet contains a modest number of mitochondria, the mitochondria make up a relatively large proportion of the platelet volume. Platelets, in turn, are the most abundant mitochondria-carrying cells in blood, and blood is easily sampled in large volumes. Taken together, this makes platelets a convenient source of mitochondria for experimental purposes in human studies.

Mitochondrial disease and diagnosis

General background

Mitochondrial diseases (MDs) are a heterogenous group of disorders caused by inborn errors of mitochondrial respiration that vary greatly in severity and presentation.

The first known case of MD was described in 1962 by Swedish endocrinologist Rolf Luft. He described a woman who presented in her late teens with symptoms of hypermetabolism that progressed and did not respond to medical or surgical thyroid treatment. Biochemical studies of skeletal muscle revealed abnormal respiratory uncoupling and altered mitochondrial morphology, that explained her symptoms.[122,123] MDs have most likely existed among humans long before we were aware of them. Luft's clinical discovery was made at the vanguard of contemporary mitochondrial science and the methods he used to analyse his muscle samples had not been available just a few years earlier.

Since the 1960s, our knowledge has expanded vastly. It is now clear that MDs constitute a prominent category of inborn metabolic diseases, with an estimated incidence of around 1 in 11 000 preschool children.[124] Since mitochondria are present in all tissues of the body, any organ can theoretically be affected and there is a wide spectrum of possible symptoms.

Generally, organs with high energy demand tend to be most affected and generate the most severe symptoms. This characteristically includes symptoms from skeletal muscles and the central nervous system (CNS). Leigh syndrome is one of the most common presentations of MD, with several possible genetic causes that give rise to the same phenotype: early-onset intellectual and motor retardation, usually with regression, and signs of brainstem dysfunction.[125,126]

There are also less typical examples of mitochondrial disease, isolated to a single organ, or even to a part of an organ. Leber hereditary optic neuropathy (LHON), caused by mutations in mtDNA, produces acute and severe bilateral blindness, but rarely affects other parts of the central nervous system.[127]

Severity and natural history in MDs are highly variable. Some patients present late or have a mild course, only temporarily exacerbated by exercise or infections. It is possible that mild or "oligosymptomatic" mitochondrial disease is underdiagnosed.[128] However, the characteristic clinical course of MD is that of debilitating and progressive symptoms with high risk of childhood mortality. Early presentation, in particular, tends to be associated with worse prognosis.[129]

Sadly, there is presently no approved and effective treatment for mitochondrial disease. (with the possible exception of idebenone in certain cases of LHON).[130,131] Treatment for mitochondrial disease is an area of much active

research and pharmacological agents with a wide array of mechanisms are currently in clinical development.[132]

Even in the absence of a cure, confirming the diagnosis is crucial for proper management and counselling.[133] With the discovery of new treatments, the importance of a timely diagnosis will likely increase further.

Current diagnostic process and challenges

The diagnosis of MD can be difficult. The diagnostic process can be summarised as a long journey of collecting symptoms, signs, and findings, that ideally will end with a confirmatory genetic finding that corresponds to the disease phenotype. In the absence of clear molecular pathology, the physician may still confirm the MD diagnosis by a combination of clinical, radiological, and laboratory findings, where results from a muscle biopsy usually plays a major part. The *Bernier criteria* were once proposed as a standard for both molecular and non-molecular diagnosis.[134] The criteria have mainly been used in the context of research, and were more applicable historically, but illustrate well how major and minor findings may be synthesised into a final diagnosis.

Clinical presentation and physical examination

Because MDs have a highly variable presentation and are relatively uncommon in general practice, the first challenge is to even consider the diagnosis.

Despite the otherwise technological nature of the MD diagnosis, a proper history is indispensable. Some symptoms and signs are considered "red flags", which means that even when occurring in isolation they should prompt a baseline diagnostic evaluation for MD.[133] This list includes many neurological findings, such as myoclonus, ataxia, and stroke-like cerebral lesions without obvious vascular pathology. Other signs and symptoms, such as failure to thrive, pigmentary retinopathy, or intractable epilepsy, are less specific to MD. In these cases, the combination of several, seemingly unrelated symptoms from different organ systems, or the absence of another more plausible diagnosis, should raise the suspicion of MD.[133]

Some clinical presentations are quite characteristic and will promptly point the clinician in the right direction. Other times, even in the hands of specialists, the investigation may continue for years without a definitive diagnosis.[135]

Progressive symptoms are indicative but not obligatory. A positive family history, especially with a maternal inheritance pattern, increases the likelihood of MD – but a negative one does not exclude it.[136]

The physical examination should include a thorough neurological examination, and a general physical examination to identify involvement of other organ systems.

Plasma lactate

Elevated plasma lactate is a considered a classic finding in MD, either with concurrent acidosis (lactic acidosis) or without (hyperlactataemia). It is very logical for ETS dysfunction to cause an increase in lactate, by feedback mechanisms affecting the enzyme lactate dehydrogenase (LDH, Figure 4d).

Plasma lactate is very easy to test but suffers both from low specificity and sensitivity. A high lactate may be caused by many other acute conditions, and some patients with MD do not exhibit elevations. In some instances of MD, lactate will rise only during acute metabolic decompensation and otherwise stay low.[133] (The pathophysiology of elevated lactate will be further explained in the last part of the Introduction section.) According to a summary statement by the Mitochondrial Medicine Society (MMS), the sensitivity of elevated lactate is 34-62%.[84]

Other non-invasive or accessible initial tests

In addition to plasma lactate, there are several tests that are recommended in the basal investigation.[133] Some of these are tests that may exclude or confirm alternative diagnoses (such as organic acids in urine), or identify additional organ dysfunction (such as infectious panels, and tests of liver and kidney dysfunction). Pyruvate is sometimes measured along with lactate, as the pyruvate:lactate ratio may sometimes help elucidate the cause of the elevated lactate and separate MD from PDH deficiency.[137] (PDH deficiency is usually not classified as an MD.)

If the patient has neurological symptoms, lactate may also be measured in cerebrospinal fluid (CSF) from a lumbar puncture. This is slightly more invasive than a blood test but, on the other hand, CSF lactate is considered less prone to spurious elevations and poor sampling technique. However, CSF lactate may rise in several other conditions included in the differential diagnosis (such as meningitis and malignancy), and may also be normal in some cases of MD.[138,139]

Neuroradiology

Neuroradiology is important part of the diagnostic process. Useful modalities include magnetic resonance imaging (MRI) and magnetic resonance spectroscopy (MRS). In some cases, the patterns seen on MRI may be very characteristic. For example, focal symmetrical lesions of the basal ganglia, diencephalon and/or the brainstem on MRI are strongly associated with Leigh syndrome.[140]

In many other instances, neuroradiological findings are present, and compatible with MD, but too unspecific to exclude other diagnoses. In a minority of MD cases, neuroradiology is normal.[136,140] Normal findings are more likely when patients have isolated myopathy.[141]

Muscle biopsy

Ever since Luft's first case of MD, muscle studies have been a cornerstone of MD diagnostics. As mentioned earlier, a muscle biopsy is considerably more invasive than a blood sample, especially for paediatric patients.

A muscle biopsy can be used for histological studies, enzyme assays and respirometry, and to extract material for genetic testing (see below). Typical histological findings include the appearance of ragged red-fibres (RRF) and COX-negative fibres on histochemical staining.[142] Spectrophotometric enzyme assays are used to analyse the activity of the ETS enzyme complexes individually, while respirometry mimics in vivo conditions more closely, measuring the efficiency of enzyme complexes in cooperation with each other.

An advantage of these studies is that they can visualise the core pathological mechanism directly, identifying a single enzyme complex as the culprit. A clear pathological finding that is in line with the phenotype, together with a causative mutation, leave little doubt about the MD diagnosis.

Muscle biopsies are often important but not always conclusive. It is important to note that a muscle biopsy is not a binary test but rather a series of analyses rich with nuance. In a certain clinical context, certain combinations of histological and enzymatic findings may be highly specific for MD. Even so, there are examples of false positives, where diseases with similar presentations cause secondary mitochondrial dysfunction, and of false negatives.[72,136,142,143]

Genetic testing

There are around 1 500 known nuclear genes encoding mitochondrial proteins. Most MDs are caused by nDNA mutations with a classic Mendelian inheritance pattern, normally autosomal recessive inheritance. Autosomal dominant and X-linked inheritance also exist.[144] Mutations may be point mutations, deletions, or insertions, either directly affecting the subunits of the ETS or, indirectly, by affecting factors such as protein assembly and transport. When the translation of mitochondrial genes is affected, this sometimes leads to a phenomenon called mtDNA depletion (quantitative loss of mtDNA), which can vary a lot in phenotype.[145]

The possibility of mtDNA mutations further complicates genetic studies. Mutations in mtDNA normally have a maternal inheritance pattern. Identification of these mutations can be difficult, as not all tissues, nor all mitochondria within one tissue or cell, may carry the pathological mutation (heteroplasmy). As a rule, mutations are more likely to be found in tissues that give rise to symptoms, which poses a problem in diagnostics. For example, if the disease manifestations are confined to the CNS, genetic confirmation may only be possible post mortem.[136]

The existence of an mtDNA mutation, conversely, may not always correlate to disease manifestation. A minority of mitochondria in a certain tissue may carry a pathological mutation below a certain "threshold", where metabolism remains unaffected.

In recent decades, there have been striking technological advances in molecular diagnostics. Testing has moved from testing panels of genes to whole-exome sequencing (WES), which, in turn, is being successively replaced by whole genome sequencing (WGS).[144] This development has made the diagnostic process less reliant on muscle biopsy, with some suggesting it may be omitted in as many as half of the cases.[144,146,147] Even so, some cases still elude molecular confirmation.[148,149]

Diagnostic challenges and possible improvements

Despite recent advances, the diagnosis of MD remains a challenge. In a recent study, the mean time from symptom onset to MD diagnosis was 10 years. The patients, on average, saw seven different physicians during the process.[135] In Sweden, an extensive MD investigation, including a muscle biopsy, can only be performed at one of two specialised MD centres in Gothenburg and Stockholm. Neither one of the main diagnostic pillars – muscle biopsy and genetic testing – is easy and accessible.

There are many conceivable ways to improve the complex diagnostic process. While improvements at the back end of the process – molecular testing – have been impressive, there has been little progress at the front end. The MMS has pointed out in a consensus statement that it is a problem that, despite advances in other areas of diagnostics, MDs "still lack sufficiently sensitive and specific biomarkers".[84] New biomarkers, particularly in blood, was one of the top 10 research priorities in the field of mitochondrial diseases recently outlined by a priority setting partnership (PSP), where both clinicians and patients participated.[150]

A relatively easy, accessible, and non-invasive test, that could be performed at an early stage of the investigation and still provide relatively high diagnostic accuracy, would be helpful. A test that could easily exclude MD would be most valuable, but early and accurate confirmation could also be an improvement.

Lactate as a clinical test

Lactate in health and disease

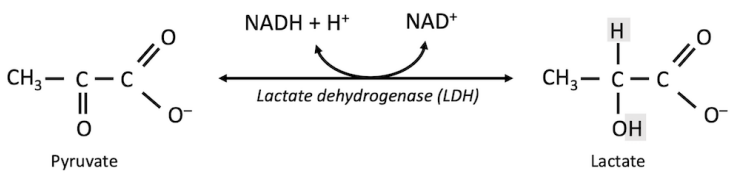
An old metabolite in a new suit

Lactic acid was first discovered in sour milk in 1780 by the Swedish chemist Karl Wilhelm Scheele.[151] Later it was found in the tissues of animals and humans, where it mainly exists in the form of lactate, its conjugate base.

Though extensively studied for over two centuries, recent discoveries have led to a re-evaluation of the traditional view on lactate – which is no longer seen as a deleterious waste-product of anaerobic metabolism.[152-155] Aerobic lactate production has turned out to be common in both health and disease, and lactate appears to be not a "waste product", but an important energy substrate, whose role we likely do not yet fully appreciate.[28]

After Scheele's initial report, a series of different discoveries were made during the 19th century to elucidate the role of lactate in animal biology. Several discoveries were made independently by different scientists, apparently unaware (or even wilfully ignorant) of each other's findings, and oftentimes the results were conflicting.[156] Nonetheless, a picture gradually emerged that lactate was produced in muscle tissue during intense muscle contractions, accompanied by widespread speculation that this was caused by local tissue hypoxia.

Exercise-induced lactate elevation in muscles was eventually confirmed experimentally by Fletcher and Hopkins, in Cambridge in 1907.[157] Their publication was very influential and also provided some indirect evidence that hypoxia causes elevated lactate. The mechanism for this is by now well-known. Lactate is the terminal product of glycolysis (Figure 4a). In the absence of oxygen, the ETS comes to a halt, inhibiting the further and complete oxidation of pyruvate in the Krebs cycle to CO_2 and H_2O. Instead of entering the mitochondrion, pyruvate is converted into lactate, by the enzyme lactate dehydrogenase (LDH), simultaneously oxidising NADH + H^+:



There is no doubt that tissue hypoxia can cause elevated lactate. Unfortunately, this fact was for many years supplemented by an erroneous inference of the reverse: that elevated lactate must always be a sign of hypoxia.

When Warburg discovered that cancer cells are prone to aerobic glycolysis (as mentioned earlier), the discovery was based on elevated lactate, found in venous blood draining tumour-infested limbs.[158] Thus, the phenomenon of aerobic lactate production was known already in the 1920s – but it did not fit into the contemporary understanding of normal physiology. Instead, the enigmatic Warburg effect was long thought of as a special case of cancer pathology (and, as it turned out, was not even applicable to all types of cancer cells).

However, several successive studies from the 1960s and onward showed that lactate production in exercised muscle is not primarily caused by hypoxia, but by a combination of other mechanisms, including accelerated aerobic glycolysis (Figure 4a) and insufficient lactate removal (Figure 4g–f).[155]

The realisation that lactate is often produced under non-hypoxic conditions opened up the possibility that lactate may not just be metabolic waste, or, in the context of exercise, an unsolicited inducer of muscle fatigue. The LDH reaction being bidirectional, circulating lactate can also be taken up by cells and reconverted into pyruvate. The reaction was originally emphasised mainly in the liver, which can use lactate to regenerate glucose and release it back into the bloodstream. This is known as the Cori cycle (Figure 4e).[159] But pyruvate, regenerated from lactate, can also fuel the Krebs cycle for complete oxidation. This happens not just in liver cells but in all tissues in the body (Figure 4g). In fact, isotope tracing studies indicate that the whole-body utilisation of lactate as an energy substrate may be on par with glucose, even exceeding it under certain conditions.[160]

Why would cells sometimes use lactate rather than glucose? According to the lactate shuttle theory, pioneered by George A. Brooks in the 1980s, lactate is shuttled from cells and tissues with a net lactate production, and consumed and oxidised by other cells with capacity to do so (Figure 4g).[161] This happens for several postulated reasons, including redox regulation and energy redistribution to vital organs. The value of lactate as energy substrate explains why normally just a few percent of it will be excreted by the kidneys in urine, this fraction representing the only "true" case of lactate as a waste product (Figure 4f).[162]

Lactate elevation in human diseases

While the overall turnover of lactate is high, whole-body production and elimination are normally balanced, keeping the lactate concentration in blood steady at around 1 mmol/L. Increased blood concentrations occur when homeostasis is disturbed: by physical exercise, medications, inborn errors of metabolism in various pathologic states, including – but not limited to – tissue hypoxia.

In clinical medicine, just like in physiology, the view of lactate has shifted and become more nuanced. The German physician Johann Joseph Scherer was the first to demonstrate increased lactate concentrations in diseased patients. In

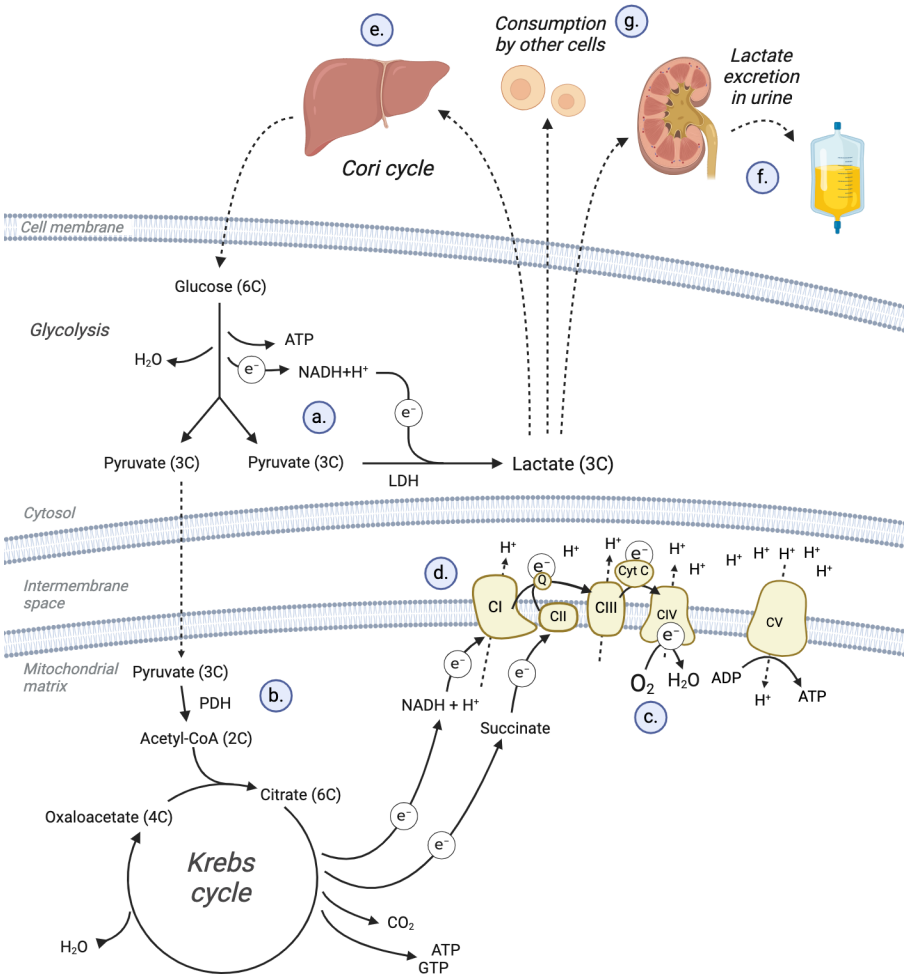


Figure 4 Lactate metabolism and mechanisms of lactate elevation.
A schematic illustration of lactate metabolism and principally different mechanisms of elevated lactate: Increased pyruvate production beacause of **a)** increased rate of glycolysis or shifts in redox balance; decreased pyruvate utilisation because of **b)** deficient or dysfunctioning PDH **c)** cellular hypoxia due to tissue hypoperfusion (shock, arterial insufficiency), **d)** dysfunciton of the ETS; or, decreased lactate "clearence" because of **e)** liver failure/dysfunction, **f)** kidney failure/dysfunction, or **g)** insufficient compensatory uptake by other local or distant cells (the lactate shuttle). ATP = adenosine triphosphate. ADP = adenosine diphosphate. C = carbon. CI-CV = complex I-V. CoA = Co-ezyme A. Cyt C = cytochrome C. e^- = electron. $FADH_2$ = flavin adenine dinucleotide (reduced). GTP = guanosine triphosphate. $NADH+H^+$ = nicotinamide adenine dinucleotide (reduced). PDH = pyruvate dehydrogenase. Please note that the illustration is a simplified summary of metabolic pathways and is not meant to represent balanced biochemical reactions (for example the numbers of electrons or protons). *Created in BioRender.*

Table 1 Mechanisms of lactate elevation
The letters in parentheses refer to Figure 4. Adapted and modified from Müller et al.[163]

Mechanism	Clinical examples
Increased lactate production	
Increased pyruvate production	
Increased glycolysis (a)	Endogenous catecholamine stimulated glycolysis (sepsis[164], phaeocromocytoma[165], stress), Warburg effect in cancer[158], inhaled beta agonists[166] or iv adrenaline[167], redox shift (ethanol intoxication[168]), respiratory alkalosis (from hyperventilation[169])
Decreased pyruvate utilisation	
Deficiency of PDH (b)	Inherited pyruvate dehydrogenase (PDH) deficiency[170], aquired thiamine deficiency (alcoholism, malnutrision[171]), sepsis-induced PDH dysfunction[82]
Insufficient intracellular oxygen (c)	Tissue ischaemia from shock or regional hypoperfusion, (profound) hypoxia or anemia, carbon monoxide (CO) poisoning[172], relative oxygen deficiency in intense muscle contraction (physical exercise, seizures)
Dysfunction of the ETS (d)	Primary mitochondrial disease (MD)[170], aquired ETS dysfunction because of drugs (metformin[81], antiretroviral drugs[173]) or poisoning (cyanide[174])
Decreased lactate removal	
Impaired liver function (e)	Liver failure[175]
Impaired renal function (f)	Renal failure[176]
Insufficient consumption (g)	Multifactorial, including liver and kidney failure, and unknown mechanisms

1843, he published a series of cases of septic and haemorrhagic shock with elevated lactate in blood, a finding that he had not made in healthy people.[156,177]

In the mid 20th century, tissue hypoxia due to hypoperfusion was popularised as the main mechanistic explanation behind hyperlactataemia.[178,179] Also, an association was noted between elevated lactate and severe acidosis in critically ill patients, leading to the assumption that lactate causes harm by inducing acidosis ("lactic acidosis").[178]

However, cases of elevated lactate in diseases without apparent signs of hypoxia or hypoperfusion had also been noted repeatedly since the 19th century (for example, in leukaemia patients). For a while, this category was referred to as "idiopathic" lactic acidosis. In 1970, Cohen and Woods renamed hypoxic and idiopathic lactic acidosis as Type A and Type B lactic acidosis, respectively.[180] This nomenclature is still in widespread use today and works in theory, but it has become gradually clear that lactate elevation is, in many cases, multifactorial, and that pure Type A lactic acidosis is the clinical exception rather than a rule.

As an alternative to the Type A/B-classification, another framework for understanding lactate elevation is to divide the causes into increased production

and decreased elimination. Increased production can, in turn, be divided into factors that increase pyruvate production, and those that decrease pyruvate utilisation, respectively (Table 1).[163] (To be noted, several disease states have multifactorial causes of elevated lactate and some of the pathophysiological explanations in Table 1 are contested or sparsely studied.)

Hyperlactataemia vs. lactic acidosis

The expressions "hyperlactataemia" and "lactic acidosis" are sometimes used interchangeably in clinical practice, possibly because patients often have both. Hyperlactataemia is defined as an elevated concentration of lactate in plasma, usually above 2 mmol/L. Lactic acidosis is defined as hyperlactataemia and a concomitant acidosis, i.e., the assumed existence of an acidifying process in the bodily fluids. Unless a simultaneous alkalotic process is occurring, acidosis will manifest itself as acidaemia, defined as a lowered plasma pH, usually ≤7.35 (in arterial blood).

Does lactate cause acidosis? This is a complex question which has been the subject of repeated scientific debates.[181-185] Since lactate itself is not an acid, but a weak base, the answer is not as straightforward as one might think. Biochemical theory aside, lactate levels do not correlate strongly with acid-base status neither in clinical settings nor in animal models.[186,187] As for interventional experiments, results point both ways. An in vitro study showed an undeniable correlation between lactate titration and markers of acidosis. On the other hand, considerable amounts had to be added before it would actually result in acidaemia.[188] A clinical study found that the administration of exogenous lactate to a group of patients only resulted in acidaemia for less than half of them.[189]

In any case, it is safe to conclude that in clinical settings elevated lactate may occur both with and without acidaemia, and it is possible that the two entities have different causes and outcomes.

Elevated lactate in the emergency department

Lactate as a prognostic marker

Plasma lactate is a popular test in the ED, probably in part because it is a very accessible test included in most modern blood gas analyses. In emergency medicine, it has mainly been studied in relation to sepsis, where its association with poor prognosis is well established.[190]

Outside of sepsis, lactate is less studied in the ED, and the clinical interpretation tends to be less straightforward. Recent clinical reviews on lactate promote the consideration of a broad differential, beyond tissue hypoxia and sepsis.[191-193] In light of our evolved understanding of lactate metabolism, lactate

could be a potential diagnostic or prognostic tool in a wide variety of acute conditions. However, since lactate is now known to have several beneficial functions in normal physiology, it is not obvious that elevated lactate should always be a sign of poor prognosis.

While there is a stronger tradition of studying lactate in the intensive care setting, there are also several studies on lactate and mortality in a general ED population. A systematic literature search conducted in October 2024 yielded 43 studies on the subject (for details, see Appendix).[194-236] Many of the older studies have small or moderate population sizes but in recent years there has been an addition of several larger studies.

All studies referenced above found an association between elevated lactate and mortality in general ED populations. The strength of the association and the prognostic accuracy varied.

This may partly be explained by variations in study objectives (in hospital-mortality, 30-day mortality, etcetera) and study populations (unselected patients, patients ≥65 years, critically ill patients, etcetera). This also makes formal meta-analysis difficult; instead, the main findings will be summarised here narratively. The area under the receiver operating characteristic (AUROC) values for arterial or venous lactate as a predictor for mortality, when reported, ranged from 0.64 to 0.89 across 18 studies.[194-211] In the three largest of these studies, comprising 108 553 cases (more than two thirds of the combined study populations), AUROC values were 0.69, 0.71, and 0.72, respectively.[202,209,210] The optimal diagnostic cut-off value for lactate, when reported, ranged from 1.4 to 6.0 mmol/L across 13 studies.[194-204,232,236] The largest study reporting an optimal cut-off value for the initial lactate in the ED (Park et al., 2018) put it at 2.6 mmol/L, with a resulting sensitivity of 57%, and a specificity of 74%.[202]

Despite an unquestionable association to mortality, it is still not obvious exactly when and how lactate is of clinical use in the undifferentiated ED population. The positive and negative likelihood ratios extrapolated from the aforementioned 2.6 mmol/L cut-off would be 2.19 and 0.58, respectively, values usually considered small in terms of clinical impact.[237] (For elaboration on likelihood ratios, please see Methods.)

In an effort to enhance its utility, a handful of studies have compared the prognostic accuracy of lactate depending on aetiology. Some were only able to compare large subcategories because of constraints in sample size, but could at least show that, in addition to infectious aetiologies, lactate is also predictive in non-infectious medical conditions, and trauma.[202,206,210,218,238] Contenti et al. (2019) compared the subgroups of "infection" and "fainting/seizures" and showed that lactate predicted a severe outcome in the former but not in the latter.[218] Pedersen et al. (2015) have made the most detailed aetiological analysis. Examining 12 aetiology subgroups separately, they saw that lactate was predic-

tive in infections, trauma, cardiac diseases, and intestinal diseases, but not in respiratory or neurological diseases (including seizures). Many of the remaining subgroups, including the important subgroup of renal and liver diseases, were too small to yield conclusive results.[231]

Only a minority of the studies in a general ED setting have integrated acid-base status in their analysis of lactate. Base excess, pH, and HCO_3, have all been shown to predict mortality, but it has been unclear to what degree these associations were independent from lactate.[220,228] In a recent study by D'Abrantes et al. (2021), where lactate and acid-base status were the main focus, lactate's predictive value on mortality was much lower in patients without concomitant acidosis.[219] They did not, however, adjust for confounders or assess the prognostic utility.

Unanswered questions

From other research, as summarised above, there is reason to believe that both aetiology and acid-base status are important factors that could influence the interpretation of lactate in the ED.

With a sufficiently large population of patients with hyperlactataemia, it would be possible to both corroborate previous findings, and to add knowledge on the smaller, less well-described aetiological subcategories. In addition, the relationships between lactate, acid-base status, and mortality, could benefit from further clarification.

Aims

This thesis investigates new possibilities in the assessment of mitochondrial disease, and in other types of metabolic dysfunction.

The aims of the thesis were:

- To investigate the blood cell respirometry as a biomarker for mitochondrial function in muscle fibres and for systemic conditions (Paper II and Paper III).

- To investigate the utility of blood cell respirometry and lactate in the diagnosis of mitochondrial disease (Paper I).

- To investigate the prognostic value of elevated lactate in the emergency department, and how aetiology and acid-base status could influence the interpretation (Paper IV).

Methods

Study participants

Paper I

Blood samples were collected from all participants, including both patients and healthy controls. Paediatric patients with suspected mitochondrial disease presenting to Skåne University Hospital (Lund, Sweden) from July 2008 through December 2013 were eligible for inclusion. Reference values for the platelet respirometry method were established in a group of otherwise healthy children scheduled for minor elective surgery.

Paper II and III

For the comparison of respiration between blood cells and muscle fibres, muscle biopsies were taken from a group of 24 healthy volunteers, in addition to blood samples. Included in the group were five semi-professional athletes, used in one subgroup comparison.

For the comparisons between platelets and PBMCs, for the ageing study, and for other methodological assessments, a larger data set was used. Samples were compiled retroactively from all experiments performed at our lab during a 10-year period. One might describe this as a kind of "internal" meta-analysis. Part of the data from these experiments has been used in other publications from our lab (including in Paper I).[239-244] The pooling of data was possible since the same SUIT protocol (see Methods) had been used in our lab across various studies during the period.

Paper IV

This was a registration-based cohort of patients in EDs across the Skåne Healthcare Region (Region Skåne) in southern Sweden. All adult patients with an elevated plasma lactate (≥2 mmol/L) analysed during an emergency department visit from January 2015 through December 2016 were eligible for inclusion.

Assessment of mitochondrial function

High-resolution respirometry in blood cells

General background and principles

For this thesis, mitochondrial function was primarily assessed by high-resolution respirometry in permeabilised blood cells (platelets and PBMCs), using the Oroboros Oxygraph 2k (Oroboros Instruments, Innsbruck, Austria).

There are many ways to assess mitochondrial function and the most appropriate method will depend on the context. First, one must decide which function to test. As was discussed earlier, mitochondria have many functions beside metabolism. However, mitochondria's main function is to generate ATP by oxidative phosphorylation, and this is the traditional and most common function to assess.

Figure 5A shows a conceptual illustration of mitochondrial metabolism adapted from Brand and Nicholls (2011), as a framework for understanding different methods of mitochondrial assessment.[245] As a simplified description, substrate oxidation will generate a protonmotive force (pmf), which will be consumed by a combination of processes related to either ATP turnover or proton leak. The schematic modules in the figure can be broken down into several smaller components, but the pmf is a common intermediate between all of them. The pmf drives the inward proton current across the IMM, which, in turn, is tightly coupled to the consumption of oxygen within complex IV (CIV) of the ETS. Thus, oxygen consumption is a good proxy for the pmf and the proton current.

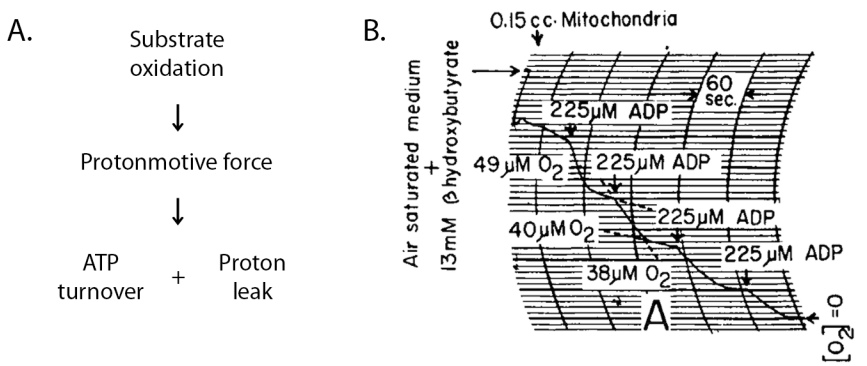


Figure 5 Mitochondrial functional assessment by respirometry
A. A conceptual, simplified illustration of the main 'modules' of mitochondrial metabolism, adapted from Brand and Nicholls (2011).[245] **B.** Example of a graph depicting oxygen concentration over time (oxygraphy) from Chance and Williams (1955) (licenced under CC by 4.0).[246]

By measuring oxygen consumption and controlling one or more of the remaining three modules (substrate oxidation, ATP turnover, proton leak), it is possible to design experiments that assess not just mitochondrial metabolism in general, but also many of its individual components.

The modern assessment of mitochondrial function was pioneered by Chance and Williams in the 1950s, who used a microelectrode to measure oxygen levels in a mitochondrial suspension.[246] Oxygen is consumed at the cathode of the electrode, generating a measurable electrical current that correlates to the concentration of oxygen dissolved in the liquid. The method is known as polarographic respirometry and can be used to plot the oxygen concentration over time ("oxygraphy"). An illustrative trace, from one of Chance and Williams 1955 publications, is shown in Figure 5B. As an example, the addition of ADP increases the rate of oxygen consumption, which can be seen in the graph as a steepening of the slope beginning at the arrow.

Since the 1950s, polarographic technology has advanced considerably and, in the 1990s, it was developed into the modern concept of high-resolution respirometry.[247,248] New technologies, such as the Oroboros, provides air-tight chambers that minimise oxygen diffusion, high-accuracy electrodes, and powerful software for live recordings and calculations.[249] A key advantage of the new methodological standard is the ability to measure very subtle changes in respiration. For this purpose, modern oxygraphy allows the measurement of the time derivative of the oxygen concentrations (the oxygen flux, usually measured in pmol $O_2 \cdot s^{-1} \cdot$ n of cells, volume, or other reference unit), greatly enhancing precision compared to earlier methods.

Nevertheless, the main principles are still the same: the measurement of mitochondrial oxygen consumption in the unaltered state, and/or the addition of certain substances followed by measurements of resulting changes in the rate of oxygen consumption.

Sample acquisition and preparation

Samples for mitochondrial assessment presented in Paper I-III were drawn from venous blood, collected in EDTA vials, and analysed within 3–5 hours. The methods of centrifugation and preparation of platelets and PBMCs are described in detail in the methods section of respective paper (most extensively in Paper II) and were essentially the same as in earlier publications from our lab.[239,250]

Conveniently, platelets and PBMCs can be prepared from the same blood sample and 6–12 ml generally yields enough of both cell types to perform all intended analyses. For the respirometry protocols in permeabilised cells, cells were suspended in a physiological mitochondrial medium (MiR05).[247] The chamber volume of the Oxygraph was 2 ml. For all platelet experiments, a

chamber concentration of 200 · 10^6 cells/ml was used. For the PBMC experiments, the concentrations were 2.5–5.0 · 10^6/ml (usually 5.0).

The SUIT protocol

Mitochondrial function was assessed by what is known as a substrate-uncoupler-inhibitor titration (SUIT) protocol. There are many versions of such protocols used in various settings and with different objectives, which all have their roots in the work by Chance and Williams. The protocol used in our lab, for the experiments in Paper I-III, was developed by several of my lab colleagues and current supervisors, including Dr Fredrik Sjövall, and in collaboration with Dr Prof Erich Gnaiger, based on his previous work in muscle respirometry.[239,250,251]

Figure 6 shows the principal effects of the additions made during this protocol on different parts of the ETS. In short, the substrates increase respiration by reducing enzyme complexes (CI or CII), the inhibitors stop electron transfer in respective enzyme complex and "upstream" from that complex, and the uncouplers allow protons to be released back across the IMM independently of ATP generation.

When added in a certain sequence, the SUIT-components will generate a trace (oxygraphy), which is schematically illustrated in Figure 7. The grey line and the right y-axis represent oxygen concentration over time (corresponding to the graph in Figure 5B). The black line and the left y-axis show the negative time derivative of the oxygen concentration.

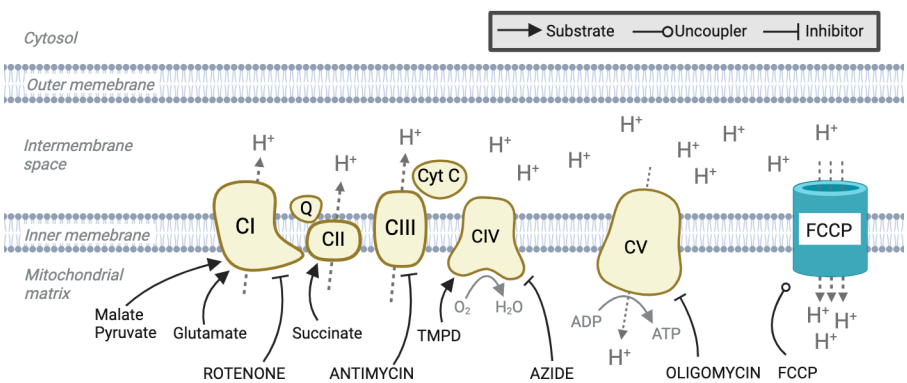


Figure 6 Substrates, uncouplers and inhibitors
Illustration of the components of the SUIT-protocol and their respective effects. For abbreviatinos, please see the list at the beginning of the thesis summary. *Created in BioRender.*

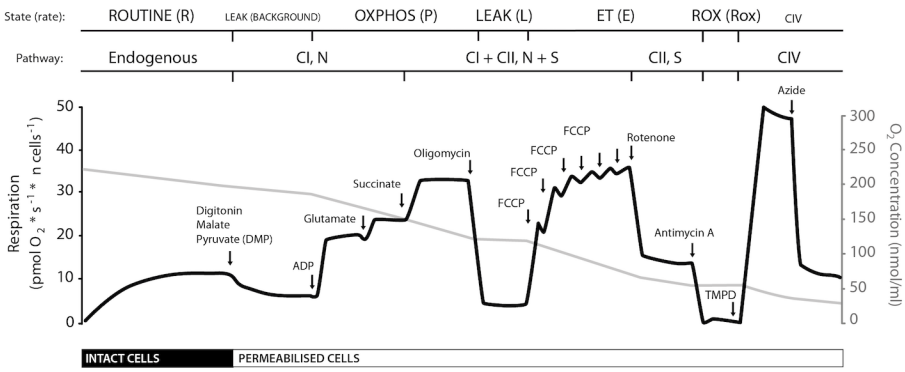


Figure 7 SUIT protocol for permeabilised blood cells

An illustration of the study protocol used for platelets and PBMCs. The gray line indicates the O2 concentration in the chamber, corresponding to the right y-axis. The black line indicates the O2 flux measured per n number of cells (n = 10^{-8} platelets, n = 10^{-6} PBMCs, respectively), corresponding to the left y-axis. Additions of substrates, inhibitors and uncouplers to the chamber are indicated by the arrows. The first top bar illustrates respiratory states and corresponding rates (except "CIV", which is not a respiratory state). The second top bar illustrates main pathways of electron transfer and the respiratory complex through which the electrons are donated. For abbreviations, see the list at the beginning. The figure and caption are adapted from Paper II.[252]

The top bar indicates the coupling state of the mitochondria, and the pathway or pathways through which electrons are transferred to the ETS. The terminology used in this text is mainly based on the consensus developed by the MitoEAGLE task group, but some deviations and compromises were made.[253]

The following text describes the protocol from left to right (Figure 7). First, routine respiration is measured in intact cells, oxidising endogenous substrates. The addition of digitonin permeabilises the cells, making them accessible to the experimental additions of substrates, the first two of which are the CI substrates malate and pyruvate. Since electrons are transferred to CI via NADH + H^+, this is also known as the N pathway. With the addition of ADP, respiration through the N pathway is stimulated, and increases further with the addition of glutamate.

Succinate transfers electrons to CII, which is known as the S pathway. When succinate is added, maximum OXPHOS capacity is achieved, with electron transfer converging from the N+S pathways (CI+CII).

The addition of oligomycin inhibits CV, and as ADP is no longer being phosphorylated, this brings the ETS almost to a halt. However, there are still some protons leaking across the IMM, and some electrons are still transferred through CI-CIV to compensate for the leak, resulting in a low but measurable rate of oxygen consumption. This is known as the LEAK state.

An uncoupler (FCCP) is then titrated to achieve a maximally noncoupled state, where electrons are transferred through the ETS (CI-CIV) at high rates to compensate for the backflow of protons created by the uncoupler. This is known as the ET (electron transfer) state, as the O_2 consumption is no longer limited by oxidative phosphorylation, but by the capacity to transfer electrons. The ET capacity will normally exceed the OPXHOS capacity. It should, theoretically, never be below it.

In the next step, rotenone is added to inhibit CI, revealing the individual contribution of CII, or the S pathway, in the ET state. (This might sometimes be a slight overestimation, due to incomplete additivity.)

The addition of antimycin A inhibits CIII, bringing electron transfer to a complete halt. The remaining oxygen consumption rate (Rox, residual oxygen consumption) is assumed to be non-mitochondrial (unrelated to the ETS) and is subtracted from all previous values.

Lastly, TMPD and azide are added to oxidise and inhibit CIV, respectively. The difference between the TMPD and azide measurments is an estimate of CIV function. (In the studies included in the thesis, this step was sometimes omitted from the protocol.)

Normalisation methods

In the protocol as described above, respiration is normalised to cell count ($O_2 \cdot s^{-1} \cdot 10^{-8}$ platelets, $O_2 \cdot s^{-1} \cdot 10^{-6}$ PBMCs, respectively). It is sometimes desirable to know the respiration per cell (or cells) but without further normalisation it is impossible to tell whether observed changes in respiration are due to changes in mitochondrial content (number and size of mitochondria per cell) or intrinsic changes in the function of each mitochondrion.

For this purpose, there are several different ways to normalise respiration to mitochondrial content. Two of the common approaches, which both have been used in our lab, is to measure the activity of citrate synthase (CS), or the amount of mtDNA. Citrate synthase is a protein which is abundant in the matrix and its activity can be measured easily with commercially available kits. In a study by Larsen et al. (2012), CS activity was shown to be superior to mtDNA as a biomarker of mitochondrial content in muscle.[254] It should, however, be noted that the performance of normalisation methods may vary across tissues.[255] In a previous publications from our lab, there was a relatively high correlation between CS activity and mtDNA in PBMCs, but less so in platelets.[242,250] CS activity was chosen over mtDNA for normalisation in Paper II and III.

Another normalisation method is the use of internal respiratory ratios. The basic principle is to divide one respiratory rate from the protocol with another. This normalisation method may also be more informative of intrinsic mitochondrial effects than cell normalisation (revealing, for example, an isolated

defect in a single ETS complex), but is strictly speaking a way to normalise respiration independent of both cell number *and* mitochondrial content.

The respiratory control ratio (RCR) is a classic respiratory ratio used since the 1950s. In the original terminology, the ratio was defined as State 3/State 4 respiration in isolated mitochondria, which is similar (but not entirely equal) to OXPHOS capacity/LEAK respiration in Figure 7.[256] A high ratio usually indicates that substrate oxidation is tightly coupled to ATP generation and was originally used as a quality metric when isolating mitochondria. The RCR is still widely used today, as many types of mitochondrial dysfunction – both laboratory errors and actual pathology – affect the RCR.[245]

In Paper I, respiratory ratios were the only method of normalisation used. We used a combination of established and unestablished ratios, to cover different aspects of ETS dysfunction, and selected these a priori for the assessment of diagnostic accuracy.

Some of the ratios in Paper I overlap with the ratios in Paper II and III. The best summary of all relevant ratios in this thesis summary is found in Table 2 of Paper II (where several of the ratio names are based on the MitoEAGLE consensus terminology).[253]

Limitations and alternative methods

There are many ways to assess mitochondrial metabolic function and all methods have their advantages and limitations. As a rule, employing several methods in parallel will strengthen a study. A general limitation of this thesis is that the experiments used in Paper I-III were made only in permeabilised cells, with respirometry as the only method.

From a broad perspective, mitochondrial function can be studied *in vivo* and *ex vivo*; the latter includes studies in tissue samples, intact or permeabilised cells, or in isolated mitochondria.[245] As that list descends to smaller and smaller units, the ability to control the mitochondria experimentally increases, and non-mitochondrial sources of error decrease. These advantages come at a price: with smaller units of study, the conditions become less physiological.

Historically, much mitochondrial research was performed in isolated mitochondria, which are easily controlled and well suited for mechanistic inquires. However, much of mitochondrial function is dependent on their movements, and their interaction with each other, with non-mitochondrial cellular substrate metabolism, and with other organelles, including the ER (as exemplified by the MAM-theory, described earlier). Furthermore, there is concern that the isolation process itself may damage the mitochondria.[257]

The use of permeabilised cells, as in our protocol, decreases the risk of damage while still allowing access for exogenous substrates. It also leaves some of the intracellular interactions intact. However, permeabilised cells are still less

physiological than intact cells.[245] Nonetheless, when aiming at intrinsic dysfunctions of the ETS, or individual complexes of the ETS, as with MDs in Paper I, the physiologic milieu may be less important.

An even less physiological and more easily performed type of study are individual enzyme assays of ETS complexes. This is standard to include in the clinical muscle biopsy studies when diagnosing MD.[142] Except that they are easy, there are, however, few other advantages over respirometry.[245]

In terms of the SUIT protocol itself, the consistent use of a uniform protocol was an advantage in Paper II and III. On the other hand, the protocol completely ignores some very important metabolic processes both outside of the mitochondria (such as glycolysis) and within them (such as lipid oxidation). When studying a complex systemic issue such as ageing, this is clearly limiting. So is the omission of genetic studies, as mtDNA is thought to have a central role in mitochondria and ageing.

High-resolution respirometry in muscle fibres

In Paper II, muscle biopsies were obtained in addition to blood samples for a subset of the participants. (The method of sampling and preparation is naturally different from blood samples, see Paper II for details.)

A protocol similar to the one presented in Figure 7 was used for the muscle fibres, with a few exceptions. The fibres were permeabilised with saponin during preparation (not with digitonin while in the chamber, as blood cells) and doses of ADP and oligomycin (in relation to chamber volume) were different. Respiratory measurements were adjusted to the respiratory rate before substrate addition (corresponding to Routine in Figure 7), not to the rate after addition of Antimycin A.

Clinical measurements of plasma lactate

All lactate measurements, both in Paper I (the paediatric cohort) and in Paper IV (the adult cohort), were made as part of the usual clinical management and values were extracted retrospectively. Analyses were made on local ABL machines (ABL800 Flex and ABL90 Flex, both by Radiometer).

There are several potential sources of error and variation in the clinical measurements of lactate, some of greater importance than others. Poor handling and phlebotomy tourniquets are often accused of causing falsely elevated lactate. Lactate concentrations will generally increase with storage time of the sample, but if the analysis is performed within 15 minutes, the effect is small.[258] As for

the possible interference of phlebotomy tourniquets, such fears are not supported by the available evidence.[258,259] A more reasonable concern is probably that intense physical struggling during sampling (young children, psychotic or intoxicated patients) could cause a spurious lactate elevation. It is unclear how much of a problem this is in practice.

Lactate can be sampled both venously and arterially. Usually, most lactate measurements in the ED are venous, with arterial samples reserved for the sickest patients.[218] Venous lactate measurements tend to be slightly higher than the arterial ones and there their interchangeability in the clinic has been debated. In general, differences are small, and values correlate well.[260,261] However, disagreement increases with higher lactate values.[262] In emergency medicine, venous and arterial lactate are considered close enough for clinical purposes, and preceding studies similar to ours have included lactate values from both sampling sites.[218,231,263]

Clinical patient data

The patients in Paper I were enrolled prospectively but much of the clinical data were collected retrospectively during the extended follow-up. It was mainly extracted from the patients' electronic health records, but also directly at the muscle pathology lab at Sahlgrenska University Hospital (Gothenburg), and, in one case, by correspondence with the mitochondrial centre at Karolinska University Hospital (Stockholm).

Retrospective data collection, even if done manually, is always susceptible to some degree of error, and especially to instances of incomplete data, which may introduce bias. Still, the 33 extended mitochondrial investigations were documented with high completeness. The categorisation of final diagnoses was usually clear from the records. In ambiguous cases, the interpretation of the mitochondrial investigations were discussed with one or more clinical paediatricians specialised in MD.

Paper IV, had a patient sample on a completely different magnitude, compared to Paper I (tens of thousands instead of 113). Patient data were extracted from registers, laboratory records and health records on an automated basis. As with all retrospective, register-type studies, the advantage of a large sample size is balanced against lower precision in the data. This applies both to lab data and patient records. While ICD discharge diagnoses have a comparatively high predictive values in Scandinavian countries, they are far from perfect in the individual case (as any Scandinavian clinician could probably attest to).[264]

Diagnostic test accuracy

The accuracy of a diagnostic test can be assessed and compared in different ways. Normally, the accuracy will be compared to a gold standard test. The results of this test will categorise patients into two categories: those who have the disease, and those who do not have the disease. The new test will be judged by how closely it can replicate the categorisation of the gold standard test.

The classic measurements of test accuracy are sensitivity and specificity. This terminology was developed in the first half of the 19th century with respect to certain immunological reactions, so the meanings of the words are unfortunately not intuitive in their modern context.[265] Sensitivity means "true positive rate", i.e., the share of patients with the disease who test positive. Specificity means "true negative rate", i.e., the share of patients without the disease who test negative. These values can be easily calculated from a 2 × 2 contingency table (here with fictitious numbers as an example):

		Status according to gold standard			
		Has the disease:	Does not have the disease:	Row total:	Calculations:
Diagnostic test	Positive test:	**15** (true positives)	**8** (false positives)	23	15/23 = **65% PPV**
	Negative test:	**5** (false negatives)	**72** (true negatives)	77	72/77 = **94% NPV**
	Column total:	20	80		
	Calculations:	15/20 = **75% sensitivity**	72/80 = **90% specificity**		

Sensitivity and specificity are good for comparison between tests and studies but are not the most meaningful clinical measurements. For example, a test with excellent sensitivity (100%) and abysmal specificity (0%) is clinically useless for any purpose – everyone will test positive regardless of disease and the test cannot be used to rule in, nor to rule out, the disease.

Clinically useful measurements need to integrate sensitivity and specificity in the same parameter and answer a clinically relevant question. For example, a clinician will normally want to know how much a positive test increases the likelihood of the disease, compared to how likely it was before ordering the test, and conversely, how much a negative test will lower the likelihood of the disease. Another way to put it is: How much does the test alter our current state of knowledge? These properties of a test are reflected in positive and negative likelihood ratios (PLR and NLR). A PLR should be greater than 1.0 and reflects

the ability to rule in disease based on a positive test. The NLR should be lower than 1.0 and reflect the ability to rule out disease based on a negative test. The PLR and NLR are calculated as follows:

PLR = true positive rate / false positive rate = sensitivity / (1–speciticity)

NLR = false negative rate / true negative rate = (1–sensitivity) / specificity

Likelihood ratios (LRs), like sensitivity and specificity, are measurements independent of disease prevalence, which facilitates comparison between different settings and studies. As a rule of thumb, the clinical impact of a test with a LR >10 or <0.1 is considered high, 5–10 or 0.1–0.2 is moderate, and 2–5 or 0.2–0.5 is small (but not necessarily useless). LRs of 1–2 or 0.5–1 rarely alter probability to a clinically significant degree, with a LR of 1 signifying no alteration.[237] Using the example numbers from the table above, the PLR would be 7.5 (a moderate impact on clinical decision making) and the NLR would be 0.3 (a small impact).

The receiver operating characteristics (ROC) curve is another, visual way of integrating sensitivity and specificity. It is a graphical plot that depicts the relationship between the true positive rate (sensitivity) and the false positive rate (1–specificity). The area under the curve (AUC) of the ROC (AUROC) measures the combined performance of sensitivity and specificity across the range of possible diagnostic cut-off values (and can also be used to find the optimal cut-off value). It is less clinically applicable than PLR and NLR, but a useful summary measurement for comparing tests or studies with each other.

The individual clinician, in the individual case, is probably most interested in knowing exactly how likely the patient in front of them is to have the disease. To know this, it is not enough to know the accuracy of the test, it is necessary to also know the prevalence of the disease. The positive predictive value (PPV) and the negative predictive value (NPV) combine accuracy and prevalence. The PPV tells the probability of having the disease if the test is positive, and the NPV tells the probability of not having the disease if the test is negative. Since the measurements are dependent on prevalence, they are less generalisable across studies and settings. Calculations are shown in the table above. Assuming a prevalence of 20%, the PPV would be 65% and the NPV 94%.

Diagnostic accuracy was assessed in Paper I. Paper IV is concerned with prognostic accuracy, which can be assessed by the same methods, but instead of a gold standard test, an outcome is used as reference (in this case, mortality).

It is important to note that the assessment of a test can never rely on numbers alone, regardless of which metric is being used. Two tests with equal accuracy can have very different value depending on the disease and the clinical context.

Study designs and other statistical methods

These are very brief, general comments. For comprehensive descriptions of statistical methods, please see the respective methods sections of Paper I–IV.

Paper I

Patients were enrolled prospectively. The result of the entire clinical investigation was used as gold standard, not an individual test. In practice, MD was confirmed by genetic testing in most cases. Test accuracy was presented as sensitivity, specificity, PLR, NLR, PPV and NPV, to facilitate interpretation in different contexts. The main tests were defined a priori and separated from the exploratory analyses, that were made after the main results were known.

Paper II and III

In Paper II, the Pearson correlation coefficient was calculated to assess correlation between respiratory parameters in platelets, PBMCs and muscle fibres. A limitation of assessing simple correlation, as opposed to using regression models, is the inability to properly adjust for confounders. Simple correlation was chosen, nonetheless, as the assumptions for regression analysis were not satisfied.

In Paper III, multiple linear regression was used to assess the association between ageing and blood cell respiratory function. This allowed the addition of patient samples to the healthy samples, with adjustment, thus increasing sample size. However, mixing patients and healthy participants may still be a source of confounding.

Paper IV

This was a retrospective cohort study. Multiple logistic regression was used to assess the value of lactate and pH as independent predictors of mortality. ROC-curves and AUCs were used to compare differences in the predictive accuracy of lactate, depending on pH and aetiology. Additional measurements of prognostic accuracy (such as LRs) were avoided in this study, as the inclusion of only patients with hyperlactataemia would likely cause spectrum bias.

Ethical considerations

Study approvals

The included studies were approved by the Ethical Review Board of Lund, Sweden (113/2008, 59/2009, 97/2009, 89/2011, 320/2011) and the scientific ethical committee of Copenhagen County, Denmark (H–C-2008-023).

Conflicts of interest

I have no financial conflict of interest. Several co-authors of the papers included in the thesis (including my supervisor, Eskil Elmér) have equity interests in and/or have received salary from Abliva AB (formerly NeuroVive Pharmaceutical AB), a public company developing medications in the field of mitochondrial medicine. For specific declarations, please see each paper respectively. I have referenced publications by these co-authors on the topic of MD treatment in the thesis summary.[27,132]

Results

Blood cell respirometry as a biomarker

For these studies, we performed high-resolution respirometry and measured CS activity in PBMCs, platelets and muscle fibres from healthy volunteers. We also pooled data from several earlier studies in our lab to be able to analyse normalisation methods, quality metrics and effects of ageing in a relatively large number of blood cells. This part of the results section references Paper II and III.

Correlation of respiration within and across tissues

Correlation of respiration between blood cells and muscle fibres

The study population for direct comparison between blood cell and muscle respiration comprised 24 healthy, non-smoking volunteers. Their ages ranged from 19 to 42 years, 10 were female and 14 were male.

The hypothesis was that there would be some degree of correlation, but this was not what we found.

In summary, mitochondrial respiration did not correlate significantly between blood cells and muscle fibres for neither PBMCs, nor platelets (Table 2). This was the case both for non-normalised values and for the normalised ones: both when using internal ratios (Table 2), and when normalising to CS activity (not shown, see Paper II).

The negative results prompted us to re-check the muscle respirometry traces manually for technical sources of error and this was done by a lab member who had not participated in the analyses. No obvious issues were found.

Correlation of respiration within blood tissue

To put the across tissue correlations in context, we also examined correlations within the same tissue. For this analysis we had a significantly larger dataset, paired samples of PBMCs and platelets from 318 unique individuals.

We had hypothesised that the within tissue correlation would be higher than across tissues, which it was.

Table 2 Correlation of respiration between blood cells and muscle fibres
For abbreviations, see list on p. 11 and Table 2, Paper II.

	PBMCs and muscle fibres		Platelets and muscle fibres	
	Pearson rho (95% CI)	p	Pearson rho (95% CI)	p
Absolute values				
Routine*	n/a	n/a	n/a	n/a
DMP*	0.31 (-0.63 – 0.11)	0.145	0.28 (-0.62 – 0.14)	0.180
ADP	0.28 (-0.14 – 0.61)	0.186	0.04 (-0.37 – 0.44)	0.849
Glutamate	0,20 (-0,23 – 0,56)	0.358	0.02 (-0.39 – 0.42)	0.945
Succinate	0.17 (-0.25 – 0,54)	0.430	0.17 (-0.26 – 0.53)	0.441
Oligomycin	-0.44 (-0.72 – 0.04)	0.032	0.29 (-0.13 – 0.62)	0.175
FCCP	0.03 (-0.38 – 0.43)	0.880	-0.03 (-0.42 – 0.38)	0.906
Rotenone	-0.16 (-0.53 – 0.26)	0.451	0.13 (-0.29 – 0.51)	0.545
TMPD-azide	0.27 (-0.16 – 0.61)	0.214	-0.25 (-0.60 – 0.17)	0.233
Internal ratios				
CI-RR	-0.40 (-0.69 – 0.01)	0.056	-0.01 (-0.41 – 0.39)	0.959
RCR	-0.38 (-0.68 – 0.03)	0.070	-0.02 (-0.42 – 0.39)	0.936
L/E CCR	-0.05 (-0.44 – 0.36)	0.821	-0.01 (-0.41 –0.39)	0.958
OCR	-0.19 (-0.55 – 0.23)	0.366	-0.37 (-0.67 – 0.04)	0.075
CI-CII-RR	0.14 (-0.28 – 0.52)	0.508	0.14 (-0.28 – 0.51)	0.530
L/P CCR	-0.33 (-0.65 – 0.09)	0.117	0.23 (-0.19 – 0.58)	0.285
N/NS RCR	0.31 (-0.11 – 0.64)	0.139	-0.03 (-0.43 – 0.38)	0.902
S/NS RCR	0.16 (-0.26 – 0.53)	0.451	0.35 (-0.07 – 0.66)	0.096

The within tissue correlation between different types of blood cells was significant but generally weak. Normalised values were generally more significant both for internal ratios and CS-normalisation (Figure 8).

Normalisation methods and other comparisons

Normalising by internal ratios was, as hypothesised, as good as CS normalisation, or possibly slightly better, when comparing the resulting degree of correlation between blood cell types (Figure 8).

We also analysed the correlation between CS activity and respiration within each cell type, which was higher for platelets than for PBMCs, and there were no significant differences in this parameter for patients, as opposed to healthy volunteers (not shown, see Paper II).

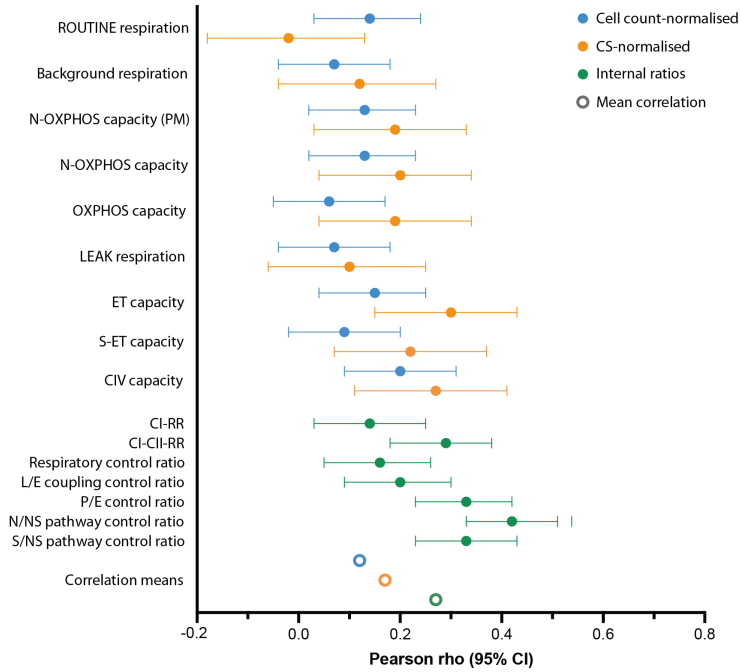


Figure 8 PBMCs and platelet correlation and comparison of normalisation methods. Filled squares shows correlation for cell count normalised values ($O_2 \cdot s^{-1} \cdot 10^{-8}$ platelets, and $O_2 \cdot s^{-1} \cdot 10^{-6}$ PBMCs), black dots show the same measurements normalised to citrate synthase activity, and hollow dots shows a selection of internal ratios. Bars represent 95% confidence intervals (CI). Bottom three dots show mean values (without CI).

Physical exercise and ageing

Functional mitochondrial changes in athlete blood cells

The group of 24 healthy volunteers, from whom muscle fibres were sampled in addition to PBMCs and platelets, included a group of semi-professional athletes (n=5) active in endurance sports (long-distance running and orienteering).

Respiration in the three cell types was analysed and compared between the athletes and non-athletes. It is known previously that physical exercise is associated with increased mitochondrial respiration in muscle fibres, primarily due to mitochondrial biogenesis.

Our hypotheses were 1) to replicate these findings in muscle and 2) that there would be some degree of corresponding changes in blood cells.

Despite small sample sizes in the subgroups, we found that respiration, both the maximum coupled and non-coupled respiration (NS-OXPHOS capacity, and NS-ET capacity, respectively) were significantly higher in the athlete group

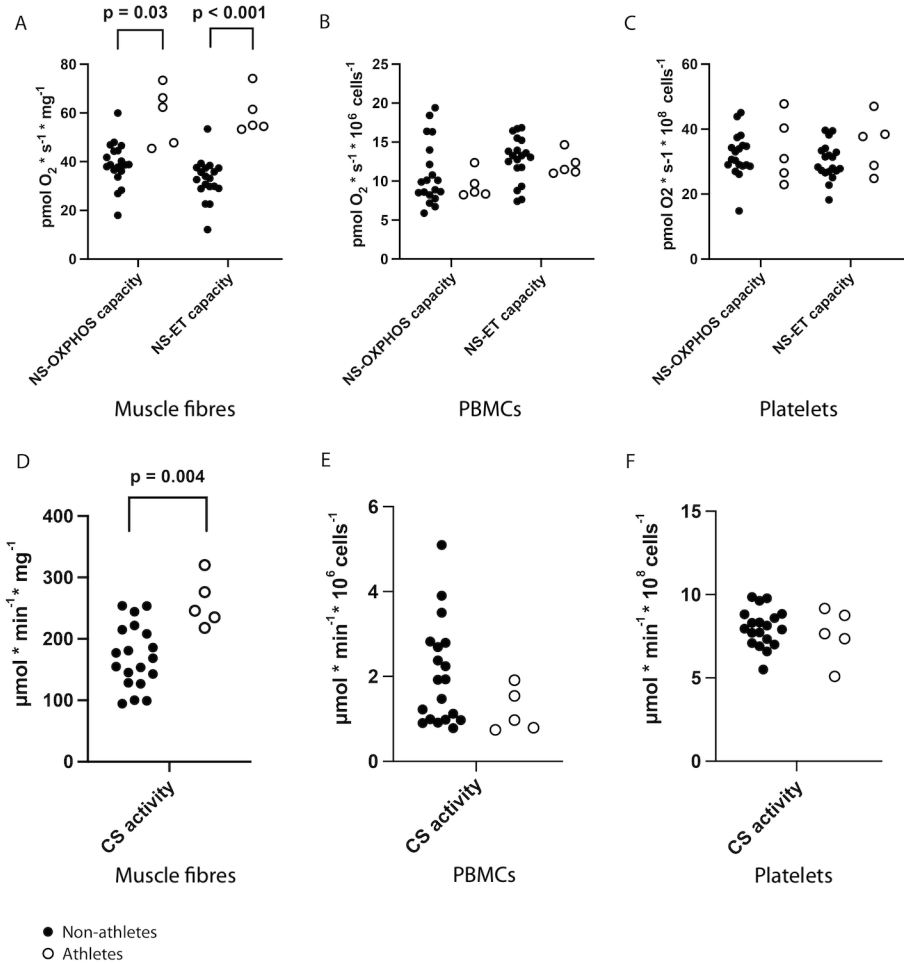


Figure 9 Muscle and blood respiration in athletes compared to non-athletes.
Filled dots = non-athletes (n=19). Hollow dots = non-athletes (n=5). **A-C** shows cell-count normalised mitochondrial respiration, **D-F** shows citrate synthase (CS) activity. P values for significant group differences (P < 0.05 according to Mann-Whitney *U* test) are labelled.

compared to non-athletes (Figure 9A) As expected, CS activity was also higher in the muscle fibres of the athletes, indicating that the increased respiration was due to increased mitochondrial content (Figure 9D).

These changes were not present in the blood cells of the athletes, neither in non-normalised respiratory capacities (Figure 9B-C), nor in CS activity (Figure 9E-F).

Age-dependent changes of mitochondrial function in blood cells

For this analysis we again used the retroactively pooled data from our lab, comprising 308 cases when including data from various previous studies. (Nine cases with known primary MD, and one case where the exact age was uncertain, were excluded, as compared to the 318 cases described previously.)

With the inclusion of patient samples, as opposed to only healthy participants, it was possible to greatly increase sample size at the price of possible bias. To counteract this bias, a multiple regression model was made with adjustment for the health status (patient cohort/healthy volunteer), in addition to sex. The age range of the included participants was 3 months to 86 years.

As there has been several reports of age-dependent decline in mitochondrial respiration in other human tissues, and in blood cells from animals, our hypothesis was to find an age-dependent decline in respiration.

In summary, age-dependent changes in mitochondrial function were mainly absent or small (Figure 11). No significant changes in mitochondrial content were found (not shown here, see Figure 2, Paper III).

There was an age-dependent decline in the N/NS pathway control ratio (complex I) in PBMCs, and a (possibly compensatory) rise in the S/NS pathway control ratio (complex II), but these effects were small.

Possible sources of a type II-error were further explored but not encountered (for details, see Paper III).

Changes in blood cells from patients with mitochondrial disease

Group level changes in respiratory ratios were compared in blood cells from a small group of paediatric patients with mitochondrial disease (n=9) and patients with other, similarly presenting diseases (n=50). Several ratios differed significantly between the groups in both cell types, more so in platelets (Figure 10).

Summary of biomarker assessment

We have not found mitochondrial respiration in blood cells to correlate with muscle fibres in healthy controls. Nor have we, in blood cells, seen the typical exercise-induced or age-associated changes present in other tissues. The sum of results does not support the use of blood cell respirometry as a universal biomarker for less accessible tissues, nor for systemic mitochondrial function.

While blood respirometry does not appear a good biomarker in a general sense, patients with pronounced inherited mitochondrial defects may have detectable changes in blood cells despite mainly exhibiting symptoms from other organs. The possible application of this phenomenon in clinical diagnosis is further investigated in the following part of the results section.

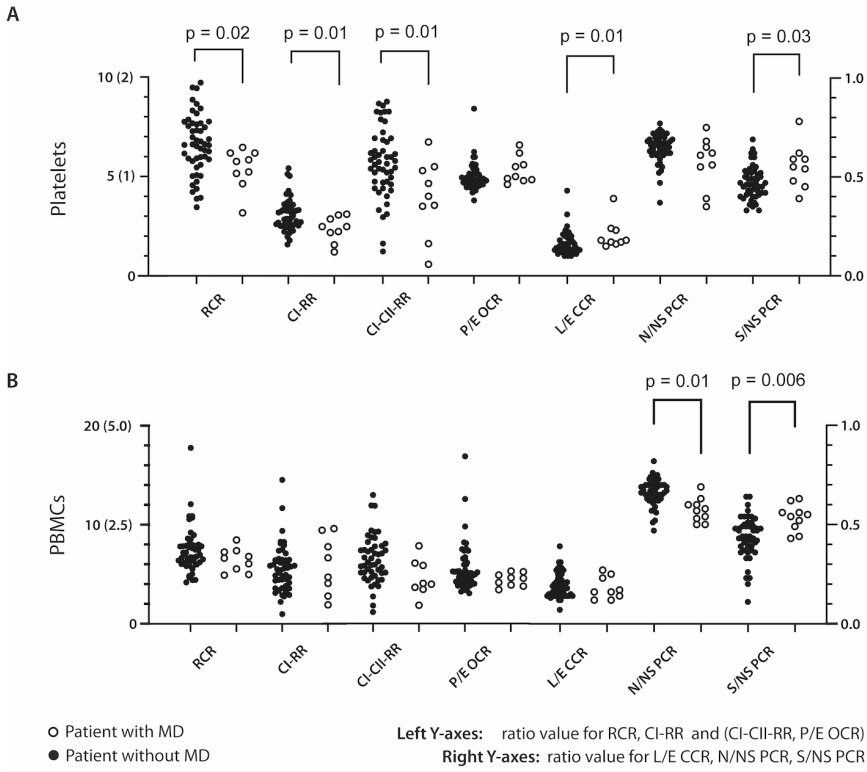


Figure 10 Respiratory ratios in paediatric patients with and without MD
Internal respiratory ratio values are compared between patients with confirmed mitochondrial disease (MD) (n=9) and patients without MD (n=49–50), in platelets (A), and PBMCs (B), respectively. Significant group differences are noted in the figure ($P<0.05$ according to Mann-Whitney *U* test). For abbreviations, see Table 1 in Paper II. The left y-axis shows the scale of RCR and CI-RR (0–20), and CI-CII-RR and P/E OCR (0-5.0). The right y-axis (0.0–1.0) corresponds to the values of the remaining ratios.

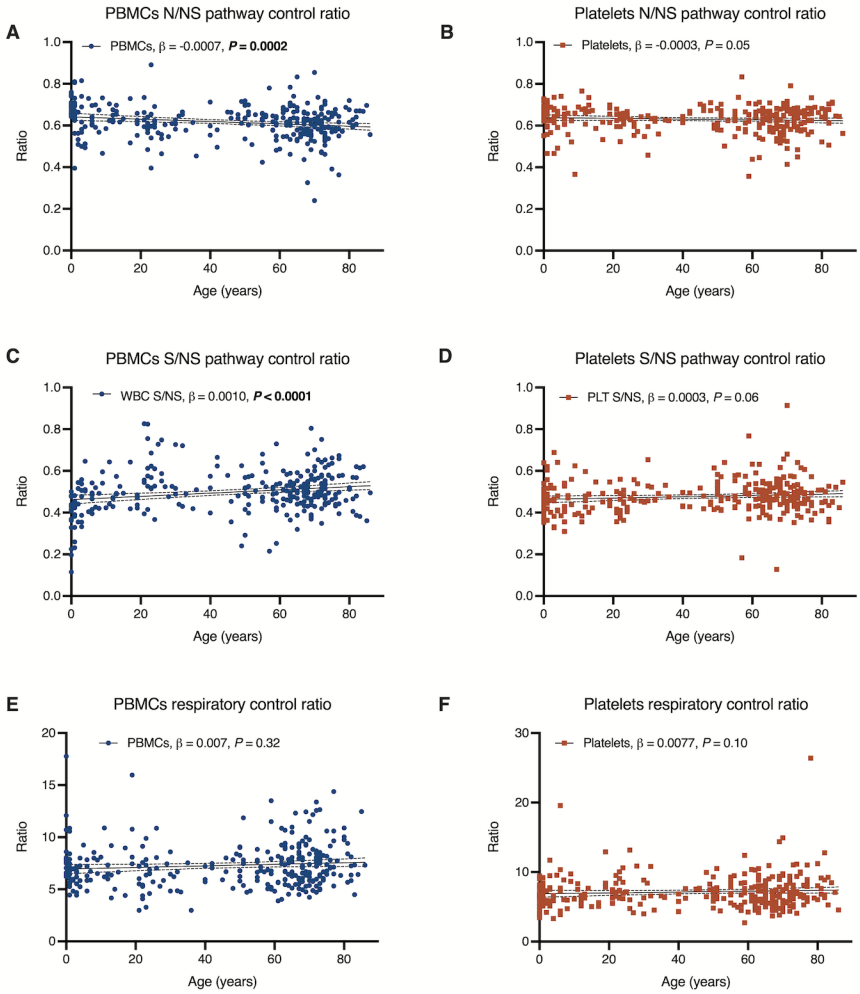


Figure 11 Mitochondrial function across the human lifespan
Respiratory ratios as a function of age. Plots represent simple linear regression for each ratio; the slope is depicted as a straight line with its 95% confidence interval as a dotted line. In respecitve graph legend, the age effect from a multiple regression model, adjusted for health status and sex, and its P-value, is shown. P-values in bold were classified as true discoveries after adjustment for false discovery rate. The overall MR model was significant for the N/NS pathway control ratio (R2 = 0.047, P = 0.002) and S/NS pathway control ratio (R2 = 0.086, P < 0.0001) for PBMCs. None of the MR models were significant for platelets.

The diagnosis of mitochondrial disease in blood

This was a clinical study (Paper I) that included paediatric patients who presented with suspected mitochondrial disease (MD) at Skåne University Hospital. A set of diagnostic respiratory ratios for platelets were chosen and reference ranges were established in 25 healthy controls, prior to analysing the patients. The diagnostic accuracy of the platelet model was compared to the results of standard clinical investigation after extended follow-up.

Clinical features and routine investigation

113 paediatric patients with clinically suspected MD were included during a five-year period (July 2008-December 2013). The ages ranged from less than a month to 17 years. The age distribution was skewed to the younger side, with a median age of 3 years and about half of the included patients being younger that 5 years.

Neurological symptoms were heavily featured, but the range of symptoms was wide, including the heart, the skeletal muscles, vision, hearing, the gastro-intestinal tract, and the endocrine system. The most common presenting symptoms were intellectual disability (n=46), seizures (n=40), hypotonia/muscle weakness (n=35), psychomotor regression (n=31) and vomiting (n=14).

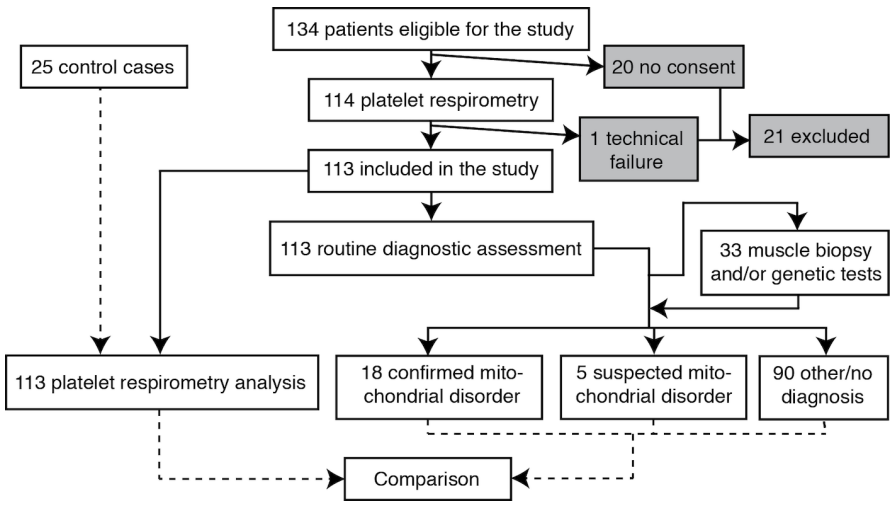


Figure 12 Diagnostic study design
Two additional cases with techical failure could be included after reanalysis.

Blood samples from all patients were collected for platelet respirometry analysis. The patients continued their normal clinical paths, and the end results of the standard investigations were recorded and used as gold standard to evaluate the accuracy of the platelet method (Figure 12).

Table 3 Cases of confirmed mitochondrial disease

For a more detailed version of this table, also explaining abbreviations, see Table 3, Paper I.

Case no	Diagnosis	Gene	Mutation	Main supportive findings if no genetic diagnosis
1	Leigh syndrome	MT-ND5	m.13513G>A	
2	MELAS	MT-TL1	m.3260A>G	
3	Alpers syndrome	POLG	N.A.	
4	PDH deficiency	PDHA1	c.1142_1145 dupATCA	
5	Mitochondrial encephalopathy	-	-	Plasma lactate, MR spectroscopy, muscle histology
6	PDH deficiency	PDHA1	c.871G>A	
7	Alpers-Huttenlocher syndrome	POLG	p.W748S, p.Q497H, p.R852C, p.E1143G	
8	Mitochondrial encephalopathy	-	-	Muscle histology
9	CPEO and myopathy	MT-TN	m.5703G>A,	
10	Mitochondrial depletion syndrome	MPV17	c.191C>G, c.503A>G	
11	Mitochondrial hepatopathy	-	-	Plasma and CSF lactate, enzyme deficiency in liver mitochondria
12	Kearns-Sayre syndrome	-	-	CSF protein, muscle histology, mt-DNA depletion of probable pathogenicity
13	LHON plus	MT-ND4	m.11778G>A	
14	Leigh syndrome	-	-	CSF lactate, MRI findings
15	PDH deficiency	PDHA1	c.1068-1078delTCCT GAGCCAC	
16	Leigh syndrome	SLC19 A3	C153A>G, c.157A>G	
17	MDS	SUCLA -2	c.751G>A	
18	Mitochondrial encephalopathy	SARS2	c.691C>G, c.868C>T	

For the purposes of the study, confirmed MD was defined as a diagnosis made by the patient's physician after follow-up and investigation. 33 of the patients (about a third of all cases) went through a more extensive mitochondrial investigation including either muscle biopsy, genetic testing, or both. 18 of these were confirmed to have MD; 13 of them had a genetic diagnosis, another three had pathological findings in muscle and the last two had other supportive evidence of mitochondrial disease (Table 3).

In five cases, mitochondrial disease could neither be confirmed nor completely ruled out despite extensive investigation and follow-up. These were defined has not having MD for the purpose of the study. In the group of patients who did not go through extensive mitochondrial investigation, common reasons for ruling out mitochondrial disease were the confirmation of an alternative diagnosis or sustained clinical improvement.

Diagnostic accuracy of platelet respirometry

Accuracy of the main diagnostic model

To assess different parts of the electron transport system, a selection of five pre-defined respiratory ratios were examined. A ratio value outside of the reference ranges established in the healthy controls were defined as a positive test, and a value inside the range was defined as negative. Four ratios were unidirectional (only a low value was considered pathological, not a high one).

The CI-CII response ratio (CI-CII RR, ΔADP/Δsuccinate) was bidirectional since a low value was hypothesized to indicate relative complex I-dysfunction, and a high value a relative complex II-dysfunction. This ratio was counted as two separate tests, depending on the direction. Unless otherwise specified, CI-CII RR here refers to the test detecting CI-dysfunction (a low value).

Finally, the accuracy of combining all six tests was examined as a separate test (called the "combined model"), making the total number of pre-defined tests seven. The purpose of making a combined model was, of course, a hope to reach sufficient sensitivity to rule out mitochondrial disease.

In summary, the single test with the best performance was the CI response ratio (CI RR, OXHPOS CI_{MP}/DMP), with a specificity of 96% (95% CI 90-99%) and a PLR of 5.3 (95% CI 1.5-19) (Table 4). In other words, this test had a moderate utility for ruling in mitochondrial disease.

No test was useful for ruling out disease. As expected, the combined model performed best for this purpose, with a sensitivity of 72% (95% CI 47-90%) and a NLR of 0.5 (95% CI 0.23-1.07). This can be classified as a low utility for ruling out disease, and the statistical uncertainty included the possibility of no utility (i.e., LR 1.0).

Table 4 Accuracy of platelet respirometry and routine tests

A selection of the three best performing platelet tests and routine test performance. (For all seven platelet tests and explanation of the combined model, see Paper I, Table 5.) The 95% confidence intervals (CI) for sensitivity/specificity and for LRs are based on the Clopper-Pearson method and the Log method respectively, using MedCalc online software.[266] Number of cases (*n*) for respirometry was 113, for plasma lactate 95. For *n* in other tests, see Table 6 of paper I. Abbreviations: see list at the beginning of the thesis.

Diagnostic test	Sensitivity % (95% CI)	Specificity % (95% CI)	PLR (95% CI)	NLR (95% CI)
Platelet respirometry				
CI response ratio	22 (6-48)	96 (90-99)	5.3 (1.5-19)	0.8 (0.6-1.0)
CI-CII response ratio	33 (13-59)	88 (80-94)	2.9 (1.2-6.8)	0.8 (0.5-1.0)
Combined model	72 (47-90)	56 (45-66)	1.6 (1.1-2.4)	0.5 (0.2-1.1)
Routine investigation				
Plasma lactate	78 (52-94)	67 (56-78)	2.4 (1.6-3.6)	0.3 (0.1-0.8)
CSF lactate	67 (38-88)	-	-	-
Organic acids in urine	62 (32-86)			
Radiology (MR)	78 (52-94)	-	-	-
Muscle biopsy	75 (47-93)	-	-	-
Genetic testing	93 (69-100)	-	-	-

Comparison with plasma lactate and other routine clinical tests

It is important to note that the accuracy of platelet respirometry in the main analysis was measured against the results of the entire routine investigation and not against another single test. For context, none of the other tests, including the often used "gold standard" of genetic testing, succeeded in identifying every case of MD in this study population. Sensitivities ranged from the low 60s to mid 90s (Table 4).

The study was designed to evaluate the diagnostic performance of platelet respirometry, which was the only test performed on all included patients. Thus, comparisons of specificity and other tests dependent on data from patients without the disease (likelihood ratios), would be even less reliable for many of the other individual tests, particularly the more specialised ones that were only performed in selected patients under extended investigation, such as genetic testing and muscle biopsy. These two (especially genetic testing) are also exposed to incorporation bias, which likely inflates their accuracy.

However, plasma lactate was measured in 95 out of the 113 patients (84%), including in all that were subsequently confirmed to have MD. From the available data, sensitivity, specificity, and likelihood ratios, can be estimated for lactate, which has an NLR of 0.3 (95% CI 0.1-0.8) (Table 4). This is slightly superior to the combined platelet model for ruling out disease but still

insufficient. The PLR for lactate was 2.4 (1.6-3.6), slightly worse than the platelet CI response ratio.

Diagnostic accuracy of respirometry combined with lactate

Plasma lactate is an even simpler test than blood cell respirometry, available from the same blood sample and already in widespread clinical practice. In post-hoc analyses, lactate was combined with platelet respirometry tests in an attempt to increase accuracy, which it did (Table 5).

A pathological lactate and CI response ratio had a specificity of 99% (95% CI 94-100) and a PLR of 21 (95% CI 2.5–178). This is usually classified as a high utility for ruling in disease. The test, however, only identified four of the 18 MD patients and the statistical uncertainty was large.

Combining lactate with the combined platelet model did not improve the ability to rule out disease, compared to lactate alone. Two of the 18 mitochondrial patients were negative in both lactate and platelet respirometry and NLR remained at 0.3 (95% CI 0.1–1.0).

Table 5 Accuracy of platelet respirometry combined with plasma lactate

*Tests were considered positve only if both respirometry and lactate were pathological. Lactate was not available in 18 cases without confirmed mitochondrial disease, which were counted positive if only respirometry was postive, to generate a conservative specificity estimate. **Test were considered positive if either test was pathological, only cases with available lactate were used. 95% CI in parenthesis are calculated by the same method as in Table 4. CI-RR = CI response ratio. CI-CII RR = CI-CII response ratio. CM = combined model.

Diagnostic test combination	Sensitivity % (95% CI)	Specificity % (95% CI)	PLR (95% CI)	NLR (95% CI)
CI-RR + lactate*	22 (6.4–48)	99 (94–100)	21 (2.5–178)	0.8 (0.6–1.0)
CI-CII RR + lactate*	28 (9.7–53)	96 (90–99)	6.6 (2.0–22)	0.8 (0.6–1.0)
CM + lactate**	89 (65–99)	40 (29–52)	1.5 (1.2–1.9)	0.3 (0.1–1.0)

Summary of the diagnostic study

Platelet respirometry was not sensitive enough to be useful for ruling out mitochondrial disease. Some of the respirometry tests had moderate utility for ruling in disease, the CI-RR performing best with a PLR of 5.3.

Combining platelet respirometry with lactate, in a post-hoc exploratory analysis, greatly increased their ability to rule in mitochondrial disease, but the combination was still insufficient for ruling out disease.

Blood lactate is a theoretically interesting and very accessible metabolic test which is not specific to mitochondrial disease. Its utility in a wider clinical context will be explored in the last part of the results section.

Lactate in the emergency department

This was a clinical study (Paper IV) that investigated aspects related to the use of lactate as a prognostic test in the emergency department, in a large register-based, retrospective cohort. The rationale for the study was partly based on experiences from Paper I, that lactate is an unspecific but sensitive and very accessible test, that could be of use in wide variety of diseases.

Patient characteristics and general aspects

The study included 39 388 adult patients who visited the ED and had hyperlactataemia (lactate ≥2.0 mmol/L) (Figure 13).

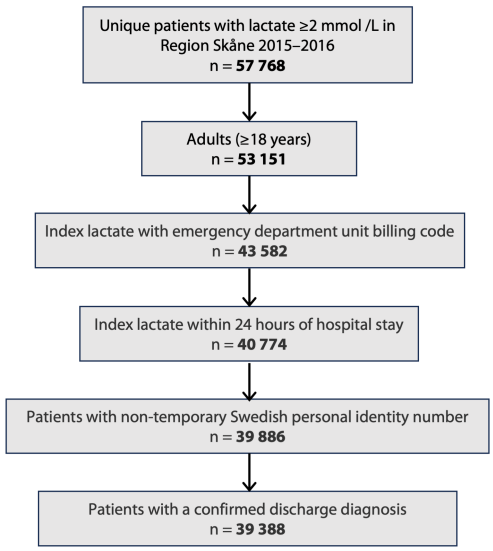


Figure 13. Study flow chart
Summary of data extraction and patient inclusion.

Most patients (86.5%) had a lactate level <4.0 mmol/L (Table 6). Patients were distributed across a wide spectrum of discharge categories, with the most common ones (apart from 'other diseases') being infections (12.9%), cardiovascular diseases (10.3%) and trauma (8.7%).

Table 6 Patient characteristics

Patients' column % indicate share of total study population. SD = standard deviation. Within subgroup differences in mortality were significantly different for all subgroups, according to Pearson Chi-Square test at the P <0.001 level, except for sex difference (P = 0.96).

	Patients, n (%)	Lactate, mean mmol/L (SD)	pH, mean (SD)	30-day mortality, %
All patients	39 388	3.1 (1.9)	7.39 (0.09)	7.1
Lactate				
2.00–3.99 mmol/L	34 065 (86.5)	2.5 (0.5)	7.40 (0.07)	5.4
4.00–9.99 mmol/L	4 699 (11.9)	5.4 (1.4)	7.36 (0.11)	15.2
≥10 mmol/L	620 (1.6)	14.0 (3.6)	7.09 (0.21)	43.4
Acidaemia				
No acidaemia (pH>7.30)	32 644 (91.6)	2.8 (1.1)	7.40 (0.06)	5.4
Mild-moderate (7.10<pH≤7.30)	2 533 (7.1)	4.7 (3.3)	7.25 (0.05)	20.0
Severe acidaemia (pH≤7.10)	455 (1.3)	11.4 (6.0)	6.94 (0.13)	58.0
Charlson Comorbidity Index (CCI)				
0	26 851 (68.2)	3.1 (1.9)	7.39 (0.09)	5.0
1	4 109 (10.4)	3.0 (1.6)	7.38 (0.08)	6.5
≥2	8 424 (21.4)	3.0 (1.8)	7.38 (0.09)	14.4
Age				
<65 years	16 901 (42.9)	3.1 (2.0)	7.40 (0.09)	1.5
≥65 years	22 483 (57.1)	3.0 (1.8)	7.38 (0.08)	11.3
Sex				
Female	17 596 (44.7)	2.9 (1.6)	7.39 (0.08)	7.1
Male	21 787 (55.3)	3.2 (2.1)	7.38 (0.09)	7.1
Sample type				
Venous	37 887 (96.2)	2.9 (1.7)	7.39 (0.08)	6.4
Arterial	1 497 (3.8)	4.7 (4.0)	7.32 (0.18)	25.8
Discharge category				
Allergy	224 (0.6)	2.9 (1.1)	7.40 (0.06)	0
Cardiac arrest	232 (0.6)	11.5 (5.2)	6.97 (0.21)	87.9
Cardiovascular diseases	4 049 (10.3)	2.9 (1.5)	7.38 (0.08)	8.3
Endocrine diseases	1 117 (2.8)	3.4 (2.5)	7.36 (0.11)	5.7
Haematological malignancies	71 (0.2)	2.9 (1.4)	7.41 (0.07)	22.5
Haematological diseases	345 (0.9)	2.9 (1.5)	7.38 (0.06)	6.1
Hepatological diseases	139 (0.4)	3.7 (2.9)	7.41 (0.08)	14.4
Hypovolemic conditions	1 038 (2.6)	3.4 (2.2)	7.38 (0.08)	12.8
Infections	5 070 (12.9)	2.9 (1.4)	7.40 (0.07)	9.9
Intestinal diseases	1 993 (5.1)	2.9 (1.2)	7.41 (0.07)	4.7
Intoxications	851 (2.2)	3.2 (1.9)	7.35 (0.07)	0.7
Solid malignancies	754 (1.9)	3.2 (1.9)	7.41 (0.08)	31.3
Nephrological diseases	1 145 (2.9)	3.0 (1.7)	7.40 (0.10)	4.5
Neurological diseases	2 064 (5.2)	2.9 (1.4)	7.39 (0.07)	11.3
Psychiatric disorders	944 (2.4)	3.6 (2.2)	7.39 (0.09)	1.8
Respiratory diseases	1 359 (3.5)	3.0 (1.6)	7.36 (0.10)	11.3
Seizures	1 061 (2.7)	5.5 (3.9)	7.34 (0.10)	1.1
Trauma	3 421 (8.7)	3.1 (1.8)	7.37 (0.07)	3.9
Other diseases	13 506 (34.3)	2.7 (1.2)	7.40 (0.07)	4.3

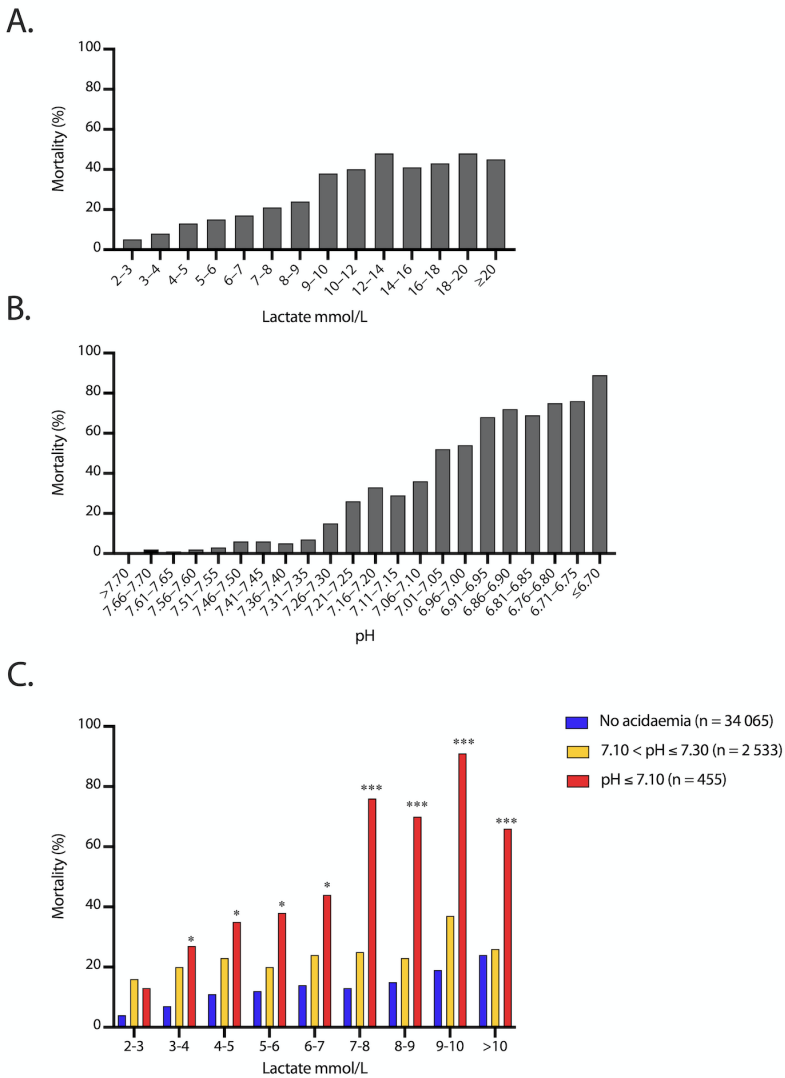


Figure 14. Mortality according to lactate and pH

30-day mortality at different levels of **A.** lactate, **B.** pH, and **C.** at different levels of lactate, depending on pH. Even lactates are sorted into the lower group (2–3 means 2.00–2.99, etcetera). ***Mortality of severe acidaemia significantly different from both mild-moderate and no acidaemia, using two-tailed independent sampled T test, assuming unequal variance, at the P <0.001 significance level. *Mortality of sever acidaemia significantly different from no acidaemia at the P < 0.05 significance level.

Mortality in the entire study population was 7.1%. This is markedly higher than the general mortality for Swedish ED patients, which, depending on the setting, is seldom higher than 2.5%.[267,268] Mortality increased with a linear appearance with increasing lactate levels up to around a lactate level of 10 mmol/L (Figure 14A). After that, mortality plateaued around 45–50%, but it should be noted that patients with severe hyperlactataemia (lactate ≥10 mmol/L) were relatively few (n=620).

Lactate and pH

Mortality increased with a linear appearance as pH dropped, starting at a pH around 7.30, and surpassed 70% at the extreme levels of acidaemia (Figure 14B). Stratifying lactate according to pH level, mortality was increasingly higher for patients with mild-moderate acidaemia (7.10 < pH ≤ 7.30) and severe acidaemia (pH < 7.10), compared to no acidaemia (Figure 14C).

In multivariate logistic regression, the relationship between lactate and mortality was examined, with pH level, sex, age, Charlson comorbidity index (CCI), and sample type (arterial/venous) as covariates. Both lactate and pH level were independently associated with mortality, and the OR for lactate was slightly smaller in the model where pH level was added as a covariate (for details, see Paper IV, Table 2). When further examining the relationship by adding an interaction term between lactate and pH level, the interaction turned out to be significantly less than 1 (for details, see Paper IV), which was not expected. The interpretation is that, even though mortality is generally lower when acidaemia is not present, paradoxically, the lactate concentration itself predicts mortality better in this group. This was illustrated by stratifying adjusted ORs for lactate by pH level, where the OR was highest in the non-acidaemic group (Table 7).

In summary, the presence and degree of acidaemia affects mortality in patients with elevated lactate. However, even in the non-acidaemic group, lactate significantly predicts mortality.

Table 7 Adjusted OR for lactate and mortality at different pH levels

Multivariate logistic regression with 30-day mortality, separately calculated for each pH category. ORs are adjusted for Charlson Comorbidity Index (CCI), age, sex, and sample site (venous/arterial). Significant ORs at the P <0.001 level are marked in bold. OR = odds radio. CI = confidence interval.

	Adjusted OR (95% CI) for lactate (mmol/L)	P=	N=
pH			
No acidaemia (pH > 7.30)	**1.40** (1.35–1.44)	<0.001	32 644
7.10 < pH ≤ 7.30	**1.13** (1.09–1.16)	<0.001	2 533
pH ≤ 7.10	**1.08** (1.04–1.13)	<0.001	455

Lactate and aetiology

Calculating adjusted ORs for lactate and mortality, for each presumed aetiology separately, the ORs ranged from 1.53 (95% CI 1.43–1.64) for cardiovascular disease to non-significant in the categories of seizures, intoxications, and haematological malignancies (see Table 3, Paper IV). ('Allergy' had no mortalities and could not be calculated.)

In the ROC curves for different aetiologies, there was also variation in the AUCs (Figure 15). The highest AUCs for lactate were in the categories of cardiac arrest, cardiovascular diseases, and hepatological diseases. These were all equal to or above 0.70. Many others were in the 0.60–0.69 range, which is generally considered to be weak or moderate.[269]

The AUCs were not significantly different from 0.5 (no predictive value) for seizures, intoxications, endocrinological diseases, psychiatric disorders, and haematological malignancies.

Negative results should always be interpreted with caution. Still, several subgroups were rather large in size and still no significant predictive value was found, this includes seizures, intoxications and endocrinological diseases.

Another limitation in the interpretation of the AUC values is that only patients with hyperlactataemia were included, which will cause spectrum bias. Still, the AUC for the whole study population was 0.67 (95% CI 0.65-0.68), which is fairly close to previous studies that have included the whole spectrum of lactate concentrations.

In the subgroup of patients with severe hyperlactataemia, without severe acidosis, the three most common categories of aetiologies (apart from 'other diseases') were seizures, infections, and trauma (Figure 16). Seizures were much more common in this group than in the whole study population and mortality for seizures in this group, as in general for seizures, was very low (1 out of 95 patients).

Summary of the lactate study

In a large population of general ED patients, lactate concentration was linearly associated with mortality up to a lactate level about 10 mmol/L, after which mortality plateaued around 45–50 %. Most patients with an elevated lactate did not have acidaemia.

The presence and degree of acidaemia affected mortality in patients with elevated lactate, but even in the non-acidaemic group, lactate did significantly predict mortality. The predictive value of lactate varied depending on the presumed aetiology of lactate elevation. In several categories of disease, lactate did not significantly predict mortality.

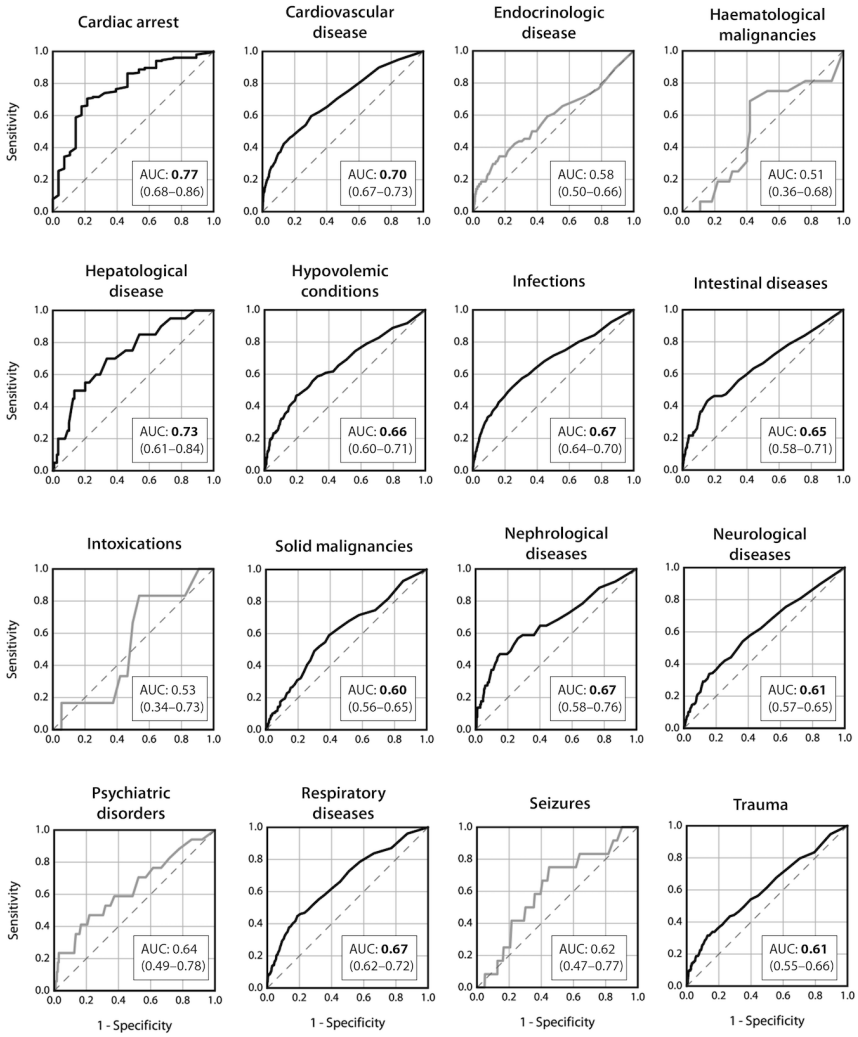


Figure 15. Predictive value of lactate for different discharge categories
Receiver operating characteristics (ROC) curves for the predictive value of lactate on 30-day mortality according to discharge category. Area under the curve (AUC) with the 95% confidence interval (within parentheses) is displayed in respective graph. AUCs significantly greater than 0.50 ($P < 0.05$) are marked in bold. Not shown in figure: allergy (no mortality), other diseases (AUC 0.66, 95% CI 0.63–0.68) and haematological diseases (AUC 0.64, 95% CI 0.52–0.75).

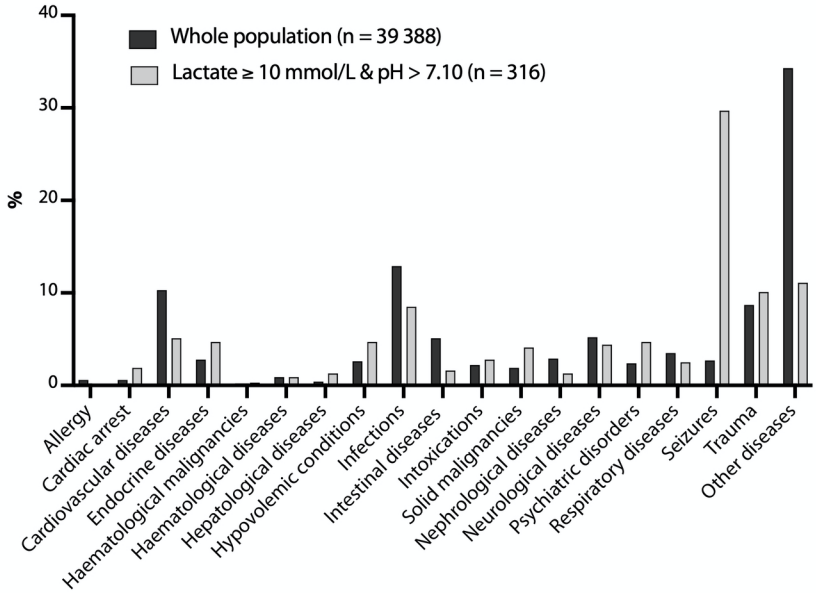


Figure 16 Severe hyperlactataemia without severe acidaemia
Distribution of discharge categories with severe hyperlactataemia (lactate ≥ 10 mmol/L), without severe acidaemia (pH > 7.10), compared to the whole study population. Y-axis shows percent within respective group.

Discussion

Blood cell mitochondria as biomarkers

The biomarker at the rainbow's end

According to old Irish folklore, the mythical leprechauns buried pots of gold at the rainbow's end. Many searched for this gold in vain, only to discover that the rainbow's end was an optical illusion – it moves or disappears whenever one gets closer. To my knowledge, there is no empirical evidence that the gold ever existed in the first place.

Is the search for a mitochondrial biomarker in blood cells also in vain? As with the pot of gold in the legend, many would surely like it to be true. Since blood cell mitochondria are easy to obtain from humans, it would be very convenient to use them as substitutes for muscle mitochondria, or for systemic mitochondrial function, both in research and for clinical applications.

The general theme of the Paper II and III was to find out whether blood cell mitochondria could be used as biomarkers for other tissues or systemic conditions. In the context of physiological conditions (exercise, ageing, correlation in healthy individuals), results were largely negative. Does this mean that blood cell mitochondria are poor biomarkers? The interpretation of negative results should always be cautious, especially if a study is not designed specifically to prove the absence of an association. There is always the possibility that such an association exists even though it was not found. The comparisons between blood cell and muscle mitochondria were made in a relatively small sample, which may account for the non-significant results. On the other hand, sample size was similar to other, previous studies, some of which had positive results.[104,110] Technical errors in the laboratory is another potential source of falsely negative results. To the best of our ability, we tried to check the quality of the experiments to exclude this. Furthermore, significant differences were found in muscle between athletes and non-athletes, which gives some, indirect support to the quality of the experiments.

The results in this thesis are not in sharp contrast to the previous body of research. As was described previously, the few previous studies in humans are a

mix of positive and negative results, and the positive studies are not in agreement with each other.[86,88,110,111,118] Methodological differences and differences in cell choice may account for some of the variation in the results.

In Paper II, we also examined the correlation of mitochondrial function within the same tissue: between different types of blood cells. Significant correlations were present but generally weak. The fact that the correlation is not strong even between different types of blood cells gives some, indirect support to the notion that mitochondrial function varies a lot across the body. Since the publication of Paper II, Devine et al. have made a mixed animal-human study (currently in a preprint version) with similar findings, and have drawn similar conclusions.[270] In that study, correlation in mitochondrial respiration between tissues in mice was largely absent, and the expression patterns of mitochondrial genes in different human tissues did not correlate strongly either. There were small to moderate correlations between different regions within the brain, but not between brain mitochondria and the rest of the body.

In a review article from 2020, Braganza et al. stated that "accumulating evidence suggests that peripheral blood cells can be used as a surrogate for other cell types or tissues to assess systemic mitochondrial health".[88] This was supported mainly by circumstantial evidence and a few positive studies with direct comparisons between muscle and blood cells, while several negative studies were not mentioned. Considering the state of evidence at the time, the statement seems a bit too optimistic. The addition of negative studies after 2020 (including Paper II) may not have settled the question but, at any rate, evidence cannot be said to currently "accumulate" in favour of blood cells as biomarkers.

At least not in a general, healthy population, which is an important point. To be fair, this point was also made by Braganza in the review.[88]

What about blood cell mitochondria as biomarkers for pathological conditions? In Paper II, there were significant, group-wise differences in blood cell respiration between paediatric patients with, and without MD. We also had significant results in the diagnostic study of MD in platelets in Paper I (albeit of uncertain clinical relevance). While mitochondrial respiration in blood cells may not be a good universal biomarker of mitochondrial function, it is possible that in some extreme cases, or in certain systemic conditions, it could be of value. Apart from MD, this may include, for example, systemic intoxications and side-effects of drugs. To use a metaphorical example: The colour of people's clothes probably correlates poorly to their favourite football team in the general population, but in a subset of the population attending a live football game on a Saturday afternoon, the correlation will naturally be stronger.

In conclusion, blood cell mitochondria should probably not be assumed to reflect other tissues and systemic changes in general. However, they may be used

as biomarkers in certain contexts, with caution, and preferably with the support of specific, empirical evidence for each application.

The mystery of ageing

What is the cause of ageing? Why do we age? Can the process of ageing be slowed, halted or even – reversed?

These questions are intriguing from a purely scientific perspective, and the answers seem less obvious the more you consider them. At a glance, nothing could be more natural and inevitable than the ageing and eventual death of the individual organism. Then again, why do different animals age biologically at different rates? Are there evolutionary benefits and costs? Is the rate of ageing in humans the result of natural selection or of other evolutionary forces?

Besides scientific intrigue, the question of ageing is of practical interest to the clinical and political sciences. Ageing and age-related chronic diseases are increasingly seen as two aspects of the same problem. There is widespread hope that scientific breakthroughs will both alleviate the individual suffering of ageing, and the economic burdens of the changing demographic landscape.[271,272]

Moreover, longevity research is not merely of academic and political interest, but also seen potential source of profit. The allure of healthy lifespan expansion is gaining traction within the biotech industry, marketed with bold claims of an imminent scientific revolution.[273] But when? It seems that we are constantly at the cusp of revolution – but never quite there. A prominent industry voice promised his readers in 2010 that within 15 years (the current year) "we'll be adding more than a year every year […] to your lifetime expectancy".[274]

Why the hold-up? It is probably linked to the fact that the most basic questions of ageing – the "why" and the "how" – have not yet been answered. In a recent survey, a hundred leading ageing researchers were asked about the cause of ageing. Six of them said they did not know – the rest "knew" but gave widely different answers.[275]

How does our study on blood cell mitochondria (Paper III) fit into the broader context of ageing research? Contrary to our hypothesis, mitochondrial function did not generally decline with age in blood cells. This is contrary to several human studies on muscle mitochondria, but not to preceding research in blood cells, where results have been discrepant.[104-107] An additional study, by Fišar et al., also in platelets but with different methods, was published during the submission process of Paper III and did show age-dependent decline of respiration.[276]

As always, the possibility of falsely negative results must be considered but there are also other, reasonable explanations for the lack of a decline. One is that different tissues and organs may age at different rates. A study in rats measured

respiratory function in different organs from ageing rats, where mitochondrial function declined significantly in muscle cells but not in platelets. In other organs, results varied.[277] The constant renewal of peripheral blood cells from progenitor cells (and ultimately stem cells) in the bone marrow adds an additional layer to the issue, as age-related changes in stem cell mitochondria may not be reflected in their progeny. (Again, this calls into question the use of blood cell mitochondria as universal biomarkers.)

How can the results of Paper III be interpreted in relation to the MFRTA? The theory states that ROS cause mtDNA mutations, that impairs mitochondrial respiration, leading to a vicious cycle of further ROS production and the ageing phenotype. Several findings are at odds with the theory. As mentioned, experimental anti-oxidant interventions failed to slow ageing.[56,57] In mutator mouse model (also previously mentioned), where an increased accumulation of mtDNA mutations correlated with a premature ageing phenotype, a corresponding increase in ROS production was not evident.[278] This calls the concept of a "vicous cycle" into question. Furthermore, when the mtDNA mutational load has been measured in tissue samples, it has been shown to be much too small (<1%) to affect the overall respiratory function.[279] The results of Paper III, where respiratory function did not decline with age, would seem to be in line with these findings and contradict the MFRTA.

However, even if the original MFRTA did not hold up, a new version has been proposed, that accounts for the empirical evidence and still points to mitochondrial dysfunction as the culprit of ageing.[11,280] One reason that anti-oxidant interventions do not work could be that ROS is not uniformly bad for the cell. Variations is ROS production (for instance during physical exercise) have an important signalling function to the nucleus to adapt to changing energy demands.[281] While *all* ROS production may not be bad, it would still be possible that improper or prolonged ROS production damages respiration by way of mtDNA damage. The reason that the tissue mutational load is generally low could be that cells with too many dysfunctional mitochondria are selected for apoptosis.[279] An increased apoptosis rate is consistent with the atrophy of muscle and brain tissue seen in old age.[282]

Still, all the above is merely a hypothesis – one among many. It has generally been easy to find biological phenomena that are *correlated with* ageing. It has been harder, but sometimes possible, to prove that they *cause* ageing, at least experimentally. But what is *the* cause of ageing? That mystery remains unsolved.

Blood cell respirometry in mitochondrial disease

Blood respirometry as a diagnostic test

There is a genuine need to improve the process of diagnosing mitochondrial disease (MD), especially its early stages. The elusive presentation patterns and the complexity of a full investigation means that some patients may have to wait a long time for a diagnosis to be confirmed or excluded.[135]

While genetic testing technology has advanced greatly, attempts to find successful, non-invasive biomarkers have not been as successful. Despite numerous studies and several promising candidates, there has not yet been a definite, game-changing test translated into clinical practice.[90,283] One candidate for an easy, non-invasive test is high-resolution respirometry (HRR) in blood cells. The rationale for the test is clear: blood cells are easily obtained and may harbour the core pathology of MD, which can be directly assessed by HRR.

Groupwise differences between patients with and without MD have been demonstrated in numerous studies, both before and after the publication of Paper I in 2018.[90,284] However, surprisingly few have reported measurements of diagnostic accuracy in a clinically relevant setting. In Paper I, we found that blood respirometry could not independently rule out MD, but that several tests increased the likelihood of MD enough to have possible clinical utility. For example, if the CI-RR test was positive, the probability of MD increased from 16% (the pre-test probability in the cohort) to 50%.

According to reference literature for diagnostic test evaluation, the accuracy of blood respirometry (PLR) would usually be classified as "moderate".[237] But it is probably more relevant to discuss the numbers in the specific context of MD diagnosis. First, there is no gold standard test for MD and none of components of the traditional work-up have perfect accuracy, not even muscle biopsy and genetic testing. It is difficult to present precise and generalisable numbers for all tests; even in a systematic review by the MMS, accuracy estimates are only sparsely mentioned.[84] Table 8 presents rough estimations from a few relevant sources. The numbers for muscle biopsy studies are especially difficult to pinpoint, especially for specificity, as studies are seldom designed to measure it. The diagnostic accuracy of genetic tests (mtDNA, WES) is even more difficult to determine since genetic confirmation is increasingly seen as a gold standard for a confirmed diagnosis. Some genetic findings are highly specific but even after WES, a number of probable cases will remain without a certain diagnosis.[285]

It would be surprising for any non-invasive biomarker to clearly outperform the traditional tests and perhaps this is not a realistic goal, at least not for ruling

Table 8 Diagnostic accuracy compared to other tests

Comparison of the diagnostic accuracy of several standard tests, as evaluated in other comparable studies or systematic reviews. It should be noted that included studies have different methods and populations (for instance either paediatric or adult patients, or both). The ranges represent the ranges of values presented in or extrapolated from the references studies without formal meta-analysis, nor assessment of bias. CSF = cerebrospinal fluid.

Diagnostic test	Sensitivity %	Specificity %
Blood lactate[84,139]	34–72	83–100
CSF lactate[139,286,287]	67–94	89–100
Neuroradiology[140,285,287]	63–90	24–87
Muscle histopathology[139,285,287]	63–80	6–83
Muscle enzymes/respirometry[285,287]	65–73	30–75

in disease. Even tests of moderate utility, such as blood respirometry, may contribute to the composite assessment along with other, traditional tests.

For the purpose of quickly ruling out MD, any new biomarker that claims to add value must reasonably outperform blood lactate. Lactate is easy to analyse in a standardised way, is already in widespread clinical use, and has decent, if not perfect, accuracy.[84,287] In Paper I, blood respirometry did not outperform lactate for ruling out disease, and few other biomarkers that have been studied did so either.

Among the other biomarkers that have been studied, the most promising ones have been fibroblast growth factor-21 (FGF-21) and growth differentiation factor-15 (GDF-15).[90,283] The function of these substances is not yet fully known but they are both released from cells in response to cellular stress, for example because of mitochondrial dysfunction. They are not yet widely used in the clinic but have received much attention in research as several studies have showed that their accuracy is greater than lactate (especially in terms of sensitivity). One study combined FGF-21 with lactate (in a similar way that blood respirometry was combined with lactate in Paper I) to boost accuracy even further.[288] Another advantage of these biomarkers is that they can be easily analysed with standardised kits. This contrasts with blood cell respirometry, which is relatively more expensive and complicated, and would be much more difficult to standardise across non-specialised laboratories. Another crucial limitation of blood respirometry is of course that, because of heteroplasmy, the genetic defect and corresponding ETS pathology may not even be present in blood cells in some cases.[289]

Despite all this, there could still be a future place for blood respirometry in the clinical evaluation of MD. One major advantage of respirometry, over many other biomarkers, is that that it – ideally – examines the specific pathology behind the phenotype. As such, it could potentially point to defects in specific respiratory complexes, which correlate to and corroborate genetic findings. This could prove even more important as genetic studies get cheaper and more

accessible. If, hopefully, more therapeutic agents are developed to treat MD, blood respirometry could also be of use to evaluate treatment effects, as repeated sampling in the same patient is relatively harmless.

Future research

Looking to the future of MD biomarkers, in a recent review by Hubens et al., the authors expressed hope that even better alternatives may be found by AI and omics studies.[90] That is entirely reasonable but other factors are also important to move forward. Presently, many studies in the field of MD diagnosis are repeated, with slight variations and in various small cohorts, but few results are truly validated.

Even though the heterogeneity and relative rarity of MD are challenging features in diagnostic studies, it is important to aim for proper validation and high accuracy. There are certain risks involved with translating tests of less-than-optimal accuracy into clinical practice, as clinicians are notoriously bad at interpreting diagnostic probability.[290,291] The misconception that a patient has MD, or even that the patient has "possible MD", can have real and unfortunate consequences.[292]

With better collaboration, the most promising biomarkers could be selected and validated in clinically relevant, multicentre settings, preferably prospectively. As it seems difficult to find a single high-accuracy biomarker, a well-selected combination of biomarkers may be an option. Lactate, on the merits of already being a well-established test, should be strongly considered for such combinations. Variations on lactate testing, such as post-exercise measurements, merit further exploration.[293]

Lastly, better incorporation of clinical information with laboratory tests to enhance accuracy should also be further explored. After all, in terms of non-invasiveness and accessibility, the patient history is hard to beat.

Lactate in the ED

Uses and interpretation of lactate in the ED

When lactate is elevated in the blood of an ED patient, it will generally give rise to these two questions: What is causing it? Is it bad?

As a clinical test in emergency medicine, the use of lactate has been popularised by sepsis guidelines and related awareness campaigns.[191] Its use in sepsis work-up is well-established and exhaustively studied and has resulted in its use also in many non-septic patients.[190,294] Furthermore, lactate it is often included in arterial and venous blood gas panels, which are widely used in emergency medicine in many conditions, including hypoxia, cardiac arrest, intoxications, and generally in resuscitation.[295] As a result, lactate is often available even when its indication or clinical value is not completely clear.

How can lactate best be used in the ED? As a general marker of acute illness, some have suggested its use in triage. The current body of evidence, indirectly supported by the results of Paper IV, does not favour its use in isolation as a triage tool, as the prognostic accuracy is only moderate in the general ED population. But there have also been suggestions that lactate is added as an extra parameter in already established triage tools. One example of this is NEWS+L (National Early Warning Score+Lactate), and its variants.[224,225] NEWS is a composite score based on the four classic vital signs, oxygen saturation, and conscious level. According to some (but not all) studies, NEWS+L is superior to NEWS without lactate in predicting mortality.[196,197,207,225]

In this application, lactate can be seen as 'another vital sign'. Indeed, the predictive value of lactate seems to be similar to several of the non-invasive vital signs – possibly even superior to some of them.[200,296,297] However, the invasiveness of lactate is a big drawback compared to the other measurements. There are suggested non-invasive additions to triage scales that may improve the accuracy just as well.[298] The addition of a blood test in triage needs strong motivation, especially since the evidence for systemic triage is not very strong in in the first place.[299,300]

If lactate is not to be used in triage, or in the unselected ED population, it might still be used by the clinician during the work-up patients with certain conditions. The purpose of lactate would then be to estimate the prognosis and need for further management in the context of a certain aetiology, and other relevant clinical information. The results of Paper IV has corroborated previous research showing that the differential for an elevated lactate is broad, and that its prognostic value is highly dependent on the aetiology of the lactate elevation. For some categories of patients, such as those with seizures, lactate levels may be

extreme without relation to mortality. Such findings are not surprising considering recent perspective shifts in the scientific literature on lactate, whose physiological role in the body is being more and more appreciated.[152]

Should clinicians also shift their perspective on lactate? On the one hand, elevated lactate is consistently shown to be present in disease and high levels are, in general, associated with high mortality (Figure 14). On the other hand, this is not true in all cases. Is the lactate making the patient ill – or is it merely a sign of illness? Is lactate the fire – or the fire *alarm*? (Does it even matter? One reviewer begrudgingly admitted that many clinicians seem to make very good use of lactate despite their "gross mechanistic misinterpretation" (!) of lactate pathophysiology.[155])

To understand lactate clinically, perhaps it could be likened to C-reactive protein (CRP) – a general sign of illness that is elevated in many pathological conditions, and whose prognostic implications is very context dependent. Also, like CRP, lactate has the potential of broad applications if properly studied.

Future research

Plasma lactate concentration is already being widely analysed in the ED. It has the potential to be even more informative in decision making than it is today in many areas outside of sepsis evaluation. Since the prognostic implications are very different in different conditions, future studies should focus on conditions where the prognostic value is most promising. Elevated lactate is generally prognostic for mortality regardless of the patient's acid-base status. Still, the addition of pH, which is often available on same blood gas panel, may further enhance the use of lactate to stratify risk.

As the prognostic value of lactate in isolation is not excellent, not even in selected conditions, the inclusion of lactate into new or existing composite scoring tools is generally a sound idea. Future studies in the ED should focus on clinically meaningful questions and any retrospective results of interest should ideally be validated prospectively before introduced into clinical practice.

Conclusions

High-resolution respirometry is a detailed and nuanced method for assessing mitochondrial dysfunction. Mitochondrial function may differ in different tissues, and even in different cell types within the same tissue. Both in clinical and preclinical settings, blood cell mitochondria should not be assumed to be good biomarkers in a general sense but may have specific applications in certain pathological or systemic conditions.

High-resolution respirometry in blood cells cannot be used alone to rule out mitochondrial disease. The method may play some part in the composite assessment of suspected MD and could have possible future applications in monitoring treatment. However, for improving the early diagnostic process by non-invasive testing, especially for ruling out disease, it would probably be more fruitful to pursue other avenues of research.

Lactate is an unspecific but accessible test which acts as a general signal of metabolic distress, including mitochondrial dysfunction and many other aetiologies. It has several potential uses in emergency medicine but its interpretation is highly dependent of context. An elevated lactate is not always a sign of poor prognosis. Future studies should focus on specific, high-yield hypotheses and include clinically useful measurements.

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References

1. Mathews CK, van Holde K, Appling D. Biochemistry. Pearson; 2013.
2. Lane N. Transformer : the deep chemistry of life and death. Profile Books; 2022.
3. Mitchell P. Coupling of phosphorylation to electron and hydrogen transfer by a chemi-osmotic type of mechanism. Nature. Jul 8 1961;191:144-8. doi:10.1038/191144a0
4. Mitchell P, Moyle J. Chemiosmotic hypothesis of oxidative phosphorylation. Nature. Jan 14 1967;213(5072):137-9. doi:10.1038/213137a0
5. Demine S, Renard P, Arnould T. Mitochondrial Uncoupling: A Key Controller of Biological Processes in Physiology and Diseases. Cells. Jul 30 2019;8(8)doi:10.3390/cells8080795
6. Hengartner MO. The biochemistry of apoptosis. Nature. Oct 12 2000;407(6805):770-6. doi:10.1038/35037710
7. Bernardi P. Mitochondrial transport of cations: channels, exchangers, and permeability transition. Physiol Rev. Oct 1999;79(4):1127-55. doi:10.1152/physrev.1999.79.4.1127
8. Ryan DG, Murphy MP, Frezza C, et al. Coupling Krebs cycle metabolites to signalling in immunity and cancer. Nat Metab. Jan 2019;1:16-33. doi:10.1038/s42255-018-0014-7
9. Schwartz RM, Dayhoff MO. Origins of prokaryotes, eukaryotes, mitochondria, and chloroplasts. Science (New York, NY). Jan 27 1978;199(4327):395-403. doi:10.1126/science.202030
10. Sagan L. On the origin of mitosing cells. J Theor Biol. Mar 1967;14(3):255-74. doi:10.1016/0022-5193(67)90079-3
11. Lane N. Power, sex, suicide : mitochondria and the meaning of life. Oxford University Press; 2005.
12. Yahalomi D, Atkinson SD, Neuhof M, et al. A cnidarian parasite of salmon (Myxozoa: Henneguya) lacks a mitochondrial genome. Proc Natl Acad Sci U S A. Mar 10 2020;117(10):5358-5363. doi:10.1073/pnas.1909907117
13. Sjostrand FS. Electron microscopy of mitochondria and cytoplasmic double membranes. Nature. Jan 3 1953;171(4340):30-2. doi:10.1038/171030a0

Note: This publication by Swedish histologist Fritiof S. Sjöstrand features some of the earliest electron microscopy images of mitochondria (George E. Palade also published his work around the same time).

14. Grichine A, Jacob S, Eckly A, et al. The fate of mitochondria during platelet activation. Blood Advances. 2023/10/24/ 2023;7(20):6290-6302. doi:https://doi.org/10.1182/bloodadvances.2023010423
15. Parlakgül G, Pang S, Artico LL, et al. Spatial mapping of hepatic ER and mitochondria architecture reveals zonated remodeling in fasting and obesity. Nature Communications. 2024/05/10 2024;15(1):3982. doi:10.1038/s41467-024-48272-7
16. Alberts B. Molecular biology of the cell. Garland Science; 2002.
17. Wallace DC. The mitochondrial genome in human adaptive radiation and disease: on the road to therapeutics and performance enhancement. Gene. Jul 18 2005;354:169-80. doi:10.1016/j.gene.2005.05.001
18. Camus MF, Alexander-Lawrie B, Sharbrough J, Hurst GDD. Inheritance through the cytoplasm. Heredity (Edinb). Jul 2022;129(1):31-43. doi:10.1038/s41437-022-00540-2
19. Prole DL, Chinnery PF, Jones NS. Visualizing, quantifying, and manipulating mitochondrial DNA in vivo. The Journal of biological chemistry. Dec 18 2020;295(51):17588-17601. doi:10.1074/jbc.REV120.015101
20. Kevles DJ, Hood LE. The code of codes : scientific and social issues in the human genome project. Harvard Univ. Press; 1992.
21. O'Mahony S. After the golden age: what is medicine for? Lancet (London, England). May 4 2019;393(10183):1798-1799. doi:10.1016/s0140-6736(19)30901-8
22. Gannett L. The Human Genome Project. In: Edward NZ, Uri N, eds. The Stanford Encyclopedia of Philosophy. Fall 2023 ed. Metaphysics Research Lab Stanford University; 2023.
23. Klein RJ, Zeiss C, Chew EY, et al. Complement factor H polymorphism in age-related macular degeneration. Science (New York, NY). Apr 15 2005;308(5720):385-9. doi:10.1126/science.1109557
24. Jansen PR, Watanabe K, Stringer S, et al. Genome-wide analysis of insomnia in 1,331,010 individuals identifies new risk loci and functional pathways. Nat Genet. Mar 2019;51(3):394-403. doi:10.1038/s41588-018-0333-3
25. Manolio TA, Collins FS, Cox NJ, et al. Finding the missing heritability of complex diseases. Nature. Oct 8 2009;461(7265):747-53. doi:10.1038/nature08494
26. Wallace DC. A mitochondrial paradigm of metabolic and degenerative diseases, aging, and cancer: a dawn for evolutionary medicine. Annu Rev Genet. 2005;39:359-407. doi:10.1146/annurev.genet.39.110304.095751
27. Ehinger J. Mitochondrial dysfunction and metabolic intervention. Lund University, Faculty of Medicine; 2017.

28. Voices: The Future of Metabolism. Cell Metab. Jan 7 2020;31(1):3-5. doi:10.1016/j.cmet.2019.12.011

29. Harrison SA, Palmeira RN, Halpern A, Lane N. A biophysical basis for the emergence of the genetic code in protocells. Biochim Biophys Acta Bioenerg. Nov 1 2022;1863(8):148597. doi:10.1016/j.bbabio.2022.148597

30. Kim JA, Wei Y, Sowers JR. Role of mitochondrial dysfunction in insulin resistance. Circ Res. Feb 29 2008;102(4):401-14. doi:10.1161/circresaha.107.165472

31. Nassir F, Ibdah JA. Role of mitochondria in nonalcoholic fatty liver disease. Int J Mol Sci. May 15 2014;15(5):8713-42. doi:10.3390/ijms15058713

    Note: NAFLD has recently been renamed metabolic dysfunction-associated steatotic liver disease (MASLD), which further highlights its presumptive pathophysiology.

32. Zurlo F, Larson K, Bogardus C, Ravussin E. Skeletal muscle metabolism is a major determinant of resting energy expenditure. The Journal of clinical investigation. Nov 1990;86(5):1423-7. doi:10.1172/jci114857

33. Boushel R, Gnaiger E, Schjerling P, Skovbro M, Kraunsøe R, Dela F. Patients with type 2 diabetes have normal mitochondrial function in skeletal muscle. Diabetologia. Apr 2007;50(4):790-6. doi:10.1007/s00125-007-0594-3

34. Patti ME, Corvera S. The role of mitochondria in the pathogenesis of type 2 diabetes. Endocr Rev. Jun 2010;31(3):364-95. doi:10.1210/er.2009-0027

35. Ashcroft FM, Proks P, Smith PA, Ammälä C, Bokvist K, Rorsman P. Stimulus-secretion coupling in pancreatic beta cells. J Cell Biochem. 1994;55 Suppl:54-65. doi:10.1002/jcb.240550007

36. Ballinger SW, Shoffner JM, Hedaya EV, et al. Maternally transmitted diabetes and deafness associated with a 10.4 kb mitochondrial DNA deletion. Nat Genet. Apr 1992;1(1):11-5. doi:10.1038/ng0492-11

37. Miles WR, Root HF. Psyhologic tests applied to diabetic patients. Archives of Internal Medicine. 1922;30(6):767-777. doi:10.1001/archinte.1922.00110120086003

38. Biessels GJ, Staekenborg S, Brunner E, Brayne C, Scheltens P. Risk of dementia in diabetes mellitus: a systematic review. Lancet Neurol. Jan 2006;5(1):64-74. doi:10.1016/s1474-4422(05)70284-2

39. Gillardon F, Rist W, Kussmaul L, et al. Proteomic and functional alterations in brain mitochondria from Tg2576 mice occur before amyloid plaque deposition. Proteomics. Feb 2007;7(4):605-616. doi:10.1002/pmic.200600728

40. Area-Gomez E, Schon EA. On the Pathogenesis of Alzheimer's Disease: The MAM Hypothesis. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. Mar 2017;31(3):864-867. doi:10.1096/fj.201601309

41. Area-Gomez E, Schon EA. Towards a Unitary Hypothesis of Alzheimer's Disease Pathogenesis. J Alzheimers Dis. 2024;98(4):1243-1275. doi:10.3233/jad-231318

42. Warburg O, Wind F, Negelein E. Über den Stoffwechsel von Tumoren im Körper. Klinische Wochenschrift. 1926/05/01 1926;5(19):829-832. doi:10.1007/BF01726240

Note: The article is in German, an English version from 1927 is available with the title "The Metabolism of Tumors in the Body" (ref. 158).

43. Warburg O. On the origin of cancer cells. Science (New York, NY). Feb 24 1956;123(3191):309-14. doi:10.1126/science.123.3191.309

44. DeBerardinis RJ, Chandel NS. We need to talk about the Warburg effect. Nat Metab. Feb 2020;2(2):127-129. doi:10.1038/s42255-020-0172-2

45. Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell. Jun 2006;9(6):425-34. doi:10.1016/j.ccr.2006.04.023

Note: This mechanism has mainly been associated to hepatocellular carcinomas, phaeochromocytomas and paragangliomas. (Phaeochromocytomas may raise blood lactate by other mechanisms too, see ref. 165.)

46. López-Otín C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. Jun 6 2013;153(6):1194-217. doi:10.1016/j.cell.2013.05.039

47. Hanahan D, Weinberg RA. The hallmarks of cancer. Cell. Jan 7 2000;100(1):57-70. doi:10.1016/s0092-8674(00)81683-9

48. Gems D, de Magalhães JP. The hoverfly and the wasp: A critique of the hallmarks of aging as a paradigm. Ageing Res Rev. Sep 2021;70:101407. doi:10.1016/j.arr.2021.101407

49. Harman D. Aging: a theory based on free radical and radiation chemistry. J Gerontol. Jul 1956;11(3):298-300. doi:10.1093/geronj/11.3.298

Note: The name "mitochondrial free radical theory of ageing" was a later modification of the "free radical theory of ageing" and does not appear in this publication.

50. Goncalves RL, Quinlan CL, Perevoshchikova IV, Hey-Mogensen M, Brand MD. Sites of superoxide and hydrogen peroxide production by muscle mitochondria assessed ex vivo under conditions mimicking rest and exercise. The Journal of biological chemistry. Jan 2 2015;290(1):209-27. doi:10.1074/jbc.M114.619072

51. Gruber J, Schaffer S, Halliwell B. The mitochondrial free radical theory of ageing--where do we stand? Front Biosci. May 1 2008;13:6554-79. doi:10.2741/3174

52. Richter C, Park JW, Ames BN. Normal oxidative damage to mitochondrial and nuclear DNA is extensive. Proc Natl Acad Sci U S A. Sep 1988;85(17):6465-7. doi:10.1073/pnas.85.17.6465

53. Cortopassi GA, Shibata D, Soong NW, Arnheim N. A pattern of accumulation of a somatic deletion of mitochondrial DNA in aging human tissues. Proc Natl Acad Sci U S A. Aug 15 1992;89(16):7370-4. doi:10.1073/pnas.89.16.7370

54. Barja G, Herrero A. Oxidative damage to mitochondrial DNA is inversely related to maximum life span in the heart and brain of mammals. FASEB journal : official publication of the Federation of American Societies for Experimental Biology. Feb 2000;14(2):312-8. doi:10.1096/fasebj.14.2.312

55. Trifunovic A, Wredenberg A, Falkenberg M, et al. Premature ageing in mice expressing defective mitochondrial DNA polymerase. Nature. May 27 2004;429(6990):417-23. doi:10.1038/nature02517

56. Zhang Y, Ikeno Y, Qi W, et al. Mice deficient in both Mn superoxide dismutase and glutathione peroxidase-1 have increased oxidative damage and a greater incidence of pathology but no reduction in longevity. The journals of gerontology Series A, Biological sciences and medical sciences. Dec 2009;64(12):1212-20. doi:10.1093/gerona/glp132

57. Van Raamsdonk JM, Hekimi S. Superoxide dismutase is dispensable for normal animal lifespan. Proc Natl Acad Sci U S A. Apr 10 2012;109(15):5785-90. doi:10.1073/pnas.1116158109

58. Weindruch R, Walford RL, Fligiel S, Guthrie D. The retardation of aging in mice by dietary restriction: longevity, cancer, immunity and lifetime energy intake. J Nutr. Apr 1986;116(4):641-54. doi:10.1093/jn/116.4.641

59. Kealy RD, Lawler DF, Ballam JM, et al. Effects of diet restriction on life span and age-related changes in dogs. J Am Vet Med Assoc. May 1 2002;220(9):1315-20. doi:10.2460/javma.2002.220.1315

60. Colman RJ, Anderson RM, Johnson SC, et al. Caloric restriction delays disease onset and mortality in rhesus monkeys. Science (New York, NY). Jul 10 2009;325(5937):201-4. doi:10.1126/science.1173635

61. Krebs HA. Nobel Lecture - The Citric Acid Cycle. The Nobel Foundation. Accessed 12 November, 2024. https://www.nobelprize.org/prizes/medicine/1953/krebs/lecture/

62. Paul MH, Sperling E. Cyclophorase system. XXIII. Correlation of cyclophorase activity and mitochondrial density in striated muscle. Proc Soc Exp Biol Med. Mar 1952;79(3):352-4. doi:10.3181/00379727-79-19375

63. Holloszy JO. Biochemical adaptations in muscle. Effects of exercise on mitochondrial oxygen uptake and respiratory enzyme activity in skeletal muscle. The Journal of biological chemistry. May 10 1967;242(9):2278-82.

64. Gollnick PD, Armstrong RB, Saubert CWt, Piehl K, Saltin B. Enzyme activity and fiber composition in skeletal muscle of untrained and trained men. Journal of applied physiology. Sep 1972;33(3):312-9. doi:10.1152/jappl.1972.33.3.312

65. Gollnick PD, Armstrong RB, Saltin B, Saubert CWt, Sembrowich WL, Shepherd RE. Effect of training on enzyme activity and fiber composition of human skeletal muscle. Journal of applied physiology. Jan 1973;34(1):107-11. doi:10.1152/jappl.1973.34.1.107

66. Jacobs RA, Lundby C. Mitochondria express enhanced quality as well as quantity in association with aerobic fitness across recreationally active individuals up to elite athletes. Journal of applied physiology (Bethesda, Md : 1985). Feb 2013;114(3):344-50. doi:10.1152/japplphysiol.01081.2012

67. Puigserver P, Wu Z, Park CW, Graves R, Wright M, Spiegelman BM. A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. Cell. Mar 20 1998;92(6):829-39. doi:10.1016/s0092-8674(00)81410-5

68. Oliveira AN, Hood DA. Exercise is mitochondrial medicine for muscle. Sports Med Health Sci. Dec 2019;1(1):11-18. doi:10.1016/j.smhs.2019.08.008

69. Dybjer E, Nilsson PM, Engström G, Helmer C, Nägga K. Pre-diabetes and diabetes are independently associated with adverse cognitive test results: a cross-sectional, population-based study. BMC Endocr Disord. Dec 4 2018;18(1):91. doi:10.1186/s12902-018-0318-3

70. Baumgartner RN, Koehler KM, Gallagher D, et al. Epidemiology of sarcopenia among the elderly in New Mexico. Am J Epidemiol. Apr 15 1998;147(8):755-63. doi:10.1093/oxfordjournals.aje.a009520

71. Studenski S, Perera S, Patel K, et al. Gait speed and survival in older adults. Jama. Jan 5 2011;305(1):50-8. doi:10.1001/jama.2010.1923

72. Tulinius MH, Holme E, Kristiansson B, Larsson NG, Oldfors A. Mitochondrial encephalomyopathies in childhood. I. Biochemical and morphologic investigations. J Pediatr. Aug 1991;119(2):242-50. doi:10.1016/s0022-3476(05)80734-6

73. Sciacco M, Bonilla E, Schon EA, DiMauro S, Moraes CT. Distribution of wild-type and common deletion forms of mtDNA in normal and respiration-deficient muscle fibers from patients with mitochondrial myopathy. Hum Mol Genet. Jan 1994;3(1):13-9. doi:10.1093/hmg/3.1.13

74. Di Lisa F, Menabò R, Canton M, Petronilli V. The role of mitochondria in the salvage and the injury of the ischemic myocardium. Biochim Biophys Acta. Aug 10 1998;1366(1-2):69-78. doi:10.1016/s0005-2728(98)00121-2

75. Kuznetsov AV, Javadov S, Margreiter R, Grimm M, Hagenbuchner J, Ausserlechner MJ. The Role of Mitochondria in the Mechanisms of Cardiac Ischemia-Reperfusion Injury. Antioxidants (Basel). Oct 6 2019;8(10)doi:10.3390/antiox8100454

76. Huang J, Chen L, Yao ZM, Sun XR, Tong XH, Dong SY. The role of mitochondrial dynamics in cerebral ischemia-reperfusion injury. Biomed Pharmacother. Jun 2023;162:114671. doi:10.1016/j.biopha.2023.114671

77. Beţiu AM, Noveanu L, Hâncu IM, et al. Mitochondrial Effects of Common Cardiovascular Medications: The Good, the Bad and the Mixed. Int J Mol Sci. Nov 7 2022;23(21)doi:10.3390/ijms232113653

78. Varga ZV, Ferdinandy P, Liaudet L, Pacher P. Drug-induced mitochondrial dysfunction and cardiotoxicity. American journal of physiology Heart and circulatory physiology. Nov 2015;309(9):H1453-67. doi:10.1152/ajpheart.00554.2015

79. Jaeschke H, McGill MR, Ramachandran A. Oxidant stress, mitochondria, and cell death mechanisms in drug-induced liver injury: lessons learned from acetaminophen hepatotoxicity. Drug Metab Rev. Feb 2012;44(1):88-106. doi:10.3109/03602532.2011.602688

80. Garrabou G, Inoriza JM, Morén C, et al. Mitochondrial injury in human acute carbon monoxide poisoning: the effect of oxygen treatment. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev. 2011;29(1):32-51. doi:10.1080/10590501.2011.551316

81. Piel S, Ehinger JK, Elmér E, Hansson MJ. Metformin induces lactate production in peripheral blood mononuclear cells and platelets through specific mitochondrial complex I inhibition. Acta physiologica (Oxford, England). Jan 2015;213(1):171-80. doi:10.1111/apha.12311

82. Fink MP. Cytopathic hypoxia. Mitochondrial dysfunction as mechanism contributing to organ dysfunction in sepsis. Crit Care Clin. Jan 2001;17(1):219-37. doi:10.1016/s0749-0704(05)70161-5

83. Moskowitz A, Berg KM, Grossestreuer AV, et al. Thiamine for Renal Protection in Septic Shock (TRPSS): A Randomized, Placebo-controlled, Clinical Trial. Am J Respir Crit Care Med. Sep 1 2023;208(5):570-578. doi:10.1164/rccm.202301-0034OC

    Note: It was unsuccessful.

84. Parikh S, Goldstein A, Koenig MK, et al. Diagnosis and management of mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. Genet Med. Sep 2015;17(9):689-701. doi:10.1038/gim.2014.177

85. Strimbu K, Tavel JA. What are biomarkers? Curr Opin HIV AIDS. Nov 2010;5(6):463-6. doi:10.1097/COH.0b013e32833ed177

86. Tyrrell DJ, Bharadwaj MS, Jorgensen MJ, Register TC, Molina AJ. Blood cell respirometry is associated with skeletal and cardiac muscle bioenergetics: Implications for a minimally invasive biomarker of mitochondrial health. Redox biology. Dec 2016;10:65-77. doi:10.1016/j.redox.2016.09.009

87. Petrus AT, Lighezan DL, Danila MD, et al. Assessment of platelet respiration as emerging biomarker of disease. Physiol Res. Jun 30 2019;68(3):347-363. doi:10.33549/physiolres.934032

88. Braganza A, Annarapu GK, Shiva S. Blood-based bioenergetics: An emerging translational and clinical tool. Molecular Aspects of Medicine. Feb 2020;71100835. doi:10.1016/j.mam.2019.100835

89. Acin-Perez R, Benincá C, Shabane B, Shirihai OS, Stiles L. Utilization of Human Samples for Assessment of Mitochondrial Bioenergetics: Gold Standards, Limitations, and Future Perspectives. Life (Basel, Switzerland). Sep 10 2021;11(9)doi:10.3390/life11090949

90. Hubens WHG, Vallbona-Garcia A, de Coo IFM, et al. Blood biomarkers for assessment of mitochondrial dysfunction: An expert review. Mitochondrion. Jan 2022;62:187-204. doi:10.1016/j.mito.2021.10.008

91. Bergstrom J. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. Scandinavian journal of clinical and laboratory investigation. Nov 1975;35(7):609-16.

92. Gallo A, Abraham A, Katzberg HD, Ilaalagan S, Bril V, Breiner A. Muscle biopsy technical safety and quality using a self-contained, vacuum-assisted biopsy technique. Neuromuscul Disord. May 2018;28(5):450-453. doi:10.1016/j.nmd.2018.02.006

93. Newmire DE, Willoughby DS. The skeletal muscle microbiopsy method in exercise and sports science research: A narrative and methodological review. Scand J Med Sci Sports. Nov 2022;32(11):1550-1568. doi:10.1111/sms.14215

94. Gibreel WO, Selcen D, Zeidan MM, Ishitani MB, Moir CR, Zarroug AE. Safety and yield of muscle biopsy in pediatric patients in the modern era. J Pediatr Surg. Sep 2014;49(9):1429-32. doi:10.1016/j.jpedsurg.2014.02.079

Note: Studies on safety usually report a low incidence of mainly non-serious complications, such as local haematoma (ref. 92). This study reported one incident of a surgeon fainting (!) during the procedure and, in the fall, accidentally cutting off the patient's femoral vein, prompting immediate surgical repair and extended follow-up with repeated duplex sonography. Traumatic fall injuries contracted by the surgeon, if any, were unmentioned and not featured in the list of complications.

95. Rumsey WL, Kendrick ZV, Starnes JW. Bioenergetics in the aging Fischer 344 rat: effects of exercise and food restriction. Experimental gerontology. 1987;22(4):271-87. doi:10.1016/0531-5565(87)90006-4

96. Trounce I, Byrne E, Marzuki S. Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing. Lancet (London, England). Mar 25 1989;1(8639):637-9. doi:10.1016/s0140-6736(89)92143-0

97. Cooper JM, Mann VM, Schapira AH. Analyses of mitochondrial respiratory chain function and mitochondrial DNA deletion in human skeletal muscle: effect of ageing. Journal of the neurological sciences. Nov 1992;113(1):91-8. doi:10.1016/0022-510x(92)90270-u

98. Boffoli D, Scacco SC, Vergari R, Solarino G, Santacroce G, Papa S. Decline with age of the respiratory chain activity in human skeletal muscle. Biochim Biophys Acta. Apr 12 1994;1226(1):73-82. doi:10.1016/0925-4439(94)90061-2

99. Rasmussen UF, Krustrup P, Kjaer M, Rasmussen HN. Experimental evidence against the mitochondrial theory of aging. A study of isolated human skeletal muscle mitochondria. Experimental gerontology. Aug 2003;38(8):877-86. doi:10.1016/s0531-5565(03)00092-5

100. Short KR, Bigelow ML, Kahl J, et al. Decline in skeletal muscle mitochondrial function with aging in humans. Proc Natl Acad Sci U S A. Apr 12 2005;102(15):5618-23. doi:10.1073/pnas.0501559102

101. Picard M, Ritchie D, Wright KJ, et al. Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. Aging Cell. Dec 2010;9(6):1032-46. doi:10.1111/j.1474-9726.2010.00628.x

102. D'Aurelio M, Merlo Pich M, Catani L, et al. Decreased Pasteur effect in platelets of aged individuals. Mech Ageing Dev. Jun 2001;122(8):823-33. doi:10.1016/s0047-6374(01)00239-1

103. Shi C, Guo K, Yew DT, et al. Effects of ageing and Alzheimer's disease on mitochondrial function of human platelets. Experimental gerontology. Jun 2008;43(6):589-94. doi:10.1016/j.exger.2008.02.004

104. Braganza A, Corey CG, Santanasto AJ, et al. Platelet bioenergetics correlate with muscle energetics and are altered in older adults. JCI insight. May 23 2019;5doi:10.1172/jci.insight.128248

105. Jedlička J, Kunc R, Kuncová J. Mitochondrial respiration of human platelets in young adult and advanced age - Seahorse or O2k? Physiol Res. Dec 31 2021;70(S3):S369-s379. doi:10.33549/physiolres.934812

106. Alonso M, Zabala C, Mansilla S, et al. Blood cell respiration rates and mtDNA copy number: A promising tool for the diagnosis of mitochondrial disease. Mitochondrion. Nov 2021;61:31-43. doi:10.1016/j.mito.2021.09.004

107. Chacko BK, Smith MR, Johnson MS, et al. Mitochondria in precision medicine; linking bioenergetics and metabolomics in platelets. Redox biology. Apr 2019;22:101165. doi:10.1016/j.redox.2019.101165

108. Gupta R, Kanai M, Durham TJ, et al. Nuclear genetic control of mtDNA copy number and heteroplasmy in humans. Nature. Aug 2023;620(7975):839-848. doi:10.1038/s41586-023-06426-5

109. Little JP, Safdar A, Benton CR, Wright DC. Skeletal muscle and beyond: the role of exercise as a mediator of systemic mitochondrial biogenesis. Appl Physiol Nutr Metab. Oct 2011;36(5):598-607. doi:10.1139/h11-076

110. Rose S, Carvalho E, Diaz EC, et al. A comparative study of mitochondrial respiration in circulating blood cells and skeletal muscle fibers in women. American journal of physiology Endocrinology and metabolism. Sep 1 2019;317(3):E503-e512. doi:10.1152/ajpendo.00084.2019

111. Hedges CP, Woodhead JST, Wang HW, et al. Peripheral blood mononuclear cells do not reflect skeletal muscle mitochondrial function or adaptation to high-intensity interval training in healthy young men. Journal of applied physiology (Bethesda, Md : 1985). Feb 1 2019;126(2):454-461. doi:10.1152/japplphysiol.00777.2018

112. Kaushansky K, Lichtman MA, Prchal JT, et al. Williams Hematology. 9 ed. McGraw-Hill Education; 2015.

113. Fulcher DA, Basten A. B cell life span: a review. Immunol Cell Biol. Oct 1997;75(5):446-55. doi:10.1038/icb.1997.69

114. Macallan DC, Borghans JA, Asquith B. Human T Cell Memory: A Dynamic View. Vaccines (Basel). Feb 4 2017;5(1)doi:10.3390/vaccines5010005

115. Faas MM, de Vos P. Mitochondrial function in immune cells in health and disease. Biochim Biophys Acta Mol Basis Dis. Oct 1 2020;1866(10):165845. doi:10.1016/j.bbadis.2020.165845

116. Garraud O, Cognasse F. Are Platelets Cells? And if Yes, are They Immune Cells? Front Immunol. 2015;6:70. doi:10.3389/fimmu.2015.00070

117. Kaushansky K. Thrombopoiesis. Semin Hematol. Jan 2015;52(1):4-11. doi:10.1053/j.seminhematol.2014.10.003

118. Chacko BK, Kramer PA, Ravi S, et al. Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood. Laboratory investigation; a journal of technical methods and pathology. Jun 2013;93(6):690-700. doi:10.1038/labinvest.2013.53

119. Kholmukhamedov A, Jobe S. Platelet respiration. Blood Adv. Feb 26 2019;3(4):599-602. doi:10.1182/bloodadvances.2018025155

120. Weyrich AS, Schwertz H, Kraiss LW, Zimmerman GA. Protein synthesis by platelets: historical and new perspectives. J Thromb Haemost. Feb 2009;7(2):241-6. doi:10.1111/j.1538-7836.2008.03211.x

121. Richardson JL, Shivdasani RA, Boers C, Hartwig JH, Italiano JE, Jr. Mechanisms of organelle transport and capture along proplatelets during platelet production. Blood. Dec 15 2005;106(13):4066-75. doi:10.1182/blood-2005-06-2206

122. Luft R, Ikkos D, Palmieri G, Ernster L, Afzelius B. A case of severe hypermetabolism of nonthyroid origin with a defect in the maintenance of mitochondrial respiratory control: a correlated clinical, biochemical, and morphological study. The Journal of clinical investigation. Sep 1962;41(9):1776-804. doi:10.1172/jci104637

Note: The same case was initially mentioned in a 1959 article with speculation of a mitochondrial aethiology but the 1962 article provided more conclusive biochemical support.

123. Ganetzky RD, Markhard AL, Yee I, et al. Congenital Hypermetabolism and Uncoupled Oxidative Phosphorylation. The New England journal of medicine. Oct 13 2022;387(15):1395-1403. doi:10.1056/NEJMoa2202949

Note: Luft's disease has turned out to be rare even among mitochondrial diseases. This recent paper reports one of few known (possible) cases and also sheds light on the possible cause.

124. Darin N, Oldfors A, Moslemi AR, Holme E, Tulinius M. The incidence of mitochondrial encephalomyopathies in childhood: clinical features and morphological, biochemical, and DNA abnormalities. Annals of neurology. Mar 2001;49(3):377-83.

125. Rahman S, Blok RB, Dahl HH, et al. Leigh syndrome: clinical features and biochemical and DNA abnormalities. Annals of neurology. Mar 1996;39(3):343-51. doi:10.1002/ana.410390311

126. Lake NJ, Compton AG, Rahman S, Thorburn DR. Leigh syndrome: One disorder, more than 75 monogenic causes. Annals of neurology. Feb 2016;79(2):190-203. doi:10.1002/ana.24551

127. Yu-Wai-Man P, Griffiths PG, Chinnery PF. Mitochondrial optic neuropathies - disease mechanisms and therapeutic strategies. Prog Retin Eye Res. Mar 2011;30(2):81-114. doi:10.1016/j.preteyeres.2010.11.002

128. Savvidou A, Sofou K, Eklund EA, Aronsson J, Darin N. Manifestations of X-linked pyruvate dehydrogenase complex deficiency in female PDHA1 carriers. Eur J Neurol. Jul 2024;31(7):e16283. doi:10.1111/ene.16283

129. Sofou K, De Coo IF, Isohanni P, et al. A multicenter study on Leigh syndrome: disease course and predictors of survival. Orphanet J Rare Dis. Apr 15 2014;9:52. doi:10.1186/1750-1172-9-52

130. Tinker RJ, Lim AZ, Stefanetti RJ, McFarland R. Current and Emerging Clinical Treatment in Mitochondrial Disease. Mol Diagn Ther. Mar 2021;25(2):181-206. doi:10.1007/s40291-020-00510-6

131. Carelli V, Carbonelli M, de Coo IF, et al. International Consensus Statement on the Clinical and Therapeutic Management of Leber Hereditary Optic Neuropathy. J Neuroophthalmol. Dec 2017;37(4):371-381. doi:10.1097/wno.0000000000000570

132. Åsander Frostner E, Simón Serrano M, Chamkha I, Donnelly E, Elmér E, Hansson M. Towards a treatment for mitochondrial disease: current compounds in clinical development. Bioenerg Commun. 2022;4doi:10.26124/bec:2022-0004

133. Haas RH, Parikh S, Falk MJ, et al. Mitochondrial disease: a practical approach for primary care physicians. Pediatrics. Dec 2007;120(6):1326-33. doi:10.1542/peds.2007-0391

134. Bernier FP, Boneh A, Dennett X, Chow CW, Cleary MA, Thorburn DR. Diagnostic criteria for respiratory chain disorders in adults and children. Neurology. Nov 12 2002;59(9):1406-11. doi:10.1212/01.wnl.0000033795.17156.00

135. Thompson JLP, Karaa A, Pham H, et al. The evolution of the mitochondrial disease diagnostic odyssey. Orphanet J Rare Dis. Jun 22 2023;18(1):157. doi:10.1186/s13023-023-02754-x

Note: Even though the publication date is recent, the time to diagnosis is based on retrospective data over an extended period of time. The duration of a diagnostic investigation initiated today will probably be shorter.

136. Sofou K. Genotypic and phenotypic spectrum of mitochondrial diseases with focus on early onset mitochondrial encephalopathies. Department of Pediatrics, The Queen Silvia Children's Hospital, Institute of Clinical Sciences, Sahlgrenska Academy at University of Gothenburg; 2014.

137. Debray FG, Mitchell GA, Allard P, Robinson BH, Hanley JA, Lambert M. Diagnostic accuracy of blood lactate-to-pyruvate molar ratio in the differential diagnosis of congenital lactic acidosis. Clin Chem. May 2007;53(5):916-21. doi:10.1373/clinchem.2006.081166

138. Nazir M, Wani WA, Malik MA, et al. Cerebrospinal fluid lactate: a differential biomarker for bacterial and viral meningitis in children. J Pediatr (Rio J). Jan-Feb 2018;94(1):88-92. doi:10.1016/j.jped.2017.03.007

139. Debray FG, Lambert M, Chevalier I, et al. Long-term outcome and clinical spectrum of 73 pediatric patients with mitochondrial diseases. Pediatrics. Apr 2007;119(4):722-33. doi:10.1542/peds.2006-1866

140. Sofou K, Steneryd K, Wiklund LM, Tulinius M, Darin N. MRI of the brain in childhood-onset mitochondrial disorders with central nervous system involvement. Mitochondrion. Jul 2013;13(4):364-71. doi:10.1016/j.mito.2013.04.008

141. Dinopoulos A, Cecil KM, Schapiro MB, et al. Brain MRI and proton MRS findings in infants and children with respiratory chain defects. Neuropediatrics. Oct 2005;36(5):290-301. doi:10.1055/s-2005-872807

142. Haas RH, Parikh S, Falk MJ, et al. The in-depth evaluation of suspected mitochondrial disease. Molecular genetics and metabolism. May 2008;94(1):16-37. doi:10.1016/j.ymgme.2007.11.018

143. Yamamoto M, Koga Y, Ohtaki E, Nonaka I. Focal cytochrome c oxidase deficiency in various neuromuscular diseases. Journal of the neurological sciences. Jun 1989;91(1-2):207-13. doi:10.1016/0022-510x(89)90088-9

144. McCormick EM, Zolkipli-Cunningham Z, Falk MJ. Mitochondrial disease genetics update: recent insights into the molecular diagnosis and expanding phenotype of primary mitochondrial disease. Curr Opin Pediatr. Dec 2018;30(6):714-724. doi:10.1097/mop.0000000000000686

145. Spinazzola A, Zeviani M. Disorders of nuclear-mitochondrial intergenomic communication. Biosci Rep. Jun 2007;27(1-3):39-51. doi:10.1007/s10540-007-9036-1

146. Liang C, Ahmad K, Sue CM. The broadening spectrum of mitochondrial disease: shifts in the diagnostic paradigm. Biochim Biophys Acta. Apr 2014;1840(4):1360-7. doi:10.1016/j.bbagen.2013.10.040

147. Ng YS, Bindoff LA, Gorman GS, et al. Mitochondrial disease in adults: recent advances and future promise. Lancet Neurol. Jul 2021;20(7):573-584. doi:10.1016/s1474-4422(21)00098-3

148. Puusepp S, Reinson K, Pajusalu S, et al. Effectiveness of whole exome sequencing in unsolved patients with a clinical suspicion of a mitochondrial disorder in Estonia. Mol Genet Metab Rep. Jun 2018;15:80-89. doi:10.1016/j.ymgmr.2018.03.004

149. Schon KR, Horvath R, Wei W, et al. Use of whole genome sequencing to determine genetic basis of suspected mitochondrial disorders: cohort study. BMJ (Clinical research ed). Nov 3 2021;375:e066288. doi:10.1136/bmj-2021-066288

150. Thomas RH, Hunter A, Butterworth L, et al. Research priorities for mitochondrial disorders: Current landscape and patient and professional views. J Inherit Metab Dis. Jul 2022;45(4):796-803. doi:10.1002/jimd.12521

151. Scheele CW. Om Mjölk, och dess syra [About milk, and its acid]. Kongliga Vetenskaps Academiens Nya Handlingar [New Proceedings of the Royal Academy of Science]. 1780;1:116-124.

    Note: In addition to lactic acid, Scheele discovered several other organic acids and chemical elements. He discovered and, reportedly, ate arsenic, which contributed to his fame and death, respectively. His most famous discovery was oxygen.

152. Rabinowitz JD, Enerbäck S. Lactate: the ugly duckling of energy metabolism. Nat Metab. Jul 2020;2(7):566-571. doi:10.1038/s42255-020-0243-4

153. Vavřička J, Brož P, Follprecht D, Novák J, Kroužecký A. Modern Perspective of Lactate Metabolism. Physiol Res. Aug 31 2024;73(4):499-514. doi:10.33549/physiolres.935331

154. Brooks GA. The Science and Translation of Lactate Shuttle Theory. Cell Metab. Apr 3 2018;27(4):757-785. doi:10.1016/j.cmet.2018.03.008

155. Ferguson BS, Rogatzki MJ, Goodwin ML, Kane DA, Rightmire Z, Gladden LB. Lactate metabolism: historical context, prior misinterpretations, and current understanding. Eur J Appl Physiol. Apr 2018;118(4):691-728. doi:10.1007/s00421-017-3795-6

156. Kompanje EJ, Jansen TC, van der Hoven B, Bakker J. The first demonstration of lactic acid in human blood in shock by Johann Joseph Scherer (1814-1869) in January 1843. Intensive Care Med. Nov 2007;33(11):1967-71. doi:10.1007/s00134-007-0788-7

157. Fletcher WM. Lactic acid in amphibian muscle. The Journal of physiology. Mar 27 1907;35(4):247-309. doi:10.1113/jphysiol.1907.sp001194

158. Warburg O, Wind F, Negelein E. THE METABOLISM OF TUMORS IN THE BODY. J Gen Physiol. Mar 7 1927;8(6):519-30. doi:10.1085/jgp.8.6.519

159. Cori CF, Cori GT. Glycogen formation in the liver from d- and l-lactic acid. Journal of Biological Chemistry. Feb 1929;81(2):389-403.

    Note: This is not the first publication of what later became known as the Cori cycle, but the concept of a cycle is more clearly expressed here than in previous publications.

160. Hui S, Ghergurovich JM, Morscher RJ, et al. Glucose feeds the TCA cycle via circulating lactate. Nature. Nov 2 2017;551(7678):115-118. doi:10.1038/nature24057

161. Brooks GA. Cell-cell and intracellular lactate shuttles. The Journal of physiology. Dec 1 2009;587(Pt 23):5591-600. doi:10.1113/jphysiol.2009.178350

162. McKelvie RS, Lindinger MI, Heigenhauser GJ, Sutton JR, Jones NL. Renal responses to exercise-induced lactic acidosis. Am J Physiol. Jul 1989;257(1 Pt 2):R102-8. doi:10.1152/ajpregu.1989.257.1.R102

163. Müller J, Radej J, Horak J, et al. Lactate: The Fallacy of Oversimplification. Biomedicines. Dec 1 2023;11(12)doi:10.3390/biomedicines11123192

164. Levy B, Desebbe O, Montemont C, Gibot S. Increased aerobic glycolysis through beta2 stimulation is a common mechanism involved in lactate formation during shock states. Shock. Oct 2008;30(4):417-21. doi:10.1097/SHK.0b013e318167378f

165. Madias NE, Goorno WE, Herson S. Severe lactic acidosis as a presenting feature of pheochromocytoma. American journal of kidney diseases : the official journal of the National Kidney Foundation. Sep 1987;10(3):250-3. doi:10.1016/s0272-6386(87)80182-8

166. Koul PB, Minarik M, Totapally BR. Lactic acidosis in children with acute exacerbation of severe asthma. Eur J Emerg Med. Feb 2007;14(1):56-8. doi:10.1097/01.mej.0000224430.59246.cf

167. Grip J, Jakobsson T, Hebert C, et al. Lactate kinetics and mitochondrial respiration in skeletal muscle of healthy humans under influence of adrenaline. Clin Sci (Lond). Aug 2015;129(4):375-84. doi:10.1042/cs20140448

168. Wilson DF, Matschinsky FM. Ethanol metabolism: The good, the bad, and the ugly. Med Hypotheses. Feb 19 2020;140:109638. doi:10.1016/j.mehy.2020.109638

169. Baettig SJ, Fend RR, Gero D, Gutschow C, Schlaepfer M. Postoperative exacerbated cough hypersensitivity syndrome induces dramatic respiratory alkalosis, lactatemia, and electrolyte imbalance. BMC Anesthesiol. Sep 11 2024;24(1):323. doi:10.1186/s12871-024-02695-3

170. Clarke C, Xiao R, Place E, et al. Mitochondrial respiratory chain disease discrimination by retrospective cohort analysis of blood metabolites. Molecular genetics and metabolism. Sep-Oct 2013;110(1-2):145-52. doi:10.1016/j.ymgme.2013.07.011

171. Madl C, Kranz A, Liebisch B, Traindl O, Lenz K, Druml W. Lactic acidosis in thiamine deficiency. Clin Nutr. Apr 1993;12(2):108-11. doi:10.1016/0261-5614(93)90060-h

172. Moon JM, Shin MH, Chun BJ. The value of initial lactate in patients with carbon monoxide intoxication: in the emergency department. Hum Exp Toxicol. Aug 2011;30(8):836-43. doi:10.1177/0960327110384527

173. Claessens YE, Chiche JD, Mira JP, Cariou A. Bench-to-bedside review: severe lactic acidosis in HIV patients treated with nucleoside analogue reverse transcriptase inhibitors. Critical care (London, England). Jun 2003;7(3):226-32. doi:10.1186/cc2162

174. Baud FJ, Borron SW, Mégarbane B, et al. Value of lactic acidosis in the assessment of the severity of acute cyanide poisoning. Crit Care Med. Sep 2002;30(9):2044-50. doi:10.1097/00003246-200209000-00015

175. Kruse JA, Zaidi SA, Carlson RW. Significance of blood lactate levels in critically ill patients with liver disease. The American journal of medicine. Jul 1987;83(1):77-82. doi:10.1016/0002-9343(87)90500-6

176. Bellomo R. Bench-to-bedside review: lactate and the kidney. Critical care (London, England). Aug 2002;6(4):322-6. doi:10.1186/cc1518

177. Scherer JJ. Chemische und Mikroskopische Untersuchungen zur Pathologie angestellt an den Kliniken des Julius-Hospitales zu Würzburg. C.F. Winter; 1843.

    Note: Pdf online (https://wellcomecollection.org/works/sh44nk3z), fetched on 13 September 2024. The book is in German; Kompanje et al. (ref. 156) summarise the contents in English and elaborates on the historical context.

178. Huckabee WE. Abnormal resting blood lactate: II. Lactic acidosis. The American journal of medicine. 1961/06/01/ 1961;30(6):840-848. doi:https://doi.org/10.1016/0002-9343(61)90172-3

179. Weil MH, Afifi AA. Experimental and clinical studies on lactate and pyruvate as indicators of the severity of acute circulatory failure (shock). Circulation. Jun 1970;41(6):989-1001. doi:10.1161/01.cir.41.6.989

180. Cohen RDW, H.F. Clinical and Biochemical Aspects of Lactic Acidosis. Blackwell Scientific Publications Ltd; 1976.

181. Hochachka PW, Mommsen TP. Protons and anaerobiosis. Science (New York, NY). Mar 25 1983;219(4591):1391-7. doi:10.1126/science.6298937

182. Mainwood GW, Renaud JM. The effect of acid-base balance on fatigue of skeletal muscle. Can J Physiol Pharmacol. May 1985;63(5):403-16. doi:10.1139/y85-072

183. Robergs RA, Ghiasvand F, Parker D. Biochemistry of exercise-induced metabolic acidosis. Am J Physiol Regul Integr Comp Physiol. Sep 2004;287(3):R502-16. doi:10.1152/ajpregu.00114.2004

184. Böning D, Maassen N. Point: Lactic acid is the only physicochemical contributor to the acidosis of exercise. Journal of applied physiology (Bethesda, Md : 1985). Jul 2008;105(1):358-9. doi:10.1152/japplphysiol.00162.2008

185. Qian Q. Reply to Robergs et al. Physiology (Bethesda, Md). Jan 1 2018;33(1):13. doi:10.1152/physiol.00034.2017

186. Deshpande SA, Platt MP. Association between blood lactate and acid-base status and mortality in ventilated babies. Arch Dis Child Fetal Neonatal Ed. Jan 1997;76(1):F15-20. doi:10.1136/fn.76.1.f15

187. Paschen W, Djuricic B, Mies G, Schmidt-Kastner R, Linn F. Lactate and pH in the brain: association and dissociation in different pathophysiological states. J Neurochem. Jan 1987;48(1):154-9. doi:10.1111/j.1471-4159.1987.tb13140.x

188. Morgan TJ, Hall JA. Hyperlactaemia without acidosis - an investigation using an in vitro model. Crit Care Resusc. Dec 1999;1(4):354-9.

189. Davenport A, Will EJ, Davison AM. Hyperlactataemia and metabolic acidosis during haemofiltration using lactate-buffered fluids. Nephron. 1991;59(3):461-5. doi:10.1159/000186609

190. Casserly B, Phillips GS, Schorr C, et al. Lactate measurements in sepsis-induced tissue hypoperfusion: results from the Surviving Sepsis Campaign database. Crit Care Med. Mar 2015;43(3):567-73. doi:10.1097/ccm.0000000000000742

191. Reid C, Rees V, Collyer-Merritt H. Non-septic hyperlactataemia in the emergency department. Emerg Med J. May 2010;27(5):411-2. doi:10.1136/emj.2009.087379

192. Andersen LW, Mackenhauer J, Roberts JC, Berg KM, Cocchi MN, Donnino MW. Etiology and therapeutic approach to elevated lactate levels. Mayo Clinic proceedings. Oct 2013;88(10):1127-40. doi:10.1016/j.mayocp.2013.06.012

193. Wardi G, Brice J, Correia M, Liu D, Self M, Tainter C. Demystifying Lactate in the Emergency Department. Ann Emerg Med. Feb 2020;75(2):287-298. doi:10.1016/j.annemergmed.2019.06.027

194. Aksu A, Gulen M, Avci A, Satar S. Adding lactate to SOFA and qSOFA scores predicts in-hospital mortality better in older patients in critical care. Eur Geriatr Med. Jun 2019;10(3):445-453. doi:10.1007/s41999-019-00179-z

195. Donoso Calero MI, Mordillo-Mateos L, Martín-Conty JL, et al. Modified Rapid Emergency Medicine Score-Lactate (mREMS-L) performance to screen non-anticipated 30-day-related-mortality in emergency department. Eur J Clin Invest. Aug 2023;53(8):e13994. doi:10.1111/eci.13994

196. Dundar ZD, Kocak S, Girisgin AS. Lactate and NEWS-L are fair predictors of mortality in critically ill geriatric emergency department patients. Am J Emerg Med. Feb 2020;38(2):217-221. doi:10.1016/j.ajem.2019.02.006

197. Durantez-Fernández C, Martín-Conty JL, Polonio-López B, et al. Lactate improves the predictive ability of the National Early Warning Score 2 in the emergency department. Aust Crit Care. Nov 2022;35(6):677-683. doi:10.1016/j.aucc.2021.10.007

198. Edward U, Sawe HR, Mfinanga JA, et al. The utility of point of care serum lactate in predicting serious adverse outcomes among critically ill adult patients at urban emergency departments of tertiary hospitals in Tanzania. Trop Med Health. 2019;47:61. doi:10.1186/s41182-019-0186-1

199. Hori T, Aihara KI, Watanabe T, et al. The Respiratory Adjusted Shock Index at Admission Is a Valuable Predictor of In-Hospital Outcomes for Elderly Emergency Patients with Medical Diseases at a Japanese Community General Hospital. Article. Journal of Clinical Medicine. 2024;13(16)doi:10.3390/jcm13164866

200. Jansen TC, van Bommel J, Mulder PG, Rommes JH, Schieveld SJ, Bakker J. The prognostic value of blood lactate levels relative to that of vital signs in the pre-hospital setting: a pilot study. Critical care (London, England). 2008;12(6):R160. doi:10.1186/cc7159

201. Ma X, Li J, Zhou Q, Wang J. Serum lactate and the mortality of critically ill patients in the emergency department: A retrospective study. Exp Ther Med. Aug 2023;26(2):371. doi:10.3892/etm.2023.12070

202. Park YJ, Kim DH, Kim SC, et al. Serum lactate upon emergency department arrival as a predictor of 30-day in-hospital mortality in an unselected population. PloS one. 2018;13(1):e0190519. doi:10.1371/journal.pone.0190519

203. Seker YC, Bozan O, Sam E, Topacoglu H, Kalkan A. The role of the serum lactate level at the first admission to the emergency department in predicting mortality. Am J Emerg Med. Jul 2021;45:495-500. doi:10.1016/j.ajem.2020.09.088

204. Zhang Y, Wang J, Yu BZ, et al. Predictive Value of Heart-Type Fatty Acid-Binding Protein for Mortality Risk in Critically Ill Patients. Dis Markers. 2022;2022:1720414. doi:10.1155/2022/1720414

205. Brio-Ibañez PD, López-Izquierdo R, Martín-Rodríguez F, et al. Clinical Utility of Delta Lactate for Predicting Early In-Hospital Mortality in Adult Patients: A Prospective, Multicentric, Cohort Study. Diagnostics (Basel). Nov 17 2020;10(11)doi:10.3390/diagnostics10110960

206. del Portal DA, Shofer F, Mikkelsen ME, et al. Emergency department lactate is associated with mortality in older adults admitted with and without infections. Academic emergency medicine : official journal of the Society for Academic Emergency Medicine. Mar 2010;17(3):260-8. doi:10.1111/j.1553-2712.2010.00681.x

207. Graham CA, Leung LY, Lo RSL, Yeung CY, Chan SY, Hung KKC. NEWS and qSIRS superior to qSOFA in the prediction of 30-day mortality in emergency department patients in Hong Kong. Ann Med. Nov 2020;52(7):403-412. doi:10.1080/07853890.2020.1782462

208. Hucker TR, Mitchell GP, Blake LD, et al. Identifying the sick: can biochemical measurements be used to aid decision making on presentation to the accident and emergency department. Br J Anaesth. Jun 2005;94(6):735-41. doi:10.1093/bja/aei122

209. Kaçar AA, Aksay E, Bayram B, Kıran E, Güldalı BE. Identifying high-risk undifferentiated emergency department patients with hyperlactatemia: Predictors of 30-day in-hospital mortality. Turk J Emerg Med. Jul-Sep 2024;24(3):158-164. doi:10.4103/tjem.tjem_34_24

210. Susur O, Yesilaras M, Eyler Y. The Relationship Between Lactate and Lactate Clearance with In-Hospital Mortality in Unselected Emergency Department Patients. Prehosp Disaster Med. Apr 2024;39(2):178-183. doi:10.1017/s1049023x24000141

211. Zelis N, Hundscheid R, Buijs J, et al. Value of biomarkers in predicting mortality in older medical emergency department patients: a Dutch prospective study. BMJ Open. Jan 31 2021;11(1):e042989. doi:10.1136/bmjopen-2020-042989

212. Aduen J, Bernstein WK, Khastgir T, et al. The use and clinical importance of a substrate-specific electrode for rapid determination of blood lactate concentrations. Jama. Dec 7 1994;272(21):1678-85.

213. Barfod C, Lundstrøm LH, Lauritzen MM, et al. Peripheral venous lactate at admission is associated with in-hospital mortality, a prospective cohort study. Acta Anaesthesiol Scand. Apr 2015;59(4):514-23. doi:10.1111/aas.12503

214. Bernhard M, Döll S, Kramer A, et al. Elevated admission lactate levels in the emergency department are associated with increased 30-day mortality in non-trauma critically ill patients. Scand J Trauma Resusc Emerg Med. Aug 17 2020;28(1):82. doi:10.1186/s13049-020-00777-y

215. Bou Chebl R, El Khuri C, Shami A, et al. Serum lactate is an independent predictor of hospital mortality in critically ill patients in the emergency department: a retrospective study. Scand J Trauma Resusc Emerg Med. Jul 14 2017;25(1):69. doi:10.1186/s13049-017-0415-8

216. Bou Chebl R, Jamali S, Mikati N, et al. Relative Hyperlactatemia in the Emergency Department. Front Med (Lausanne). 2020;7:561. doi:10.3389/fmed.2020.00561

217. Cannon CM, Miller RT, Grow KL, Purcell S, Nazir N. Age-adjusted and Expanded Lactate Thresholds as Predictors of All-Cause Mortality in the Emergency Department. West J Emerg Med. Aug 20 2020;21(5):1249-1257. doi:10.5811/westjem.2020.5.46811

218. Contenti J, Occelli C, Lemoel F, Ferrari P, Levraut J. Blood lactate measurement within the emergency department: A two-year retrospective analysis. Am J Emerg Med. Mar 2019;37(3):401-406. doi:10.1016/j.ajem.2018.05.065

219. D'Abrantes R, Dunn L, McMillan T, Cornwell B, Bloom B, Harris T. Evaluation of the Prognostic Value of Lactate and Acid-Base Status in Patients Presenting to the Emergency Department. Cureus. Jun 2021;13(6):e15857. doi:10.7759/cureus.15857
220. Datta D, Walker C, Gray AJ, Graham C. Arterial lactate levels in an emergency department are associated with mortality: a prospective observational cohort study. Emerg Med J. Sep 2015;32(9):673-7. doi:10.1136/emermed-2013-203541
221. Demircan S, Ergin M, Tanriverdi F, et al. Combination of lactate with modified early warning score and rapid emergency medicine score in geriatric patients admitted to emergency department to predict 28-day mortality. Article. Turk Geriatri Dergisi. 2018;21(3):354-364. doi:10.31086/tjgeri.2018344050
222. Fukumoto Y, Inoue Y, Takeuchi Y, et al. Utility of blood lactate level in triage. Acute Med Surg. Apr 2016;3(2):101-106. doi:10.1002/ams2.130
223. Haidl F, Brabrand M, Henriksen DP, Lassen AT. Lactate is associated with increased 10-day mortality in acute medical patients: a hospital-based cohort study. Eur J Emerg Med. Aug 2015;22(4):282-4. doi:10.1097/mej.0000000000000210
224. Jo S, Lee JB, Jin YH, et al. Modified early warning score with rapid lactate level in critically ill medical patients: the ViEWS-L score. Emerg Med J. Feb 2013;30(2):123-9. doi:10.1136/emermed-2011-200760
225. Jo S, Yoon J, Lee JB, Jin Y, Jeong T, Park B. Predictive value of the National Early Warning Score-Lactate for mortality and the need for critical care among general emergency department patients. J Crit Care. Dec 2016;36:60-68. doi:10.1016/j.jcrc.2016.06.016
226. Kramer A, Urban N, Döll S, et al. Early Lactate Dynamics in Critically Ill Non-Traumatic Patients in a Resuscitation Room of a German Emergency Department (OBSERvE-Lactate-Study). The Journal of emergency medicine. Feb 2019;56(2):135-144. doi:10.1016/j.jemermed.2018.10.033
227. López-Izquierdo R, Martín-Rodríguez F, Santos Pastor JC, et al. Can capillary lactate improve early warning scores in emergency department? An observational, prospective, multicentre study. Int J Clin Pract. Apr 2021;75(4):e13779. doi:10.1111/ijcp.13779
228. Meral G, Ardıç Ş, Günay S, et al. Comparative analysis of Glasgow Coma Scale, quick Sepsis-related Organ Failure Assessment, base excess, and lactate for mortality prediction in critically ill emergency department patients. Turkish Journal of Emergency Medicine. 2024;24(4)
229. Oskay A, Eray O, Dinç SE, Aydın AG, Eken C. Prognosis of Critically ill patients in the ED and value of perfusion index measurement: a cross-sectional study. Am J Emerg Med. Aug 2015;33(8):1042-4. doi:10.1016/j.ajem.2015.04.033

230. Patel KI, Patel RN, Patel PP. Role of Arterial Blood Lactate Level to Predict Outcome of Critically Ill Patients. Article. International Journal of Pharmaceutical and Clinical Research. 2024;16(8):520-524.

231. Pedersen M, Brandt VS, Holler JG, Lassen AT. Lactate level, aetiology and mortality of adult patients in an emergency department: a cohort study. Emerg Med J. Sep 2015;32(9):678-84. doi:10.1136/emermed-2014-204305

232. Soupison T, Piednoël Y, Provoost L, Sicot C. Lactatemia: A new non specialized indicator of the severity of illness for patients in an emergency department. Article. Urgences Medicales. 1996;15(6):251-254. doi:10.1016/S0923-2524(97)84292-0

233. Tangpaisarn T, Drumheller BC, Daungjunchot R, Kotruchin P, Daorattanachai K, Phungoen P. Severe hyperlactatemia in the emergency department: clinical characteristics, etiology and mortality. BMC emergency medicine. Aug 20 2024;24(1):150. doi:10.1186/s12873-024-01071-1

234. van den Nouland DP, Brouwers MC, Stassen PM. Prognostic value of plasma lactate levels in a retrospective cohort presenting at a university hospital emergency department. BMJ Open. Jan 30 2017;7(1):e011450. doi:10.1136/bmjopen-2016-011450

235. Vroonhof K, van Solinge WW, Rovers MM, Huisman A. Differences in mortality on the basis of laboratory parameters in an unselected population at the Emergency Department. Clin Chem Lab Med. 2005;43(5):536-41. doi:10.1515/cclm.2005.093

236. Wilson W, Selvaraj DR, Ramya Kumar MV, Jain V, Umra S, Murty S. Arterial lactate as a prognostic tool to predict mortality and disposition in the emergency department: A prospective observational single-centre study. Hong Kong Journal of Emergency Medicine. 2023/01/01 2020;30(1):16-22. doi:10.1177/1024907920964177

237. Jaeschke R, Guyatt GH, Sackett DL. Users' guides to the medical literature. III. How to use an article about a diagnostic test. B. What are the results and will they help me in caring for my patients? The Evidence-Based Medicine Working Group. Jama. Mar 2 1994;271(9):703-7. doi:10.1001/jama.271.9.703

238. Meral G, Ardıç Ş, Günay S, et al. Comparative analysis of Glasgow Coma Scale, quick Sepsis-related Organ Failure Assessment, base excess, and lactate for mortality prediction in critically ill emergency department patients. Turk J Emerg Med. Oct-Dec 2024;24(4):231-237. doi:10.4103/tjem.tjem_45_24

239. Sjövall F, Morota S, Hansson MJ, Friberg H, Gnaiger E, Elmér E. Temporal increase of platelet mitochondrial respiration is negatively associated with clinical outcome in patients with sepsis. Critical care (London, England). 2010;14(6):R214. doi:10.1186/cc9337

240. Sjövall F, Ehinger JK, Marelsson SE, et al. Mitochondrial respiration in human viable platelets--methodology and influence of gender, age and storage. Mitochondrion. Jan 2013;13(1):7-14. doi:10.1016/j.mito.2012.11.001

241. Sjövall F, Morota S, Asander Frostner E, Hansson MJ, Elmér E. Cytokine and nitric oxide levels in patients with sepsis--temporal evolvement and relation to platelet mitochondrial respiratory function. PloS one. 2014;9(5):e97673. doi:10.1371/journal.pone.0097673

242. Ehinger JK, Morota S, Hansson MJ, Paul G, Elmér E. Mitochondrial dysfunction in blood cells from amyotrophic lateral sclerosis patients. Journal of neurology. Jun 2015;262(6):1493-503. doi:10.1007/s00415-015-7737-0

243. Ehinger JK, Morota S, Hansson MJ, Paul G, Elmér E. Mitochondrial Respiratory Function in Peripheral Blood Cells from Huntington's Disease Patients. Movement disorders clinical practice. Sep-Oct 2016;3(5):472-482. doi:10.1002/mdc3.12308

244. Westerlund E, Marelsson SE, Ehinger JK, et al. Oxygen consumption in platelets as an adjunct diagnostic method for pediatric mitochondrial disease. Pediatric research. Feb 2018;83(2):455-465. doi:10.1038/pr.2017.250

245. Brand MD, Nicholls DG. Assessing mitochondrial dysfunction in cells. The Biochemical journal. Apr 15 2011;435(2):297-312. doi:10.1042/bj20110162

246. Chance B, Williams GR. Respiratory enzymes in oxidative phosphorylation. I. Kinetics of oxygen utilization. The Journal of biological chemistry. Nov 1955;217(1):383-93.

247. Gnaiger E, Kuznetsov AV, Schneeberge rS, et al. Mitochondria in the cold. In: Heldmaier G, Klingenspor M, eds. Life in the Cold. Springer; 2000:431-442.

248. Gnaiger E, Steinlechner-Maran R, Méndez G, Eberl T, Margreiter R. Control of mitochondrial and cellular respiration by oxygen. J Bioenerg Biomembr. Dec 1995;27(6):583-96. doi:10.1007/bf02111656

249. Hütter E, Unterluggauer H, Garedew A, Jansen-Dürr P, Gnaiger E. High-resolution respirometry--a modern tool in aging research. Experimental gerontology. Jan 2006;41(1):103-9. doi:10.1016/j.exger.2005.09.011

250. Sjövall F, Morota S, Persson J, Hansson MJ, Elmér E. Patients with sepsis exhibit increased mitochondrial respiratory capacity in peripheral blood immune cells. Critical care (London, England). Jul 24 2013;17(4):R152. doi:10.1186/cc12831

251. Gnaiger E. Capacity of oxidative phosphorylation in human skeletal muscle: new perspectives of mitochondrial physiology. Int J Biochem Cell Biol. Oct 2009;41(10):1837-45. doi:10.1016/j.biocel.2009.03.013

252. Westerlund E, Marelsson SE, Karlsson M, et al. Correlation of mitochondrial respiration in platelets, peripheral blood mononuclear cells and muscle fibers. Heliyon. 2024/03/15/ 2024;10(5):e26745. doi:https://doi.org/10.1016/j.heliyon.2024.e26745

253. Gnaiger E, Åsander Frostner E, Abdul KN, et al. MitoEAGLE Task Group (2020) Mitochondrial physiology. Bioenerg Communications. 2020;2020(2020.1)

254. Larsen S, Nielsen J, Hansen CN, et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. The Journal of physiology. Jul 15 2012;590(14):3349-60. doi:10.1113/jphysiol.2012.230185

255. McLaughlin KL, Hagen JT, Coalson HS, et al. Novel approach to quantify mitochondrial content and intrinsic bioenergetic efficiency across organs. Sci Rep. Oct 19 2020;10(1):17599. doi:10.1038/s41598-020-74718-1

256. Divakaruni AS, Jastroch M. A practical guide for the analysis, standardization and interpretation of oxygen consumption measurements. Nat Metab. Aug 2022;4(8):978-994. doi:10.1038/s42255-022-00619-4

257. Picard M, Taivassalo T, Ritchie D, et al. Mitochondrial structure and function are disrupted by standard isolation methods. PloS one. Mar 28 2011;6(3):e18317. doi:10.1371/journal.pone.0018317

258. Jones AE, Leonard MM, Hernandez-Nino J, Kline JA. Determination of the effect of in vitro time, temperature, and tourniquet use on whole blood venous point-of-care lactate concentrations. Academic emergency medicine : official journal of the Society for Academic Emergency Medicine. Jul 2007;14(7):587-91. doi:10.1197/j.aem.2007.03.1351

259. Balakrishnan V, Wilson J, Taggart B, Cipolla J, Jeanmonod R. Impact of Phlebotomy Tourniquet Use on Blood Lactate Levels in Acutely Ill Patients. Cjem. Sep 2016;18(5):358-62. doi:10.1017/cem.2016.6

260. Gallagher EJ, Rodriguez K, Touger M. Agreement between peripheral venous and arterial lactate levels. Ann Emerg Med. Apr 1997;29(4):479-83.

261. Lavery RF, Livingston DH, Tortella BJ, Sambol JT, Slomovitz BM, Siegel JH. The utility of venous lactate to triage injured patients in the trauma center. J Am Coll Surg. Jun 2000;190(6):656-64. doi:10.1016/s1072-7515(00)00271-4

262. Van Tienhoven AJ, Van Beers C, Siegert C, Nanayakkara P. The utility of peripheral venous lactate in emergency department patients with normal and higher lactate levels: A prospective observational study. Acute Med. 2020;19(3):125-130.

263. Kruse O, Grunnet N, Barfod C. Blood lactate as a predictor for in-hospital mortality in patients admitted acutely to hospital: a systematic review. Scand J Trauma Resusc Emerg Med. Dec 28 2011;19:74. doi:10.1186/1757-7241-19-74

Note: This review was broader in scope than the literature review made for Paper IV (Appendix), including prehospital and ICU settings, and also studies focused on a single diagnosis or condition, such as sepsis or trauma.

264. Prins H, Hasman A. Appropriateness of ICD-coded diagnostic inpatient hospital discharge data for medical practice assessment. A systematic review. Methods Inf Med. 2013;52(1):3-17. doi:10.3414/me12-01-0022

265. Binney N, Hyde C, Bossuyt PM. On the Origin of Sensitivity and Specificity. Annals of internal medicine. Mar 2021;174(3):401-407. doi:10.7326/m20-5028

266. MedCalc Software Ltd Diagnostic test evaluation calculator. Accessed 22/10/2024, https://www.medcalc.org/calc/diagnostic_test.php

267. Af Ugglas B, Djärv T, Ljungman PLS, Holzmann MJ. Emergency department crowding associated with increased 30-day mortality: a cohort study in Stockholm Region, Sweden, 2012 to 2016. J Am Coll Emerg Physicians Open. Dec 2020;1(6):1312-1319. doi:10.1002/emp2.12243

268. Wretborn J, Wilhelms DB, Ekelund U. Emergency department crowding and mortality: an observational multicenter study in Sweden. Front Public Health. 2023;11:1198188. doi:10.3389/fpubh.2023.1198188

269. de Hond AAH, Steyerberg EW, van Calster B. Interpreting area under the receiver operating characteristic curve. Lancet Digit Health. Dec 2022;4(12):e853-e855. doi:10.1016/s2589-7500(22)00188-1

270. Devine J, Monzel AS, Shire D, et al. Brain-body mitochondrial distribution patterns lack coherence and point to tissue-specific and individualized regulatory mechanisms. bioRxiv. Sep 22 2024;doi:10.1101/2024.09.20.614152

271. Scott AJ, Ellison M, Sinclair DA. The economic value of targeting aging. Nat Aging. Jul 2021;1(7):616-623. doi:10.1038/s43587-021-00080-0

272. Iwarsson S, Jönson H, Deierborg T, et al. 'Proactive aging' is a new research approach for a new era. Nat Aging. Jul 2023;3(7):755-756. doi:10.1038/s43587-023-00438-6

273. de Magalhães JP. Longevity pharmacology comes of age. Drug Discov Today. Jul 2021;26(7):1559-1562. doi:10.1016/j.drudis.2021.02.015

274. Fahy GM, West MD, Coles LS, Harris SB. The Future of Aging [Elektronisk resurs] Pathways to Human Life Extension. Springer Netherlands; 2010.

275. Gladyshev VN, Anderson B, Barlit H, et al. Disagreement on foundational principles of biological aging. PNAS Nexus. Dec 2024;3(12):pgae499. doi:10.1093/pnasnexus/pgae499

276. Fišar Z, Hroudová J, Zvěřová M, Jirák R, Raboch J, Kitzlerová E. Age-Dependent Alterations in Platelet Mitochondrial Respiration. Biomedicines. May 28 2023;11(6)doi:10.3390/biomedicines11061564

277. Jedlička J, Tůma Z, Razak K, et al. Impact of aging on mitochondrial respiration in various organs. Physiol Res. Dec 31 2022;71(S2):S227-s236. doi:10.33549/physiolres.934995

278. Trifunovic A, Hansson A, Wredenberg A, et al. Somatic mtDNA mutations cause aging phenotypes without affecting reactive oxygen species production. Proc Natl Acad Sci U S A. Dec 13 2005;102(50):17993-8. doi:10.1073/pnas.0508886102

279. Hebert SL, Lanza IR, Nair KS. Mitochondrial DNA alterations and reduced mitochondrial function in aging. Mech Ageing Dev. Jul-Aug 2010;131(7-8):451-62. doi:10.1016/j.mad.2010.03.007

280. Zhang J, Shi Y. In Search of the Holy Grail: Toward a Unified Hypothesis on Mitochondrial Dysfunction in Age-Related Diseases. Cells. Jun 12 2022;11(12)doi:10.3390/cells11121906

281. Henriquez-Olguin C, Meneses-Valdes R, Jensen TE. Compartmentalized muscle redox signals controlling exercise metabolism - Current state, future challenges. Redox biology. Aug 2020;35:101473. doi:10.1016/j.redox.2020.101473

282. Marzetti E, Leeuwenburgh C. Skeletal muscle apoptosis, sarcopenia and frailty at old age. Experimental gerontology. Dec 2006;41(12):1234-8. doi:10.1016/j.exger.2006.08.011

283. Shayota BJ. Biomarkers of mitochondrial disorders. Neurotherapeutics. Jan 2024;21(1):e00325. doi:10.1016/j.neurot.2024.e00325

284. Korandová Z, Pecina P, Pecinová A, et al. Cryopreserved PBMCs can be used for the analysis of mitochondrial respiration and serve as a diagnostic tool for mitochondrial diseases. Anal Biochem. Mar 2025;698:115745. doi:10.1016/j.ab.2024.115745

285. Kerr M, Hume S, Omar F, et al. MITO-FIND: A study in 390 patients to determine a diagnostic strategy for mitochondrial disease. Molecular genetics and metabolism. Sep-Oct 2020;131(1-2):66-82. doi:10.1016/j.ymgme.2020.08.009

286. Yamada K, Toribe Y, Yanagihara K, Mano T, Akagi M, Suzuki Y. Diagnostic accuracy of blood and CSF lactate in identifying children with mitochondrial diseases affecting the central nervous system. Brain Dev. Feb 2012;34(2):92-7. doi:10.1016/j.braindev.2011.08.004

287. Forny P, Footitt E, Davison JE, et al. Diagnosing Mitochondrial Disorders Remains Challenging in the Omics Era. Neurol Genet. Jun 2021;7(3):e597. doi:10.1212/nxg.0000000000000597

288. Morovat A, Weerasinghe G, Nesbitt V, et al. Use of FGF-21 as a Biomarker of Mitochondrial Disease in Clinical Practice. J Clin Med. Aug 21 2017;6(8)doi:10.3390/jcm6080080

289. de Laat P, Koene S, van den Heuvel LP, Rodenburg RJ, Janssen MC, Smeitink JA. Clinical features and heteroplasmy in blood, urine and saliva in 34 Dutch families carrying the m.3243A > G mutation. J Inherit Metab Dis. Nov 2012;35(6):1059-69. doi:10.1007/s10545-012-9465-2

290. Arkes HR, Aberegg SK, Arpin KA. Analysis of Physicians' Probability Estimates of a Medical Outcome Based on a Sequence of Events. JAMA Netw Open. Jun 1 2022;5(6):e2218804. doi:10.1001/jamanetworkopen.2022.18804

291. Morgan DJ, Pineles L, Owczarzak J, et al. Accuracy of Practitioner Estimates of Probability of Diagnosis Before and After Testing. JAMA Intern Med. Jun 1 2021;181(6):747-755. doi:10.1001/jamainternmed.2021.0269

292. Parikh S, Karaa A, Goldstein A, et al. Diagnosis of 'possible' mitochondrial disease: an existential crisis. Journal of medical genetics. Mar 2019;56(3):123-130. doi:10.1136/jmedgenet-2018-105800

293. Jeppesen TD, Orngreen MC, Van Hall G, Vissing J. Lactate metabolism during exercise in patients with mitochondrial myopathy. Neuromuscul Disord. Aug 2013;23(8):629-36. doi:10.1016/j.nmd.2013.05.007

294. Dellinger RP, Levy MM, Carlet JM, et al. Surviving Sepsis Campaign: international guidelines for management of severe sepsis and septic shock: 2008. Intensive Care Med. Jan 2008;34(1):17-60. doi:10.1007/s00134-007-0934-2
295. Jörg M, Öster M, Wretborn J, Wilhelms DB. Agreement of pCO(2) in venous to arterial blood gas conversion models in undifferentiated emergency patients. Intensive Care Med Exp. Nov 21 2023;11(1):80. doi:10.1186/s40635-023-00564-w
296. Candel BG, Duijzer R, Gaakeer MI, et al. The association between vital signs and clinical outcomes in emergency department patients of different age categories. Emerg Med J. Dec 2022;39(12):903-911. doi:10.1136/emermed-2020-210628
297. Ladde JG, Miller S, Chin K, et al. End-tidal carbon dioxide measured at emergency department triage outperforms standard triage vital signs in predicting in-hospital mortality and intensive care unit admission. Academic emergency medicine : official journal of the Society for Academic Emergency Medicine. Aug 2023;30(8):832-841. doi:10.1111/acem.14703
298. Wretborn J, Munir-Ehrlington S, Hörlin E, Wilhelms DB. Addition of the clinical frailty scale to triage tools and early warning scores improves mortality prognostication at 30 days: A prospective observational multicenter study. J Am Coll Emerg Physicians Open. Oct 2024;5(5):e13244. doi:10.1002/emp2.13244
299. Zachariasse JM, van der Hagen V, Seiger N, Mackway-Jones K, van Veen M, Moll HA. Performance of triage systems in emergency care: a systematic review and meta-analysis. BMJ Open. May 28 2019;9(5):e026471. doi:10.1136/bmjopen-2018-026471
300. Sztaniszláv K, Asgari S, Henning L, Klang R, Olsson L. A Systematic Review on Triage Based on Specific scales/Instruments in Emergency Departments for Adults. 2024:34. 2024:74. 2024-12-16. Accessed 2025-01-23. https://www.regionorebrolan.se/siteassets/media/forskning/hta-camto/rapporter/rapporter-2024/2024.74-en-systematisk-oversikt-om-triage-med-specifika-skalor-eller-instrument-pa-akutmottagning-for-vuxna.pdf

Paper I

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Clinical Investigation | **Articles**

Oxygen consumption in platelets as an adjunct diagnostic method for pediatric mitochondrial disease

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BACKGROUND: Diagnosing mitochondrial disease (MD) is a challenge. In addition to genetic analyses, clinical practice is to perform invasive procedures such as muscle biopsy for biochemical and histochemical analyses. Blood cell respirometry is rapid and noninvasive. Our aim was to explore its possible role in diagnosing MD.

METHODS: Blood samples were collected from 113 pediatric patients, for whom MD was a differential diagnosis. A respiratory analysis model based on ratios (independent of mitochondrial specific content) was derived from a group of healthy controls and tested on the patients. The diagnostic accuracy of platelet respirometry was evaluated against routine diagnostic investigation.

RESULTS: MD prevalence in the cohort was 16%. A ratio based on the respiratory response to adenosine diphosphate in the presence of complex I substrates had 96% specificity for disease and a positive likelihood ratio of 5.3. None of the individual ratios had sensitivity above 50%, but a combined model had 72% sensitivity.

CONCLUSION: Normal findings of platelet respirometry are not able to rule out MD, but pathological results make the diagnosis more likely and could strengthen the clinical decision to perform further invasive analyses. Our results encourage further study into the role of blood respirometry as an adjunct diagnostic tool for MD.

For the clinician facing a severely sick child with indistinct but serious symptoms such as seizures, hypotonia, or liver failure, mitochondrial disease (MD) is one of the conditions that need to be considered.

While certain diagnoses have a characteristic clinical picture, and some cases can be rapidly confirmed by targeted genetic testing, diagnosing MD is often a challenge (1,2).

Standardized clinical criteria have been proposed to facilitate a general mitochondrial diagnosis and rely on a combination of symptoms and tests of varying difficulty (3,4). Elevated blood lactate is an important indicator for MD, but is nonspecific and may be caused by numerous systemic conditions such as hypoxia or sepsis (5). Cerebrospinal fluid lactate is more specific in diagnosing mitochondrial encephalopathy, but requires an invasive procedure (6). Magnetic resonance imaging of the brain may show typical patterns (as in Leigh syndrome) or less specific pathology. Analysis of organic acids in urine, like lactate, is noninvasive but may have low sensitivity if the patient is clinically stable (7).

A muscle biopsy is often necessary to confirm the diagnosis. It is used for biochemical, histochemical, and, in some cases, genetic analyses (1,8,9). Muscle biopsy is an invasive procedure requiring sedation or most often general anesthesia in children. Fresh muscle tissue is preferred over frozen and patients need to be transferred to centers with accredited laboratories for such analyses (7). Physicians can obviously not order this investigation on too wide a suspicion, nor expect expeditious results. While new methods of genetic testing facilitate large nuclear gene panels and whole-exome sequencing, and will likely play an increasingly important role in the future, they are not considered first-line diagnostic tools.

An accessible mitochondrial test derived from a normal blood sample would be of value to direct the early stages of diagnostic investigations. Mitochondrial function in platelets

Table 1. Demographic profile of patients and controls

	Patients ($n = 113$)	Controls ($n = 25$)
Median age in years (IQR)	3 (0.5–9.5)	3 (1–7)
Age groups, n *(%)*		
Under 1 month	12 (11)	0 (0)
1 month to 1 year	24 (21)	2 (8)
1–5 years	30 (27)	12 (48)
5–15 years	40 (35)	11 (44)
15 years or older	7 (6)	0 (0)
Female/male, *n* (%)	62 (55)/51 (45)	7 (28)/18 (72)

Abbreviation: IQR, interquartile range.

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Table 2. Symptoms and findings

Clinical data		Test results	
Symptom or finding	*n*	Type of test	Pathologic (*n*): normal (*n*)
Total patients reported	113	*Metabolic tests*	
Birth complications	5	P-lactate	39:56
CNS		Organic acids in urine	9:6
Intellectual disability	46	Organic acids in blood	1:0
Seizures	40	Amino acids in urine	1:8
Hypotonia/muscle weakness	35	Amino acids in blood	0:7
Psychomotor regression	31	Triglyceride or carnitine	4:3
Ataxia	8	*CSF*	
Episodic vomiting	8	Lactate	10:6
Pain/polyneuropathy	8	Protein	7:5
Dystonia	5	Pyruvate	2:4
Headache/migraine	5	Lactate/pyruvate ratio	2:1
Nystagmus	5	*Imaging studies*	
Spasticity	5	MRI or CT findings	24:13
Apnea	4	*Other clinical physiology studies*	
Hypoventilation	2	EEG	14:4
Microcephaly	2		
Stroke	2	ECG	5:5
Hypertonia	1	UCG	4:6
Other CNS symptoms	6	NCS	3:1
Skeletal muscle and heart		EMG	1:0
Cardiomyopathy	8		
Hypotension	3		
Myopathy	3		
Hypertension	2		
Other cardiac symptoms	4		
Ophthalmologic symptoms and findings			
Ptosis	5		
Visual loss	5		
External ophtalmoplegia	3		
Optic nerve atrophy	2		
Retinitis pigmentosa	1		
Other ophtalmologic symptoms	3		
Gastrointestinal symptoms and findings			
Vomiting	14		
Dysphagia	3		
Other GI symptoms	3		
Endocrine symptoms and findings			
Growth retardation	11		
Diabetes mellitus	1		
Other endocrine symptoms and findings	1		
Other symptoms and features			
Liver symptoms and findings	10		
Dysmorphism	5		
Hearing loss	5		
Scoliosis	3		
Airway symptoms and findings	2		
Renal symptoms and findings	2		
Anemia	1		
Fever	1		
Psychiatric symptoms and findings	1		

Abbreviations: CNS, central nervous system; CSF, cerebrospinal fluid; CT, computed tomography; ECG, electrocardiography; EEG, electroencephalography; EMG, electromyography; GI, gastrointestinal; MRI, magnetic resonance imaging; NCS, nerve conduction studies; UCG, echocardiography.

has been suggested as a marker of systemic mitochondrial function and has been studied in a variety of conditions where mitochondrial dysfunction is part of the pathology, such as amyotrophic lateral sclerosis, Alzheimer's disease, and sepsis (10–12).

Only a few studies have investigated blood cells from patients with primary MD, mostly in lymphocytes. Results have indicated that blood cell mitochondria are affected in some primary MDs, but study populations have often been small (13–16). Two studies on lymphocyte respirometry suggest candidate measurements for diagnostic use (14,16).

To our knowledge, no published study has evaluated respirometry in blood cells as a diagnostic method on a larger group of patients where MD was a differential diagnosis.

The aim of this study was to investigate the value of platelet respirometry as a complement to existing diagnostic tests for MD. The method is rapid and noninvasive and could be performed in the early stages of investigation.

RESULTS

Clinical data

The study included 113 patients where MD was considered a differential diagnosis. A wide pediatric age span was represented in the material, but most patients were under 5 years (**Table 1**). Central nervous system symptoms were heavily featured among reported symptoms (**Table 2**).

Table 3. Cases with confirmed mitochondrial disease

	Mitochondrial disease	Main clinical findings	Genetic findings		Main supportive diagnostic findings for patients without known genetic defect
			Gene	Mutation[a]	
1	Leigh syndrome	Ophthalmoparesis, central deafness, congenital heart defect	MT-ND5	m.13513G>A	
2	MELAS	Myopathy, cardiomyopathy, stroke	MT-TL1	m.3260A>G	
3	Alpers syndrome	Intellectual disability, epilepsy, hypotonia, cardiomyopathy	POLG	NA	
4	PDH deficiency	Hypotonia, delayed psychomotor development, microcephaly	PDHA1	c.1142_1145 dupATCA	
5	Mitochondrial encephalopathy	Hypotonia, cardiomyopathy, hepatopathy	—	—	Elevated plasma lactate, increased lactate on MR spectroscopy, COX-negative fibers in muscle biopsy
6	PDH deficiency	Psychomotor regression, microcephaly, hypotonia	PDHA1	c.871G>A	
7	Alpers–Huttenlocher syndrome	Hypotonia, psychomotor regression, liver failure, epilepsy, adrenal insufficiency	POLG	p.W748S, p.Q497H, p. R852C, p.E1143G	
8	Mitochondrial encephalopathy	Visual loss, cardiomyopathy, pancreas insufficiency, apnea, hypotonia, polyneuropathy	—	—	10–20% COX-negative fibers in muscle biopsy
9	CPEO and myopathy	External ophtalmoplegia, ptosis, muscle weekness	MT-TN	m.5703G>A	
10	Mitochondrial depletion syndrome	Liver failure, polyneuropathy, dystonia	MPV17	c.191C>G, c.503A>G	
11	Mitochondrial hepatopathy	Respiratory failure, progressive hypotonia, hepatopathy, cholestasis	—	—	Elevated plasma and CSF lactate, decreased amount of complexes I, III and IV in BNGE of isolated liver mitochondria
12	Kearns–Sayre syndrome	External ophtalmoplegia, retinitis pigmentosa, ptosis	—	—	Elevated CSF protein, 5–10 % COX-negative fibers and RRF in muscle biopsy, large mtDNA deletion of probable pathogenicity
13	LHON plus	Vision loss, psychomotor regression, hypotonia, epilepsy	MT-ND4	m.11778G>A	
14	Leigh syndrome	Psychomotor regression, progressive hemiparesis, dystonia, respiratory failure	—	—	Elevated CSF lactate and bilateral basal ganglia lesions on MRI
15	PDH deficiency	Intellectual disability, hypotonia, microcephaly, epilepsy, cyclic vomiting	PDHA1	c.1068-1078delTCCTGAGCCAC	
16	Leigh syndrome[b]	Ataxia, spasticity, psychomotor regression	SLC19A3	C153A>G, c.157A>G	
17	Mitochondrial depletion syndrome	Psychomotor regression, bilateral sensorineural hearing loss, dyskinesia	SUCLA-2	c.751G>A	
18	Mitochondrial encephalopathy[c]	Epilepsy, hypotonia, lactic acidosis, pulmonary hypertension	SARS2	c.691C>G, c.868C>T	

Abbreviations: BNGE, Blue Native gel electrophoresis; COX, cytochrome oxidase; CPEO, chronic progressive external ophthalmoplegia; CSF, cerebrospinal fluid; HUPRA, hypertension, renal failure, and alkalosis; LHON, Leber's hereditary optic neuropathy; MELAS, mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episode; MRI, magnetic resonance imaging; mtDNA, mitochondrial DNA; NA, not available; PDH, pyruvate dehydrogenase; RRF, ragged red fibers.
Case numbers correspond to **Table 6**.
[a]At least one of listed mutations assessed to be pathogenic.
[b]Thiamine transporter-2 deficiency.
[c]Suspected HUPRA syndrome.

Thirty-three patients went through a more extensive clinical investigation including muscle biopsy, genetic testing, or both. Eighteen patients were confirmed to have MD and the basis for these diagnoses is presented in Table 3. Five patients still had suspected but unconfirmed MD after extended investigation. These patients were considered as not having MD for all calculations in the study. In the remaining 90 cases, routine clinical investigation did not indicate MD (Figure 1).

Evaluation of diagnostic respiratory ratios

Absolute values for platelet respirometry in the reference group and established reference bounds for respiratory ratios are shown in Table 4. Diagnostic evaluation of the six separate tests (4 one-sided ratios and 1 two-sided ratio) and the combined model is presented in Table 5. Oxidative phosphorylation (OXPHOS) $CI_{malate\ and\ pyruvate}$/digitonin, malate and pyruvate successfully identified 4 out of 18 MD patients, with few false positives resulting in 96% specificity and a positive-likelihood ratio (PLR) of 5.3. Low values of Log (ETS CI+II/Routine) and ΔADP (adenosine diphosphate)/ΔSuccinate identified 9 and 6 MD patients, respectively. The remaining two tests had no true positives. The sensitivity for the combined model (positive here being defined as testing positive in any of the six tests) was 72% and the specificity 56%. No single test had higher sensitivity than 50%.

Table 6 shows an overview of the 18 MD patients' individual respirometry results in relation to other clinical tests. Thirteen out of the 18 MD patients were identified by at least one respiratory ratio.

Explorative analyses

A correlation between high blood lactate and pathologic respirometry results has previously been described (14). We plotted respiratory ratios in relation to lactate values (Figure 2), and while no linear correlation was seen, selected tests in combination with high lactate improved specificity (Table 7). A low OXPHOS CI_{MP}/DMP ratio in combination with high lactate had 99% specificity and a PLR of 21. Also, Log(ETS CI+II/Routine) and ΔADP/ΔSuccinate, respectively, combined with high lactate raised specificity and PLR.

Positive result in all of the three best tests had 100% specificity for MD, but this model only identified three of the patients. A scaled down version of the main model, using only the two most sensitive tests and counting a positive result on either test or on both tests as positive, retained a sensitivity of 67% and raised specificity to 73% (Table 7). Finally, when applying stricter criteria for true positive, defining confirmed diagnosis as confirmed by genetic testing, sensitivity and specificity for the three best tests and the combined model did not change conspicuously (Table 8).

DISCUSSION

We show that platelet respirometry provides useful diagnostic information through a rapid and noninvasive procedure in pediatric patients, in whom MD was clinically considered a differential diagnosis. Positive results substantially increase the likelihood of MD.

Diagnosis of MD is a clinical challenge. MD is the most common group of inherited metabolic disorders, but their diverse presentation and complex pathophysiology make them hard to investigate. Many of the patients are young children with severe symptoms and the invasiveness or inaccessibility of the best diagnostic methods may delay or even prevent confirmation of diagnosis.

The study population is a representative cohort of pediatric patients with clinically suspected MD at a tertiary hospital, making the study highly relevant to clinicians investigating these often severely sick children. The prevalence of MD in the study population was 16%, in line with previous reports from patient cohorts with similar clinical presentation (6,14,23).

Oxygen consumption in platelets was assessed to evaluate mitochondrial function. We used ratios rather than absolute

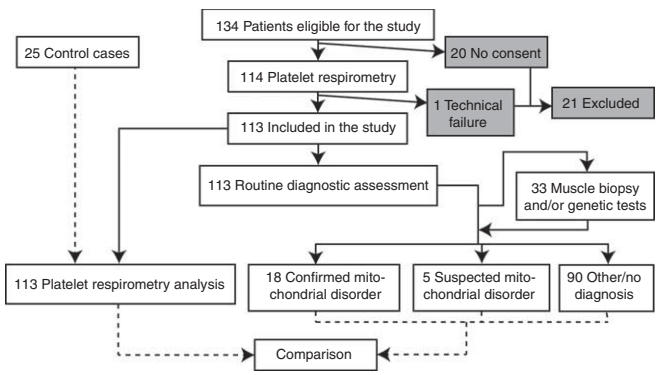

Figure 1. Study design. Two additional patients with technical failure were reanalyzed and the cases included.

Table 4. Reference group respirometry and reference ratios

Platelet respirometry (pmol O_2/s/10^8 cells)									Ratio means and reference intervals					
	Routine respiration	DMP	OXPHOS CI_{MP}	OXPHOS CI_{MPG}	OXPHOS CI+II	LEAK CI+II	ETS CI+II	ETS CII	CIV Activity		OXPHOS CI_{MP}/DMP	Log(ETS CI +II /Routine)	ETS CI+II /OXPHOS CI +II	ΔADP/ ΔSuccinate	OXPHOS CI_{MP} /OXPHOS CI_{MPG}
Mean ($n = 25$)	7.48	6.34	19.0	21.3	33.3	4.83	34.7	15.1	42.5	Upper bound	4.13	0.88	1.12	**1.36**	0.98
										Mean	3.03	0.67	0.96	1.03	0.89
SD	2.98	1.68	5.73	6.45	9.05	1.54	9.63	4.16	10.2	Lower bound	**1.92**	**0.45**	**0.81**	**0.69**	**0.81**

Abbreviations: ADP, adenosine diphosphate; CI+II, complex I and II; DMP, digitonin, malate and pyruvate; ETS, electron transport system; MD, mitochondrial disease; MP, malate and pyruvate; MPG, malate, pyruvate and glutamate; OXPHOS, oxidative phosphorylation; SD, standard deviation.
Bold numbers represent reference bounds used in the main model. Platelet respirometry (absolute values) for the control group have previously been published (17).

values of oxygen consumption, circumventing the need to calculate and adjust for mitochondrial content in the samples. A low OXPHOS CI_{MP}/DMP ratio had high specificity (96%) and a PLR of 5.3, meaning that in this sample the likelihood of disease rose from 16 to 50% after testing positive. The ratio was constructed to be sensitive to CI dysfunction, as inadequate response to ADP in the presence of CI substrates would lower the ratio (14,16). While all true-positive findings had confirmed CI dysfunction according to the current standard investigation, some patients with CI dysfunction were not caught by the test.

The ratio OXPHOS CI_{MP}/DMP had a low sensitivity, possibly only detecting severe dysfunction. Log(ETS CI+II/Routine) and ΔADP/ΔSuccinate appear to better detect mild dysfunction; they were more sensitive and their true positives overlap those of OXPHOS CI_{MP}/DMP. Pathological results in all three best tests made the diagnosis of mitochondrial disorder very likely (in this material 100% specificity). Each ratio was chosen to test a different aspect of the ETS (electron transport system) and thus a relatively low sensitivity for MD of any kind is expected when looking at the ratios individually. The combined model had a higher sensitivity at 72% but suffered from many false positives.

Although not enough to rule out disease on its own (negative likelihood ratio (NLR) of 0.5), platelet respirometry may be a useful part of an investigation. With the currently used diagnostic procedures, each individual test is in most cases insufficient to rule out MD, and that is also the case with this method. Blood respirometry has the advantage of swiftness, low invasiveness, and potential high availability and could be performed early in an investigation. In this material, two patients with normal muscle biopsy that later were confirmed to have MD by genetic testing had pathologic respirometry.

Plasma lactate is known to be a sensitive indicator of MD and can be obtained easily. A combination of high lactate and a low OXPHOS CI_{MP}/DMP ratio had 99% specificity (**Table 7**), comparable to the highest estimates of cerebrospinal fluid lactate, which is a markedly more invasive method (6). The likelihood for disease in a patient testing positive rose from 16 to 80%. Cerebrospinal fluid lactate is primarily suitable to detect MD affecting the central nervous system and has lower specificity in acutely ill patients (24,25). Blood lactate values, in contrast to platelet respirometry results, were technically not independent from the clinical findings that informed the final diagnosis. However, elevated lactate did not have a pivotal role for any of the MD diagnoses (**Table 3**).

We hypothesized that a limitation of OXPHOS at complex V (CV) would yield higher ETS than OXPHOS and result in a low OXPHOS CI+II/ETS CI+II ratio, indicating CV dysfunction. No patient had biochemically or genetically confirmed dysfunction in CV, so this hypothesis could not be tested.

A low OXPHOS CI_{MP}/OXPHOS CI_{MPG} ratio failed to single out any of the three patients with pyruvate dehydrogenase (PDH) deficiency, despite them being identified by other ratios.

Table 5. Diagnostic evaluation of the main model

Test ($n = 113$)	Confirmed MD ($n = 18$)	No confirmed MD ($n = 95$)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	PLR (95% CI)	NLR (95% CI)
OXPHOS CI_{MP}/DMP low								
Positive (8)	4	4	22 (6–48)	96 (90–99)	50 (16–84)	87 (79–93)	5.3 (1.5–19)	0.81 (0.63–1.04)
Negative (105)	14	91						
Log(ETS CI+II/Routine) low								
Positive (27)	9	18	50 (26–74)	81 (72–88)	33 (17–54)	90 (81–95)	2.6 (1.4–4.9)	0.62 (0.38–0.99)
Negative (86)	9	77						
OXPHOS CI+II/ETS CI+II low								
Positive (1)	0	1	0	99 (94–100)	0	84 (76–90)	0	1.01 (0.99–1.03)
Negative (112)	18	94						
ΔADP/ΔSuccinate low								
Positive (17)	6	11	33 (13–59)	88 (80–94)	35 (14–62)	88 (79–93)	2.9 (1.2–6.8)	0.75 (0.54–1.05)
Negative (96)	12	84						
ΔADP/ΔSuccinate high								
Positive (21)	2	19	11 (1–34)	80 (71–88)	9.5 (1.2–30)	83 (73–90)	0.56 (0.14–2.2)	1.1 (0.92–1.4)
Negative (92)	16	76						
OXPHOS CI_{MP}/OXPHOS CI_{MPG} low								
Positive (4)	0	4	0	96 (90–99)	0	83 (75–90)	0	1.0 (1.0–1.1)
Negative (109)	18	91						
Combined model (6 tests)								
Any test positive (55)	13	42	72 (47–90)	56 (45–66)	24 (13–37)	91 (81–97)	1.6 (1.1–2.4)	0.50 (0.23–1.07)
All tests negative (58)	5	53						

Abbreviations: ADP, adenosine diphosphate; DMP, digitonin, malate and pyruvate; 95% CI, 95% confidence interval; CI+II, complex I and II; ETS, electron transport system; MD, mitochondrial disease; MP, malate and pyruvate; MPG, malate, pyruvate and glutamate; OXPHOS, oxidative phosphorylation; PLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.
Disease prevalence in the study population was 16%.

Study limitations include insufficient information on any ongoing, potentially confounding medical treatment at the time of sampling (14). Further, the diversity of MD makes categorizing at all levels problematic. Although a clinical definition of confirmed MD was thought to best serve the aims of this study, a strict biochemical or genetic definition may have facilitated more detailed comparisons. (A strict genetic definition of true positive would arguably have had the benefit of less ambiguity. We included a *post hoc* analysis with such a definition, in which the main findings largely remains the same.) Also, the number of MD patients in the study was limited and many of the subdiagnoses were only represented by one patient each. The results therefore need to be confirmed by further studies. Another reason such studies are warranted is that the circumstances of data collection and analysis in this pilot project did not allow for strict blinding, something that would have to be readdressed in a follow-up investigation.

Lastly, the inherent problem of tests with significant false-negative rates needs to be taken into account (if future models do not improve sensitivity), making sure a negative result in platelet testing alone does not unintentionally refrain the diagnostician from pursuing further investigations when needed. We do not expect blood cell respirometry to replace any part of the current diagnostic workup, but believe that the addition of this rapid test could potentially reduce time to diagnosis for certain patients.

When discussing the possible role of blood respirometry, it is important to consider not just the current clinical practices but also how they are projected to evolve. As mentioned above, genetic methods such as next-generation sequencing, either in the form of large-scale gene panels, whole-exome

Table 6. Respirometry and routine tests for cases with confirmed MD

			Platelet respiration (pmol O_2/s/10^8 cells)									Routine diagnostics					Platelet diagnostics				
			Routine respiration	DMP	OXPHOS CI_{MP}	OXPHOS CI_{MPG}	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_{MP}/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.

sequencing or in some settings whole-genome sequencing, are currently gaining traction (2,26). Interestingly, as one recent review pointed out, improved genetic testing may actually place greater emphasis on less invasive tests and methods to confirm pathogenicity (26). Blood cell respirometry may potentially be a part of the first-line screening arsenal, and as such it would not likely be replaced by large-scale genetic testing any time soon. Additionally, respirometry being a functional analysis, we see it as a complement rather than a competitor to genetic tests.

CONCLUSION

We have shown that pathologic platelet respirometry, as defined in this study, increased the likelihood of MD in a clinically relevant situation. Combining blood respirometry with blood lactate further increased its diagnostic yield. As it is a fast method with low invasiveness and potentially high availability, these results encourage further study into the method's possible role as an adjunct diagnostic tool for MD.

METHODS

Patients and control subjects

Patient samples were collected at the Skåne University Hospital (Lund, Sweden). Patients under the age of 18 years presenting from July 2008 through December 2013, where MD was clinically considered as a differential diagnosis by the attending physician, were eligible for the study. Patients with malignant disease were excluded. The pediatric control group comprised samples from patients undergoing anesthesia for minor elective surgery (inguinal hernia repair or phimosis surgery). Control group samples were drawn before induction of anesthesia. Written informed consent was obtained from parents or guardians. The regional ethical review board of Lund, Sweden, approved this study (59/2009 and 97/2009). All patients were given standard care and the study was conducted according to the Declaration of Helsinki.

Clinical data and routine diagnostic assessment

The standard care of patients included in the trial was not affected by the results of this study, as no patient was excluded from further clinical investigation based on a negative blood cell respirometry. Clinical data were reported by clinicians and supplemented with review of medical records in Lund and at Sahlgrenska University Hospital, Gothenburg (where a majority of the muscle biopsies and genetic analyses were performed). Muscle biopsies were analyzed according to established practices (including histology, spectrophotometry, and oximetry/respirometry, normally all three) (7,8). Genetic analyses included screening for suspected mutations with or without the addition of whole-exome sequencing.

Patients were considered to have confirmed disease when a specialist in pediatric neurology had documented the diagnosis of MD as confirmed in the patient journal, after compiling the results of the investigation.

This definition was, as with the inclusion criteria, chosen to most closely adhere to the clinical reality. Although in practice the Bernier criteria are generally used at the participating hospitals in this study, there are neither national nor regional guidelines for diagnosing MD. A suspected case of MD not disproven during early investigation (due to, for instance, confirmation of an alternative diagnosis or

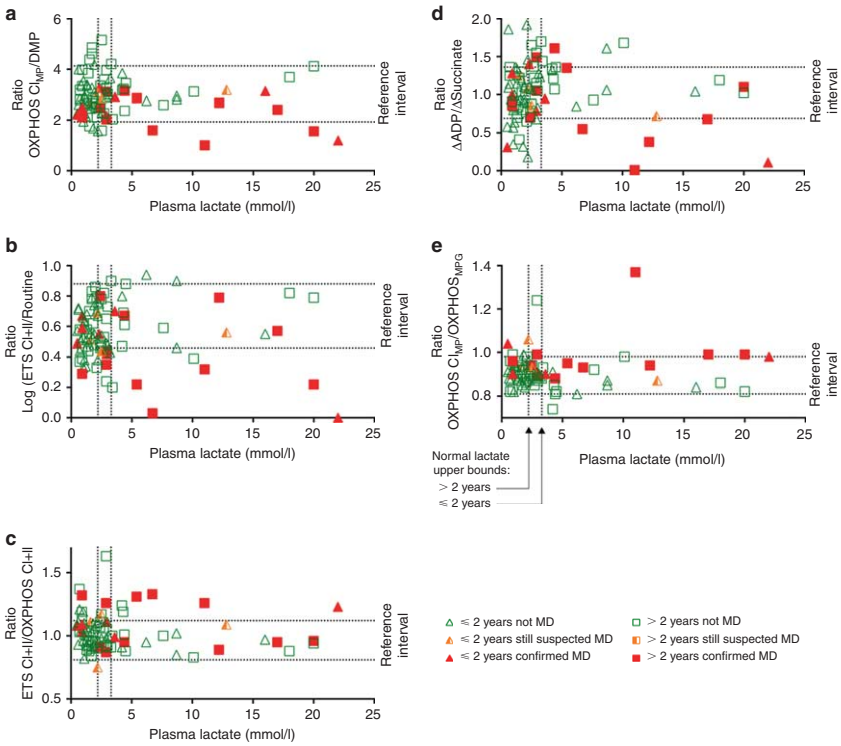

Figure 2. Plasma lactate and diagnostic ratios. Horizontal dotted lines: respirometry ratio reference interval. Vertical dotted lines: Plasma lactate upper reference bounds (for patients <2 years old: 3.3 mmol/l; ≥2 years old: 2.2 mmol/l). Red: confirmed MD; yellow: still suspected MD; green: no MD. Squares: >2 years old; triangles: ≤ 2 years old.

clinical recovery), was usually further evaluated with muscle biopsy, genetic testing, or both. The final diagnosis was preceded by discussion among several specialists and was made independently of the experimental platelet model.

Patients were defined as having "still suspected" MD if routine diagnostics could neither confirm nor rule out a diagnosis. Patients where disease was "still suspected" were considered as not having MD in all calculations of diagnostic accury in platelets, unless specified otherwise.

Platelet preparation

Depending on age and weight of the patient, 6–12 ml venous blood was drawn to EDTA vials. Blood samples were analyzed within 3–5 h as described previously (11,17.)

High-resolution respirometry

Mitochondrial respiration was measured with an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) following a previously described protocol (11,17).

The platelet pellet was dissolved in a mitochondrial respiration medium (MiR05) containing sucrose 110 mM, HEPES 20 mM, taurine 20 mM, K-lactobionate 60 mM, $MgCl_2$ 3 mM, KH_2PO_4 10 mM, EGTA 0.5 mM, bovine serum albumin 1 g/l, at pH 7.1 (ref. 18). The final cell concentration in the chamber was 19–200×10^6 cells per ml. Data were recorded with the DatLab Software 4.3 (Oroboros Instruments, Innsbruck, Austria).

Experimental protocol for permeabilized platelets

The analytic protocol is described in **Figure 3**. At the onset, the complexes of the respiratory system are coupled to the process of OXPHOS, phosphorylating ADP to adenosine triphosphate (ATP) as oxygen is consumed. The respiratory state in which the respiratory system is active but artificially uncoupled from OXPHOS is termed ETS (electron transport system). If neither OXPHOS nor artificial uncoupling drives the respiratory system, a certain amount of protons leaking over the inner membrane may still drive the respiratory system to some extent, as the mitochondria pump the protons back to maintain the electrochemical potential over the inner mitochondrial membrane. This state is called LEAK.

First, Routine respiration was established. Subsequently, the detergent digitonin was added to permeabilize the plasma membrane and allow the mitochondria to access exogenous (added) substrates. Malate (5 mM) and pyruvate (5 mM) were added simultaneously (respiratory state DMP). ADP (1 mM) was then added to induce OXPHOS with these substrates (OXPHOS CI_{MP}). Further, addition of glutamate (5 mM) provided additional electrons for complex I (CI), also via NADH (OXPHOS CI_{MPG}). Convergent input of electrons through both CI and complex II (CII) was achieved by the addition of succinate (10 mM), which is oxidized by CII. This

Table 7. Explorative analyses

Test ($n = 113$)	Confirmed MD ($n = 18$)	No confirmed MD ($n = 95$)	Sensitivity (95% CI)	Specificity (95% CI)	PPV (95% CI)	NPV (95% CI)	PLR (95% CI)	NLR (95% CI)
Scaled down model [a]								
Any test positive (38)	12	26	67 (41–87)	73 (63–81)	32 (18–49)	92 (83–97)	2.4 (1.5–3.9)	0.46 (0.24–0.89)
All tests negative (75)	6	69						
OXPHOS CI+II/LEAK								
Positive (21)	6	15	33 (13–59)	84 (75–91)	29 (11–52)	87 (78–93)	2.1 (1.0–4.7)	0.79 (0.56–1.1)
Negative (92)	12	80						
Three best tests 3/3 [b]								
3/3 positive (3)	3	0	17 (3.6–41)	100 (96–100)	100 (29–100)	86 (79–92)	—	0.83 (0.68–1.0)
Any test negative (110)	15	95						
Test combined with plasma lactate [c]								
OXPHOS CI_{MP}/DMP low								
Positive and high lactate (5)	4	1	22 (6.4–48)	99 (94–100)	80 (28–99)	87 (79–93)	21 (2.5–178)	0.79 (0.61–1.0)
Negative or low lactate (90)	14	94						
Log(ETS CI+II/Routine) low								
Positive and high lactate (14)	6	8	33 (13–59)	92 (84–96)	43 (18–71)	88 (80–94)	4.0 (1.6–10)	0.73 (0.52–1.0)
Negative or low lactate (99)	12	87						
ΔADP/ΔSuccinate low								
Positive and high lactate (9)	5	4	28 (9.7–53)	96 (90–99)	56 (21–86)	88 (80–93)	6.6 (2.0–22)	0.75 (0.56—1.0)
Negative or low lactate (104)	13	91						

Abbreviations: ADP, adenosine diphosphate; 95% CI, 95% confidence interval; CI+II, complex I and II; ETS, electron transport system; DMP, digitonin, malate and pyruvate; MD, mitochondrial disease; MP, malate and pyruvate; MPG, malate, pyruvate and glutamate; OXPHOS, oxidative phosphorylation; PLR, positive likelihood ratio; NLR, negative likelihood ratio; PPV, positive predictive value; NPV, negative predictive value.
[a]Two most sensitive tests: Log(ETS CI+II/Routine) low and ΔADP/ΔSuccinate low.
[b]OXPHOS CI_{MP}/DMP low and Log(ETS CI+II/Routine) low and ΔADP/ΔSuccinate low.
[c]Lactate was not available in 18 cases, all without confirmed MD, these cases were counted as positive if only respirometry was positive to generate conservative specificity estimate.

achieves maximum OXPHOS capacity through CI and CII (OXPHOS CI+II).

Next, proton leak over the mitochondrial membrane was measured by adding the ATP synthase inhibitor oligomycin (1 µg/ml), blocking OXPHOS (LEAK CI+II).

This was followed by titration of the uncoupler protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone creating a chemically induced leak of protons over the inner mitochondrial membrane, thereby stimulating the ETS to maintain the chemiosmotic proton gradient (ETS CI+CII). Subsequently, by adding CI inhibitor rotenone, the CII-dependent uncoupled respiration was measured (ETS CII).

To adjust for nonmitochondrial oxygen consumption by the cells, the complex III (CIII) inhibitor antimycin-A was added and this value was subtracted from each of the other parameters.

Finally, tetramethylphenylenediamine (0.5 mM) was added, driving complex IV (CIV) activity through the reduction of cytochrome *c*. To control oxygen consumed through the autoxidation of tetramethylphenylenediamine, CIV is inhibited by azide (10 mM) and the remaining respiration is subtracted from the former value (CIV activity).

From the measured parameters we constructed five ratios to cover different aspects of mitochondrial dysfunction. We used ratios of respirometry measurements, as opposed to absolute values, thus circumventing the need to adjust for mitochondrial content in the sample. This keeps the protocol simple, rapid, and more compatible with routine clinical use.

A low OXPHOS CI_{MP}/DMP ratio (in the absence of other findings indicating CII–V dysfunction) was assumed to reflect dysfunction in CI or upstream (of CI) processes, such as pyruvate dehydrogenase deficiency, as only CI substrates are present in this state (13,16). A low ETS CI+CII/Routine ratio was assumed to reflect general dysfunction for all complexes except CV and a reduced reserve capacity of the respiratory system. The OXPHOS CI+II/ETS CI+II was assumed to reflect CV dysfunction as respiration in the numerator but not in the denominator is limited by CV capacity. The ΔADP/ΔSuccinate ratio was defined to reflect either CI or CII dysfunction. A high response to ADP (in the presence of CI substrates) in comparison with the sequential response to succinate would indicate CII dysfunction and raise the ratio. The opposite would imply CI dysfunction. Finally, we assumed that a low OXPHOS CI_{MP}/OXPHOS CI_{MPG} ratio would indicate PDH deficiency. Transaminases in the malate-aspartate shuttle convert glutamate and oxaloacetate to α-ketoglutarate and aspartate, replenishing the tricarboxylic acid cycle downstream of PDH. Increased oxygen consumption with the addition of glutamate could thus indicate a limitation to mitochondrial respiration by PDH (19).

Table 8. Explorative model with stricter true-positive criteria

Test ($n = 113$)	Confirmed MD ($n = 13$)	No confirmed MD ($n = 100$)	Sensitivity (95% CI)	Specificity (95% CI)
OXPHOS CI_{MP}/DMP low				
Positive (8)	3	5	23 (5.0–54)	95 (89–98)
Negative (105)	10	95		
Log(ETS CI+II/Routine) low				
Positive (27)	7	20	54 (25–81)	80 (71–87)
Negative (86)	6	80		
OXPHOS CI+II/ETS CI+II low				
Positive (1)	0	1	0	99 (95–100)
Negative (112)	13	99		
ΔADP/ΔSuccinate low				
Positive (17)	5	12	39 (14–68)	88 (80–94)
Negative (96)	8	88		
ΔADP/ΔSuccinate high				
Positive (21)	1	20	7.7 (0.2–36)	80 (71–87)
Negative (92)	12	80		
OXPHOS CI_{MP}/OXPHOS CI_{MPG} low				
Positive (4)	0	4	0 (0–25)	96 (90–99)
Negative (109)	13	96		
Combined model (6 tests)				
Any test positive (55)	10	45	77 (46–95)	55 (45–65)
All tests negative (58)	3	55		

Abbreviations: ADP, adenosine diphosphate; CI+II, complex I and II; ETS, electron transport system; DMP, digitonin, malate and pyruvate; MD, mitochondrial disease; MP, malate and pyruvate; MPG, malate, pyruvate and glutamate; OXPHOS, oxidative phosphorylation.
Disease prevalence in the study population was 12% with stricter criteria for true positive (only cases confirmed by molecular testing).

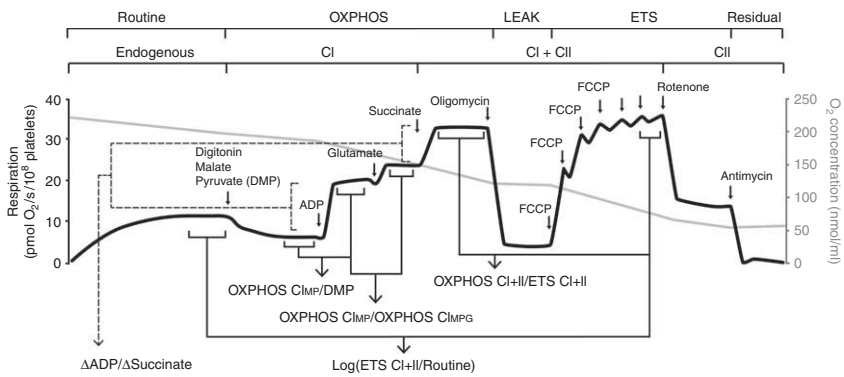

Figure 3. Platelet respirometry protocol. This is an illustration of the main part of the experimental protocol for platelets. The gray curve represents oxygen concentration over time (during around 1 h). The black curve represents rate of change in oxygen concentration (respiration). Small arrows mark the addition of substrates, uncouplers, and inhibitors. The diagram below the curve illustrates which respiratory states are compared in each diagnostic ratio for four of the five ratios ΔADP/ΔSuccinate (not shown) compares the change in respiration after addition of ADP with the change after addition of succinate.

Statistical methods

Respiratory parameters from a control group of 25 pediatric patients were used to establish reference intervals for the respiratory ratios.

The method for establishing reference intervals was adapted from the Clinical and Laboratory Standards Institute (CLSI) guidelines (20). Outliers were detected with Tukey's outlier labeling rule and two ratios had one outlier each removed (21).

The five chosen ratios were tested for normal distribution with D'Agostinos test for skewness and the Anscombe–Glynn test of kurtosis. All were found to have normal distribution except ETS CI +II/Routine. Logarithmic transformation (base 10) of that ratio produced normal distribution and was used. The reference interval for each ratio was defined as the mean $\pm$ 1.96 standard deviations.

With one-sided hypotheses for four ratios and a two-sided hypothesis for one, ratios were treated as six separate (but not independent) tests with binary outcomes. Diagnostic evaluation was performed for each of the tests separately and for a combined model where positive test result was defined as a positive result in one or more out of the six tests. The relation between test ratios and lactate value was examined with scatter plots.

Diagnostic accuracy was presented as sensitivity, specificity, positive and negative predictive value, and PLR and NLR. Likelihood ratios describe how much information a given test result adds compared with pretest knowledge (22). High PLR indicates that the probability of disease rises after testing positive. A low NLR means a negative result lowers probability of disease. These ratios are independent of disease prevalence.

Statistical analyses were mainly performed with SPSS (IBM SPSS Statistics Software, version 23, IBM Corp., Armonk, NY) and GraphPad PRISM (GraphPad Software, version 6.0d, GraphPad Software, Inc., La Jolla, CA). Tests of normality were performed using Free Statistics Software (Wessa, P. 2015, Office for Research Development and Education, version 1.1.23-r7, URL http://www.wessa.net/) and confidence intervals for the diagnostic evaluation results were calculated with MedCalc (online version 15.11.4, MedCalc Software, Ostend, Belgium). Statistical figures were constructed with GraphPad PRISM.

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REFERENCES

1. McFarland R, Taylor RW, Turnbull DM. A neurological perspective on mitochondrial disease. Lancet Neurol 2010;9:829–40.
2. Parikh S, Goldstein A, Koenig MK, et al. Diagnosis and management of mitochondrial disease: a consensus statement from the Mitochondrial Medicine Society. Genet Med 2015;17:689–701.
3. Bernier FP, Boneh A, Dennett X, Chow CW, Cleary MA, Thorburn DR. Diagnostic criteria for respiratory chain disorders in adults and children. Neurology 2002;59:1406–11.
4. Wolf NI, Smeitink JA. Mitochondrial disorders: a proposal for consensus diagnostic criteria in infants and children. Neurology 2002;59:1402–5.
5. Haas RH, Parikh S, Falk MJ, et al. Mitochondrial disease: a practical approach for primary care physicians. Pediatrics 2007;120:1326–33.
6. Yamada K, Toribe Y, Yanagihara K, Mano T, Akagi M, Suzuki Y. Diagnostic accuracy of blood and CSF lactate in identifying children with mitochondrial diseases affecting the central nervous system. Brain Dev 2012;34:92–7.
7. Haas RH, Parikh S, Falk MJ, et al. The in-depth evaluation of suspected mitochondrial disease. Mol Genet Metab 2008;94:16–37.
8. Tulinius MH, Holme E, Kristiansson B, Larsson NG, Oldfors A. Mitochondrial encephalomyopathies in childhood. I. Biochemical and morphologic investigations. J Pediatr 1991;119:242–50.
9. Dinopoulos A, Smeitink J, ter Laak H. Unusual features of mitochondrial degeneration in skeletal muscle of patients with nuclear complex I mutation. Acta Neuropathol 2005;110:199–202.
10. Mancuso M, Calsolaro V, Orsucci D, et al. Mitochondria, cognitive impairment, and Alzheimer's disease. Int J Alzheimers Dis 2009: 2009.
11. Sjovall F, Morota S, Hansson MJ, Friberg H, Gnaiger E, Elmer E. Temporal increase of platelet mitochondrial respiration is negatively associated with clinical outcome in patients with sepsis. Crit Care 2010;14:R214.
12. Ehinger JK, Morota S, Hansson MJ, Paul G, Elmer E. Mitochondrial dysfunction in blood cells from amyotrophic lateral sclerosis patients. J Neurol 2015;262:1493–503.
13. Kunz D, Luley C, Fritz S, et al. Oxygraphic evaluation of mitochondrial function in digitonin-permeabilized mononuclear cells and cultured skin fibroblasts of patients with chronic progressive external ophthalmoplegia. Biochem Mol Med 1995;54:105–11.
14. Artuch R, Colome C, Playan A, et al. Oxygen consumption measurement in lymphocytes for the diagnosis of pediatric patients with oxidative phosphorylation diseases. Clin Biochem 2000;33:481–5.
15. Pecina P, Gnaiger E, Zeman J, Pronicka E, Houstek J. Decreased affinity for oxygen of cytochrome-*c* oxidase in Leigh syndrome caused by SURF1 mutations. Am J Physiol Cell Physiol 2004;287:C1384–8.
16. Pecina P, Houšťková H, Mráček T, et al. Noninvasive diagnostics of mitochondrial disorders in isolated lymphocytes with high resolution respirometry. BBA Clin 2014;2:62–71.
17. Sjovall F, Ehinger JK, Marelsson SE, et al. Mitochondrial respiration in human viable platelets—methodology and influence of gender, age and storage. Mitochondrion 2013;13:7–14.
18. Gnaiger E, Kuznetsov AV, Schneeberger S, et al. Mitochondria in the coldIn:Heldmaier G, Klingenspor Meds. Life in the Cold. Heidelberg, Berlin, Germany and New York: Springer, 2000: 431–42.
19. Ehinger JK, Morota S, Hansson MJ, Paul G, Elmér E. Mitochondrial respiratory function in peripheral blood cells from Huntington's disease patients. Mov Disord Clin Pract 2016;3:472–82.
20. Clinical and Laboratory Standards Institute (CLSI). Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory: Approved Guideline3rd ednCLSI Document C28-A3 Wayne, PA: Clinical and Laboratory Standards Institute, 2008.
21. Hoaglin DC, Iglewicz B. Fine-tuning some resistant rules for outlier labeling. J Am Stat Assoc 1987;82:1147–9.
22. McGee S. Simplifying likelihood ratios. J Gen Intern Med 2002;17:646–9.
23. Lieber DS, Calvo SE, Shanahan K, et al. Targeted exome sequencing of suspected mitochondrial disorders. Neurology 2013;80:1762–70.
24. Hutchesson A, Preece MA, Gray G, Green A. Measurement of lactate in cerebrospinal fluid in investigation of inherited metabolic disease. Clin Chem 1997;43:158–61.
25. Chow SL, Rooney ZJ, Cleary MA, Clayton PT, Leonard JV. The significance of elevated CSF lactate. Arch Dis Child 2005;90:1188–9.
26. Davison JE, Rahman S. Recognition, investigation and management of mitochondrial disease. Arch Dis Child 2017 (doi:10.1136/archdischild-2016-311370).

Paper II

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Research article

Correlation of mitochondrial respiration in platelets, peripheral blood mononuclear cells and muscle fibers

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ABSTRACT

There is a growing interest for the possibility of using peripheral blood cells (including platelets) as markers for mitochondrial function in less accessible tissues. Only a few studies have examined the correlation between respiration in blood and muscle tissue, with small sample sizes and conflicting results.

This study investigated the correlation of mitochondrial respiration within and across tissues. Additional analyses were performed to elucidate which blood cell type would be most useful for assessing systemic mitochondrial function.

There was a significant but weak within tissue correlation between platelets and peripheral blood mononuclear cells (PBMCs). Neither PBMCs nor platelet respiration correlated significantly with muscle respiration.

Muscle fibers from a group of athletes had higher mass-specific respiration, due to higher mitochondrial content than non-athlete controls, but this finding was not replicated in either of the blood cell types. In a group of patients with primary mitochondrial diseases, there were significant differences in blood cell respiration compared to healthy controls, particularly in platelets. Platelet respiration generally correlated better with the citrate synthase activity of each sample, in comparison to PBMCs.

In conclusion, this study does not support the theory that blood cells can be used as accurate biomarkers to detect minor alterations in muscle respiration. However, in some instances, pronounced mitochondrial abnormalities might be reflected across tissues and detectable in blood cells, with more promising findings for platelets than PBMCs.

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1. Introduction

There is growing interest in using peripheral blood cells as markers for mitochondrial function in less accessible tissues or for systemic mitochondrial function. This applies both to physiological research and clinical medicine [1,2].

Mitochondrial respiration plays an important role in physiological processes such as aging, exercise and metabolic regulation [3,4]. Naturally, there has been a tradition of studying these phenomena in organs with high energy demand and large amounts of mitochondria, especially skeletal muscle. Also in clinical medicine, where the assessment of mitochondrial respiration is currently used to diagnose primary (inherited) mitochondrial disease, skeletal muscle is the preferred tissue to sample [5].

While generally safe for a healthy adult, a muscle biopsy is an invasive procedure that has to be performed by a trained physician, requires local anesthesia and may cause pain and local soreness for a few days after [6]. This poses problems if a study requires large sample sizes or repeated measurements [7]. In clinical medicine there are additional problems associated with muscle biopsies. Mitochondrial disease is generally suspected and diagnosed in early childhood but a child – as opposed to an adult – will often need general anesthesia, or at least sedation, for the procedure [5].

A blood sample, in contrast to a muscle biopsy, can easily be obtained through a minimally invasive technique and used for mitochondrial analyses [8]. While potential practical benefits are obvious, it is still unclear how reliable blood cell respiration is as a biomarker for muscle or systemic respiration as most studies have examined this relationship indirectly.

Only a few studies have examined the direct correlation of mitochondrial respiration between muscle fibers and blood cells (including platelets, which, for the purposes of this article will be labeled 'cells' as opposed to 'cell fragments'). In these studies, sample sizes have been small and the results conflicting. One study reported a strong correlation between mitochondrial respiration in monocytes and skeletal muscle in monkeys (n = 18) but weaker correlation between platelets and muscle cells [9]. A study on humans featuring young healthy men (n = 10) found no correlation between respiration in peripheral blood mononuclear cells (PBMCs), including a small fraction of monocytes, and permeabilized muscle cells [10]. Another study, where participants were a mix of healthy and diseased women (n = 32), found no correlation between respiration of permeabilized muscle fibers and intact platelets, nor intact PBMCs. The only significant correlations were found in two out of 12 tested parameters ('CI LEAK' and 'OXPHOS coupling efficiency') in a subset of the participants (n = 12–13), and only for permeabilized platelets [11]. These findings are in some sense contrasted by another study, similar in size (n = 32), that found more consistent correlations (several respiratory states correlated in the main study population) between platelets and muscle cells (but did not examine other blood cell types). However, this study differed from the former as it used intact platelets and included older participants of both sexes [12].

To our knowledge, no study has so far examined the correlation of mitochondrial respiration between different types of blood cells. Indeed, since all types of blood cells are equally accessible it would rarely be relevant in practice to use one as a biomarker for the other. But the question of correlation within blood tissue (i.e. between different blood cells or blood cell types) is still interesting for theoretical purposes. PBMCs (lymphocytes and monocytes) and platelets are being studied individually as candidate biomarkers, and it is unlikely that they both correlate highly with muscle tissue respiration unless they correlate with each other.

In this study the first aim was to examine if respiration in platelets and PBMCs correlate with one another. Some degree of within tissue correlation was assumed to be a prerequisite for finding a correlation across tissues. The within-tissue correlation was hypothesized to be higher than that across tissues, or more specifically, in the present study, between cells from blood and muscle tissue.

Accordingly, the second aim was to make an across tissue comparison to directly compare platelets and PBMCs to muscle fibers in a subgroup of healthy volunteers.

Additional analyses were made to further explore the results of the main analyses. We analyzed the correlation between respiration and mitochondrial content in the different sample types and made groupwise analyses in two subgroups, each subgroup representing a possible application area of blood cells as biomarkers and also representing two conceptually different forms of metabolic alterations. The first of these subgroups included semi-professional athletes, who were expected to have physiological, acquired alterations of their muscle respiration as a result of exercise, in comparison with non-athlete controls. We hypothesized that these alterations would appear to some degree also in blood cells. The second subgroup were pediatric patients with confirmed primary mitochondrial disease. They were hypothesized to have some degree of altered blood cell respiration due to genetic defects of the enzymes of the respiratory system, when compared to other pediatric patients without mitochondrial disease.

2. Results

2.1. Characteristics of the study population

An overview of the study population and the different subpopulations used for different analyses is presented in Table 1. In total, 318 individuals were included. Data from either PBMCs or plalelets, or both, for these individuals (but not the data from the 24 muscle biopsies) have been published before in other contexts [13–20]. The ages ranged from 3 days to 86 years with a roughly equal sex distribution (148 females). Of the total study population, 226 participants were patients and 92 were healthy volunteers. Adult patients primarily had neurological diseases (n = 153) such as Parkinson's disease (n = 57), essential tremor (n = 18), amyotrophic lateral sclerosis (n = 16) and Huntington's disease (n = 13), and another subset were patients admitted to the ICU with sepsis (n = 14). Healthy adult participants were composed of neurologically healthy age-matched controls to the neurological cases, healthy student volunteers and semi-professional athletes [14,15,21]. There were 82 children (under the age of 18 years) in this study. Out of these, 23 were otherwise healthy patients undergoing minor elective surgery [13]. There were 59 pediatric patients, nine of whom had

Table 1
Study population and subgroups. CS = citrate synthase SD = standard deviation *Adult defined as ≥ 18 years, child as < 18 years **Exact age was unavailable for one adult participant.

	Related table/figure	n	Female/male (not recorded)	Adults/ children*	Median age in years (range); mean age in years (SD)*	Patients/healthy participants
Total study population	Table 3, Fig. 2	318	148/167 (3)	236/82	58 (0–86); 45.1 (28.4)	226/92
All healthy participants	–	92	51/38 (3)	69/23	26 (0–80); 35.0 (26.0)	0/92
All patients	–	226	116/110	167/59	63 (0–86); 49.2 (28.4)	226/0
All cases where CS activity was measured	Table 3, Figs. 2–4	162	85/74 (3)	94/68	23 (0–84); 30.1 (27.2)	78/84
All healthy particiapts where CS activity was measured	Fig. 3B	84	47/34 (3)	62/22	24 (0–80); 32.4 (24.7)	0/84
All patients where CS activity was measured	Fig. 3B	78	38/40 (–)	32/46	12.5 (0–84); 29.0 (29.8)	78/0
Cases where muscle respiration was measured, i.e. athletes (n = 5) and non-athlete controls (n = 19)	Table 3, Fig. 3A–. 4	24	10/14 (–)	24/0	24 (19–42); 25.5 (5.4)	0/24
Pediatric patients with (n = 9) and without (n = 50) mitochondrial diseae	Fig. 5	59	33/26 (–)	0/59	3 (0–17); 4.8 (5.1)	59/0

confirmed primary mitochondrial disease. The remaining 50 patients were acutely ill patients where mitochondrial disease was included in the differential diagnosis. Many of these turned out to have other – mainly neurological or metabolic – diseases, while others eventually recovered. In addition to blood samples, which were obtained from all participants, muscle biopsies were collected from 24 of the healthy adult participants. The muscle respiration data have not been published previously.

Table 2
Overview of respiratory rates and internal ratios. P = OXPHOS; L = LEAK; E = ET; N = NADH; S = succinate; CI = complex I; CII = complex II; CIV = complex IV; DMP = digitonin, malate, pyruvate; PM = pyruvate, malate; PGM = pyruvate, glutamate, malate; PGMS = pyruvate, glutamate, malate, succinate; TMPD = tetramethylphenylenediamine; FCCP = protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; ADP = adenosine diphosphate *Measurements made after respective additions to the chamber during the experiment (and corrected for antimycin A) as illustrated in Fig. 1.

Respiratory rates and ratios	Abbreviation	Corresponding measurement or calculation*	Explanation
ROUTINE respiration	*R*	Routine	Routine respiration in intact cells.
Background respiration	*L*(n)	DMP	Background respiration in permeabilized cells with malate and pyruvate. This rate may alternatively be called LEAK respiration as it reflects the same coupling state as *L*(Omy) (see below) but it is labeled *L*(n) to indicate that the absence of ADP is limiting phosphorylation, as opposed to inhibition of ATP synthase for *L*(Omy).
N-OXPHOS capacity (PM)	PM_P	ADP	Maximal ADP stimulated respiration with pyruvate and malate (N-pathway/CI-linked pathway).
N-OXPHOS capacity	PGM_P	glutamate	Maximal ADP stimulated respiration with pyruvate, malate and glutamate (N-pathway/ CI-linked pathway).
NS-OXPHOS capacity	$PGMS_P$	succinate	Maximal ADP stimulated respiration with pyruvate, malate, glutamate and succinate (NS pathway/CI–CII-linked pathway).
LEAK respiration	*L*(Omy)	oligomycin	Background respiration (with oligomycin-inhibited ATP synthase) with pyruvate, malate, glutamate and succinate. Consists mainly of proton leak over the inner mitochondrial membrane.
NS-ET capacity	$PGMS_E$	FCCP	Maximal noncoupled respiration with pyruvate, malate, glutamate and succinate (NS pathway/CI–CII-linked pathway).
S-ET capacity	S_E	rotenone	Maximal noncoupled respiration with succinate, CI inhibited by rotenone (S-linked pathway (CII-linked).
CIV capacity	CIV_E	TMPD-azide	Maximal complex IV activity
Respiratory control ratio	RCR	succinate/oligomycin	Coupling efficiency (OPXHOS capacity relative LEAK respiration)
L/E coupling control ratio	L/E CCR	oligomycin/FCCP	Intrinsic uncoupling at constant ET capacity (LEAK respiration relative ET-capacity)
P/E OXPHOS control ratio	P/E OCR	succinate/FCCP	The limitation of OXPHOS capacity by the phosphorylating system
CI response ratio	CI-RR	ADP/DMP	ADP-stimulated response to malate and pyruvate, hypothesized to measure CI-linked phosphorylation
CI–CII response ratio	CI–CII-RR	(ADP-DMP)/(succinate-glutamate)	Hypothesized to measure alterations in either CI- or CII-linked phosphorylation relative to each other
N/NS pathway control ratio	N/NS PCR	glutamate/succinate	Relative N-pathway/CI-linked pathway function (in the coupled state)
S/NS pathway control ratio	S/NS PCR	rotenone/FCCP	Relative S-pathway/CII-linked pathway function (in the noncoupled state)

For mean blood cell respiratory data for the healthy population (n = 92) and the healthy adult population (n = 69), we refer to Supplementary Table 1.

2.2. Correlation within and across tissues

Table 3 and Fig. 2A summarize correlation within and across tissues. There was a general but very weak correlation between PBMCs and platelets and the correlation was stronger when normalizing for CS (mitochondrial content) or using internal ratios (calculating ratios between different respiratory rates is another way of normalizing the measurements, as the value of such ratios are independent of cellular mitochondrial content). The N/NS-pathway control ratio displayed the strongest correlation of 0.42 (95% CI 0.33–0.51, P < 0.001). The correlations between PBMCs and platelets were also tested for several subgroups of the main study population. These post hoc-analyses are presented in Supplementary Table 3 and Supplementary Table 4.

In general, neither PBCM nor platelet respiration correlated with muscle fiber respiration and that pattern did not change by any method of normalization. Only three parameters out of 48 tested were found to correlate significantly and these correlations were negative (NS-LEAK-respiration correlated negatively between cell count-normalized PBMCs and mass-normalized muscle fibers, and CS-normalized background respiration and NS-OXPHOS capacity correlated negatively between platelets and muscle fibers). In accordance with the study plan, Spearman rank correlations were also tested for all parameters in Table 2, in general yielding slightly higher (but occasionally lower) correlation coefficients than the Pearson tests but no major difference in the general pattern (Supplementary Table 2).

Fig. 2B visualizes how the correlation between PBMCs and platelets is distributed over quartiles for three of the normalized measurements (the top one is CS-normalized and lower two are normalized using internal ratios). The overlap in the top and bottom quartiles (bottom right and top left squares, respectively) is bigger than the overlap in the second and third quartiles, indicating that when a value deviates further from the median it is more likely to do so across cell types.

2.3. Correlation between respiration and citrate synthase

When examining the correlation between cell count-normalized respiratory values and CS activity in the same sample, there was generally a higher degree of correlation in platelet and muscle fiber samples compared to PBMCs (Fig. 3A). This finding was more prominent for NS-OXPHOS capacity and NS-ET capacity than for routine respiration and the former two were significantly higher in

Table 3
The correlation between parameters of respiration in PBMCs, platelets and muscle fibers. CS = citrate synthase; CI = confidence interval; Other abbreviations, see Table 1. *PBMC and platelet respiration was normalized to cell count, muscle respiration was normalized to mass. ** Muscle fibers did not have digitonin added to them.

	PBMCs and platelets			PBMCs and muscle fibers			Platelets and muscle fibers		
	Pearson's r (95% CI)	p	n	Pearson's r (95% CI)	p	n	Pearson's r (95% CI)	p	n
Cell count-normalized*									
ROUTINE respiration	**0.14 (0.03–0.24)**	0.02	318	n/a	n/a	n/a	n/a	n/a	n/a
Background respiration**	0.07 (−0.04–0.18)	0.20	318	0.31 (−0.63–0.11)	0.15	24	0.28 (−0.62–0.14)	0.18	24
N-OXPHOS capacity(PM)	**0.13 (0.02–0.23)**	0.03	318	0.28 (−0.14–0.61)	0.19	24	0.04 (−0.37–0.44)	0.85	24
N-OXPHOS capacity	**0.13 (0.02–0.23)**	0.03	318	0.20 (−0.23–0.56)	0.36	24	0.02 (−0.39–0.42)	0.95	24
NS-OXPHOS capacity	0.06 (−0.05–0.17)	0.27	318	0.17 (−0.25–0.54)	0.43	24	0.17 (−0.26–0.53)	0.44	24
LEAK respiration	0.07 (−0.04–0.18)	0.21	318	**−0.44 (-0.72–-0.04)**	0.03	24	0.29 (−0.13–0.62)	0.18	24
NS-ET capacity	**0.15 (0.04–0.25)**	0.01	318	0.03 (−0.38–0.43)	0.88	24	−0.03 (−0.42–0.38)	0.91	24
S-ET capacity	0.09 (−0.02–0.20)	0.10	318	−0.16 (−0.53–0.26)	0.45	24	0.13 (−0.29–0.51)	0.55	24
CIV capacity	**0.20 (0.09–0.31)**	0.001	283	0.27 (−0.16–0.61)	0.21	23	−0.25 (−0.60–0.17)	0.23	24
CS-normalized									
ROUTINE respiration	−0.02 (−0.18–0.13)	0.75	162	n/a	n/a	n/a	n/a	n/a	n/a
Background respiration**	0.12 (−0.04–0.27)	0.13	162	−0.23 (−0.58–0.19)	0.28	24	**−0.45 (-0.72–-0.06)**	0.03	24
N-OXPHOS capacity(PM)	**0.19 (0.03–0.33)**	0.02	162	−0.34 (−0.08–0.65)	0.11	24	−0.08 (−0.47–0.34)	0.73	24
N-OXPHOS capacity	**0.20 (0.04–0.34)**	0.01	162	−0.21 (−0.56–0.21)	0.33	24	−0.40 (−0.69–0.00)	0.05	24
NS-OXPHOS capacity	**0.19 (0.04–0.34)**	0.01	162	−0.38 (−0.68–0.03)	0.07	24	**−0.46 (-0.73–-0.07)**	0.02	24
LEAK respiration	0.10 (−0.06–0.25)	0.23	162	−0.08 (−0.47–0.34)	0.72	24	0.04 (−0.37–0.43)	0.87	24
NS-ET capacity	**0.30 (0.15–0.43)**	<0.001	162	−0.04 (−0.44–0.37)	0.85	24	−0.39 (−0.69–0.02)	0.06	24
S-ET capacity	**0.22 (0.07–0.37)**	0.004	162	−0.18 (−0.55–0.24)	0.34	24	−0.31 (−0.63–0.11)	0.14	24
CIV capacity	**0.27 (0.11–0.41)**	0.001	141	0.40 (−0.02–0.70)	0.06	23	−0.14 (−0.52–0.28)	0.51	24
Internal ratios									
CI-RR	**0.14 (0.03–0.25)**	0.01	317	−0.40 (−0.69–0.01)	0.06	24	−0.01 (−0.41–0.39)	0.96	24
RCR	**0.16 (0.05–0.26)**	0.006	317	−0.38 (−0.68–0.03)	0.07	24	−0.02 (−0.42–0.39)	0.94	24
L/E CCR	**0.20 (0.09–0.30)**	<0.001	317	−0.05 (−0.44–0.36)	0.82	24	−0.01 (−0.41–0.39)	0.96	24
P/E OCR	**0.33 (0.23–0.42)**	<0.001	318	−0.19 (−0.55–0.23)	0.36	24	−0.37 (−0.67–0.04)	0.08	24
CI–CII-RR	**0.29 (0.18–0.38)**	<0.001	317	0.14 (−0.28–0.52)	0.51	24	0.14 (−0.28–0.51)	0.53	24
L/P CCR	**0.12 (0.01–0.23)**	0.03	317	−0.33 (−0.65–0.09)	0.12	24	0.23 (−0.19–0.58)	0.29	24
N/NS RCR	**0.42 (0.33–0.51)**	<0.001	318	0.31 (−0.11–0.64)	0.14	24	−0.03 (−0.43–0.38)	0.90	24
S/NS RCR	**0.33 (0.23–0.43)**	<0.001	318	0.16 (−0.26–0.53)	0.45	24	0.35 (−0.07–0.66)	0.10	24

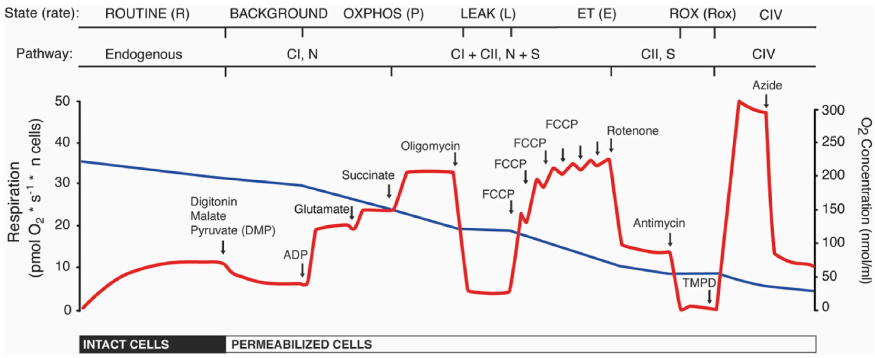

Fig. 1. Study protocol. Illustration of the study protocol for permeabilized platelets and PBMCs. Additions of substrates, inhibitors and uncouplers to the chamber are indicated by the arrows. The first top bar illustrates respiratory states and corresponding rates (except "CIV", which is not a respiratory state). The second top bar illustrates main substrate pathways and respiratory complex through which the electrons are donated. The gray line indicates the O_2 concentration in the chamber, corresponding to the right y-axis. The black line indicates the O_2 flux measured per n number of cells ($n = 10^{-8}$ platelets, $n = 10^{-6}$ PBMCs, respectively), corresponding to the left y-axis. Labels on the y-axes before the addition of ADP do not apply to the muscle fibre protocol. CI = complex I; CII = complex II; CIV = complex IV; N = NADH-linked substrates; S = succinate; OXPHOS = oxidative phosphorylation; ETS = electron transport system. ROX = residual oxygen consumption.

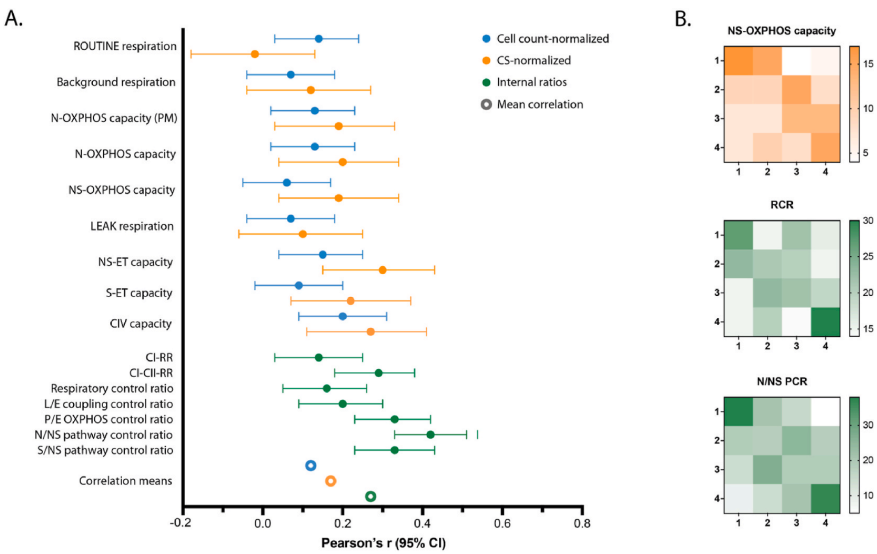

Fig. 2. Platelet and PBMC correlations. **A** Comparison of different methods of normalization. The plot shows correlation between platelet and PBMC cell count normalized respiratory values ($O_2 * s^{-1} * 10^{-8}$ platelets and $O_2 * s^{-1} * 10^{-6}$ PBMCs; blue filled dots), the same values normalized to citrate synthase (CS) activity (orange filled dots) and a selection of internal ratios (green filled dots). Bars represent 95% confidence interval (CI). The hollow dots show mean correlation (without CI) for the respective groups corresponding in color. For n refer to Table 2. **B** Frequency distribution of respiratory parameters grouped as quartiles (1–4) to illustrate the association of values far from the median (quartiles 1 and 4, respectively) in comparison with values near the median (quartiles 2 and 3). The tables are crosstabulations of a CS normalized respiratory value (orange heat map) and a selection of ratios (green heat maps) for platelets (y-axes) and PBMCs (x-axes). The color of each square represents the overlap in absolute numbers for each quartile; darker color for higher overlap. The right-hand scale translates color intensity in each square into the absolute number of overlapping cases. X- and y-axes represent the quartiles of respiratory values for platelets and PBMCs. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

platelets as compared to PBMCs (Fishers r to z transformation, $p < 0.001$, Supplementary Table 5). The mean correlation for these two parameters were also higher in muscle, compared to PBMCs, but the differences were not significant. When comparing correlation between CS activity and respiration for healthy participants and patients there were no significant differences for PBMCs. Routine respiration and NS-OXPHOS capacity, but not NS-ET capacity, in platelets correlated slightly more with CS in patients (Fig. 3B–Supplementary Table 6).

2.4. Blood cell and muscle fiber alterations in athletes

Mass-specific mitochondrial respiration in muscle fibers in a group of five semi-professional athletes (n = 5, mean age: 25 years, 2 females/3 males) compared to a control group of non-athletes (n = 19, mean age: 26 years, 8 females/11 males) was significantly higher (Fig. 4A) but such differences were seen neither in PBMCs nor in platelets (Fig. 4B–C). The muscle fibers from the athletes had significantly higher CS activity (Fig. 4D) as opposed to CS activity in blood cells, which was not affected in the athletes (Fig. 4E–F). When normalizing respiration to CS, the differences in respiration were eliminated in athletes and remained absent in the blood cells (Supplementary Table 7).

2.5. Blood cell alterations in patients with mitochondrial disease

Platelets and PBMCs from nine pediatric patients with mitochondrial disease were compared to samples from another group of pediatric patients where mitochondrial disease was initially included in the differential diagnosis. (The patients in this analysis are a subset of the patients presented in a previous publication [13]. The subset comprises all cases where both platelet and PBMC data were available. For more information on the individual mitochondrial diagnoses we refer to Table 3 of that publication; the patients included in this study are the cases indexed as 2, 3, 5, 6, 10, 11, 13, 14 and 15 [13].) Several of the respiratory ratios were significantly altered in the group with mitochondrial disease, mainly in platelets (Fig. 5A, 5/7 ratios) but also in PBMCs (Fig. 5B and 2/7 ratios) (see also Supplementary Table 8).

3. Discussion

In this study, within and across tissue correlations of mitochondrial respiration were examined, to further the understanding of blood cell respiration as a biomarker for less accessible tissues or systemic metabolic alterations. The main findings were that correlation within blood tissue was generally statistically significant, but weak, while neither platelets nor PBMCs correlated with muscle fibers. However, additional analyses provided nuances to these largely negative findings.

While the majority of the correlations in our main analysis between PBMCs and platelets were weak or very weak, they were nonetheless significant and, as expected, normalizing to CS or normalizing by the use of internal ratios increased both the correlation coefficients and the significance level (in several cases to the <0.001 level). One potential source of error for the correlations is the impurity of the blood samples inherent to the isolation method. The potential effect is difficult to estimate but we did measure mean contamination levels, which in the PBMC samples was 4 platelets (SD ± 3, n = 308) per PBMC and in the platelet samples 37 PBMCs (SD ± 42, n = 305) per 10 000 platelets, numbers which are common according to recent methodological literature [1,22]. Contaminations in PBMCs and platelets likely make the correlations inaccurate, but without additional experiments it is difficult to calculate their exact contributions to respiration.

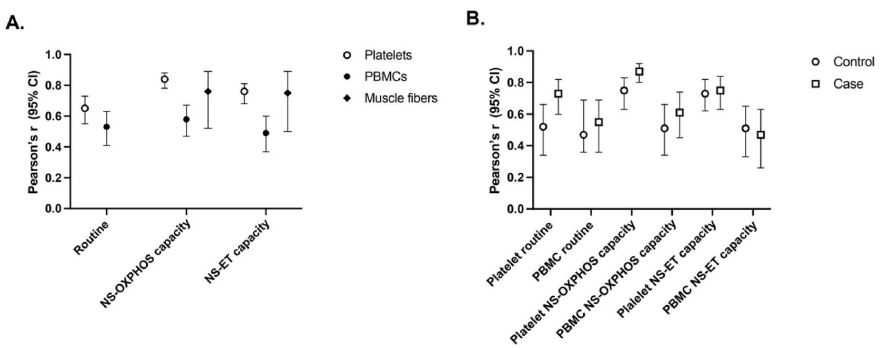

Fig. 3. Correlations between respiration and citrate synthase activity. **A** Correlation between CS activity ($\mu mol*min^{-1}*ml^{-1}$), and basal, succinate and FCCP respiration (pmol $O_2*s^{-1}*ml^{-1}$), for platelets (n = 163), PBMCs (n = 165) and between CS activity ($\mu mol*min^{-1}*mg^{-1}$) and the corresponding respiratory values (pmol $O_2*s^{-1}*mg^{-1}$) in muscle fibers (n = 24). **B** The same PBMC and platelet correlations as in panel A, comparing patients (n = 84) and healthy controls (n = 73).

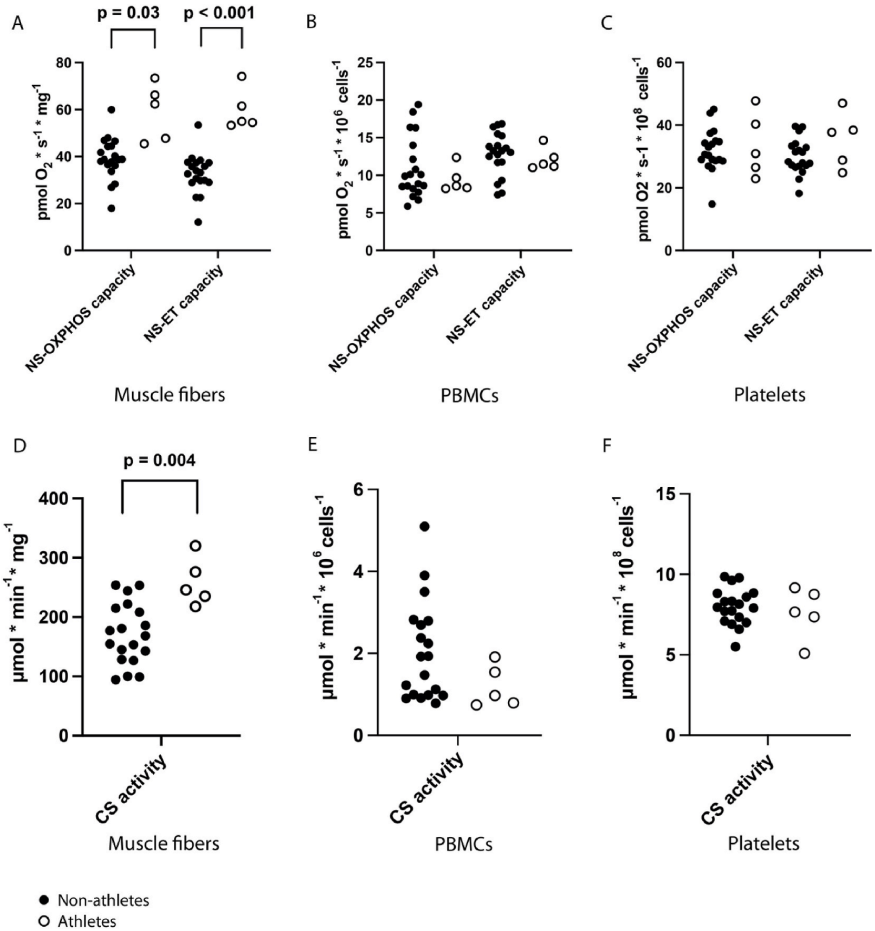

Fig. 4. Respiration in a group of athletes compared to non-athletes. Significant group differences ($P < 0.05$ according to Mann-Whitney *U* test) are displayed. Black dots = non-athletes (n = 19). White dots = athletes (n = 5). Graphs A-C show mitochondrial respiration in **A** muscle fibers, **B** PBMCs and **C** platelets. Graphs D-F show citrate synthase (CS) activity in **A** muscle fibers, **B** PBMCs and **C** platelets.

Our main results do not support the hypothesis that both blood cell types correlate strongly with muscle as they correlate weakly with each other. To our knowledge there is no previous study on PBMCs and platelets of this size but the conclusion is compatible with previous work comparing blood cell respiration with muscle. Two studies comparing PBMCs with muscle did not find any correlation [10,11] and the correlations that have been found were found in platelets or monocytes [8,9,11].

In this study neither PBMCs nor platelets correlated with muscle except for very few negative correlations, to which no other plausible explanation outside of statistical noise have been found. The general lack of correlation is compatible with our hypothesis that correlations across tissues would be smaller than within the same tissue but should still be interpreted very cautiously since the sample size of the subgroup with muscle biopsies (n = 24) was much smaller than the main study population (n = 318). To conclude a real lack of correlation could be to inflict a type II error. The negative results may not be able to settle the question of whether blood cells can be used as biomarkers for the bioenergetic profile of muscle fibers, but they add disfavor to the conflicting results of previous publications [8–11]. It should be noted that the conflicting results were produced by study populations differing substantially in their characteristics and that the population most similar to the one in this study, the young healthy males examined by Hedges et al., had the most negative results [10]. Muscle samples from a more widely representative study population or a separate one with children would have expanded the applicability of the results but the latter was not feasible for this study for logistical and ethical reasons.

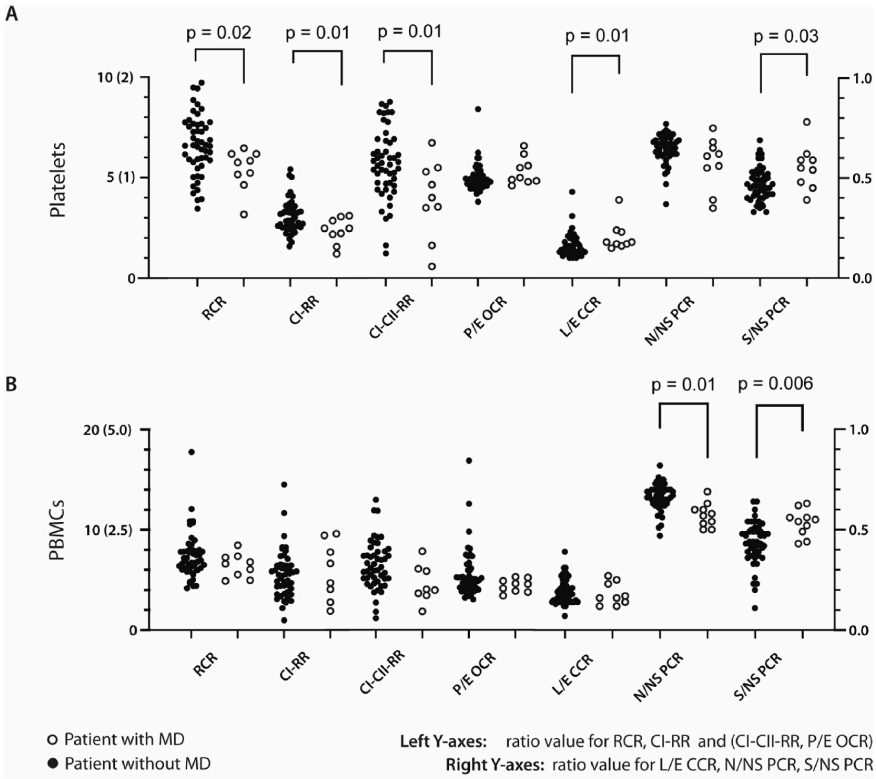

Fig. 5. Respiration in in a group of pediatric patients with mitochondrial disease compared to other pediatric patients. Significant group differences ($P < 0.05$ according to Mann-Whitney *U* test) are displayed. For abbreviations, see Table 1. **A** Comparison of internal ratios in platelet mitochondria from pediatric patients without mitochondrial disease (left side dots, n = 50) and patients with confirmed mitochondrial disease (right side dots, n = 9). Left y-axis shows the scale of RCR and CI-RR (0–10) and in parenthesis for CI–CII-RR and P/E OCR (0–2). Right y-axis shows the scale for the remaining ratios (0.0–1.0). **B** Corresponding analysis in PBMCS from patients without mitochondrial disease (n = 50, except CI–CII-RR n = 49) and with mitochondrial disease (n = 9, except CI-RR n = 8). Left y-axis shows the scale of RCR and CI-RR (0–20) and in parentheses for CI–CII-RR and P/E OCR (0–5.0). Right y-axis shows the scale for the remaining ratios (0.0–1.0).

Furthermore, when Hedges et al. failed to reproduce the significant monocyte-muscle correlations of Tyrell et al. using PBMCs they noted that their samples contained only a small fraction of monocytes and that these cells may be better suited as biomarkers than other mononuclear cells [9,10]. Differential count on a subset of PBMC samples (n = 251) in our study revealed that monocytes in average comprises at most 7.4% of the analyzed cells, leading us to the same assessment. Mean lymphocyte count was 85.6% (SD ± 9.2). Metabolic changes in mitochondria are highly involved in hematopoietic stem cell differentiation and previous research has indicated that different peripheral blood cells and platelets have distinctly different bioenergetic profiles [23,24].

The importance of differing bioenergetic profiles was further explored in Fig. 3. The correlation between cell count-normalized – or in the case of muscle fibers, mass-normalized – respiration and CS activity was examined. CS activity is an often used and reliable marker of mitochondrial content [25]. We assumed that stronger correlation between CS and respiration values would indicate a more reliable assessment of mitochondrial function. Platelets and muscle fibers had higher correlation with CS activity than did PBMCs. The differences in degree of correlation were only significant for platelets but the lack of significance for muscle fibers could be due to the small sample size (Fig. 3A–Supplementary Table 5). The NS-OXPHOS and NS-ET capacities correlated stronger than routine respiration, which was expected, as the maximum coupled and uncoupled capacities are limited by mitochondrial content whereas routine respiration is not (it is limited by, among other things, the amount of endogenous substrates). The correlations with CS activity were moderate to strong both in healthy participants and patients (Fig. 3B).

In a subgroup of the participants, we compared semi-professional athletes to non-athletes on a group level. NS-OXPHOS and NS-ET capacities per mass unit of muscle fiber were significantly higher for the athletes (Fig. 4A). CS activity was also significantly higher (Fig. 4D) in athletes compared to non-athletes. Since normalizing the respiratory values to CS eliminated the significant differences in

respiration (Supplementary Table 7), it is likely that the increased respiration in the muscles of the athletes was related to increased mitochondrial content (measured as CS activity). No differences between athletes and non-athletes in respiration or mitochondrial content were detected in either blood cell type (Fig. 4B–C, E-F, Supplementary Table 7). The results in muscle agree with previous research [26,27]. Much less is known about exercise-induced changes in blood cell respiration. Recent studies have detected increased fatty-acid dependent respiration in PBMCs and increased LEAK state respiration in platelets as acute post-exercise effects [28,29]. We are not aware of any previous study on permanent exercise-induced changes in the mitochondrial content of blood cells.

In another subgroup analysis, blood cells in a group of pediatric patients with confirmed mitochondrial disease were compared to patients that were initially suspected to have mitochondrial disease but without a final diagnosis. As is shown in Fig. 2B, there is higher across-sample overlap for values deviating further from the average and this supports the theory that conspicuous respiratory dysfunction – such as mitochondrial disease – is more likely to manifest itself across tissues than do slight alterations. Since our analysis showed that internal ratios increased the correlation as well as, or better than CS normalization, we only examined the ratios. (This method of normalization was also used in our previous related work on patients with mitochondrial disease [13].) On a group level, the comparisons showed significant differences in several ratios. These findings are in line with previous research, as group level differences in mitochondrial respiration in blood cells or platelets for patients with MD has previously been shown in small samples [30–32]. Most differences were seen in platelets, but the N/NS PCR was significant only in PBMCs and not platelets.

Strengths of this study include a large study population used in the main analysis, which together with the precision of high-resolution respirometry enabled the detection of weak correlations. Another strength of the study is that citrate synthase normalization was performed for a large subset of the main population.

A major limitation of the study is that it is mainly a retrospective, single-center study. Power calculations were not possible. Though we believe the study population as a whole is well-balanced and diverse enough for the purposes of the analyses, it is inevitable that the retrospective nature of the study and non-randomized selection of participants, including selection for several sub-analyses that were made on the basis of available data (CS-normalization, muscle correlation, assessment of CIV-activity), introduce possible bias.

Another major limitation of this study is the lack of adjustment for important contributors to the correlation, such as age, sex, and metabolic disease. It is possible that several such factors contributed to the correlation found, but adjusting for them would more likely lower than raise the already weak correlation. Such factors are sometimes adjusted for in a multivariate regression model, but regression was not performed here since the causality condition was not believed to be satisfied for these data (i.e. that one variable is clearly dependent on the other). While it might have been possible to use partial correlation to single out some individual factors contributing to the correlation found, we opted instead to present the results of several relevant subgroups, among which some differences can be noted, in Supplementary Tables 3 and 4

Data on BMI, smoking status, medications and psychological comorbidities, which would also have been interesting denominators, were lacking for most subjects. For the comparison of athletes versus non-athletes, it would have been desirable to be able to present more detailed data, such as exercise regimens. On the other hand, the highly significant differences seen in the muscle comparisons indirectly confirm a successful selection of participants into the two groups. The fact that intact study protocols were not included may be a limitation of the study but the permeabilized protocol was preferred as it was assumed that the maximal capacities (NS-OXPHOS and NS-ET) would correlate best. Digitonin and saponin, which are used to permeabilize cell membranes, introduce a possible risk of mitochondrial toxicity that is not present in the intact protocols but this risk can be kept low by using validated concentrations. Another limitation was that the results could not be adjusted for differential count, this might have strengthened correlations.

In conclusion, the weak within tissue correlation coupled with the lack of across-tissue correlation found in this study argues against the notion that blood cell respiration can be used as accurate markers for muscle respiration in a general population. However, additional results support the hypothesis that blood cells, especially platelets, may reflect certain, pronounced metabolic alterations in less-accessible tissues or systemically in the body.

4. Methods

4.1. Study population

Blood samples were collected from patients and healthy volunteers including both sexes at a wide age span. Most samples were collected as part of other studies and platelet and PBMC data from the participants have been published in other contexts [13–20]. Some samples, including all muscle biopsies, were collected principally for this study. There were 318 participants in total, of which 226 were patients, with diseases such as neurodegenerative diseases, pediatric neurological conditions and sepsis, and 92 were healthy volunteers. Out of the 318 participants, 82 were children. PBMCs and platelets were analyzed in all 318 participants. In 24 of the adult participants, muscle biopsies were obtained in addition to the blood samples.

4.2. Sample preparations

Venous blood was collected in K2 ethylene diamine tetraacetic acid (EDTA) tubes. Blood cells were separated through consecutive centrifugations and further prepared as described in previous publications and analyzed within three to 5 h [16,19]. The EDTA tubes were centrifuged 15 min at 300 g to yield a platelet rich plasma (PRP), which was pipetted and re-centrifuged for 5 min at 4600 g. This yielded a layer of close to cell free plasma and a platelet pellet. The pellet was dissolved in 1–3 ml of the study participant's own plasma by gentle pipetting to obtain a highly concentrated PRP [16]. The PBMC preparations were made from the remaining blood, after the first PRP had been pipetted off following the initial centrifugation (see above). This blood was diluted in saline (NaCl 9 mg/ml) up to 6

ml and carefully added to tubes containing 3 ml Lymphoptep™, which were centrifuged for 25–30 min at 800 g. The mononuclear cell layer and lymphoprep fraction were collected and washed in saline (~10 x dilution), then recentrifuged for 10 min at 250 g. The supernatant layer was aspirated and resuspended in 0.5–1 ml the participant's own plasma. All centrifugations described above were made at room temperature [19].

Muscle biopsies were obtained under sterile conditions after a subcutaneous injection of 5 ml mepivacaine/norepinephrine (10 mg/ml + 5 μg/ml) through a 14 gauge needle in m. vastus lateralis. The local anesthetic was applied carefully not to expand down to the muscle, as local anesthetics are known to effect mitochondrial function [28]. A sample of approximately 20 mg tissue was taken directly under the muscle fascia and was immediately transferred to an ice-cold biopsy preservation solution (BIOPS; 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 $MgCl_2$, 5.77 mM ATP, 15 mM phosphocreatine, pH 7.1). The sample was then dissected under a microscope using forceps to mechanically separate fibers and to remove fat and connective tissue. The fiber bundles were permeabilized for 30 min in 2 ml BIOPS + Saponin (20 μl of 5 mg/ml) and afterwards washed in MiR05 (0.5 mM EGTA, 3 mM $MgCl_2$, 60 mM k-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM sucrose, 1 g/l BSA, pH 7.1) [33] for another 10 min. Before respiratory analyses, the biopsy wet weight was measured with a digital precision scale (Precisa 40SM-200A).

The contents of the chamber (cells/fibers in MiR05; 2 ml) were frozen and stored at −80 °C after each experiment and later thawed on ice for citrate synthase (CS) measurements. CS activity was measured, as previously described, using a commercially available kit in accordance with the manufacturer's instructions (Citrate Synthase Assay Kit, CS0720, Sigma–Aldrich, St Louis, MO, USA) [14]. Samples were sonicated (PBMCs 30 s, platelets and muscle fibres, 2 × 60 s) and transferred onto a 96 well plate mixed with assay buffer, acetyl-CoA and 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB). The optimal sonication times were determined in a different set of experiments (data not shown). A spectrophotometer set to 412 nm recorded the absorbance before and after the addition of oxaloacetic acid, as the formation of 5-thio-2-nitrobenzoic acid (TNB) can be used to estimate the activity of CS. The absorbance was followed for 2 min and the activity of the sample was calculated using the extinction coefficient of TNB, which is 13.6 $mM^{-1} \cdot cm^{-1}$ at 412 nm.

4.3. High-resolution respirometry and respiratory ratios

Mitochondrial respiration was measured at 37 °C in high-resolution oxygraphs (Oxygraph-2k Oroboros Instruments, Innsbruck, Austria). The experiment design for blood components has previously been published in detail and was identical for platelets and PBMCs, except that the software (DatLab Software 4.3, Oroboros Instruments, Innsbruck, Austria) was set to record respiration per 10^8 platelets and 10^6 PBMCs respectively to make graphic comparisons more convenient [16].

In summary, the cells were suspended in an airtight chamber, in MiR05 [33], and changes in the rate of oxygen consumption (O_2 flux) were measured with a high precision sensor following subsequent additions of substrates, uncouplers and inhibitors to assess different pathways and other aspects of mitochondrial respiration. This type of protocol is called substrate-inhibitor-uncoupler-titration (SUIT) [34].

The details of the study protocol are outlined in Fig. 1. The graph illustrates a typical experiment; all measurements were made consecutively in the same sample. Mitochondrial respiration was categorized by the respiratory state of the mitochondria (first top bar) and by substrate pathways (second top bar).

Initially, routine respiration was measured in the intact cells after reaching a stable plateau. Digitonin (6 μg per 10^6 PBMCs, 1 μg per 10^6 platelets) was added to permeabilize the cell membrane, after which malate (5 mM) and pyruvate (5 mM) were added followed by ADP (1 mM) to induce oxidative phosphorylation (OXPHOS). At this point electrons are provided to complex I of the electron transfer system (ETS) by the electron carrier NADH, which is why the measured respiration can be said to represent the N-linked pathway. The addition of glutamate (5 mM) provides further electrons via NADH. The addition of succinate (10 mM) provides additional electrons via complex II, also known as the S-linked pathway.

The combined pathways (N + S) were measured and represent the maximal coupled respiration, known as the NS-OXPHOS capacity (or alternatively just 'OXPHOS capacity', without the prefix). Next, the ATP synthase inhibitor oligomycin (1 μg/ml) was added, inhibiting OXPHOS and allowing the measurement of LEAK respiration (LEAK) over the inner mitochondrial membrane.

This was followed by titration of the uncoupler protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), to uncouple the ETS from ATP synthesis and the measurement of respiration at the titration plateau represents the maximal uncoupled respiration, known as the NS-ET capacity (or alternatively just 'ET capacity').

Subsequently, the complex I inhibitor rotenone (2 μM) was added to measure the S-linked pathway separately from the combined pathway. The complex III inhibitor antimycin A (1 μg/ml) was added after this to adjust for residual oxygen consumption in the cells. This O_2 flux was subtracted from all previous measurements before further calculations were made.

In select cases, tetramethylphenylenediamine (TMPD, 0.5 mM) followed by sodium azide (10 mM) were added at the end of the experiment to assess the activity of complex IV. The addition of ascorbate was tested (data not show) in a prototype version of the protocol but caused too much auto-oxidation and was omitted from the protocol. (This was an unexpected finding, as the purpose of ascorbate is to keep TMPD in a reduced state. The auto-oxidation was likely catalyzed by different metal-containing proteins in the platelet samples. TMPD without ascorbate in platelets was shown to give reproducible results [16].) Ascorbate was likewise omitted from the PBMC and muscle preparations to harmonize the protocols.

The muscle fiber protocol was similar to the protocol for platelets and PBMCs, except for a few details: Respiration was recorded per mg of muscle tissue. Digitonin was not added, as the fibers were saponin-permeabilized during preparation. ADP was given at a dose of 2 mM instead of 1 mM and oligomycin in the dose of 2 μg/ml instead of 1 μg/ml. The muscle respirometry was performed in two chambers for each individual and mean values were calculated. Respiratory values were corrected not for antimycin but for respiration

before adding substrates (malate, pyruvate) and ADP, since this was assumed to represent residual oxygen consumption (ROX) more reliably than the levels after the addition of antimycin A.

Table 2 explains the terminology used in text, tables and figures, and its relation to the additions and measurements made during the experiment. For a more comprehensive description of the states, rates and pathways we refer to other literature [34]. In addition to normalizing for cell count and CS, we have normalized values by calculating a number of internal ratios, which are further explained in Table 2. Two of the ratios (CI response ratio, CI–CII response ratio) are not in common use but are based on our previous publication where they displayed a promising diagnostic accuracy for primary mitochondrial disease [13].

4.4. Statistics

Correlation between platelets, PBMCs and muscle fibers was examined for cell count-normalized values (or, in the case of muscle fibers, mass-normalized values), CS-normalized values and internal ratios using Pearson correlation. As a sensitivity measure, we also analyzed Spearman rank correlation to exclude any large discrepancies. Mean correlations for cell count-normalized and CS-normalized values, and internal ratios respectively were calculated to ease visual comparison in the diagram. To analyze whether extreme respiratory values correlated better than average values we compared quartile overlap for selected parameters and ratios.

As a quality control of the measurements of mitochondrial respiration we analyzed the correlation between CS values (as a measure of mitochondrial content) and respiration in each sample, comparing the sample types (PBMCs, platelets, muscle fibers) to one another, and, for platelets and PBMCs, comparing healthy participants with patients. For assessing the significance of the difference between two correlation coefficients the Fisher r-to-z transformation was applied.

In a subgroup of the healthy participants we made groupwise comparison of athletes and non-athletes, examining respiration and citrate synthase activity in muscle, platelets and PBMCs.

Finally, we compared platelet and PBMC respiration on a group level between pediatric patients with confirmed mitochondrial disease to pediatric patients without mitochondrial disease, using internal respiratory ratios.

To test for statistical significance in the analyses concerning athletes and mitochondrial disease patients respectively, the Mann-Whitney *U* test, was selected due to small and unequally distributed sample sizes in the groups compared.

In addition to the analyses mentioned above, descriptive blood cell and muscle fiber respirometry values (mean values for cell-count or mass normalized respiration, and CS-normalized respiration) for the adult participants, both for the whole group and for the healthy adults separately, were calculated.

Statistical significance was defined as $p < 0.05$ for all analyses. For the correlation coefficiants (Pearson's r or Spearman's rho), we defined 0.00–0.19 as very weak, 0.20–0.39 as weak, 0.40–059 as moderate, and 0.60–0.79 as strong and 0.80–1.00 as very strong correlation.

4.5. Study approval

This study has been approved by the regional ethical review board of Lund, Sweden (113/2008, 59/2009, 97/2009, 89/2011, 320/2011) and the scientific ethical committee of Copenhagen County, Denmark (H–C-2008-023). Written informed consent was obtained from each participant or from the participant's parent or guardian or, in applicable cases, from other relative.

CRediT authorship contribution statement

Emil Westerlund: Writing – review & editing, Writing – original draft, Visualization, Validation, Software, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Sigurður E. Marelsson:** Writing – review & editing, Investigation, Formal analysis. **Michael Karlsson:** Writing – review & editing, Methodology, Formal analysis, Data curation, Conceptualization. **Fredrik Sjövall:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Imen Chamkha:** Writing – review & editing, Validation, Methodology, Formal analysis, Data curation. **Eleonor Åsander Frostner:** Writing – review & editing, Validation, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Johan Lundgren:** Writing – review & editing, Formal analysis. **Vineta Fellman:** Writing – review & editing, Formal analysis. **Erik A. Eklund:** Writing – review & editing, Formal analysis. **Katarina Steding-Ehrenborg:** Writing – review & editing, Formal analysis, Data curation. **Niklas Darin:** Writing – review & editing, Formal analysis. **Gesine Paul:** Writing – review & editing, Formal analysis. **Magnus J. Hansson:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Johannes K. Ehinger:** Writing – review & editing, Writing – original draft, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Eskil Elmér:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

Imen Chamkha, Johannes K. Ehinger, Eskil Elmér, Magnus J. Hansson, Michael Karlsson, and Eleonor Åsander Frostner have equity interests in, and/or have received salary support and/or travel reimbursements and/or grants from Abliva AB (formerly NeuroVive Pharmaceutical AB), a public company developing pharmaceuticals in the field of mitochondrial medicine. The other authors declare no financial or commercial conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e26745.

References

[1] R. Acin-Perez, et al., Utilization of human samples for assessment of mitochondrial bioenergetics: gold standards, limitations, and future perspectives, Life 11 (9) (2021).
[2] W.H.G. Hubens, et al., Blood biomarkers for assessment of mitochondrial dysfunction: an expert review, Mitochondrion 62 (2022) 187–204.
[3] C. Granata, N.A. Jamnick, D.J. Bishop, Training-induced changes in mitochondrial content and respiratory function in human skeletal muscle, Sports Med. 48 (8) (2018) 1809–1828.
[4] H.N. Carter, C.C. Chen, D.A. Hood, Mitochondria, muscle health, and exercise with advancing age, Physiology 30 (3) (2015) 208–223.
[5] R.H. Haas, et al., The in-depth evaluation of suspected mitochondrial disease, Mol Genet Metab 94 (1) (2008) 16–37.
[6] J. Bergstrom, Percutaneous needle biopsy of skeletal muscle in physiological and clinical research, Scand. J. Clin. Lab. Invest. 35 (7) (1975) 609–616.
[7] M. Jacques, et al., Mitochondrial respiration variability and simulations in human skeletal muscle: the Gene SMART study, Faseb j 34 (2) (2020) 2978–2986.
[8] A. Braganza, G.K. Annarapu, S. Shiva, Blood-based bioenergetics: an emerging translational and clinical tool, Mol. Aspect. Med. (2020) 71.
[9] D.J. Tyrrell, et al., Blood cell respirometry is associated with skeletal and cardiac muscle bioenergetics: implications for a minimally invasive biomarker of mitochondrial health, Redox Biol. 10 (2016) 65–77.
[10] C.P. Hedges, et al., Peripheral blood mononuclear cells do not reflect skeletal muscle mitochondrial function or adaptation to high-intensity interval training in healthy young men, J. Appl. Physiol. 126 (2) (2019) 454–461.
[11] S. Rose, et al., A comparative study of mitochondrial respiration in circulating blood cells and skeletal muscle fibers in women, Am. J. Physiol. Endocrinol. Metab. 317 (3) (2019) E503–e512.
[12] A. Braganza, et al., Platelet bioenergetics correlate with muscle energetics and are altered in older adults, JCI Insight 5 (2019).
[13] E. Westerlund, et al., Oxygen consumption in platelets as an adjunct diagnostic method for pediatric mitochondrial disease, Pediatr. Res. 83 (2) (2018) 455–465.
[14] J.K. Ehinger, et al., Mitochondrial dysfunction in blood cells from amyotrophic lateral sclerosis patients, J. Neurol. 262 (6) (2015) 1493–1503.
[15] J.K. Ehinger, et al., Mitochondrial respiratory function in peripheral blood cells from huntington's disease patients, Mov Disord Clin Pract 3 (5) (2016) 472–482.
[16] F. Sjövall, et al., Mitochondrial respiration in human viable platelets–methodology and influence of gender, age and storage, Mitochondrion 13 (1) (2013) 7–14.
[17] F. Sjövall, et al., Cytokine and nitric oxide levels in patients with sepsis–temporal evolvement and relation to platelet mitochondrial respiratory function, PLoS One 9 (5) (2014) e97673.
[18] F. Sjövall, et al., Temporal increase of platelet mitochondrial respiration is negatively associated with clinical outcome in patients with sepsis, Crit. Care 14 (6) (2010) R214.
[19] F. Sjövall, et al., Patients with sepsis exhibit increased mitochondrial respiratory capacity in peripheral blood immune cells, Crit. Care 17 (4) (2013) R152.
[20] J.K. Ehinger, et al., Mitochondrial function in peripheral blood cells across the human lifespan, NPJ Aging 10 (1) (2024) 10.
[21] K. Steding-Ehrenborg, et al., Determinants of kinetic energy of blood flow in the four-chambered heart in athletes and sedentary controls, Am. J. Physiol. Heart Circ. Physiol. 310 (1) (2016) H113–H122.
[22] M. Urata, et al., Platelet contamination causes large variation as well as overestimation of mitochondrial DNA content of peripheral blood mononuclear cells, Ann. Clin. Biochem. 45 (Pt 5) (2008) 513–514.
[23] A. Wanet, et al., Connecting mitochondria, metabolism, and stem cell fate, Stem Cells Dev 24 (17) (2015) 1957–1971.
[24] B.K. Chacko, et al., Methods for defining distinct bioenergetic profiles in platelets, lymphocytes, monocytes, and neutrophils, and the oxidative burst from human blood, Lab. Invest. 93 (6) (2013) 690–700.
[25] S. Larsen, et al., Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects, J Physiol 590 (14) (2012) 3349–3360.
[26] R.A. Jacobs, C. Lundby, Mitochondria express enhanced quality as well as quantity in association with aerobic fitness across recreationally active individuals up to elite athletes, J. Appl. Physiol. 114 (3) (2013) 344–350.
[27] A.K. Meinild Lundby, et al., Exercise training increases skeletal muscle mitochondrial volume density by enlargement of existing mitochondria and not de novo biogenesis, Acta Physiol. 222 (1) (2018).
[28] F. Hoppel, et al., Effects of ultramarathon running on mitochondrial function of platelets and oxidative stress parameters: a pilot study, Front. Physiol. 12 (2021) 632664.
[29] E. Liepinsh, et al., Low-intensity exercise stimulates bioenergetics and increases fat oxidation in mitochondria of blood mononuclear cells from sedentary adults, Physiol Rep 8 (12) (2020) e14489.
[30] P. Pecina, et al., Noninvasive diagnostics of mitochondrial disorders in isolated lymphocytes with high resolution respirometry, BBA Clin. 2 (2014) 62–71.
[31] R. Artuch, et al., Oxygen consumption measurement in lymphocytes for the diagnosis of pediatric patients with oxidative phosphorylation diseases, Clin. Biochem. 33 (6) (2000) 481–485.
[32] V. Kaplanová, et al., Segregation pattern and biochemical effect of the G3460A mtDNA mutation in 27 members of LHON family, J. Neurol. Sci. 223 (2) (2004) 149–155.
[33] D. Pesta, E. Gnaiger, High-resolution respirometry: OXPHOS protocols for human cells and permeabilized fibers from small biopsies of human muscle, Methods Mol. Biol. 810 (2012) 25–58.
[34] E. Gnaiger, et al., MitoEAGLE Task Group, Mitochondrial physiology, Bioenerg Commun 1 (2020), 2020.

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Mitochondrial function in peripheral blood cells across the human lifespan

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Mitochondrial dysfunction is considered a hallmark of aging. Up to now, a gradual decline of mitochondrial respiration with advancing age has mainly been demonstrated in human muscle tissue. A handful of studies have examined age-related mitochondrial dysfunction in human blood cells, and only with small sample sizes and mainly in platelets. In this study, we analyzed mitochondrial respiration in peripheral blood mononuclear cells (PBMCs) and platelets from 308 individuals across the human lifespan (0–86 years). In regression analyses, with adjustment for false discovery rate (FDR), we found age-related changes in respiratory measurements to be either small or absent. The main significant changes were an age-related relative decline in complex I-linked respiration and a corresponding rise of complex II-linked respiration in PBMCs. These results add to the understanding of mitochondrial dysfunction in aging and to its possible role in immune cell and platelet senescence.

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INTRODUCTION

The role of mitochondria in aging has been recognized at least since the 1950s when the free radical theory of aging was first proposed[1]. While the causal relationship between free radicals and aging, as it was originally described, has been contradicted by more recent animal studies[2,3], the complex association between mitochondria and aging has been consolidated and further explored[4]. Several other theories on how mitochondrial dysfunction may influence aging are currently being investigated. One such theory proposes that a gradual increase in mutations or deletions in the mitochondrial DNA (mtDNA) during the normal lifespan causes dysfunction in the electron transfer system (ETS), which leads to aging phenotypes[5,6]. This theory is supported by studies of knock-in mice with defective mtDNA polymerase[7].

A gradual decline in mitochondrial respiration with age has been shown in human muscle tissue in several studies[8–11]. The magnitude of the decline is contested[12,13]; nonetheless, its potential link to sarcopenia, an important phenotype of frailty in aging, has been the focus of much research in recent years[14].

Outside of muscle tissue, age-related mitochondrial dysfunction is far less studied, with differing profiles of declining function reported in human tissues such as the brain, liver, and intestine[15–17]. As for blood cells, only a handful of studies, with modest sample sizes and almost exclusively featuring platelets, have examined age-related mitochondrial decline, and the results have been inconclusive[18–23]. Hence, the question of mitochondrial function with aging in blood cells is still largely open. The aim of this study was to investigate the hypothesis that mitochondrial function declines with age in human PBMCs and platelets.

RESULTS

In 317 individuals aged 3 months to 86 years, mitochondrial function was analyzed separately in isolated PBMCs and isolated platelets. Nine participants with confirmed primary mitochondrial disease were excluded, and the remaining 308 individuals were analyzed for the effect of age on mitochondrial respiration and mitochondrial content. Data for 153 of the 308 participants have previously been published elsewhere in articles not focused on the effects of aging[24–30]. The study population comprised patient cohorts formed to study specific conditions, including patients with neurodegenerative disorders and sepsis, and healthy controls (for further details, see Methods). The patient cohorts consisted of 217 cases, and the remaining 91 were healthy controls (Table 1). Mitochondrial respiratory function was assessed by high-resolution respirometry using oxygraphy in intact and permeabilized cells (Supplementary Fig. 1, Supplementary Tables 1 and 2). These protocols allow for a comprehensive analysis of integrated function as well as separate pathways of electron transfer, i.e., through different enzyme complexes. In addition to routine (endogenous) respiration, measured in intact cells, maximum phosphorylating capacity (OXPHOS capacity), maximum electron transfer capacity (ET capacity), and the non-phosphorylating respiration needed to compensate for proton leak (leak respiration) were measured in permeabilized cells. Ratios of measurements were calculated to assess the relative contribution of CI-linked respiration (N/NS pathway control ratio; S = succinate, N = NADH), CII-linked respiration (S/NS pathway control ratio), and coupling efficiency (respiratory control ratio; RCR) (Supplementary Table 1).

Unadjusted respiratory measurements normalized to cell count were plotted against age (Fig. 1, Supplementary Table 3). Multiple regression analyses were performed where sex and health status

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Table 1. Study population.

	n	Female/male (not recorded)	Adults/children*	Age, median years (range)	Patients/healthy participants
Main study population	308	162/143 (3)	235/73	59 (0–86)	217/91
Population where CS activity was measured	159[a]	85/71 (3)	93/66	23 (0–84)	91/68

[a]CS was measured in both cell types in 157 cases. CS was measured only in platelets in two cases and only in PBMCs in one case.

were used as binary covariates in addition to age for each respiratory parameter. The possibility of sex differences in mitochondrial respiration has garnered much attention and speculation, but a recent meta-analysis, including 2258 participants from 50 studies, demonstrated an overall lack of sex differences[31]. Therefore, sex differences were not expected to confound the age effect substantially. However, one of the few slight sex differences detected in the meta-analysis was a higher mitochondrial content in women's leukocytes[31]. Allowing the possibility of confounding effects, sex was included as a covariate in all regression models. Since around two-thirds of the participants were selected from patient cohorts, and since several of these patients have diseases that may be associated with mitochondrial alterations, health status (patient cohort or healthy controls) was included as another covariate. The inclusion of the patient cohorts permitted a much greater sample size and higher statistical power. (simple regression for the healthy subgroup without adjustment for sex is provided in Supplementary Fig. 2, Supplementary Fig. 3, and Supplementary Table 9).

After adjusting for sex and health status, the only significant relationships in the respiratory parameters, normalized to cell count, were a slight age-dependent increase in routine respiration (slope = 0.004, SD ± 0.002, $P = 0.03$) and ET capacity (slope = 0.026, SD ± 0.010, $P = 0.01$) in PBMCs (Fig. 1, Supplementary Table 4). In platelets, none of the cell count-normalized respiratory measurements varied with age (Fig. 1, Supplementary Table 4). A notable fact is that the significant results in PBMCs showed an increase in respiration with advancing age, contrary to the hypothesis of a respiratory decline. However, the overall effect was slight, as the variation explained by the combined regression model accounted for under 5% of the total variation in respiration in the two significant parameters ($R^2 = 0.027$ for routine respiration and $R^2 = 0.047$ for ET capacity, respectively, in PBMCs). After calculating the false discovery rate (FDR) to control for the influence of multiple comparisons, these results were not classified as true discoveries.

The three respiratory ratios calculated from the respiratory measurements were plotted in the same manner as described above (Fig. 2, Supplementary Table 5). After adjusting for sex and health status, age-dependent changes remained highly significant in the N/NS pathway control ratio (slope = −0.0007, SD ± 0.0002, $P = 0.0002$) and the S/NS pathway control ratio for PBMCs (slope = 0.0010, SD ± 0.0002, $P = < 0.0001$) (Fig. 2, Supplementary Table 5). This can be summarized as a pattern of an age-dependent decrease in the relative contribution of CI-linked respiration, mirrored by an almost equal increase in the relative contribution of CII-linked respiration. Again, the explained variance was small for the combined model ($R^2 = 0.047$ for N/NS pathway control ratio and $R^2 = 0.086$ for S/NS pathway control ratio, respectively, in PBMCs) (Fig. 2, Supplementary Table 5), indicating that the variability in these parameters is mainly explained by factors other than age. The significant results for the age covariate and for the combined model for the N/NS and S/NS pathway control ratios were all classified as true discoveries after calculating FDR.

The RCR was not affected by age in PBMCs, and none of the ratios changed with age in platelets. CS activity (mitochondrial content) did not change with age in neither blood cell type. Also of note, after adjustment for age and health status, there were no significant sex differences in any parameter in neither PBMCs nor platelets, which is in line with recent evidence[31].

Principal component analysis, using all respiratory rates and derived ratios in the regression analyses (Supplementary Table 1) and additional measurements from the study protocol (Supplementary Table 2) were performed. In the loadings plots (Fig. 3C, D), the measurements not selected for regression analysis had a relatively high level of collinearity with the ones used (bundled arrows pointing right in the graph), confirming that no important age-dependent respiratory change in the study protocol was overlooked when selecting the representative measurements for further analysis.

DISCUSSION

In summary, blood cells from a large human study population showed no age-dependent decline in mitochondrial respiration in platelets and only small changes in PBMCs. This is reflected in the PC score plots for PBMCs (Fig. 3C) and platelets (Fig. 3D), which did not reveal prominent clustering when the two first principal components were plotted with labeling for age. This finding runs contrary to, and nuances, the general notion of mitochondrial decline in aging but is not unique. As was previously touched upon, even the well-studied mitochondrial decline in skeletal muscle has turned out to be less evident than originally perceived. While several studies have found a general age-dependent decline in respiration in muscle mitochondria[8–11], several comparable studies have found little or no decline[13,32,33]. One reason for the discrepancies may be methodological. Picard et al. found that the aging effects were much greater in isolated muscle mitochondria from rats when compared to mitochondria in permeabilized muscle bundles, suggesting that the isolation procedure itself more easily damages the frail (but otherwise functional) mitochondria of aged individuals[12]. A strength of the present study is that all experiments were made in both intact and permeabilized cells rather than in isolated mitochondria, more closely mimicking in vivo conditions.

One should also be cautious not to generalize the mitochondrial function and rate of decline across the entire organism, as these changes may be tissue-specific. Previous studies on human blood cells are the most relevant ones in comparison to the present study, but results from these have been inconsistent. Two studies found no age-dependent decline in platelet respiration[19,23]. Two other studies found a comparable decline in some respiratory parameters but contradictory results in others[21,22]. Differences in underlying study populations may account for the diverging results, as well as differences in statistical methods. The two latter studies compared group means and only one of the two former ones used a linear regression model. The statistical methods may have been limited by sample sizes, which were 27, 85, 59, and 64, respectively[19,21–23]. The sample size in the present study was 308, and the linear regression models allowed, in addition to examining the strength of the correlations, a quantification of the age effect. In a recent animal study with a large sample size,

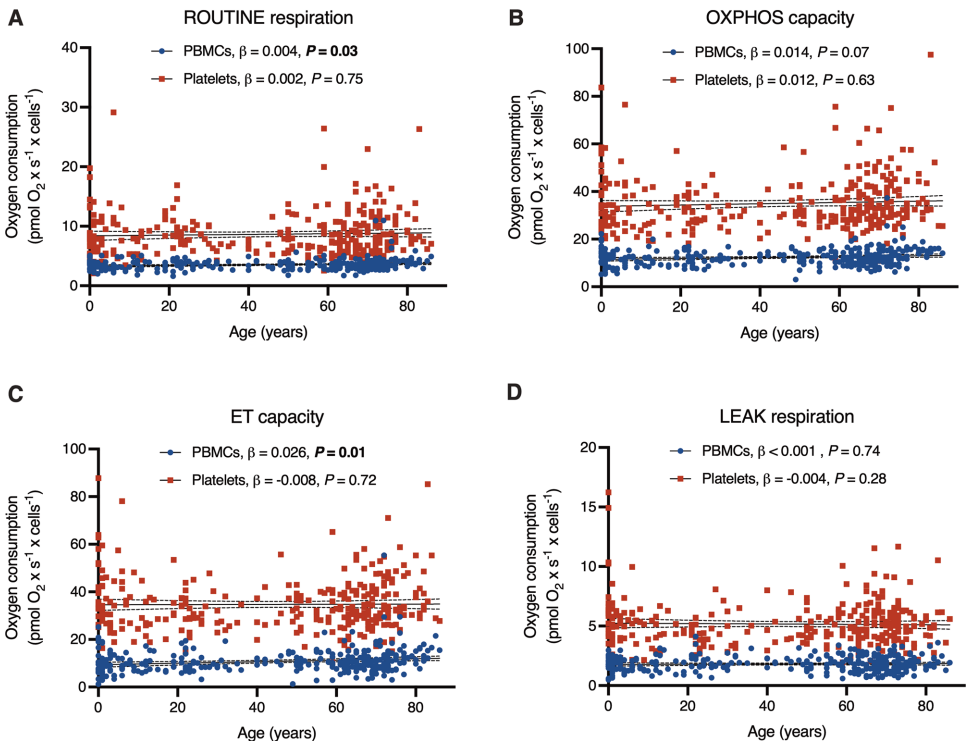


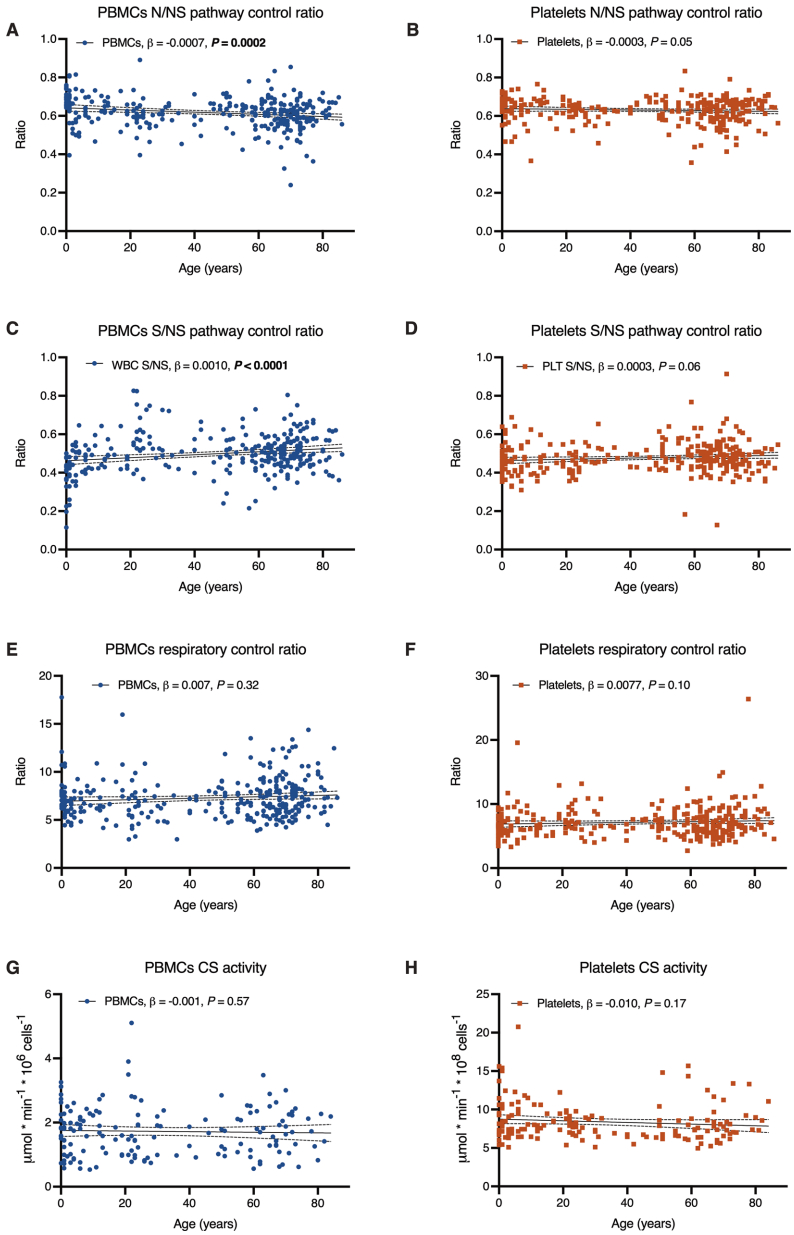
Fig. 1 Cell count-normalized respiration as a function of age, with adjustment for confounders. (**A**–**D**) Plots depict simple linear regression for each respiratory parameter (*y*-axes) depending on age (*x*-axes); the slope is depicted as a straight line with its 95% confidence interval as a dotted line (complete data in Supplementary Table 3). The age effect, and its *P*-value, adjusted for sex and health status in a multiple regression (MR) model, is displayed in the respective graph legend. None of the age effects were classified as true discoveries after adjustment for the false discovery rate. The overall MR model was significant for routine respiration ($R^2 = 0.027$, $P = 0.04$), OXPHOS capacity ($R^2 = 0.036$, $P = 0.01$), and ET capacity ($R^2 = 0.047$, 0.002) for PBMCs; none of the MR models were significant for platelets (complete MR data in Supplementary Table 4).

there was no decline in platelet respiratory function with age in 344 rats, even though such changes were seen in mitochondria from skeletal muscle and kidney cortex from the same rats[34]. We share the authors' conclusion in that publication, further supported by the present findings, that one should be cautious not to generalize mitochondrial function across tissues.

To our knowledge, only one previous study has examined age-related decline in mitochondrial respiration in PBMCs. That study, in agreement with the present one, reported no decline in cell count-normalized respiration in a small human population ($n = 38$). However, when normalizing to mtDNA content, several parameters, such as routine respiration and ET capacity, did decline with age[19]. Although mtDNA was not analyzed for this study, another marker of mitochondrial content, CS activity, was analyzed for about half of the study population (Table 1). As a sensitivity analysis, the cell count-normalized respiratory parameters for PBMCs (Fig. 1, Supplementary Table 3) were normalized to CS activity and reanalyzed. Multiple regression analyses of this subset ($n = 156$) did not reveal an age-dependent decline for any parameter (Supplementary Table 8). It should be noted that even though CS activity and mtDNA are both widely used markers for mitochondrial content, their accuracy and mutual correlation vary in previous research[27,35].

It is well known that blood differential count changes with age in humans[36]. The cells used in the PBMC experiments were isolated by a commercially available method, ensuring a high share of lymphocytes in the final suspension regardless of whole blood differential count. To make sure that the participants' ages had not influenced the isolation process in a way that would have skewed the results, age-dependent variations in differential count were analyzed (for a majority of cases, $n = 262$). The proportion of lymphocytes did not change significantly depending on age, nor did the proportion of granulocytes (Fig. 3A). The proportion of midsize cells did slightly increase with age (Fig. 3A), but the explained variance was low ($R^2 = 0.027$) and midsize cells, on average, only made up 7.0% (SD ± 7.2) of the analyzed PBMC samples (Fig. 3B). Taken together, it is unlikely that variations in PBMC composition could have influenced the results strongly in either direction, particularly in such a way that a large age-dependent respiratory decline would have gone undetected.

One should always be careful not to overinterpret negative results. Nonetheless, the findings of this study are interesting in relation to other advances in the understanding of blood cell senescence. While hematopoietic stem cell (HSC) function is known to decline with age, peripheral blood cells are continuously renewed from HSCs via progenitor cells in the bone marrow, and it



is unclear at what level the age-related effects are determined[37]. Animal cell studies have described mechanisms by which stem cells can protect their progeny from damaged proteins, containing the effects of aging to the mother cell[38]. The immune system deterioration seen in aging (such as chronic inflammation and impaired immunity) may not be closely linked to the senescence of the individual peripheral cells. For example, in a recent study, T cells from mice were able to substantially outlive their species

Fig. 2 Ratios and CS activity as a function of age, with adjustment for confounders. Plots depict simple linear regression for each ratio (**A**–**F**) and citrate synthase (CS, **G**–**H**) activity (*y*-axes) depending on age (*x*-axes); the slope is depicted as a straight line with its 95% confidence interval as a dotted line (complete data in Supplementary Table 3). The age effect, and its *P*-value, adjusted for sex and health status in a multiple regression (MR) model, is displayed in the respective graph legend. Age effect *P*-values in bold were significant and classified as true discoveries after adjustment for false discovery rate. The overall MR model was significant for the N/NS pathway control ratio ($R^2 = 0.047$, $P = 0.002$) and S/NS pathway control ratio ($R^2 = 0.086$, $P < 0.0001$) for PBMCs; none of the MR models were significant for platelets (complete MR data in Supplementary Table 5).

with retained ability to proliferate and fight infections[39]. Furthermore, it is known that the composition of peripheral lymphocyte subpopulations shifts with advancing age, and this could affect immune function through mechanisms unrelated to mitochondrial impairment or stem cell deterioration[40].

As for platelets, it is known that they become hyperreactive with age, and this contributes to an increased risk for clotting and cardiovascular diseases[41]. The reason for this is not fully known, but the cause may lie outside of the platelets themselves, driven by dysregulated inflammatory pathways in the aged organism[41]. If that were the case, the altered function of platelets in aging would not need to be linked to a decline in aerobic respiratory function in the non-activated state, which was studied here.

A few significant age-dependent effects were observed in this study. The fact that CII-respiration increased relative to CI-respiration with age in PBMCs is of interest, even though the effect itself was small. Complex II differs from complex I in that it is completely determined by nuclear DNA, which in theory should make it unsusceptible to the mutations in mtDNA that are known to increase with age[5]. The CI–CII balance shift may represent compensation for a slight age-dependent decline in CI function that does not reach the threshold to influence overall metabolic or immune cell function.

There are several important limitations. The participants included are not representative of a healthy aging population and this could bias the results even though measures were taken to adjust for the influence of the patient cohorts. Another major limitation is the lack of data on several potential confounders, such as BMI, history of smoking, and exercise habits. While the age span of this study population is similar to the ones of many preceding studies of mitochondrial function and aging, the oldest participant was 86 years old, limiting the applicability of the results to mitochondrial function in advanced age (>80 years). Nonlinear relationships between aging and mitochondrial function were not examined; this decision was based on previous findings of linear relationships in other tissues.

In conclusion, this is, to date, the largest study on aging and mitochondrial respiration in human PBMCs and platelets. The results do not support a decline in respiratory function in peripheral blood cells during the normal human lifespan.

METHODS

Participants

The study includes samples from 308 participants collected between 2008 and 2016 at Skåne University Hospital, Sweden, and Rigshospitalet, Denmark. They comprise all individuals (both patients and healthy controls) sampled for blood cell respirometry in our laboratory during that period, for which data for both PBMCs and platelets exist. Part of the data has been used in previous publications that have not focused on age-related effects on mitochondrial respiration[24–30]. The dataset includes both healthy controls and patients. The latter group includes patients with neurodegenerative movement disorders and with sepsis (sampled at the time of admittance) and a group of pediatric patients with diffuse complaints seen at a pediatric A&E department. Patients with known primary mitochondrial disease were not included.

The included projects were subject to approval by the regional ethical review board of Lund University, Sweden (No 2008/113, 2009/97, 2011/89, and 2011/320) and the scientific ethical committee of Copenhagen County, Denmark (H-C-2008-023) and all participants, or guardian/parent or next of kin, as stipulated by the respective ethical permit, signed informed consent.

Sample preparation

Participants were sampled through a venous puncture or through an existing arterial line and peripheral blood drawn into K_2EDTA tubes. Samples were analyzed within 3–5 hours. Cell concentrations in whole blood and cell suspensions were measured using a Swelab Alfa automated hemocytometer (Swelab, Stockholm, Sweden).

Cell isolation was performed at room temperature. Erythrocytes and leukocytes were loosely pelleted by centrifugation at 300–400*g* for 10–15 min, leaving a platelet-rich plasma (PRP). The pellet was resuspended in saline, and lymphocytes were isolated using Lymphoprep (axis-shield PoC AS). The resuspended cells were layered on top of the Lymphoprep and centrifuged at 800*g* for 20–30 min. The resulting lymphocyte layer was pipetted off and resuspended in saline, and centrifuged at 250*g* for 5 min. After removing the supernatant, the lymphocyte pellet was resuspended in 100–200 µL saline. The final suspension contained 20–30% plasma. The lymphocyte suspension contained up to 30% granulocytes and midsize cells (monocytes, eosinophils, basophils, etc.).

The PRP (from the initial centrifugation) was collected and centrifuged for 5 minutes at 4 600*g*, producing close to cell-free plasma and a platelet pellet. The platelet pellet was resuspended in 1–3 mL of the participant's own plasma by gentle pipetting to obtain a highly enriched PRP.

High-resolution respirometry

Measurement of mitochondrial respiration was performed in an oxygraph (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria) at a constant temperature of 37 °C. Platelets were suspended at a concentration of 200×10^6/ml, except for six cases where there was insufficient sample (primarily for pediatric participants, conc. 19, 126, 152, 154, 190, and 190×10^6 platelets/mL, respectively). PBMCs were suspended at a concentration of $2.5–5 \times 10^6$/ml, as the sample amount allowed. Calibration with air-saturated Millipore water was performed daily. A mitochondrial respiration medium (MiR05) containing sucrose 110 mM, HEPES 20 mM, taurine 20 mM, K-lactobionate 60 mM, $MgCl_2$ 3 mM, KH_2PO_4 10 mM, EGTA 0.5 mM, BSA 1 g/l, pH 7.1 was used for all experiments. Oxygen solubility factors relative to pure water were set to 0.92, and stirrer speed to 200. Data were recorded in DatLab version 4.3 (Oroboros Instruments, Innsbruck, Austria).

A schematic description and a representative trace of a standard experiment are shown in Supplementary Fig. 1, and the respiratory parameters measured in or derived from the experiment are listed in Supplementary Tables 1 and 2.

Routine respiration was measured in intact platelets with endogenous substrates. Cells were permeabilized using digitonin (1 µg per 10^6 platelets or 6 µg per 10^6 PBMCs), and simultaneously, the N-pathway substrates (or CI substrates) malate and pyruvate

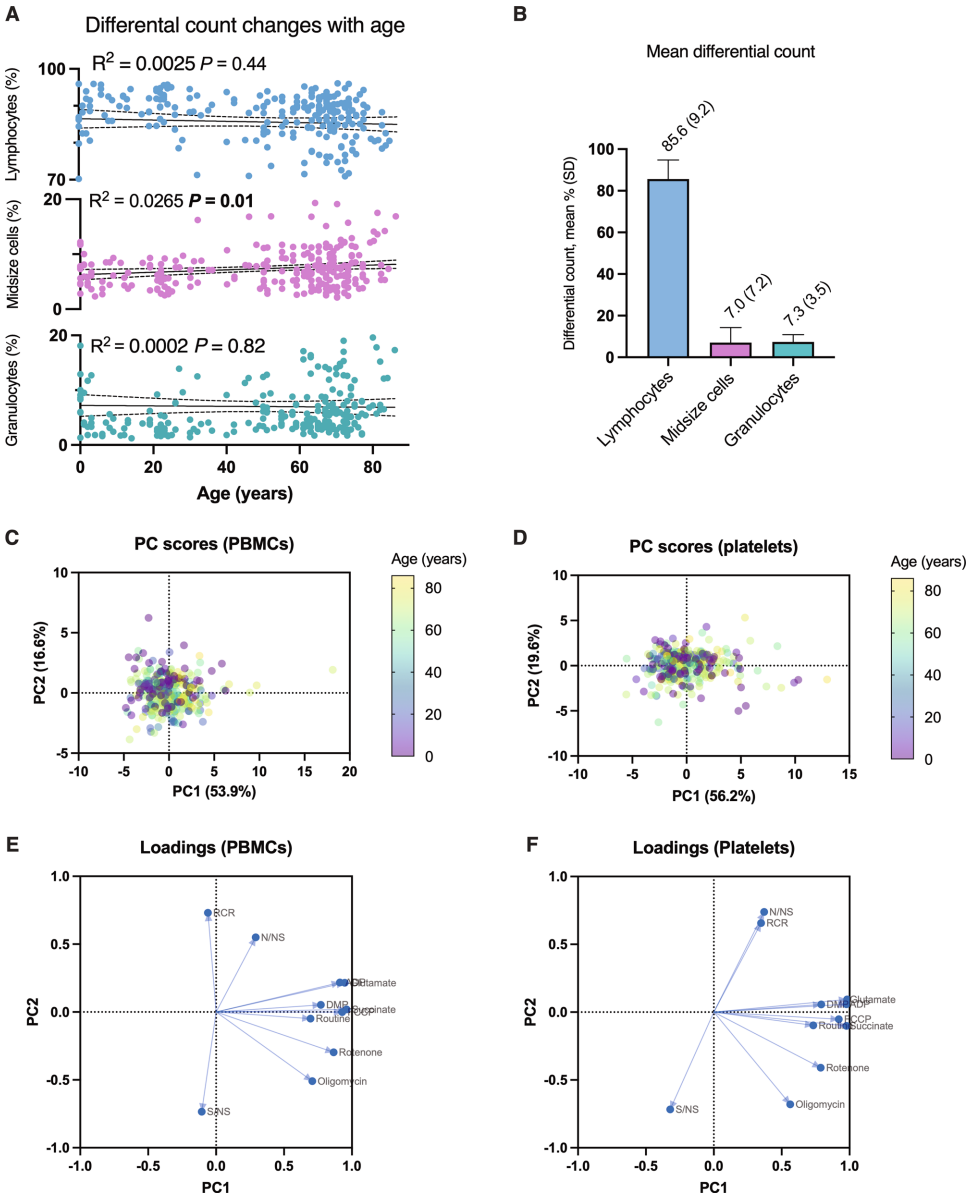


Fig. 3 Differential count and principal component analysis. Linear regression of differential count (%) depending on age (**A**) for lymphocytes, midsize cells, and granulocytes ($n = 262$; slopes in Supplementary Table 6). 12, 1, and 8 data points, respectively, were removed from the lymphocyte, midsize cell, and granulocyte plots to compress the figure and enhance legibility. Mean differential count (%) and standard deviation (SD) for the same sample (**B**). Principal component (PC) scores for all respiratory measurements and derived ratios (Supplementary Tables 1 and 2) for PBMCS (**C**) and platelets (**D**) labeled by age. Corresponding loading plots for all parameters, for PBMCs (**E**) and platelets (**F**) (table of loadings in Supplementary Table 5).

were added at saturating concentrations (5 mM each). Subsequently, adenosine diphosphate (ADP) was added (1 mM), followed by another N-pathway substrate, glutamate (5 mM). This was followed by S-pathway substrate (CII substrate) succinate (10 mM) to reach maximum phosphorylating capacity through both N- and S-pathways (both CI and CII). This respiration rate is labeled OXPHOS capacity (alternatively known as NS-OXPHOS capacity).

Next, the ATP synthase was inhibited by oligomycin (1 µg/ml), revealing LEAK respiration. This was followed by the titration of carbonyl cyanide-p-tri-fluoromethoxyphenylhydrazone (FCCP) to induce maximal capacity of the electron transport system (ETS) in the uncoupled state, labeled ET capacity (alternatively known as NS-ET capacity). This was followed by the inhibition of CI by rotenone (2 µM) to measure the S-pathway (CII) in the uncoupled state. After this, the CIII-inhibitor antimycin A (1 µg/ml) was added to halt electron transfer through the ETS altogether, and the residual oxygen consumption (Rox) measured at this point was subtracted from all other measurements before any further analyses were made.

The N/NS pathway control ratio (N/NS PCR) reflects the relative contribution of the N-pathway or CI-linked respiration. The S/NS-pathway control ratio (S/NS PCR) reflects the relative contribution of the S-pathway or CII-linked respiration. The RCR reflects the coupling efficiency of the ETS. In contrast to the other respiratory measurements, which were normalized to cell count, respiratory ratios are a way of normalizing results to mitochondrial content, providing qualitative indices of mitochondrial function. Supplementary Table 1 describes how each ratio was calculated.

Citrate synthase activity

After completion of the respirometry, the content of each oxygraph chamber (2 ml) was stored at −80 °C. A subset of the samples was later thawed for analysis of CS activity using a commercial kit (Citrate Synthase Assay Kit, CS0720, Sigma-Aldrich, St Louis, MO, USA). Before the enzyme assay, the thawed samples were sonicated on ice for 30 s for PBMCs, and 2 sets of 30 s for platelets (Ultrasonic homogenizer 4710 Series; Cole-Parmer Instrument Company LLC, Vernon Hills, IL). The assay was performed in accordance with the manufacturer's instructions, as previously described[29].

Statistical methods

All statistical analyses were performed, and all data figures were created using PRISM GraphPad version 9.1.2 (La Jolla, USA) unless otherwise specified. Four measurements of respiratory function and three ratios of respiratory rates were selected based on previous literature in order to cover relevant aspects of ETS function. Together with CS activity, a marker of mitochondrial content, there were eight parameters selected for the main analysis. These parameters were plotted against age in scatterplots with a straight line illustrating the slope of simple linear regression and dotted lines to illustrate its 95% confidence interval. Multiple regressions were performed for each parameter, adjusting the results for sex (coded as females = 0, males = 1) and health status (coded as healthy cohort = 0, disease cohort = 1).

Principal component analyses (PCA) were made separately for PBMCs and platelets using a correlation matrix and including all parameters used in the regression analysis (Supplementary Table 1), as well as additional parameters measured in the study protocol (Supplementary Table 2). Data were not transformed prior to ordination. The principal components were selected using parallel Monte Carlo analysis with 1000 simulations and a percentile level of 95%. The purpose of the PCA was to illustrate the overall effect of age on respiratory parameters available for selection and to analyze collinearity to control for unintended omission of important age-dependent parameters.

To further control for possible sources of error, a differential count was performed in the PBMC suspension after the lymphocyte isolation procedure. Linear regressions were made to see whether differential count varied across age. Mean cell contamination of platelets in the PBMC samples, and vice versa, was calculated in a subset of the samples.

For all regression analyses described above, a P-value < 0.05 was considered statistically significant at the first stage of interpretation. To control for multiple comparisons, a false discovery rate (FDR) analysis was performed using the adaptive linear step-up procedures with Q set to 5 on all 112 P-values from both the linear (Figs. 1 and 2; Supplementary Tables 3 and 8) and the multiple (Supplementary Tables 4, 5, and 8) regressions[42].

Reporting summary

Further information on research design is available in the Nature Research Reporting Summary linked to this article.

DATA AVAILABILITY

The data that support the findings of this study are available from the corresponding author, J.E., upon reasonable request.

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REFERENCES

1. Harman, D. Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298–300 (1956).
2. Zhang, Y. et al. Mice deficient in both Mn superoxide dismutase and glutathione peroxidase-1 have increased oxidative damage and a greater incidence of pathology but no reduction in longevity. *J. Gerontol. Ser. A* **64**, 1212–1220 (2009).
3. Van Raamsdonk, J. M. & Hekimi, S. Superoxide dismutase is dispensable for normal animal lifespan. *Proc. Natl Acad. Sci. USA* **109**, 5785–5790 (2012).
4. Sun, N., Youle, R. J. & Finkel, T. The mitochondrial basis of aging. *Mol. Cell* **61**, 654–666 (2016).
5. Bratic, A. & Larsson, N. G. The role of mitochondria in aging. *J. Clin. Investig.* **123**, 951–957 (2013).
6. López-Otín, C., Blasco, M. A., Partridge, L., Serrano, M. & Kroemer, G. The hallmarks of aging. *Cell* **153**, 1194–1217 (2013).
7. Trifunovic, A. et al. Premature ageing in mice expressing defective mitochondrial DNA polymerase. *Nature* **429**, 417–423 (2004).
8. Cooper, J. M., Mann, V. M. & Schapira, A. H. Analyses of mitochondrial respiratory chain function and mitochondrial DNA deletion in human skeletal muscle: effect of ageing. *J. Neurol. Sci.* **113**, 91–98 (1992).
9. Boffoli, D. et al. Decline with age of the respiratory chain activity in human skeletal muscle. *Biochim. Biophys. Acta* **1226**, 73–82 (1994).
10. Trounce, I., Byrne, E. & Marzuki, S. Decline in skeletal muscle mitochondrial respiratory chain function: possible factor in ageing. *Lancet* **1**, 637–639 (1989).
11. Short, K. R. et al. Decline in skeletal muscle mitochondrial function with aging in humans. *Proc. Natl Acad. Sci. USA* **102**, 5618–5623 (2005).
12. Picard, M. et al. Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. *Aging Cell* **9**, 1032–1046 (2010).
13. Rasmussen, U. F., Krustrup, P., Kjaer, M. & Rasmussen, H. N. Experimental evidence against the mitochondrial theory of aging. A study of isolated human skeletal muscle mitochondria. *Exp. Gerontol.* **38**, 877–886 (2003).
14. Johnson, M. L., Robinson, M. M. & Nair, K. S. Skeletal muscle aging and the mitochondrion. *Trends Endocrinol. Metab.* **24**, 247–256 (2013).
15. Yen, T. C., Chen, Y. S., King, K. L., Yeh, S. H. & Wei, Y. H. Liver mitochondrial respiratory functions decline with age. *Biochem. Biophys. Res. Commun.* **165**, 944–1003 (1989).
16. Ojaimi, J., Masters, C. L., Opeskin, K., McKelvie, P. & Byrne, E. Mitochondrial respiratory chain activity in the human brain as a function of age. *Mech. Ageing Dev.* **111**, 39–47 (1999).
17. Greaves, L. C. et al. Defects in multiple complexes of the respiratory chain are present in ageing human colonic crypts. *Exp. Gerontol.* **45**, 573–579 (2010).
18. D'Aurelio, M. et al. Decreased Pasteur effect in platelets of aged individuals. *Mech. Ageing Dev.* **122**, 823–833 (2001).

19. Alonso, M. et al. Blood cell respiration rates and mtDNA copy number: a promising tool for the diagnosis of mitochondrial disease. *Mitochondrion* **61**, 31–43 (2021).
20. Shi, C. et al. Effects of ageing and Alzheimer's disease on mitochondrial function of human platelets. *Exp. Gerontol.* **43**, 589–594 (2008).
21. Braganza, A. et al. Platelet bioenergetics correlate with muscle energetics and are altered in older adults. *JCI Insight* **5**, e128248 (2019).
22. Jedlička, J., Kunc, R. & Kuncová, J. Mitochondrial respiration of human platelets in young adult and advanced age—seahorse or O2k? *Physiol. Res.* **70**, S369–s379 (2021).
23. Chacko, B. K. et al. Mitochondria in precision medicine; linking bioenergetics and metabolomics in platelets. *Redox Biol.* **22**, 101165 (2019).
24. Sjövall, F. et al. Mitochondrial respiration in human viable platelets–methodology and influence of gender, age and storage. *Mitochondrion* **13**, 7–14 (2013).
25. Sjövall, F., Morota, S., Asander Frostner, E., Hansson, M. J. & Elmér, E. Cytokine and nitric oxide levels in patients with sepsis–temporal evolvement and relation to platelet mitochondrial respiratory function. *PloS ONE* **9**, e97673 (2014).
26. Sjövall, F. et al. Temporal increase of platelet mitochondrial respiration is negatively associated with clinical outcome in patients with sepsis. *Crit. Care* **14**, R214 (2010).
27. Sjövall, F., Morota, S., Persson, J., Hansson, M. J. & Elmér, E. Patients with sepsis exhibit increased mitochondrial respiratory capacity in peripheral blood immune cells. *Crit. Care* **17**, R152 (2013).
28. Westerlund, E. et al. Oxygen consumption in platelets as an adjunct diagnostic method for pediatric mitochondrial disease. *Pediatr. Res.* **83**, 455–465 (2018).
29. Ehinger, J. K., Morota, S., Hansson, M. J., Paul, G. & Elmér, E. Mitochondrial dysfunction in blood cells from amyotrophic lateral sclerosis patients. *J. Neurol.* **262**, 1493–1503 (2015).
30. Ehinger, J. K., Morota, S., Hansson, M. J., Paul, G. & Elmér, E. Mitochondrial respiratory function in peripheral blood cells from Huntington's disease patients. *Mov. Disord. Clin. Pract.* **3**, 472–482 (2016).
31. Junker, A. et al. Human studies of mitochondrial biology demonstrate an overall lack of binary sex differences: a multivariate meta-analysis. *FASEB J.* **36**, e22146 (2022).
32. Kent-Braun, J. A. & Ng, A. V. Skeletal muscle oxidative capacity in young and older women and men. *J. Appl. Physiol.* **89**, 1072–1078 (2000).
33. Hütter, E. et al. Oxidative stress and mitochondrial impairment can be separated from lipofuscin accumulation in aged human skeletal muscle. *Aging Cell* **6**, 245–256 (2007).
34. Jedlička, J. et al. Impact of aging on mitochondrial respiration in various organs. *Physiol. Res.* **71**, S227–s236 (2022).
35. Larsen, S. et al. Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J. Physiol.* **590**, 3349–3360 (2012).
36. Valiathan, R., Ashman, M. & Asthana, D. Effects of ageing on the immune system: infants to elderly. *Scand. J. Immunol.* **83**, 255–266 (2016).
37. Morrison, S. J., Wandycz, A. M., Akashi, K., Globerson, A. & Weissman, I. L. The aging of hematopoietic stem cells. *Nat. Med.* **2**, 1011–1016 (1996).
38. Bufalino, M. R., DeVeale, B. & van der Kooy, D. The asymmetric segregation of damaged proteins is stem cell-type dependent. *J. Cell Biol.* **201**, 523–530 (2013).
39. Soerens, A. G. et al. Functional T cells are capable of supernumerary cell division and longevity. *Nature* **614**, 762–766 (2023).
40. Sadighi Akha, A. A. Aging and the immune system: an overview. *J. Immunol. Methods* **463**, 21–26 (2018).
41. Davizon-Castillo, P. et al. TNF-α-driven inflammation and mitochondrial dysfunction define the platelet hyperreactivity of aging. *Blood* **134**, 727–740 (2019).
42. Benjamini, Y., Krieger, A. M. & Yekutieli, D. Adaptive linear step-up procedures that control the false discovery rate. *Biometrika* **93**, 491–507 (2006).

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AUTHOR CONTRIBUTIONS

J.E., E.E., F.S., and G.P.V. conceived the study. M.K., J.E., E.Å.F., F.S., and E.E. developed methods and performed the experiments. J.E., E.W., and E.E. designed the analyses and interpreted the results. J.E. and E.W. wrote the paper and made the figures with critical feedback from the remaining authors. J.E. and E.W. contributed equally.

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COMPETING INTERESTS

J.E., E.Å.F., M.K., and E.E. have equity interests in and/or have received salary support from Abliva AB (formerly NeuroVive Pharmaceutical AB), a public company developing pharmaceuticals in the field of mitochondrial medicine. The remaining authors declare no competing interests.

ADDITIONAL INFORMATION

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Paper IV

Appendix

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Clinical tests of metabolic dysfunction

High-resolution respirometry in blood cells is a relatively noninvasive method for assessing mitochondrial function. This thesis examines its potential for diagnosing primary mitochondrial disease, which is sometimes complicated.

Elevated lactate in blood is an unspecific sign of metabolic dysfunction and cellular stress. This thesis examines its use in the assessment of mitochondrial disease, and of various other conditions in the emergency department.

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