Mitochondrial dysfunction and metabolic intervention

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Abstract

This thesis comprises projects related to the role of mitochondria in disease: methods to evaluate mitochondrial function in patient samples, a drug-development project targeting mitochondria, and development of a large animal model of metabolic decompensation due to mitochondrial dysfunction.

In paper I, mitochondrial respiration related to respiratory chain complex IV was found to be decreased in blood cells from patients with amyotrophic lateral sclerosis, with a possible compensatory increase in cellular mitochondrial content. In paper II, the same methodology was applied to patients with Huntington's disease, with a finding of decreased respiration related to respiratory chain complex I. The results from the studies, blood cell respirometry in general, and the validity of findings in blood cells in neurodegenerative disease patients are discussed in this thesis.

In paper III, we showed how newly developed cell-permeable prodrugs of succinate can be used to improve mitochondrial function in cells and tissues with complex I dysfunction. Mitochondrial respiration and membrane potential were increased, and lactate production reduced when cells with rotenone-induced complex I dysfunction were treated with prodrugs of succinate. The respiratory function of fibroblasts from a Leigh syndrome patient was improved. The role of succinate in physiology, and the potentials and limitations of succinate prodrugs as therapy for mitochondrial disease are discussed.

In paper IV we described a new pig model of rotenone-induced metabolic decompensation. Pigs infused with rotenone developed acute energy crisis with lactic acidosis and reduced tissue oxygen extraction. We proposed that this model may be suitable for evaluation of therapeutic intervention for acute mitochondrial disease.

In summary, this thesis highlights the important role of mitochondria in health and disease, and suggests that metabolism is an emerging paradigm in disease understanding and drug development.

Key words
mitochondria, mitochondrial disease, inborn errors of metabolism, neurodegenerative disease, amyotrophic lateral sclerosis, Huntington's disease, animal models, succinate

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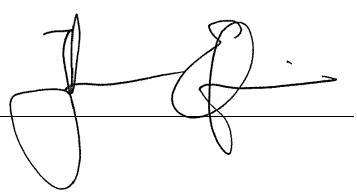
Signature  Date 2017/04/19

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To my family

Content

Original articles

This thesis comprises the following original articles, referred to in the text by their respective roman numerals (I–IV):

I. JK Ehinger, S Morota, MJ Hansson, G Paul, E Elmér. Mitochondrial dysfunction in blood cells from amyotrophic lateral sclerosis patients, *Journal of Neurology* 2015;262:1493–1503

II. JK Ehinger*, S Morota*, MJ Hansson, G Paul, E Elmér. Mitochondrial respiratory function in peripheral blood cells from Huntington's disease patients, *Movement Disorders Clinical Practice* 2016;3:472–482

*contributed equally

III. JK Ehinger, S Piel, R Ford, M Karlsson, F Sjövall, E Åsander-Frostner, S Morota, RW Taylor, DM Turnbull, C Cornell, C Metzsch, MJ Hansson, H Fliri, E Elmér. Cell-permeable succinate prodrugs bypass mitochondrial complex I deficiency, *Nature Communications* 2016;7:12317

IV. M Karlsson*, JK Ehinger*, S Piel, F Sjövall, J Henriksnäs, U Höglund, MJ Hansson, E Elmér. Energy decompensation due to acute mitochondrial complex I dysfunction – an *in vivo* large animal model, *Mitochondrion* 2016;31:56–62

*contributed equally

List of abbreviations

AD	Alzheimer's disease
ADP	adenosine diphosphate
ALA	α-lipoic acid
ATP	adenosine triphosphate
CI	complex I
CII	complex II
CIII	complex III
CIV	complex IV
CV	complex V
CS	citrate synthase
CoQ_{10}	coenzyme Q_{10}
DCA	dichloroacetate
DMF	dimethyl fumarate
FAD	flavin adenine dinucleotide
GDP	guanosine diphosphate
GSH	glutathione
GTP	guanosine triphosphate
HD	Huntington's disease
HIF-1α	hypoxia-inducing factor 1α
IMM	inner mitochondrial membrane
IPMDS	international paediatric mitochondrial disease scale
KO	knock-out
LHON	Leber's hereditary optic neuropathy
MELAS	mitochondrial encephalo-myopathy, lactic acidosis and strokelike episodes
MMF	monomethyl fumarate
MMP	mitochondrial membrane potential
MS	multiple sclerosis
mtDNA	mitochondrial DNA
NAC	*N*-acetylcysteine
NAD	nicotinamide adenine dinucleotide
nDNA	nuclear DNA
NO	nitric oxide
NPMDS	Newcastle paediatric mitochondrial disease scale
OAA	oxaloacetate
PBMC	peripheral blood mononuclear cells
PD	Parkinson's disease
PDH	pyruvate dehydrogenase
PEP	phosphoenolpyruvate
PEPCK	phosphoenolpyruvate carboxinase
PET	positron emission tomography
RC	respiratory chain
RET	reverse electron transfer
ROS	reactive oxygen species
SDH	succinate dehydrogenase
TBI	traumatic brain injury
TCA	tricarboxylic acid
VHL	von Hippel-Lindau

Summary

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In paper I, mitochondrial respiration related to respiratory chain complex IV was found to be decreased in blood cells from patients with amyotrophic lateral sclerosis, with a possible compensatory increase in cellular mitochondrial content. In paper II, the same methodology was applied to patients with Huntington's disease, with a finding of decreased respiration related to respiratory chain complex I. The results from the studies, blood cell respirometry in general, and the validity of findings in blood cells in neurodegenerative disease patients are discussed in this thesis.

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Svensk sammanfattning

Den näring som människor får i sig via maten behöver omvandlas till en form av energi som kroppens celler kan hantera, i huvudsak molekylen ATP. ATP kan beskrivas som kroppens energivaluta och merparten av allt ATP produceras i mitokondrierna. Mitokondriernas energiproduktion är mycket effektiv och man tror att mitokondrierna är så viktiga för cellers funktion att de var en av de evolutionära förutsättningarna för att avancerat liv skulle uppstå.

En central del av mitokondriernas energiproduktion är den serie av kemiska reaktioner som kallas citronsyracykeln. Nedbrytningsprodukter från näringsämnen går in i citronsyracykeln i mitokondrien och därifrån skickas sedan elektroner med högt energivärde till en serie proteiner i mitokondriens innermembran, andningskedjan. De olika delarna av andningskedjan brukar benämnas komplex I till V. Energin i elektronerna används av komplex I, III och IV för att pumpa protoner över mitokondriens innermembran, ut ur mitokondrien, och därmed bygga upp en elektrokemisk gradient. Protonerna vill ta sig tillbaka in i mitokondrien, ungefär som vatten i en kraftverksdamm vill röra sig nedåt med gravitationen. På samma sätt som ett kraftverk utnyttjar vattnets benägenhet att röra sig nedåt till att producera elektricitet i en turbin så använder mitokondrien den elektrokemiska gradienten till att producera ATP i komplex V i andningskedjan. När protonerna passerar komplex V och tar sig igenom mitokondriens innermembran, in till mitokondriens insida så används den frigjorda energin för att skapa ATP.

Om mitokondrierna inte fungerar som de ska kan det ge upphov till sjukdom. Det finns sjukdomar vars direkta orsak är att mitokondrierna inte fungerar fullt ut, men man tror att bristande funktion i mitokondrierna är en del av sjukdomsprocessen i andra sjukdomar, såsom blodförgiftning, traumatisk hjärnskada och neurodegenerativa sjukdomar. I artikel I och II i denna avhandling så har vi tagit blodprover från patienter med de neurodegenerativa sjukdomarna amyotrofisk lateralskleros (ALS) och Huntingtons sjukdom och undersökt den mitokondriella funktionen i blodcellerna med metoden högupplöst respirometri. Respirometri innebär mätning av syrgaskonsumtion, och i och med att mitokondrierna använder syrgas när de producerar energi kan detta ge en bild av mitokondriernas funktion.

Vi har visat att i blodceller från ALS-patienter är funktionen av komplex IV i andningskedjan nedsatt jämfört med friska personer i samma ålder, något vi kunde se

både i blodplättar och i vita blodkroppar. I blodplättar från patienter fanns det mer mitokondrier än i kontrollproverna, något som skulle kunna vara en kompensationsmekanism för försämrad mitokondriell funktion. Vi såg också att funktionen i komplex I var sämre hos svårt sjuka patienter än hos patienter som befann sig tidigare i sjukdomsförloppet. När vi genomförde en liknande analys i patienter med Huntingtons sjukdom så såg vi tecken till nedsatt komplex I-funktion i blodproverna, och mitokondriernas maximala kapacitet var sänkt.

Resultaten är intressanta då cellerna i blodet inte är den del av kroppen varifrån man får symptom vid ALS eller Huntingtons sjukdom. Att mitokondrierna i blodet är påverkade kan tyda på att sjukdomarna inte är begränsade till nervceller, utan att hela kroppen är sjuk, men att sjukdomarna primärt ger symptom från nervsystemet. Samtidigt är detta en begränsning med studierna, vi vet inte om fynden i blodceller verkligen är relevanta för vad som händer i nervsystemet på dessa patienter.

I artikel III i denna avhandling presenterar vi resultaten från ett projekt där vi utvecklat en ny klass av potentiella läkemedel för mitokondriell sjukdom. Citronsyracykeln levererar i huvudsak elektroner till komplex I i andningskedjan, men metabolismen av ämnet succinat (bärnstenssyra) ger elektroner direkt till komplex II. Vi upptäckte att när vi gav succinat till celler från ett barn med mitokondriell sjukdom på grund av fel i komplex I, så kunde mitokondrierna producera energi igen. Problemet var att vi var tvungna att öka genomsläppligheten i cellmembranen för att succinat skulle komma in i cellerna. Vi bestämde oss för att utveckla prodroger till succinat, ämnen som kan ta sig igenom cellmembran och väl inne i cellen frisätta succinat. I artikeln visar vi att var och en av tre nyutvecklade prodroger kan förbättra mitokondriefunktionen i intakta celler med bristande funktion i komplex I, bland annat i fibroblaster från en patient med Leighs syndrom, en allvarlig barnsjukdom som beror på bristande mitokondriell funktion.

Innan ett läkemedel under utveckling för första gången provas på människor måste det ha visat effekt i djurmodeller av den sjukdom som läkemedlet är avsett för. Det finns ett relativt litet antal modeller för mitokondriella sjukdomar, i huvudsak musmodeller. I artikel IV i denna avhandling beskriver vi utvecklingen av en ny djurmodell för akut energikris på grund av bristande funktion i komplex I. Vi valde gris som modelldjur då det är ett av de djur vars anatomi och fysiologi mest påminner om människans. Vi försämrade mitokondriernas funktion genom att behandla djuren med rotenon, ett ämne som minskar komplex I-funktionen. Det resulterade i minskad konsumtion av syrgas i djuren och en ökning av laktat i blodet, tecken på sänkt funktion i mitokondrierna.

Denna avhandling beskriver flera forskningsprojekt som relaterar till mitokondrier, dels metoder för att undersöka mitokondriell funktion i patientprover, dels läkemedelsutveckling riktad mot att förbättra mitokondriernas funktion och dels utveckling av nya djurmodeller som beskriver mitokondriell sjukdom. Mitokondriernas roll i olika sjukdomstillstånd får alltmer uppmärksamhet och det första läkemedlet mot en mitokondriell sjukdom har registrerarats i EU (mot Lebers hereditära optiska neuropati,

en ovanlig ögonsjukdom). Fältet mitokondriell medicin är ännu i sin linda och jag tror att vi i framtiden kommer att få se fler läkemedel som syftar till att förbättra mitokondriell funktion, såväl för ovanliga primära mitokondriesjukdomar som för vanligare sjukdomar där mitokondriens funktion spelar en roll. Vi kan vara i början av ett metabolt paradigm.

Introduction

A metabolic paradigm

Since the advent of modern medicine, the function of the human body and the root to disease have been described anatomically, with an organ-specific focus. This paradigm has advanced our understanding of physiology and pathophysiology, and introduced pharmaceuticals for a plethora of medical conditions. Drugs are traditionally developed by identifying a macromolecular target in a physiological process of interest in a specific organ; a ligand for a receptor, an inhibitor of an enzyme or a modulator of the function of an ion channel[1]. A compound that specifically interacts with the chosen targets in the desired way is identified, and the compound is brought through the many phases of drug development and clinical testing toward the goal of benefitting patients. It is increasingly acknowledged that this reductionist view on biological processes fails to consider the multifactorial background to most diseases, and many drugs fail during clinical testing due to unforeseen effect outside the target process or organ of interest. Various systems biology approaches are being employed to advance medicine beyond organ-centered and reductionist biology, to a more integrated investigation of the biological system as a whole[1].

The presence of mitochondria is a signature feature of the eukaryotic cell, to the extent that development of mitochondrial metabolism is suggested as the key evolutionary step required for complex life to emerge[2]. Mitochondria were probably formed through endosymbiosis between two prokaryotes, an archaeon and an α-protobacteria, leading to an energetic advantage as the internal presence of an expandable biomembrane surface allowed for far more efficient energy conversion and subsequently increasing gene expression[2].

Mitochondria are at the intersection of most energy-producing pathways in the cell, and are involved also in intracellular signaling, Ca^{2+} homeostasis and the regulation of cell death pathways. Mitochondria are present in all tissues in the human body, with the notable exception of erythrocytes. Given the key role of mitochondria in the evolution of advanced life forms, and the critical importance of cellular energy for normal function of cells, tissues and organs, it is reasonable to believe that mitochondrial metabolism is a decisive factor for sustained health.

In the 1920's, the German physicist, biochemist and Nobel prize laureate Otto Warburg described how cancer cells are prone to anaerobic glycolysis rather than aerobic oxidation of substrates by mitochondria, a phenomenon since termed "the Warburg effect". Even though it has been shown that this is not uniformly true for all tumors, the fact that metabolism in cancer tissue is different from healthy tissue is utilized for diagnostic purposes, such as through positron emission tomography (PET), and is being suggested as an avenue for intervention[3]. Mitochondrial function and metabolism are not only of interest for cancer research. It has been suggested that many age-related diseases (such as neurodegenerative disorders) and life style diseases (such as diabetes) that burden society are at least partly due to factors relating to mitochondrial function. If this holds true, it opens new avenues for intervention, not directly focusing on macromolecular targets in the primary organs affected, but rather targeting the signature metabolic processes of the diseases[4]. Thus, we may be at the rise of a metabolic paradigm[5].

An introduction to mitochondrial physiology

The product of two genomes

The mitochondria comprise building blocks and functional elements from two sets of DNA, the nuclear genome (nDNA) and the distinct mitochondrial genome (mtDNA) harbored within the mitochondrial matrix. The mtDNA is ring shaped (a residue of the mitochondria's prokaryotic origin) and encodes only 37 genes, the 12S and 16S mitochondrial ribosomes, 22 tRNAs required for mitochondrial protein synthesis, and 13 subunits of the protein complexes in the mitochondrial respiratory chain (RC). The remaining 1500 or so proteins required for functional mitochondria are nDNA-derived and imported to the mitochondria. The mitochondrial genome is almost exclusively maternally inherited, and even though spermatozoa do have mitochondria, only the mtDNA of the oocyte is passed to the resulting offspring[5].

The power plant of the cell

Mitochondria convert the food nutrients we consume into chemical energy, primarily in the form of adenosine triphosphate (ATP), reducing molecular oxygen to water in the process. Most energy substrates are processed through glycolysis (carbohydrates), β-oxidation (fatty acids) or deamination (proteins) before entering the set of reactions termed the tricarboxylic acid (TCA) cycle, which takes place in the mitochondrial matrix. The TCA cycle generates reduced nicotinamide adenine dinucleotide (NADH) which in

turn donates two electrons to the first protein complex of the mitochondrial RC, complex I (CI; NADH dehydrogenase).

The mitochondrial RC comprises four protein complexes (complex I through IV) that use the energy from electrons from the TCA cycle to pump proton over the inner mitochondrial membrane (IMM), thus creating an electrochemical gradient over the IMM. The electrons are ultimately used to reduced oxygen to water at complex IV (CIV; cytochrome c oxidase). The electrochemical gradient is utilized by RC complex V (CV; the ATP-synthase) to phosphorylate adenosine diphosphate (ADP) into ATP.

The tricarboxylic acid cycle: fueling the mitochondria

Mitochondrial metabolism centers around a set of chemical reactions in the mitochondrial matrix, the TCA cycle[6], that supplies reducing equivalents, or electron, to the RC. The cycle can be understood by tracking the carbon atoms that form the skeletons of the acids in the cycle.

A series of oxidative reactions

Citrate has six carbons (6C) whereof three form carboxyl-groups (hence the name tricarboxylic acid cycle). Citrate is formed when two carbons from acetyl-CoA (2C) are added to the four-carbon oxaloacetate (OAA) (4C), the end-product of the TCA cycle. The continuous influx of carbons in form of acetyl-CoA constitutes the fuel for the TCA cycle to continue spinning. The main sources of carbons for the TCA cycle are (i) the glycolysis, which converts glucose in the cytosol to pyruvate (which in turn is converted to acetyl-CoA by pyruvate dehydrogenase, PDH)[7], (ii) the β-oxidation, which degrades fatty acids to two-carbon segments that form acetyl-CoA[8], and (iii) the catabolism of certain amino acids[9]. Citrate (6C) undergoes confirmation changes via aconitase (6C) to isocitrate (6C) whereafter one carbon is lost as CO_2, one molecule of NAD^+ is reduced to NADH, and α-ketoglutarate (5C) is formed. Another carbon is then lost as CO_2 and another molecule of NADH is formed as α-ketoglutarate (5C) is converted to succinyl-CoA (4C). Succinyl-CoA loses its coenzyme and forms succinate (4C) and a molecule of ATP or guanosine triphosphate (GTP, energetically equivalent to ATP) is formed. Succinate, in turn, is oxidized by the flavin adenine dinucleotide (FAD) part of succinate dehydrogenase (SDH), complex II (CII) of the mitochondrial RC, thereby donating electrons to the RC[10]. Succinate turns into fumarate (4C) in the process, then onward to malate (4C) and ultimately OAA. This last conversion also generates a molecule of NADH. Thus, the six carbons in citrate have stepwise been reduced to four in OAA, the two lost ones forming the CO_2 that we exhale, and 3 NADH have been generated. Additionally, one molecule of ADP or guanosine diphosphate (GDP) has been phosphorylated into ATP or GTP, and succinate has reduced FAD of CII in the RC to $FADH_2$.

Anaplerosis: replenishing the tricarboxylic acid cycle

The TCA cycle does not only generate electrons for the RC, its intermediates also serve important functions as biosynthetic precursors of other metabolic compounds such as amino acids, nucleotides and fatty acids[9]. Intermediates are not only extracted from the TCA cycle, they are also replenished by various reactions, termed anaplerosis. A key anaplerotic reaction is catalyzed by pyruvate carboxylase, in which pyruvate is converted into OAA by the addition of a carboxylic group, consuming one molecule of ATP in the process[11]. OAA can then be imported into the mitochondria as malate through the malate–aspartate shuttle.

The malate–aspartate shuttle: importing energy from the outside

OAA, α-ketoglutarate and malate, along with the amino acids glutamate and aspartate, partake in the set of reactions known as the malate–aspartate shuttle. The malate–aspartate shuttle is necessary to fully use all energy produced by the glycolysis. The shuttle translocates reducing equivalents (NADH) generated in the cytosol by the glycolysis to the mitochondrial matrix, where they can be utilized for energy production by the RC[12]. OAA is transported out of the mitochondrial matrix into the cytosol, and is there converted to malate through the reverse action of malate dehydrogenase[13], accepting electrons from cytosolic NADH in the process. Malate is then transported back into the mitochondrial matrix. Malate constitutes the step before OAA in the TCA cycle and when malate is converted to OAA in the mitochondrial matrix a molecule of NADH is generated. Now the reducing power of the cytosolic NADH has been moved into the mitochondria and made accessible to the RC. In short, malate carries the reducing power of NADH into the mitochondria as IMM is impermeable to NADH.

Malate is transferred over the IMM by the malate–α-ketoglutarate antiporter[14], and for each molecule of malate that enters the mitochondrial matrix, one molecule of α-ketoglutarate goes in the opposite direction. For the shuttle to keep going, OAA, the receiver of the electrons from NADH, must be present in the cytosol. OAA can be acquired through the deamination of aspartate by aspartate aminotransferase, and when this takes place in the cytosol, the amino group is transferred to the α-ketoglutarate that was extruded from the mitochondria, forming glutamate. This glutamate can then enter the mitochondria again through the action of the glutamate–aspartate antiporter which as the name implies simultaneously extrudes aspartate for the formation of OAA. The reverse reactions occur in the mitochondrial matrix, as aspartate aminotransferase transfer an amino group from glutamate to OAA, forming aspartate and α-ketoglutarate, thus balancing the reaction.

Primary mitochondrial disease

The definition of the term "mitochondrial disease" is not clear, and it could be argued that mitochondrial disease may be any disease in which the mitochondria do not function normally and this contribute or lead to a pathophysiological state. As mitochondrial dysfunction has been shown in numerous conditions with few similarities between them, such as sepsis[15-17], traumatic brain injury (TBI)[18-20] and presbyacusis[21], such a definition would however be of little practical use. More commonly, mitochondrial disease is thought of as conditions where dysfunction of the mitochondria is the pathological driver or and not an epiphenomenon.

The difficulties of diagnosing mitochondrial disease

There are no uniformly accepted diagnostic algorithms for mitochondrial disease, but different sets of diagnostic criteria have been introduced for adults and children respectively[22-24]. Primary mitochondrial disease may be caused by mutations in nDNA or mtDNA, either inherited or acquired, and as mitochondria are sensitive to a multitude of toxins, likely also by environmental factors. A definite diagnosis can in most patients be confirmed through sequencing of the nuclear and mitochondrial genome, but a significant part of the patients remains without an identified genetic cause of their disease, and are diagnosed through the combined results of an investigation including enzymatic, functional and histologic analysis of tissue samples, as well as various biomarkers.

The organs that need energy the most will fail first

Patients with primary mitochondrial disease normally exhibit symptoms from organs requiring lots of energy to function, such as skeletal muscles, the heart, the brain and specialized sensory organs such as the retina and the cochlea. Severity of disease varies from discrete symptoms to lethal disorders that frequently result in childhood death, such as Leigh syndrome[25]. A near total lack of mitochondrial function is hardly compatible with extra-uterine life, and newborns with severely reduced mitochondrial dysfunction rarely survive the neonatal period. Patients may present with very diverse symptoms, such as failure to thrive, fatigue, cardiomyopathy, seizures, stroke-like episodes, encephalopathy, vision impairment or sensorineural hearing loss. Otherwise unexplained symptoms from several different organ systems should make the investigating physician consider mitochondrial disease[26]. Many patients do not show signs of disease until burdened with an additional stressor, such as an infection. As the patient lacks the ability to energetically compensate for the additional stress, a metabolic decompensation ensues. Patients with mitochondrial disease typically decease due to intercurrent illness, sepsis being the most common cause followed by pneumonia[25], as the metabolic system is unable to handle additional inflammatory stress.

Neurodegenerative disease: the role of mitochondria

Gradual decline in mitochondrial function has been proposed as the explanation for aging, as efficiency of the mitochondrial RC declines with accumulating somatic mutations in the mtDNA, due to errors during mtDNA replication[27, 28]. In parallel, mitochondrial dysfunction is part of the pathogenesis of common age-related neurological diseases, such as Parkinson's disease (PD) and Alzheimer's disease (AD), and also amyotrophic lateral sclerosis (ALS)[29-32]. Whether mitochondrial dysfunction is the cause or a result of these diseases is debated. For almost all forms of familial PD, the identified gene is directly related to mitochondrial function[33]. In AD, one potential model depicts mitochondrial dysfunction as the trigger for amyloid β plaque and neurofibrillary tangle formation in the brain[32]. In addition, in Huntington's disease (HD), where a well-defined genetic cause not directly related to mitochondria is known, mitochondrial dysfunction is a persistent pathogenic response[34-40]. Mutant huntingtin directly interacts with mitochondrial membranes and receptors and negatively affects mitochondrial trafficking, integrity and calcium handling, and reduces ATP concentration in synapses[33].

Age is the primary risk factor for most neurodegenerative diseases, and the accumulating mutations in mtDNA[28] and decreased capacity of the RC that come with higher age[28, 41] may constitute a gradually lowered threshold for disease. With decreasing mitochondrial function, the ability for cells to cope with and compensate for other pathogenic processes

is reduced and hence, even if the mitochondria are not the culprits in neurodegenerative disease, they may be the decisive factor for when disease ensues.

Complex I: the front door to the respiratory chain

CI of the mitochondrial RC (NADH dehydrogenase) is an L-shaped multisubunit protein complex assembled in the mitochondrial matrix and embedded in the IMM. CI is the main entry point, the front door, for the reducing equivalent NADH, which is generated from the metabolism of most substrates in the TCA cycle. CI comprises polypeptides both of mtDNA (7 subunits) and nDNA origin (38 subunits) and is the largest protein complex of the RC (approximately 1 MDa)[42, 43].

Dysfunction in CI is the most common signature in primary mitochondrial disease, either due to mutations in nDNA or mtDNA affecting CI assembly or function[25, 44, 45], and CI is the most commonly implicated RC complex in the pathogenesis of neurodegeneration, first described in 1989 for PD[46-48]. As CI catalyzes the shuttling of two electrons from NADH onward to ubiquinone (the oxidized state of coenzyme Q_{10}, CoQ_{10}) and reducing it to ubiqunol, the energy released is used to relocate 4 protons over the IMM, building up the electrochemical gradient used by CV to produce ATP[49]. As the majority of reducing equivalents generated from the glycolysis and the TCA cycle use CI as the entry point to the RC, it is a key protein complex for normal mitochondrial function.

Modeling complex I-disease: shutting the front door

Dysfunction of CI is involved in many pathologic processes and there exist numerous animal models mimicking various human diseases by compromising CI with toxins or genetic alterations in either the nuclear or the mitochondrial genome. This section touches upon a few models highlighting different aspects of mitochondrial pathophysiology relevant to the current work.

Phenotypes mimicking PD can be induced by toxins inhibiting mitochondria. Due to epidemiologic indications that PD may have a link to pesticide exposure, rats were exposed to the toxin rotenone (used commercially as an insecticide and to eradicate fish). When exposed to 2-3 mg/kg bodyweight of intravenous rotenone, rats developed motor symptoms and postural traits characteristic for PD such as hunched posture, hypokinesia and rigidity, and pathologic examination revealed dopaminergic nigrostriatal lesions[50]. The model has since been developed further and has been widely utilized for PD research[51]. Other neurotoxins compromising CI have also been utilized to produce PD-like symptoms in animal models[52].

Knock-out (KO) in mice of the nuclear encoded CI subunit NDUFS4 results in a phenotype that displays several similarities with human Leigh syndrome, with symptoms appearing at about 5 weeks of age. The mice develop signs of encephalomyopathy with ataxia and lethargy, reduced growth rate and loss of motor skills. This is due to failure of CI to assemble properly, resulting in reduced mitochondrial function shown by decreased enzymatic CI activity, tissue oxygen consumption, and increased serum lactate[53]. There exist several different versions of NDUFS4 KO mice with conditional or tissue-specific KO[54].

Not specific to CI, a mouse model known as the "mutator mouse" merits attention due to its importance for the current theories linking aging to mitochondrial senescence[28]. The mouse, described in 2004, has a defect mtDNA polymerase POLG, resulting in lack of proofreading of the replicating mitochondrial genome. The model accumulates mtDNA mutations and the resulting phenotype exhibits signs of accelerated aging with weight loss, hearing loss, grey hairs and reduced fertility, and have a shortened life span compared to wild type litter mates[55]. Data from this mouse model constitute a strong link between accumulating mtDNA mutations and aging, but there is controversy on the subject[56].

Treating primary mitochondrial disease

Current and putative treatments for primary mitochondrial disease

Coenzyme Q_{10} and its derivatives

There is a paucity of high-quality scientific evidence regarding pharmacological treatment of mitochondrial disease. Only one drug is approved in the EU for mitochondrial disease, idebenone for Leber's hereditary optic neuropathy (LHON), a disease leading to progressive loss of visual acuity primarily in young males. Idebenone can be considered a water-soluble analogue of the mitochondrial CoQ_{10}, the site in the RC where the pathways from CI and CII merge. Its beneficial effects are considered to be due to its antioxidant capacities, but also due to its ability to act as an electron carrier in the RC, accepting electrons from CI, CII and other electron donors, and donating them to complex III (CIII; CoQ–cytochrome c reductase). This may bypass CI, which is dysfunctional in LHON, improving ATP production and viability in the retinal ganglion cells that are inactive but viable, and thereby reducing symptoms[57].

CoQ_{10} and analogues thereof have long been a research focus when investigating treatment for primary mitochondrial disease, with similar rationale as for ideboneone in LHON. CoQ_{10} has been evaluated in a number of clinical trials of variable quality but no clinically relevant effects have been reported[58, 59]. CoQ_{10} has also been evaluated in

combination with creatine and α-lipoic acid (ALA) without any convincing evidence of efficacy[60]. Although there is no formal evidence for its use, there is relative consensus among experts in the field that CoQ_{10}-treatment should be attempted in most patients with primary mitochondrial disease[61, 62].

Another related compound, the para-benzoquinone EPI-743 is under development for mitochondrial disease, and initial clinical trials for Leigh syndrome indicated positive functional outcomes, but results are from open-label, non-randomized studies using historic data as control, which makes the level of scientific evidence provided low[63, 64].

Mitochondrial cocktail

In current practice, a plethora of food supplements is being employed in various combinations by physicians treating mitochondrial disease. They are usually available off-label as food supplements, and self-medication is common. There are but anecdotal reports of efficacy for most. A consensus document from the Mitochondrial medicine society[65] recommends that CoQ_{10}, riboflavin and ALA are given to most patients with mitochondrial disease, while treatment with B-vitamins, L-arginine, L-carnitine, vitamins C and E, and folinic acid should be considered independently in each case. ALA is given due to its antioxidant properties, but there is no evidence for any beneficial clinical effect[60, 65, 66]. Riboflavin, vitamin B2, is a key building block in CI and CII and a cofactor in several key enzymatic reactions in the TCA cycle and β-oxidation. Similarly, there are reports of clinical improvements in patients treated with riboflavin but no effects have been proven in controlled clinical trials[67, 68]. L-carnitine is an essential co-factor for fatty acid transport into the mitochondria, and buffers mitochondrial acetyl CoA-levels[69]. There are no clinical trials evaluating its use in mitochondrial disease[65]. Muscular fatigue is a commonly reported symptom in patients with mitochondrial disease. As decreased levels of phosphocreatine at baseline and postactivity have been associated with this, several studies have evaluated creatine as an intervention, however without any clinical benefit for the patients[70-72]. Creatine is nevertheless used in practice as part of a mitochondrial cocktail[61]. Thiamine has also been used as a part of a drug cocktail for a case of Leigh syndrome, where the given medication improved neurological disease and the obstructive sleep apnea that the patient suffered from[73].

Do no harm

In addition to the therapies described above, treating coexisting illnesses, managing symptoms, optimizing diet and encouraging physical activity are key components of treatment of primary mitochondrial disease[61]. Avoiding medications and toxins with known negative effect on mitochondria is also crucial. An illustrative example is sensitivity to aminoglycoside antibiotics in patients with certain mtDNA alterations, typically the m.1555A>G point mutation. This mutation alters the secondary structure of the 12S subunit of the mitochondrial ribosome so it more resembles the 16S

ribosome of *Escherichia coli*, the target for aminoglycosides. In these patients, even a single dose of aminoglycosides may cause severe sensorineural hearing loss[74].

Management of acute exacerbations

The mainstay of treatment in acute exacerbations of mitochondrial disease is supportive care, as no evidence based treatments exist[58]. Stressors such as dehydration, fasting, trauma, infections or surgery may induce a catabolic state where the cells try to maintain energy production by breakdown of proteins and fat stores. Intravenous glucose is frequently given to provide energy substrates but should be used with care or avoided in certain patients with defects in glucose metabolism (such as PDH-deficiency)[65].

In MELAS (mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes), arginine and citrulline have been observed to have a therapeutic role in management of stroke-like episodes[75, 76]. Arginine and citrulline are nitric oxide (NO) precursors and endothelial dysfunction due to reduced NO production is believed to play a role in the stroke-like episodes. Possibly, treatment with arginine and citrulline can increase NO production and cerebral blood flow[65]. Few other disease specific medications are available.

Attempted treatments for mitochondrial disease

Glutathione (GSH) is an important intracellular antioxidant and the biosynthesis of GSH is dependent on the availability of cysteine. Based on observations of GSH deficiency in mitochondrial disease, supplement with cysteine was attempted in a clinical trial in mitochondrial disease but with disappointing results[77]. *N*-acetylcysteine (NAC) prevents depletion of GSH by systemically increasing cysteine, and supplementation with NAC has also been attempted[78]. The most widely used antioxidant is ALA[60]. Dichloroacetate (DCA) activates PDH by inhibiting pyruvate dehydrogenase kinase (an enzyme which normally inhibits PDH by phosphorylation), this way reducing lactate accumulation. The use of DCA in mitochondrial disease has been evaluated in a number of randomized clinical trials with some effects on surrogate markers like blood lactate, but without any effect on function or disease outcome[79-82] but peripheral nerve toxicity was seen in a long-term trial[83]. Dimethyl glycine[84] and vitamin K in the form of menadione[85] have also been tested without any therapeutic effects[86].

Targeting drugs to mitochondria

Due to its proton pumping function, the mitochondrion has a highly negatively charged interior, which has been utilized to direct molecules toward mitochondria. By linking a positively charged molecule to a compound, it will accumulate in the

mitochondrial matrix if it is lipophilic enough to cross the cytoplasmic membrane and the IMM. A known example is the CoQ_{10} analogue MitoQ[87], a compound that has been evaluated in clinical trials for PD without any beneficial effects for the patients[88]. Another related compound is the plastoquinone-containing antioxidant SkQ1, that similarly is targeted to the mitochondria through a positively charged side-chain. SkQ1 in eye-drops has shown efficacy in phase 2 clinical trials for dry-eye syndrome[89, 90] and is being marketed in Russia for that indication. SkQ1 is being considered for other indications related to mitochondria and aging, and has been shown to increase lifespan in mice when given systemically[91]. Yet another compound targeting mitochondria is the tetrapeptide elamipretide[92]. Elamipretide is believed to interact with the phospholipid cardiolipin, stabilizing the IMM and enhancing mitochondrial function[93]. Elamipritide has been evaluated in a phase 2 clinical trial for mitochondrial myopathy with preliminary reports being positive, but the results are not yet published[94].

Succinate as treatment for mitochondrial disease: taking the back door

There are case reports in the scientific literature where succinic acid (or the anion form, succinate) has been attempted as a therapy for mitochondrial disease involving RC CI. In 1989, a 61-year-old female with Kaerns-Sayre syndrome, a mitochondrial disease characterized by chronic eye muscle paralysis (and in its syndromic form also involving other organ systems), was treated with coenzyme CoQ_{10} (300 mg/day) and succinate (6 g/day) based on the finding of reduced mitochondrial CI enzyme activity. This patient experienced respiratory symptoms as part of her disease, and her condition was markedly improved while on medication[95]. In 2004, it was reported that a patient with MELAS, who had been suffering severe neurological symptoms refractory of attempted treatment, improved with daily treatment with 6 g of succinate. He remained free of most neurological symptoms for 30 months, except progressing hearing loss[96]. There is a brief report of another MELAS patient similarly benefitting from such treatment[97]. Succinate treatment (250 mg/kg/day) could also halt the progress of Leigh-like MRI lesions in a 7-year-old boy diagnosed with progressive dystonia due to a m.14459G>A mtDNA mutation during 4 years of follow-up. The patient had previously been progressing on supplement with CoQ_{10} (2 mg/kg/day) and L-carnitine (90 mg/kg/day)[98]. There are no reports of systematic evaluation of succinate treatment, or treatment with any related compound.

Methods

Measuring mitochondrial function

Respirometry: a live view of the active mitochondria

Oroboros O2k

The Oroboros O2k is a refined version of the classic Clark electrode where a membrane permeable to oxygen covers an electrode that is reducing oxygen[99]. Oxygen concentration in the medium can be determined by the current that ensues. The Oroboros O2k is using the same principle but has undergone improvements relative to the classic instrument. Reflux of oxygen into the sample has been minimized, and the instrument provides meticulous temperature control and exact readings. These improvements allow for real-time measurements of oxygen consumption at pmol/s resolution (figure 1). It is a versatile instrument where the sample can be easily accessed for additions, and the operator can hence respond to biological changes to previous additions, allowing for easy titration of substrates, toxins or uncouplers within the same experiment.

Through a series of publications authored by Dr. Fredrik Sjövall, our group has led the process of developing and optimizing protocols for analysis of blood cells in the Oroboros O2k[16, 100-102]. The instrument is very well suited for this application, as samples analyzed are in suspension, the natural environment for blood cells. In addition to providing the most accessible source of patient cells, blood cells from healthy donors may also be used as a model system toxicity evaluation of chemicals or drugs[103] and for drug development applications, as shown in this thesis.

Seahorse

A complementary instrument to the Oroboros O2k is any of the Extracellular Flux Analyzer instruments manufactured by Seahorse Bioscience (since 2015 a part of Agilent). The Seahorse simultaneously measures oxygen consumption and acidification rate in a 24- or 96-well plate in adhered cells, using a different technology than the Oroboros O2k. The Seahorse has certain advantages especially when it comes to

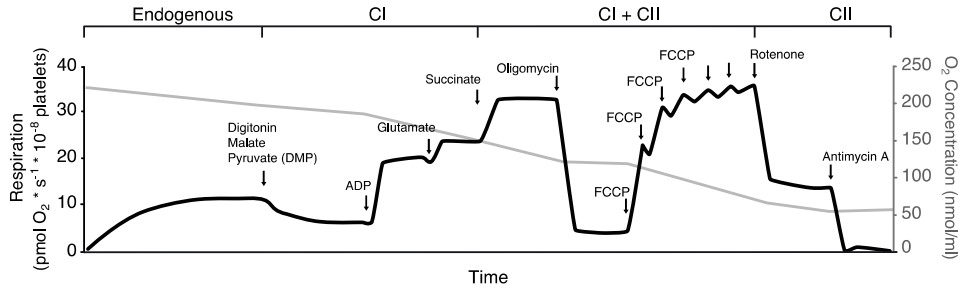

Figure 1. Typical trace from a respiratory experiment in the Oroboros O2k with permeabilized cells. The grey trace shows actual oxygen concentration in the experimental chamber, while the black trace depicts oxygen consumption. The time point of each addition is marked by arrows. In the top panel, the substrate availability is given: endogenous, complex I (CI) and/or complex II (CII) substrates respectively. Manipulation of mitochondrial function is performed by permeabilization of the cell membranes (digitonin) to allow for exogenous substrates to be administered, addition of the CI substrates malate, pyruvate and glutamate respectively, the addition of the CII substrate succinate, addition of the ATP-synthase inhibitor oligomycin, titration of the uncoupler FCCP until maximum respiration, the addition of rotenone to inhibit CI and the addition of antimycin A to inhibit CII. For further details regarding the experimental conditions, see the methods sections in paper I through IV.

cultured cells in monolayers. Less cells are required to get a stable signal, and with careful design, relatively large amounts of data can be gathered in a single experiment. The instrument does however suffer from limited versatility, as an experiment must be exactly predefined; only up to 4 additions are possible in one experiment, and the experimental environment is not as stable as in the Oroboros O2k. Due to its lack of flexibility, piloting is more difficult and less precise in the Seahorse; an efficient approach may be designing experiments in the Oroboros O2k and executing them in the Seahorse where throughput is higher. A further downside with the Seahorse is the high price of the plastic consumables. In paper III of this thesis we employed the Seahorse to assess mitochondrial respiration in cultured fibroblasts, an application where the Seahorse is preferable to the Oroboros O2k.

Limitations

Blood cell respirometry is a versatile, powerful and reproducible method to assess mitochondrial function in cell and tissue samples but does exhibit certain limitations. In a protocol with serial additions of mitochondrial substrates, inhibitors and uncouplers, it is reasonable to assume that the variation will increase later in the protocol, e.g. as accumulating additions may affect more fragile cells (such as patient cells with defect mitochondria) to a higher degree than healthier cells.

The oxygen tension the cells and tissues are exposed to under these experimental circumstances is very different from what can be expected *in vivo*. An aqueous solution at 37° C and 100 kPa have a pO_2 of 147 mmHg[104], while estimates of the mitochondrial pO_2 amount to 30–40 mmHg[105]. Although respiration rates in mitochondria traditionally have been considered to be constant down to very low oxygen concentration (2 μM)[106], the artificial *in vitro* oxygen concentration used (50

and 210 μM in a typical experiment) should still be considered a potential source of bias[107]. This is however a universal problem in most *in vitro* science applications, as *in vivo* tissue pO_2 rarely is taken into consideration. When comparing two samples with different oxygen consumption rates, this problem may be even more pronounced toward the late stages of an experimental protocol when the media for one sample could have an oxygen concentration very different from the one it is being compared to. Even though the software corrects for the chemical background, it naturally cannot compensate for a biological effect if such exists.

An additional limitation with the protocols utilized in the studies in this thesis is that they do not take into account the metabolism of fatty acids. This is possible to do in respirometry by supplying fatty acids as substrates[108].

Using intact or permeabilized cells

Respirometry can be applied to most cell or tissue samples such as intact cells, permeabilized cells, permeabilized muscle fibers, tissue homogenates or isolated mitochondria, and any result should be interpreted taking into account what sample is being analyzed[109]. Key discoveries in mitochondrial physiology were made using isolated mitochondria[49], but today the problem with isolating the mitochondria from their intracellular milieu is increasingly acknowledged, as crucial intracellular signaling pathways are lost when organelles are separated. Intact cells on the other hand provide only an indirect view of the mitochondria, as anything that is being observed is filtered through the intricate functions of the cytoplasm and the cytoplasmic membrane. Further, the ability for the researcher to manipulate mitochondria is severely hampered by the integrity of the cell; throughout an experiment the mitochondria are relying on endogenous substrates, or substrates that the cell actively takes up.

In papers I and II of this thesis, we employed respirometry to intact and permeabilized blood cells to analyze mitochondrial function in neurodegenerative disease. Permeabilized cells allow for control of substrate supply, while maintaining more of normal cellular physiology. The approaches were somewhat different between the papers. In paper I, the data were analyzed without any specific a priori analysis, using both data from permeabilized and intact cells. When designing our analysis for paper II, we decided to analyze data based on a few predefined hypotheses to make the analysis even more stringent. Altogether, the key conclusions drawn from both paper I and paper II were based on data from permeabilized cells, demonstrating the relative bluntness of respiration in intact cells. On the other hand, in paper III the utilization of intact cells was instrumental as the pharmacological concept introduced is based on the ability of the suggested compounds to permeate biomembranes, and here permeabilized cells were merely used as a negative control. In paper IV, in addition to blood cell respirometry, we also utilized permeabilized muscle fibers to

evaluate *ex vivo* mitochondrial function in the animal model of mitochondrial decompensation.

Mitochondrial membrane potential

One way to describe mitochondrial energy production through coupling of the electrochemical gradient over the IMM to the ATP synthase is through the analogy of an electric circuit[109]. The electrochemical gradient over the IMM, the mitochondrial membrane potential (MMP), can be likened to the charge of a battery and can similarly be quantified in terms of volts. The reflux of protons into the mitochondrial matrix through the ATP-synthase is driven by the MMP, in a similar way to how the current in a circuit is driven by the voltage of a battery. We do not have a direct measurement available of the proton flux, but a close approximation is provided by quantifying oxygen consumption, as is done in respirometry[109].

To gain further knowledge about mitochondrial function a measurement of the MMP, the voltage of the battery in the circuit analogy, is required. MMP can be assessed by utilizing the fact that the mitochondrial matrix is negatively charged. By applying a membrane permeable positively charged fluorescent probe (in paper III, TMRM is used) the MMP can be quantified, as the uptake of the probe will vary with the polarization of the IMM. A source of potential bias is any variations in charge over the cytoplasmic membrane, as this also will influence the fluorescence signal from the probe[109]. Most instruments with lasers able to excite the probe on the required wavelength, and record the emissions can be utilized, in our case the flow cytometer FACSAria III (BD, Franklin Lakes, USA).

Lactate production

If mitochondria fail to produce sufficient ATP to meet cell requirements, glycolysis is compensatively upregulated. When mitochondria do not incorporate the end-product of glycolysis, pyruvate, into the TCA cycle, lactate dehydrogenase will convert pyruvate to lactate and in the process make NAD^+ out of NADH[110]. NAD^+ is required for the glycolysis to continue, and thus by conversion of pyruvate to lactate, cells can continue to produce ATP despite mitochondrial dysfunction, even though the ATP yield is dramatically lower than if the substrates would be fully oxidized through mitochondrial metabolism.

In paper III of this thesis, the ability of the cell-permeable prodrugs of succinate NV118, NV189 and NV241 to attenuate rotenone-induced lactate production in platelets was tested using a Lactate ProTM 2 blood lactate meter (Arkray, Alere AB, Lidingö, Sweden)[111]. Platelets incubated with rotenone increase lactate production

due to decreased mitochondrial function and compensatory increase in glycolysis. Co-incubation with either succinate prodrug returned lactate production to control levels. As a control, an experiment was performed where also the CIII-inhibitor antimycin A was added to the incubation. This again increased lactate production, demonstrating that the attenuation of lactate production indeed was due to succinate-related electron flux through the RC. Further, this control experiment ascertained cell viability and patency of glycolysis also after addition of drug compounds.

Metabolomics

The omics revolution of biomedicine and life science has led to a previously unparalleled amount of data on e.g. nucleotide or peptide sequences being generated in short time. This has challenged many widely employed concepts of data analysis and statistics, as many statistical methods in standard use are unsuitable to handle this type of data[112]. The neologism "omics" refers to studying something in its whole, something which in applied practice is not always the actual case. In paper III and IV of this thesis, we applied metabolomics to investigate the abundance of the different metabolites relevant for mitochondrial metabolism. Metabolomics can be described as the most downstream of the omics in current use, with genomics, the study of the genome being at the top of the stream (figure 2).

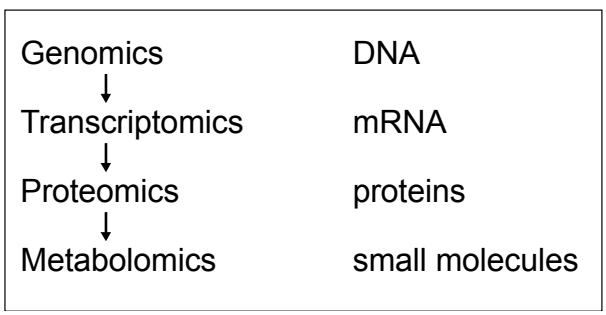

Figure 2. Hierarchic relation of omics applications. Metabolomics assesses the thousands of metabolites that partake in cellular metabolism, such as fatty acids, sugars and amino acids, which in turn is depending on the protein expression, the translation and the genes of the cell.

Metabolomics quantifies metabolites and small molecules in a biological sample, but does not in itself provide inference regarding the cause of any specific finding. One of the major challenges with interpreting metabolomics is relating identified metabolites to their biologic role[113]. Genomics on the other hand provides the basic template to which all events occurring in a cell in one way or another are related, but do not necessarily bestow information regarding what impact this has on cellular function

and the physiology of the organism. As always, it is all about proper analysis and interpretation of data.

We used capillary electrophoresis to separate metabolites in the samples. Time-of-flight mass spectrometry for cationic compounds and tandem mass spectrometry for anionic compounds were then used to determine the mass charge ratio *m/z* of each metabolite. The identities of the metabolites were determined using a database of known metabolites, based on *m/z* from mass spectrometry and capillary electrophoresis migration time. The absolute concentrations of the metabolites detected could be determined by normalization to a calibration curve calculated from an internal standard. Two separate set-ups were used for anions and cations respectively[114]. Mass spectrometry is a very good method of metabolomics analysis, as it is sensitive, reproducible and versatile. The prior separation of metabolites with capillary electrophoresis permits using also migration time for species identification, providing even better sensitivity[113]. Further information regarding metabolic pathways can be obtained from metabolomics studies where a biologic system is metabolizing a substrate labeled with a stable isotope such as ^{13}C[115]. Downstream metabolites containing ^{13}C can be distinguished from non-labeled metabolites, allowing for detection of breakdown products specifically from the added substrate.

Developing a large animal model of mitochondrial disease

The disease course for patients with mitochondrial disease is usually characterized by periods of deterioration and recovery, with significant variability between patients. A patient may present with a metabolic decompensation but may also maintain a stable condition over years. We identified a lack of disease models that directly focused on metabolic decompensation due to mitochondrial dysfunction, and in paper IV of this thesis we aimed to develop such a model. The most common site of dysfunction in the RC is CI and the CI toxin rotenone has successfully been used in other disease models involving CI-dysfunction. Pigs have (compared to rodents) relatively similar physiology to humans, and a large animal allows for similar kind of invasive monitoring and supportive care as used in clinical practice.

The monitoring and sampling in the model were organized to acquire information on different levels: whole animal, tissue and cell level. We applied continuous indirect calorimetry using a Quark RMR ICU (Cosmed, Rome, Italy), measuring whole animal O_2 consumption (VO_2) and CO_2 production (VCO_2) to assess global metabolism and utilization of oxygen. We hypothesized that lactate production due to a shift to anaerobic metabolism would be an important marker of induced mitochondrial function. Lactate production can also be due to hypoperfusion and consequential tissue hypoxia whereas in mitochondrial dysfunction maintained normoxia or even hyperoxia would be expected.

Hence assessment of tissue oxygenation provided means to differentiate between the two, and this was accomplished through insertion of an oxygen probe in a peripheral muscle of the modeled animal. Physiological reading at cell level were provided by respirometry in blood cells and permeabilized muscle fibers, and through a metabolomics assay in muscle samples.

Results and discussion

Mitochondria in neurodegenerative disease

Using respirometry, we evaluated mitochondrial function in blood cells from two different hematopoietic origins, platelets and peripheral mononuclear blood cells (PBMCs), in patients with neurodegenerative disease: ALS and HD.

Amyotrophic lateral sclerosis

Mitochondrial content

Mitochondrial content was quantified using two independent complementary methods: citrate synthase (CS) activity and mtDNA copy number. CS is an nDNA-encoded enzyme that catalyzes the reaction forming citrate from OAA and acetyl-CoA, and is often considered a rate-controlling step of the TCA cycle. The activity of the enzyme is widely used as an indicator of how much mitochondria are present in a sample[116]. We also used the approach of quantifying the mtDNA copy number. In contrast to nDNA that exists in just one copy per cell, mtDNA is present in up to thousands of copies per cell. In ALS patient platelets, both markers of mitochondrial content were significantly elevated compared to control; in PBMCs, there was a similar trend for CS activity. Interestingly, even though the two methods are completely independent, there was a good correlation, especially in PBMCs, between the two markers (paper I figure 1e,f, paper II figure 3c,d).

Decreased complex IV activity

More mitochondria in ALS patient blood cells compared to control resulted in respiratory activity normalized for cell count being increased in ALS patients throughout most respiratory states measured. When respiration was normalized to mitochondrial content however, and expressed as oxygen consumption per CS activity or per mtDNA copy, there were tendencies for reduced respiration in most respiratory states, more so in PBMCs than platelets. The only respiratory state that was significantly decreased was CIV activity, which was decreased in PBMCs when normalized to CS activity, and in platelets using either normalization method.

Disease staging

We plotted mitochondrial function as a function of temporal disease duration without finding evidence for progressing mitochondrial alterations. However, when we grouped the patients per disease stage[117], we observed a gradual increase in markers of mitochondrial content in the cells, and a gradual decrease in CI function. The increase in mitochondrial content may be compensatory due to energetic failure involving dysfunction in either CI or CIV.

Huntington's disease

How to reduce the risk of data fishing: predefined hypotheses

Based on previous findings in the literature, we predefined hypotheses regarding mitochondrial function in blood cells from patients with HD. The primary hypothesis was that mitochondrial respiration linked to succinate metabolism through CII would be impaired. As secondary hypotheses, we explored if there were impairments in respiration of substrates through CI or CIV, decreased maximum capacity of oxidative phosphorylation, if mitochondrial content was changed and if PDH function was reduced.

Mitochondrial function

Maximum capacity of oxidative phosphorylation was significantly reduced in platelets when normalized for CS, but not using other normalization methods, and not in PBMCs. Similarly, respiration linked to CI substrates was reduced only in platelets

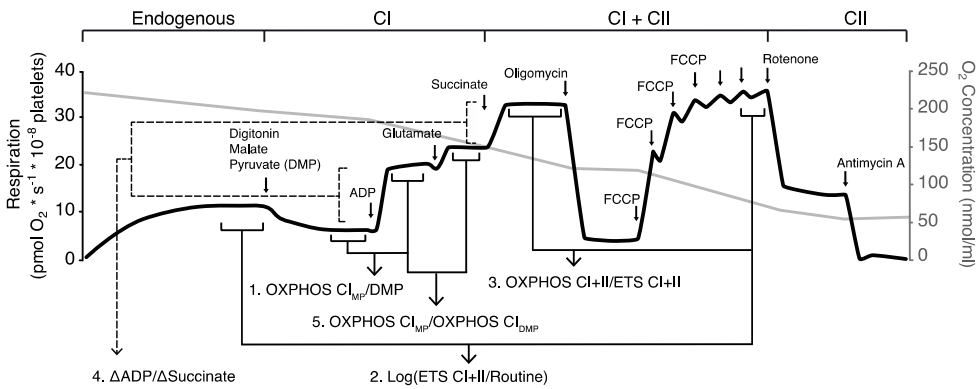

Figure 3. Respiratory ratios in a respiratory protocol in permeabilized cells. Five different respiratory ratios, each providing information about a specific aspect of mitochondrial function. Ratios from within the same experiment benefit from not being dependent of normalization to cell number or mitochondrial mass to provide information from the experiment. For definitions, please see figure 1 and the text. ETS, respiration associated with maximal protonophore-stimulated flux through the respiratory chain (also termed the electron transport system); OXPHOS, respiration associated with ATP synthesis by oxidative phosphorylation.

normalized for CS activity. However, the presence of CI dysfunction was supported by the fact that when calculating a normalization-independent respiratory ratio, expressing CI function as part of the maximum capacity of the RC, this ratio was significantly reduced in the HD patients.

Disease severity and mitochondrial function

Patients were grouped clinically based on total functional capacity (TFC) score, into early manifest HD and middle-to-end manifest HD. When analyzing the above results at TFC group level, most of the above reported alterations where driven by the later disease-stage group.

Additional data analysis

Respiratory ratios

There are means to gain knowledge about mitochondrial function from a respiratory experiment in addition to absolute values normalized for cell count or markers of mitochondrial content. When relying on a normalization factor an additional source of error and variation is introduced. By calculating ratios from data acquired within the same experiments, the need for normalization is avoided.

Within the scope of another project (Westerlund *et al*. unpublished data) we have defined respiratory ratios based on the data acquired in a respiratory Oroboros O2k protocol in permeabilized cells (figure 3).

1. OXPHOS CI_{MP}/DMP reflects the ability of the mitochondria to increase respiration when ATP is added to cells with access to CI substrates, thus assessing CI function. A low ratio would indicate decreased function in CI or upstream thereof.
2. ETS CI+CII/Routine demonstrates the maximum respiratory capacity of the cells in relation to their basal activity, the reserve capacity. A low value would indicate generally insufficient mitochondrial health.
3. OXPHOS CI+II/ETS CI+II evaluates the difference between two maximum rates of mitochondrial respiration in the protocol, with active ATP-synthase (OXPHOS CI+II) or maximum uncoupled respiration (ETS CI+II). If a dysfunctional CV is rate limiting for mitochondrial function, this would result in a low ratio as the cells could respire at a higher rate when the RC is uncoupled from ATP-production.
4. The ΔADP/ΔSuccinate ratio looks at the relative function of CI and CII. ΔADP is the change in respiration when ADP is added to cells that have CI substrates available but lack ADP. ΔSuccinate is the change in respiration when the CII

substrate succinate is added to cells that already are respiring on CI substrates with ADP. A low ratio would indicate CI dysfunction and a high ratio CII dysfunction.

5. We also hypothesized that a low OXPHOS CI_{MP}/OXPHOS CI_{MPG} ratio would indicate PDH deficiency. The malate aminotransferase converts glutamate to α-ketoglutarate in the mitochondria, providing anaplerosis to the TCA cycle downstream of PDH. Thus, if respiration increases when glutamate is added to cells respiring on malate and pyruvate, that could indicate a limitation in the ability of PDH to provide carbons to the TCA cycle.

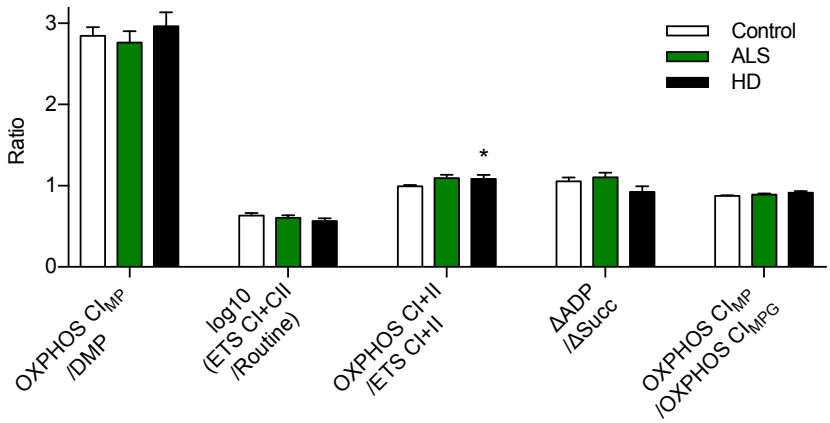

Figure 4. Respiratory ratios in platelets from patients with amyotrophic lateral sclerosis or Huntington's disease. A set of ratios between different respiratory states in a respirometric protocol with permeabilized cells in the Oroboros O2K were defined, each designed to reflect a certain aspect of mitochondrial function. For definitions of ratios please see text and figure 3. ALS, amyotrophic lateral sclerosis; HD, Huntington's disease. * indicate p-value < 0.05 compared to control using students t-test. Data presented as mean and standard error.

Respiratory Ratio	Control	HD	ALS
OXPHOS CI_{MP}/DMP	0%	0%	0%
log10(ETS CI+CII /Routine)	10%	21%	9%
OXPHOS CI+II /ETS CI+II	0%	0%	0%
ΔADP/ΔSucc (below reference)	0%	4%	4%
ΔADP/ΔSucc (above reference)	10%	4%	9%
OXPHOS CI_{MP}/OXPHOS CI_{MPG}	19%	4%	22%

Table 1. Percentage of subjects outside of reference intervals for respiratory ratios in amyotrophic lateral sclerosis patients, Huntington's disease patients and the control group. Data from blood cell respirometry on permeabilized cells. Reference values calculated from an independent set of healthy control subjects. For definitions of respiratory ratios, see figure 3 and the text. ALS, amyotrophic lateral sclerosis; HD, Huntington's disease.

Reference values for all respiratory ratios were established. Outliers were detected with Tukey's outlier labeling rule and removed. Based on a previous data material, the above ratios have all been found to follow Gaussian distribution, except ETS CI+II/Routine that required logarithmic transformation (base 10). The reference interval for each ratio was defined as the mean ± 1.96 standard deviations. The results from analyses of these respiratory ratios in this patient material are provided in figure 4 and table 1. In summary, the number of individual patients that had ratios outside of the reference values did not differ in any consistent way between the control group and either of the disease groups (table 1). Neither did the summary data differ in any consistent way, although there was a significant but slight difference in mean OXPHOS CI+II/ETS CI+II between HD and control subjects (figure 4). No correction for multiple comparisons were made and this finding does not correspond to any finding in the main data analysis in paper II, hence it is not likely this represents any relevant pathophysiological phenomena.

Statistically significant is not the same as clinically relevant

Properly performed statistical analysis of a data set is crucial to delineate between random findings and consistent patterns in data. Statistical analysis in biomedical research is burdened with problems due to selective reporting and improper methodology[118], but even when stringent analyzes are performed, statistically *significant* does not equal biologically or clinically *relevant*. For a patient, it is not very interesting that the treatment being offered is statistically significantly better than another treatment or no treatment, what is interesting is *how much better* the treatment offered is compared to other available options, the effect size. Even if a treatment is significantly better than the alternative, the *effect size* could potentially be so small that it is not relevant[119, 120]. This is increasingly discussed for clinical trials but should also be considered when interpreting results in preclinical life science. Are my statistically significant findings relevant for the process or disease I am studying?

In article I and II of this thesis we reported statistically significant findings regarding alterations in mitochondrial function. Other scientific reports regarding ALS and HD describe findings related to mitochondrial function that are not the same as the ones we present, as outlined in the introduction to the respective articles. In this light, we cannot say for certain that the effects that we are seeing in our two articles are large enough and consistent enough to claim that our findings are clinically relevant, although statistically significant.

Drug development

A patient unable to respire on complex I substrates

The blood cells respirometry method outlined and reported above have also been used by our group in a research program, Mitochondrial Disease in Children (MIDIC), where it was evaluated as a potential diagnostic tool for mitochondrial disease (Westerlund *et al*, unpublished data), and findings within that study led to the drug development program that resulted in paper III in this thesis. Permeabilized blood cells from a patient with suspected mitochondrial disease exhibited severely hampered respiration when metabolizing only CI substrates, but as the CII substrate succinate was added, the cells responded with an increase in respiration similar to control cells (figure 5). Inducing CII-linked respiration with succinate is not possible in intact cells, as succinate does not permeate the cytoplasmic membrane, hence we initiated a drug development and screening program to identify cell-permeable prodrugs of succinate that could support mitochondrial respiration independent of CI.

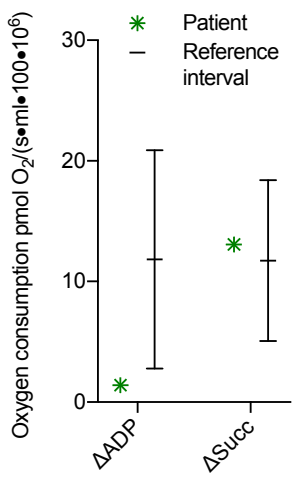

Figure 5. Inadequate response to ADP with complex I substrates present, but normal response to the subsequent addition of the complex II substrate succinate in blood cells from a patient with suspected mitochondrial disease. In an Oroboros O2k respirometer, permeabilized platetets from a 10-year-old female with clinically suspected mitochondrial disease were evaluated. ΔADP, the increase in respiration prompted by the addition of ADP to cells with malate and pyruvate present; ΔSucc, the increase in respiration prompted by the addition of succinate to cells with malate, pyruvate, glutamate and ADP present. The reference interval is mean and 1.96 standard deviation of values from 31 healthy pediatric subjects.

We evaluated over 50 newly designed and synthesized chemical entities using an Oroboros O2k and several respiratory protocols evaluating the effect of the compounds on mitochondrial respiration in intact human cells. The primary protocol used is shown in figure 6.

Ideal properties for a screened compound being identified as a potential drug when evaluated in intact cells with inhibited CI are:

1. The compound should when added induce higher respiration than the succinate reference (a bigger than a´). If a is similar to a´ that indicates the compound does not enter the cell.
2. It should induce as high respiration in intact cells as succinate does in permeabilized cells (a similar to b´).
3. The maximum respiration should be similar (c similar to c´) after succinate is added to *both* samples and the cells have been permeabilized.
4. After inhibition of CIII with antimycin A, the compound should not exhibit auto-oxidation or induce non-mitochondrial oxygen consumption (d similar to d´).
5. Effect of the compound should be reached at the lowest possible concentration.

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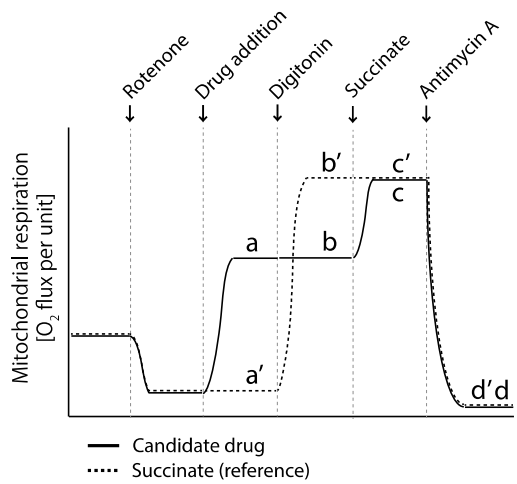

Figure 6. Schematic representation of screening protocol for cell-permeable succinate prodrugs. The primary screening protocol utilized to screen newly synthesized compounds for ability to support mitochondrial complex II (CII)-linked respiration. In platelets with complex I inhibition due to rotenone, drug compound to be evaluated or the reference succinate was added in increasing doses. The cells were then permeabilized and succinate was added to both experiments with drug compound and reference, to assess maximum CII-linked respiration. The experiments were terminated with the addition of antimycin A.

Cell-permeable prodrugs of succinate

Cell-permeable prodrugs of succinate support complex II respiration

The succinate prodrugs NV118, NV189 and NV241, identified using the screening protocol in figure 5, were extensively evaluated for cell-permeability and for potential to sustain mitochondrial function independent of CI. In paper III of this thesis, we demonstrated that all three compounds provided succinate to the mitochondria in various cell types and tissues. Mitochondrial CI dysfunction, either induced by rotenone or inherent in cells from a patient with Leigh syndrome, could be compensated for, and oxidative phosphorylation restored. Increase in MMP was observed with the addition of a succinate prodrug, further demonstrating increased substrate supply.

Intracellular succinate metabolism

In addition to quantification of intracellular metabolites in PBMCs after incubation with succinate prodrugs (paper III figure 2a and supplementary figure 3) we performed experiments with isotope-labeled compounds. By enriching the four carbons in the succinate-structure of the prodrug NV118 with the stable naturally occurring isotope ^{13}C and incubating the labeled compound with human platelets, we could trace the intracellular metabolic fate of the succinate released from the prodrug using capillary electrophoresis time of flight mass spectrometry. Of special interest is the presence of [$^{13}C_6$]citrate in the [1, 2, 3, 4-$^{13}C_4$]NV118-treated samples. For [$^{13}C_6$]citrate to form, [$^{13}C_4$]OAA have to react with [$^{13}C_2$]acetyl-CoA and as the natural abundance of [^{13}C] is about 1%[121], only 1 in every 10 000 molecules of acetyl-CoA can normally be expected to be [$^{13}C_2$]acetyl-CoA. For [$^{13}C_2$]acetyl-CoA to be present in any detectable concentration it must have been formed from [$^{13}C_4$]succinate released from [1, 2, 3, 4-$^{13}C_4$]NV118 (paper III figure 2a and supplementary figure 4). The likely pathway for this is formation of pyruvate from phosphoenolpyruvate (PEP) that in turn is derived from OAA. Pyruvate could then be transported into the mitochondria by the mitochondrial pyruvate carrier. A previous observation that the activities of pyruvate carboxylase and phosphoenolpyruvate carboxinase (PEPCK) are negligible in platelets[122] shrouds the picture, as PEPCK is catalyzing the reaction from OAA to PEP.

Succinate oxidation alleviates rotenone-induced lactate production

The CI inhibitor rotenone induces lactate production in human platelets, from a baseline production of 1.73 ± 0.5 (regression slope ± SD) mmol lactate per 10^9 cells per hour to 4.30 ± 0.24. Co-incubation with NV189 reduced the rotenone-induced lactate production to levels like control. When also the CIII inhibitor antimycin A was added to the incubation, effectively blocking respiration downstream of CII, lactate production was again increased (paper III figure 1m,n).

Lactate production is a hallmark of mitochondrial dysfunction, as when mitochondria fail to meet the energy requirements of the cells, a compensatory increase in glycolysis ensues. Dysfunctions in the RC result in decreasing mitochondrial NAD^+/NADH, which slows down the TCA cycle as there is not enough NAD^+ to accept electrons. The TCA cycle fails to incorporate pyruvate produced by the glycolysis, and to keep cytosolic NAD^+/NADH ratios low, to be able to maintain glycolysis, pyruvate is converted to lactate by lactate dehydrogenase, converting NADH to NAD^+ in the process. When mitochondria resume ATP production through delivery of electrons from succinate to the RC, the need for ATP provision by the glycolysis is reduced.

Reducing equivalents generated in the cytosol (NADH) are transferred to the mitochondria through by the malate–aspartate shuttle. The reducing power of cytosolic NADH is brought into the mitochondrial matrix in form of malate, that is derived from the OAA in the cytosol. OAA from the mitochondria is converted to aspartate, transported out of the mitochondria and again deaminated to OAA. With a halted TCA cycle, possibly there is an insufficient amount of mitochondrial OAA. As OAA also is consumed outside of the TCA cycle, such as gluconeogenesis, depletion of OAA is plausible. Reduced aspartate synthesis has been shown to be the reason why RC dysfunction inhibits cell growth, and aspartate can be derived from OAA in the cytosol under conditions with reduced RC function through the reverse action of cytosolic aspartate aminotransferase, which further could reduce OAA[123, 124]. When succinate is provided to cells and metabolized to fumarate, malate and OAA in turn, the availability of these metabolites will increase (paper III, figure 2a). This constitutes a putative complementary aspect of the alleviation of the lactate over-production upon administration of succinate, as the malate–aspartate shuttle may function more efficiently with better substrate availability. Arguing against this is the possibility that OAA is reduced rather than increased in the mitochondria due to CI-inhibition; regrettably we do not have data quantifying OAA. OAA can also be replenished through the action of pyruvate carboxylase, generating OAA from pyruvate[11]. In figure 6, the incorporation of ^{13}C from [1, 2, 3, 4-$^{13}C_4$]NV118 into metabolites in the malate–aspartate shuttle is depicted. The similar ratio of isotope-labeled carbons between malate and aspartate indicates activty of the aspartate aminotransferase and a funcioning malate–aspartate shuttle.

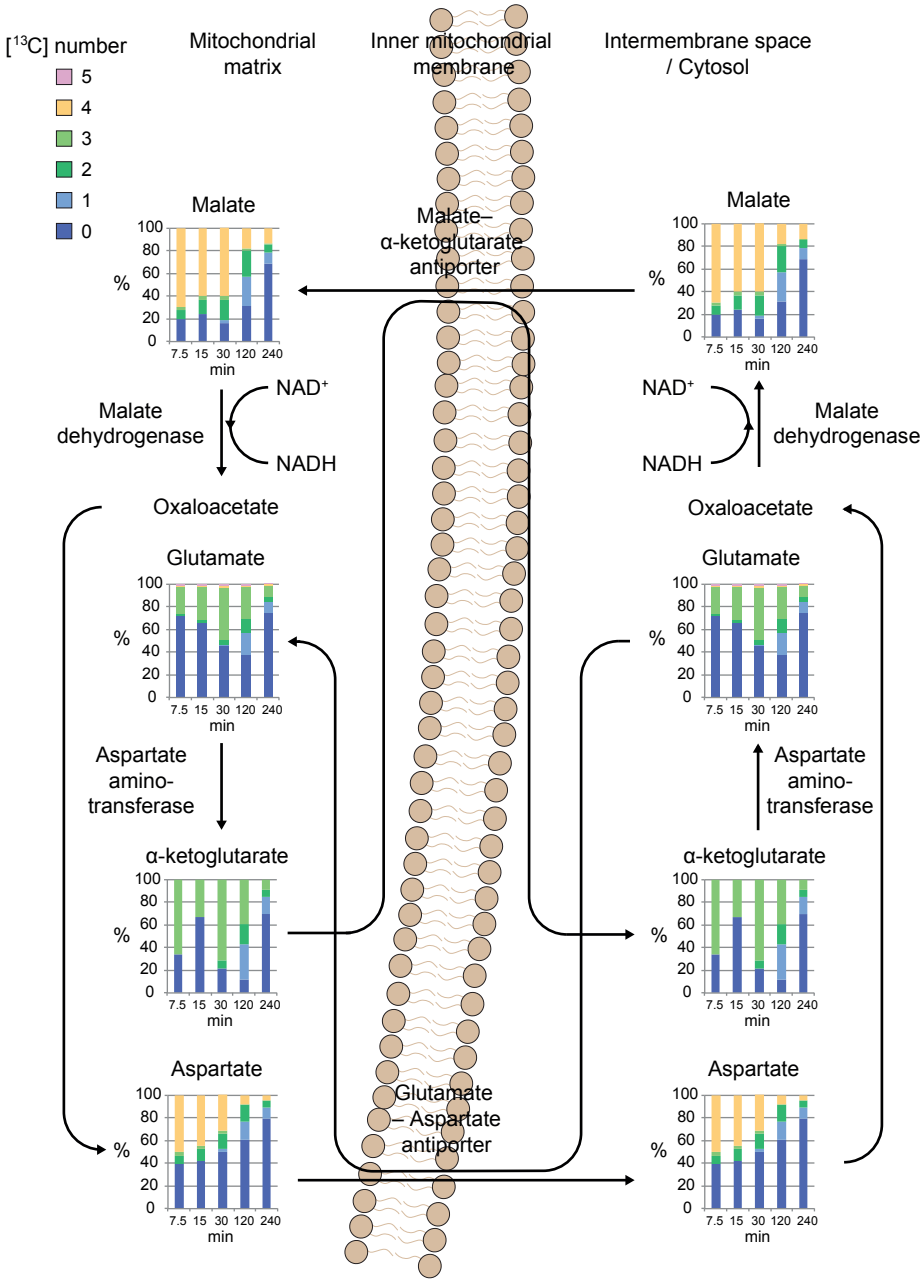

Figure 7. Incorporation of ^{13}C into the malate–asparate shuttle. Relative abundance of the stable isotope 13C in the intermediates of the malate–asparate shuttle in human platelets after incubation with [1, 2, 3, 4-13C4]NV118 for 7.5 to 240 minutes. The carbons delivered to the TCA cycle by [1, 2, 3, 4-13C4]NV118 are incorporated also into the malate–asparate shuttle as can be seen by similarites in isotope-labeling in glutamate and α-ketoglutarate, and malate and asparate respectively. No assessment of oxaloacetate was performed but the isotope-labeling can be expected to be similar to that of malate and aspartate, as it acts as an intermediate between them. The cells are not complex I-inhibited. Cytosolic and mitochondrial metabolite levels are not measured individually. Mean of n = 2.

A potential drug for mitochondrial complex I disease

There are case reports regarding successful treatment of patients with mitochondrial disease due to RC CI dysfunction with succinate or disodium succinate, but no clinical systematic evaluation has been performed. Succinate has very limited cell-permeability (paper III figure 1), and a therapeutic approach with succinate is more feasible with a pharmacologically optimized molecule, yielding relatively more succinate available for mitochondrial respiration.

A large animal model of mitochondrial energy crisis

To expand the toolbox available for research in mitochondrial disease, we developed and characterized a large animal model for metabolic decompensation due to mitochondrial RC CI dysfunction. We utilized pigs due to the similarities between humans and pigs in physiology[125], and we induced energetic crisis through intravenous infusions of the toxin rotenone, a substance previously used in models of PD[51].

Physiology of complex I dysfunction

Whole animal oxygen utilization

At whole organism level, indirect calorimetry demonstrated decreasing whole body oxygen fluxes due to rotenone exposure. For best performance of indirect calorimetry, a stable metabolic state is preferable and in our experiments, at least two critical factors in addition to increasing mitochondrial dysfunction affected the balance of inhaled and exhaled gases. The pigs that were subject to rotenone-infusion gradually developed lactic acidosis, with a parallel decrease in blood pH. Blood pH affects hemoglobin's oxygen binding affinity, shifting the oxygen-hemoglobin dissociation curve with acidification, decreasing the amount of oxygen that the erythrocytes bind[126]. This naturally will affect exhaled gases, and might be confounding when interpreting data. Further, as part of the experimental protocol, inhaled oxygen in the animals was adjusted to maintain arterial normoxia. These changes introduced variation in the calorimetry data and had to be adjusted for in the data handling. Despite these methodological limitations, the measurements provided evidence on whole organism level of mitochondrial dysfunction and decreased oxygen utilization.

Hemodynamics, organ function and lactate production

Beside a gradual decline in mean arterial pressure, more pronounced in the rotenone-treated animals, the hemodynamics remained largely unaffected during the first hours of the experiments. After the increase in rotenone infusion rate at three hours, the animals showed further signs of metabolic stress with increasing tachycardia. The rotenone-treated animals gradually developed lactic acidosis despite adequate tissue oxygenation. The oxygen extraction decreased, seen by increasing oxygen tension and saturation of venous blood.

Mitochondrial respiration

Ex vivo analysis of mitochondrial respiration in muscle samples and thrombocytes did not yield any statistically significant results, but the results from thrombocytes trended toward decreased respiration with CI substrates at the end of experiment compared to prior to start of infusion, and the control animals trended in the opposite direction. No consistent trends were observed in muscle.

Metabolomics

Metabolomics evaluation, the most downstream step in the hierarchically structured sampling scheme for this model, demonstrated increased levels of all measured TCA cycle intermediates except succinate. This is consistent with decreased flux of electrons through CI but maintained or upregulated metabolism of CII-substrates.

An interesting additional finding was that the level of aspartate was significantly reduced in animals treated with rotenone compared to control (1.59 nmol/g tissue in the rotenone groups compared to 5.28 in the control group), while no other amino acids differed between rotenone and control animals. As previously discussed, it has been shown that one of the crucial functions of the RC in proliferating cells is to maintain a high NAD^+/NADH ratio to allow for sufficient NAD^+ for aspartate synthesis, and CI inhibition inhibit cell proliferation through depletion of NAD^+[123, 124]. This finding suggests that maintaining sufficient aspartate levels may not only be an important task for the RC in proliferating cells, but also in skeletal muscles during time of metabolic stress.

Metabolic decompensation

The data from this model demonstrate that rotenone can be used to induce mitochondrial dysfunction *in vivo* and provoke an energetic crisis. Increased blood lactate with maintained tissue oxygenation is consistent with mitochondrial dysfunction as the cause for the apparent shift to anaerobic ATP-production, rather than insufficient oxygen availability. The trend toward decreased ability to respire on CI substrates in platelets *ex vivo* further strengthen this, as do the metabolomics results where all CI-linked TCA cycle

intermediates build up, and glycolysis pathways are upregulated. This model may provide a valuable additional tool for evaluating therapies for metabolic decompensation in patients with mitochondrial disease.

Concluding remarks and future perspectives

Data handling in mitochondrial research

Although the amount of data generated through respirometric experiments of the kind utilized in this thesis differ by several orders of magnitude compared to omics data, there are common challenges regarding data handling between them, and respirometric data would benefit from similar considerations regarding reproducibility and validation of results as omics data. The difficulties with achieving reproducibility in omics research have been highlighted repeatedly[112]. A surprisingly high percentage of the conclusions drawn in the scientific literature is believed to be incorrect due to e.g. inappropriate statistic methodology and publication bias (the negative results do not get published, the positive do)[118, 127]. This problem is in no way unique for respirometry though, but exists throughout medical science.

When we have worked with the projects in this thesis, and developed the method of blood cells respirometry, we have in parallel developed the methodologies used for analyzing the data acquired. Based on experience from paper I, we did in paper II decide to base our data analysis on a few predefined hypotheses, to minimize the risk of reporting incidental findings as outcomes of our study. Based on an extensive literature search we bulleted previous findings and reports regarding mitochondrial activity in HD, and deducted analyses to test these hypotheses specifically, even if that meant leaving some of our data out of the analysis.

This, and similar approaches, should be more consistently used in mitochondrial research and medical science in general. Any primary data set where there is not a statistical plan for how to mine for findings in a statistical sound way should only provide the investigator with hypotheses to evaluate in an independent data set, and should not be used to draw inference.

Assessing mitochondrial function at group level

Primary or secondary mitochondrial disease

Although respirometry is a precise modality to measure mitochondrial function, there are a few considerations to make regarding its application in investigation of mitochondrial involvement in disease processes at group level. In the MIDIC project described above, we have demonstrated a potential of this method to detect mitochondrial dysfunction in pediatric patients with primary mitochondrial disease. In that patient category, mitochondrial dysfunction is clearly an upstream event in the pathological process, and many of those patients have severely impaired mitochondrial respiration. The situation is different in patients with systemic diseases like neurodegenerative disease, where it is unknown at what stage in the pathological process mitochondria are involved (if they are), and variations can be expected to be subtler.

Neurodegenerative disease

Reports regarding dysfunction of different parts of the mitochondria are abundant for both ALS and HD and are only consistent regarding that there *is* an alteration in mitochondrial function. Few reports are available that attempt to take in the full picture, and it must be admitted that neither does paper I nor II of this thesis. I believe that to reach any conclusive evidence on the matter, approaches investigating multiple aspects of mitochondrial function simultaneously in the same sample is required (respiration, metabolomics, protein expression etc.). Further, I believe that more than one tissue ideally should be investigated. Blood cells have been suggested as a marker for global mitochondrial function, but are yet not proven as such. In primary mitochondrial disease, there may be a large variation between different tissues in mitochondrial function, for example due to mtDNA heteroplasmy, and similar differences may shroud the picture in systemic diseases.

Amyotrophic lateral sclerosis

In paper I of this thesis, we reported decreased CIV function in blood cells from ALS patients. Even though CIV activity previously has been reported to be reduced in patients with ALS[128], the general tendency of the results in this study toward decreased respiratory function per mitochondria, and the fact that the measurement of CIV activity is late in the experimental protocol, call for caution before irrefutably concluding that CIV dysfunction is a specific pathological feature for ALS. The observations regarding CIV could be an effect of a generally upregulated mitochondrial content in the cells, a normalization effect, which is unspecific to ALS. The ambiguity between the gradual

decline in activity related to CI seen when stratifying ALS patients per disease stage and the general decrease in CIV function described above further indicate that these findings could be normalization effects.

Huntington's disease

To avoid reporting incidental findings in the HD material, we predefined hypotheses and performed analyses specifically to answer the hypotheses, attempting to utilize all aspects of the data to do so. For a result to be considered biologically relevant, ideally a consistency through most analyses relating to a specific hypothesis should be seen. Even though dysfunctions of CI-linked respiration and maximum respiration were demonstrated there was insufficient consistency throughout the data to unequivocally confirm any hypothesis. Most differences seen in HD compared to control were driven by patients in later disease stages which puts into question whether mitochondrial dysfunction specifically is a part of the early pathological process in HD. The alterations in mitochondrial function could be an epiphenomenon, disease-unspecific metabolic strain on the organism related to severe disease in general.

Modeling mitochondrial failure

Although the number of genetic mouse models available for mitochondrial disease continues to increase, the heterogeneity of mitochondrial disease makes it practically impossible to cover the full disease spectra. Compared to e.g. PD, where there is an abundance of proposed models for that specific disease, in the field of primary mitochondrial disease, the investigator is lucky if there is even one model for the specific disease of interest. All disease models are just that, models, and it is very rare that a model considers all or even most of the characteristics for the modeled disease normally seen in human patients. For a disease like PD, a pathway can be investigated or a therapy evaluated in several different models to mitigate risk that any finding is just confined to that model; that option is rarely available in mitochondrial disease. An example is a proposed model of LHON that also exhibits several general symptoms of neurodegeneration that is typically not a part of LHON in human patients[129].

Of the more commonly used research animals, pigs are the species that most resembles humans regarding anatomy and physiology[125]. The rate of drugs that translate from promising rodent results to proven efficacy in humans is low[130] and one way to improve translation is likely to demonstrate *in vivo* efficacy of a drug in phylogenetically more advanced species. A larger animal also facilitates the mimicking of a clinical setting in which the proposed drug compound may be evaluated, as further testing and monitoring is available for a pig compared to a rodent.

Models should address an expected clinical situation for a patient category. In mitochondrial disease, there is a need for medications for long term treatment, but also for acute treatment of energy crisis, and hence, there is a need for models mimicking this clinical situation.

The many faces of succinate

In paper III of this thesis, we showed how cell-permeable prodrugs of succinate deliver succinate to cells, and how reduced respiratory activity due to CI-dysfunction can be improved. As all metabolic processes in the human body are interlinked, succinate plays other important roles than merely being an intermediate of the TCA cycle and providing reducing equivalents to the RC. Succinate metabolism is believed to be an important source of mitochondrial reactive oxygen species (ROS) under different conditions and is implied in both inflammation and carcinogenesis. Further it is the natural agonist for the G-protein-coupled receptor GPR91.

The potential problem with mitochondrial reactive oxygen species

The generation of mitochondrial ROS is an important process, as ROS when produced in excess are believed to cause oxidative damage to DNA and other critical cell structures[131]. Mitochondria metabolizing succinate under conditions with decreased ATP-synthase activity are generally considered to produce ROS. The ROS production can be at least partly abolished through inhibition of CI by rotenone, demonstrating that the ROS production is due to reverse electron transfer (RET) through CI, although this theory has been challenged[132]. ROS are usually considered a major source of cellular oxidative damage, but potential beneficial effects of ROS production are beginning to attract attention[133]. In a patient with mitochondrial dysfunction due to CI deficiency, CV will likely function normally, striving to produce more ATP to sustain cellular function, and CI will be defect. This argues against that ROS formation through RET will be a major factor in these patients, but this may also vary depending on the nature of the CI deficiency. The $ND6^{P25L}$ mouse model of CI mitochondrial disease (with a neurodegenerative phenotype resembling LHON) displays total lack of RET[129]. The proposed sites for ROS generation at CI are the ubiquinone binding site and the flavin mononucleotide (FMN) that oxidizes NADH[131]. The finding that the $ND6^{P25L}$ mouse does not exhibit RET indicates that the site rather is upstream of the ubiquinone binding site, most likely the FMN, at least in this model[129]. In addition to CI, the other suggested major source of ROS is RC CIII[134].

Succinate as a hypoxia signal

Succinate is reported to act as a pro-inflammatory factor by stabilization of the hypoxia-induced transcription factor HIF-1α, causing a state sometimes termed "pseudohypoxia". Upon activation of Toll-like receptors by lipopolysaccharide, macrophages downregulate mitochondrial oxidative phosphorylation and upregulate glycolysis. Simultaneously succinate levels are increased despite reduced TCA cycle activity, possibly by anaplerosis from glutamine through α-ketoglutarate[135]. The pro-inflammatory properties of succinate are believed to be due to succinate oxidation by SDH rather than high levels of succinate in itself. Succinate oxidation is reported to be a key factor in repurposing the mitochondria in activated macrophages toward producing ROS through RET, which in turn is key for the inflammatory phenotype of macrophages and release of pro-inflammatory cytokines[136].

Succinate and SDH have also been implicated in carcinogenesis. SDH is sometimes considered a tumor suppressor[137], as there are pro-carcinogenic mutations described in the gene[138, 139]. Impaired succinate oxidation leads to succinate build-up and subsequent stabilization of HIF-1α[140] through inhibition of the α-ketoglutarate-dependent prolyl hydroxylases that normally tag HIF-1α for proteosomal degradation. HIF-1α in turn acts as a signal for switch to glycolysis, a characteristic of tumor cells (the Warburg effect). This connection has however been challenged[141]. Interestingly, the proposed pathways for pro-carcinogenic effects and pro-inflammatory effects of succinate are, albeit both conveyed through stabilization of HIF-1α, somewhat incoherent, as the former is linked to the oxidation of succinate and the latter to its presence in high concentration[136, 142]. HIF-1α is an important pathway in hypoxia sensing and inflammation and is being investigated as a drug target for several different conditions, targeted both for stabilization and inhibition[143]. Succinate-containing compounds have been proposed to pharmacologically counter brain hypoxia due to both their signaling effect for adaptation to hypoxia, and through their energotropic effects through CII[144].

The HIF-1α pathway has a therapeutic potential also for primary mitochondrial disease. In a Cas9-mediated genome-wide screening putative targets rescuing from mitochondrial dysfunction due to the CIII-inhibitor antimycin A, KO of the von Hippel-Lindau (VHL) factor was identified as a rescuing factor promoting cell growth. VHL stabilizes HIF-1α, and beneficial effects on cell survival were shown from treatment with a small molecule that also stabilize HIF-1α[145]. *In vivo* relevance of the pathway was shown using the NDUFS4 KO mouse model of Leigh syndrome, that when exposed to continuous low oxygen environment dramatically improved its phenotype[145].

Succinate's own G-protein-coupled receptor

Succinate was in 2004 identified as the natural ligand for a previously orphan G-protein-coupled receptor, GPR91, and receptor activation was shown to cause increased blood pressure in mice through the renin–angiotensin system[146]. Expression of GPR91 has subsequently been demonstrated in retina[147], dendritic cells[148], hematopoietic cells and blood cells[149, 150], adipose tissue[151] and heart[152]. In one study, GPR91 mRNA was detected in almost all organs in mice[153], and its expression have been shown to be momentarily increased in face of hypoxia, most pronounced in the brain[154].

Succinate is normally present in plasma in the range from 2–3 μM up to 20 μM, with concentration below 10 μM being the norm[155, 156], but succinate is known to accumulate up to 20-fold under ischemic conditions in brain[157, 158], liver[159] and heart[160]. The EC_{50} for succinate-induced activation of human GPR91 has been reported to be 56 ± 8 μM or 28 ± 5 μM in different assays[146], leaving the receptor largely inactivated at baseline.

In addition to its renal hypertensive effects, activation of the receptor has been linked to retinal angiogenesis[147] and to ischemic retinopathy in diabetes[161]. In cardiomyocytes, the receptor is believed to play a role in apoptotic signaling in ischemia[152] and succinate signaling through GPR91 causes cardiac hypertrophy[162, 163].

Future perspective

Succinate clearly exerts effects beyond reducing FAD, both as an intracellular signal molecule and as a receptor ligand. The current literature regarding GPR91 activation is largely linking the activation of the receptor to pathways negative for cellular health, but it is not unlikely that this view will be diversified with a growing body of evidence emerging for the role of the receptor in different tissues, species and pathways. Illustrating this, prolonged agonistic targeting of the GPR91 has been proposed to improve post stroke adaptation[164]. Comparably, the stabilization of HIF-1α is in cancer research described as detrimental as it induces carcinogenic pathways, but this pathway is believed to be beneficial in certain inflammatory diseases[143]. As several other pathologies feature mitochondrial CI dysfunction, succinate prodrugs have potential as a treatment option also in e.g. PD[165], renal tubular damage[166], and TBI[19, 20]. For the specific topic of mitochondrial disease, it is an interesting prospect that succinate delivered to cells in addition to providing reducing equivalents to CII of the RC also could induce a beneficial effect by the hypoxia-sensing pathways. Neither HIF-1α stabilization, nor the reactive oxygen species formation, GPR91-binding or inflammatory effects of the prodrug-released succinate are covered experimentally in this thesis, but are all matters that will require attention onward.

Energetic deficiency and redox imbalance

Mitochondrial dysfunction caused by decreased CI function is likely not only due to reduced flow through the RC and subsequent reduction in coupled ATP synthesis. Altered redox status is likewise a potential reason for mitochondrial dysfunction. In the ideal mitochondrial system, the protein complexes in the RC represent a balanced stoichiometric system, and when one part of the system becomes perturbed, imbalance ensues. The glycolysis and the TCA cycle convey energy from dietary nutrients to cellular energy by producing reducing equivalents, i.e. they convey electrons to (reduce) other molecules, normally NAD^+ or FAD to NADH and $FADH_2$ respectively, that in turn convey their electrons (energy) to the RC. A dysfunctional CI may be unable to act as an electron acceptor which causes build-up of NADH (as NADH normally pass electrons to CI) and a lack of NAD^+. This decreasing mitochondrial NAD^+/NADH (increasingly reduced state) stops the TCA cycle, the influx of cytosolic NADH through the malate–aspartate shuttle, and other cellular processes.

Succinate delivered to the mitochondria allows for electron supply to FAD and subsequent RC activity. However, the TCA cycle may still be perturbed due to redox imbalance, an unfavorable NAD^+/NADH ratio, as the following steps in the TCA cycle, like the oxidation of malate to OAA, require NAD^+. Ways to mitigate this have been suggested by introducing an acceptor of electrons from NADH, just regenerating NAD^+ without actually using the energy for producing ATP[167, 168]. An appealing thought is a combined therapeutic intervention; a succinate prodrug providing electrons to the TCA cycle for proton pumping and an electron acceptor for NADH may act synergistically.

Other tricarboxylic acid cycle intermediates as drugs

Fumarate

Fumarate is the next metabolic step in the TCA cycle after succinate. The dimethyl ester of fumarate, dimethyl fumarate (DMF), was in 2013 launched as a drug for the relapsing form of the neurodegenerative disease multiple sclerosis (MS). DMF appears to act immunomodulatory through Nrf2 expression and by promoting a shift from T-helper cell type 1 to 2, but the therapeutic benefit on MS by DMF is pleiotropic[169] and therapeutic effect has been shown also in Nrf2 null mice[170]. Mitochondrial dysfunction has been reported as a part of the pathology of MS[171] and as DMF acts

anaplerotic on the TCA cycle, with increased levels of fumarate, malate and succinate, this may mediate parts of its beneficial biological effect[172].

Fumarate hydratase, the enzyme that mediates the metabolism of fumarate into malate in the TCA cycle, has similar to SDH been reported as a tumor suppressor gene, as fumarate build-up due to dysfunctional fumarate hydratase stabilizes HIF-1α[173]. It remains to be seen if there is an increased cancer incidence with the use of DMF (which eventually is integrated in the TCA cycle as fumarate through anaplerosis); currently DMF is rather considered as a putative anti-cancer agent[174].

DMF is an interesting illustration of the influence of metabolic intermediates on physiology, and how diverse the spectra of molecular interactions may be. I believe that we onward will adjust the way we describe the effects of compounds such as DMF on physiology, leaving the focus on ligand-target interactions and instead use system descriptors. To understand the pharmacokinetic fate of drugs incorporated into the metabolism like DMF, metabolomics studies using isotope labeled compound should be used more extensively.

Oxaloacetate

OAA is the last step in the TCA cycle, just before new carbons are added as acetyl-CoA. OAA or analogues thereof have been proposed as a treatment for PD and AD[4], diseases where mitochondrial CI and CIV dysfunction are implicated respectively. As OAA is converted to malate in the cytosol by malate dehydrogenase, the cytosolic ratio of NAD+/NADH will increase which promote glycolysis. The produced malate may be imported to the mitochondria and contribute to mitochondrial respiration[175]. A potential limitation to this approach is the reported deficiency of CI in PD, which could limit electron transfer to CI and cause a low NAD+/NADH ratio in the matrix, slowing down the TCA cycle and the malate–aspartate shuttle as a result. Preclinical studies have also provided evidence for a potential therapeutic effect of OAA in other conditions. In ALS, over-expression of cytosolic aspartate amino-transferase together with OAA administration scavenge glutamate and reduce excitotoxicity[176]. Scavenging of glutamate by OAA may also be beneficial after TBI[177].

To trace a drug that is not there anymore

Pharmacokinetics, the up-take, distribution and elimination of a drug to, in and from a biological system, is an essential part of pharmacology. For compounds that serve as intermediates in metabolism, such as the drug compounds described in paper III of this thesis, pharmacological studies pose an extra challenge. A molecule otherwise not present in the body can readily be traced and quantified also in small quantities, but a metabolic intermediate derived from a drug compound is not normally distinguishable from the endogenous pool of that metabolite.

The pharmacokinetics of DMF are interesting and provide a parallel to the succinate prodrugs presented in paper III of this thesis, as the compound similarly is integrated in cellular metabolism rather than being eliminated via renal clearance or hepatic breakdown. DMF is largely broken down to its mono-ester monomethyl fumarate (MMF) in first-pass metabolism[178], and no DMF is detectable in blood after oral administration of DMF. MMF is believed to be the biologically active metabolite and is pharmacokinetically assessed[179]. The ultimate fate of DMF could only be assessed by administering of [^{14}C]DMF, as DMF is incorporated into the TCA cycle. Metabolism to primarily glucose then could be shown, with 60 % of the compound ultimately being eliminated as exhaled CO_2[180].

In paper III, we described *in vitro* methods of tracing metabolism of pro-drug delivered succinate using an isotope-labeled drug compound. By enriching the four central carbons in the compound, the carbons that form succinate once the drug is cleaved, with ^{13}C the fingerprint in mass spectrometry of the molecule will change. This way, by measuring the relative abundance of ^{13}C in putative intracellular metabolites, the fate of the drug and its incorporation into cellular metabolism can be traced. This will be even more crucial *in vivo*, when evaluating uptake, organ distribution and elimination.

The delineation between pharmacokinetics and pharmacodynamics (a drug's interaction with its target) of a drug that will be integrated in metabolism, and potentially modulate metabolic fluxes is not clear cut, and thus metabolomics analysis of isotope-labeled compound also provides critical information of the drug's action.

Clinical trial endpoints for mitochondrial disease

The perfect endpoint

Although mitochondrial diseases as a group affect approximately 1 person in 8 500[181], every specific disease or syndrome is rare. LHON and Leigh syndrome are two of the more common diagnoses[182, 183], which is probably an important reason why many drug development projects for mitochondrial disease focus on these two disorders. CI dysfunction is implicated in both diseases, although other perturbations in the RC also can cause Leigh syndrome. The relative ease with which a therapeutic effect can be quantified is likely another reason why LHON is the first mitochondrial disease for which a medication is approved. Visual acuity is an ideal endpoint. It is not a surrogate marker, it is reproducible, it has a small inter-investigator variation, it is cheap and it provides a biometric value. Still, with a perfect endpoint and a relatively large cohort for a rare disease (85 patients enrolled), the primary endpoint was not met in the clinical phase III trial evaluating idebenone as treatment for LHON (and neither were most of the secondary endpoints)[184]. The fact that idebenone got market authorization in the EU despite a negative phase III result, based on non-significant trends, sub-group analyzes, a follow-up trial[185] and retrospective patient data[186] illustrates the huge unmet medical need in this field.

Study endpoints in rare diseases

In rare diseases, such as mitochondrial disease, hard endpoint such as mortality are difficult to employ in a clinical trial, as large cohorts of patients or dramatic clinical effects are required to properly power a study for such an endpoint. Even though more subjective, quality of life is a very relevant endpoint for the patients but must rely on crude questionnaires. Demonstrating significant change is challenging in small trials[187]. Biomarkers of disease, such as a value obtained from a blood sample, is in certain diseases though to be relevant from disease progress, such as low density lipoprotein in cardiovascular disease[188], but are even so usually not considered sufficiently relevant to prove efficacy in a phase III clinical trial. They may however be of value in early stage investigations of a new drug. There are currently no validated biomarkers for mitochondrial disease that correlate with clinical outcome[187]. An interesting approach to endpoints in clinical trials for rare diseases are individualized endpoints, something currently being included as secondary endpoint in a clinical trial evaluating EPI-743. The rationale behind individualized endpoints is that due to the rarity of each disorder and the heterogeneity of clinical presentation and symptoms, an endpoint uniformly relevant for all patients cannot be defined[189].

Among the more feasible approaches for clinical trial endpoints for mitochondrial disease are disease specific outcome scales, considering various aspects of the disease including both subjective patient concerns and objective biometric data. The currently most accepted scale is the Newcastle paediatric mitochondrial disease scale (NPMDS)[190], that primarily was conceived to describe the natural history of mitochondrial disease, and provide a tool for follow-up. An updated more detailed adaptation of this scale, the International paediatric mitochondrial disease scale (IPMDS), has been suggested as a tool more suitable as clinical trial endpoint[191]. Scales like NPMDS and IPMDS provide a way to get summarized numerical data out of a complex and heterogeneous clinical situation and subjective patient views, and this is likely the most feasible approach for clinical endpoint design in mitochondrial disease. A major limitation of disease scales relevant for the pharmacological concept outlined in paper III of this thesis is clinical situations like the one modeled in paper IV of this thesis: acute illness and metabolic decompensation. To identify parameters of relevance for the acute phases of mitochondrial disease, further studies are needed regarding the natural history of the disease at time of metabolic decompensation, hospitalization and intensive care.

Conclusion

Mitochondria are at the junction of most cellular metabolic pathways in eukaryotic cells and mitochondrial dysfunction is implicated in numerous diseases, however very few medical interventions targeting mitochondria are available. Further investigation into the role of mitochondria in human disease is required to understand the pathophysiology centering around mitochondria, and methods to assess mitochondrial function and diagnose mitochondrial disease need to be developed further.

Prodrugs of succinate are a theoretically appealing approach for treating mitochondrial disease due to CI dysfunction. In this thesis, *in vitro* data demonstrate beneficial effects on mitochondrial function by such compounds. Efficacy and safety now must be shown *in vivo*, before a prodrug of succinate can enter clinical trials and eventually benefit patients with mitochondrial disease.

As an addition to the toolbox in drug development for mitochondrial disease, we present a clinically highly relevant large animal model with a distinct phenotype of metabolic decompensation due to mitochondrial dysfunction at RC CI, which could be used to evaluate therapeutics for acute energy crisis.

I believe that we are only seeing the beginning of mitochondrial medicine, and that we onward will gain deeper understanding of the role of mitochondria in health and disease. Shifting focus toward metabolic processes rather than macromolecular targets and single organs may constitute a new paradigm of medicine.

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ORIGINAL COMMUNICATION

Mitochondrial dysfunction in blood cells from amyotrophic lateral sclerosis patients

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Abstract Mitochondrial dysfunction is implicated in amyotrophic lateral sclerosis, where the progressive degeneration of motor neurons results in muscle atrophy, paralysis and death. Abnormalities in both central nervous system and muscle mitochondria have previously been demonstrated in patient samples, indicating systemic disease. In this case–control study, venous blood samples were acquired from 24 amyotrophic lateral sclerosis patients and 21 age-matched controls. Platelets and peripheral blood mononuclear cells were isolated and mitochondrial oxygen consumption measured in intact and permeabilized cells with additions of mitochondrial substrates, inhibitors and titration of an uncoupler. Respiratory values were normalized to cell count and for two markers of cellular mitochondrial content, citrate synthase activity and mitochondrial DNA, respectively. Mitochondrial function was correlated with clinical staging of disease severity. Complex IV (cytochrome c-oxidase)-activity normalized to mitochondrial content was decreased in platelets from amyotrophic lateral sclerosis patients both when normalized to citrate synthase activity and mitochondrial DNA copy number. In mononuclear cells, complex IV-activity was decreased when normalized to citrate synthase activity. Mitochondrial content was increased in amyotrophic lateral sclerosis patient platelets. In mononuclear cells, complex I activity declined and mitochondrial content increased progressively with advancing disease stage. The findings are, however, based on small subsets of patients and need to be confirmed. We conclude that when normalized to mitochondria-specific content, complex IV-activity is reduced in blood cells from amyotrophic lateral sclerosis patients and that there is an apparent compensatory increase in cellular mitochondrial content. This supports systemic involvement in amyotrophic lateral sclerosis and suggests further study of mitochondrial function in blood cells as a future biomarker for the disease.

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Abbreviations

ALS	Amyotrophic lateral sclerosis
BSA	Bovine serum albumin
BZD	Benzodiazepines
CI	Complex I
CII	Complex II
CIV	Complex IV
CNS	Central nervous system
CS	Citrate synthase
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycoltetraacetic acid
ETS	Electron transport system
FCCP	Carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone
HEPES	4-(2-hydroxyethyl)-1-Piperazineethanesulfonic acid
mtDNA	Mitochondrial DNA
OXPHOS	Respiration associated with ATP synthesis by oxidative phosphorylation
PBMC	Peripheral blood mononuclear cell
PSMA	Progressive spinal muscular atrophy
SD	Standard deviation
SSRI	Selective serotonin re-uptake inhibitor
TMPD	Tetramethylphenylenediamine

Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder affecting motor neurons, resulting in progressive muscle weakness and ultimately leading to respiratory failure and death in the majority of patients [14]. Mitochondrial abnormalities have been implicated in the pathogenesis of ALS, although the exact role of mitochondrial dysfunction is not known [29]. In human post mortem brain tissue, increase in mitochondrial electron transport system (ETS) complex I (CI) enzymatic activity per mitochondrial content has been reported [4, 6] and in post mortem spinal cord, reduction of cytochrome c-oxidase [ETS complex IV (CIV)]-activity has been shown in patients with ALS [11]. The evidence for mitochondrial dysfunction in ALS has been reviewed repeatedly, e.g., by Cozzoloni and Carri [7].

Systemic involvement has been suggested in ALS [1, 20, 28], and mitochondrial alterations have been reported in muscle, liver and blood cells from ALS patients [8, 9, 12, 17, 19, 28]. Here, we assess mitochondrial function in blood cells [platelets and peripheral blood mononuclear cells (PBMCs)] from ALS patients using high-resolution respirometry to elucidate whether previously reported mitochondrial perturbations in blood cells affect mitochondrial respiration. We measure mitochondrial oxygen utilization with live respiring mitochondria ex vivo in intact and permeabilized cells in order to evaluate the integrated mitochondrial respiration. This will provide further evidence of whether systemic mitochondrial dysfunction is present and detectable in ALS patients. Furthermore, we explore if measures of mitochondrial dysfunction in blood cells could serve as diagnostic or prognostic biomarkers for the disease.

Materials and methods

Patients

The study was approved by the regional ethical review board at Lund University (EPN no. 2011/89). The study population comprised patients diagnosed with motor neuron disease showing clinical phenotypes of upper and/or lower motor neuron affection, herein referred to as ALS [14]. Twenty-four patients (9 females and 15 males, age 64.9 ± 12.3 years) were included in the study between May 2011 and October 2012. Patients were recruited at the Neurology Clinic at Skåne University Hospital, Lund, Sweden. As controls, relatives (primarily spouses) of patients with chronic neurological disorders admitted to the same clinic were recruited. Written informed consent was acquired prior to inclusion. Demographic and clinical data are summarized in Table 1. Any prescription drugs or class of prescription drugs taken by 5 or more patients are listed, and the diagnostic certainty according to the El Escorial criteria is given [5]. The control group consisted of 21 individuals and was age matched (64.6 ± 11.6 years), but not gender matched (15 females and 6 males).

Staging

Patients were staged by an experienced specialist blinded to the experimental results at time of staging, based on criteria recently suggested by Roche et al. [23]. Stage 1, symptom onset (involvement of first region); stage 2A, diagnosis; stage 2B, involvement of a second region; stage 3, involvement of a third region; stage 4A, need for gastrostomy; stage 4B, need for respiratory support (non-invasive ventilation). Referral to surgery for gastrostomy at the time of blood sampling was considered as stage 4A. To allow for statistical analysis, subgroups A and B were pooled.

Table 1 Research subject characteristics

				Age (mean ± SD)	Males/females	Duration (mean ± SD)	
ALS patients				65 ± 12	15/9	6 ± 8 Years	
Control subjects				65 ± 11	6/15		
No.	Age	Gender	Duration	Medication	Symptoms (alive at follow-up[a])	El Escorial	Stage
1	68	Female	1½ years	Riluzole, BZD	Reduced muscle function left arm (onset), walks by herself	Probable	2B
2	82	Female	10 years	Riluzole, SSRI	Dysarthria (onset) and dysphagia, in wheelchair	Definite	3
3	79	Male	2 months	Riluzole	Dysphagia, lower limb weakness (onset), in wheelchair	Probable	2B
4	50	Male	10 years	Riluzole, SSRI	Onset was increased muscular tonus in lower limb, in wheelchair[a,c]	Definite[b]	4B
5	50	Female	9 years		Difficulties walking (onset), walks on crutches[a]	Possible	2B
6	40	Male	1½ years	Riluzole	Weakness in left foot (onset), Walks on crutches[a]	Possible	2B
7	56	Female	2 years	Riluzole	Pseudobulbar symptoms, onset was dysarthria[a]	Definite	3
8	81	Male	2 years	Riluzole	Anarthria (onset) and gastrostomy	Probable	4A
9	67	Male	1 years	Riluzole	Reduced muscle function in the upper limbs (onset)	Probable	2B
10	60	Male	1 years	Riluzole, BZD	Dysarthria, dysphagia and dementia. Walks with walker. Upper limb onset	Definite	3
11	54	Male	16 years	BZD	Spastic paraparesis, dysphonia (onset), slow progress. In wheelchair[a]	Possible	2B
12	69	Female	1 years	Riluzole	Upper limb weakness, dysarthria and dysphagia (onset). Gastrostomy[a]	Definite	4A
13	67	Male	4 years	Riluzole	Dysarthria, right side hemiplegia (onset). Walks by herself[a]	Definite	3
14	72	Male	37 years		Slow progress, tetraparesis (onset), wheelchair[a]	PSMA	2B
15	70	Female	13 years	BZD	Wheelchair, gastrostomy	Definite[b]	4A
16	53	Male	1 year	Riluzole	Weak left foot (onset), walks by himself, can manage daily life[a]	Probable	2B
17	78	Male	2 years	Riluzole, BZD	Bilateral lower limb weakness (onset)[a]	Probable	2B
18	73	Female	3 years	Riluzole	Left side hemiparesis (onset), walks with walker[a]	Possible	2B
19	51	Female	5 years	Riluzole, SSRI	Lower limb weakness (onset), walks on crutches[a]	PSMA	2A
20	59	Male	3 years	Riluzole, SSRI	Tetraplegia (bedridden), right side onset. Tracheostomy and gastrostomy[a]	Possible	4B
21	84	Female	5 years	Riluzole, BZD, SSRI	In wheelchair, anarthria (dysarthria onset symptom), gastrostomy	Definite	4A
22	74	Male	6 years	Riluzole	Bulbar symptoms, weak left arm (onset), needs help with daily life[a]	Definite	3
23	71	Male	2 years	Riluzole, SSRI	Mild dysarthria, weak right hand and arm (onset), wheelchair[a]	Definite	3
24	50	Male	4 years	Riluzole, BZD,	In wheelchair with ventilator and gastrostomy. Lower limb onset	Definite	4B

Clinical data at time of blood sampling. Subject no. 10 was excluded from the analysis of platelet data due to technical uncertainty of cell number in the sample, and in subject no. 4, 10, 11, 12, 14 and 16 there were insufficient PBMCs available for analysis

BZD benzodiazepines, *SSRI* selective serotonin re-uptake inhibitor, *PSMA* progressive spinal muscular atrophy

[a] Alive at follow-up

[b] Familial amyotrophic lateral sclerosis

[c] Known SOD1-mutation

Chemicals

Monopotassium phosphate was acquired from Merck KGaA (Darmstadt, Germany). All other chemicals were purchased from Sigma–Aldrich (St Louis, MO, USA).

Isolation of peripheral blood cells

From each patient or control, a total of approximately 20 ml of blood was drawn to K_2EDTA tubes (Vacutainer®, BD, Franklin Lakes, NJ, USA) via venous puncture. Platelets and PBMCs were isolated with consecutive centrifugation steps as previously described [26, 27].

High-resolution respirometry

Measurements were performed with an Oxygraph-2k (Oroboros Instruments, Innsbruck, Austria) as previously reported [26, 27]. The oxygen concentration in the medium during an experiment was between 210 and 50 μM O_2. The intracellular-mimicking 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 [13] was used for all experiments with permeabilized cells. Experiments with intact cells were performed in the subject's own plasma.

For both platelets and PBMCs experiments were performed as previously published [26, 27] using two main protocols, one for intact and one for permeabilized cells. Cells were counted using an automated haemocytometer (SweLab Alfa, Boule Diagnostics, Stockholm, Sweden) and the cell count used in the experiments was 200 $\times$ 10^6 cells/ml for platelets and 3.5–5.0 $\times$ 10^6 cells/ml for PBMCs. The amount of digitonin used to permeabilize cells was 6 µg/1 $\times$ 10^6 cells for PBMCs and 1 µg/1 $\times$ 10^6 cells for platelets. Where material supply was insufficient for all analyses, measurements of permeabilized cells were prioritized.

Intact cells with endogenous substrates suspended in the patient's own plasma were allowed to stabilize at routine respiration ($ROUTINE_{plasma}$) and the proton leak over the inner mitochondrial membrane was measured by adding the ATP-synthase inhibitor oligomycin ($LEAK_{intact}$). By subsequent titration of the protonophore carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP), the maximum flux through the ETS (ETS_{intact}) was achieved. The experiment was terminated by inhibition of CI and complex III, revealing non-mitochondrial oxygen consumption. Data were corrected for non-mitochondrial oxygen consumption, and control ratios ($ETS_{intact}/LEAK_{intact}$, $ROUTINE_{plasma}/LEAK_{intact}$ and $ETS_{intact}/ROUTINE_{plasma}$) were calculated.

Permeabilized cells were assayed in MiR05 using a protocol with sequential addition of substrates, inhibitors, and titration of uncoupler. Specific mitochondrial CI-respiration was measured with CI-substrates (pyruvate, malate and glutamate) and ADP present, i.e., active oxidative phosphorylation ($OXPHOS_{CI}$). Convergent CI + CII-respiration associated with ATP synthesis ($OXPHOS_{CI+II}$) was then measured by addition of the CII-substrate succinate. Subsequently, the complex V-inhibitor oligomycin was added to induce $LEAK_{CI+II}$ (oxygen consumption due to proton leak over the inner mitochondrial membrane in presence of CI- and CII-substrates) and maximal flux through the electron transport system (ETS_{CI+II}) was achieved by titrating the uncoupling agent FCCP. Specific CII-dependent uncoupled respiration was then assessed through the addition of the CI-inhibitor rotenone (ETS_{CII}). CIV-activity was evaluated by addition of the artificial electron donor tetramethylphenylenediamine (TMPD) followed by the CIV inhibitor azide. Before evaluation of CIV, non-mitochondrial oxygen consumption was recorded as described for intact cells. All data were corrected for non-mitochondrial oxygen consumption, and uncoupling control ratio ($ETS_{CI+II}/LEAK_{CI+II}$) and phosphorylating control ratio ($OXPHOS_{CI+II}/LEAK_{CI+II}$) were calculated. For concentrations of toxins and substrates, please see methods in the two articles from 2013 by Sjövall et al. [26, 27].

Citrate synthase activity measurement

Measurements of citrate synthase (CS)-activity were performed with a commercially available assay kit (CS0720, Sigma–Aldrich, St Louis, MO, USA). After sonication (30 s for PBMCs and 2 $\times$ 60 s for platelets), samples were loaded into a 96-well plate in assay buffer with addition of 300 µM acetyl CoA and 100 µM 5,5-dithiobis-(2-nitrobenzoic acid). In a spectrophotometric plate reader set to 412 nm on a kinetic program with 1.5-min duration and 10-s interval, the absorbance of the baseline reaction was measured. Following this, 500 µM of oxaloacetate was added to each well and absorbance was measured. Calculations of CS-activity were then performed according to the manufacturer's instructions.

Mitochondrial DNA measurements

Quantification of cellular mitochondrial DNA (mtDNA) content was performed as previously reported [26, 27]. Frozen samples were thawed and diluted 500 times in a lysis buffer (10 mM TRIS–HCl, 1 mM EDTA, salmon sperm DNA 1 ng/µl, pH 8.0). 10 µl of this dilution was amplified in a 25 µl PCR reaction containing 1× Power SYBR® Green PCR Master Mix using an ABI Prism 7000 real-time PCR machine (Applied Biosystems Inc., Foster City, CA, USA) and 100 nM of primers directed to the human mitochondrial COX-1 gene (Eurofins MWG-operon, GmbH, Ebersberg, Germany). The threshold cycle (Ct) values were related to a standard curve using cloned PCR products.

Statistical analysis

All analyses were performed with SPSS (version 21 and 22, IBM, New York, USA) and all figures were generated using GraphPad PRISM (GraphPad Software version 5.01 and 6.0d, La Jolla, CA, USA). All values are presented as mean ± standard error of the mean, except for demographic data where standard deviation (SD) are used. Comparisons of respiratory values between ALS and the control group, and between survivors and non-survivors, were performed with unpaired Student's *t* test, not assuming equal distribution between groups. All ranges are 95 % confidence intervals of difference from the unpaired *t* tests. Comparisons between respiratory values at different disease stages were performed with Jonckheere–Terpstra's test, a non-parametric test for ordered alternatives, with post hoc correction for multiple comparisons between all pairs. Results were considered significant if $p < 0.05$. One data point, one of two CS replicates in subject no. 10, deviated more than 5 SD from the mean and was excluded as an outlier.

Results

Cellular mitochondrial content is increased in platelets of ALS patients

CS-activity in ALS patient platelets was significantly increased by 28 % (95 % confidence interval 11–50 %) compared to the control group and the mtDNA copy-number per cell was 38 % higher (7–68 %). Similarly, in PBMCs, ALS patients showed a 19 % higher CS-activity [(−8) to 45 %] and 6 % more mtDNA copies [(−21) to 33 %]; however, the differences in PBMCs were not significant (Fig. 1a–d; Table 2). For both cell types, CS and mtDNA correlated well (r^2 values ranging from 0.66 to 0.89), indicating the validity of both markers as a measure of mitochondrial content in peripheral blood cells (Fig. 2e, f).

Mitochondrial respiration dependent on complex IV is reduced in ALS

Data were first analyzed on a respiration per cell count basis (Table 2). In intact platelets $ROUTINE_{plasma}$ respiration was increased by 36 % (8–64 %) in ALS patients compared to control and ETS_{intact} respiration by 23 % (3–44 %). $LEAK_{intact}$, oxygen consumed to uphold mitochondrial inner membrane potential and compensate for endogenous proton escape, was close to zero for both groups. Analyses of respiration in intact PBMCs were

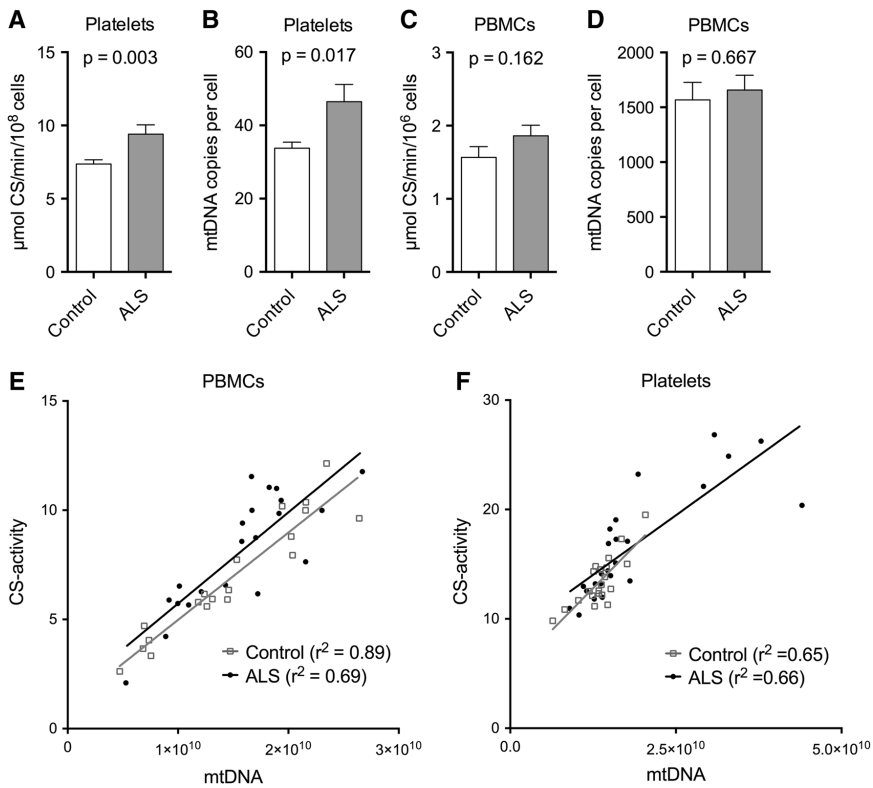

Fig. 1 Mitochondrial content in peripheral blood cells from ALS patients. Mitochondrial content per cell, measured using CS-activity (**a**) and mtDNA copies per cell (**b**), is significantly increased in platelets from ALS patients. In PBMCs no significant alteration could be demonstrated (**c** and **d**). For both PBMCs (**e**) and platelets (**f**), CS-activity and mtDNA correlate well, indicating the validity of these parameters as markers for mitochondrial content in peripheral blood cells. Two-tailed *p* values from unpaired Student's *t* test, not assuming equal distribution. *ALS* amyotrophic lateral sclerosis, *PBMC* peripheral blood mononuclear cells, *CS* citrate synthase, *mtDNA* mitochondrial DNA

excluded due to limited data (only 5 ALS and 3 control samples were analyzed due to insufficient amount of cells).

Next, we further characterized the nature of the altered mitochondrial function using permeabilized cells to be able to control the substrate supply to the mitochondria. Respiratory data were normalized to cell count (which is presented in Table 2) and mitochondrial content, respectively (CS and mtDNA) (presented in Fig. 2). Normalized to cell count, respiration was increased in $OXPHOS_{CI}$ [38 % (9–66 %)], ETS_{CII} [16 % (0–32 %)], $OXPHOS_{CI+II}$ [31 % (8–54 %)] and ETS_{CI+II} [19 % (1–36 %)] in permeabilized platelets of ALS patients. These differences were not found in PBMCs (Table 2).

When we analyzed data as oxygen consumed per mitochondrial content (by normalization for CS-activity and mtDNA) instead of per cell, mitochondrial CIV was significantly affected, but no other respiratory parameter. In platelets, the CIV-activity was significantly reduced in ALS patients both when normalized to CS-activity [24 % (5–42 %)] (Fig. 2b) and for mtDNA content [24 % (3–45 %)] (Fig. 2d). In PBMCs, the CIV-activity was significantly lower in ALS patients when normalized to CS [35 % (5–65 %)] (Fig. 2a), while when data were normalized to mtDNA (30 % [(−3) to 64 %]) the CIV-activity was reduced but did not reach statistical significance ($p = 0.075$) (Fig. 2c). There was no difference between the ALS and control group in any of the analyzed respiratory control ratios, either for intact or permeabilized cells (data not shown). The similar level of respiration in $OXPHOS_{CI+II}$ and ETS_{CI+II} states indicates that complex V is not a major rate limiting factor in platelets and PBMCs, which is in accordance with previous studies [27].

With progressing disease stage, mitochondrial complex I-dependent respiration declines and mitochondrial content increases

Patients were grouped according to clinical disease stage [23] (see "Materials and methods") and key data on mitochondrial function were analyzed for significant continuous trends. In PBMCs, $OXPHOS_{CI}$ and $OXPHOS_{CI+II}$ normalized to CS both displayed a significant decline with progressing disease, each with a 43 % decline in respiration at stage 4 compared to controls, whereas patients in stage 2 of the disease only displayed a 2 and 8 % decrease, respectively (Fig. 3a, c). Mitochondrial content in PBMCs gradually increased with progressing disease stage, with a 61 % increase at stage 4 compared to controls (Fig. 3g). Further, CIV in PBMCs measured per cell was gradually increased from 62 % in stage 2–92 % of control in stage 4. No progressive change was shown for CIV when normalized to mitochondrial content (Fig. 3e). These findings could not be detected in platelets (Fig. 3b, d, f, g).

Non-survivors have lower complex I-linked respiration in PBMCs than survivors

Post hoc, patients were retrospectively grouped into survivors and non-survivors based on medical records at the end of study [follow-up time 13 ± 7 months (Mean ± SD)] and evaluated for differences in mitochondrial function with respect to survival. In PBMCs, $ROUTINE_{MiR05}$ respiration per cell was 21 % (1–41 %, 3.35 ± 0.16 versus 4.04 ± 0.26 pmol $O_2/s/10^6$ cells, $p = 0.043$) higher in non-survivors and, when investigated at the level of individual respiratory complexes, $OXPHOS_{CI}$ and $OXPHOS_{CI+II}$ was 22 % (3–42 %, 7.31 ± 0.47 versus 8.94 ± 0.48, $p = 0.027$) and 27 % (5–49 %, 11.39 ± 0.86 versus 14.47 ± 0.81, $p = 0.019$) higher, respectively, compared to survivors. Further, $LEAK_{CI+II}$ was 44 % (9–79 %, 1.50 ± 0.17 versus 2.16 ± 0.18, $p = 0.019$) higher in non-survivors (data not shown).

To control for influence of medication on mitochondrial function, ALS patients were grouped according to their prescribed medication for all drugs taken by 5 or more study subjects. For all parameters where a significant difference was found between the ALS and the control group in this study, the results for treated and non-treated patients in each drug class were compared (using non-parametric methods due to unequal and small groups). No significant differences were found (data not shown). Further, all significant differences between the ALS and the control group reported were reanalyzed excluding patients not meeting the revised El Escorial criteria for ALS [5], without any change in result or conclusion of the study (data not shown).

Discussion

In the current study we demonstrate increased mitochondrial content in ALS patient platelets, seen also in PBMCs with increasing disease stage. With this finding, it is imperative to normalize respiration to cellular mitochondrial content in order to be able to draw conclusions regarding the mitochondrial respiratory capacity of blood cells from ALS patients.

Using normalization, we provide evidence of mitochondrial dysfunction in ALS patients, a dysfunction related primarily to CIV of the electron transport system. We also report a progressive decline in mitochondrial CI-dependent respiration with advancing stage of the disease. These findings were detectable in peripheral blood cells suggesting that mitochondrial dysfunction affects cells beyond the CNS and muscles in ALS.

CIV, or cytochrome c oxidase, is the final electron receiver of the electron transport system in the mitochondrial

Table 2 Respiratory data and markers of mitochondrial content

	$ROUTINE_{plasma}$	$LEAK_{intact}$	ETS_{intact}	*n*					
Intact platelets									
ALS patients	14.15 ± 1.28	0.65 ± 0.32	20.53 ± 1.50	22					
Control	10.41 ± 0.57	−0.044 ± 0.15	16.65 ± 0.77	21					
p value	0.012	0.056	0.029						
	$ROUTINE_{MiR05}$	$LEAK_{CI+II}$	$OXPHOS_{CI}$	$OXPHOS_{CI+II}$	ETS_{CI+II}	ETS_{CII}	CIV-activity	CS-activity	mtDNA	*n*
Permeabilized platelets										
ALS patients	10.79 ± 1.21	5.58 ± 0.45	26.58 ± 2.49	44.27 ± 3.51	40.54 ± 2.60	18.70 ± 1.08	36.11 ± 3.01	9.63 ± 0.63	47 ± 5.5	23
Control	8.27 ± 0.66	4.98 ± 0.20	19.32 ± 1.07	33.84 ± 1.58	34.20 ± 1.48	16.14 ± 0.61	39.55 ± 2.06	7.37 ± 0.29	34 ± 1.8	21
p value	0.076	0.234	0.012	0.011	0.042	0.049	0.353	0.003	0.017	
Permeabilized PBMCs										
ALS patients	3.65 ± 0.16	1.79 ± 0.14	8.04 ± 0.38	12.76 ± 0.69	10.22 ± 0.96	5.27 ± 0.56	6.57 ± 0.65	1.86 ± 0.15	1643 ± 142	18
Control	3.29 ± 0.14	1.94 ± 0.13	7.66 ± 0.30	12.57 ± 0.42	10.84 ± 0.55	5.37 ± 0.22	8.17 ± 0.51	1.57 ± 0.15	1568 ± 159	19
p value	0.108	0.467	0.437	0.815	0.577	0.869	0.064	0.108	0.667	

Data are presented adjusted for cell count; data normalized to mitochondrial content are presented in Fig. 2. $OXPHOS_{CI}$, $OXPHOS_{CI+II}$, ETS_{CII} and ETS_{CI+II} are increased per cell in platelets from ALS patients. Mitochondrial content (CS-activity and mtDNA) in platelets from ALS patients is significantly increased compared to control. All respiratory data are corrected for non-mitochondrial respiration and given as mean and SEM. Data are expressed in pmol O_2/s/100 × 10^6 cells from experiments in platelets and per 10^6 cells in PBMCs. Similarly, CS-activity is depicted as µmol CS/min/100 × 10^6 cells in platelets and per 10^6 cells in PBMCs. mtDNA is given as copies per cell. Two-tailed *p* values from Student's *t* test. Equal variance not assumed

ALS amyotrophic lateral sclerosis, *CS* citrate synthase, *OXPHOS* respiration associated with ATP synthesis by oxidative phosphorylation, *ETS* respiration associated with maximal protonophore stimulated flux through the electron transport system, *LEAK* idle respiration without ATP-synthase activity, *CI* complex I, *CII* complex II, *CIV* complex IV, *mtDNA* mitochondrial DNA, *n* number of patients, *PBMC* peripheral blood mononuclear cells, *ROUTINE* endogenous respiration in plasma or MiR05 medium, *SEM* standard error of mean

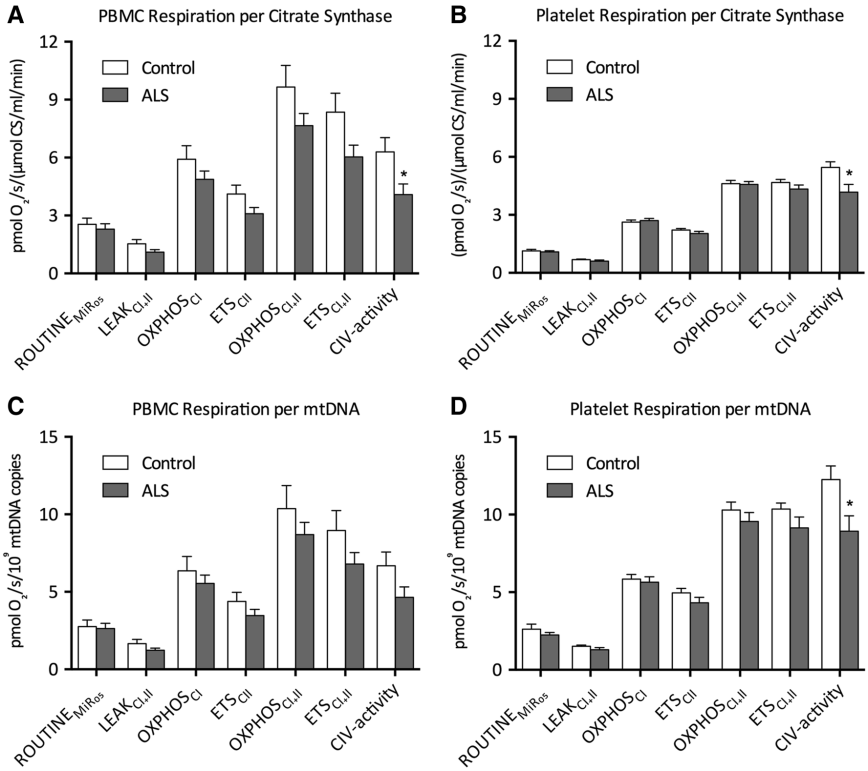

Fig. 2 Respiration of platelets and peripheral blood cells from ALS patients normalized to mitochondrial content. Respiratory activity was normalized to mtDNA and CS-activity, respectively. In platelets, CIV-dependent respiration was decreased both when normalized to CS-activity (**b**) and mtDNA (**d**), while in PBMCs CIV-activity was reduced when normalized to CS-activity (**a**) but not significantly so when normalized to mtDNA (**c**). Data are presented as mean and standard error. * $p \leq 0.05$ using unpaired Student's t test with two-tailed p values without assuming equal distribution. *ALS* amyotrophic lateral sclerosis, *CI* complex I, *CII* complex II, *CIV* complex IV, *CS* citrate synthase, *ETS* respiration associated with maximal protonophore stimulated flux through the electron transport system, *LEAK* idle respiration without ATP-synthase activity, *mtDNA* mitochondrial DNA, *OXPHOS* respiration associated with ATP synthesis by oxidative phosphorylation, *PBMC* peripheral blood mononuclear cells, *ROUTINE* endogenous respiration in MiR05 medium

inner membrane and is in turn donating electrons to oxygen, in the process pumping electrons over the mitochondrial inner membrane to build up the electrochemical gradient that powers ATP synthesis at complex V. A reduction of CIV respiration was present in both platelets and PBMCs, cells from different hematopoietic origins. Previously, mitochondrial CIV deficiency has been reported both in post mortem CNS tissue [2, 31] and muscle fibers [8, 30] from ALS patients. Here we show that CIV deficiency extends beyond the previously described tissues and is also detectable in peripheral blood cells in ALS.

Furthermore, we found that mitochondrial content per cell measured either as mtDNA copies per cell or activity of the matrix enzyme CS was increased in platelets from ALS patients. It is conceivable that the increase in mitochondrial content and the detected increase in mitochondrial respiration per cell are due to allostatic compensation for the inadequacy of the mitochondria to comply with cellular energy demands, possibly due to decreased CIV function. Mitochondria have recently been proposed as a key mediator of cellular damage due to allostatic load in chronic systemic stress responses [22], and similar mechanisms are plausible to play a role in progressing neurodegeneration. At this stage it is, however, uncertain what the biological implications of reduced CIV-activity and increased mitochondrial content are. This and the causative mechanisms of these alterations warrant further investigation.

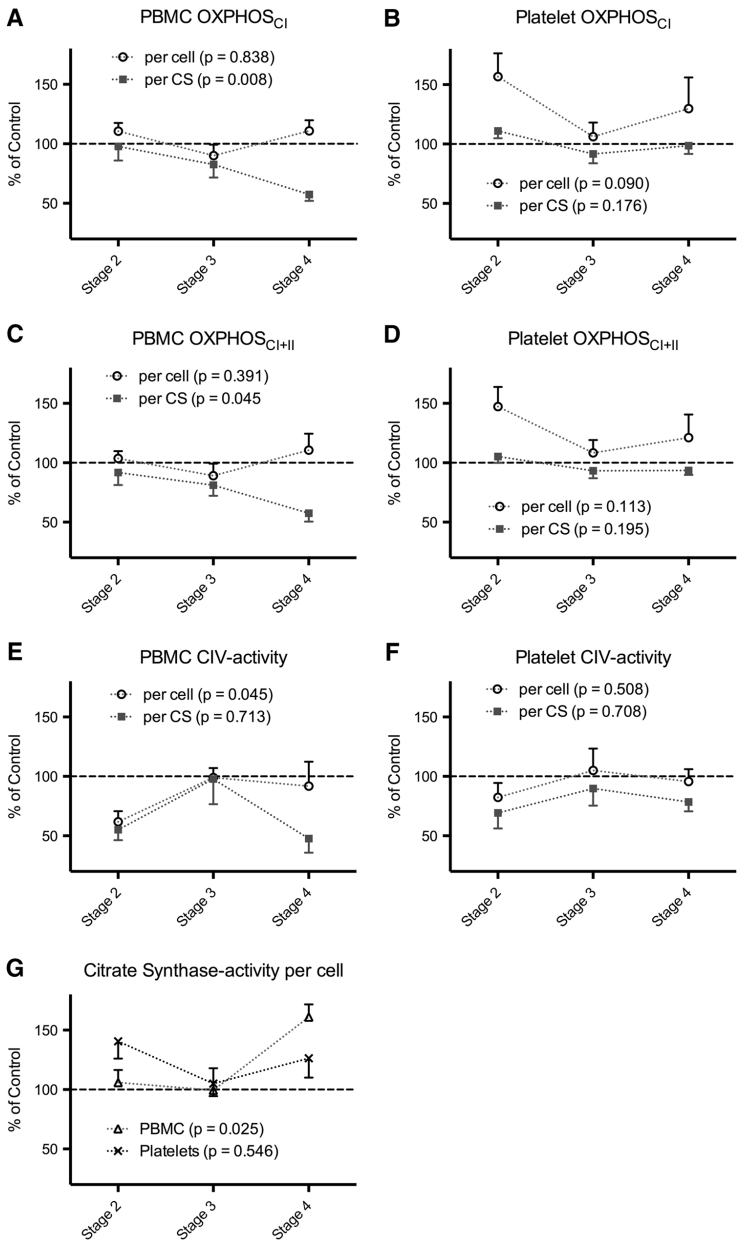

Fig. 3 Mitochondrial function and ALS disease stage. With progressing ALS disease stage, respiration coupled to ATP synthesis in PBMCs using convergent CI- and CII-linked substrate oxidation is gradually reduced (**c**), as is CI-linked respiration (**a**) when normalized to mitochondrial content. CIV-activity measured per cell displays an increasing trend with advancing stage in PBMCs, while no progression can be detected when normalized to mitochondrial content (**e**). Mitochondrial content, measured as CS-activity, is significantly increased with advancing ALS disease stage in PBMCs (**g**). In platelets, no significant trends could be demonstrated (**b**, **d**, **f**, **g**). All data are presented as mean and SEM, and expressed as percent of control. The zero-hypothesis that the distribution of data was equal across all ALS disease stage was rejected at p values <0.05 using the non-parametric Jonckheere–Terpstra test for ordered alternatives, with post hoc test of all pairwise comparisons. The number of subjects in each group (ALS Stage 2, 3 and 4, respectively) was 8, 5 and 5 for PBMCs and 11, 5 and 7 for platelets. *PBMC* peripheral blood mononuclear cells, *CS* citrate synthase, *OXPHOS* respiration associated with ATP synthesis by oxidative phosphorylation, *CI* complex I, *CII* complex II, *CIV* complex IV

When assessing the function of mitochondria in tissue or cells, it is crucial to discriminate between functional mitochondrial activity per cell (or mg tissue or protein) and activity per mitochondrial content. Several measures of mitochondrial respiration were increased in ALS patient cells per cell (Table 2), both in intact and in permeabilized

cells. At the level of mitochondria, however, the measure of mitochondrial function that was significantly altered was the activity of CIV, which was decreased. Hence, the alterations seen at cell level can be attributed to the increased mitochondrial content in the cells. The two independently measured markers of mitochondrial content used in this study, mtDNA copies per cell and CS-activity, correlated well.

Platelets have previously been proposed as markers for global mitochondrial function in other neurodegenerative disorders [3, 16, 18, 21, 24] and a few previous studies analyzing peripheral blood cells in ALS patients are relevant to the present findings. One study reported altered Ca^{2+} metabolism, but no ETS complex alterations in ALS patient lymphocytes [9], and the other study suggested a decrease in enzymatic CI-activity per mg of protein in the same cell type. In the latter study the investigators also saw a correlation between disease duration and reduced enzymatic CI-activity [12]. In cybrids generated from ALS patients' platelets, altered mitochondrial ultrastructure and decreased enzymatic activity of CI per mg have been shown [28]. These findings could not be confirmed in this study, but a relation between disease stage and CI-activity was found (see below). Altered mitochondrial membrane potential in platelets from ALS patients has also been reported [25], which further strengthens the notion of mitochondrial dysfunction in blood cells from ALS patients. Recently, reduced expression of mtDNA encoded genes has been reported in PBMCs from ALS subjects, and a corresponding alteration in post mortem spinal cord tissue was seen [17].

When patients were grouped according to clinical disease stage as described above, a significant progressive decrease in CI-associated respiratory parameters (normalized to mitochondrial content) was seen in PBMCs with advancing disease stage, suggesting a progressive alteration in mitochondrial function with the progression of the disease. Interestingly, this recently suggested staging [23] is solely based on hallmark clinical events for the individual patient, without any biomedical markers or biometric data. The progressing mitochondrial impairment with higher staging seen in the current study suggests biomedical validity to this staging. However, it should be noted that no change in CI-activity per mitochondrial content was observed when the total group of 24 was considered. Albeit different at group level, no changes in CIV function at the level of mitochondria could be shown with progressing disease stage, which argues for caution when interpreting the results. However, CIV function in ALS patients remained lower than in controls for all disease stages indifferent of normalization to mitochondrial content or cells. Echaniz-Laguna et al. [10] and colleagues investigated temporal alterations in muscle mitochondrial function in ALS patients using repeated biopsies, and showed increased CI-activity per dry weight from first to second sampling. No correction for mitochondrial content was made, and thus it can be postulated that this increase is due to increase in mitochondrial content, similar to our findings in platelets with progressing disease stage. In the current study, no correlation between any measure of mitochondrial function and disease duration could be shown.

In contrast to analyzing enzymatic activities of isolated respiratory complexes, we used ex vivo measurements of integrated mitochondrial activity of living respiring cells either suspended in their normal physiological environment (patients' own blood plasma) or permeabilized cells, in a buffer mimicking intracellular conditions, to enable for control of substrate access. By supplying the mitochondria with either CI- or CII-substrates or both, the role of individual complexes of the integrated mitochondrial function is analyzed. This approach has several advantages compared to measurements of the enzymatic activity of individual complexes, where the physiological context and function of the mitochondria are disrupted.

There are differences in cellular metabolism and mitochondrial function between platelets and PBMCs, differences that are reflected in the findings of this study. We show reduced CIV activity normalized to mitochondrial content in ALS patient blood cells in both platelets and PBMCs but in PBMCs only statistically significant for one of the two markers used. Under physiological conditions, there are lower levels of the catalytic subunit of CIV in platelets than in PBMCs, and thus platelets could provide a more sensitive marker for CIV dysfunction [15]. Further, platelet mitochondria habitually operate at a higher percentage of their maximum capacity relative to PBMCs, and a failure to comply with energy requirements would possibly result in a signal of mitochondrial biogenesis affecting platelets as they are produced and released to the circulation [15]. Platelets and lymphocytes also differ due to their hematopoietic origin, and through the lack of nuclei in platelets. PBMCs have all transcription mechanisms intact and can respond to external stimuli and stressors affecting metabolism by more elaborate means.

We conclude that mitochondrial content is increased and mitochondrial CIV-function/mitochondria is impaired in peripheral blood cells of ALS patients, further supporting the proposition that ALS is a systemic disease affecting mitochondria in cells outside the CNS rather than being a strictly neurodegenerative disorder [1, 20].

Further investigations in larger cohorts are warranted to elucidate the pathophysiological significance of the observed changes in mitochondrial content and function in ALS and to evaluate the potential of mitochondrial function as a future candidate biomarker for ALS.

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Conflicts of interest Johannes Ehinger, Magnus J. Hansson and Eskil Elmér own shares in NeuroVive Pharmaceutical AB, a public company active in the field of mitochondrial medicine. Saori Morota, Johannes Ehinger, Magnus J. Hansson and Eskil Elmér received salary support from NeuroVive Pharmaceutical AB during parts of the study. Gesine Paul has no conflict of interest to disclose.

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Paper II

Movement Disorders
CLINICAL PRACTICE

RESEARCH ARTICLE

Mitochondrial Respiratory Function in Peripheral Blood Cells from Huntington's Disease Patients

Johannes K. Ehinger, MD,[1,2,] Saori Morota, PhD,[1,3] Magnus J. Hansson, MD, PhD,[1,4] Gesine Paul, MD, PhD,[5,6] Eskil Elmér, MD, PhD[1,7]*

Abstract: **Background:** Patients with Huntington's disease display symptoms from both the central nervous system and peripheral tissues. Mitochondrial dysfunction has been implicated as part of the pathogenesis of the disease and has been reported in brain tissue and extracerebral tissues, such as muscle and blood cells, but the results are inconsistent. Therefore, the authors performed a refined evaluation of mitochondrial function in 2 types of peripheral blood cells from 14 patients with Huntington's disease and 21 control subjects. Several hypotheses were predefined, including impaired mitochondrial complex II function (primary), complex I function (secondary), and maximum oxidative phosphorylation capacity (secondary) in patient cells.
Methods: High-resolution respirometry was applied to viable platelets and mononuclear cells. Data were normalized to cell counts, citrate synthase activity, and mitochondrial DNA copy numbers.
Results: Normalized to citrate synthase activity, platelets from patients with Huntington's disease displayed respiratory dysfunction linked to complex I, complex II, and lower maximum oxidative phosphorylation capacity. No difference was seen in mononuclear cells or when platelet data were normalized to cell counts or mitochondrial DNA. The ratio of complex I respiration through maximum oxidative phosphorylation was significantly decreased in patients compared with controls. The corresponding ratio for complex II was unaffected.
Conclusions: The data indicate decreased function of mitochondrial complex I in peripheral blood cells from patients with Huntington's disease, although this could not be uniformly confirmed. The results do not confirm a systemic complex II dysfunction and do not currently support the use of mitochondrial function in blood cells as a biomarker for the disease.

Huntington's disease (HD) is an autosomal-dominant, progressive, neurodegenerative disease with a phenotype dominated by characteristic chorea, cognitive decline, and psychiatric symptoms. HD is caused by a cytosine-adenine-guanine (CAG) repeat expansion in the huntingtin (*HTT*) gene, resulting in contiguous glutamine residues and subsequent gain of function in the translated protein.[1] Mitochondrial dysfunction, as for several other neurodegenerative disorders, has been implicated in HD.[2] Several aspects of mitochondrial function, such as trafficking,[3] dynamics,[4] calcium handling,[5,6] and susceptibility for membrane permeability transition,[7] have been proposed to be affected by mutant *HTT*. In postmortem tissues from the central nervous regions (CNS) primarily affected in HD (putamen and nucleus caudatus), defects in electron transport system (ETS) complexes have been described. Several authors report dysfunction in respiration or enzymatic activity related to ETS complex

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II (CII), whereas there is controversy regarding the function of ETS complex IV (CIV).[8–11] A dysfunction of pyruvate dehydrogenase has also been suggested.[12]

HD is suggested to be a systemic disorder rather than a disease strictly confined to the CNS. In addition to brain pathology, HD patients exhibit symptoms from peripheral tissues, such as muscle wasting, weight loss, and testicular atrophy, arguably due to a direct effect of mutant HTT, because the expression of the protein is not restricted to the brain.[13] However, there is some controversy regarding whether mitochondrial dysfunction is present or detectable in tissues outside the primarily affected areas of the CNS. Several studies in postmortem tissue have failed to show general CNS defect of mitochondria in HD patients, and the reported findings are confined to the putamen and/or nucleus caudatus.[9,10,14] However, some authors do report mitochondria-related metabolic deficiencies in skeletal muscles, with increased lactate levels and decreased phosphocreatine recovery rate upon exercise,[15,16] and also mitochondrial ultrastructural alterations in HD fibroblasts and myoblasts.[17] Several studies report that HD-related alterations present in the CNS also can be detected in peripheral blood cells,[18–21] but others fail to do so.[8] In 1 study, primary fibroblasts from HD patients did not display any energetic deficiencies.[11] To our knowledge, there are 3 published reports on mitochondrial ETS function in peripheral blood from HD patients, 1 study reporting increased enzymatic CIV activity in patient platelets,[8] a second study reporting decreased ETS complex I (CI) function,[22] and a third refuting the findings in the latter study in a larger patient cohort.[23] Mitochondrial abnormalities not directly related to the function, expression, or structure of the ETS have repeatedly been demonstrated in lymphoblasts.[17,24–26] When platelets were used to generate cybrid (cytoplasmic hybrid) cells, no ETS dysfunction could be demonstrated.[27] In the current study, we set out to investigate the function of the ETS in HD patient blood cells using refined methodology applied to cells from 2 different hematopoietic origins.

With a cross-sectional approach, we have used high-resolution respirometry (oxygen consumption of mitochondria in living cells) to measure mitochondrial function ex vivo in human platelets and peripheral blood mononuclear cells (PBMCs) either in the patients' own plasma or in an assay buffer allowing for cell permeabilization and detailed substrate control. In contrast to the more widely used approach of assaying enzymatic activities of individual respiratory complexes in disrupted cells and mitochondria, when using respirometry, the integrated function and more of the physiologic and regulatory pathways of mitochondria are left intact. The objective of the study was to determine whether there are signs of mitochondrial dysfunction in peripheral blood cells from patients with HD and to evaluate whether this may serve as a biomarker for the disease.

We hypothesized that mitochondrial dysfunction in patients with HD could be demonstrated in peripheral blood cells using high-resolution respirometry ex vivo and that the dysfunction would correlate with the number of CAG repeats and/or clinical severity. Based on previously published observations, our primary hypothesis was that mitochondrial respiration based on CII-linked respiration of succinate is impaired in blood cells from HD patients. Furthermore, as secondary hypotheses, in blood cells from HD patients, we explored whether there were alterations in the respiration of nicotinamide adenine dinucleotide/CI-linked substrates, impairments in the maximum capacity of oxidative phosphorylation (OXPHOS), alterations in CIV function, changes in mitochondrial content, or reductions in pyruvate dehydrogenase function.

Patients and Methods

Chemicals

Monopotassium phosphate was purchased from Merck KGaA (Darmstadt, Germany), and Lymphoprep was purchased from Axis-Shield PoC AS (Oslo, Norway). All other chemicals were purchased from Sigma-Aldrich (St Louis, MO, USA).

Experimental Groups

The study was approved by the regional ethical review board of Lund University, Sweden (EPN 2011/89). Fourteen HD mutation-positive (40–46 uninterrupted CAG repeats) patients (ages 45–65 years; 5 men, 9 women) and 21 control subjects were recruited from the Neurology Clinic at Lund University Hospital after informed consent was obtained. Patients were divided into 2 groups based on a total functional capacity (TFC) score: early manifest HD (defined here as a TFC score of 7–13, stage I–II) and middle-to-end manifest HD (a TFC score of 0–6, stage III–V).[28,29] The TFC score is a global functional measurement with scale ranging from 0 to 13, with higher scores indicating better functionality. The control cohort consisted of relatives or caretakers (ages 38–82 years) of patients admitted to the Neurology Clinic without known neurological disorder. The control group was not specifically age or gender matched to the HD cohort. For demographic data, see Table 1.

Sample Preparation

Venous blood samples were drawn (24 mL) in K_2 ethylene diamine tetraacetic acid (EDTA) tubes (BD Vacutainer; BD, Plymouth, United Kingdom) and analyzed within the same day (4–9 hours). Erythrocytes and leukocytes were loosely pelleted by centrifugation at 400*g* for 10 minutes at room temperature, leaving a platelet-rich plasma (PRP). The PRP was pipetted off and centrifuged at 4600*g* for 5 minutes at room temperature, producing a close to cell-free plasma and a platelet pellet. The platelet pellet was dissolved in 1 or 2 mL of its own plasma by gentle pipetting to obtain a highly enriched PRP.

The loose pellet containing erythrocytes and leukocytes 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

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TABLE 1 Clinical and Demographic Data of Research Subjects

Subjects	Men/Women	Mean ± SD (Range)					
		Age, y	CAG Repeats	Duration, y	TFC Score (0–13)	FA Score (0–25)	IS Score (10–100)
Controls	6/15	64.2 ± 11.7 (38–82)					
All HD patients	5/9	56.6 ± 6.7 (45–65)	42.7 ± 1.7 (40–46)	6.5 ± 4.9 (0–16)	5.4 ± 5.3 (0–13)	11.3 ± 11.2 (0–25)	56.8 ± 37.1 (15–100)
TFC score							
7–13	4/1	50.2 ± 4.8 (45–58)	42.8 ± 1.3 (41–44)	2.2 ± 1.6 (0–4)	12.0 ± 1.2 (10–13)	24.4 ± 0.9 (23–25)	100.0 ± 0.0 (100)
0–6	4/5	60.1 ± 4.8 (50–65)	42.6 ± 2.0 (40–46)	8.9 ± 4.5 (4–16)	1.8 ± 1.9 (0–5)	4.0 ± 6.1 (0–16)	32.8 ± 20.6 (15–60)

SD, standard deviation; CAG, cytosine-adenine-guanine; TFC, total functional capacity; FA, functional assessment; IS, independence scale; HD, Huntington's disease.

20 to 30 minutes at room temperature to yield a lymphocyte layer. The layer was pipetted off and resuspended in saline, followed by centrifugation at 250*g* for 5 minutes at room temperature. The supernatant was removed, and the lymphocyte pellet was resuspended in 100 to 200 µL saline containing approximately 20% to 30% plasma. The lymphocyte suspension contained up to 30% granulocytes and midsize cells (monocytes, eosinophils, and basophils, etc.). Cell concentrations were measured using a Swelab Alfa automated hemocytometer (Swelab, Stockholm, Sweden).

Mitochondrial Respiration

The mitochondrial respiratory capacity of platelets and PBMCs was measured using a high-resolution respirometer (Oxygraph-2k; Oroboros Instruments, Innsbruck, Austria) to monitor real-time oxygen consumption rate. Two protocols were used, either with intact cells in their own plasma or with permeabilized cells in a buffer mimicking intracellular conditions (MiR05)[30] at a final concentration of 200 × 10^6 platelets/mL or 5 × 10^6 lymphocytes/mL.

The experimental protocol with intact cells in plasma examines mitochondrial respiratory capacity with endogenous substrates. Cells were suspended in their own plasma and allowed to stabilize at routine respiration ($ROUTINE_{plasma}$) controlled by cellular energy demand and turnover of the oxidative phosphorylation. Adenosine triphosphate (ATP) synthase was inhibited by oligomycin (1 µg/mL) to examine the oxygen consumption rate independent of ADP phosphorylation, predominantly due to proton leak over the inner mitochondrial membrane ($LEAK_{intact}$). Subsequently, carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) was titrated to induce maximal capacity of the electron transport system (ETS_{intact}). The experiment was terminated by adding rotenone (2 µM), antimycin (1 µg/mL), and azide (10 mM), which are CI, CIII, and CIV inhibitors, respectively, revealing nonmitochondrial oxygen consumption. The data on mitochondrial function that were generated using this protocol are subject to influence from any upstream factor present in plasma or cells that could affect the mitochondrial function.

We used a protocol with permeabilized cells in MiR05 buffer, the addition of substrates and inhibitors, and the titration of an uncoupler to examine mitochondrial respiratory capacity with electron flow through CI and CII separately as well as convergent electron input via the Q-junction (CI + CII).[30,31] After stabilization of routine respiration ($ROUTINE_{MIR05}$), digitonin (1 µg per 10^6 platelets or 6 µg per 10^6 lymphocytes) was added to permeabilize the plasma membrane and allow mitochondria to access exogenous substrates. The CI substrates malate and pyruvate were added at saturating concentration (5 mM each), and oxidative phosphorylation (OXPHOS) capacity was evaluated by adding adenosine diphosphate (ADP) (1 mM) ($OXPHOS_{CI\ [MP]}$) and subsequently adding 5 mM of glutamate ($OXPHOS_{CI\ [MPG]}$). Thereafter, succinate (10 mM) was added to induce maximum OXPHOS capacity with convergent input through both CI and CII ($OXPHOS_{CI\ +\ CII}$). The ATP synthase was inhibited by oligomycin (1 µg/mL), revealing leak respiration ($LEAK_{CI\ +\ CII}$) followed by FCCP titration, inducing maximal convergent respiratory capacity of the ETS ($ETS_{CI\ +\ CII}$). CI was inhibited by rotenone (2 µM) to evaluate ETS capacity with electron flow through CII (ETS_{CII}), followed by antimycin (1 µg/mL) to inhibit CIII and stop electron transfer through the ETS (revealing nonmitochondrial oxygen consumption). CIV activity was evaluated by adding tetramethylphenylenediamine (TMPD) (10 mM), followed by the CIV inhibitor sodium azide at 10 mM. CIV activity was calculated by deducting the value after the addition of azide to that before the addition. The content of each oxygraph chamber (2 mL) was stored at −20°C and subsequently thawed and sonicated on ice (30 seconds for lymphocytes and 2 sets of 30 seconds for platelets; Ultrasonic homogenizer 4710 Series; Cole-Parmer Instrument Company LLC, Vernon Hills, IL) for further analyses.

The contribution of CII to convergent oxidative phosphorylation was calculated by subtracting $OXPHOS_{CI\ (MPG)}$ (the maximum ADP-stimulated respiration with only CI substrates) from $OXPHOS_{CI\ +\ CII}$ (the same conditions but with succinate present), yielding a net increase in oxygen consumption when succinate was added ($OXPHOS_{CII}$).

Respiratory Ratios

To further investigate the role of the metabolic pathways through either CI or CII in the ETS, respiratory ratios were

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calculated. $OXPHOS_{CI\ (MPG)}/OXPHOS_{CI\ +\ CII}$ indicates the relative contribution of CI to convergent phosphorylating respiration, and $ETS_{CII}/ETS_{CI\ +\ CII}$ is the contribution of CII to maximum uncoupled respiration. The calculated ratios are independent of cellular and mitochondrial content of the analyzed samples.

Pyruvate Dehydrogenase Function

The substrate combination of malate and pyruvate supports CI-linked mitochondrial respiration through the conversion of malate to oxaloacetate by malate dehydrogenase (measured by $OXPHOS_{CI\ [MP]}$, as described above) and the conversion of pyruvate to acetyl-coenzyme A (CoA) via pyruvate dehydrogenase (PDH). Condensation of acetyl-CoA and oxaloacetate yields citrate. It has been reported that PDH is defective in HD[12] and if so, it could be rate limiting for this reaction. To evaluate this possibility, glutamate was added to cells respiring on malate and pyruvate (yielding the respiratory state $OXPHOS_{CI\ [MPG]}$). Through transaminases in the malate-aspartate shuttle, glutamate and oxaloacetate are converted to α-ketoglutarate and aspartate, replenishing the TCA cycle and potentially relieving the limitation by a reduced PDH capacity. A ratio was calculated between the respiratory states without and with glutamate present ($OXPHOS_{CI\ [MP]}/OXPHOS_{CI\ [MPG]}$) to assess the function of PDH.

Citrate Synthase Activity

The enzymatic activity of citrate synthase (CS) was measured spectrophotometrically (model 680 Microplate Reader; Bio-Rad Laboratories, Hercules, CA, USA) using the Citrate Synthase Assay Kit (Sigma-Aldrich, St Louis, MO, USA). Samples were analyzed in at least duplicate in a 96-well plate in an assay buffer containing 300 μM acetyl CoA and 100 μM 5,5-dithiobis-(2-nitrobenzoic acid). In a plate reader set to 412 nm on a kinetic program with 1.5-minute duration and 10-second intervals, the absorbance of the baseline reaction was measured. Subsequently, 500 μM of oxaloacetate were added to each well, and absorbance was again measured. Calculations of CS activity were performed according to the manufacturer's instructions.

Mitochondrial DNA Content

Copy numbers of mitochondrial DNA (mtDNA) were measured as previously described.[32] Frozen samples were thawed and diluted 500 times in TE buffer (10 mM TRIS-HCl; 1 mM EDTA; and 1 ng/μL salmon sperm DNA, pH 8.0). Ten microliters of this dilution were amplified in a 25-μL polymerase chain reaction (PCR) reaction containing 1 × Power SYBR Green PCR Master Mix using an ABI Prism 7000 real-time PCR machine (Applied Biosystems Inc., Foster City, CA, USA) and 100 nM of each primer (Eurofins MWGoperon, GmbH, Ebersberg, Germany). The primers targeted the human mitochondrial cytochrome c oxidase I (COX1) gene (forward: CCC CTG CCA TAA CCC AAT ACC A; reverse: CCA GCA GCT AGG ACT GGG AGA GA). The threshold cycle (Ct) values were related to a standard curve using cloned PCR products (kindly provided by P. Schjerling, University of Copenhagen, Copenhagen, Denmark). Samples were analyzed in pentaplicates.

Statistics

Statistical comparisons were performed with *GraphPad PRISM* (versions 5.01 and 6.0d; GraphPad Software, La Jolla, CA, USA) using nonparametric analyses because of the small group sizes (the Mann–Whitney test was used for comparisons of 2 groups, and the Kruskal–Wallis test with Dunn's multiple comparison test was used for comparisons of 3 groups). Statistical analysis was only performed for the parameters that were considered relevant for answering 1 or more of the hypotheses of the study. The results were considered statistically significant at $P < 0.05$.

Results

Only data that were considered instrumental to answer 1 or more of the predefined hypotheses were analyzed statistically; as a result, only data from the permeabilized cell protocol were further processed. Complete raw data from the assessment of respiratory function in both intact and permeabilized cells are provided in Table 2. All respiratory data were adjusted for nonmitochondrial respiration.

CII-Linked Mitochondrial Respiration

Both the net increase in oxygen consumption when succinate was added to cells respiring on CI substrates with active ATP-synthase ($OXPHOS_{CII}$) and rotenone-inhibited, uncoupled respiration (ETS_{CII}) were analyzed and normalized to cell count, CS activity, and mtDNA copy number, respectively. In all, 2 of 12 measures of CII activity differed significantly between the HD group and the control group. In platelets, ETS_{CII} normalized for CS activity was decreased in HD patients; and, in PBMCs, $OXPHOS_{CII}$ normalized for cell count was increased (Fig. 1A,C). When patients with HD were grouped based on TFC score, the same 2 parameters were significantly different between the control group and the group with TFC scores from 0 to 6 (Fig. 1B,D).

CI-Linked Mitochondrial Respiration

CI-linked respiration with a combination of the substrates malate, pyruvate, and glutamate ($OXPHOS_{CI\ [MPG]}$) was significantly reduced in platelets from patients with HD compared with platelets from the control group when normalized for CS

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activity, but not in PBMCs. When grouped according to TFC score, $OXPHOS_{CI\ (MPG)}$ in platelets was decreased both in the group with TFC scores from 0 to 6 and in the group with TFC scores from 7 to 13. There was no difference between the 2 disease-severity groups. When normalizing for cell count and for mtDNA content, there were no significant differences in CI-linked respiration (Fig. 1E,F).

Maximum OXPHOS Capacity

$OXPHOS_{CI\ +\ CII}$ depicts the maximum respiratory capacity of the mitochondria under conditions with saturating levels of ADP and substrates supplied to both the CI and the CII pathways. Both platelets and PBMCs were analyzed and normalized to cell count, CS activity, and mtDNA copy number. The $OXPHOS_{CI\ +\ CII}$ normalized for CS activity was reduced in HD platelets, but no other differences could be observed. To further analyze this finding, the remaining platelet respiratory parameters normalized for CS activity were analyzed. The finding was not specifically driven by either CI-linked or CII-linked respiration, because parameters linked to both pathways were reduced (Fig. 1G,H).

CIV Activity

No significant differences in mitochondrial CIV activity between the patient and control groups could be demonstrated in either platelets or PBMCs, regardless of mode of normalization, and this also applied when patients were grouped according to TFC score (Fig. 1I,J).

Relative Respiratory Complex Contributions to Maximal Respiration Rates

Calculated respiratory ratios were independent of cellular and mitochondrial content of the analyzed samples and were used to qualitatively assess the relative function of CI and CII to maximal phosphorylating and nonphosphorylating respiration. The ratio $OXPHOS_{CI\ (MPG)}/OXPHOS_{CI\ +\ CII}$ was significantly lower in platelets from the HD group compared with the control group, but not so in PBMCs. However, when grouped according to TFC score, this ratio was significantly lower in the group with TFC scores from 0 to 6 than in both the group with TFC scores from 7 to 12 and the control group in PBMCs, but not in platelets (Fig. 2A,B). The ratio $ETS_{CII}/ETS_{CI\ +\ CII}$ did not differ between the HD and control groups in any of the cell types (Fig. 2C,D).

Cellular Mitochondrial Content

There were no significant differences in mtDNA copy number per cell or CS activity in either PBMCs or platelets from the HD patient group compared with the control group (Table 2, Fig. 3A). No differences could be detected when patients were grouped according to TFC score (Fig. 3B). There was no difference in fractions of different types of PBMCs (percentages of lymphocytes and monocytes) between the control and HD groups (data not shown). CS activity and mtDNA copy number were correlated for both the control group and the HD patient

TABLE 2 Mitochondrial Respiration and Mitochondrial Content of Peripheral Blood Cells from Huntington's Disease Patients and Control Subjects*

Variable	Mean ± SD			
	Platelets		PBMCs	
	Control	HD	Control	HD
Intact cells				
No.	21	14		
$ROUTINE_{plasma}$	10.41 ± 2.63	10.06 ± 3.93		
$LEAK_{intact}$	−0.04 ± 0.66	0.26 ± 0.86		
ETS_{intact}	16.65 ± 3.51	15.15 ± 4.61		
Permeabilized cells				
No.	21	14	19	13
$ROUTINE_{MiR05}$	8.27 ± 3.03	8.95 ± 4.27	3.28 ± 0.62	3.53 ± 0.88
$LEAK_{CI\ +\ CII}$	4.98 ± 0.90	5.08 ± 1.49	1.93 ± 0.58	2.21 ± 0.52
$OXPHOS_{CI\ (MP)}$	19.32 ± 4.91	18.58 ± 6.74	7.65 ± 1.28	8.13 ± 1.62
$OXPHOS_{CI\ (MPG)}$	22.08 ± 5.50	20.24 ± 7.09	7.86 ± 1.37	8.28 ± 2.06
$OXPHOS_{CI\ +\ CII}$	33.84 ± 7.23	33.66 ± 11.01	12.56 ± 1.83	13.79 ± 2.52
$ETS_{CI\ +\ CII}$	34.20 ± 6.79	31.68 ± 11.35	10.84 ± 2.40	11.61 ± 3.05
ETS_{CII}	16.14 ± 2.81	15.84 ± 4.69	5.36 ± 0.98	6.19 ± 1.82
CIV activity	39.55 ± 9.45	38.49 ± 15.27	8.15 ± 2.24	8.87 ± 2.71
CS activity	6.71 ± 1.14	7.10 ± 2.78	1.46 ± 0.57	1.92 ± 0.87
mtDNA[a]	34 ± 7	36 ± 13	1.568 ± 694	1.939 ± 745

*Respiratory data are corrected for nonmitochondrial respiration and are expressed in pmol O_2/second/100 × 10^6 platelets or 10^6 peripheral blood mononuclear cells (PBMCs). Citrate synthase (CS) activity is depicted as µmol CS/min/100 × 10^6 platelets or 10^6 PBMCs.
[a]Mitochondrial DNA (mtDNA) values indicate copies per cell.
SD, standard deviation; HD, Huntington's disease; ROUTINE, endogenous respiration in plasma or MiR05 medium; LEAK, idle respiration without ATP-synthase activity; ETS, respiration associated with maximal protonophore-stimulated flux through the electron transport system; CI, electron transport system complex I; CII, electron transport system complex II; OXPHOS, respiration associated with ATP synthesis by oxidative phosphorylation; MP, malate and pyruvate; MPG, malate, pyruvate, glutamate and ADP; CIV, electron transport system complex IV.

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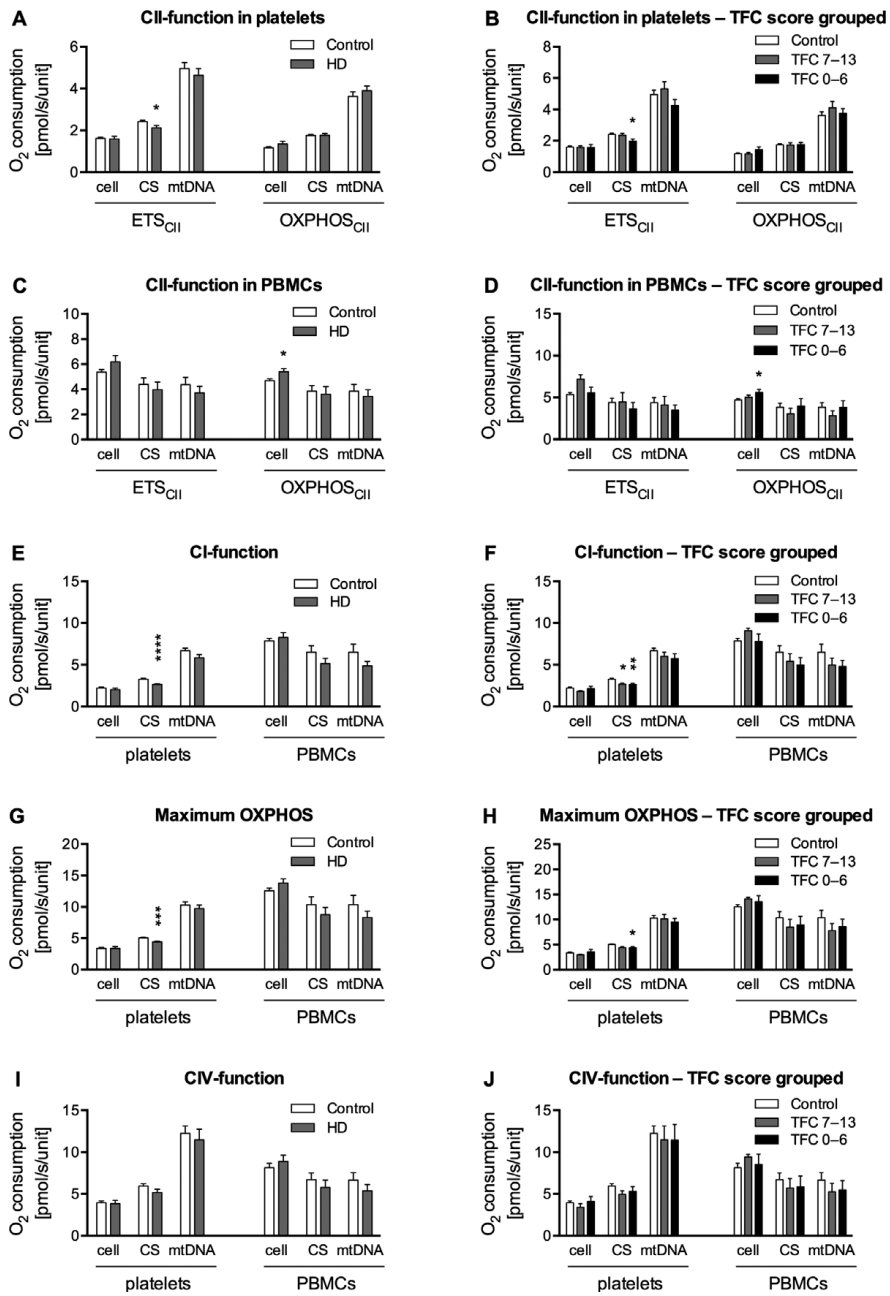

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Figure. 1 A–J: Respiratory data are expressed either as pmol O_2/second/10 × 10^6 platelets or 10^6 peripheral blood mononuclear cells (PBMCs) (oxygen consumption per cell), as pmol O_2/second/(μmol citrate synthase [CS]/mL/minute) (oxygen consumption normalized for citrate synthase [CS] activity), or as pmol O_2/second/10^9 mitochondrial DNA (mtDNA) copies (oxygen consumption normalized for mitochondrial DNA [mtDNA] copy number). All respiratory data are corrected for nonmitochondrial respiration. *P* values are from nonparametric Mann–Whitney *U* tests for comparisons of 2 groups and from nonparametric Kruskal–Wallis tests of variance with Dunn's post hoc test for comparison between all groups for analyses of 3 groups. The oxidative phosphorylation capacity (respiration associated with ATP synthesis by oxidative phosphorylation [OXPHOS]) for electron transport system complex II (CII) ($OXPHOS_{CII}$) is calculated by subtracting the OXPHOS for electron transport system complex I (CI) with the addition of glutamate ($OXPHOS_{CI\ [MPG]}$) from $OXPHOS_{CI\ +\ CII}$. Data are presented as means and standard errors. ETS, respiration associated with maximal protonophore-stimulated flux through the electron transport system; HD, Huntington's disease; TFC, total functional capacity; CIV, electron transport system complex IV.

group in both cell types, more strongly so in PBMCs than in platelets (Fig. 3C,D).

Pyruvate Dehydrogenase Function

No difference in the ratio ($OXPHOS_{CI\ [MP]}$/$OXPHOS_{CI\ [MPG]}$) was observed in PBMCs between the control and HD groups; whereas, in platelets, the ratio was significantly higher in the HD group; thus, the results did not support the hypothesis of decreased PDH capacity in HD patient cells (Fig. 2E,F).

Correlation Between Disease Progression and Mitochondrial Function

Results from stratification of patients according to TFC score are reported under each of the sections above. In addition, correlations between the different measures of mitochondrial function were made to CAG repeats and disease duration without any significant findings. There was a correlation ($R^2 = 0.56$; $P < 0.01$) between TFC score and disease duration (data not shown).

Discussion

In the current study, we assessed mitochondrial respiratory function in blood cells (platelets and PBMCs) from patients with HD compared with a cohort of healthy volunteers. Before analyzing data, we defined a primary hypothesis and a set of secondary hypotheses based on previous reports of mitochondrial dysfunction in HD. We evaluated respiration with substrates for CII and CI, maximum OXPHOS capacity, maximum CIV activity, relative CI-dependent and CII-dependent respiratory ratios, cellular mitochondrial content, and PDH function. None of the hypotheses could be uniformly confirmed.

Our primary hypothesis was that a defect in mitochondrial CII-linked succinate metabolism could be shown in peripheral blood cells from patients with HD. It has repeatedly been reported that CII enzymatic activity is reduced in the basal ganglia of HD patients and that the levels of structural proteins of the same complex are lower than those for relevant controls.[8–10,14]

Peripheral blood cells have been proposed as markers for global mitochondrial function in neurodegenerative disorders,[33–40] and platelets also have been evaluated previously in HD.[8,22,23] The results regarding ETS function, however, are inconsistent and are not consonant with findings in the affected brain regions, because reports regarding the ETS in blood cells suggest impaired function of CI rather than CII. Here, we have used cells from 2 hematopoietic origins, PBMCs and platelets, to more comprehensively evaluate whether systemic mitochondrial alterations are present in blood cells from patients with HD. In addition to data normalized to cell count, we normalized to 2 independent measures of mitochondrial content: CS activity and mtDNA copy number.

In previous reports regarding mitochondrial function in blood cells from patients with HD, the results are contradictory. One study reported a marked decrease in enzymatic CI activity per milligram of mitochondrial protein in isolated mitochondria from platelets in 5 patients with HD,[22] whereas another study that included 11 patients did not find any mitochondrial alterations in platelet CI activity normalized for either protein content or CS activity.[8] A later study comprised a larger number of patients (n = 21) than the previous studies and was powered to exclude a reduction in CI activity by >10% with 80% confidence, but as it appears, not a priori. CI was assayed enzymatically but not normalized to CS activity.[23] The present study differs from previous reports, in that we are using living cells with respiring mitochondria, measuring integrated mitochondrial function with less disruption of intracellular pathways, and analyzing data using several methods of normalization.

Permeabilized platelets respiring on CI substrates displayed a highly significant ($P < 0.0001$) decrease in activity per cell compared with control cells; and, when patients were grouped according to disease severity, there were significant differences between each of the patient groups and the control group. However, when using mtDNA as the normalization method, no significant differences were detectable. No significant differences were present in PBMCs. In platelet data, CII function and maximum OXPHOS also were significantly decreased when normalized for CS activity, but there was no consistency using mtDNA as the normalization method. To further evaluate the role of the 2 main metabolic pathways into the ETS, via CI or CII, we calculated respiratory ratios for the contribution of either pathway. Data indicated lower activity of CI in HD cells, but the results were not consistent for the 2 cell types.

The significant findings in platelets appear to be a normalization effect driven by generally higher CS values in HD patients' platelets, because no significant difference was observed in any

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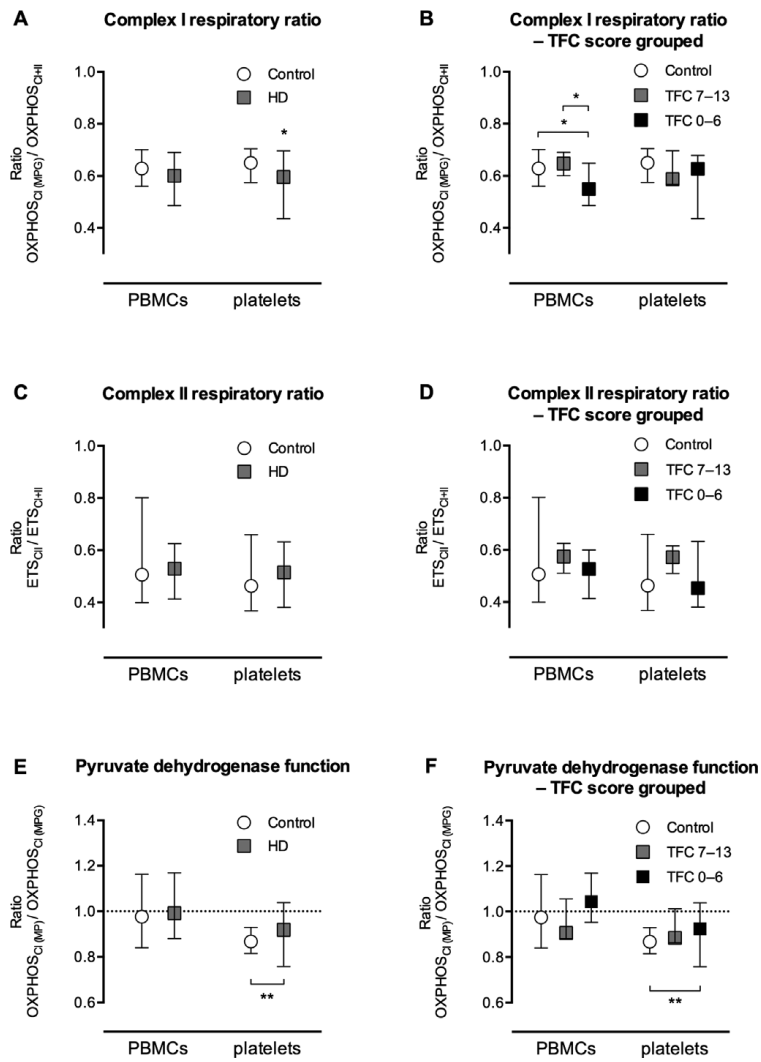

Figure. 2 A–D: Respiratory ratios are illustrated for the relative contribution of metabolism of electron transport system complex I (CI) and electron transport system complex (CII) substrates, respectively, in patients with Huntington's disease (HD) compared with controls. E,F: The function of pyruvate dehydrogenase was assessed by comparing the ratio of the respiration associated with ATP synthesis by oxidative phosphorylation (OXPHOS) for CI with the addition of ADP and the OXPHOS for CI with the addition of glutamate ($OXPHOS_{CI\ [MP]}/OXPHOS_{CI\ [MPG]}$) between HD patients and controls. If pyruvate dehydrogenase is rate-limiting to the mitochondrial respiration for patients with HD, then an increase in respiration should be seen by the addition of glutamate, yielding a low ratio. Quite the contrary, an increased ratio compared with controls was seen in platelets, and no difference could be detected in peripheral blood mononuclear cells (PBMCs). Data are presented as medians and ranges of ratios. ETS, respiration associated with maximal protonophore stimulated flux through the electron transport system; TFC, total functional capacity.

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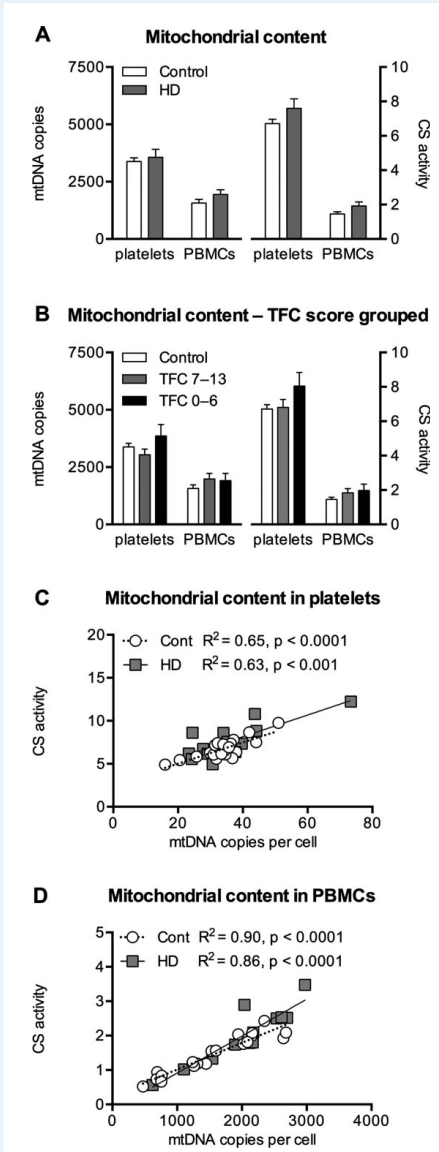

Figure. 3 Citrate synthase (CS) activity is depicted in μmol CS/minute/100 × 10^6 cells in platelets and per 10^6 cells in peripheral blood mononuclear cells (PBMCs). Mitochondrial DNA (mtDNA) in A and B is given as copies per 100 cells in platelets and as copies per cell in PBMCs. C, D: Correlation between CS activity and mtDNA copy number in platelets and PBMCs, respectively. Data are presented as means and standard errors. HD, Huntington's disease; TFC, total functional capacity; Cont, control; R^2, correlation coefficient.

parameter when we used mtDNA as the normalization method. The 2 markers of mitochondrial content were correlated (Fig. 3), and we would expect a mitochondrial dysfunction of biologic relevance to be detectable using either normalization method. However, the respiratory ratios indicated a lower contribution from CI-linked substrate metabolism in HD patients than in controls, but these results were not unequivocal (Fig. 2A–D).

Mutant *HTT* is considered to be ubiquitously expressed in peripheral tissue in HD, and peripheral symptoms observed in HD are generally attributed to this.[41] Expression of mutant *HTT* has been experimentally demonstrated in lymphoblasts,[42] and there is evidence that HTT plays a role in hematopoiesis[43]; however, to our knowledge, there are no data published regarding the expression of mutant *HTT* in megakaryocytes or presence of the translated protein in platelets. Few findings from basal ganglia in HD patients have been replicated in blood cells, but there is 1 report of upregulation of a set of mRNAs in both putamen and platelets discerning HD patients from controls, which could indicate common pathophysiological mechanisms. The study did not link the mRNA subset presented to any specific changes in cell metabolism and was not related to mitochondrial function.[19]

Limitations of the current study include relatively small group sizes, which increased the risk of both type I and type II errors. Because the study was not a priori power calculated, no definite refutation of any of the hypotheses can be made. The control group was not age or gender matched because it was recruited as a joint control group for a research project comprising other neurodegenerative disorders. We previously demonstrated that there was no detectable difference in mitochondrial respiration in blood cells between males and females.[31]

We conclude that, using high-resolution respirometry, no recurrently reported alteration of the ETS in affected brain regions could be uniformly confirmed in peripheral blood cells from patients with HD, even though we observed indications of decreased function of mitochondrial complex I. The results can neither confirm nor exclude mitochondrial dysfunction in peripheral tissues in HD and thus do not currently support the use of mitochondrial function in blood cells as a biomarker for the disease.

Author Roles

1. Research Project: A. Conception, B. Organization, C. Execution; 2. Statistical Analysis: A. Design, B. Execution, C. Review and Critique; 3. Manuscript Preparation: A. Writing the First Draft, B. Review and Critique.

J.K.E.: 1A, 1B, 1C, 2A, 2B, 3A, 3B
S.M.: 1B, 1C, 2A, 2B, 3A, 3B
M.J.H.: 1A, 1B, 1C, 2C, 3B
G.P.: 1A, 1B, 1C, 2C, 3B
E.E.: 1A, 1B, 1C, 2C, 3B

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Disclosures

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Cell-permeable succinate prodrugs bypass mitochondrial complex I deficiency

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Mitochondrial complex I (CI) deficiency is the most prevalent defect in the respiratory chain in paediatric mitochondrial disease. This heterogeneous group of diseases includes serious or fatal neurological presentations such as Leigh syndrome and there are very limited evidence-based treatment options available. Here we describe that cell membrane-permeable prodrugs of the complex II substrate succinate increase ATP-linked mitochondrial respiration in CI-deficient human blood cells, fibroblasts and heart fibres. Lactate accumulation in platelets due to rotenone-induced CI inhibition is reversed and rotenone-induced increase in lactate:pyruvate ratio in white blood cells is alleviated. Metabolomic analyses demonstrate delivery and metabolism of [^{13}C]succinate. In Leigh syndrome patient fibroblasts, with a recessive *NDUFS2* mutation, respiration and spare respiratory capacity are increased by prodrug administration. We conclude that prodrug-delivered succinate bypasses CI and supports electron transport, membrane potential and ATP production. This strategy offers a potential future therapy for metabolic decompensation due to mitochondrial CI dysfunction.

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Paediatric mitochondrial disease due to complex I (CI) deficiency is a heterogeneous group of disorders, and can be due to alterations in either the nuclear or mitochondrial genome. It is the most prevalent defect in the respiratory chain in paediatric patients and often leads to serious or fatal neurological presentations, such as Leigh syndrome[1]. There are currently very limited evidence-based treatment options directed towards mitochondrial respiratory chain dysfunction[2,3]. Succinate is a mitochondrial substrate metabolized through complex II (CII). It is not cell membrane-permeable and exogenously given succinate has limited uptake into cells.

Here we describe that cell membrane-permeable prodrugs of succinate provide increased ATP-linked mitochondrial oxygen consumption in CI-deficient human cells and tissues, which offers a potential future intervention for patients with metabolic decompensation due to mitochondrial CI dysfunction.

Results

Drug development and screening. In a drug discovery program, >50 different prodrugs of succinate[4] were designed, synthesized and evaluated for cell permeability and ability to support respiration independent of CI in human peripheral blood cells from healthy donors (platelets and mononuclear cells (PBMCs)) using an Oroboros O2k respirometer. Three compounds were selected for further evaluation: NV101-118 (NV118, diacetoxymethyl succinate), NV101-189 (NV189, bis-(1-acetoxy-ethyl) succinate) and NV101-241 (NV241, 1-acetoxyethyl acetoxymethyl succinate) (Fig. 1a). This article focuses on NV189, but qualitatively the results for all three prodrugs were similar and data on the other compounds are presented as Supplementary Figs.

Increased CII-linked respiration. At 100 μM, NV189 increased mitochondrial oxygen consumption in intact platelets with CI inhibition induced by the mitochondrial toxin rotenone (2 μM). Neither succinate nor monomethyl succinate, a monoester of succinate previously reported to be cell permeable[5], increased mitochondrial respiration (Fig. 1b; Supplementary Fig. 1a). In cells with normal CI function, oxygen consumption was also increased upon addition of 100 μM NV189 (Fig. 1c; Supplementary Fig. 1b). To exclude the possibility that increased respiration was due to an induction of proton leak through the mitochondrial inner membrane (uncoupling), the platelets were treated with the ATP synthase inhibitor oligomycin. This prompted a significant decrease in oxygen consumption, which indicates the extent of respiration linked to ADP phosphorylation (Fig. 1c; Supplementary Fig. 1b). Increased substrate supply, rather than uncoupling, was further demonstrated by measuring mitochondrial inner membrane potential with the positively charged membrane-permeable probe tetramethylrhodamine methyl ester (TMRM) in non-quench mode using fluorescence-activated cell sorting. TMRM fluorescence was increased in CI-inhibited human platelets upon addition of 250 μM NV189 and fluorescence increased further with ATP synthase inhibition, indicating mitochondrial membrane hyperpolarization (Fig. 1d). Cells with maximal uncoupled respiratory chain activity via titration of the protonophore carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) increased oxygen consumption even more with addition of 250 μM NV189, further indicating increased substrate supply to the respiratory chain (Fig. 1e; Supplementary Fig. 1c). In blood cells, pre-permeabilized with the detergent digitonin, 250 μM NV189 did not induce any increase in respiration, while succinate control did, showing the need for intracellular metabolism for succinate to be released and made available to the mitochondria (Fig. 1f; Supplementary Fig. 1d). To confirm that the increase in respiration is specifically due to respiration through CII, a cell-permeable prodrug of the CII inhibitor malonate, NV01-161, (NV161, diacetoxymethyl malonate, Fig. 1h) was designed, synthesized and evaluated (Supplementary Fig. 2). Intact cells exposed to succinate prodrugs were treated with NV161 with ensuing decrease in respiration (Fig. 1g; Supplementary Fig. 1e). The applicability of the platelet data to other cell types was evaluated by assessing respiration in PBMCs treated with NV189 with or without CI inhibition with similar results to those in platelets (Fig. 1i,j; Supplementary Fig. 1f,g).

Paediatric mitochondrial diseases primarily display symptoms from energy intense organs such as the liver, brain, muscles, retina and cochlea. In some reports, 30–40% of paediatric patients with respiratory chain CI dysfunction present with cardiomyopathy[6,7], a condition that is linked to higher mortality[8]. Human atrial heart muscle biopsies from elective surgery were acquired and the fibres gently separated using forceps. The fibres were incubated with the CI inhibitor rotenone and subsequently treated with succinate prodrug, eliciting an increase in oxygen consumption (Fig. 1k; Supplementary Fig. 1h).

Attenuated lactate production. A hallmark of mitochondrial disease is lactic acidosis. When the mitochondrial energy production fails to comply with demand, pyruvate is converted to lactate to maintain the NAD^+ pool, causing increased lactate levels and decreased pH in blood and cerebrospinal fluid in the patients. About 80% of patients with mitochondrial disease show signs of lactate accumulation[6,8,9]. We incubated human platelets with or without 2 μM rotenone and measured lactate accumulation in media over time. With CI inhibition, the cells displayed a significantly higher lactate production than control, 4.30 ± 0.24 μmol lactate per 10^9 cells per hour compared with control level 1.73 ± 0.5 (regression slope ± s.d.), but with incubation with NV189 the rotenone-induced lactate production was similar to control level (1.26 ± 0.19). To verify the viability of the cell preparation, the glycolytic pathway upon drug addition and the specificity of CII-mediated ATP supply, cells were incubated with NV189, rotenone and an inhibitor of the downstream respiratory chain complex III (antimycin A, 1 μg ml^{-1}), eliciting lactate production at the level of that of rotenone alone (4.44 ± 0.19; Fig. 1m,n; Supplementary Fig. 1i).

Metabolomics confirms metabolism of delivered succinate. To elucidate the intracellular metabolism of NV189, a metabolomic assay was performed on PBMCs from four healthy donors. Cells were incubated with or without rotenone and with or without NV189 for 20 min. Using quantitative capillary electrophoresis mass spectrometry (CE-MS), the concentrations of 116 metabolites were determined. Delivery of intracellular succinate and anaplerosis of tricarboxylic acid (TCA) cycle intermediates were confirmed (Fig. 2a; Supplementary Fig. 3). The lactate:pyruvate ratio was increased when cells were inhibited with rotenone and normalized when the cells were treated with NV189 (Fig. 1l). No conclusive alterations due to drug treatment in metabolism of succinyl-CoA-related amino acids or glycolysis could be shown. Levels of cysteine were decreased, which could indicate oxidative stress. To investigate the time course of intracellular metabolism of delivered succinate, [1, 2, 3, 4-$^{13}C_4$]NV118 was synthesized, whereby the carbon atoms in NV118 that upon release would comprise the four carbon atoms in succinate were enriched with the stable isotope ^{13}C. This distinguishes between endogenous TCA cycle intermediates and metabolites originating from the prodrug-delivered succinate. NV118 rather than NV189 was used due to relatively less complex synthesis. Human platelets were then

incubated with [1, 2, 3, 4-$^{13}C_4$]NV118 for 7.5, 15, 30, 120 and 240 min. Even at the first time point, [$^{13}C_4$]malate and [$^{13}C_4$]citrate were observed, demonstrating rapid entry of [$^{13}C_4$]succinate into the TCA cycle (Fig. 2b). There was also [$^{13}C_6$]citrate present, which indicates that [^{13}C]oxaloacetate or [^{13}C]malate had converted to pyruvate and through acetyl-CoA formed citrate with [$^{13}C_4$]oxaloacetate (Fig. 2b; Supplementary Fig. 4), demonstrating continuous metabolism in the TCA cycle. The ratio of labelled species gradually declined with time but still after 240 min, there was a supply of labelled succinate available.

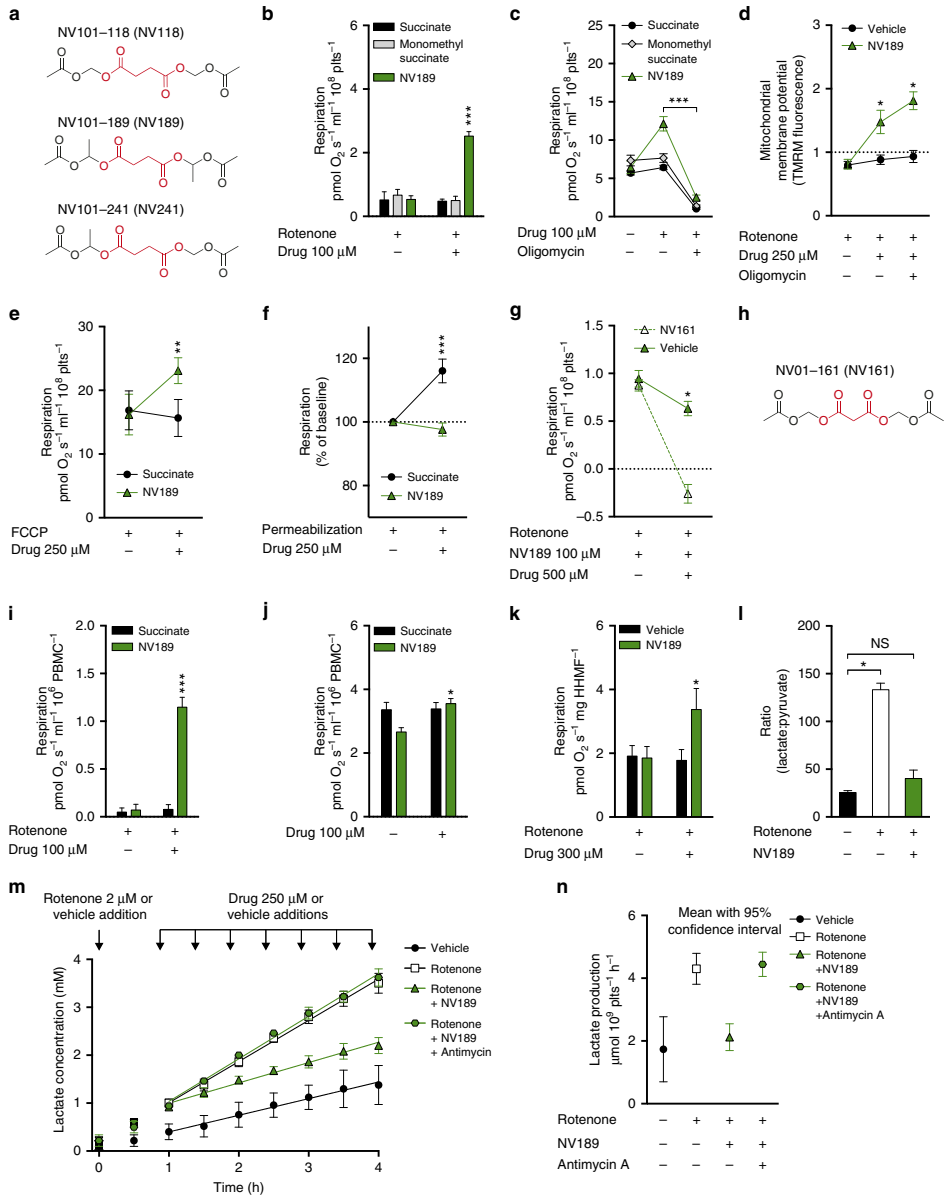

Figure 1 | Effects of mitochondrial complex II stimulation by the succinate prodrug NV189. (**a**) Structures of NV118, NV189 and NV241, succinate highlighted in red. (**b**) Respiration in platelets (plts) with rotenone-induced mitochondrial complex I (CI) inhibition. (**c**) ATP-generating respiration in platelets. (**d**) Mitochondrial membrane potential in complex I-inhibited platelets, ratio of basal TMRM fluorescence, $n=4$. (**e**) Respiration in platelets with FCCP-induced uncoupling. (**f**) Respiration in digitonin-permeabilized platelets. (**g**) Effect on respiration in platelets with addition of the cell-permeable complex II inhibitor NV161, * indicate significant difference between NV161 and vehicle, $n=4$. (**h**) Structure of NV161, malonate highlighted in red. (**i**) Respiration in peripheral blood mononuclear cells (PBMCs) with rotenone-induced CI inhibition, $n=4$. (**j**) Convergent respiration in PBMCs, $n=4$, * indicate significant difference between pre and post dosing. (**k**) Respiration in human heart muscle fibres (HHMFs), $n=5$. (**l**) Lactate:pyruvate ratio in PBMCs at baseline, after rotenone CI inhibition and after treatment with NV189, $n=4$. * indicates significant difference using Friedmans non-parametric paired test with Dunn's multiple comparisons test of all groups against control. For three data points, pyruvate was below detection limit and the estimated lower-quantification limit was used for calculating the ratio. (**m**) Lactate accumulation in 2 ml buffer containing 400×10^6 platelets, incubated with or without rotenone, antimycin A and NV189, $n=5$. (**n**) Lactate production in platelets, data quantification from previous panel. Mean with 95% confidence interval. All respirometric experiments in human platelets were performed with $n=6$ individuals donors if not otherwise stated. All data presented as mean and s.e. if not otherwise stated. In all experiments, blood cells from separate donors are used for each *n*. $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$ (two-tailed paired or unpaired Student's *t*-test as appropriate, difference between test compound and control if not otherwise stated).

Respiration increased in Leigh syndrome patient fibroblasts. To evaluate the effect of NV189 on patient cells, fibroblasts from a patient with Leigh syndrome due to recessive nuclear DNA mutations in the structural CI gene *NDUFS2* and three control cell lines were investigated using a Seahorse Bioscience XF^e 96 Extracellular Flux Analyzer (Fig. 3; Supplementary Fig. 5). The patient fibroblasts have previously been shown to exhibit severely decreased activity of CI, decreased CI assembly and lower expression of CI structural proteins[10]. Pooled data from all experiments (Fig. 3c,d; Supplementary Fig. 5c,d) revealed a 25% decrease in basal oxygen consumption rate (OCR) and a 42% reduction in maximum uncoupled respiration in the Leigh syndrome patient cells compared with the mean of the control cell lines. After addition of NV189, the OCR was similar between

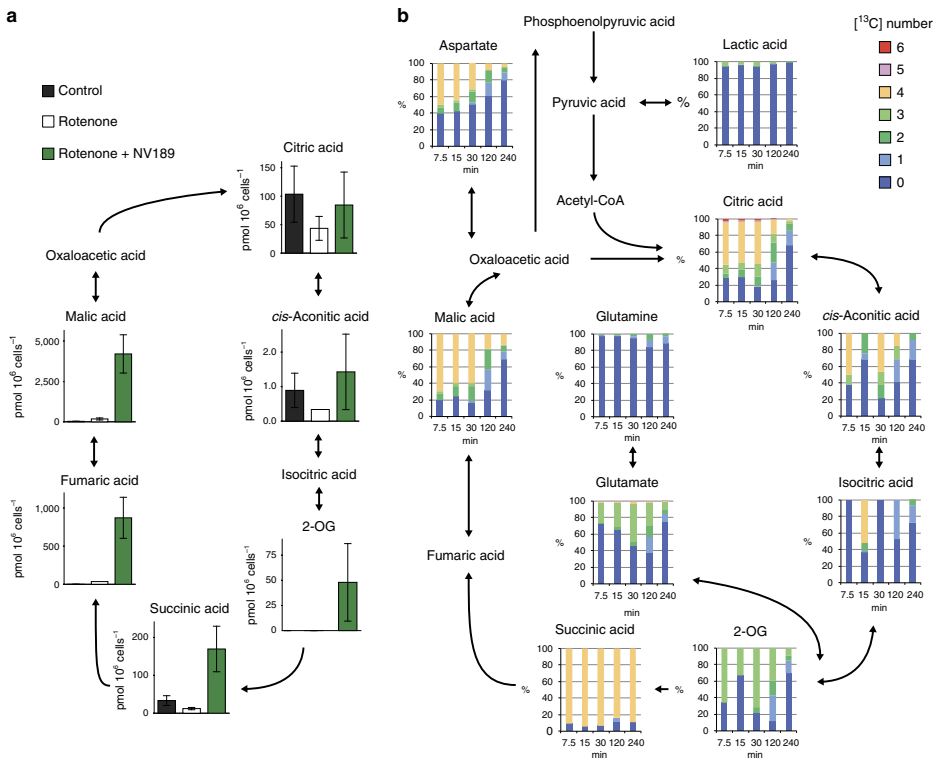

Figure 2 | Intracellular metabolism of exogenous prodrug-delivered succinate. (**a**) TCA cycle intermediates in peripheral blood mononuclear cells after 20 min incubation with or without rotenone and NV189 quantified using capillary electrophoresis mass spectrometry, $n=4$. Data presented as mean and s.d. (**b**) Fraction of [^{13}C] isotope labelled carbons in TCA cycle intermediates and related metabolites in human platelets incubated with [1, 2, 3, 4-$^{13}C_4$]NV118 for 7.5, 15, 30, 120 or 240 min. Mean of $n=2$. 2-OG, 2-oxoglutaric acid.

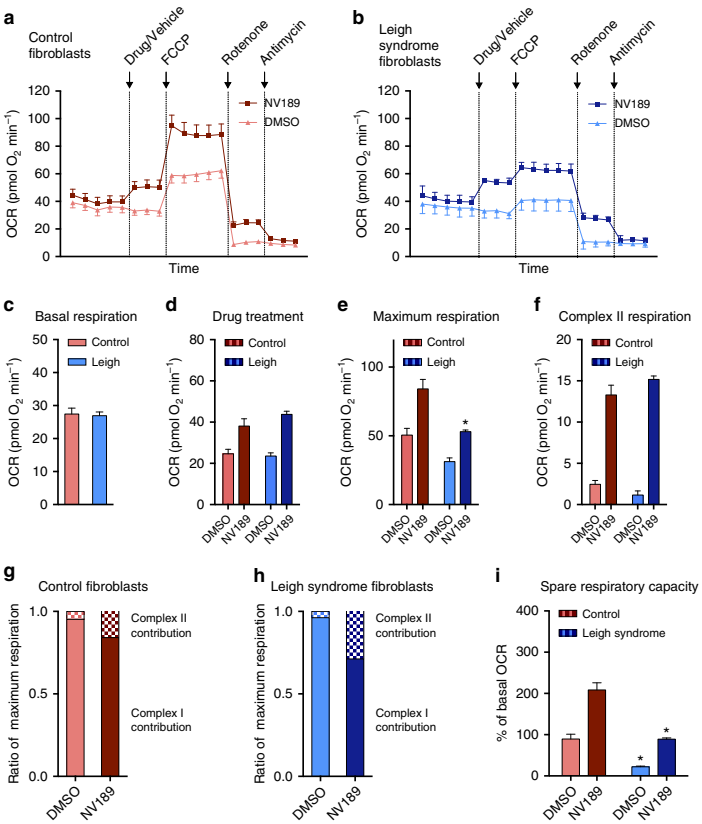

Figure 3 | Succinate prodrug treatment of mitochondrial complex I-deficient Leigh syndrome patient fibroblasts. (**a**,**b**) Oxygen consumption rate (OCR) in three control fibroblast cell lines and a mitochondrial complex I-deficient cell line (recessive *NDUFS2* mutation) treated with NV189 or vehicle. (**c**–**f**) Quantification of OCR in control and patient fibroblasts for each respiratory state. (**g**,**h**) Relative contribution of complex I- and complex II-linked respiration to maximum uncoupled respiration in patient cells and control cell lines. (**i**) Spare respiratory capacity, defined as per cent increase from endogenous baseline to maximum uncoupled respiration. Data presented as mean and s.e. of $n=3$ experiments from separate cell culture flasks performed with eight technical replicates each time for each cell lines. Data from the three control cell lines are pooled. *$P<0.05$ (two-tailed unpaired Student's *t*-test, difference between Leigh and control cell lines).

patient and controls (Fig. 3d; Supplementary Fig. 5d). The patient cells had lower maximum respiration compared with control cells, but in the presence of NV189 the OCR of patient cells was similar to that of untreated control cells (Fig. 3e and Supplementary Fig. 5e). After rotenone inhibition of CI, both cell types elicited clear remaining respiratory activity in cells treated with NV189 (Fig. 3f; Supplementary Fig. 5f). The relative contribution of flux through CII to maximum uncoupled respiration for NV189 was 4.8% in the control cell lines and 3.8% in the Leigh syndrome cells. With treatment, this increased to 15.9% in control cells and to 28.8% in patient cell (Fig. 3g,h; Supplementary Fig. 5g,h), illustrating the dependence of CII substrates in the patient cells to reach normal respiratory function. When patient cells were treated with the prodrugs, the spare respiratory capacity (respiratory reserve, the ability of the cells to increase respiration from the endogenous baseline) as percentage of the endogenous baseline was similar to that of the control cell lines (Fig. 3i; Supplementary Fig. 5i). Succinate or dimethyl succinate (an ester previously suggested to be cell permeable[11,12]) did not exert any effects on either cell type (Supplementary Fig. 6).

Discussion

Mitochondrial disorders frequently present early in life with failure to thrive, myopathy and neuropathy, but the symptoms are very diverse[13]. At least 1 in 8,000 births will develop a mitochondrial disease[14]. Mitochondrial diseases are usually progressive and have a fluctuating clinical course. Periods of deterioration, such as during an intercurrent viral infection, are prompted by the increase in metabolic demand that the mitochondria cannot compensate for, resulting in metabolic

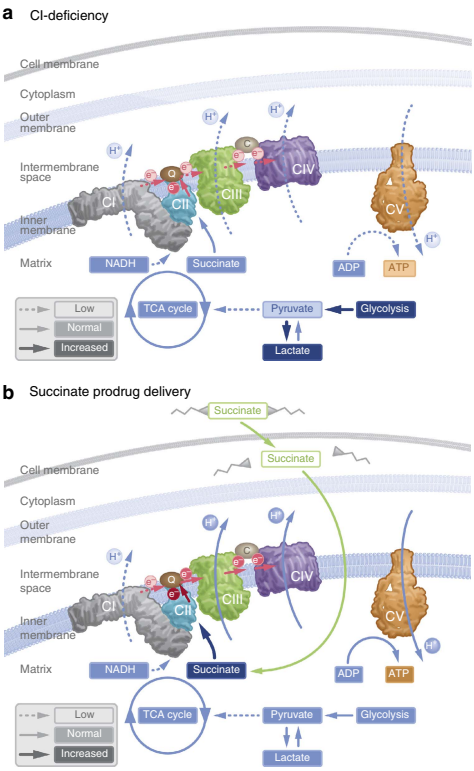

Figure 4 | Delivery of succinate to the intracellular space via a prodrug strategy. (**a**) Dysfunction in mitochondrial complex I reduces electron flow through the respiratory chain, shift metabolism towards glycolysis, induce lactate accumulation and limit ATP production. (**b**) Cell membrane-permeable prodrugs of succinate access the intracellular space and release succinate, enabling increased electron transport, respiration and ATP production through complex II, thus bypassing the deficiency in mitochondrial complex I.

decompensation[15]. It is an area of large unmet medical need as few evidence-based treatment options are available[2]. We describe here three model compounds of the first generation of a new pharmacological strategy to metabolically support these patients during time of metabolic decompensation. The current compounds lack sufficient plasma stability to be suitable for *in vivo* use. A cell-permeable prodrug of succinate can enter the cell independent of active uptake and subsequently release succinate. By supplying the mitochondria with substrates for CII, cells that are unable to comply with metabolic demand due to limitations at CI, or upstream thereof, may increase ATP production through oxidative phosphorylation, demonstrated here by the normalization of spare respiratory capacity in metabolically defect patient cells (Fig. 3i). By supporting aerobic metabolism, the relative dependence on glycolysis for ATP generation is alleviated and lactate production is attenuated (Figs 1n and 4). Utilizing a cell-permeable prodrug strategy to deliver a TCA cycle intermediate to the intracellular space is a feasible pharmacologic strategy with potential benefit in conditions affecting mitochondrial function, such as CI dysfunction or TCA cycle intermediate depletion in organic acidemias. Here we demonstrate that prodrug-delivered succinate can alleviate metabolic decompensation due to CI-related mitochondrial dysfunction.

Methods

Human peripheral blood cells. The blood cell protocols were approved by the regional ethics committee of Lund University, Sweden (permit no. 2013/181), and written informed consent was acquired from each participant. From healthy volunteers, venous blood was drawn to K_2EDTA tubes (Vacutainer, BD, Franklin Lakes, USA) via venous puncture. Platelets were isolated with consecutive centrifugation steps as previously described[16]. Peripheral blood mononuclear cells (PBMCs) were isolated using Lymphoprep (Axis-Shield, Dundee, Scotland). Erythrocytes and PBMCs were loosely pelleted by 10 min centrifugation at 500*g*. The pellet was resuspended in saline, layered on a Ficoll gradient and centrifuged at 800*g* for 20–30 min. The resulting leukocyte layer was collected, resuspended in saline and pelleted by 5 min centrifugation at 250*g*. The supernatant was removed and the pellet resuspended in 100–200 µl of saline. Blood cells were counted using an automated hematocytometer (SweLab Alfa, Boule Diagnostics, Sweden). The number of biological replicates (blood cells derived from different individual donors) are provided in the respective figure legends for all experiments.

Human cardiac muscle samples. Biopsies of human cardiac muscle were obtained at the Department of Cardiothoracic Surgery, Skåne University Hospital, Lund, Sweden. Pre-surgery informed consent was obtained from patients undergoing planned open-heart surgery such as mitral valve repair or maze procedure for treatment of atrial fibrillation. Only superfluous tissue that otherwise would have been discarded or located behind the suture line for the cannulation catheter was collected (up to 2 g was collected, 50–100 mg used for each experiment). Ethical permission was granted by the regional ethical review board of Lund, Sweden (permit no. 2013/271, 2013/701). The biopsy was immediately transferred to ice-cold 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 dithiothreitol, 6.56 mM $MgCl_2$, 5.77 mM ATP and 15 mM phosphocreatine, pH 7.1). It was thereafter dissected under microscope using forceps to gently separate the fibres and remove any fat and connective tissue. Biopsy wet weight was obtained before respiratory measurements (Precisa 40SM-200A, Abbot, USA).

Cultured fibroblasts. Permit for research on fibroblasts was granted by the Newcastle and North Tyneside 1 NRES Committee (REC reference 2002/205). A cultured skin fibroblast cell line from a patient with clinical Leigh syndrome due to a deficiency in the nuclear encoded structural mitochondrial CI protein *NDUFS2* (p.Arg118Gln; p.Met292Thr mutations), and relevant control cell lines from healthy donors were provided by the Wellcome Trust Centre for Mitochondrial Research at Newcastle University, UK[10]. The fibroblasts were cultured in minimum essential medium (MEM) supplemented with 10% fetal bovine serum, 1% MEM vitamins, 1% MEM non-essential amino acids, 2 mM L-glutamine, 50 µg ml^{-1} streptomycin, 50 U ml^{-1} penicillin, 50 µg ml^{-1} uridine and 1 mM sodium pyruvate at 37 °C and 5% CO_2. Cells were collected using trypsin and split or used for analysis at ~70–80% confluence and counted using an automated cell counter (TC20, Bio-Rad, Hercules, USA).

Respirometry. For cells in monolayers, the Seahorse Bioscience XF^e 96 Extracellular Flux Analyser (Seahorse Bioscience, North Billerica, USA) was the instrument of choice, and for cells in suspension such as blood cells the Oroboros O2k (Oroboros Instruments, Innsbruck, Austria) was used. Respiratory measurements using Oroboros O2k were performed in stirred (750 r.p.m.) 2 ml glass chambers at 37 °C. The media MiR05 (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 and bovine serum albumin 1 g l^{-1}, pH 7.1) was used in all experiments[16,17]. Data were recorded using the DatLab software version 4, 5 or 6 (Oroboros Instruments). Correction for instrumental background and air calibration was performed according to the manufacturer's instructions.

All experiments with platelets were performed with cell concentrations of 200×10^6 cells per ml and all experiments with PBMCs with 5×10^6 cells per ml. In experiments with human heart fibres, ~10 mg of tissue was used in each run. To inhibit mitochondrial CI, rotenone (2 µM) was used and to inhibit mitochondrial complex III, antimycin A (1 µg ml^{-1}) was used. ATP synthase was inhibited using oligomycin (1 µg ml^{-1}), evaluating the contribution of respiration independent of ADP phosphorylation. Maximum uncoupled respiration of the electron transport system was induced by titration of the protonophore carbonyl cyanide FCCP until no further increase in respiration was detected. The test compound or control substances (succinate, dimethyl succinate, monomethyl succinate, malonate, dimethyl malonate or dimethylsulphoxide (DMSO)) were dosed as indicated in each figure.

Respirometric measurements in fibroblasts were performed using a Seahorse Bioscience XF^e 96 Extracellular Flux Analyzer. The day before the experiment, fibroblasts were seeded out at 25,000 cells per well in cell growth medium in collagen-coated 96-well plates and kept at 37 °C and 5% CO_2 overnight. Before the experiment, the growth medium was replaced by XF-Base Medium containing 2 mM L-glutamine, 5 mM sodium pyruvate and 10 mM glucose (pH 7.4) and the cells were kept at 37 °C 1 h at atmospheric O_2 and CO_2. Oxygen consumption was measured at routine state and after addition of 500 µM of NV241 or NV189, its vehicle DMSO, dimethyl succinate or disodium succinate, followed by different concentrations of FCCP (0.125, 0.5, 1.0 and 1.5 µM), 2 µM rotenone and 1 µg ml^{-1} antimycin A. After FCCP and drug addition, the first data point was generally used, if not another data point was clearly higher, and for the remaining states the last data point before the subsequent addition was used. The FCCP dosing resulting in the highest uncoupled respiration was chosen for analysis for each experiment with each cell line and treatment.

All respirometric measurements, with the exception of the human heart fibre data, were corrected for non-mitochondrial oxygen consumption, obtained after the addition of antimycin A.

Lactate. Platelets ($n = 5$ individual donors, 200×10^6 cells per ml) were incubated in PBS for 4 h with rotenone (2 µM), rotenone and antimycin A (1 µg ml^{-1}) combined or the vehicle for rotenone (DMSO). At $t = 60$ min, additions of 250 µM NV118, NV189, NV241 or vehicle (DMSO) were initiated and repeated every 30 min throughout the experiment. Lactate levels were determined every 30 min using a Lactate ProTM 2 blood lactate meter (Arkray, Alere AB, Lidingö, Sweden)[18]. Incubation was performed at 37 °C at a stirrer speed of 750 r.p.m.

Mitochondrial membrane potential. Mitochondrial membrane potential in isolated human platelets (200×10^6 ml^{-1}) was measured using a flow cytometer FACSAria III (BD, Franklin Lakes, USA) with Diva version 7.0 acquisition and analysis software, using the probe TMRM (Life Techologies, Ref: T668), in non-quench mode (30 nM)[19] excited by 561 nM 40 mW laser and collected on 582/15 band pass filter. CD41a-APC (BD Pharmingen, Clone HIP8, Ref: 559777) at 18 times dilution was used to assess platelet activation. Samples were incubated with the probes in MiR05 for 30 min at room temperature. CI was inhibited using 2 µM rotenone. NV189 (250 µM) or DMSO control was added to the samples, followed by oligomycin (1 µg ml^{-1}), FCCP (20 µM) and antimycin A (1 µg ml^{-1}), the two latter additions as internal controls. Data software used was FlowJo 10 (Tree Star, Ashland, USA). Statistical analyses were performed, and all figures were generated using Prism 6 (GraphPad Software).

Metabolomics. Isolated human PBMCs ($16–25 \times 10^6$ ml^{-1}) were incubated at 37 °C in 2 ml MiR05 with 5 mM glucose and with rotenone 2 µM or DMSO control. NV189 (250 µM; 0.5 mM total) or DMSO control was added in two subsequent additions. Samples were centrifuged at 4,600g for 4 min and the supernatant discarded in two cycles with resuspension of pellet in 1.5 ml of 5% mannitol solution before the second run. To each sample, 800 µl of methanol and 550 µl of solution of the internal standard (H3304-1002, Human Metabolome Technologies Inc., Tsuruoka, Japan) were added and 1 ml of the extracted solution was taken for centrifugation at 2,300g at 4 °C for 5 min. Thereafter, 400 µl of the supernatant was filtered at 9,100g at 4 °C until no liquid remained. The extract was dried in a centrifugal evaporator (1,500 r.p.m., 1,000 Pa) and put in −80 °C until analysis. Samples were analysed using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) for cationic compounds and capillary electrophoresis tandem mass spectrometry (CE-MS/MS) for anionic compounds (Agilent Technologies, Santa Clara, USA), as previously described[20]. Peaks detected in CE-TOFMS analysis were extracted using automatic integration software (MasterHands ver.2.16.0.15 developed at Keio University) and those in CE-MS/MS analysis were extracted using automatic integration software (MassHunter Quantitative Analysis B.06.00, Agilent Technologies) to obtain peak information including m/z, migration time and peak area. The peak area was then converted to relative peak area. The peaks were annotated based on the migration times in CE and m/z values determined by TOFMS. Putative metabolites were then assigned from Human Metabolome Technologies (HMT) metabolite database on the basis of m/z and migration time. All the metabolite concentrations were calculated by normalizing the peak area of each metabolite with respect to the area of the internal standard and using standard curves, which were obtained by three-point calibrations. The lactate:pyruvate ratio was analysed using Friedman's non-parametric paired test for comparison between three groups or more with Dunn's multiple comparisons test of all groups against control. For three data points (one data point in the group treated with only rotenone and two data points in the group treated with rotenone and NV189), pyruvate was below the quantification limit. The estimated lower-quantification limit for pyruvate was between 16.96 and 20.55 pmol per 10^6 cells and a mean of these two values was used for calculating the lactate:pyruvate ratio for the missing data points. Experiments were performed by the service provider Human Metabolome Technologies Inc. (Tsuruoka, Japan). Cells from the same four healthy volunteers were used for each experimental group.

Isotope labelling. NV118 was synthesized incorporating all four carbons in the central succinate structure of the molecule with [^{13}C] isotopes. Isolated platelets (800×10^6 ml^{-1}) were kept at 37 °C in 2 ml MiR05 containing 5 mM glucose. [1, 2, 3, 4-$^{13}C_4$]NV118 was added in two boluses to a final concentration of 0.5 mM and the samples were incubated for 15, 30, 120 or 240 min. Extracts were prepared as described above. Metabolome measurements were carried out through Human Metabolome Technology Inc., Tsuruoka, Japan. Target metabolites and their isotopomers were annotated based on their theoretical m/z value and migration time[21]. Cells from the same two healthy volunteers were used for each experimental group.

Statistics. Statistical analyses were performed, and all figures generated, using Prism 6 (GraphPad Software, La Jolla, USA) if not otherwise stated. A P value of <0.05 was considered statistically significant. No blinding or randomization was performed, except for the metabolomics assays, where the lab performing the analyses was blinded to the intervention allocated to the samples. Data from blood cell respirometry have previously been reported to be normally distributed and parametric tests were used[16].

Data availability. All relevant data are contained within the paper and Supplementary Information files or available from the authors upon request.

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Author contributions

E.E., M.J.H., F.S. and J.K.E. conceived the study. R.F., C.C., H.F. and S.J.M. designed new chemical entities. R.W.T. and D.M.T. provided cell lines. S.M., S.P., J.K.E., M.K., C.M., R.W.T., D.M.T., S.J.M. and E.Å.F. evaluated the properties of the compounds. J.K.E., S.P. and M.K. performed the statistical analysis. J.K.E. drafted the manuscript. J.K.E., S.P., M.J.H. and M.K. prepared figures. J.K.E., M.J.H. and E.E. directed the study. All authors critically reviewed the manuscript and approved of the final version.

Additional information

Supplementary Information accompanies this paper at http://www.nature.com/naturecommunications

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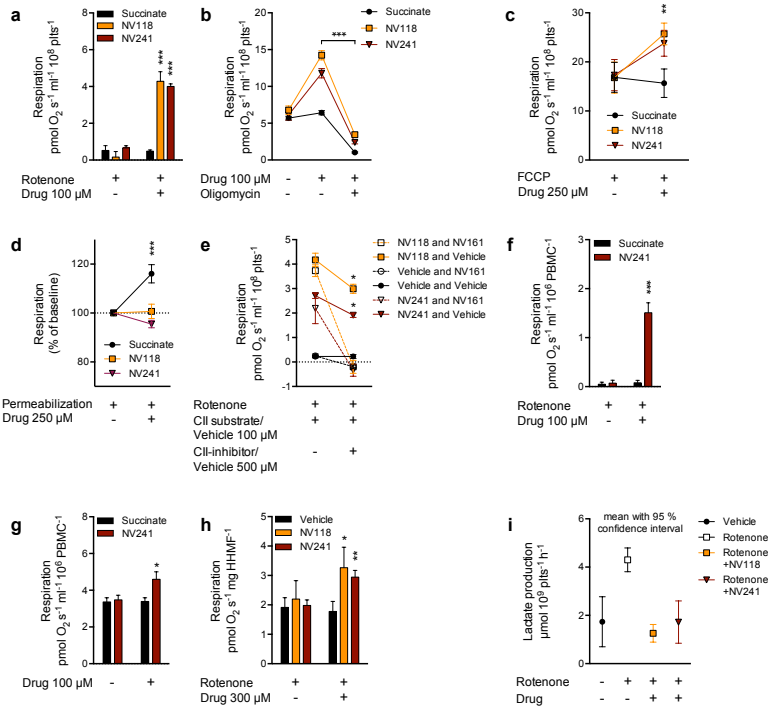
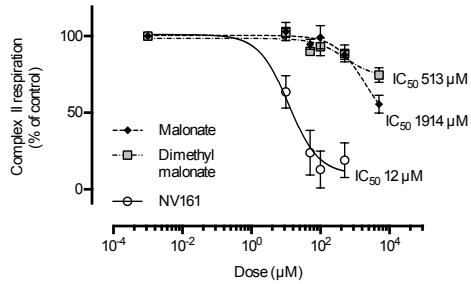
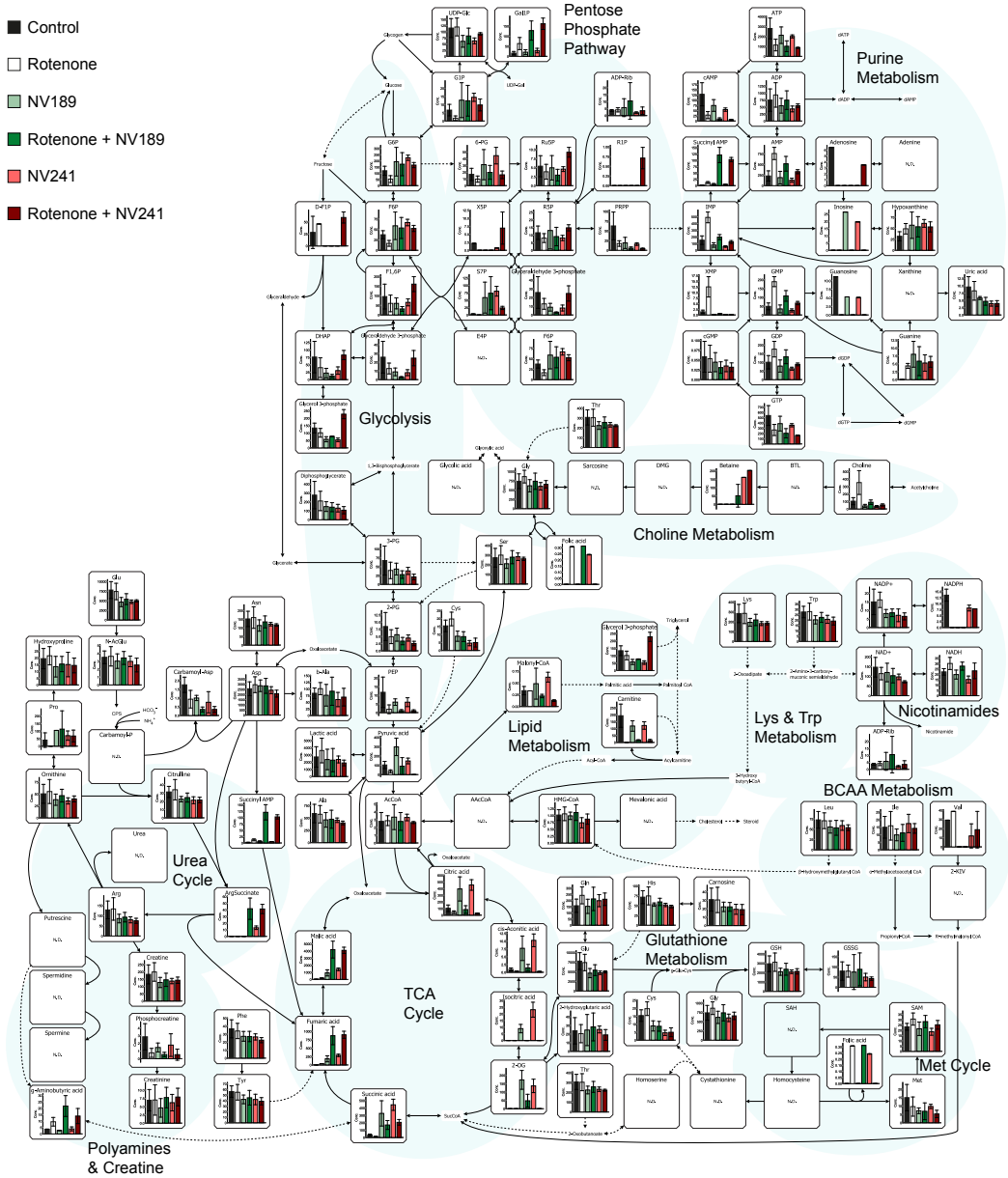


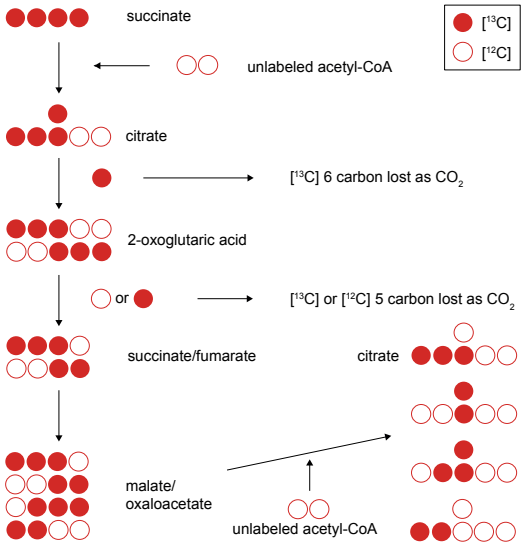
Supplementary Figure 1. Effects of mitochondrial complex II stimulation by succinate prodrugs NV118 and NV241. (**a**) Respiration in platelets (plts) with rotenone-induced mitochondrial complex I (CI) inhibition. (**b**) ATP-generating respiration in platelets, *** indicate significant reduction in drug treated sample pre and post oligomycin. (**c**) Respiration in platelets with FCCP-induced uncoupling. (**d**) Respiration in digitonin-permeabilized platelets. **(e**) Effect on respiration in platelets with addition of the cell-permeable CII inhibitor NV161, * indicate significant difference between NV161 and vehicle for each drug, n=4. (**f**) Respiration in peripheral blood mononuclear cells (PBMCs) with rotenone-induced CI inhibition, n = 4. **(g**) Convergent respiration in PBMCs, n = 4. **(h**) Respiration in human heart muscle fibres (HHMF), n = 5. (**i**) Lactate production in 2 ml buffer containing 400 × 10^6 platelets, incubated with or without rotenone and NV118 or NV241, n = 5. All respirometric experiments in human platelets were performed with n = 6 individuals donors if not otherwise stated. All data presented as mean and standard error if not otherwise stated. In all experiments blood cells from separate donors are used for each n. * = $p < 0.05$, ** = $p < 0.01$, *** = $p < 0.001$ (two-tailed paired or unpaired Student's *t*-test as appropriate, difference between succinate prodrug and control if not otherwise stated).



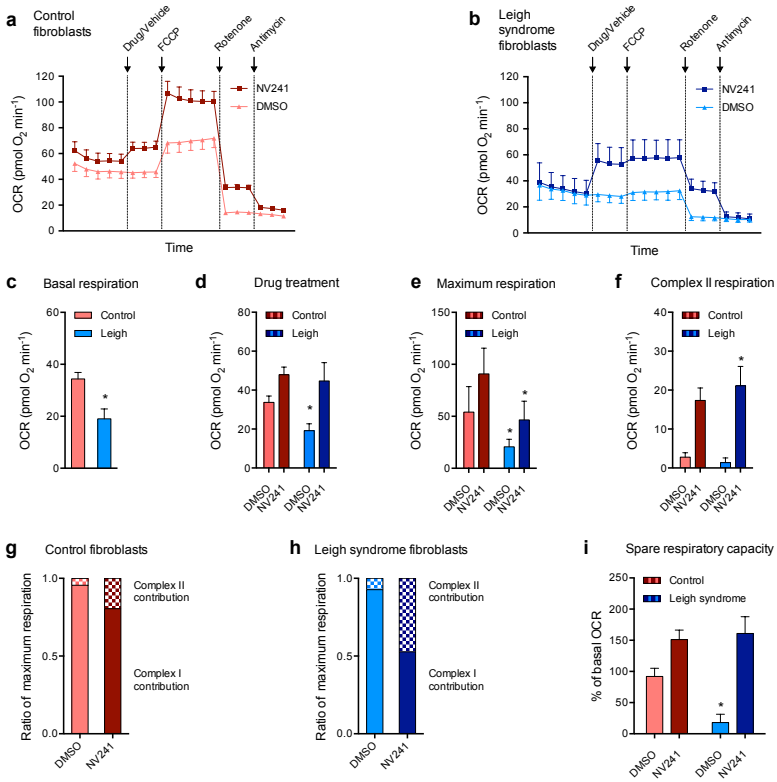
Supplementary Figure 2. Dose-response of three mitochondrial complex II-inhibitors in intact cells. Human platelets (200×10^6 ml^{-1}) were incubated as described in the methods section. Rotenone (2 µM) was added to inhibit Complex I and NV241 (100 µM) was added to allow for Complex II-linked respiration. Malonate, dimethyl malonate or NV161 was titrated (from 10 µM to 5 mM final concentration) and rate of oxygen consumption assessed. IC_{50}-values for each compounds was calculated using Prism GraphPad 6.0. NV161 inhibited complex II-supported respiration at lower concentrations than malonate or dimethyl malonate, as depicted by the IC_{50}-values provided in the figure.


Control
Rotenone
NV189
Rotenone + NV189
NV241
Rotenone + NV241
Pentose Phosphate Pathway
Purine Metabolism
Glycolysis
Choline Metabolism
Lipid Metabolism
Lys & Trp Metabolism
Nicotinamides
BCAA Metabolism
Urea Cycle
TCA Cycle
Glutathione Metabolism
Met Cycle
Polyamines & Creatine

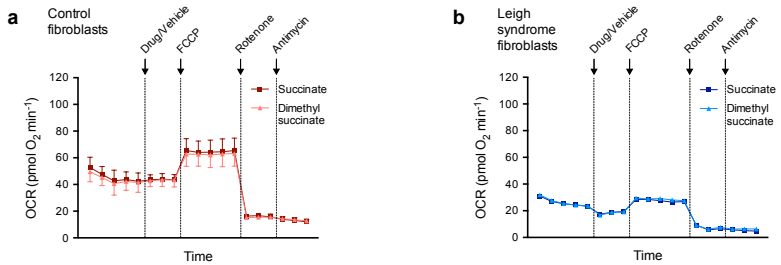
Supplementary Figure 3. Intracellular metabolism of exogenous succinate. Overview of metabolic pathways in peripheral blood mononuclear cells incubated with or without rotenone (2 μM) and with or without NV189 or NV241. Metabolites were quantified using capillary electrophoresis time-of-flight mass spectrometry for cationic compounds and capillary electrophoresis tandem mass spectrometry for anionic compounds. Delivery of intracellular succinate and anaplerosis of TCA cycle intermediates were confirmed. The branched-chain amino acids valine (Val) and isoleucine (Ile) can be metabolized to succinyl-CoA but are primarily metabolized in muscle tissue. Valine was only detected in very few samples and altogether these data were inconclusive. Total succinyl CoA-related amino acids revealed no difference between the treatment groups. The levels of reduced glutathione (GSH) in samples treated with NV189 or NV241 were marginally decreased, but the glutathione redox ratio was unaltered. Cysteine (Cys), a precursor to glutathione, which in itself also holds antioxidant properties, was decreased in samples treated with NV189 or NV241. Altogether the data cannot exclude oxidative stress. ATP levels were decreased similarly in all rotenone-treated samples. ADP levels changed in the opposite direction, and thus the interrelated adenylate energy charge clearly indicated aggravated energy status of the rotenone-treated cells. Guanylate energy charge also indicates the energy status and displays equivalent results. Furthermore, NADH was increased in all samples treated with rotenone. Creatine levels, also linked to energy status of the cell, though primarily in muscle, did neither differ significantly. Overall the drug treated samples did not display a significant change in the energy status of the cells as measured by metabolomics. The data related to glycolytic flux is not conclusive. Glucose 6-phosphate (G6P) displayed somewhat higher values in the drug-treated samples, as did fructose 6-phosphate (F6P). All data presented as mean and SD using cells from same 4 individuals for each treatment group.



Supplementary Figure 4. Metabolic route of isotope labeled compounds. Putative metabolic incorporation of carbons from [1,2,3,4-$^{13}C_4$]NV118 through the TCA cycle. In order to achieve [$^{13}C_6$]citrate, [$^{13}C_2$]acetyl-CoA must be generated.



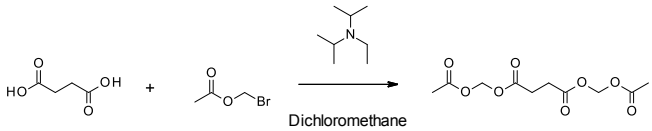
Supplementary Figure 5. Treatment of mitochondrial complex I-deficient Leigh syndrome patient fibroblasts with NV241. (**a-b**) Oxygen consumption rate (OCR) in three control fibroblast lines and a mitochondrial complex I-deficient cell line (recessive *NDUFS2* mutation) treated with NV241 or vehicle. (**c-f**) Quantification of OCR in control and patient fibroblasts for each respiratory state. (**g-h**) Relative contribution of CII (and CI)-linked respiration to maximum uncoupled respiration in patient cells and control cell lines (4.4 % in the control cell lines and 7.3 % in the Leigh syndrome cells; with treatment, this increased to 19.6 % in control cells, and to 47.3 % in patient cells). (**i**) Spare respiratory capacity, defined as percent increase from endogenous baseline to maximum uncoupled respiration. Data presented as mean and standard error of n = 3 separate experiments performed with eight technical replicates for each cell lines. Data from the three control cell lines are pooled. * = $p < 0.05$ (two-tailed unpaired Student's *t*-test, difference between Leigh and control cell lines).



Supplementary Figure 6. Effects of succinate and dimethyl succinate on complex I-deficient Leigh syndrome patient fibroblasts. Oxygen consumption rate (OCR) in three control fibroblast lines (**a**) and a mitochondrial complex I-deficient cell line (**b**) (recessive *NDUFS2* mutation) treated with succinate or dimethyl succinate. Each experiment performed with eight technical replicates and n = 1 for each cell line. Data from the three control cell lines are pooled.

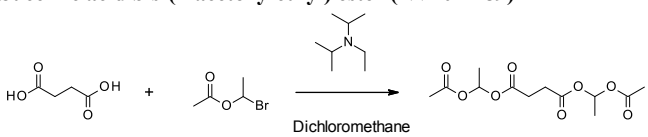
Supplementary Methods

Succinic acid diacetoxymethyl ester (NV101-118)



Succinic acid (58.6 g, 0.496 mol) was added to dichloromethane (2 L) and the mixture cooled to 0°C. Diisopropylethylamine (201 mL, 1.154 mol) was added during 20 minutes followed by bromomethyl acetate (159.4 g, 1.042 mol) during 30 minutes and the solution stirred overnight under an atmosphere of nitrogen. The solution was cooled to 0°C and washed successively with 1 L of cold 1% hydrochloric acid, 0.6% hydrochloric acid and water (x3). The solution was treated with decolourizing charcoal, dried with magnesium sulphate and concentrated to an oil which was crystallized from diethyl ether (200 mL)/*iso*hexane (10 mL) to afford 92 g of succinic acid diacetoxymethyl ester as a white solid. m.p.: 59–60°C.
^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.76 (s, 4H), 2.72 (s, 4H), 2.13 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ 170.8, 169.6, 79.2, 28.5, 20.7. LCMS (m/z) 263.2 $[M+H]^+$, 285.1 $[M+Na]^+$.A further 8 g of pure material was obtained from concentration of the liquors.

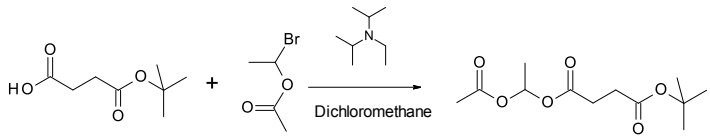
Succinic acid bis-(1-acetoxy-ethyl) ester (NV101-189)



Succinic acid (58.6 g, 0.496 mol) was added to dichloromethane (2 L) and the mixture cooled to 0°C. Diisopropylethylamine (201 mL, 1.154 mol) was added during 20 minutes followed by 1-bromoethyl acetate (175 g, 1.05 mol) during 30 minutes and the solution stirred overnight under an atmosphere of nitrogen. The solution was cooled to 0°C and washed successively with cold (1.5 L quantities) of water, 1% hydrochloric acid (twice), sodium bicarbonate solution and water. The solution was dried with magnesium sulphate and concentrated to an oil which was crystallized from *t*-butylmethyl ether to afford 41 g of succinic acid bis-(1-acetoxy-ethyl) ester as a white solid. m.p.: 63–64°C.
^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 6.87 (q, *J*=5.5 Hz, 2H), 2.66 (m, 4H), 2.07 (s, 6H), 1.48 (d, *J*=5.4 Hz, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ 170.5, 169.3, 89.1, 29.1, 21.4, 20.0. LCMS (m/z) 313.0 $[M+Na]^+$

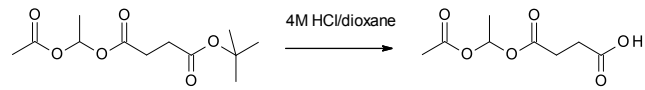
Succinic acid 1-acetoxy-ethyl ester acetoxymethyl ester (NV101-241)

i) Succinic acid 1-acetoxy-ethyl ester *tert*-butyl ester



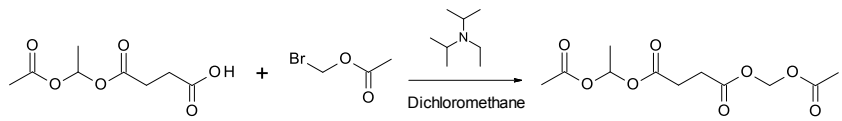
To a solution of succinic acid mono-*tert*-butyl ester (136.15 g, 781 mmol) in dichloromethane (1 L) at -5°C was added di-isopropylethylamine (163.36 mL, 121.21 g, 938 mmol, 1.2 eq). Acetic acid 1-bromo-ethyl ester (143.57 g, 860 mmol, 1.1 eq) was then added dropwise. The reaction was then stirred for 16 hours at room temperature. The reaction was concentrated *in vacuo*, and the residue was dissolved in EtOAc (500 mL) and this was washed sequentially with 0.5 M aq HCl (2 x 450 mL), saturated aq $NaHCO_3$ (500 mL), and brine (400 mL). The organics were then dried over $MgSO_4$. The residue was purified by vacuum distillation (vapour temperature 109-115°C, pressure <20 mbar) to give the title compound as a colourless oil (140.5 g, 539.8 mmol, 69%).
^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 6.88 (q, *J*=5.5 Hz, 1H), 2.50-2.65 (m, 4H), 2.07 (s, 3H), 1.48 (d, *J*=5.5 Hz, 3H), 1.45 (s, 9H).

ii) Succinic acid mono-(1-acetoxy-ethyl) ester



Succinic acid 1-acetoxy-ethyl ester *tert*-butyl ester (74.8 g, 288 mmol) was dissolved in 4 M HCl in dioxane (720 mL, 2.88 mol, 10 eq), and the reaction was stirred for 5 hours at 40°C. The reaction was concentrated *in vacuo*, and the residue was azeotroped with toluene (500 mL), to give the desired product as a clear oil (58 g).
^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 10-14 (br, 1H), 6.73 (q, *J*=5.5 Hz, 1H), 2.40-2.60 (m, 4H), 2.03 (s, 3H), 1.40 (d, *J*=5.5 Hz, 3H).

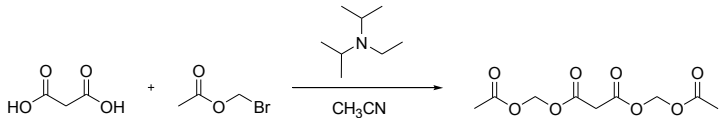
iii) Succinic acid 1-acetoxy-ethyl ester acetoxymethyl ester (NV101-241)



To a solution of succinic acid mono-(1-acetoxy-ethyl) ester (58.7 g, 287 mmol) in dichloromethane (600 mL) at -5°C was added di-isopropylethylamine (60.1 mL, 44.6 g, 345 mmol, 1.2 eq). Acetic acid 1-bromo-methyl ester (31.0 mL, 48.36 g, 316 mmol, 1.1 eq) was then added dropwise. The reaction was then stirred for 4 hours at room temperature. The reaction was concentrated in vacuo, and the residue was dissolved in EtOAc (450 mL) and this was washed sequentially with cold (5°C) 0.5 M aq HCl (400 mL), saturated aq $NaHCO_3$ (400 mL), and brine (350 mL). The organics were then dried over $MgSO_4$. The residue was purified by vacuum distillation (vapour temperature 154-169°C, pressure 3.2 mbar) to give the title compound as a pale yellow oil (57.45 g, 208 mmol).

^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 6.87 (q, J=5.5 Hz, 1H), 5.76 (s, 2H), 2.60-2.77 (m, 4H), 2.13 (s, 3H), 2.08 (s, 3H), 1.49 (d, J=5.5 Hz, 3H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ 170.9, 170.0, 169.6, 168.9, 88.7, 79.2, 28.6, 20.8, 20.7, 19.4. LCMS (m/z) 277.0 $[M+H]^+$, 299 $[M+Na]^+$.

Malonic acid diacetoxymethyl ester (NV01-161)



To a mixture of malonic acid (0.30 g, 2.9 mmol) and di-isopropylethylamine (1.50 g, 11.5 mmol) in acetonitrile (5 mL) was added bromomethyl acetate (0.96 g, 6.4 mmol). The reaction was stirred at room temperature overnight. After removing the solvent, the residue was purified by silica gel chromatography (eluting with petroleum ether and ethyl acetate (9/1 to 3/1)) to give the title compound as a colourless oil (119 mg, 17% yield).
^{1}H NMR (300 MHz, $CDCl_3$, ppm) δ 5.78 (s, 4H), 3.50 (s, 2H), 2.14 (s, 6H). ^{13}C NMR (100 MHz, $CDCl_3$, ppm) δ 169.4, 164.6, 79.5, 40.8, 20.6. LCMS (m/z) 266.2 $[M+NH_4]^+$, 271.1 $[M+Na]^+$.

Paper IV

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Changes in energy metabolism due to acute rotenone-induced mitochondrial complex I dysfunction – An *in vivo* large animal model

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ABSTRACT

Metabolic crisis is a clinical condition primarily affecting patients with inherent mitochondrial dysfunction in situations of augmented energy demand. To model this, ten pigs received an infusion of rotenone, a mitochondrial complex I inhibitor, or vehicle. Clinical parameters, blood gases, continuous indirect calorimetry, *in vivo* muscle oxygen tension, *ex vivo* mitochondrial respiration and metabolomics were assessed. Rotenone induced a progressive increase in blood lactate which was paralleled by an increase in oxygen tension in venous blood and skeletal muscle. There was an initial decrease in whole body oxygen utilization, and there was a trend towards inhibited mitochondrial respiration in platelets. While levels of succinate were decreased, other intermediates of glycolysis and the TCA cycle were increased. This model may be suited for evaluating pharmaceutical interventions aimed at counteracting metabolic changes due to complex I dysfunction.

1. Introduction

NADH:ubiquinone oxidoreductase, more commonly referred to as Complex I (CI), is the largest complex of the mitochondrial electron transport chain (ETC) (Carroll et al., 2006). CI dysfunction is a common pathophysiological mechanism involved in several mitochondrial disorders. Various other inborn errors of metabolism (IEM) may also cause secondary inhibition of CI (Brusque et al., 2002). Further, in a general patient population drug-induced CI dysfunction may occur, for example in metformin-induced lactic acidosis (Brunmair et al., 2004; Dykens et al., 2008; Piel et al., 2015; Protti et al., 2012b). CI dysfunction is also implicated in the pathophysiology of several other conditions, including neurodegenerative disorders such as Parkinson's disease, cardiac ischemia-reperfusion injury, traumatic brain injury and sepsis (Kilbaugh et al., 2015; Paradies et al., 2004; Protti et al., 2007; Schapira et al., 2014).

Even though our knowledge about the pathophysiology underlying mitochondrial respiratory chain disorders in recent years has increased considerably, this has not been paralleled by the development of new pharmaceutical treatment options (Dimauro and Rustin, 2009). In a Cochrane review from 2012 it is concluded that there is presently no evidence supporting the use of any pharmacological intervention in mitochondrial disorders (Pfeffer et al., 2012).

A cornerstone of effective pharmaceutical development is a relevant and reliable animal model. A few models related to mitochondrial respiratory diseases exists, for instance transgenic mice (Koene et al., 2011; Kruse et al., 2008; Tyynismaa and Suomalainen, 2009; Wallace and Fan, 2009), among them specific CI models such as the WB NDUFS4$^{-/-}$ KO mouse, the CWB NDUFS4$^{-/-}$ KO mouse (Roestenberg et al., 2012) and the NDUFS6$^{-/-}$ KO mouse with cardiac specific defects (Ke et al., 2012). There is also the *Harlequin* mouse, where the phenotype of the natural mutant has been attributed to CI dysfunction (Vahsen et al., 2004). In order to allow for adequate clinical monitoring with invasive measurements and control of the ventilation, resembling an intensive care unit, a large-animal model is preferable. Further, a larger animal also grants the possibility of collecting multiple blood samples, where the blood volume of a rodent would be insufficient. A pig model of metformin-induced lactic acidosis has recently been published (Protti et al., 2012a), where inhibition of mitochondrial CI respiration by metformin is likely a major mechanism for lactate accumulation (Piel et al., 2015).

In general, mitochondrial disorders affect organs with high energy requirements. The development of lactic acidosis is a common feature related to the shift from aerobic mitochondrial respiration to anaerobic glycolysis as the cells strive to uphold ATP production (Jackson et al.,

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1567-7249/

1995; Sue et al., 1998). Lactate overproduction can be due to either hypoxia or dysoxia, with the former representing true lack of oxygen in the tissue regardless of cause and the latter representing abnormal tissue oxygen metabolism in the presence of normal tissue oxygen supply (Robin, 1977).

The objective of this study was to establish an *in vivo* pig model of acute mitochondrial CI dysfunction with associated changes in metabolism to be used to evaluate pharmaceutical interventions aimed at counteracting these changes, and to show that the detected systemic effects, and specifically the increase in lactate, were primarily due to a shift towards non-mitochondrial metabolism and not only due to tissue hypoperfusion or other secondary cardiovascular effects of rotenone. The model aims at mimicking a clinical situation of acute metabolic crisis involving CI dysfunction.

2. Material and methods

2.1. Animals and rotenone infusion

The regional animal experimental ethics committee of Stockholm approved the study (N29/13). Ten female Yorkshire/landrace hybrid pigs (mean body weight 37.8 kg range 30.4–46 kg, age 12–14 weeks) were included. Before transportation to the test facility the animals were pre-medicated with intramuscular administration of Zoletil (tiletamine; 1.5 mg/kg and zolazepam; 1.5 mg/kg), Domitor (medetomidine hydrochloride; 0.06 mg/kg) and atropin (0.033 mg/kg).

The pigs were anaesthetized by a combination of pentobarbital (120 mg bolus) and fentanyl (100 μg bolus). Thereafter the animals were intubated and supplied with assisted ventilation. The rate and tidal volume of the ventilator was kept constant during the experiment. $ETCO_2$ and SaO_2 were measured continuously and recorded every 15 min. Arterial blood pO_2 was kept constant at approximately 13 kPa by regulating the inspired oxygen concentration, measurements were done every 15 min and FiO_2 was adjusted accordingly. A urine catheter with a temperature sensor was placed in the urine bladder and a pulse-oximeter was positioned on either lip or auricle to monitor oxygen saturation. Anesthesia was maintained with continuous intravenous infusions of pentobarbital 7–18 mg/kg/h and fentanyl 2–3 μg/kg/h. Ringer-Acetate was infused at a rate of 300–1000 mL/h, adjusted according to the clinical assessment of urinary output and MAP. Heparin (5000 IE) bolus doses was administered hourly.

In five of the pigs, after stabilization, the rotenone infusion was initially 0.25 mg/kg/h for 3 h, thereafter at 0.5 mg/kg/h for 1 h through a single lumen catheter in the left jugular vein. The remaining five pigs were infused with vehicle (71% H_2O, 25% *N*-methylpyrrolidone, 4% polysorbate). The selected rotenone dosing regiment and tolerability of the vehicle were determined in a prior set of dose-escalation experiments in a total of ten pigs with a rotenone dose starting at 0.3 μg/kg/h (data not shown).

2.2. Hemodynamic parameters and blood gases

Arterial blood pressure was measured continuously through a catheter in the femoral artery. A Swan-Ganz catheter (CCOmbo V, Edwards Life sciences, Irvine, USA) was inserted through the right external jugular vein into the pulmonary artery. Pulmonary artery pressure and central venous pressure were recorded every 15 min; pulmonary wedge pressure was measured every 30 min. Cardiac output (CO) was measured by thermo-dilution from the Swan-Ganz catheter and recorded every 15 min. Animals were also monitored by ECG during the experiment to detect serious arrhythmias.

Arterial blood samples were collected from the femoral artery and venous blood samples were collected from the Swan-Ganz catheter in the pulmonary artery. Blood gases were analyzed every 15 min (ABL725 blood gas analyzer, Radiometer Medical Aps, Brønshøj, Denmark) measuring pH, pCO_2, pO_2, HCO_3, sO_2, tCO_2(B), tHb, Hct, K^+, Na^+, Ca^{2+}, Cl^-, glucose, lactate and base excess.

2.3. Continuous indirect calorimetry

Continuous indirect calorimetry was performed using a Quark RMR ICU (Cosmed, Rome, Italy) measuring whole animal oxygen consumption (VO_2) and carbon dioxide production (VCO_2). The Quark RMR ICU measures VCO_2 and VO_2 using a breath-by-breath technique through an infrared CO_2 and paramagnetic O_2 analyzer. The gas is sampled through a tube attached to the ventilator. Minute ventilation is measured by a turbine flow meter attached to the ventilator expiratory port. The Quark software detects any bias flow from the ventilator and compensates for this when calculating VCO_2 and VO_2 (Sundstrom et al., 2013). Data was recorded every 15 min.

2.4. Oxygen tension in skeletal muscle in vivo

Measurement of oxygen tension was conducted with large area surface (LAS)™ oxygen-sensing luminescent optodes (0.7 mm diameter; 8 mm^2) in *m. pectineus* and *m. sternocleidomastoideus* for continuous monitoring of tissue PO_2 with data recorded every 15 min. The skin incisions for placement of probes were sutured. The probes were pre-calibrated by the manufacturer (Oxylite Pro, Oxford Optronic Ltd, Abingdon, UK). These fiber optic probes send pulses of light to a luminophore at the tip of the cable and the emitted light is then carried back to a monitoring system with automatic temperature compensation (Dyson et al., 2012).

2.5. Mitochondrial respiration ex vivo

In four pigs per treatment group mitochondrial respiratory function was analyzed *ex vivo* in platelets and skeletal muscle biopsies using high-resolution respirometry (Oxygraph-2k, Oroboros Instruments, Innsbruck, Austria). Oxygen flux was monitored and recorded in real-time using DatLab 5.1 software (Oroboros Instruments, Innsbruck, Austria). Samples were collected and analyzed both immediately prior to infusion and after 3 h.

Using the catheter in the femoral artery, 20 mL of blood was collected in K_2EDTA tubes (Vacuette®, Greiner Bio-One GmbH, Kremmünster, Austria). Platelets were isolated using sequential centrifugation steps, as previously described (Sjovall et al., 2013). Mitochondrial function of intact platelets was analyzed in both the animal's own plasma and MiR05 solution (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). Analysis was executed using an adapted version of a previously published protocol (Sjovall et al., 2010). After stabilization of routine respiration with endogenous substrates, carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) was titrated until no further respiratory increase. Rotenone (2 μM) was added to completely inhibit CI and lastly antimycin-A (1 μg/mL), a mitochondrial complex III inhibitor, was added to reveal residual oxygen consumption.

Muscle biopsies were obtained using a 14 G micro-biopsy needle from the *m. pectineus* (contralateral to the *in vivo* oxygen tension probe described above). The samples were instantaneously transferred to 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) and thereafter gently dissected using forceps to remove any fat and connective tissue. The mitochondrial function was then analyzed in MiR05 (Pesta and Gnaiger, 2012). A titration protocol was implemented specified at detecting complex I (CI) dysfunction using NADH-linked substrates at concentrations that were semipermeable in muscle biopsies. First malate (5 mM), pyruvate (5 mM) and glutamate (5 mM) were added, thereafter, FCCP was titrated until no further

increase in respiration was achieved (mean concentration 1.5 μM). Subsequently rotenone (2 μM) and finally antimycin-A (1 μg/mL) was added.

Citrate synthase activity was used as a biomarker for mitochondrial content, as a complement to cell-count and tissue weight for normalization of respiration (Larsen et al., 2012). Citrate synthase activity was measured using a commercially available kit according to the manufacturer's instructions (Citrate Synthase Assay Kit, CS0720, Sigma).

2.6. Metabolomics in muscle biopsies

Assessment of metabolomics profile was performed in a subset of three control samples and two rotenone treated samples. Muscle biopsies were collected as described in the previous section after 3 h of rotenone or vehicle infusion.

A targeted quantitative analysis of 116 metabolites was performed using capillary electrophoresis time-of-flight mass spectrometry (CE-TOFMS) for cationic compounds and capillary electrophoresis tandem mass spectrometry (CE-MS/MS) for anionic compounds. Focus was on metabolites involved in glycolysis, pentose phosphate pathway, TCA cycle and the urea cycle, as well as polyamine, creatine, purine, glutathione, nicotinamide, choline, and amino acid metabolism. The analysis (CARCINOSCOPE™) was carried out by Human Metabolome Technologies Inc. (HMT), Tsoruka, Japan. Samples were handled according to their instructions and sent to HMT in frozen condition. At HMT the samples were mixed with 750 μL of 50% acetonitrile in water (v/v) containing internal standards (40 μM for cation and 10 μM for anion measurement) and homogenized. Then 750 μL of 50% acetonitrile in water (v/v) was added and subsequently centrifuged. The supernatant was filtrated through a 5-kDa cut-off filter (ULTRAFREE-MC-PLHCC, HMT) The filtrate was once again centrifuged and thereafter resuspended in 50 μL of ultrapure water before metabolome analysis using CE-MS. All the metabolite concentrations were calculated by normalizing the peak area of each metabolite with respect to the area of the internal standard and by using standard curves, which were obtained by three-point calibrations.

2.7. Statistical analysis

Statistical analyses of hemodynamic parameters and blood gases (means, SD, and 2-way repeated measures (RM) ANOVA of interaction between time and drug) were performed using Prism 7 (GraphPad Software, San Diego, USA). In the repeated measures analysis missing values were replaced by carrying forward (or backward) the last sampled data. Data of indirect calorimetry revealed that parameter values differed substantially between animals at start of the sampling periods and data was normalized group-wise to the highest initial value. A running median with a window of 3 min was used to filter drastic spikes in data caused by adjustments of the inspired oxygen concentration. Statistical analyses for mitochondrial respiration were performed using paired *t*-test. In the analysis of platelet respiration one value was excluded due to evident laboratory error. A p-value <0.05 was considered significant. As the magnitude of changes in evaluated parameters were not pre-specified, power calculation for sample size was not applicable in the present study.

3. Results

3.1. Hemodynamic parameters

All animals survived the 4 h infusion period. In both the rotenone and vehicle treated groups, the major hemodynamic parameters such as body temperature, oxygen saturation (SaO_2), heart rate (HR) and cardiac output (CO) remained stable throughout the first 3 h of rotenone infusion at 0.25 mg/kg/h. There was a trend of decreased mean arterial pressure (MAP) with time for both groups for the first 3 h. With the higher infusion rate at 0.5 mg/kg/h, the clinical status of the pigs treated with rotenone rapidly became unstable in regards to vital hemodynamic parameters (Table 1).

3.2. Lactate, pH and oxygen tension

Rotenone infusion induced a progressive increase in lactate concentration up to 6.1 mmol/L (range: 4.9–7.6 mmol/L) during the first 3 h ($p < 0.05$ at 45 min) and up to 10.5 mmol/L (range: 9.4–11.4 mmol/L)

Table 1
Clinical parameters. Rot – Rotenone, Temp – Temperature, SaO_2 – Oxygen saturation, HR – Heart rate, MAP – Mean arterial pressure, CO – Cardiac output, CVP – central venous pressure, MPAP – Mean pulmonary arterial pressure, PCWP – Pulmonary wedge pressure. Data presented as mean (SEM).

		Start of infusion (0.25 mg/kg/h)				Increased rate (0.5 mg/kg/h)					
Time (min)		0	30	60	90	120	150	180	210	240	270
Temp °C	Control	38.8 (0.29)	38.8 (0.37)	38.8 (0.33)	38.8 (0.31)	39.0 (0.33)	39.0 (0.42)	39.1 (0.46)	39.2 (0.45)	39.3 (0.42)	39.4 (0.42)
	Rot	39.3 (0.36)	39.3 (0.43)	39.1 (0.45)	39.2 (0.46)	39.5 (0.41)	39.6 (0.39)	39.6 (0.38)	39.7 (0.33)	39.6 (0.33)	39.5 (0.33)
SaO_2%	Control	97.4 (0.81)	98.2 (0.49)	98.0 (0.55)	98.0 (0.77)	96.8 (0.86)	97.2 (0.49)	96.6 (0.75)	96.2 (1.46)	97.0 (1.05)	96.6 (0.93)
	Rot	98.4 (1.12)	98.0 (1.14)	98.2 (1.32)	97.4 (1.86)	98.6 (0.68)	98.4 (0.87)	97.8 (0.73)	98.0 (0.84)	96.8 (1.02)	92.8 (2.06)
HR Beats/min	Control	118.6 (9.91)	116.2 (13.3)	104.0 (9.47)	103.2 (5.44)	101.0 (4.39)	103.6 (3.26)	103.8 (3.18)	105.8 (3.48)	106.0 (3.62)	109.8 (4.87)
	Rot	114.0 (8.44)	114.8 (6.85)	104.4(8.39)	107.8 (6.34)	109.0 (10.5)	114.4(7.09)	119.2 (5.83)	122.8 (5.96)	173.6 (5.22)	185.0 (16.1)
MAP mm Hg	Control	101.4 (5.36)	109,2 (4.42)	102.2 (5,36)	96.2 (6.60)	91.0 (6.51)	87.0 (7.08)	84.2 (6.89)	81.2 (7.66)	77.4 (7.38)	80.2 (7.78)
	Rot	109.2 (4.13)	105.6 (4.01)	94.60 (3.98)	94.4 (2.71)	82.8 (5.81)	77.6 (4.13)	75.2 (4.73)	71.6 (3.91)	97.4 (4.17)	63.5 (10.1)
CO L/min	Control	5.18 (0.46)	5.38 (0.23)	4.93 (0.36)	4.80 (0.43)	4.83 (0.53)	4.88 (0.50)	4.88 (0.45)	4.88 (0.46)	4.78 (0.48)	4.98 (0.47)
	Rot	4.50 (0.41)	4.62 (0.24)	4.12 (0.36)	4.44 (0.26)	4.70 (0.18)	4.70 (0.22)	4.92 (0.29)	5.16 (0.43)	6.26 (0.50)	5.88 (0.63)
CVP mm Hg	Control	7.0 (0.63)	7.8 (0.37)	7.8 (0.37)	8.2 (0.49)	8.2 (0.58)	8.2 (0.58)	8.2 (0.66)	8.6 (0.40)	8.0 (0.63)	7.8 (0.66)
	Rot	10.6 (2.93)	7.2 (0.80)	7.8 (0.73)	7.0 (0.55)	7.4 (0.75)	7.0 (0.63)	6.8 (0.80)	6.8 (0.80)	6.0 (1.00)	6.2 (0.97)
mPAP mm Hg	Control	18.7 (0.93)	19.7 (1.38)	19.9 (1.06)	20.0 (0.66)	21.1 (0.79)	20.5 (0.87)	22.1 (1.16)	21.9 (0.98)	21.3 (0.91)	21.5 (0.56)
	Rot	24.1 (1.41)	21.3 (1.88)	23.7 (1.71)	20.6 (1.43)	19.1 (1.47)	19.6 (1.47)	19.2 (1.31)	17.7 (1.11)	27.1 (1.37)	22.4 (3.17)
PCWP mm Hg	Control	8.2 (0.73)	10.8 (1.88)	9.0 (0.63)	9.2 (0.58)	9.0 (0.45)	8.8 (0.49)	8.8 (0.58)	9.0 (0.63)	9.0 (0.45)	8.8 (0.37)
	Rot	11.0 (3.07)	11.0 (2.37)	11.0 (2.61)	11.4 (2.29)	10.4 (2.44)	10.4 (2.73)	7.8 (0.86)	8.0 (0.63)	11.6 (3.59)	7.6 (1.29)

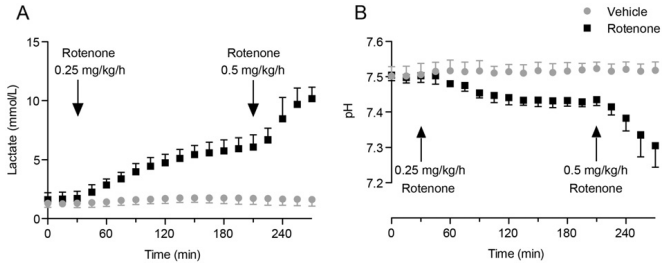

Fig. 1. Lactate and pH. Rotenone infusion induced a progressive increase in blood lactate (A) concentration up to 6.1 mmol/L during the first 3 h, reaching 10.5 mmol/L following the higher infusion rate of rotenone. Parallel to the lactate increase, arterial pH (B) progressively decreased. In control animals lactate and pH did not change significantly. $p < 0.0001$ with 2-way RM ANOVA for interaction between time and drug for both figures. Mean ± SD (one-sided shown in figure). $n = 5$ animals in each group.

following the higher infusion rate (Fig. 1A). Parallel to the lactate increase, arterial pH progressively decreased as illustrated in Fig. 1B ($p < 0.05$ at 60 min). Simultaneously, the rotenone treated pigs demonstrated an increased oxygen saturation in venous blood (Fig. 2A) and an increase in oxygen tension in skeletal muscle *in vivo* (Fig. 2B). There was a biphasic change in blood glucose levels with an initial increase (9.7 mmol/L at 75 min, $p < 0.05$) that was followed by a later decrease (3.5 mmol/L at 270 min, $p < 0.05$) compared to control (Fig. 3).

3.3. Continuous indirect calorimetry

Continuous indirect calorimetry showed that rotenone caused a decrease in whole body oxygen consumption and initially a decrease in carbon dioxide production as illustrated in Fig. 4A and B. During the first 30 min of rotenone infusion the oxygen uptake decreased by a mean of 8.7% ($p < 0.0001$) (range: −3.5% to −13.3%).

3.4. Oxygen delivery

Oxygen delivery remained steady during the lower infusion rate and increased with the higher infusion rate (Fig. 5).

3.5. Mitochondrial respiration ex vivo

Analysis of mitochondrial function *ex vivo* in viable intact platelets from rotenone treated animals displayed a statistically non-significant inhibition ($p = 0.059$) of phosphorylating capacity after 3 h of rotenone infusion. Platelets from the vehicle treated animals on the other hand displayed a non-significant increase (Fig. 6A–B). This trend was consistently present when platelets were analyzed in both the animals' own plasma and buffer solution (MiR05) (data not shown). These changes were seen regardless if respiration was normalized per cell or citrate synthase activity (data not shown). The trend seen in platelets was not detected in muscle fibers (Fig. 6C–D).

3.6. Metabolomics

In muscle biopsies from rotenone-treated animals pyruvic acid, the end product of glycolysis, was increased by 50% and lactic acid levels were almost doubled (Fig. 7); the resulting mean lactate-to-pyruvate ratio was increased from 21.9 in the controls (range 19.6–24.5) to 27.7 in the rotenone treated group (range 25.2–30.2). In the analysis of the tricarboxylic acid (TCA) cycle metabolites, succinic acid was the only intermediate that was decreased, while the others displayed increased levels. Citric acid levels were doubled and cis-aconitic acid, an intermediate in the isomerization of citrate to isocitrate, was increased several fold (Fig. 7). There was a trend towards higher levels of glycolysis intermediates in the rotenone treated group. Glucose 6-phosphate (G6P), fructose 6-phosphate (F6P) and glyceraldehyde 3-phosphate (G3P) were all several fold increased (Fig. 7). The trend was similar but less pronounced for metabolites downstream of glyceraldehyde 3-phosphate. Markers of energy state such as ATP, products of ATP hydrolysis and phosphocreatine were not altered significantly by rotenone treatment (Supplementary Table 1). The complete metabolomics data is available as supplementary material (Supplementary Table 1).

4. Discussion

The composite data indicates that intravenous rotenone infusion decreases mitochondrial utilization of oxygen and causes a switch from

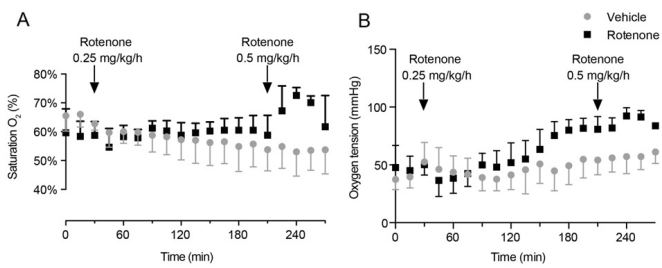

Fig. 2. Oxygen tension. The development of lactic acidosis in the rotenone treated animals was paralleled by an increase in oxygen saturation in venous blood (A) and oxygen tension in skeletal muscle (B). Measurements illustrated in Fig. 2B were acquired from *m. sternocleidomastoideus*. p < 0.0001 with 2-way RM ANOVA for interaction between time and drug for both figures. Mean ± SD (one-sided shown in figure). $n = 5$ animals in each group.

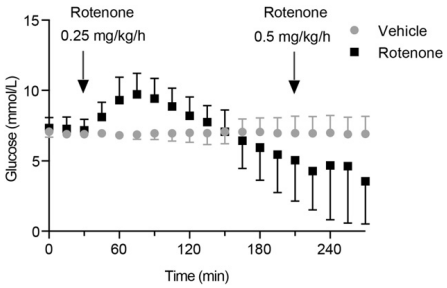

Fig. 3. Blood glucose. Rotenone infusion initially increased blood glucose levels ($p < 0.05$ at 45 min), however over time the blood glucose dropped and approached hypoglycemic levels towards the end of the experiment. $p < 0.0001$ with 2-way RM ANOVA for interaction between time and drug. Mean ± SD (one-sided shown in figure). $n = 5$ animals in each group.

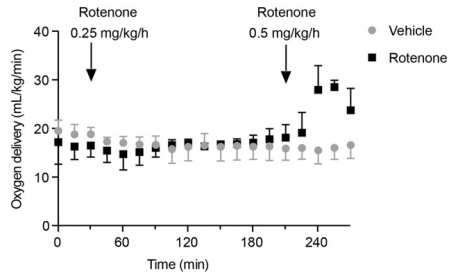

Fig. 5. Oxygen delivery. Oxygen delivery calculated as cardiac output (CO) multiplied by oxygen content ($1.39*Hb*O_2Sat/100) + (0.003*PO_2$). Mean ± SD (one-sided shown in figure). $n = 5$ animals in each group.

aerobic to anaerobic metabolism with a subsequent rise in arterial blood lactate and decrease in pH. With the start of rotenone infusion, the global oxygen consumption rapidly decreased and in the initial phase, the amount of expired carbon dioxide decreased. Venous and skeletal muscle oxygen tension displayed a progressive increase throughout the experiment, indicating a decreased oxygen extraction through decreased mitochondrial respiration. These results, together with the observed metabolic changes *in vivo* and the trend towards inhibition of mitochondrial respiration in platelets *ex vivo* suggest that the increased blood lactate levels are triggered by a mitochondrial inhibition and not secondary to decreased tissue perfusion and cellular hypoxia. The alternative interpretation that the increased tissue oxygen saturation may be due to vasodilation cannot be excluded. After the first 3 h there is no manifest energy decompensation, most likely due to accelerated glycolysis.

The increased blood lactate concentration indicates an accelerated glycolysis, a compensatory mechanism to mitigate decreased ATP production by the mitochondria. Metabolomics data demonstrates a trend towards higher levels of glycolysis intermediates in the rotenone treated group. This trend was more prominent upstream of G3P and can be related to the metabolism of G3P which is the first redox step in glycolysis as NAD+ is reduced to NADH, which can either be oxidized back to NAD+ at CI or by lactate dehydrogenase coupled to the conversion of pyruvate to lactate. There was also a trend towards an increased lactate-to-pyruvate ratio in the rotenone treated group indicating accelerated glycolysis and increased lactate production with a metabolic shift from mitochondrial respiration to glycolysis (Huckabee, 1958). Furthermore, the decrease in aspartic acid could be related to its role in the mitochondrial matrix and cytosolic redox of NADH and NAD+. All detected TCA cycle intermediates were trending towards increased levels except succinic acid, which was slightly decreased. Succinate is oxidized by complex II of the respiratory chain, which is not inhibited by rotenone. The limited group size of the metabolomics data is a limitation to the interpretation of the data.

In the *ex vivo* analyses of mitochondrial respiration, a trend towards inhibition was detected in platelets while analysis of peripheral skeletal muscle did not detect such a trend. The discrepancy between the metabolomic and respiration data of skeletal muscle could possibly be related to technical aspects of preparing the samples. As the muscle biopsies were rinsed in MiR05 buffer in preparation of respiration measurements, the rotenone was perhaps removed. The platelets, on the other hand, were suspended in plasma during the preparatory steps of the protocol and added to the final analysis as platelet-rich plasma, thereby decreasing the risk of altering the rotenone exposure during the *in vivo - ex vivo* transition. It may also be that the mitochondrial respiration in skeletal muscle was in fact not inhibited by rotenone *in vivo* and that the increase in lactate production primarily comes from other organs.

Due to the successive decrease in pH during rotenone infusion, expired VCO_2 overestimates the metabolic CO_2 production as it also encompasses the release of stored CO_2 due to a continuous leftward shift in the CO_2 - carbonic acid – bicarbonate equilibrium. The metabolic respiratory quotient (RQ) ranges from 0.7 to 1.0 depending on the relative

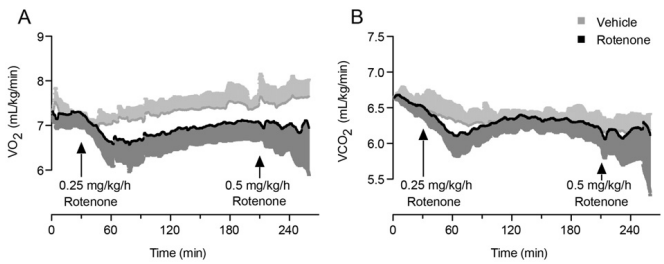

Fig. 4. Continuous indirect calorimetry. Determination of VO_2 (A) and VCO_2 (B) using indirect calorimetry demonstrated that rotenone caused a decrease in oxygen uptake and initially a decrease in carbon dioxide production which resulted in subsequent calculated increased respiratory quotient and decreased energy expenditure. During the first 30 min of rotenone infusion the oxygen uptake decreased by a mean of 8.7% ($p < 0.0001$). Data was normalized group-wise and a running median with a window of 3 min was used. Mean ± SD (one-sided shown in figure). $n = 5$ animals in each group.

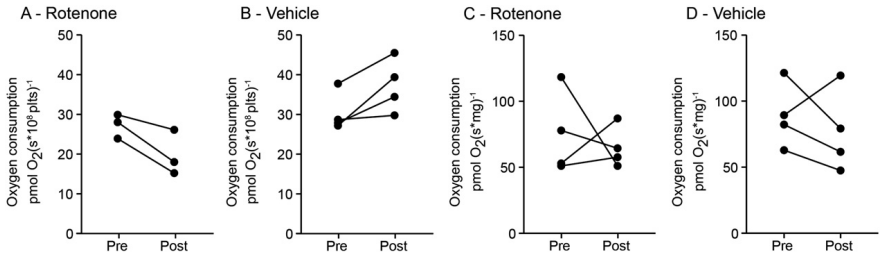

Fig. 6. Mitochondrial respiration. Mitochondrial respiration of viable platelets analyzed *ex vivo* indicated a trend towards decreased aerobic function following 3 h of rotenone infusion ($p = 0.059$) (A), compared to before start of infusion, when suspended in their own plasma. The vehicle treated group indicated a trend in the opposite direction (B). This trend was not detected in skeletal muscle fibers analyzed *ex vivo* (C–D). Individual values are shown. Paired *t*-test.

proportions of carbohydrate and fat metabolism (Krogh and Lindhard, 1920). The measured respiratory exchange ratio (RER, VCO_2/VO_2) was above 1.0 in the rotenone treated group demonstrating a partial increase in elimination of CO_2 not related to production of CO_2 in the mitochondria. As there was no steady state metabolism the decrease in energy expenditure cannot be accurately determined, but the corresponding decrease in VO_2, was as previously stated, 8.7% during the first 30 min after start of rotenone infusion.

This *in vivo* large animal model would be suitable for evaluating pharmaceutical therapies for changes in energy metabolism involving mitochondrial CI dysfunction. Our group recently published a paper presenting the concept of succinate prodrugs as a potential treatment for mitochondrial CI deficiency (Ehinger et al., 2016). It has also been suggested that standard succinate can recover mitochondrial function in septic skeletal muscle (Protti et al., 2007). Methyl succinate and heptanoate, a short odd-chain fatty acid, has also been evaluated for the treatment of CI dysfunction (Doctor et al., 1994; Hinke et al., 2007). Furthermore, methylene blue has been shown to bypass complex I and II inhibition in models of drug-induced mitochondrial dysfunction (Lee et al., 2015). These different potential pharmacological strategies have been tested *in vitro* and in small animal *in vivo* models. A potential next step in the development could be a large-animal *in vivo* model. More refined models are warranted for specific diseases, but the model presented here may be suitable for initial testing.

5. Conclusions

In conclusion, rotenone infusion decreases whole animal extraction of oxygen due to mitochondrial CI inhibition and causes a switch from mitochondrial metabolism to glycolysis. Interestingly the rather dramatic increase in lactate corresponded to a moderate decrease in VO_2. This model may be well suited for future studies of pharmacological interventions aiming at counteracting acute changes in energy metabolism due to inherited as well as drug-induced mitochondrial CI dysfunction.

Authors disclosure statement

The study was in part funded by NeuroVive Pharmaceutical AB. The listed authors, as indicated by their affiliation, have received salary

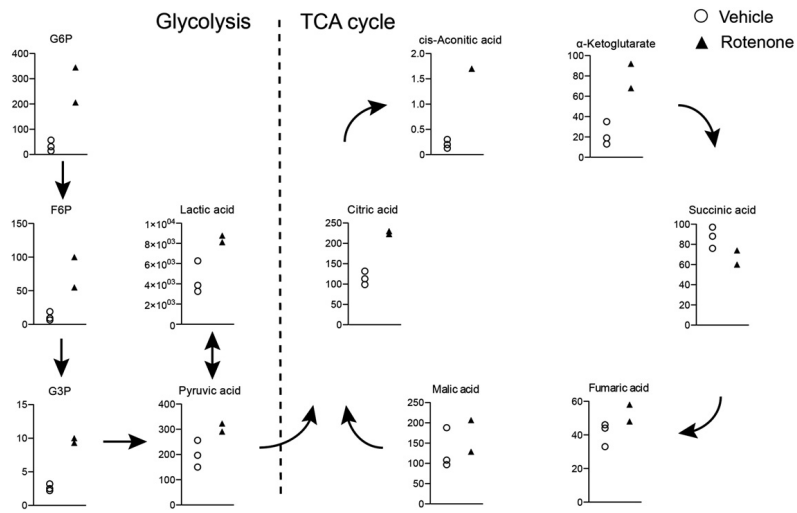

Fig. 7. Metabolomics. Analysis performed in muscle biopsies after 3 h of vehicle ($n = 3$) or rotenone ($n = 2$) infusion. G6P – Glucose 6-phosphate, F6P – Fructose 6-phosphate, G3P – Glyceraldehyde 3-phosphate. Y axis: concentration (nmol/g). Individual values shown.

support from and have equity interests in NeuroVive Pharmaceutical AB, a public company developing pharmaceuticals in the field of mitochondrial medicine.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.mito.2016.10.003.

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