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Mitochondrial dysfunction and the metabolism - studies on respiratory chain disorders

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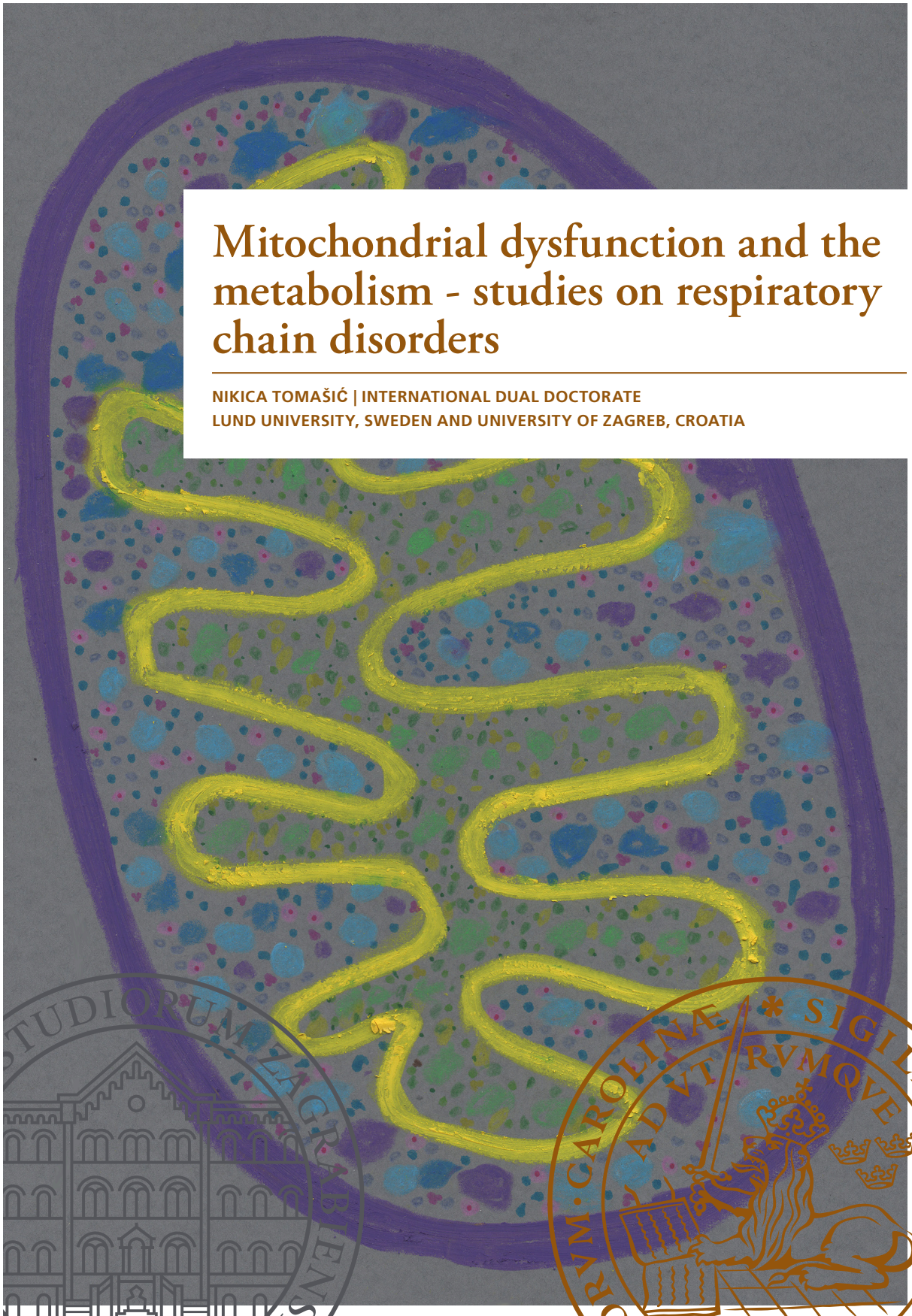

Mitochondrial dysfunction and the metabolism - studies on respiratory chain disorders

NIKICA TOMAŠIĆ | INTERNATIONAL DUAL DOCTORATE
LUND UNIVERSITY, SWEDEN AND UNIVERSITY OF ZAGREB, CROATIA

Mitochondrial dysfunction and the metabolism
- studies on respiratory chain disorders)

Mitochondrial dysfunction and the metabolism - studies on respiratory chain disorders

Nikica Tomašić

INTERNATIONAL DUAL DOCTORATE

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To be defended at Rune Grubb lecture hall, Biomedical Centre, Lund, Sweden September 23rd, 2021, at 13.00.

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Abstract

Background: Mutations of the mitochondrial assembly factor *BCS1L* disrupt assembly and function of the respiratory chain complex III (CIII) and thereby result in energy deprivation. In GRACILE syndrome, the *BCS1L* mutation causes a liver disorder that leads to metabolic disturbances associated with severe growth restriction.

Objectives: To clarify the mechanisms and affected metabolic pathways in the CIII deficiency disease progression and perform intervention trials in an experimental disease model.

Methods/research questions: A *knock-in* mouse model, carrying the same missense mutation (*Bcs1l* c.232A>G) as the GRACILE syndrome patients, is used to study disease mechanisms and pathways involved. Two randomized controlled intervention studies have been performed on homozygous mice and littermate controls: a 4-hour fasting as a metabolic pressure to assess the compensatory capabilities, and a dietary intervention to clarify whether hypoglycemia and survival can be improved by the high-carbohydrate diet (60% dextrose). Further, phenotyping and the disease mechanism in a patient with a novel compound heterozygous *BCS1L* mutation have been studied and compared to GRACILE patients and mutant mice. In the last study, a metabolic phenotype caused by a single large-scale deletion (SLSD) in mitochondrial (mtDNA), including the genes for structural components of CI and CIII, was analyzed.

Methods used in the studies include genetic analysis,WES, WGS, phenotyping, blood and urine chemistry, metabolomics, histopathology, EM, function, and expression analysis. Conventional statistics and linear mixed-effect models (MEMs) were used for the analysis of the metabolic network response to fasting.

Results: Fasting mutant animals revealed intact systemic lipid mobilization but disrupted compensatory mechanisms leading to hypoglycemia. The high-carbohydrate diet had an unexpected adverse effect on survival compared to the standard diet. The novel *BSC1L* mutation had a different phenotype compared to GRACILE syndrome. The patient with the SLSD had a complex metabolic phenotype, which emphasized the importance of diagnostics synergy of clinical awareness, genetic and funtional analysis.

Significance: By elucidating metabolic disturbances, the project increases understanding of pathophysiology in CIII deficiency. This has a potential effect on understanding other, more common mitochondrial dysfunctions and raises new queries about this complex system.

Key words
mitochondria, mitochondrial disease, Complex III, BCS1L,

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“Science is not wisdom.” *(F. J. Sheen)*

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II. Rajendran J, **Tomašić N**, Kotarsky H, Hansson E, Velagapudi V, Kallijärvi J, Fellman V. Effect of High-Carbohydrate Diet on Plasma Metabolome in Mice with Mitochondrial Respiratory Chain Complex III Deficiency. Int J Mol Sci. 2016 Nov 1;17(11).

III. Tegelberg S*, **Tomašić N***, Kallijärvi J, Purhonen J, Elmér E, Lindberg E, Nord DG, Soller M, Lesko N, Wedell A, Bruhn H, Freyer C, Stranneheim H, Wibom R, Nennesmo I, Wredenberg A, Eklund EA, Fellman V. Respiratory chain complex III deficiency due to mutated BCS1L: a novel phenotype with encephalomyopathy, partially phenocopied in a Bcs1l mutant mouse model. Orphanet J Rare Dis. 2017 Apr 20;12(1):73.

IV. **Tomašić N**, Ulinder T, Samuelsson S, Jonson T, Piccinelli P, Ehrencrona H, Forsgren M, Papadopoulou D, Ekelund C, T J de Koning, Eklund EA. Fulminant lactic acidosis and liver failure in a prenatal onset Pearson syndrome. Manuscript form.

* Equal contributors

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Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
BNGE	blue native gel electrophoresis
CYCS	cytochrome C
CYTB	cytochrome B
CI	complex I
CII	complex II
CIII	complex III
CIV	complex IV
EM	electron microscopy
ETC	electron transport chain
FFA	free fatty acid
FAD+	flavine adenine dinucleotide
FCCP	carbonyl-cyanide p-(trifluoromethoxy) phenylhydrazone
IMM	inner mitochondrial membrane
IMS	intermembrane space
MELAS	mitochondrial encephalopathy with lactic acidosis and stroke-like episodes
mRNA	messenger ribonucleic acid
mtDNA	mitochondrial DNA
nDNA	nuclear DNA
NAD+	nicotinamide adenine dinucleotide, oxidized form
NADH	nicotinamide adenine dinucleotide, reduced form
NADPH	nicotinamide adenine dinucleotide phosphate, reduced form

OMM	outer mitochondrial membrane
OxPhos	oxidative phosphorylation
PS	Pearson syndrome
RISP	Rieske iron-sulfur protein
RC	respiratory chain
SLSD	single large-scale deletion
SNV	single-nucleotide variant
SUIT	substrate-uncoupler-inhibitor-titration
TCA	tricarboxylic acid
TEM	transmission electron microscopy
WES	whole exome sequencing
WGS	whole genome sequencing
WT	wild type

Summary

Background: Mutations of the mitochondrial assembly factor *BCS1L* disrupt assembly and function of the respiratory chain complex III (CIII) and thereby result in energy deprivation. In GRACILE syndrome, the *BCS1L* mutation causes a liver disorder that leads to metabolic disturbances associated with severe growth restriction.

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Extended summary in Croatian

Uvod

Mitohondriji su eukariotski stanični organeli koji sudjeluju u energetskom metabolizmu stanice; kao proizvođači molekula ATP-a u reakcijama respiratornog lanca i mjesto su odvijanja najvažnijih metaboličkih procesa stanice. Prema endosimbiotskoj teoriji, mitohondriji potječu od neovisnog prokariota koji je endocitozom ušao u simbiozu s eukariotskim domaćinom. S vremenom, prokariot postaje stalan dio eukariotske stanice, zadržavši dvostruku membranu i vlastitu DNA. Isprva su smatrani samostalnom i odvojenom organelom, no istraživanja zadnjih nekoliko desetljeća ukazuju da su funkcionalno povezani sa susjednim organelima i u stalnoj komunikaciji sa staničnom jezgrom.

Mitohondrijske bolesti su relativno česte metaboličke bolesti koje direktno ili indirektno ometaju funkciju oksidativne fosforilacije, s prevalencijom od oko 1:5000. Izuzetno složena genetika i patofiziologija te ekstremno varijabilna klinička slika kompliciraju dijagnostiku mitohondrijskih bolesti.

Mitohondriji imaju jedinstvenu strukturu i funkciju u stanici. Njihov oblik i broj u pojedinačnim stanicama ovisi o tipu i energetskim zahtjevima stanice. Omeđeni su dvjema fosfolipidnim membranama razdvojenim intermembranskim prostorom. Vanjska i unutarnja membrana se razlikuju u permeabilnosti, sastavu i funkciji. Manje permeabilna, unutarnja mitohondrijska membrana okružuje unutrašnjost, ili matriks, i sastoji se od mnogo nabora zvanih kriste. Unutarnja membrana je i mjesto gdje se odvijaju reakcije oksidativne fosforilacije i proizvodnje ATP-a u enzimskim kompleksima nazvanim respiratorni lanac. Respiratorni lanac sastoji se od pet kompleksa, označenih kao CI-CV. Osim enzimskih kompleksa, u respiratornom lancu nalaze se još dva prijenosnika elektrona, koenzim Q i citokrom c.

Respiratorni enzimski kompleksi i oksidativna fosforilacija

Respiratorni kompleks je pod dvostrukom genskom kontrolom. Većina proteina mitohondrija kodirana je nuklearnom (nDNA) (99%), dok je manji broj, njih 37, kodirano mitohondrijskom DNA (mtDNA), dvolančanom DNA koja se nalazi u mitohondrijskom matriksu.

NADHQ-oksidoreduktaza ili kompleks I (CI), je najveći enzim oksidativno-fosforilacijskog sustava sisavaca i prvi primatelj elektrona. Podjedinice enzima kodirane su i s mtDNA (ukupno 7) i s nDNA (ukupno 45). Zbog svog složenog sastava, pogreške u CI su među najčešćim uzročnicima poremećaja respiratornog lanca, primjerice MELAS ili Leighov sindrom.

Sukcinat-Q-reduktaza ili kompleks II (CII) je još jedan ulaz za elektrone u lanac prijenosa elektrona, kodiran u potpunosti s nDNA. Djeluje na sjecištu lanca prijenosa elektrona i Krebsovog ciklusa. Poremećaji kompleksa II obično se javljaju kao nasljedni tumori, i u manjoj mjeri kao tipične manifestacije mitohondrijskih bolesti.

Q-citokrom c oksidoreduktaza ili kompleks III (CIII), čini središnji dio respiratornog lanca. Prenosi elektrone s ubikvinona na citokrom c, koristeći pritom oslobođenu energiju za pumpanje 2 protona iz matriksa u međumembranski prostor. Monomer CIII sastoji se od 11 podjedinica, od kojih su 10 kodirane s nDNA, a samo jedna s mtDNA. Struktura, slaganje i funkcija kompleksa najviše je istražena na kvascima, dok su neki dijelovi sustava identificirani i u ljudskim stanicama, a mehanizam slaganja enzima je modeliran iz strukturnih podudarnosti proteina. Za slaganje proteinskog kompleksa, osim strukturnih podjedinica potrebni su i dodatni proteini i stabilizirajući faktori. Slaganje započinje sintezom citokroma b, koji se povezuje u prijelazni oblik s dodatnim komponentama. Prijelazni oblik dimerizira, ali nije aktivan, dok se u njega ne inkorporira podjedinica nazvana Rieske centar (Fe-S protein). Inkorporacija Rieske centra je potpomognuta proteinom BCS1L, translokazom iz AAA+ porodice proteina i sklopnim faktorom (engl. assembly factor) CIII.

Citokrom c oksidaza ili kompleks IV (CIV), je enzim u kojem se elektroni prenose do molekule kisika, konačnog receptora elektrona, i reducira ga u vodu. Za reakciju su ključne dvije hem skupine i tri bakarna atoma. Poremećaji CIV su vrlo česti, zbog strukturne složenosti enzima i brojnosti popratnih faktora potrebnih za sklapanje kompleksa.

ATP sintaza ili kompleks V (CV), nije dio lanca prijenosa elektrona, već koristi stvoreni gradijent protona za fosforilaciju ADP-a i sintezu ATP-a.

Proces oksidativne fosforilacije koristi energiju iz reakcija prijenosa elektrona za stvaranje ATP molekule. Dva odvojena procesa čine sustav oksidativne fosforilacije: prijenos elektrona kroz respiratorni lanac i ATP sinteza.

NADH i FADH2 predaju elektrone CI i CII, oni zatim prolaze kroz seriju reakcija u enzimima, do koenzima Q i CIII. Kompleks III predaje elektrone CIV preko citokroma c, i konačno, prima ih molekula kisika te formira vodu. Oslobođena energija koristi se za pumpanje protona iz mitohondrijskog matriksa u intermembranski prostor. Kako se protoni vraćaju u matriks kroz energetski povoljan proces, ATP sintaza koristi tu energiju i stvara ATP. Istovremeno, NAD+

i FAD+ se re-oksidiraju kako bi se uspostavila redoks ravnoteža u mitohondrijskom matriksu. Ovaj kompleksni proces mora biti precizno kontroliran na svim razinama: transkripciji, translaciji, sintezi i transportu proteina u mitohondrije te sastavljanju respiratornog lanca. Većina informacija o funkcioniranju sustava oksidativne fosforilacije dolazi iz studija na *C. elegans*, a zbog visokog stupnja konzerviranosti procesa ta znanja prenosiva su u sustav stanica sisavaca.

Mitohondriji u metabolizmu

Metabolizam je ravnoteža između anaboličkih i kataboličkih procesa i značajan dio metabolizma odvija se upravo u mitohondrijima. Kolokalicazija i isprepletanje brojnih metaboličkih puteva unutar mitohondija ima komplekse posljedice ukoliko je jedan od puteva poremećene funkcije.

Okosnica metabolizma je proces glikolize u kojem se glukoza u stanici pretvara u piruvat. Glikoliza, zajedno s Krebsovim ciklusom i oksidativnom fosforilacijom, čini centralnu os metabolizma od koje se račvaju drugi metabolički putevi. Piruvat, konačni produkt glikolize ulazi u mitohondrije gdje se pretvara u acetil-koenzim A, koji ulazi u Krebsov ciklus. Mitohondriji kako je prethodno opisano konvertiraju energiju reducirajućih ekvivalenata za sintezu ATP-a, a Krebsov ciklus ili TCA ciklus i beta oksidacija izvor su reducirajućih ekvivalenata u mitohondrijskom matriksu. Osim toga, metaboliti iz procesa glikolize i Krebsovog ciklusa mogu biti upotrijebljeni i u druge svrhe, npr. sintezu biomolekula.

Oksidativna fosforilacija i dijelovi centralne osi metabolizma, kao i drugi metabolički procesi koji se odvijaju u mitohondriju, obavljaju svoje funkcije unutar iste organele. Stoga poremećaj oksidativne fosforilacije rezultira, osim direktno manjkom proizvodnje energije, i poremećajima metabolizma na razini organizma, kao npr. hipoglikemijom ili poremećenim metabolizmom aminokiselina. Ta se činjenica pokušava iskoristiti u dijagnostici i rasvjetljavanju patofiziologije mitohondrijskih bolesti. Različite studije istraživale su biomarkere primarnih mitohondrijskih bolesti koji bi služili u dijagnostici i eventualnom praćenju bolesti. Jedini trenutno prihvaćeni biomarker je laktat, koji nažalost zbog svoje nespecifičnosti nije dijagnostički osobito vrijedan. Osim laktata, recentne studije navode još dva potencijalno učinkovita biomarkera mitohondrijskih bolesti: GDF15 (Growth differentiation factor 15) i FGF21 (fibroblast growth factor 21).

Poremećaji CIII

Poremećaji CIII klasificirani su kao poremećaji oksidativne fosforilacije i opisani kao manjkavosti u biogenezi i/ili funkciji CIII. Rijetko se pojavljuju, a biokemijski se manifestiraju kao smanjena enzimatska aktivnost, dok je klinička prezentacija ekstremno varijabilna, kao i u drugim mitohondrijskim bolestima. BCS1L je sklopni

faktor CIII kodiran s nDNA na kromosomu II. Filogenetski je konzerviran, i sastoji se kod ljudi od 419 aminokiselina. Ima tri strukturne jedinice: AAA-ATPaze, N-terminalne regije i regije specifične za BCS1L. Mutacije gena za ovaj protein su najčešći uzrok poremećaja CIII. Klinička prezentacija poremećaja uzrokovanih *BCS1L* mutacijom variraju od blagih, kao što je Björnstad sindrom, do najtežeg oblika – GRACILE sindroma. Dio je skupine bolesti Finskog nasljeđa uzrokovan točkastom mutacijom c.232A>G u *BCS1L*, koja uzrokuje zamjenu aminokiseline serin u glicin (p.S78G) (Fellman sindrom, MIM 603358). Akronim GRACILE sumira najvažnije kliničke karakteristike sindroma: zastoj rasta (engl. growth restriction) s tjelesnom težinom -4 SD, aminoacidurija (tubulopatija po Fanconijevom tipu), kolestaza (engl. cholestasis) sa steatozom i cirozom, akumulacija željeza (engl. iron overload), teška laktacidoza i rana smrt (engl. early death). Kako bi razjasnili funkciju BCS1L-a i patofiziologiju GRACILE sindroma, uvedena je humana c.232A>G mutacija u genom miša tehnikom ciljanja gena (engl. gene targeting). Najvažnije značajke homozigotnog miša ($Bcs1l^{G/G}$) su zastoj rasta i progresivna bolest jetre nakon 24. dana života, tubulopatija i uginuće prije navršenih mjesec dana. U svim analiziranim tkivima zabilježena je snižena razina Bcs1l proteina i insuficijentna inkorporacija RISPa u CIII.

Ciljevi istraživanja

Cilj ove disertacije je pojasniti mehanizme i metaboličke poremećaje u razvoju bolesti nedostatne funkcije CIII respiratornog lanca. Disertacija je podijeljena u dva dijela: prvi (studija I i II), koji obuhvaća temeljne eksperimentalne studije na mišjem modelu s GRACILE *Bcs1l* mutacijom, jedinstvenom *knock-in* translacijskom modelu miša s nedostatnim CIII, dok su drugi dio (studija III i IV), opservacijske studije u kojima su prezentirani novi slučajevi pacijenata s bolestima respiratornog lanca i detaljno analiziran njihov metabolički fenotip.

Materijali i metode

Provedene eksperimentalne studije su randomizirani kontrolirani pokusi s usporedbom parova, dok su opservacijske studije prikazi slučaja s novim genetičkim informacijama, te genetska analiza i usporedba fenotipa kroz analizu literature.

Kao eksperimentalni model, korišteni su miševi s mutacijom c232A>G na *Bcs1l* genu, dok su kao kontrolna skupina korišteni miševi divljeg tipa i heterozigotni za mutaciju, koji su fenotipom jednaki miševima divljeg tipa. Analiza krvi životinjskih modela uključuje mjerenja glukoze, laktata, ketona, te mjerenje inzulina, HOMA IR izračun, FFA, kolesterola, triglicerida, fosfolipida, ukupnog i slobodnog kolesterola

te lipoproteina plazme. Provedena je i ciljana analiza metabolita na uzorcima plazme. Histološki uzorci jetre životinjskih modela prekontrolirani su za glikogen i trigliceride. Uzorci mozga imunohistokemijski su analizirani na prisutnost Rieske proteina, markera za mikrogliju i imunološke stanice. Uzorci jetre analizirani su na prisutnost Kupferovih stanica i makrofaga. Transmisijskim elektronskim mikroskopom analizirana je struktura i morfologija mitohondrija te strukturalne promjene uzrokovane intervencijom – četverosatnim gladovanjem.

U opservacijskim studijama, od pacijenata i roditelja su sakupljeni uzorci krvi, kao i kontrolni uzorci zdrave djece. U studiji III korišten je uzorak biopsije mišića (*m. tibialis anterior*). Uzeti su i uzorci jetre, srca, mišića i mozga iz autopsije na preminulom pacijentu. Stanična kultura fibroblasta razvijena je iz uzorka biopsije kože. Na pacijentima su provedene opće i specifične biokemijske analize.

Provjera statusa centralne metaboličke osi dio je rutinske kliničke prakse, posebno u jedinicama intenzivne njege. Učestalo kontroliranje metabolita centralne osi, npr. koncentracije glukoze, laktata i ketona, daje uvid u njezinu funkciju. Redovito su mjerene razine elektrolita, pH, amonijaka i uree, uz specifične testove aminokiselina u plazmi i cerebrospinalnoj tekućini, profil acilkarnitina u krvi i urinu te organskih kiselina u urinu. Kod pacijenata je provedena histološka analiza uzoraka mišića koristeći standardna i specifična obojenja. Dodatne histološke provjere obavljene su na uzorcima jetre, srce, mišićnog i moždanog tkiva oboljelih od GRACILE sindroma, kao i na kontrolnim uzorcima. Elektronskom mikroskopijom provjerena je struktura mišićnih mitohondrija. Osim toga učinjene su genetske analize DNA pacijenata i roditelja.

Aktivnost mitohondrija provjerena je respirometrijom visoke rezolucije u uzorcima životinja i krvi pacijenta.

Osim konvencionalnih statističkih metoda, u studiji I korištena je napredna statistička metoda linearni mixed-effect model (MEMs) za analizu metaboličkog odgovora organizma.

Rezultati

Studije na životinjskim modelima

Miševi koji nose mutacije za GRACILE sindrom pokazuju smanjenu aktivnost kompleksa III, disfunkciju metabolizma jetre i ranu smrt. Studija I pokazala je da je komunikacija između tkiva jetre i adipoznih tkiva očuvana, usprkos izraženom zastoju rasta, te da je regrutacija metabolita očuvana u slučaju metaboličkog stresa. U studijama I i II, u životinjskim modelima je uočena nemogućnost održavanja euglikemije. Hipoglikemija se pojavila gotovo u svim testiranim životinjama s

GRACILE sindromom, iako funkcija respiratornog lanca nije potpuno smanjena. U jetri se proces glukoneogeneze natječe s procesom glikolize, jer ne mogu oba puta funkcionirati u isto vrijeme. Čini se da je u mišjem modelu GRACILE sindroma prednost dana procesu glikolize. Također, bazalna razina ketona u miševima s mutacijom je viša nego u miševima divljeg tipa. To se poklapa sa stanjem poremećaja u oksidativnoj fosforilaciji, koji vodi povećanoj glikolizi i hipoglikemiji, posljedično i većoj potrebi za ketonima.

Opservacijske studije

Analiza eksona (WES) u pacijenta u studiji III otkrila je dvije točkaste mutacije u *BSC1L*, od kojih je jedna varijanta potvrđeno patogena, dok je druga okarakterizirana nepatogenom. Klinička slika, iako je bila izrazito teška s dominantnom mišićnom i neurološkom patologijom, nije bila podudarna s GRACILE sindromom. Biokemijske analize otkrile su smanjenu aktivnost CIII u mišićnim stanicama, što je potvrdilo umiješanost gena *BCS1L* i vratilo analizu na *BCS1L*. Analiza cijelog gena otkrila je mutacije c.306A>T i c399delA kod pacijenta. U usporedbi s GRACILE mutacijom c.232A>G, obje novootkrivene mutacije nalaze se u N-terminalu proteina. Kod pacijenta nisu uočeni poremećaji jetre, samo blagi oblik metaboličkog poremećaja okarakteriziranog aminoacidurijom.

Pacijent iz studije IV imao je, pak, fulminantan i kompleksan fenotip. Biokemijske analize metaboličkog fenotipa su bile zbunjujuće, čak navodeći dijagnostiku u krivom smjeru. Naprednom genetskom analizom (WGS) potvrdjena je mtDNA delecija, tzv. SLSD (single large scale deletion) koja uzrokuje Pearsonov sindrom, uz visok stupanje heteroplazmije. Ovo je prvi do sada opisani slučaj dijagnoze PS koristeći WES metodu, 72 sata od uzorkovanja, i najfulminantniji klinički tijek do sada opisan. U delecijskom segmentu bili su nekoliko podjedinica CI i tRNA te neuobičajena delecija citokroma b. Delecija gena za citokrom b utječe i na sintezu CIII te stabilnost CI što dodatno utječe na poremećaj respiratornog lanca i vjerojatno je doprinijelo fulminantnosti kliničke slike.

Zaključak

Naši rezultati pomogli su dublje okarakterizirati fenotip GRACILE sindroma i potvrditi fenotipsku heterogenost povezanu s BCS1L mutacijama.

Nadalje, kompleksni dijagnostički pristup pokazao je komplementarnost kliničke sumnje, naprednih genetskih metoda tj. sekvencioniranja nove generacije, biokemijskih, proteinskih i *in silico* analiza kod mitohondrijskih bolesti. Zbog

velike fenotipske varijabilnosti navedeni dijagnostički pristupi su neodvojivi i jedino poštujući njihovu komplementarnost možemo doći do dijagnoze.

Miševi homozigotni za BCS1L mutaciju i s poremećajem CIII pokazuju da fiziološki mehanizmi koji upravljaju prilagodbom organizma na metabolički stres izgladnjivanjem nisu poremećeni, kao ni komunikacija između tkiva. Izgladnjivanje vodi ka lošem metaboličkom odgovoru jetre i hipoglikemiji, dok hranjenje visokim udjelom ugljikohidrata ne pomaže otkloniti hipoglikemiju niti produljuje životni vijek. Osim toga, ove studije mogu poslužiti i kao referenca za metaboličke analize miševa u četvrtom tjednu života, budući da se zbog fiziološke kompleksnosti studije u ovom stadiju života miševa obično izbjegavaju.

Background

Mitochondria are ubiquitous and complex cellular organelles. Their function extends beyond the historical paradigm of being the powerhouse of the cell and exclusively contributing to the cell bioenergetics. Sitting at the nexus of the metabolism, they serve as the metabolic integrators hosting canonical metabolic pathways of oxidative phosphorylation (OxPhos), fatty acid oxidation, tricarboxylic acid (TCA) cycle, urea cycle, gluconeogenesis and ketogenesis. Crucial cellular processes are orchestrated by mitochondria, such as programmed cell death, stress, and immune responses. Moreover, they act as a factory and take part in cell signaling, production of different metabolites, such as heme, cardiolipin, and steroids. Proteins required for mitochondrial function are under dual genetic control, encoded both by the nuclear (nDNA) and mitochondrial DNA (mtDNA). By analogy, mitochondrial pathophysiology caused by a single defect in a certain pathway has a complex impact on the cellular function and disturbs the metabolism on an organismal level (Ducker and Rabinowitz 2017)(Chow et al. 2017).

This thesis work is based on the need to clarify the pathophysiology of GRACILE syndrome, hitherto the most severe CIII (CIII) OxPhos disorder (Visapää et al. 2002)(Rapola and Fellman 2002)(Fellman et al. 2002)(Fellman et al. 1998), focusing on the metabolic disturbances. The acronym describes characteristic clinical findings: fetal Growth Restriction, Aminoaciduria due to proximal tubulopathy, Cholestasis, Iron accumulation in the liver, Lactic acidosis, and Early death. It is caused by a homozygous mutation c.A232G in nDNA gene encoding BCS1L, an assembly factor for CIII leading to OxPhos CIII deficiency. A mouse model carrying the same mutation and phenocopying human disorder (Levéen et al. 2011) was used in the thesis. In addition to the animal model studies, two patients with severe clinical presentation were described in the thesis: a patient with novel compound heterozygous for BCS1L mutation and a patient with mtDNA rearrangement, a SLSD. The deleted segment included sequences for structural CI subunits, transport RNAs and, surprisingly, a deletion of a CIII subunit, cytochrome b (CYTB).

Introduction

According to the endosymbiotic theory, introduced by Lynn Margulis in 1960s, mitochondria are descendants of independent prokaryotic organisms that survived endocytosis, and were part of a symbiotic system with the hosting cell. They eventually became domesticated and merged into a single organism preserving a double membrane as a mark of their origin. Mitochondria are historically depicted as key players in energy metabolism; producing adenosine triphosphate (ATP) and hosting canonical metabolic pathways, but their function extends beyond this initial concept. Complexity of these organelles has been a subject of intense investigation during the past few decades. It is now known that they do not act as autonomous and detached organelle. On the contrary, they are functionally interconnected with co-localized organelles, in particular the endoplasmic reticulum and peroxisomes. Via bi-directional signaling they interact with the nucleus, taking on an important role in the differentiation, immune response, homeostasis and determination of cell fate (Rahman and Rahman 2018)(Van Der Bliek, Sedensky, and Morgan 2017)(McBride, Neuspiel, and Wasiak 2006)(Cagin and Enriquez 2015).

Mitochondria morphology and function

The unique and dynamic architecture of mitochondria is designed to meet their functional requirements, being able to quickly adapt to changes in the need of APT. Mitochondria can differ in shape and size; be more elongated or more circular, depending on the cell type. Energy requirements of the cell dictate the number of mitochondria per cell, hence cells with high energy demand, such as liver cells, can have up to 2000 mitochondria per cell. Rather than existing as isolated organelles, they engage to form a highly dynamic reticular network of branched tubules (Wai and Langer 2016). Mitochondrial shape is the result of events of fusion and fission that occur in response to different cellular signals. Imbalance in mitochondrial dynamics has direct impact on cellular metabolism (Wai and Langer 2016)(Cogliati et al. 2013) In case of decreased fusion or increased fragmentation, mitochondrial membrane surface area is compromised, which directly influences metabolic processes.

The double membrane system is composed of two functionally different phospholipid bilayers, creating a space between them, the inter-membrane space

(IMS) (Fig.1). The outer mitochondrial membrane (OMM) is highly permeable compared to the inner membrane (IMM), which is critical for its function. IMM incapsulates mitochondrial matrix and invaginates, forming the membrane curvatures called cristae, which increase the IMM surface area. The composition of IMM differs from the OMM. It is abundant in phospholipid cardiolipin, which is assumed to be required for positioning of protein complexes and cristae formations (Quintana-Cabrera et al. 2018). Each mitochondrial crista is considered to be a separate functional compartment communicating with OMM and cytosol through crista junctions (Quintana-Cabrera et al. 2018) (Cogliati et al. 2013).

The energy currency of the cell, ATP, is produced in the respiratory chain (RC), settled the IMM, through a process called OxPhos. OxPhos is thus a membrane dependent function. Number and shape of IMM cristae impact ATP production; more cristae will provide more respiratory machinery increasing the respiratory capacity (McBride et al. 2006) (Quintana-Cabrera et al. 2018). Moreover, when cristae tighten, in response to fasting, RC protein complexes come into close approximation to enhance electron shuttle; this furthermore increases the efficiency of OxPhos (Quintana-Cabrera et al. 2018)(Cogliati et al. 2013)(Patten et al. 2014) (Enríquez 2016).

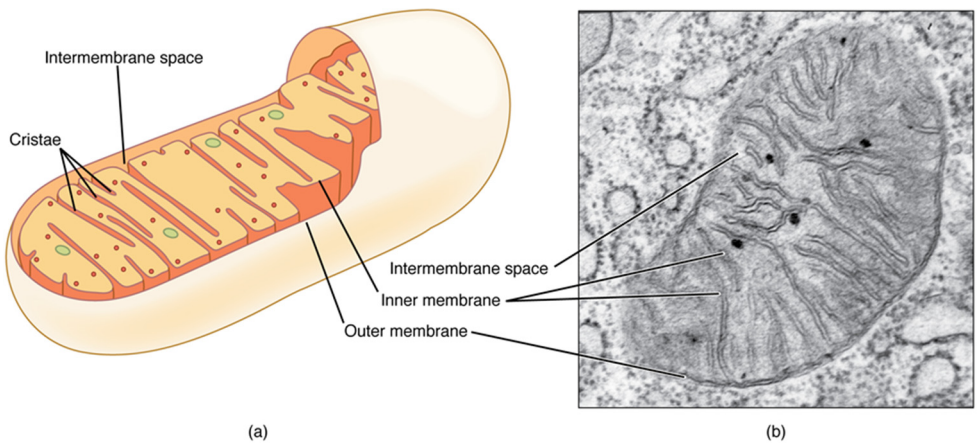

Fig.1. Structure of Mitochondria. (a) Schematic representation of a mitochondrial structure. (b) Figure on the right shows a transmission electron microscopy (TEM) image and the corresponding structure. (Copied without change from: https://commons.wikimedia.org/wiki/File:0315_Mitochondrion_new.jpg; License: https://creativecommons.org/licenses/by/4.0/deed.en)

Mitochondria in intermediary metabolism

Nutrients that undergo digestion provide cells with monosaccharides such as glucose, amino acids, and fatty acids, which undergo further processing in different metabolic pathways. Metabolism is the balance between anabolic and catabolic pathways, and it takes place in all the different parts of a cell, involving many different intersecting pathways.

The backbone of the metabolism is the process of glycolysis where glucose that enters a cell is converted through series of rection into pyruvate. It takes place in the cytosol and almost all pathways branch from glycolysis. Pyruvate, the end product of the glycolytic pathway, enters mitochondria where it is converted into acetyl-CoA by pyruvate dehydrogenase (PDH). The TCA cycle extracts electrons from acetyl-CoA and convey those electrons to oxidative phosphorylation for ATP production. This process of glucose catabolism ending in oxidative phosphorylation assures highly efficient ATP production and is considered the central axis of intermediary metabolism (Fig.2.) (Ferreira et al. 2020)(Martínez-Reyes and Chandel 2020). Also, other monosaccharides, amino acids and fatty acids can be converted, through different pathways, into intermediates able to take part in the central axis of metabolism.

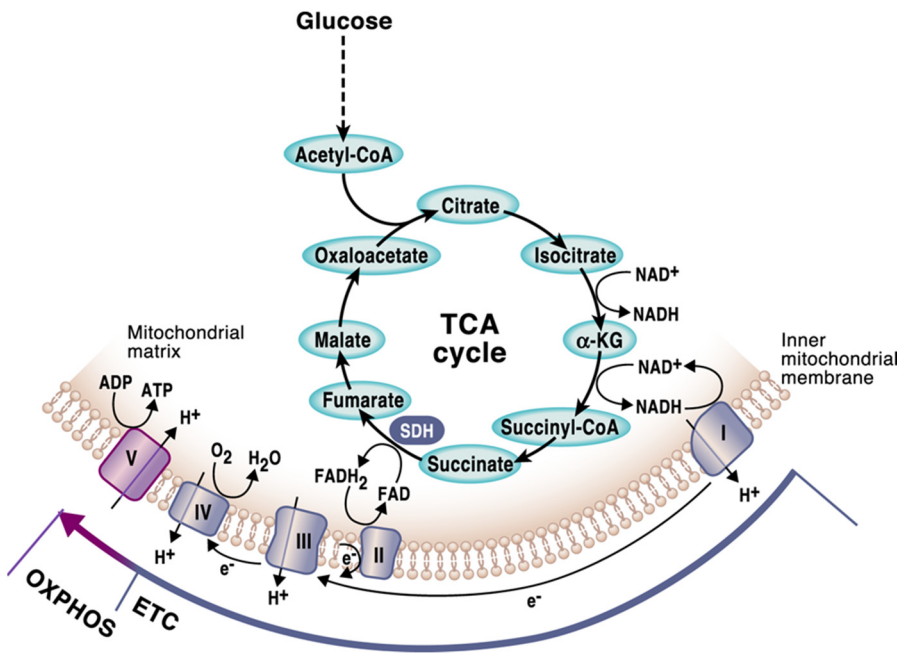

Fig.2. The TCA cycle and OxPhos system in the mitochondria, part of the central axis of intermediary metabolism. (Copied without change from (Martínez-Reyes and Chandel 2020); License: https://creativecommons.org/licenses/by/4.0/

Anions involved in TCA cycle can be redirected to other pathways. In that case, they must be replenished to ensure the proper continuous functioning of the TCA cycle. This is termed anaplerosis and it is obligatory in some biosynthetic pathways. For example, when gluconeogenesis is the prioritized pathway in a liver cell, TCA cycle intermediate oxaloacetate is diverted for gluconeogenic process. The opposite pathway, termed cataplerosis, is activated when an excess of accumulated intermediates needs to be removed from the TCA (Owen, Kalhan, and Hanson 2002). This means that pathways which are part of the central axis, glycolysis, and TCA cycle, can also be used for other purposes like synthesis of biological intermediates. This is particularly important in metabolically active cells like liver, heart, muscle, and kidney cells.

Assessment of the central axis of intermediary metabolism is a part of the routine clinical practice like measurement of blood glucose, ketone, and lactate concentration that, together with some other complementary clinical test i.e., pH, partial pressure of blood gases and complete blood count can give us a clinically relevant information about operational status the central axis.

Moreover, intermediary metabolites might have other functions besides those canonically ascribed to them (Huangyang and Simon 2018)(Huangyang and Simon 2018). There are several paradigm shifting reports discovering novel pleiotropic properties of intermediary metabolites. It has been suggested that some of them can serve as universal fuels or signaling molecules (Ducker and Rabinowitz 2017)(Rabinowitz and Enerbäck 2020)(Newman and Verdin 2014)(Martínez-Reyes and Chandel 2020).

Therefore, deficiencies in RC can result in, apart from energy deprivation, metabolic disturbances such as hypoglycemia and disrupted amino acid metabolism (Clarke et al. 2014).

Primary mitochondrial disorders and biomarkers

Primary mitochondrial disorders cause OxPhos dysfunction or other disturbances of mitochondrial structure and function with an estimated prevalence in children younger than 16 years is from 5 to 15 cases per 100,000 individuals (Rahman 2020)(Gorman et al. 2016). It can affect any organ system or any tissue in any combination, at any time. Moreover, the clinical presentation cannot be explained entirely by ATP deficiency (Gorman et al. 2016)(Parikh et al. 2019). This extreme clinical variability and multisystemic nature, as well as biochemical and genetic heterogeneity complicate the diagnostics. Treatment of mitochondrial disorders is equally challenging. Clinical studies are challenged with small sample size, genetic and phenotypic heterogeneity, lack of natural disease history and lack of outcome measures (Goldstein and Rahman 2021).

The disruption of the OxPhos has been suggested to have an impact on co-localizing pathways and disturbs the metabolism on an organismal level (Suomalainen 2011)(Khan et al. 2014)(Ducker and Rabinowitz 2017)(Kühl et al. 2017)(Costa et al. 2019)(Reinecke et al. 2012). Extensive metabolomic studies have been done on *Caenorhabditis elegans* models with mutations in different OxPhos subunits capturing abnormalities in carbohydrate, amino acid, and fatty acid metabolism (Falk et al. 2008). It has been reported that RC disorders can present only with hypoglycemia, but the underlying mechanism of the pathophysiological effect has so far not been elucidated (Mochel et al. 2005). Different OMIC studies have been used to tackle underlying pathophysiology and possible define biomarkers that might have diagnostic capabilities (Atkuri et al. 2009)(Gloerich et al. 2007)(Rahman and Rahman 2018). Lactate is considered a serum and a cerebrospinal fluid biomarker of mitochondrial disorders, but it is not specific for mitochondrial disorder thus it has insufficient diagnostic potential. Metabolomic studies have been extensively used to explore potential biomarkers with diagnostic and prognostic potential, but so far only a few of them have been proven having acceptable sensitivity and specificity, like fibroblast growth factor 21 (FGF21) and growth and differentiation factor 15 (GDF15) (Goldstein and Rahman 2021)(Rahman and Rahman 2018).

Respiratory chain complexes

The OxPhos system consists of five membrane-bound multiheteromeric complexes forming the RC placed in the IMM: complex I (CI); complex II (CII); complex III (CIII); complex IV (CIV), and the ATP synthase, also called complex V (CV). In addition, two electron shuttles which are also part of the RC, are freely moving within the IMM: coenzyme Q (CoQ, also known as ubiquinone) and cytochrome c (CYCS). (Fig.3.)

Dual genetic control

This sophisticated, finely tuned and very complex cellular system is under dual genetic control. The mitochondrial proteome is encoded by both the nDNA and the mtDNA. The majority of the proteins, almost 99%, are encoded by nDNA and only 37 proteins are encoded by mtDNA, a double-stranded circular DNA located in the mitochondrial matrix(Chacinska et al. 2009). mtDNA encodes for 13 structural subunits of the electron transport chain (ETC), and genes needed for the translation of mtDNA transcripts (22 mitochondrial tRNAs and 2 ribosomal RNAs).

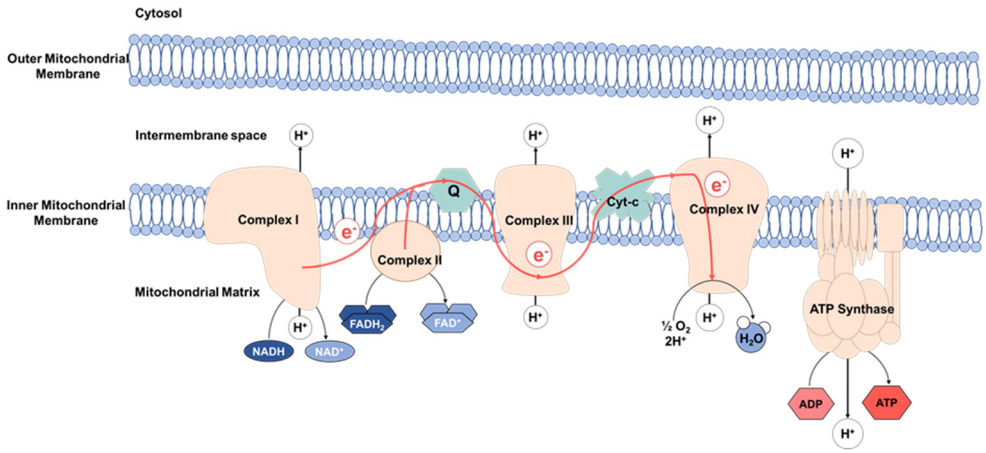

Fig.3. Respiratory chain complexes CI-CIV, ATP sythase and two electron shuttles part of the OxPhos system. (Copied without change from: https://commons.wikimedia.org/wiki/File:ElectronTransportChainDw001.png; License: https://creativecommons.org/licenses/by-sa/4.0/deed.en)

Complex I

NADH-ubiquinone oxidoreductase, CI, is the first and the largest enzyme of mammalian OxPhos system and the main electron entry port. The subunits of CI are encoded by both the mtDNA (7 subunits) and the nDNA (45 subunits) (Cogliati et al. 2018). Due to its structural complexity and difficult assembly, CI deficiencies are among most common RC disorders together with CIV deficiencies (Guo et al. 2018)(Hoefs et al. 2012)(Cogliati et al. 2018). CI deficiencies usually have late childhood or early adulthood manifestation with Leber hereditary optic neuropathy being one of the most common. Other mitochondrial phenotypes in CI deficiency include fatal infantile lactic acidosis, mitochondrial encephalopathy with lactic acidosis and stroke-like episodes (MELAS) and Leigh syndrome (Fernandez-Vizarra and Zeviani 2021).

Complex II

Succinate-ubiquinone oxidoreductase, CII, is also an entry point for electrons into the ETC, however no protons are pumped into IMS through this complex (Mazat et al. 2013). All its structural subunits are encoded by nDNA. Biochemically, it has an additional important function, since it is positioned at the intersection point between ETC and TCA cycle, operating at the same time as a TCA cycle enzyme converting succinate into fumarate. It is thought to be a separate entity anchored in the IMM (Signes and Fernandez-Vizarra 2018)(Letts and Sazanov 2017)(Guo et al. 2018)(Lapuente-Brun et al. 2013)(Enríquez 2016). Genetic disorders underlying CII deficiency often present as a hereditary tumor syndrome such as familial

pheochromocytoma or hereditary paraganglioma. Typical mitochondrial phenotypes are also being reported (Fernandez-Vizarra and Zeviani 2021).

Complex III

Cytochrome bc1 complex, CIII, constitutes the center of the RC and is the crossroad of many metabolic pathways. It is embedded into the IMM, it oxidizes coenzyme Q and reduced cytochrome c using the released energy to pump the protons into the IMS. This process is called the Q-cycle (Guo et al. 2018). A CIII monomer is composed of eleven subunits, with only one of them being encoded by mtDNA. The functionally active form of the enzyme is a symmetric dimer ($CIII_2$). CIII structure, assembly and mechanistic details have been mostly studied in yeast (*Saccharomyces cerevisiae*) (Wagener et al. 2011)(Smith, Fox, and Winge 2012b).

Complex III assembly

To fully assemble CIII, apart from structural subunits, an additional set of proteins - assembly and stabilizing factors - are required (Smith et al. 2012b). Moreover, since MT-CYB is mtDNA encoded, it is also necessary to have proper function of mitochondrial machinery for transcription and translation.

Multiprotein complexes usually do not assemble in series of reactions, but by forming subcomplexes that are later gathered to form a pre-CIII. In other words, structural components need to find one another, build up a subcomplex that is further assembled with the help of assembly proteins (Fernandez-Vizarra et al. 2007)(Sánchez et al. 2013)(Signes and Fernandez-Vizarra 2018)(Fernández-Vizarra and Zeviani 2015)(Bottani et al. 2017)(Fernandez-Vizarra and Zeviani 2018) (Sánchez et al. 2013). Some CIII subunits, 3 out of 11, have a catalytic center: cytochrome b (MT-CYB), cytochrome c1 (CYC1) and the Rieske protein. These subunits need to be properly managed to avoid excessive production of reactive oxygen species.

Assembly factors found in yeast helped identify equivalents in human samples, but only a few steps of the human mechanism have been shown in yeast. However, due to structural similarities it is deduced that the assembly must correspond to an assembly found in yeast (Fernández-Vizarra and Zeviani 2015)(Fernandez-Vizarra and Zeviani 2018).

The assembly starts by synthesis of mtDNA encoded CYTB. The early assembly factors (UQCC1, UQCC2) bind to it and guide it to the IMM. Once the first catalytic center is built in the CYTB, UQCC3 binds to it and the additional components of catalytic center are incorporated. Fully built CYTB can participate in the first subcomplex formation called an early intermediate, consisting of 3 structural components (CYTB+UQCRB+UQCRQ). After its construction, additional structural components are incorporated to form a pre-CIII which dimerizes but is

not yet activated. A final step, also considered a maturation and an activation step, is the incorporation of Rieske Fe-S protein (UQCRFS1) into the pre-$CIII_2$. After the incorporation of UQCRFS1, $CIII_2$ becomes catalytically active (Signes and Fernandez-Vizarra 2018) (Fernandez-Vizarra et al. 2007)(Fernandez-Vizarra and Zeviani 2018). This last, activating step was done with the help of BCS1L, a nDNA encoded translocase. BCS1L is a member of AAA+ (ATPases associated with diverse cellular activities) family of proteins and is required for this final step of incorporation of UQCRFS1 and UQCR11 into pre-$CIII_2$ (Fernandez-Vizarra and Zeviani 2018)(Signes and Fernandez-Vizarra 2018)(Protasoni et al. 2020)(Fernández-Vizarra and Zeviani 2015).

Complex IV

Cytochrome c oxidase (COX), CIV, where electrons are transferred from CYCS to O2 as the final acceptor, is the last complex in electron transport chain. Mammalian COX constitutes of 14 subunits, of whom 3 core subunits (COX1, COX2, COX3) are encoded by mtDNA. For redox reaction to occur in the COX, two important groups of co-factors are required: two hem groups and three copper atoms (Cogliati et al. 2018). The clinical presentation of CIV deficiency can vary in severity from mild, benign to lethal myopathies, early encephalopathies, fulminant lactic acidosis, cardiomyopathies and, interestingly, haematologic manifestations (Brischigliaro and Zeviani 2021). Compared to other OxPhos disorders, CIV deficiencies are relatively common mostly due to, as in CI, complex multiunit structure, and a myriad of assembly factors.

Complex V

Mitochondrial ATP synthase, or CV is not a part of the ETC neither the site of proton pumping. It uses the proton gradient established by CI, CII, CIV for phosphorylation of ADP and synthesis of ATP. CV is the smallest known biological motor that uses proton gradient energy to condense ADP and P+ (Mazat et al., 2013). The most common phenotypes associated with CV deficiency are Neuropathy, Ataxia, and Retinitis Pigmentosa (NARP) and Maternally Inherited Leigh Syndrome (MILS) (Fernandez-Vizarra and Zeviani 2021).

Oxidative phosphorylation

Oxidative phosphorylation, a mechanism conserved from bacteria to higher eukaryotes, captures energy from many discrete steps of electron transfer to build up an energy currency of the cell, an ATP molecule. Two separate processes define

OxPhos: first is electron transport through electron transport chain (ETC), also known as RC coupled with proton pumping, and the second is ATP synthesis (Fig.2.).

Reducing equivalents, NADH and FADH2, which come from TCA, donate electrons to CI and CII, respectively. Electrons pass along several enzymes, via series of redox reactions; from CI and CII electrons are transferred to coenzyme Q and then to CIII. CIII passes electrons to CIV via cytochrome c. At the level of CIV, oxygen molecule, a final electron acceptor, forms water. In conjunction with electron transport in ETC, deliberated energy is used to pump protons from the mitochondrial matrix across the IMM into the IMS creating a proton gradient. As those protons flow back to the mitochondrial matrix, in a very energetically favorable process, energy is captured by ATP synthase to make ATP. At the same time, redox balance is re-established in mitochondrial matrix by re-oxidizing NAD+ and FAD+ (Ghezzi and Zeviani 2018).

For OxPhos system to operate correctly, it has to be assembled and activated under well controlled mechanism which orchestrates transcription, translation, synthesis, protein translocation into mitochondria and ETC assembly (Papa et al. 2012).

Most of the studies providing information on OxPhos system functioning came from experiments on *C. elegans*. Due to high degree of conserved cellular functions between nematodes and mammals, the knowledge gained from those studies is considered transferable to mammals.

CIII deficiencies

Defects in CIII are classified as OxPhos disorders and they are described as defects in biogenesis and/or function of the CIII. They are relatively rare and manifest biochemically in reduced enzymatic activity but the clinical presentation, as in other mitochondrial diseases, is very variable. Metabolic decompensation is often reported with a different degree of severity (Fernandez-Vizarra and Zeviani 2021)(Protasoni and Zeviani 2021)(Gorman et al. 2016).

***BCS1L* mutations**

BCS1L is a CIII assembly factor, part of AAA+ family (Nouet et al. 2009). It is a nDNA encoded protein located on chromosome 2. Eight exons code for 419 amino acids in humans and it is relatively unchanged far back up the phylogenetic tree. In BCS1L protein, three regions are defined: an AAA-ATPase region, N-terminal import region and the BCS1L specific region. Mutations in encoded *BCS1L* are the most frequent cause of isolated CIII deficiency (Oláhová et al. 2019). As described

earlier, BCS1L or Bcs1, a yeast homolog, is required for maturation and activation of pre-$CIII_2$. This is done by insertion of Rieske Fe-S protein (UQCRFS1 or Rip1) (Ramos-Arroyo et al. 2009)(Wagener et al. 2011)(Wagener and Neupert 2012)(Smith, Fox, and Winge 2012a).

CIII deficiency caused by *BCS1L* mutation can have wide variety of clinical presentation ranging from mild clinical presentation like Björnstad syndrome to the most severe clinical form of CIII deficiency, GRACILE syndrome, part of the Finish heritage disease group. It is caused by a nuclear homozygous mutation in *BCS1L* causing c.A232G. The mutation is in N-terminal import auxiliary sequence, suggesting the importance of this region for function and stability. Very consistent phenotype and not very well understood pathophysiology motivated scientists to explore the mutation effect in a GRACILE mouse model (Levéen et al. 2011), the first viable mouse model for CIII deficiency (Torraco et al. 2015)(Iommarini et al. 2015). Homozygous mice (Bcs1lc.232A>G; G/G) are healthy until weaning, after which they present growth restriction, metabolic liver dysfunction, renal tubulopathy, short life span and decreased CIII activity, thus phenocopying the human disorder. Complex III activity is not completely abolished in organs investigated in mutants but decreased to a certain level of controls (20%, 40%, 40% in liver, hearth and kidney respectively) with CI function being unaffected. The mechanism for the rapid disease progress to lethality is unclear and cannot be directly explained by lack of energy.

Studies attempting to link a biochemical and/or clinical phenotype to mutation in a certain domain have been contradicting (Hinson, 2007, Kotarsky 2007, Ramos 2009). Moreover, *BCS1L* has different expression in different tissues, which complicates phenotype furthermore. It has been suggested that most probably a type of a mutation i.e. a substituted amino acid in case of a missense mutations will determine a phenotype (Kotarsky et al. 2012) (Ramos-Arroyo et al. 2009) (Kotarsky 2007, Ramos 2009).

Metabolic phenotype of GRACILE patients

Fetal growth retardation from the week 30 was observed without a pathological corelate in analyzed placental tissue. Pregnancies and deliveries were otherwise normal. No dysmorphic features were noted. The patients looked strikingly alike, partly due severe asymmetric growth restriction. Fulminant metabolic acidosis evolved after delivery. If the patient was properly fed or receiving intravenous glucose, no hypoglycemia was detected. Nonspecific hyperalaninemia was found in serum. Liver size and structure were normal on ultrasound examination and liver function tests were mildly affected with minor hepatocellular injury, but no overt liver failure. In four patients, high ketone bodies were detected: hydroxybutyrate (280–770 μmol/L, normal upper limit 150 μmol/L) and acetoacetate (380–4800 μmol/L, normal upper limit 100 μmol/L). Metabolic screening for urea cycle

disturbances and organic acidemias, mitochondrial and peroxisomal oxidation defects revealed no pathology; blood ammonia, very-long-chain fatty acid, free carnitine and phytanic acid were normal. Pyruvate-dehydrogenase activity in patient fibroblasts was normal. Fanconi-type aminoaciduria and increased excretion of lactate, hydroxyphenyl-lactate, pyruvate, phosphate, and glucose were detected in patients' urine analysis (Fellman et al. 1998)(Rapola and Fellman 2002)(Visapää et al. 2002)(Fellman 2002)(Fellman et al. 2008).

Objectives

General objectives

The overall objective of the thesis was to clarify mechanisms and affected metabolic pathways in the disease progression of complex III deficiency.

The thesis is divided into intervention basic science part (Study I, II) and observational studies (Study III, IV).

Study I and II were done on an experimental mouse model with CIII deficiency carrying *Bcs1l*^{cA232G} mutation. Whereas Study III and IV are observational clinical studies where two new cases on RC disorders are reported.

Specific aims

1. To assess mutants' compensatory capabilities under fasting condition, mimicking a metabolic pressure, using randomized intervention study with pair matched study design.
2. To assess whether extra carbohydrates (dextrose) in a diet can alleviate hypoglycemia and impact the survival in mutant mice using randomized intervention study with pair matched study design.
3. To evaluate whether novel *BCS1L* mutation shares phenotypic characteristics with GRACILE patients and Gracile mouse model in an observational study.
4. To report on a patient with mtDNA SLSD potentially affecting RC complexes, focusing on metabolic phenotyping and phenotype comparison through the literature review.

Materials and methods

Materials

Animals

An experimental animal model, mice harboring *Bcs1l* c232A>G (p.S78G) mutation were used in the thesis. Mice used in the Study I (n=90) and Study II (n=38) were >99% congenic in C57BL/6JBomTac background (Levéen et al. 2011). They were maintained in open cages at the animal facility of Lund University. Wild types (WT) Bcs1l $^{A/A}$ and heterozygous for the mutation Bcs1l $^{A/G}$, phenotypically identical to wild types, were used as controls. Mice were sacrificed by cervical dislocation after tail blood sampling. In the Study III, mice used (n=6) were backcrossed to C57BL/6JCrl genetic background, after embryo transfer from Lund. In this strain, homozygous mice live longer, for up to 6 months. Animals were housed in individually ventilated cages at the animal facility of University of Helsinki.

Patient samples

Blood samples for genomic DNA analysis were collected from patients and parents (Study III, Study IV). In addition, blood from the patients was taken for platelet respirometry and control samples were collected from healthy children undergoing elective surgery (Study III).

In the Study III, a percutaneous skeletal muscle biopsy (*m. tibialis anterior*) sample was taken for histological and biochemical investigations.

Routine autopsy was performed on a deceased patient from Study III and specimens from liver, heart, muscle, and brain were analyzed. In addition, previously obtained liver, and brain specimens from deceased GRACILE patients (n=5), homozygous for BCS1L mutation c.232A>G, and controls - infants deceased from causes other than mitochondrial disorders - were used for evaluation and comparison to a proband from Study III.

Cell culture

Fibroblasts were obtained from a skin biopsy sample from the patient and controls (n=3, one patient without mitochondrial disease, two fibroblasts from the umbilical cord of two healthy term newborns) (Study III).

Methods

Study design

Animal model studies (Study I, Study II) were randomized intervention studies with pair-matched study design. We used randomization and pair matched design to reduce bias i.e., influence of confounding variables. Observational studies were two case reports (Study III, Study IV) reporting novel genetic findings with an emphasis on phenotyping, comparison to GRACILE patients and Gracile mouse model (Study III) and genetic analysis and phenotype comparison through the literature review (Study IV).

Standardized health scoring

Detailed standardized scoring protocol for Gracile mouse assessment was used in Study II. Mutant animals have a disease-free period from birth to the time of weaning when they start to deteriorate clinically (age 19-21 days). Animals were assessed for reduced curiosity and lack of movement in the cage, wobbling gait, deterioration of balance, appearance of kyphosis, and loss of grip strength. Each behavioral trait was scored and summed daily (0 for normal, 1 for slight abnormality and 2 for clear abnormality). When the score was ≥7/12 animals were at the end-stage of the disease and scarification was performed.

Blood chemistry – an organismal level assessment

Animal model

Measurements of glucose, lactate, and ketone bodies were done from the whole tail artery blood, prior and after the intervention (Study I) and prior to scarification (Study II). Animals were anesthetized shortly before the procedure. Blood for plasma and serum analysis was collected using cardiac puncture technique after cervical dislocation and opening of the thoracic cavity. Analysis included measurement of insulin and HOMA IR calculation, FFA, cholesterol, triglycerides, phospholipids, total and free cholesterol, plasma lipoproteins.

Metabolomic analysis from plasma

Targeted metabolomics analysis (30 animals, at least 6 per group) was performed on the isolated plasma samples and quantified data was further analyzed using online tool MetaboAnalyst 3.0. Correlation and quantitative enrichment analysis were performed.

Patients

To assess the metabolic status and potential metabolic disturbances, general and specific biochemical investigation were performed, as a part of the routine clinical care (Study III, Study IV).

Intermediary metabolism was assessed regularly at the intensive care unit by measurements of, glucose, lactate, and ketone concentrations, as well as electrolyte, pH, ammonia, and urea assessment. In addition, specific metabolic tests were ordered for more comprehensive metabolic analysis: amino acid analysis in plasma and cerebrospinal fluid, acylcarnitine profile in blood and urine, and organic acids in urine.

Histology, immunohistochemistry, and electron microscopy

Animal model

Liver histology samples were collected immediately after scarification and assessed for morphology by staining with hematoxylin and eosin. Specific assessments (Study I, Study II) for glycogen and triglycerides were done by staining with periodic acid-Schiff (PAS) and oil-red-O (ORO), respectively.

Brain specimens were analyzed histologically (Study III) with immunohistochemical analysis using primary antibodies against Rieske iron sulfur protein (RISP), anti-glial fibrillary acidic protein (GFAP), a microglia marker ionized calcium-binding adaptor molecule 1 (IBA1), and anti-CD11b antibody expressed on microglia and immune cells (CD11b). Liver tissue was then assessed with IBA1 and DC11b antibodies for presence of Kupfer cells (KC), resident macrophages since microglia and KC are derived from the yolk sac's myeloid linage in early embryogenesis.

Mitochondrial architecture and morphology, as well as immediate structural changes caused by the intervention have been documented through transmission electron microscopy analysis (TEM).

Patients

Histology, using standard (hematoxilin & eosin, PAS and ORO staining) and specific staining for mitochondrial myopathies (ATPase incubation, NADH-tetrazolinum reductase, succinate dehydrogenase, cytochrome C oxidase and

combined COX/SDH as well as Gomori trichrome) was performed on skeletal muscle biopsy sample (Study III).

In addition, histology assessments were done on autopsy specimens of liver, heart, muscle, and brain tissue. Brain and liver tissue samples previously obtained on infants deceased from GRACILE syndrome, homozygous mutation c.232 A>G, were used for comparison, as well as control brain samples collected from infants deceased from other non-metabolic diagnoses.

Muscle mitochondria ultrastructure was assessed by electron microscopy.

Functional analysis

Mitochondria

Mitochondrial respiration was assessed in both animal model (Study I, Study II) and in a patient (Study III) using high resolution respirometry with Oroboros Oxygraph -2k (Pesta and Gnaiger 2017)(Makrecka-kuka, Krumschnabel, and Gnaiger 2015) (Westerlund et al. 2018) (Sjövall et al. 2013). Respiratory chain function and functional changes caused by genotype and intervention (fasting, HCD) were assessed on freshly isolated liver mitochondria. High resolution respirometry on isolated platelets from the whole blood sample was used to assess the patient RC function. Experiments were run on 37 °C in MIR05 mitochondrial respiration medium using SUIT protocol which enables a stepwise titrating of substrates and inhibitors for different individual complexes, as well as uncoupler (Fig.4).

Animal model

Measurement of enzyme activities related to hydrolyzation of triglycerides; lipoprotein lipase (LPL) and hepatic lipase (HL) activity in post heparin plasma samples. The experiment was done on fed animals to assess possible mutation effect on lipid mobilization. Two additional enzyme activities were assessed for mutation effect: a phospholipid transfer protein (PLTP) and paraoxonase – 1(PON1) activity, as described in the paper (Study I). PON1 has an important role in preserving a low oxidative state in blood circulation.

Patient

Determination of mitochondrial ATP production, RC enzymes and citrate synthase activity (Study III).

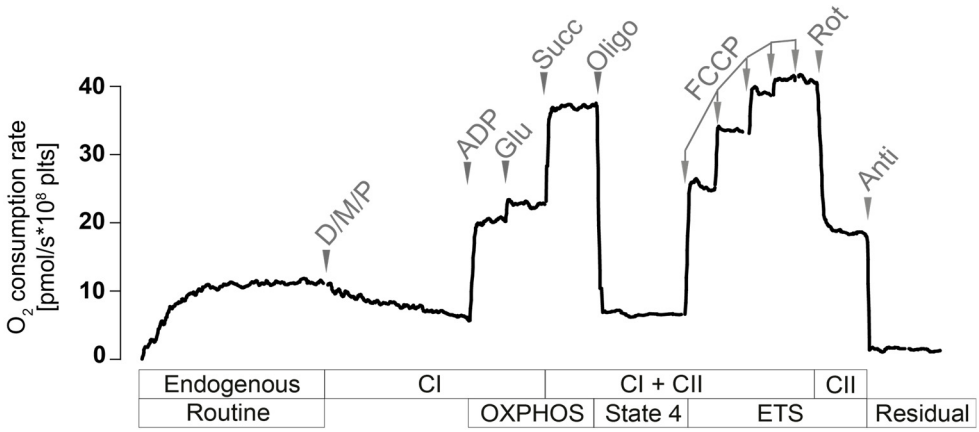

Fig. 4. Experimental protocol of permeabilized platelets. Trace from experiment displaying oxygen consumption rate using a SUIT protocol. Induced respiratory states and respiratory complexes activated are defined below the x-axis. Platelets were permeabilized with digitonin (D) and the complex I (CI) substrates malate (M) and pyruvate (P) (5 mM, respectively) were simultaneously added. Oxidative phosphorylation (OXPHOS) was stimulated by subsequent addition of ADP (1 mM) followed by the additional complex I substrate glutamate (Glu) (5 mM). Addition of the complex II (CII)-linked substrate succinate (Succ) (10 mM) enabled convergent electron input via both complex I and complex II. OXPHOS was inhibited by oligomycin (Oligo) (1 µg/ml) revealing LEAK respiration. Maximal respiratory capacity of the electron transfer system (ETS) was induced by titration of the protonophore, carbonyl cyanide p (trifluoromethoxy) phenylhydrazone (FCCP, mean concentration 6 µM)). Inhibition of complex I by rotenone (Rot) (2 µM) revealed complex II-supported respiration. The residual non-mitochondrial oxygen consumption was exposed by addition of the complex III inhibitor antimycin-A (Anti) (1 µg/ml).

Protein analysis

Basic protein expression profiling was assessed in fibroblast and liver tissue (Study III). Non-denaturing mitochondrial membrane protein complexes were analyzed from liver and fibroblast mitochondria using Blue-native polyacrylamide gel electrophoresis (BN PAGE) technique. In this analysis individual interaction between subunits of RC complexes are preserved and allow us to separate intact protein complexes using electrophoresis. Mitochondria were assessed using antibodies against BSC1L and other RC complex subunits plus more detailed assessment of CIII using two different antibodies for CIII subunits RISP and CORE1.

BN PAGE can further be combined with other analysis which assess denatured complexes as it was done in Study III using Western Blot analysis from autopsy liver samples. Antibodies used were against BCS1L, CIII (RISP, CORE1), CI (NDUFA9), CII (SDHB), CIV (COX1), mitochondrial loading control (VDAC1/porin).

Molecular analysis of BSC1L

BCS1L was sequenced using Sanger method after PCR amplification of genomic DNA. On RNA, isolated from fibroblasts, RT-PCR reaction was performed, and a subsequent sequencing of the PCR product was carried out. The mutation was additionally verified using cDNA analysis from patient and control fibroblasts.

Genetic analysis

Genomic DNA isolation from patients and parents was done in Study III and Study IV. Total RNA was isolated in Study III from patient and control fibroblasts. Whole exome sequencing (WES) on genomic DNA was done in Study III on patient and parents and only genes for congenital metabolic diseases or other monogenic diseases with a similar clinical presentation were analyzed (dbCMMS). Splice prediction tools were used for synonymous mutation assessment in *BCS1L* (SPIDEX, NetGene2). Whole genome sequencing (WGS) was performed *in trio* and the deletion was interpreted as *de novo* since it was not detected in the maternal sample. Functional genomics analyses were done in a Study III, real time PCR and RNA analysis.

Statistical analysis

Linear mixed-effect models (MEMs) were used for the analysis of the metabolic network response to fasting, using blood glucose, ketone and lactate to glucose ratio (L/G) as outcomes. The p-value used for significance testing was 0.01 if not otherwise noted. By using the same modeling technique no difference was found between WT and heterozygotes ($p>0.7$ for all models).

In addition, conventional parametric and non- parametric statistics tests were used for group differences: Wilcoxon rank sum test, Kruskal-Wallis, Man -Whitney U test.

Ethical statements

Animal experiments were conducted according to Swedish national guidelines and ARRIVE recommendations. The approval was received from Lund regional research ethics committee (M245-11, M337-12, 31-8265/08). Southern Finland Research Animal Ethics Committee (State Provincial Office of Southern Finland) approved the research with licence ESAVI-2010-07284/Ym-23 and ESAVI-6142-04.10.07-2014_paatos_05082014.

For the Study III, IV investigations were approved by Lund regional Ethics Review Board of Southern Sweden (2009/59 and 2009/97) and for the Study IV the ethical

permit number that applied was 2019-04746. Samples from GRACILE patients were analyzed with approval of Pediatrics and Psychiatry at Helsinki University Hospital, Finland (77/4/2007, updated 2011). The Declaration of Helsinki applied on clinical studies. Written inform consent to participate the study was obtained from parents (Study III, IV) for all included subject from the same family.

Results

Study I

After 4-hours of fasting glycogen reserves were depleted in WT mice and blood glucose was lower, but as expected, euglycemia was preserved. At the same time the metabolic switch towards ketogenesis occurred proving that timing and duration of fasting creates an efficient metabolic pressure.

Fed mutant animals (*Bcs1l*$^{p.S78G}$) showed decreased glycogen reserves whereas fasted mutant animals had no detectable liver glycogen. Fasted mutants had an ineffective metabolic compensation to fasting leading to hypoglycemia (<3,4 mmol/L, $p \leq 0.001$) in twelve out of fourteen mutant animals, with severe hypoglycemia (<2,5 mmol/L) in eight of them. Lower blood glucose levels for G/G mutant animals compared to WT were observed already before fasting ($p \leq 0.001$) With every day of increased age in a G/G mutant, glucose before fasting was lower by 0.56 mmol/L in average, indicating rapid clinical deterioration. With every gram of higher body weight, G/G mutant had higher glucose concentration before fasting by 0.51 mmol/L in average. The sex variable has not been shown to have a significant influence on any of the measured outcomes (glucose, ketone, lactate, lactate/glucose ratio) An adequate insulin hormonal response and normal HOMAIR ruled out insulin resistance.

Fat accumulation in mutant liver cells was detected in non-fasting group and the amount of fat in the liver was even higher in the fasting group, which was quantified by the measurement of liver triglyceride concentration.

Normal TG mobilization from the fat tissue revealed preserved tissue cross talk and supported hepatocytes to run β-oxidation, but ketogenesis in mutants was not as efficient as in WTs (mean β-hydroxybutyrate 1.2 vs 2.1 mmol/L, $p \leq 0.003$).

We assessed lipid metabolism by studying markers of metabolic crosstalk between white adipose tissue and liver during fasting. Fasting increased the blood FFA concentration in both groups, but surprisingly fasted mutants did not increase plasma glycerol concentration as WTs.

Neither fasting nor the mutation had any effect on serum levels of triglycerides, phospholipids, lipoprotein profile and apolipoprotein A1.

Isolated liver mitochondria of mutant mice displayed lower non-phosphorylating and phosphorylating respiration rates, with most pronounced deficiency in CIII-related electron transport. Short fasting increased respiratory capacity in liver mitochondria – which was especially evident in mutated animals.

Moreover, this study revealed that 4-hour fasting is sufficient to induce mitochondrial ultrastructural response with elongation and cristae remodeling. Mutants had disturbed cellular ultrastructure already before fasting with elongated mitochondria and depleted cristae, ribosomes, and endoplasmic reticulum. Fasting deranged ultrastructure even further with mitochondria swelling no observed earlier.

Study II

Previously reported metabolic disturbance in the mutant and inability to preserve the euglycemia lead us to hypothesize that high carbohydrate diet (HCD) might improve hypoglycemia and impact the survival but surprisingly, the survival was significantly shorter on the high carbohydrate diet. Assessment of other intermediary elements on the organismal level (lactate and ketones) did not show statistically significant difference between the HCD and control group. Assessment of the carbohydrate storage and lipid content in the liver found no difference HCD and the control mutant animals.

Targeted plasma metabolomics found changes in 62 out of 101 metabolites being caused by the mutation and diet intervention while the mutation alone caused 48 metabolite changes. Diet intervention caused 28 changes in the metabolites including 11 amino acids, 3 neurotransmitter intermediates and 14 other metabolites. Both groups had significantly increased plasma sucrose.

Quantitative enrichment analyses listed pathways disturbed by the mutation and changes between mutants on standard or HCD. It is obvious, and in line with previous study done on liver metabolome (Kotarsky et al. 2012), that the mutation is affecting intermediary metabolism causing changes in glycolysis, gluconeogenesis, ketone body and TCA cycle metabolism, glycerol phosphate shuttle, urea cycle, etc. Pathways, mostly affected by the intervention are glycolipid and amino acid metabolism.

Study III

A patient reported in this study is compound heterozygous for two novel mutations in *BCS1L*. The patient had a severe and a somewhat different phenotype when compared to GRACILE patients. The myopathy and encephalopathy were

predominant in the phenotype, as well as in biochemical work up like massive increase in the marker of progressive neurodegenerative disease, neurofilament light protein (NFL; 32600 ng/L, reference < 380). Except for the postpartum reversable lactic acidosis, slightly increased lactate in cerebrospinal fluid, and mild tubulopathy no other specific metabolic disturbances were detected.

Genetic analysis using WES and filtered for mitochondrial disease gens, found 2 single-nucleotide variants (SNV). First, a frameshift mutation inherited from the mother; c.399delA in the *BCS1L* gen introducing a premature stop codon after 25 amino acids leading to severe protein truncation. Another single nucleotide variant (SNV) found in *BCS1L*, inherited from the father, was a synonymous mutation, c306A>T, initially judged as non-pathogenic. However, splice predicting tools suggested a cryptic splice site in exon 3 causing protein truncation due to a frameshift in exon 4. Moreover, functional analysis of RC in muscle revealed decreased activity of CIII making the *BCS1L* gene a very strong candidate. To further asses the pathogenicity of the former SNV, RT-PCR analysis was done on the patient and control fibroblasts that confirmed frameshifted, aberrantly spliced transcript from the allele carrying apparently silent mutation. In addition, the mRNA quantification found wild type allele being less than 50%.

Mitochondrial work-up revealed limitation of electron transport downstream of CII in platelet mitochondrial respiration and decreased activity in several complexes in isolated muscle mitochondria. BCS1L protein analysis done on native (BM PAGE) and denatured (Western blot) RC complexes revealed lacking BCS1l in patient's fibroblasts and liver cell lysate confirming the pathogenicity of the mutation. Moreover, assessment of RC organization using BN PAGE revealed lack of fully assembled CIII and the lower quantities of other RC complexes.

Macroscopic autopsy finding on the brain revealed linear focal cortical damage and scarcity of white substance. However, the myelinization was unaffected. Immunohistochemical analysis found astrogliosis in the deep cortical layers and reduction on microglial cells (IBA1 positive) being most distinctive in cerebral cortex of the patient. However, no such phenotype was observed in GRACILE patients. Lack of microglia had so far not been described as part of mitochondrial neuropathology. Although liver function was not affected and no iron accumulation found, as in GRACILE patients, immunohistochemistry of the patient's liver found marked reduction in Kupffer cells, yolk sack derived macrophages, same as microglia.

Animal model histology of the brain showed similar astroglial activation localized in the Barrel filed of the primary somatosensory cortex following similar pattern to the patient's brain.

Similarities in cerebral cortical findings were observed between the proband and *Bcs1l* mutant mice with long survival.

Study IV

The patient's phenotype was so far the most severe reported Pearson syndrome (PS) (Rötig et al. 1990)(Crippa et al. 2015). Canonical PS is a disorder of infancy, whereas the reported patient was affected already prenatally with macrocytic anemia without compensatory reticulocyte response (hemoglobin 47 g/L, erythrocytes 1,1x 1012/L, hematocrit 0,15, MCV 136, reticulocytes 16x109/L). The clinical course was followed by fulminant, anion gap metabolic acidosis after birth resembling inborn error of metabolism. Interesting findings were inconclusive complex metabolic disturbances and anicteric synthetic liver failure.

WGS used for the diagnostics, so far not part of a routine clinical practice, provided the diagnosis 72 hours after sampling whereas the biochemical data were difficult to decipher. The PS diagnosis was additionally confirmed by standard clinical multiplex PCR assay (MLPA). There was 82% and 75% heteroplasmy in the blood. A deleted segment of 4961 bp involved *CYTB* gene that affects CIII assembly and CI stability. CYTB is core structural component of CIII which is usually not deleted in PS (Akesson et al. 2019)(Wild et al. 2020).

Discussion

Animal model studies

Mice carrying the human GRACILE syndrome mutation (Bcs1pS78G) have decreased CIII activity, metabolic liver dysfunction and an early death. However, the pathophysiological mechanism of the mutation remains elusive.

The liver plays the central role in metabolism and ensures blood glucose and lipid homeostasis to meet the fuel requirements of other organs, which is especially important during fasting. To uphold this, there is a tight regulation of liver metabolism by both nutrient and hormonal signals. Crosstalk between the liver and other tissues, such as the adipose tissue secreting free fatty acids (FFA) is crucial for maintenance of the homeostasis (Rui 2014). We report (Study I) that despite severe lipodystrophy in the mouse model, the tissue crosstalk is preserved and can recruit metabolites under metabolic pressure. A common interesting metabolic observation emerging from Study I and II is an inability to preserve an euglycemia, one of the imperative liver functions. RC function is not completely abolished in the GRACILE mouse model and the tissue crosstalk is preserved, but hypoglycemia evolved in almost all animals. During sudden food deprivation, liver acts as a metabolic hub bridging the gap, releasing glucose from glycogen degradation and the gluconeogenic pathway, until alternative fuels are available, and the neurons are adjusted to the use of it. Inability to preserve blood glucose concentration over the hypoglycemic level leads to coma.

Gluconeogenic pathway in the liver competes with glycolysis, the reverse catabolic pathway of vital importance. Only one of them can operate at a time. To our knowledge, the phylogenetic dominance between them, in circumstance of suboptimal ATP synthesis, has not been evaluated. It seems that glycolysis is the preferred pathway over gluconeogenesis in GRACILE mouse model. There might be several reasons for that. The main and obligate activators of anabolic direction: ATP and acetyl-CoA, are presumably decreased in our mouse model, revealing livers *locus minoris resistentiae* to keep the euglycemia in suboptimal energetic condition.

A deficiency in OxPhos would enhance the need for ketone bodies, since such a defect leads to an increased glycolytic flux and hypoglycemia at the organismal level. Our data is in accordance with this, with basal level of ketone bodies in the

mutant mice higher than in WT. Ketogenesis is not hormonally regulated. Therefore, excess acetyl-CoA together with depletion of oxaloacetate would turn the ketogenesis on. In our experiment, a failure to increase blood ketones efficiently in response to fasting, can indicate disturbances in the β-oxidation, ketogenesis, or an increased ketone body utilization. However, the test strips used to measure the ketone bodies are measuring only 3-hydroxybutyrate, leaving the possibility that part of ketone bodies in form of e.g., acetone was missed.

A rationale for the Study II was built up on the base of numerous studies exploring mechanistic rationale for nutritional interventions and their effect in different cellular and animal model systems (Kuszak et al. 2019). Unfortunately, enteral supply of extra carbohydrates was not sufficient to alleviate clinical signs in GRACILE mouse model. On the other hand, this generates a potentially useful hypothesis that parenteral supply might be more beneficial. Especially if the intermediary metabolism could be supported with different alternative fuels, e.g., ketones and glucose parenterally.

Due to sensitivity of the mouse model causing lot of missing data and relatively small number of animals per group it was not possible to reach the statistical power using conventional statistics. Linear mixed-effect models (MEMs) were used for the analysis of metabolic network response to fasting. MEMs possess several advantages compared to traditional ANOVA models, one being the provision of more power, consequently lowering the numbers of animals needed per group (Maestripieri, Lindell, and Higley 2007). Furthermore, MEMs allow for modeling of multiple levels of hierarchy existing in the data and consequently modeling intrinsic statistical dependencies existing in the data. In other words, instead of controlling the variable, by for example, pairing animals (mice of the same age and the same weight in the control and intervention groups), they were included in the model. The model corrected for the impact of the variable. Not only that, but regression analysis also allows for the insertion of interactions between confounding variables. The mix-effect models also enable correcting for the dependency coming from measurements taken from the same mouse or the same litter.

Patient studies

Diagnostic algorithms for mitochondrial disorders are loaded with difficulties due to complex genetics and extremely variable clinical presentations. Despite the diagnostic improvements through the last decades, clinical awareness is still an important loop in the diagnostic procedure (Shen et al. 2021)(Parikh et al. 2019).

Diagnostic procedure in patient from Study III was complex and not straight forward. Initial WES analysis revealed two SNVs in *BSC1L* through filtering the data for genes related to mitochondrial disorders. One variant was found clearly

pathogenic and the other deemed as non-pathogenic. Biochemical investigations found reduced CIII activity in a muscle cell and drew the diagnostics back to the *BCS1L*, a strong gene candidate. The whole gene was then analyzed and further *in silico* analysis performed lead to the diagnosis. GRACILE patients have a mutation c.232A>G, whereas the proband had c.306A>T and c399delA, all of them being in the N-terminal import sequence on the protein level. The proband's phenotype was severe, with slightly increased life span compared to GRACILE patients, but no liver phenotype was observed with only mild metabolic phenotype in form of aminoaciduria. Our patient demonstrated transient lactic acidosis that resolved within a few days of birth, leading to diagnostic challenges, as it has been reported also in some primary mitochondrial disorders (Rahman 2020).

On the contrary, the patient from study IV had severe and complex metabolic phenotype difficult to decipher. It is likely that the deletion of *CYTB* contributed to the clinical severity observed in this patient, since no signs of additional nuclear defects were observed. The deleted segment in mitochondrial DNA coded for several CI subunits (ND4L, ND4, ND5, ND6) and transport RNAs. In addition, an uncommon deletion of *CYTB* responsible for CIII assembly and CI stability (Acín-Pérez et al. 2004) potentially reduces both RC complex CI and CIII activities. Biochemical data obtained from the patient were misleading and difficult to interpret, urging molecular genetic diagnostics. Prenatal onset and fulminant acidosis were also reported in PS if the deleted segment included *CYTB* (Wild et al. 2020)(Sano et al. 1993)(Muraki et al. 1997).

Conclusions

Our studies further characterize GRACILE phenotype and provide additional proof for phenotypic heterogeneity associated with *BCS1L* mutations.

Diagnostics in mitochondrial disorders is complex and comprises complementary parts of clinical awareness and expertise, next generation sequencing, comprehensive biochemical work up, analyses of functional effects of variants, protein and *in silico* analysis.

Homozygous mice (*Bcs1l*$^{c.232A>G}$) with CIII deficiency:

- Physiologic mechanism driving metabolic adaptations to fasting and interorgan crosstalk are not compromised by CIII deficiency.
- Fasting leads to ineffective liver metabolic response and hypoglycemia.
- Enteral feeding high carbohydrates (dextrose) does not alleviate hypoglycemia.
- Papers do not bring only a novelty coming from tested hypothesis, but also can serve as a reference for metabolic analysis done in mice aged four weeks. Metabolic studies in mice avoid experiments in this age due to its physiological complexity; it is time of sexual maturation and transition from high-fat milk diet to solid, predominantly high-carbohydrate diet.

Future perspective

- It is to be evaluated weather *BSC1L* and the corresponding protein have other functions than those currently ascribed to them and listed in databases, that might contribute to phenotype heterogeneity.
- It is to be further evaluated why and how CIII deficiency affects blood glucose level in a circumstance of suboptimal energetic condition.
- Linear mixed-effect model is a valuable tool in preclinical research. This is the most advanced statistical modelling which was not used before for the similar purposes and therefore establishes a new standard in the field.
- Targeted plasma metabolomics is a valuable tool in disease progression or therapeutic intervention assessment.

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And, in the end, nothing would be possible without the huge logistics operated by my patient husband.

Populärvetenskaplig sammanfattning

Mitokondrier är cellulära organeller som finns i nästan alla celler i vår kropp. Deras primära funktion är att producera energi som används för andra cellulära processer. Dessutom möts många andra metabola vägar i mitokondrierna varför de orkestrerar och reglerar andra cellulära processer. Energiproduktion sker i andningskedjan (respiratory chain, RC) genom en process som kallas oxidativ fosforylering. Andningskedjan består av fem proteinkomplex (CI-CV) i mitokondriemembranet. Medfödda fel i RC är den vanligaste medfödda metabola dysfunktionen. Vi utforskar hur defekter i RC komplex III (CIII) leder till en störd mitokondriemetabolism på organismnivå. I våra studier använde vi en musmodell med medfödd defekt i CIII som påverkar RCs funktion och energiproduktion. Vi fann att mutantdjur inte helt klarar av att fasta (som en metabol stress) eftersom d inte kan bevara blodsockernivån utan får lågt blodsocker ledande till koma. Om vi matar dem med extra socker i maten, förbättrar det inte deras överlevnad. Vi beskrev också två patienter med något olika medfödda fel i RC -funktionen, den första hade också en CIII-brist och den andra hade en genetisk defekt som också kan påverka även andra RC -komplex förutom CIII. Båda hade störd metabolism på organismnivå. I den senare patienten efterliknade störningarna andra metabola sjukdomar, vilket var vilseledande och har klinisk betydelse i framtiden.

Vi drar slutsatsen att metabola förändringar uppstår på grund av RC -dysfunktion, men att dessa tyvärr har mycket begränsad diagnostisk potential. Klinisk medvetenhet om denna grupp av sjukdomar är fortfarande mycket viktig i diagnostiken. Klinisk medvetenhet och snabb genetisk undersökning med hjälp av avancerad genetisk teknik kan avslöja denna typ av diagnoser mycket snabbt. Tidig diagnos kan leda till att man undviker onödig och invasiv utredning och eventuellt ger möjlighet till behandling i tid för de störningar där behandling är möjlig.

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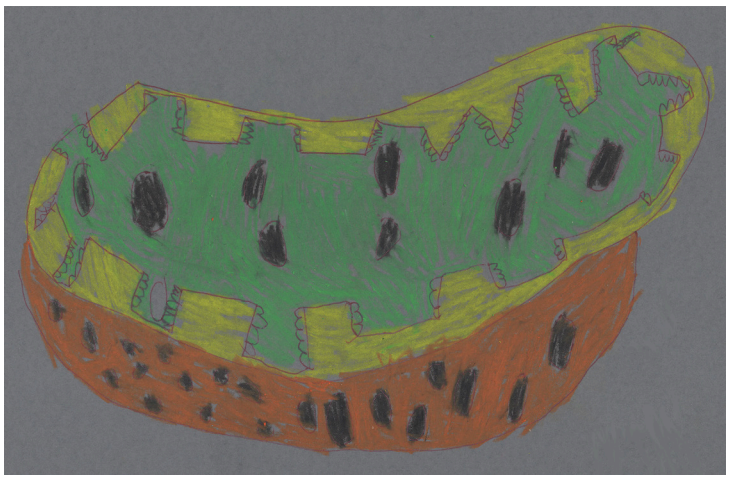

Schematic illustration of a mitochondria consisting of permeable outer mitochondrial membrane, inner mitochondrial membrane forming membrane curvatures called cristae and respiratory chain complexes anchored in the inner mitochondrial membrane.

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