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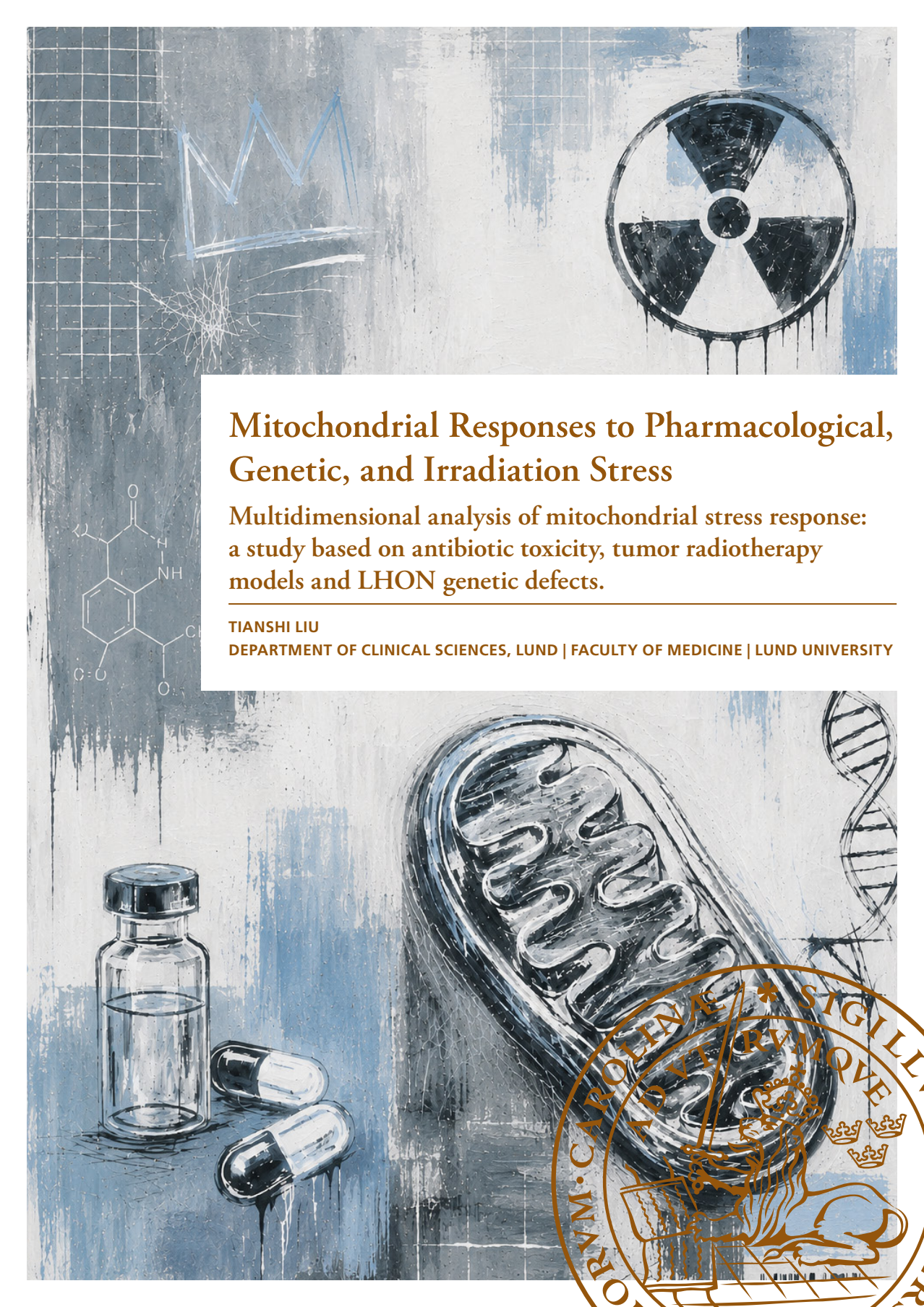
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Mitochondrial Responses to Pharmacological, Genetic, and Irradiation Stress

Multidimensional analysis of mitochondrial stress response: a study based on antibiotic toxicity, tumor radiotherapy models and LHON genetic defects.

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Abstract:

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In the third study, PBMCs and platelets from patients with Leber's hereditary optic neuropathy were used to assess complex I-related mitochondrial dysfunction. LHON PBMCs showed a reduced complex I-to-complex II respiratory ratio, suggesting a mild complex I-related phenotype, while absolute respiration was not reduced. NV189 increased respiration in partially rotenone-inhibited PBMCs and in LHON PBMCs, supporting the concept of succinate-driven complex I bypass.

The fourth study evaluated methodological factors affecting PBMC respirometry, including sample amount, storage conditions and digitonin permeabilization, and provided reference data from healthy controls.

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Abbreviations

ADP	adenosine diphosphate
ATP	adenosine triphosphate
CI	complex I
CII	complex II
CIII	complex III
CIV	complex IV
CV	complex V
CONV	conventional dose rate
DHE	dihydroethidium
DMSO	dimethyl sulfoxide
ETC	electron transport chain
ETS	electron transfer system
EUCAST	european committee on antimicrobial susceptibility testing
FCCP	carbonyl cyanide p-trifluoromethoxyphenylhydrazone
FDR	false discovery rate
GSH	reduced glutathione
GSSG	glutathione disulfide
HEI-OC1	house ear institute-organ of corti 1 cell line
HN4	head and neck squamous cell carcinoma cell line 4
HRR	high-resolution respirometry
ICUs	intensive care units
LHON	leber's hereditary optic neuropathy
mtDNA	mitochondrial DNA
mPTP	mitochondrial permeability transition pore
O ₂ k	oxygraph-2k
OCR	oxygen consumption rate

OXPPOS	oxidative phosphorylation
PBMCs	peripheral blood mononuclear cells
ROS	reactive oxygen species
RGCs	retinal ganglion cells
UHDR	ultra-high dose rate
$\Delta\Psi_m$	mitochondrial membrane potential

List of Papers

Liu T, Chamkha I, Elmér E, Sjövall F, Ehinger JK. Antibiotic-induced mitochondrial dysfunction: Exploring tissue-specific effects on HEI-OC1 cells and peripheral blood mononuclear cells. *BBA - General Subjects*. 2025;1869:130832. doi: 10.1016/j.bbagen.2025.130832

Lemos da Silva L, Liu T, Hörberger F, Chamkha I, Li N, Ceberg C, Elmér E, Adrian G, Ehinger JK. Mitochondrial remodeling after conventional and ultra-high dose rate irradiation in head and neck carcinoma cells in vitro. *Manuscript*.

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Sekine S, Chamkha I, Åsander Frostner E, Westerlund E, Liu T, Ehinger JK, Sjövall F, Uchino H, Elmér E. Mitochondrial respiration in human peripheral blood mononuclear cells: methodology and influence of permeabilization and storage. *Frontiers in Molecular Biosciences*. 2026;13:1756657. doi: 10.3389/fmolb.2026.1756657

Abstract

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In the third study, PBMCs and platelets from patients with Leber's hereditary optic neuropathy were used to assess complex I-related mitochondrial dysfunction. LHON PBMCs showed a reduced complex I-to-complex II respiratory ratio, suggesting a mild complex I-related phenotype, while absolute respiration was not reduced. NV189 increased respiration in partially rotenone-inhibited PBMCs and in LHON PBMCs, supporting the concept of succinate-driven complex I bypass.

The fourth study evaluated methodological factors affecting PBMC respirometry, including sample amount, storage conditions and digitonin permeabilization, and provided reference data from healthy controls.

Populärvetenskaplig sammanfattning

Mitokondrier finns i de flesta av kroppens celler och har som en av sina viktigaste uppgifter att omvandla näringsämnen från maten till energi som cellerna kan använda. Mitokondrier deltar också i regleringen av cellernas redoxbalans, stressreaktioner och celldöd. Eftersom olika celltyper har olika energibehov kan samma läkemedel, genetiska förändring eller fysiska påverkan få olika konsekvenser i olika vävnader. I denna avhandling undersöktes hur mitokondriernas funktion påverkas av antibiotika, strålbehandling och ärftlig mitokondriell sjukdom. Avhandlingen behandlar även hur mitokondriell respiration i mänskliga blodceller kan mätas på ett mer tillförlitligt sätt.

I den första studien jämfördes effekterna av flera kliniskt använda antibiotika i celler från innerörat och i perifera mononukleära blodceller, så kallade PBMC. Flera antibiotika minskade den mitokondriella respirationen och påverkade energirelaterade markörer i innerörecellerna. Vissa antibiotika ökade även signaler relaterade till fria syreradikaler. Blodceller som behandlades med motsvarande koncentrationer uppvisade däremot inte samma förändringar. Resultaten visar att antibiotikas effekter på mitokondrierna är beroende av celltyp och att resultat från blodceller inte direkt kan användas för att förutsäga hur celler i innerörat påverkas.

I den andra studien jämfördes strålbehandling med konventionell doshastighet med strålning vid ultrahög doshastighet, ofta kallad FLASH-strålning. Båda strålningsformerna påverkade mitokondriell respiration och andra mitokondrirelaterade markörer. I de celler och under de försöksförhållanden som användes i studien kunde dock inga signifikanta skillnader mellan de två strålningsformerna påvisas. Detta tyder på att FLASH-effekten inte kan förklaras enbart av förändringar i mitokondrierna. Även syretillgång, vävnadsstruktur och samspelet mellan olika celltyper bör vara betydelsefulla.

I den tredje studien analyserades blodceller från personer med Lebers hereditära optikusneuropati, LHON. Sjukdomen orsakas vanligen av förändringar i mitokondriellt DNA och skadar framför allt näthinnans ganglieceller. PBMC från personer med LHON uppvisade en mild förändring i respiration kopplad till mitokondriellt komplex I, men den totala respirationskapaciteten var inte tydligt nedsatt. Substansen NV189, som kan tillföra ett substrat via en väg som kringgår komplex I, ökade den mitokondriella respirationen under vissa försöksförhållanden. Resultaten motiverar fortsatt forskning om denna metabola strategi, men visar inte att NV189 kan behandla patienter. De visar inte heller att blodcellernas funktion fullt ut motsvarar förändringarna i näthinnan.

I den fjärde studien undersöktes hur provmängd, förvaringstid, temperatur och permeabilisering av cellmembranet påverkar mätningar av mitokondriell respiration i blodceller. Resultaten visade att provhanteringen före analysen och de valda mätförhållandena kan ha stor betydelse för resultatet. PBMC är lättillgängliga, kan

samlas in vid upprepade tillfällen och kan användas för att studera vissa förändringar i mitokondriell funktion. De kan däremot inte ersätta undersökningar av muskel, näthinna, inneröra eller andra vävnader. Resultat från blodceller måste därför tolkas i relation till den aktuella sjukdomen, blodets cellsammansättning och de experimentella förhållandena.

Sammantaget visar avhandlingen att mitokondriernas reaktioner på olika former av stress är starkt beroende av celltyp, sjukdomssammanhang och mätmetod. För att kunna tolka förändringar i mitokondriell funktion korrekt är det därför viktigt att välja en relevant cellmodell, standardisera provhanteringen och tydligt definiera vilka frågor studier av blodceller kan och inte kan besvara.

Introduction

Mitochondria are dynamic organelles with a semi autonomous genetic system in eukaryotic cells[1]. Since the mid-20th century, when biochemical and electron microscopy techniques revealed its structure, people's understanding of mitochondria has undergone a fundamental shift from a simple "cellular power factory"[2, 3]. Mitochondria contribute to ATP production, redox signaling, calcium handling, apoptosis regulation and intermediary metabolism[4, 5]. Through oxidative phosphorylation (OXPHOS), mitochondria generate adenosine triphosphate (ATP)[6]. They also participate in reactive oxygen species (ROS) signaling, calcium buffering, iron–sulfur cluster biogenesis and the production of metabolic intermediates[2, 3, 7]. The integrity of mitochondrial function is crucial for maintaining the normal physiological function of high-energy demanding tissues such as neurons, cardiomyocytes, auditory hair cells, and immune cells[8, 9]. Its dysfunction has become a core link in the pathophysiology of various human diseases[2, 3].

The maintenance of mitochondrial functional homeostasis faces challenges from endogenous and exogenous stress. Hereditary mitochondrial DNA (mtDNA) or nuclear DNA mutations can directly lead to structural or functional defects in respiratory chain complexes[10, 11], causing primary mitochondrial diseases such as Leber's hereditary optic neuropathy (LHON)[12]. Meanwhile, exogenous factors such as drugs, environmental toxins, and physical factors (such as ionizing radiation) can also target mitochondria as key targets, leading to cellular dysfunction and death by interfering with the electron transport chain, disrupting mitochondrial membrane potential, inducing mitochondrial permeability transition pore (mPTP) opening[13], or triggering oxidative stress[5]. Therefore, a deep understanding of how mitochondria perceive and respond to multidimensional stress from genetic, pharmacological, and physical levels is not only a core scientific question for revealing the molecular mechanisms of diseases but also provides a solid theoretical basis for developing therapeutic strategies targeting mitochondria, predicting drug toxicity, and optimizing clinical treatment plans. This thesis investigates mitochondrial respiratory and redox responses in four selected contexts: antibiotic-induced mitochondrial effects, irradiation-induced mitochondrial responses in head and neck cancer models, mitochondrial dysfunction in LHON blood cells, and methodological considerations for PBMC-based high-resolution respirometry.

Mitochondrial toxicity of antibiotics: from off target effects to cell type specificity

Antibiotics are the cornerstone of modern medicine, especially in intensive care units (ICUs), where they are essential drugs for controlling severe bacterial infections and saving patients' lives. However, the use of antibiotics often comes with a series of side effects, among which ototoxicity (hearing loss and vestibular dysfunction) and nephrotoxicity are the most well-known serious adverse reactions of aminoglycoside antibiotics, seriously affecting the quality of life[14]. For a long time, the occurrence of these toxicities has been believed to be caused by direct damage of antibiotics to cochlear hair cells or renal tubular epithelial cells. With increasing understanding of mitochondrial biology, mitochondrial injury has been proposed as one mechanism contributing to some antibiotic-associated toxicities, including ototoxicity and nephrotoxicity.

This mechanism is rooted in the endosymbiotic origin of mitochondria. According to the endosymbiotic theory developed by Lynn Margulis (then Lynn Sagan)(1967)[15], mitochondria originate from primitive alpha proteobacteria engulfed by early eukaryotic cells. After billions of years of co evolution, mitochondria still retain many features of their prokaryotic ancestors, including circular double stranded DNA, 70S ribosomes, and specific transcription and translation mechanisms. Many antibiotics are designed to target bacterial specific structures or functions, such as bacterial ribosomes (50S subunit and 30S subunit), DNA gyrases, or cell wall synthesis pathways. However, due to the high similarity between mitochondria and bacteria at the molecular level, these drugs inevitably interfere with the normal function of host cell mitochondria while killing pathogens[16].

Aminoglycoside antibiotics are a typical representative of this phenomenon. They induce mRNA misreading by binding to the 16S rRNA of the bacterial 30S ribosomal subunit, thereby inhibiting protein synthesis. Mitochondrial ribosomes and bacterial ribosomes are highly homologous in structure and sequence, especially mitochondrial 12S rRNA. Aminoglycoside susceptibility is strongly increased by variants in mitochondrial 12S rRNA, especially m.1555A>G, which makes the mitochondrial ribosome more sensitive to aminoglycoside-associated toxicity[17]. Due to the fact that multiple key subunits of respiratory chain complexes I, III, IV, and V are encoded by the mitochondrial genome, this translation inhibition directly leads to assembly defects in functional respiratory chain complexes, causing electron leakage, ROS burst, and ATP depletion, ultimately activating the apoptotic pathway, leading to hair cell death and hearing loss[18, 19]. Epidemiological studies have confirmed that individuals carrying mitochondrial 12S rRNA m.1555A>G mutations exhibit extreme ototoxicity sensitivity to aminoglycoside antibiotics, and even a single routine dose may lead to severe and irreversible hearing loss [20].

In addition to classical aminoglycosides, other classes of antibiotics have also been found to have mitochondrial toxicity potential. For example, fluoroquinolone antibiotics such as ciprofloxacin and levofloxacin interfere with DNA replication by inhibiting bacterial DNA gyrase (Topoisomerase II) and Topoisomerase IV, while mitochondrial Topoisomerase II (TopoII β) is a potential off target[21]. Fluoroquinolone drugs can induce mitochondrial DNA breakage and dysfunction, which is considered one of the important mechanisms for their side effects such as tendinitis and peripheral neuropathy. Oxazolidinone antibiotics (such as linezolid) are inhibitors of bacterial 50S ribosomal subunits, and long-term use can lead to reversible bone marrow suppression and peripheral neuropathy. There is evidence to suggest that they exert their effects by inhibiting mitochondrial protein synthesis[22, 23] . There are relatively few reports on mitochondrial toxicity of carbapenems and glycopeptides (such as vancomycin), but recent studies suggest that they may also affect mitochondrial function through indirect mechanisms, such as interfering with substrate metabolism or inducing oxidative stress.

Although the above studies have revealed the mitochondrial toxicity potential of multiple antibiotics, there are several key limitations to existing research. Firstly, most studies have focused on traditional models of cochlear hair cells or renal tubular cells, with insufficient systematic evaluation of other key cell types in the inner ear, such as supporting cells and cells of the stria vascularis, as well as circulating immune cells, such as PBMCs. Different cell types may exhibit vastly different sensitivities to antibiotic induced mitochondrial stress due to differences in their metabolic characteristics, mitochondrial reserve capacity, and drug uptake mechanisms. Secondly, the key mechanistic issues regarding the inhibitory effect of antibiotics on the mitochondrial respiratory chain, whether it occurs directly on the respiratory chain complex itself or by affecting upstream substrate supply (such as through pyruvate dehydrogenase, citric acid cycle intermediates), membrane lipid composition, or electron carriers (such as coenzyme Q10), are still not fully elucidated[16]. Therefore, evaluating how clinically relevant antibiotic concentrations affect mitochondrial respiration, membrane potential, ATP-related signals and ROS-related signals in different cell types may help clarify cell-type-specific mitochondrial vulnerability and guide further mechanistic studies.

Tumor radiotherapy: FLASH irradiation and mitochondrial response

Radiation therapy is one of the three pillars in the treatment of solid tumors such as head and neck tumors. The basic principle is to use ionizing radiation to induce double stranded DNA breaks in tumor cells, thereby killing tumor cells. However, the efficacy of radiotherapy is often limited by its dose limiting toxicity to

surrounding normal tissues (such as oral mucosal inflammation, salivary gland damage, swallowing difficulties, etc.), which becomes a bottleneck restricting the dose increase in tumor target areas and may also affect the long-term quality of life of patients[24].

In recent years, FLASH radiotherapy has attracted considerable attention as an emerging radiotherapy approach. FLASH radiotherapy delivers radiation dose in a very short period of time (milliseconds) at ultra-high dose rates (usually ≥ 40 Gy/s, about 1000 times the conventional dose rate)[25]. A series of preclinical studies have revealed an exciting "FLASH effect": compared with conventional dose rate(CONV) radiotherapy, FLASH radiotherapy can significantly reduce damage to normal tissues while maintaining the same killing effect on tumor cells, including reducing skin reactions, reducing pulmonary fibrosis, and preserving normal hematopoietic function[26-28]. These findings suggest that FLASH radiotherapy may have the potential to widen the therapeutic window, although the underlying mechanisms and clinical relevance still require further investigation[29]. (Figure.1)

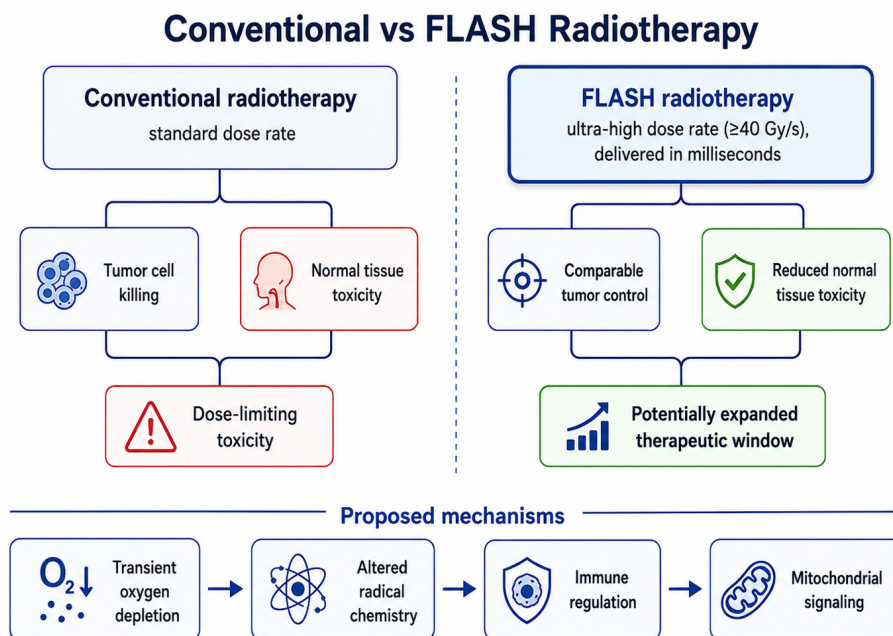


Figure 1. Schematic overview of FLASH radiotherapy.

The biological mechanisms underlying the FLASH effect remain incompletely understood. The mainstream hypothesis focuses on oxygen depletion, immune regulation, and mitochondrial signaling. The classic "oxygen fixation" hypothesis suggests that radiation-induced DNA damage is oxygen dependent, and oxygen

molecules can "fix" free radicals generated by radiation, leading to irreversible DNA damage. The ultra-high dose rate of FLASH irradiation will deplete the oxygen in the tissue in a very short period, creating an instantaneous "oxygen depletion" state and forming a protective microenvironment like hypoxia. One hypothesis is that UHDR irradiation may transiently reduce local oxygen availability during irradiation, thereby modifying radiation-induced radical chemistry [30]. In addition, FLASH radiotherapy has been reported to better preserve the activity of immune cells, induce unique immune microenvironment remodelling, and thus facilitate anti-tumor immune response[31].

Mitochondria are sensitive to changes in redox state, oxygen availability and cellular energy demand, and may therefore contribute to cellular responses after irradiation. Firstly, mitochondria are one of the main sources of intracellular ROS, which is an important mediator of radiation-induced cell damage. Mitochondria may contribute to irradiation responses through changes in ROS production, energy metabolism and cell-death signalling[32]. Whether these mitochondrial responses differ between CONV and UHDR irradiation remains unclear[7, 33]. Secondly, mitochondria are the center of radiation-induced cell apoptosis and autophagy signalling, and their mitochondrial membrane potential ($\Delta\Psi_m$), permeability transition pore (mPTP) status, and ATP production ability all significantly affect the sensitivity of cells to radiation. Conventional irradiation has been associated with changes in mitochondrial respiration, redox state, mitochondrial membrane potential and ATP-related metabolism. However, comparatively little is known about how UHDR irradiation affects mitochondrial respiration, ROS-related signals, ATP-related signals and mitochondrial membrane potential, particularly when compared with CONV irradiation under matched experimental conditions. Studies including different doses, sampling time points and cell types may help determine whether mitochondrial responses contribute to the FLASH effect.

Leber's hereditary optic neuropathy: complex I dysfunction and bypass strategies

mtDNA mutations are the main cause of a series of maternally inherited mitochondrial diseases. LHON is one of the best-known maternally inherited mitochondrial optic neuropathies and was first described by Theodor Leber in 1871[34, 35]. Clinically, LHON typically presents as painless, subacute central vision loss in young men, with sequential bilateral involvement and eventual optic nerve atrophy[36]. Most LHON cases are associated with one of three primary mtDNA variants: m.3460G>A in MT-ND1, m.11778G>A in MT-ND4, and m.14484T>C in MT-ND6. These genes encode subunits of respiratory-chain complex I[37].

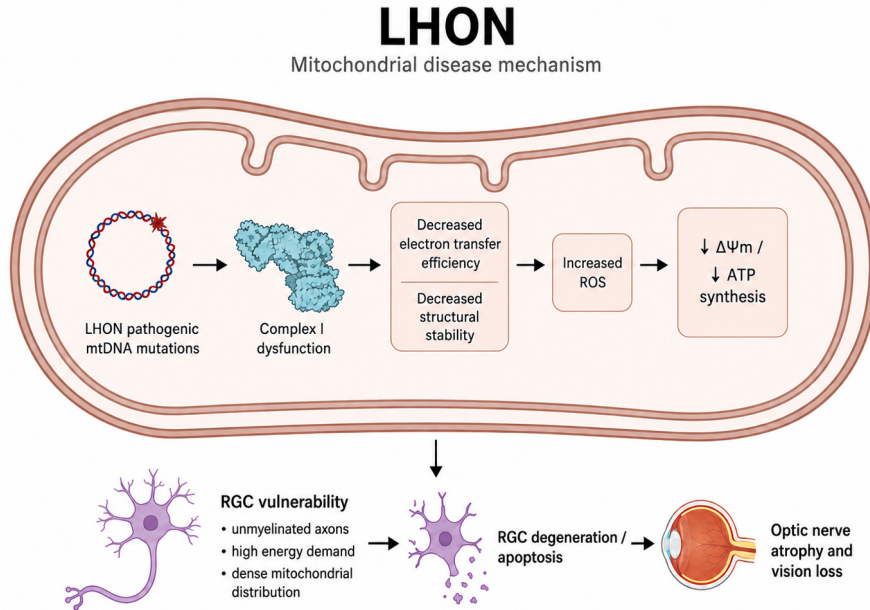


Figure 2. Overview of LHON-related mitochondrial dysfunction.[38].

Complex I is the largest and most complicated enzyme complex in the respiratory chain, consisting of 44 subunits (7 encoded by mtDNA and 37 encoded by nuclear DNA). Its function is to catalyse the electron transfer of NADH to ubiquinone and pump protons from the mitochondrial matrix to the intermembrane space during this process. It is a key rate limiting step in maintaining mitochondrial membrane potential and driving ATP synthesis[39]. The three common LHON variants affect mtDNA-encoded complex I subunits and are thought to impair complex I-linked electron transfer to varying degrees, potentially contributing to altered respiration and increased ROS production in susceptible cells. The axons of retinal ganglion cells (RGCs) have no myelin sheath, high energy requirements, and dense mitochondrial distribution[40, 41]. Their high dependence on complex I function explains why these commonly occurring mtDNA mutations have such significant tissue specificity, leading to preferential degeneration of RGCs[42]. The apoptosis and axonal degeneration of RGCs constitute the pathological basis of optic nerve atrophy and vision loss in LHON patients.

However, the pathophysiology of LHON is not limited to the optic nerve. As a systemic mtDNA mutation disease, its impact should theoretically affect all tissues dependent on OXPHOS. For a long time, the evaluation of mitochondrial functional status in peripheral blood cells (such as platelets and PBMCs) of LHON patients has

yielded inconsistent research results. Some studies have failed to detect clear respiratory chain defects, which may be due to the fact that blood cells, as circulating cells, have stronger metabolic plasticity, higher heterogeneity in mutation load, or effective compensatory mechanisms, such as upregulation of glycolysis and activation of mitochondrial unfolded protein response[43]. In addition, whether the defect of complex I manifests as a "hidden" or "mild" functional impairment in PBMCs requires more sophisticated respiratory measurement schemes (such as using different substrate inhibitor combinations) to reveal, which is a question that needs to be further explored[43].

Strategies that bypass complex I dysfunction are an important therapeutic concept in mitochondrial disease research. The core idea of this strategy is to bypass damaged complex I and restore electron flow and ATP synthesis by providing a substrate for downstream complex II, directly introducing electrons into the ubiquinone pool of the respiratory chain[44]. However, the negative charge of exogenous succinate limits its ability to cross cell and mitochondrial membranes. The development of cell-permeable succinate prodrugs, such as NV118 and NV189, has therefore opened new possibilities for translating this complex I bypass strategy into clinical applications. These prodrugs can enter cells and be hydrolyzed by non-specific esterases, releasing succinate, which accumulates in the mitochondrial matrix and activates complex II driven respiration[45]. In models of complex I deficiency and patient-derived mitochondrial disease cells, cell-permeable succinate prodrugs have been shown to support mitochondrial respiration and improve bioenergetic parameters[46].

PBMCs as an accessible but context-dependent model for mitochondrial assessment

The translation of basic research results into clinical practice highly relies on accessible, reproducible, and reliable assessment methods for the mitochondrial functional status in patients. PBMCs are easily obtained from whole blood, or leukocyte concentrates by density-gradient centrifugation and have therefore become an accessible blood-based model for assessing selected aspects of mitochondrial function in humans[47, 48]. PBMCs mainly include lymphocytes (T cells, B cells, NK cells), monocytes, and a small number of dendritic cells, which are the core executors of adaptive and innate immunity. The activation, proliferation, differentiation, and effector functions of these cells are highly dependent on metabolic reprogramming from OXPHOS to glycolysis, and their energy metabolism status is closely coupled with immune function[48-50].

The use of PBMCs as a model for studying mitochondrial function has multiple unique advantages[51]. Firstly, the sampling is convenient and minimally invasive,

allowing for longitudinal dynamic monitoring at different stages of the disease or before and after treatment. Secondly, as circulating immune cells, PBMCs can provide a snapshot of the overall metabolism, inflammation, and stress status of the body[52]. Changes in mitochondrial function have been shown to be associated with various diseases, including primary immunodeficiency or autoimmune diseases, as well as being used as biomarkers for diseases not directly related to immune disorders, such as neurodegenerative diseases (Parkinson's disease, Alzheimer's disease)[48, 53], cardiovascular diseases[54], metabolic dysfunction[55] and aging[56]. By using high-resolution respirometry such as the Oroboros O2k system[57], it is possible to evaluate endogenous substrate supported respiration in intact cells (via endogenous substrates) and cells selectively permeabilized by digitonin, as well as the maximum activity of specific respiratory chain complexes resolved by exogenous substrate and inhibitor combinations (such as complex I substrate pyruvate+malate, complex II substrate succinate), in order to obtain detailed information about OXPHOS ability.

However, standardizing the mitochondrial respiration measurement of PBMCs as a routine clinical test still faces many technical challenges. This includes: 1) Sample size limitation: The number of cells obtained from a single sample is limited, and the measurement process needs to be optimized to obtain the maximum amount of information under the limited number of cells; 2) Sample stability: The storage time, temperature, and medium after separation have a significant impact on the mitochondrial activity of PBMCs, and standardized sample processing and transportation standards need to be established[58]; 3) Quality control of permeabilization: The concentration and incubation time of digitonin must be carefully optimized to ensure effective plasma membrane permeabilization while preserving mitochondrial membrane integrity and avoiding over-permeabilization; 4) Reference value establishment: It is necessary to establish a stable and reliable range of reference values for respiratory rate and key ratios in healthy populations, taking into account factors such as age, gender, and race[59]. Standardization may improve reproducibility and help define the contexts in which PBMC respirometry provides useful translational information.

Overall Conceptual Framework of the Thesis

This thesis consists of four relatively independent but interconnected studies. At first glance, these studies address different biological questions and experimental models, including antibiotic-induced mitochondrial effects, irradiation responses in tumour cells, Leber's hereditary optic neuropathy (LHON), and methodological aspects of PBMC-based mitochondrial respiration. However, they are linked by a common central question: how mitochondrial respiration and redox-related responses change under different biological and experimental contexts.

The four studies can be viewed as three biological models of mitochondrial stress together with one methodological study. The antibiotic study represents pharmacological mitochondrial stress and examines whether different cell types show different mitochondrial sensitivity under the same drug exposure. The irradiation study represents physical stress and compares mitochondrial respiration, ROS production, ATP-related signals and $\Delta\Psi_m$ after conventional dose rate CONV and UHDR irradiation. The LHON study represents genetic mitochondrial stress and investigates whether complex I -related dysfunction can be detected in patient-derived blood cells, and whether a succinate prodrug can enhance respiration through CI bypass. The PBMC methodology study focuses on the experimental system itself, evaluating how sample amount, storage conditions and digitonin permeabilization influence HRR in PBMCs. These methodological findings may also provide useful considerations for other approaches used to assess mitochondrial respiration and cellular metabolism, such as Seahorse-based measurements.

Therefore, the main theme of this thesis is not simply to compare different diseases or stressors, but to emphasize the context-dependent nature of mitochondrial respiratory metabolism. A decrease in respiration, altered ROS production, changes in ATP-related signals or changes in $\Delta\Psi_m$ may have different biological meanings depending on cell type, substrate availability, disease mechanism, assay design and sample handling. Moreover, these readouts do not necessarily change in parallel. Intact-cell respiration reflects the overall metabolic state of the cell but does not by itself identify the specific site of respiratory impairment. In contrast, permeabilized-cell respiration allows respiratory pathways to be assessed under defined substrate conditions but requires appropriate and carefully controlled permeabilization. PBMCs are accessible human primary cells with translational value, but they should not be considered a universal substitute for mitochondrial function in the cochlea, retina, muscle or brain.

Based on this framework, the thesis argues that HRR and related redox and bioenergetic readouts can provide important information for mitochondrial research. However, their biological interpretation is reliable only when cell type, substrate entry, disease background and methodological limitations are carefully considered.

Research Purpose

Based on the above research background, mitochondria, as the core maintainer of cellular homeostasis, play a crucial role in determining cell fate when facing multiple stresses such as drugs, genetics, and physics. However, current research still lacks a systematic investigation of this issue, mechanism depth, application of new technologies, and standardization of methodologies. This study aims to systematically explore the functional changes and molecular mechanisms of

mitochondria under various stress conditions through four interrelated and focused parts, in response to these gaps:

1. Evaluation of mitochondrial toxicity of antibiotics: Five commonly used clinical antibiotics (carbapenems, fluoroquinolones, aminoglycosides, glycopeptides, and oxazolidinones) were selected to systematically evaluate their effects on mitochondrial respiration, ATP levels, membrane potential, and ROS levels in HEI-OC1 epithelial cell lines and human primary PBMCs at clinically relevant concentrations. Permeabilized-cell experiments were used to locate whether the toxic site directly acts on the respiratory chain complex or the upstream substrate supply chain, revealing the differential mechanisms of sensitivity in different cell types.
2. Compare the mitochondrial effects of CONV and FLASH irradiation: Using head and neck squamous cell carcinoma cells (LU-HNSCC4) and normal fibroblasts (VH10) as models, systematically compare the dynamic changes in mitochondrial respiration, membrane potential, ROS, and ATP levels after conventional dose rate and ultra-high dose rate irradiation at different doses (0-20 Gy) (0 h and 48 h), and combine transcriptomic analysis to reveal the mitochondrial related mechanisms of FLASH effect at the functional and molecular levels.
3. Verify the cellular pathological mechanism and potential for bypass therapy of LHON: Using primary PBMCs derived from LHON patients, accurately evaluate the presence of complex I dysfunction through high-resolution respirometry. Meanwhile, evaluating the ability of the cell-permeable succinate prodrug NV189 to improve mitochondrial respiration in patient PBMCs and in a rotenone-induced complex I inhibition model provides direct evidence supporting its translational potential.
4. Establish a standardized process for PBMCs mitochondrial respiration measurement: systematically explore the effects of sample size, storage conditions (time, temperature), and digitonin permeation conditions on the results of PBMCs mitochondrial respiration measurement, establish a standardized operating procedure, and preliminarily establish a reference range for respiratory rate in healthy populations, laying a solid foundation for the subsequent use of this technology in clinical monitoring.

Together, these studies aim to provide a structured overview of how mitochondria respond to antibiotics, Genetic and radiation-induced stress, highlighting both shared responses, such as increased ROS and impaired energy metabolism, and stressor-specific mechanisms, such as direct complex I inhibition or disturbances in upstream substrate supply. These studies provide experimental and methodological insights into mitochondrial respiratory and redox responses under different mitochondrial stress, including antibiotic exposure, complex I-related dysfunction, and FLASH radiotherapy. They also illustrate the potential value, as well as the limitations, of PBMC-based mitochondrial function testing in translational research.

Rather than providing direct clinical evidence, the findings may serve as a basis for future studies aimed at refining mitochondrial assessment and exploring disease- or treatment-related mitochondrial phenotypes.

Materials and methods

Antibiotic-induced mitochondrial dysfunction: Exploring tissue-specific effects on HEI-OC1 cells and peripheral blood mononuclear cells

This study selected five commonly used antibiotics from different antimicrobial classes: gentamicin (aminoglycoside), meropenem (carbapenem), ciprofloxacin (fluoroquinolone), vancomycin (glycopeptide), and linezolid (oxazolidinone). To avoid using arbitrary supraphysiological in vitro concentrations, antibiotic exposure was based on clinically relevant high-exposure conditions. Cells were exposed to 64 times the non-species-specific clinical breakpoint values defined by The European Committee on Antimicrobial Susceptibility Testing (EUCAST)[60]. This concentration level was chosen based on previously published ICU patient concentration data showing that antibiotic levels may reach up to 64 times the corresponding clinical breakpoint in critically ill patients. Therefore, the antibiotics were compared according to dose equivalency relative to their clinical antibacterial activity, rather than at equimolar concentrations. The final concentrations were 333 μ M meropenem, 48 μ M ciprofloxacin, 67 μ M gentamicin, 177 μ M vancomycin, and 379 μ M linezolid. HEI-OC1 mouse inner ear epithelial cell line was cultured in high glucose DMEM medium containing 10% fetal bovine serum at 33 °C and 10% CO₂. Human peripheral blood mononuclear cells (PBMCs) were separated from white blood cell concentrates of healthy blood donors by density gradient centrifugation using Lymphoprep density gradient. The specific steps were as follows: the blood was centrifuged at 800 g for 30 minutes, the mononuclear cell layer was collected, washed, and resuspended in MiR05 medium containing 50% plasma. After incubating the cells with corresponding antibiotics or solvents for 24 h, various tests were performed.

HEI-OC1 is a conditionally immortalized murine inner-ear cell line derived from the organ of Corti region and is widely used as an in vitro model in ototoxicity research. Although it does not fully reproduce mature cochlear hair cells in vivo, it provides a practical model for studying mitochondrial and oxidative stress responses in inner-ear-related cells[61].

The mitochondrial respiratory function was evaluated using an Oxygraph-2k high-resolution respirometry at 37 °C. The complete cellular respiration assay protocol is

as follows: first, record baseline routine respiration, then add oligomycin (1 µg/mL) to inhibit ATP synthase to determine leakage respiration, gradually titrate FCCP to induce maximum uncoupling respiration, and finally add rotenone (2 µM) and antimycin A (1 µg/mL) to inhibit complex I and complex III, respectively, to determine residual non mitochondrial oxygen consumption. For the permeabilization experiment of HEI-OC1 cells, after digitonin permeabilization, malate, pyruvate and glutamate were added as complex I-linked substrates. ADP was then added to induce phosphorylating respiration through complex I. Succinate was subsequently added as a complex II substrate to assess combined complex I- and complex II-linked OXPHOS. Subsequently, the titration steps of oligomycin, FCCP, and inhibitors were repeated.

The levels of superoxide anions in cells and mitochondria were stained with dihydroethidium (DHE, 10 µM) and MitoSOX Red (5 µM), respectively, and incubated at 37 °C in the dark for 20 minutes before being detected by flow cytometry[62, 63]. ATP-related changes were indirectly assessed using Magnesium Green AM, cell-permeant (MgGr, 1 µM; M3735, Thermo Fisher Scientific/Life Technologies Europe BV, Stockholm, Sweden). This acetoxymethyl ester form allows the probe to enter intact cells, where intracellular esterases cleave the AM groups and retain the dye intracellularly. Mitochondrial membrane potential was assessed using TMRM (20 nM). Fluorescence signals were quantified by flow cytometry and expressed relative to the corresponding vehicle control. [50].

Mitochondrial function in head and neck carcinoma in vitro cells after irradiation at conventional and ultra-high dose rates

Head and neck squamous cell carcinoma cells and normal fibroblasts were irradiated with CONV and UHDR, ≥ 40 Gy/s, respectively, with a total dose of 0-20 Gy. Cells were collected for detection at 0 and 48 h after irradiation. The detection methods for mitochondrial respiratory function, ROS levels, ATP levels, and membrane potential are as described above. In addition, cellular RNA was extracted for transcriptome sequencing to analyse changes in mitochondrial related gene expression profiles.

Mitochondrial function, redox status, and response to succinate prodrug treatment in blood cells from patients with Leber's Hereditary Optic Neuropathy

After venous blood collection from LHON patients and healthy controls, PBMCs and platelets were separated by density gradient centrifugation. Experiments with partial inhibition were conducted using rotenone (5 nM) to partially inhibit complex I of PBMCs from healthy blood donors, to construct an LHON cell model. The intervention drugs are NV189 (100 μ M), a precursor to succinate, and idebenone (25 μ M). High resolution respirometry was performed in intact cells and permeabilized cells, respectively. The permeabilization protocol used digitonin for permeabilization, followed by the sequential addition of complex I substrate, ADP, Succinate, as well as oligomycin FCCP, rotenone and antimycin A. To evaluate the systemic redox status, liquid chromatography tandem mass spectrometry was used to detect the levels of reduced glutathione and oxidized glutathione in whole blood. Immediately after collection, whole-blood samples were treated with N-ethylmaleimide (NEM), which derivatizes free thiol groups and thereby stabilizes glutathione species, and sulfosalicylic acid (SSA), which limits enzymatic reactions before analysis.

Mitochondrial respiration in intact PBMCs

This study optimized the key technical parameters for detecting mitochondrial respiration in PBMCs. PBMCs were isolated from white blood cell concentrates or whole blood at 8, 24, and 48 h after collection, and the effects of storage time and temperature (room temperature and 4 °C) on mitochondrial function were evaluated. The cell concentration in the chambers varies from 1×10^6 to 10×10^6 cells/mL to determine the optimal detection concentration. The permeabilization of digitonin was optimized by stepwise titration (1-15 μ g/million cells), with a ratio of oxidative phosphorylation ability to electron transfer ability close to 1.0 as the optimal permeabilization dose standard. The respiratory measurement schemes for intact cells and permeabilized cells are basically the same as before. The permeabilized-cell protocol allowed assessment of OXPHOS capacity supported by NADH-linked substrates (NP) and by combined NADH- and succinate-linked substrates (NSP), as well as the corresponding uncoupled electron-transfer capacities (NSE and SE)[64]. Use flow cytometry to identify the changes in the proportion of various subgroups of PBMC after isolation.

PBMC methodology study

Applying an uncoupler disrupts the electrochemical gradient across the inner mitochondrial membrane, stimulating the ETS to reach its maximal decoupled capacity (ET capacity). The complex I inhibitor, rotenone, and the complex III inhibitor, antimycin A, fully inhibit the ETS and reveal any residual oxygen consumption (Rox) of the cell[64-66].

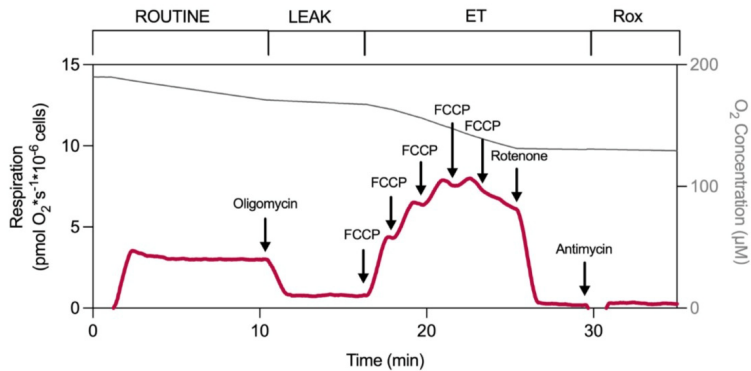


Figure 3. Experimental protocol of intact PBMCs.

Mitochondrial respiration was measured in intact PBMCs by high-resolution respirometry using a coupling-control protocol with consecutive inhibitor additions and uncoupler titration[64-67]. After establishing endogenous (ROUTINE) respiration, LEAK respiration was induced by adding the ATP synthase inhibitor oligomycin (1 μg/mL). Electron transfer pathway capacity (ET capacity), reflecting the maximally decoupled respiration, was determined by titration of the protonophore, carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP). Addition of rotenone (2 μM) followed by antimycin A (1 μg/mL), complex I and complex III inhibitors, respectively, revealed residual oxygen consumption (Rox). Induced respiratory coupling states, uncoupler, and inhibitors used are indicated in and above the graph.

Statistical analysis

Statistical analyses were performed using GraphPad Prism. Because the four studies included different experimental designs, the statistical tests were selected according to whether the data were paired or unpaired, whether two or more conditions were compared, and whether repeated measurements were included. Sample sizes

differed between studies and are therefore reported in the corresponding figure legends. A p value below 0.05 was considered statistically significant.

In the antibiotic study, HEI-OC1 cells and PBMCs were compared with their corresponding vehicle controls. Because gentamicin was dissolved in water, gentamicin-treated samples were compared with the water control using the Wilcoxon matched-pairs signed-rank test. Vancomycin, ciprofloxacin, linezolid and meropenem were dissolved in DMSO and were compared with the DMSO control using the Friedman test for paired multiple-group comparisons. The same statistical approach was used for high-resolution respirometry and flow cytometry readouts. Oxygen consumption values were corrected for residual non-mitochondrial respiration before analysis. Flow cytometry data were analysed as fluorescence intensity relative to the corresponding vehicle control.

In the irradiation study, mitochondrial respiration, ROS-related signals, ATP-related signals and mitochondrial membrane potential were analysed according to irradiation dose, dose rate and time point. GraphPad Prism 10 was used for statistical analysis. Two-way ANOVA was applied when two experimental factors were analysed, followed by Bonferroni's multiple-comparison test. To account for the number of tests performed across the study, false discovery rate correction was subsequently applied using the two-stage step-up method of Benjamini, Krieger and Yekutieli. Data were presented as mean $\pm$ standard error of the mean.

In the LHON study, respirometry data were recorded using DatLab and further processed in Microsoft Excel before statistical analysis in GraphPad Prism 10.4.1. Respiration after antimycin A, or azide in the idebenone experiments, was subtracted from all measurements as residual non-mitochondrial respiration. In experiments where LHON and control PBMCs were treated with NV189 or vehicle, respiration was normalized to the value measured before drug or vehicle addition. Oligomycin-sensitive respiration was calculated as the change in oxygen flux after oligomycin addition in the presence of the respective treatment. The relative complex I-to-complex II respiratory ratio was calculated from the ADP-stimulated NADH-linked respiratory response and the succinate-supported respiratory response. Because of the small sample size, data were presented as medians with interquartile ranges and analysed using the Mann–Whitney U test. When multiple comparisons were required, p values were adjusted using the Holm–Šidák method.

In the PBMC methodology study, GraphPad Prism 10.02 was used for statistical analysis. Data were presented as medians with interquartile ranges or as individual values. All oxygen fluxes were corrected for residual oxygen consumption. Paired comparisons between two related conditions were analysed using the Wilcoxon test. Comparisons involving more than two related conditions, such as different cell concentrations, storage times, storage temperatures or digitonin concentrations, were analysed using the Friedman test followed by Dunn's multiple-comparison test. Respiratory rates and internal ratios from healthy controls were also

summarized descriptively to provide reference values for the optimized intact-cell and permeabilized-cell PBMC protocols.

Use of Artificial Intelligence

Generative artificial intelligence (AI) tools were used during the preparation of this thesis to support the writing and editing process. Specifically, AI was used to improve grammar, spelling, punctuation, readability, and overall language clarity. AI tools also provided suggestions regarding manuscript organization, section structure, and the design of conceptual illustrations and figures. In the final stages of manuscript preparation, AI was used as an additional proofreading tool to help identify minor language, formatting, and typographical errors.

AI was not used to generate, fabricate, manipulate, or interpret research data, experimental results, statistical analyses, scientific conclusions, or references. All study design, experimental work, data collection, data analysis, interpretation of findings, and scientific conclusions were performed independently by the author. All AI-generated suggestions were critically reviewed, verified, and edited before incorporation into the thesis. The author accepts full responsibility for the accuracy, integrity, and originality of the final work.

Result

Antibiotic-induced mitochondrial dysfunction: Exploring tissue-specific effects on HEI-OC1 cells and peripheral blood mononuclear cells

The Impact of Antibiotics on Mitochondrial Respiration

First, three distinct respiratory states were evaluated in intact cells: baseline, oligomycin-induced leak, and highest FCCP-induced uncoupled respiration (Figure 4). In HEI-OC1 cells, ciprofloxacin decreased respiratory levels at baseline (-35.6%), leak (-34.5%), and highest uncoupled respiration (-34.5%) compared to controls. Linezolid reduced baseline (-45.3%), leak (-36.6%), and highest uncoupled respiration (-48.9%) levels. Meropenem decreases baseline (-37.7%), leak (-25.4%), and highest uncoupled respiration (-39.9%) levels, while gentamicin lowered baseline (-16%) and leak (-34.5%) respiratory levels, with no impact on the maximally uncoupled state. Vancomycin reduced leak respiration by 23.8% without significant effect on the other states. The only antibiotic that affected the ratio between LEAK and maximum uncoupled respiration was gentamicin, which increased the ratio, indicating higher mitochondrial coupling efficiency. In contrast, vancomycin, ciprofloxacin, linezolid, meropenem, and gentamicin did not affect mitochondrial respiratory levels in intact PBMCs.

We permeabilized intact HEI-OC1 cells using digitonin and sequentially added saturating concentrations of pathway-specific substrates and complex-specific inhibitors to measure maximal respiratory capacity (Figure 5). We found no significant differences in oxidative phosphorylation levels related to CI or CII between the antibiotic-treated groups and the control group. The only statistically significant finding was a reduction of maximum phosphorylating respiration with both CI and CII substrates of 26.3% for linezolid compared to vehicle control.

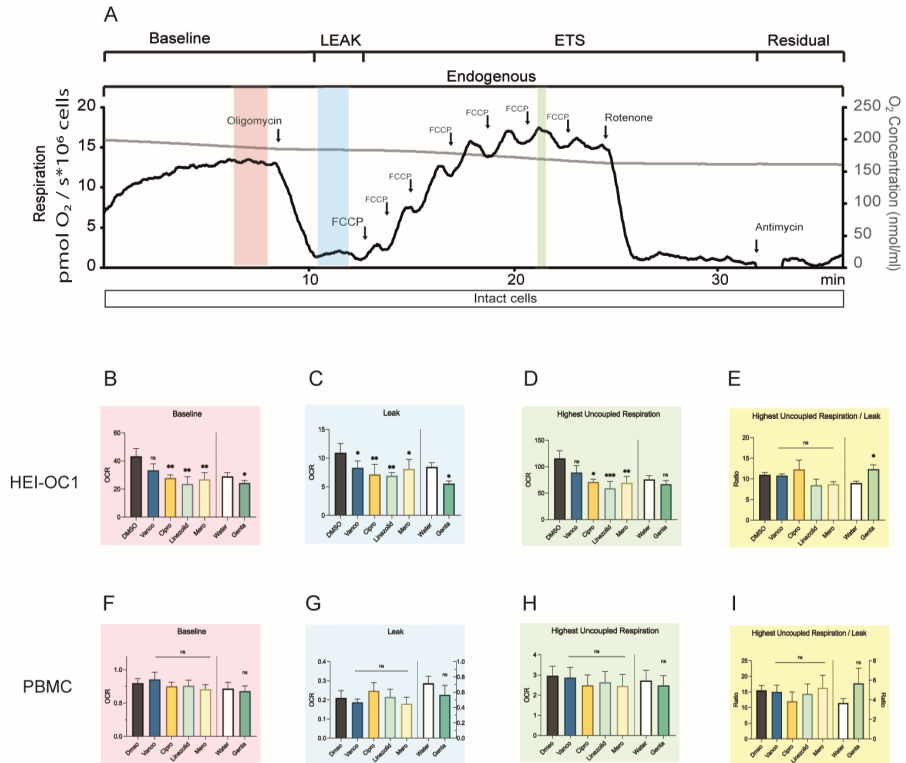


Figure 4. HEI-OC1 cells and human PBMCs were exposed to 64 times of the clinical breakpoints concentrations of vancomycin (177 μ M), ciprofloxacin (48 μ M), linezolid (379 μ M), meropenem (333 μ M), and gentamicin (67 μ M) for 24 h. Mitochondrial respiration of intact cells was evaluated in MiR05 compared to vehicle controls (DMSO and H₂O). Shown are: Representative experimental trace (A), baseline, oligomycin-induced (1 μ g/mL) LEAK respiration, highest FCCP-induced uncoupled respiration and the ratio between LEAK and maximum uncoupling for both HEI-OC1 cells (B-E) and PBMCs (F-I). Comparisons between the gentamicin group and the water control group were conducted using Wilcoxon paired rank test, while comparisons between vancomycin, ciprofloxacin, linezolid, meropenem, and the DMSO control group were performed using Friedman paired test (N=7). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.) OCR (Oxygen Consumption Rate) represents the rate of oxygen consumption as a measure of mitochondrial respiratory activity (pmol O₂ / s * 10⁶ cells).

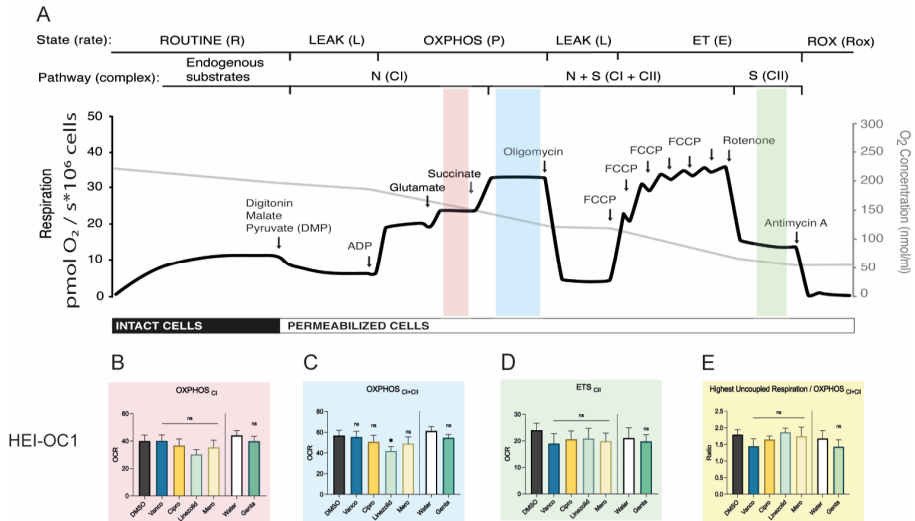


Figure 5. HEI-OC1 cells were exposed to 64 times of the the clinical breakpoints concentrations of vancomycin (177 μM), ciprofloxacin (48 μM), linezolid (379 μM), meropenem (333 μM), and gentamicin (67 μM) for 24 h. Mitochondrial respiration was evaluated in MiR05 compared to vehicle controls (DMSO and H₂O).

Shown are: Representative experimental trace (A). HEI-OC1 cells were permeabilized with digitonin with concurrent additions of malate, and pyruvate (5 mM each). After ADP (1 mM) and glutamate (5 mM) additions OXPHOSCI was recorded. OXPHOSCI+CII was recorded after succinate (10 mM) addition. Electron transfer system activity with CII substrates was revealed by rotenone (ETSCII). Comparisons between the gentamicin group and the water control group were conducted using Wilcoxon paired rank test, while comparisons between vancomycin, ciprofloxacin, linezolid, meropenem, and the DMSO control group were performed using Friedman paired test (N=7). (*p < 0.05, **p < 0.01, ***p < 0.001.) OCR (Oxygen Consumption Rate) represents the rate of oxygen consumption ($\text{pmol O}_2 / \text{s} \cdot 10^6 \text{ cells}$).

Cellular and Mitochondrial ROS ($\text{O}_2^{\bullet-}$)

Ciprofloxacin increased cellular $\text{O}_2^{\bullet-}$ by 86.2% in HEI-OC1 cells compared to the control group, and mitochondrial-related $\text{O}_2^{\bullet-}$ by 48.6%. Similarly, linezolid increased cellular $\text{O}_2^{\bullet-}$ by 94.0% and mitochondrial-related $\text{O}_2^{\bullet-}$ by 47.2%, while meropenem increased cellular $\text{O}_2^{\bullet-}$ by 45.3% and mitochondrial-related $\text{O}_2^{\bullet-}$ by 43.3%. Gentamicin showed an increase of 15.8% in cellular $\text{O}_2^{\bullet-}$ and 12.3% in mitochondria-related $\text{O}_2^{\bullet-}$ (Figure 6). Although vancomycin did not exhibit statistically significant differences compared to the control group, it displayed a trend like the other antibiotics in increasing $\text{O}_2^{\bullet-}$ levels. Conversely, in experiments with PBMCs, no statistically significant differences were observed in cellular $\text{O}_2^{\bullet-}$ or mitochondria-related $\text{O}_2^{\bullet-}$ between any of the experimental groups and the control group (Figure 7).

ATP Level and Mitochondrial Membrane Potential

Magnesium Green fluorescence is reduced as ATP levels increase[68]. In experiments with HEI-OC1 cells, linezolid decreased ATP levels by 68.9% compared to the control group, meropenem decreased ATP levels by 37.3%, and gentamicin decreased ATP levels by 39.7% (Fig. 6). There was no significant difference in ATP production between vancomycin- and ciprofloxacin-treated HEI-OC1 cells and the control group. However, the trend of decreasing ATP levels in these two groups was consistent with the other three groups. In experiments with PBMCs, none of the five antibiotics showed statistically significant differences compared to the control group (Figure 7).

Our study demonstrated a significant 32.5% reduction in mitochondrial membrane potential in HEI-OC1 cells treated with ciprofloxacin compared to the control group, and a 25.3% reduction with gentamicin. However, vancomycin, linezolid, and meropenem did not impact the mitochondrial membrane potential of HEI-OC1 cells (Figure 6). In contrast, experiments with PBMCs showed that none of the five antibiotics tested influenced mitochondrial membrane potential (Figure 7).

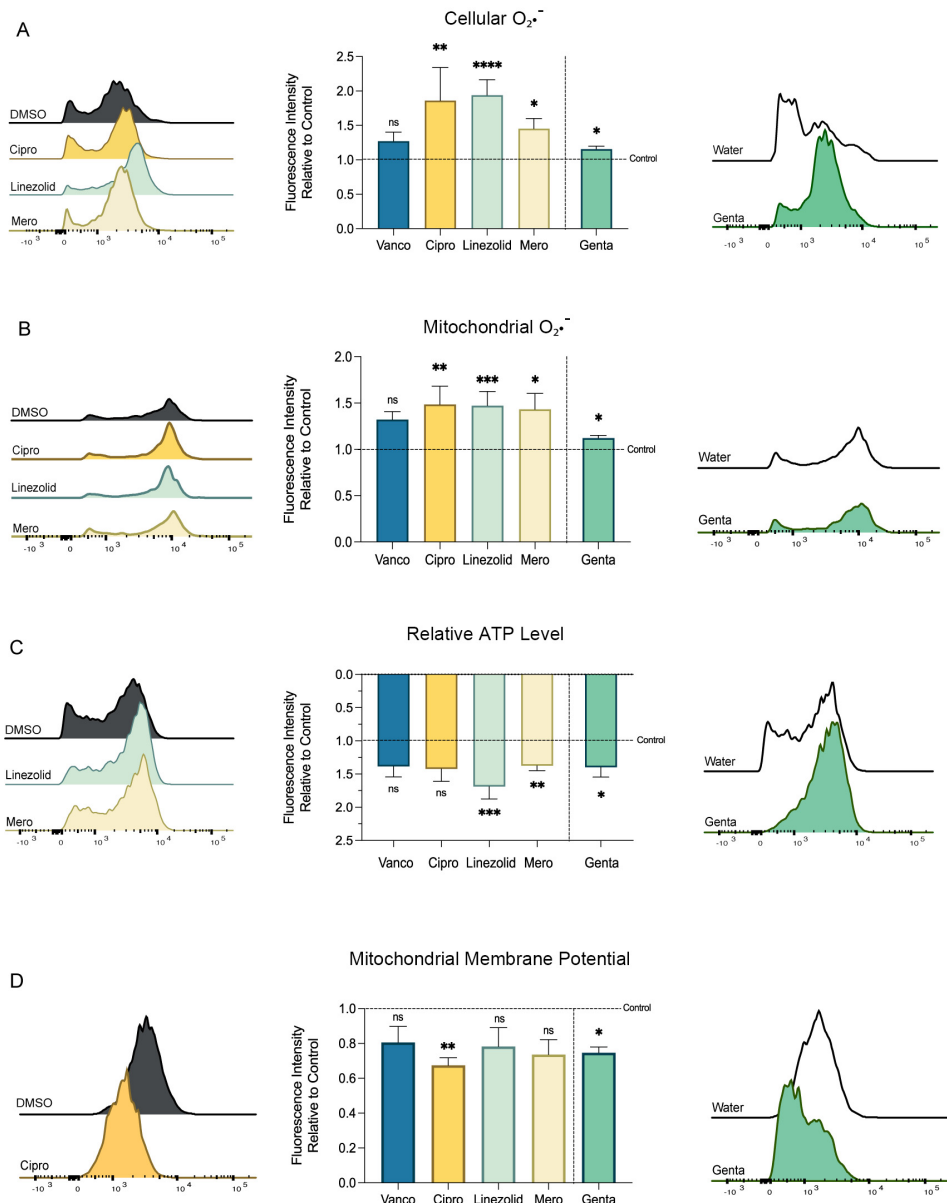


Figure 6. Cellular $O_2^{\bullet-}$, mitochondrial $O_2^{\bullet-}$, relative ATP level, and mitochondrial membrane potential (MMP) levels were assessed in HEI-OC1 cells exposed to concentrations of vancomycin (177 μ M), ciprofloxacin (48 μ M), linezolid (379 μ M), meropenem (333 μ M), and gentamicin (67 μ M) for a duration of 24 h. Cellular $O_2^{\bullet-}$, was visualized using DHE staining(A), mitochondrial $O_2^{\bullet-}$, with MitoSOX red(B), ADP/ATP with MgGr(C), and MMP with TMRM(D). Flow cytometry was utilized to analyze and compare these measurements to untreated cells. (Representative histograms on sides). Comparisons between the gentamicin group and the water control group were conducted using Wilcoxon paired rank test, while comparisons between vancomycin, ciprofloxacin, linezolid, meropenem, and the DMSO control group were performed using Friedman paired test (N=7). (*p < 0.05, **p < 0.01, ***p < 0.001).

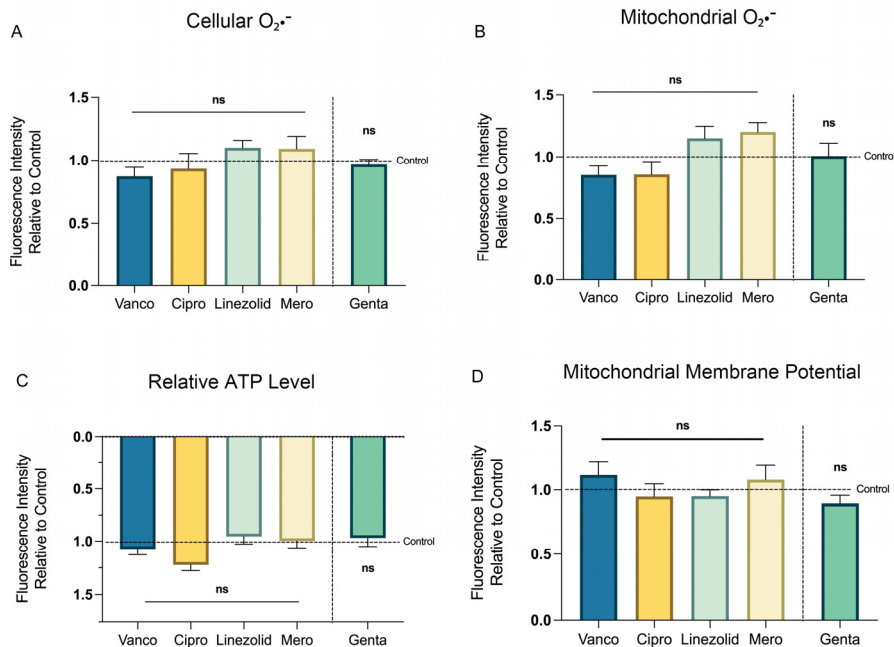


Figure 7. Cellular $O_2^{\bullet-}$, Mitochondrial $O_2^{\bullet-}$, relative ATP level, and MMP levels were assessed in PBMCs exposed to concentrations of vancomycin (177 μ M), ciprofloxacin (48 μ M), linezolid (379 μ M), meropenem (333 μ M), and gentamicin (67 μ M) for a duration of 24 h. Cellular $O_2^{\bullet-}$ was visualized using DHE staining(A), mitochondrial $O_2^{\bullet-}$ with MitoSOX red(B), relative ATP level with MgGr(C), and MMP with TMRM(D). Flow cytometry was utilized to analyze and compare these measurements to untreated cells. (Representative histograms on sides). Comparisons between the gentamicin group and the water control group were conducted using Wilcoxon paired rank test, while comparisons between vancomycin, ciprofloxacin, linezolid, meropenem, and the DMSO control group were performed using Friedman paired test (N=7). (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$)

Mitochondrial function in head and neck carcinoma in vitro cells after irradiation at conventional and ultra-high dose rates

Reactive Oxygen Species, ATP levels and Mitochondrial Membrane Potential

ROS are natural products of ET activity, functioning as signalling molecules for a variety of cellular reactions, but when in excessive concentrations can lead to several harmful effects to cells and tissues. Figure 8 shows how the investigated mitochondrial and cellular levels of ROS changed with CONV and UHDR IR in

HN4 cells, complemented with investigation of ATP levels (indirectly using Magnesium Green fluorescence) and Mitochondrial Membrane Potential (MMP).

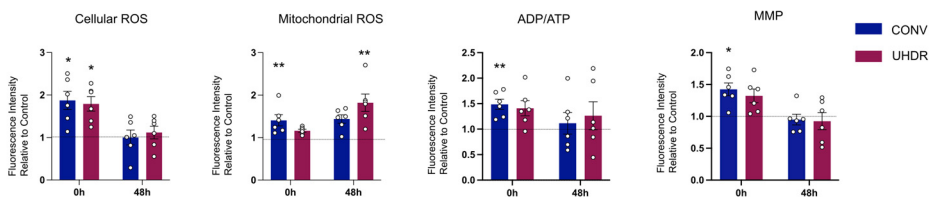


Figure 8. Mitochondrial Reactive oxygen species (mtROS) cellular ROS, ATP-levels and Mitochondrial Membrane Potential a)

Quantification of Cellular ROS using DHE (Excitation/emission wavelength 561/586 nm). b) Quantification of Mitochondrial ROS (mtROS) using MitoSOX (Ex/Em 561/610 nm). c) Quantification of ADP/ATP using MgGr (Ex/Em 405/605 nm). d) Quantification of Mitochondrial Membrane Potential (MMP) using TMRM (Ex/Em 561/586 nm). All data presented in the figure are expressed as the ratio of fluorescence intensity measured by flow cytometry in the experimental groups relative to the control group. All assays done in HN4 (1 000 000 cells/mL cells) after 0h and 48h after irradiation with CONV 20Gy and UHDR 20Gy using a BD FACSymphony A1 flow cytometer. Control samples were not irradiated, n=6 per condition. The bars represent mean values $\pm$ standard error of the mean (SEM); n=6 per condition and *p<0.05, **p<0.01, ***p<0.001 and ****p<0.0001 compared to control.

Immediately after irradiation, cellular ROS fluorescence increased by 78% in the CONV group and by 74% in the UHDR group relative to the control. At 48 h, cellular ROS fluorescence in both irradiated groups was not significantly different from the control. Mitochondrial ROS fluorescence increased by 43% in the CONV group at 0 h and returned to the control level by 48 h, whereas no significant change was observed in the UHDR group at 0 h and an increase of 78% relative to the control was observed at 48 h. ATP-related fluorescence indicated a reduction in ATP levels in the CONV group at 0 h, which was no longer detected at 48 h, while no significant change was observed in the UHDR group. Mitochondrial membrane potential also increased in the CONV group at 0 h and returned to the control level by 48 h, whereas no significant change was observed in the UHDR group. Although the patterns relative to the control differed across individual time points, direct comparisons did not identify statistically significant differences between CONV and UHDR irradiation. Therefore, these results should not be interpreted as evidence of distinct time-dependent responses between the two irradiation modalities (Figure 8).

Mitochondrial function, redox status, and response to succinate prodrug treatment in blood cells from patients with Leber's Hereditary Optic Neuropathy

Effect on oxygen consumption on intact partially rotenone-inhibited blood donor PBMCs with the succinate prodrug NV189 and idebenone

As LHON is due to pathogenic variants in mtDNA coding for subunits of complex I, we attempted to model LHON using PBMCs from leukocyte suspensions from blood donors by inducing a partial inhibition of complex I with rotenone. For these partially CI-inhibited cells a significant reduction in respiration was in basal respiration, as well as for oligomycin-sensitive respiration and maximal FCCP-stimulated respiration. NV189 was able to significantly increase respiration in these partially rotenone-inhibited cells to levels similar to cells without rotenone inhibition in respiration directly after drug addition, as well as for oligomycin-sensitive respiration and maximal FCCP-stimulated respiration. For idebenone, a small significant increase in respiration was seen directly after addition. However, for oligomycin-sensitive and maximal FCCP-stimulated respiration in idebenone-treated cells there instead was a significant decrease (Figure 9).

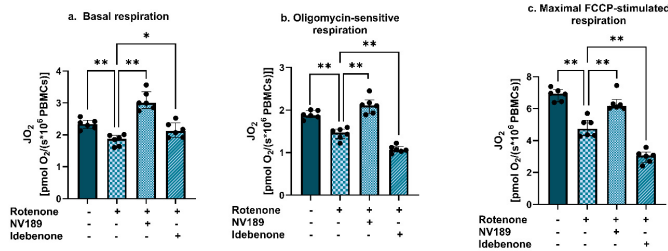


Figure 9. Oxygen consumption in a partially CI-inhibited PBMC model of LHON, treated with either the succinate prodrug NV189 or idebenone.

For each group, JO₂ with partial rotenone inhibition or addition of vehicle (DMSO) followed by treatment with NV189 or idebenone or addition of vehicle (DMSO) is shown. All values were corrected for non-mitochondrial oxygen consumption. Values are presented as medians with interquartile ranges, as well as individual data points. Adjusted p-values are calculated with the Mann-Whitney U test with multiple comparisons between groups with Holm-Šidák method. a. Respiration after drug or vehicle addition. b. Oligomycin-sensitive respiration, calculated as the difference between respiration after adding drug-treatment and respiration after inhibition of the ATP-synthase with oligomycin. c. Maximal FCCP-stimulated respiration. DMSO = Dimethyl Sulfoxide. LHON = Leber's Hereditary Optic Neuropathy. FCCP = Trifluoro-Methoxy-Carbonyl Cyanide-4-Phenylhydrazone. JO₂ = Mitochondrial Respiratory Oxygen Flux. 6 n for each experiment. * < 0.05. ** < 0.01.

In the included studies, oxygen consumption was expressed as either OCR or oxygen flux (JO₂), depending on the terminology and analytical platform used in the original studies. Both terms describe the rate at which cells consume oxygen and can therefore be regarded as indicators of mitochondrial respiratory activity. In this paper, the original terminology has been retained wherever possible.

Oxygen consumption in permeabilised PBMCs and platelets from LHON patients and healthy controls

Platelets and PBMCs were isolated from blood donated by five LHON patients. The patients were 38–77 years old with a mean age of 57 years and were all male. Four had the pathogenic variant m.11778G>A and one m.3460G>A (Table 1). For the control group the age range was 34–63 years, the mean age was 53 years old and were all male. The ratio between complex I-linked respiration and complex II-linked respiration was significantly lower for LHON PBMCs, indicating that LHON PBMCs relied more on complex II respiration compared to control (Figure 10). This was not seen in platelets. No differences could be shown between LHON and control subjects in absolute respiration for any state in neither PBMCs, nor platelets.

Table 1. Characteristics for the five LHON patients and the ten healthy controls.

	LHON Patients (n=5)	Control (n=10)
Years of Age (mean with min-max)	57 (38–77)	53 (34–63)
Sex	5 M/0 F	10 M/0 F
Pathogenic Variant	4 m.11778G>A/1 m.3460G>A	-
Smoking	None	None
BMI (kg/m ²)	29.8	25.0

M = Male. F = Female. LHON = Leber's Hereditary Optic Neuropathy.

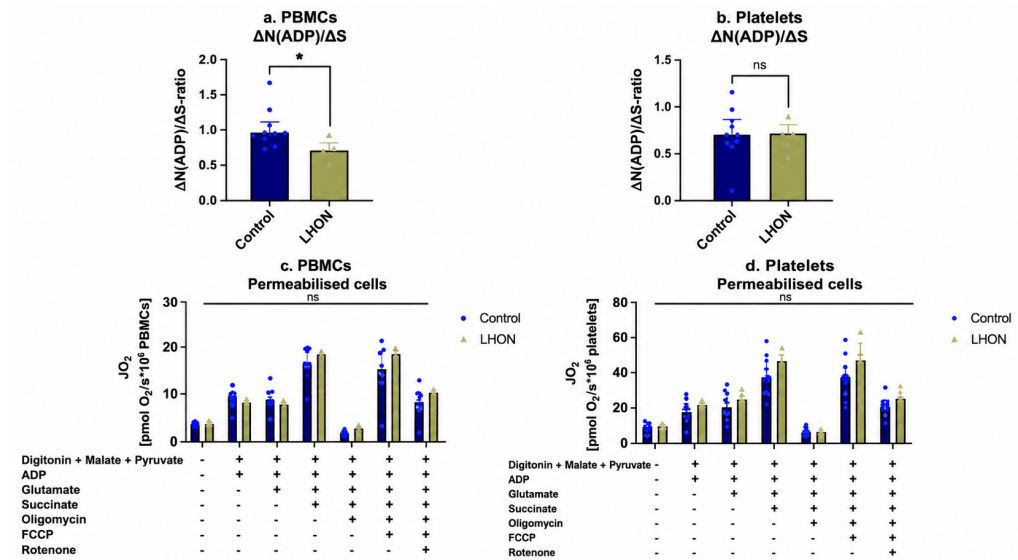


Figure 10. Oxygen consumption in permeabilised platelets and PBMCs from LHON patients and healthy controls.

All values are corrected for non-mitochondrial oxygen consumption. Values are presented as medians with interquartile ranges, as well as individual data points. Adjusted p-values are calculated with the Mann-Whitney U test and in graph c and d with multiple comparisons between groups with Holm-Šidák method a, b. The ratio between complex I-linked respiration and complex II-linked respiration for PBMCs (a) and platelets (b). c, d. Respiration after consecutive addition of substrates and inhibitors for PBMCs (c) and platelets (d). LHON = Leber's Hereditary Optic Neuropathy. PBMC = Peripheral Blood Mononuclear Cell. FCCP= Trifluoro-Methoxy-Carbonyl Cyanide-4-Phenylhydrazine. JO_2 = Mitochondrial Respiratory Oxygen Flux. N(ADP) = NADH related substrates and ADP. S = Succinate. 5 n for patients and 10 n for healthy controls for each experiment. ns = not significant. $* < 0.05$.

Effect on oxygen consumption on intact LHON and healthy control PBMCs treated with the succinate prodrug NV189

Based on the results from the partially rotenone-inhibited cell model, and due to limited availability of patient blood cells, the only drug compound evaluated in patient samples was NV189. In addition, since no decrease in the complex I/complex II ratio was seen for platelets, only PBMCs were assessed for treatment effect. For both the healthy control group and LHON-patients the respiration was significantly increased in PBMCs treated with NV189 immediately after drug

addition, as well as for oligomycin-sensitive respiration and respiration after complete rotenone inhibition (Figure 11).

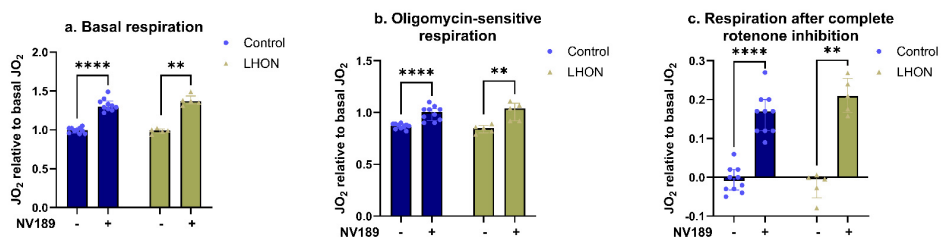


Figure 11. Oxygen consumption in intact PBMCs from LHON patients and healthy control treated with succinate prodrug NV189.

For each group, JO₂ with NV189 treatment or vehicle (DMSO) is shown. All values are corrected for non-mitochondrial oxygen consumption and are presented relative to respiration values before addition of NV189 or DMSO. Values are presented as medians with interquartile ranges, as well as individual data points. Adjusted p-values are calculated with the Mann-Whitney U test with multiple comparisons between groups with Holm-Šidák method. a. Respiration after drug or vehicle addition in PBMCs. b. Oligomycin-sensitive respiration in PBMCs, calculated as the difference between respiration after adding drug-treatment and respiration after inhibition of the ATP-synthase with oligomycin. c. Residual succinate-driven respiration after complete complex I inhibition with rotenone in PBMCs. LHON = Leber's Hereditary Optic Neuropathy. DMSO = Dimethyl Sulfoxide. PBMC = Peripheral Blood Mononuclear Cell. JO₂ = Mitochondrial Respiratory Oxygen Flux. 5 n for LHON patients and 10 n for controls for each experiment. ** <0.01. ****<0.0001.

Glutathione

All patients had blood glutathione levels, both oxidised and reduced, within previously reported reference ranges (Table 2).

Table 2. Glutathione levels for Leber's hereditary optic neuropathy patients.

Sample	Total (μM)	Glutathione	GSH (μM)	GSSG (μM)	GSH/GSSG
REF RANGE	836–1484		836–1483	<1.78	597–2584
[69]					
LHMH64	1071.5		1070.7	0.77	1390.5
LHMH60	1216.4		1215.9	0.54	2251.7
LHMH59	1280.7		1279.7	1.03	1242.4
LHMH58	1191.0		1190.3	0.74	1608.5
LHMH54	1118.0		1117.1	0.87	1284.0

GSH = Reduced Glutathione. GSSG = Oxidised Glutathione.

Methodological factors affecting PBMC respirometry

Chamber cell concentration influenced respiratory measurements in intact PBMCs (Figure 12A). Compared with measurements performed at 10×10^6 cells/mL, electron transfer capacity was significantly reduced at cell concentrations of 2.5×10^6 cells/mL or lower. In addition, LEAK respiration was increased at 1×10^6 cells/mL. These findings indicate that low chamber cell concentrations can affect the assessment of respiratory capacity even after normalization to cell number. Storage time also affected mitochondrial respiration in permeabilized PBMCs (Figure 12A). Storage of leukocyte suspensions at room temperature reduced both OXPHOS and electron transfer capacities. The convergent NADH- and succinate-linked OXPHOS capacity was already decreased after one day of storage, whereas after two days, NADH-linked OXPHOS capacity, convergent OXPHOS capacity, succinate-linked electron transfer capacity, convergent electron transfer capacity, and LEAK respiration was all reduced.

Digitonin permeabilization required careful optimisation (Figure 12B). At a sub-optimal digitonin dose, OXPHOS capacity was lower than electron transfer capacity, consistent with incomplete permeabilization and restricted access of ADP and substrates. At the optimal dose, OXPHOS and electron transfer capacities were comparable. In contrast, supra-optimal digitonin doses resulted in lower electron transfer capacity relative to OXPHOS capacity, while excessive digitonin exposure reduced both respiratory capacities. These findings show that digitonin dose should be assessed within the complete SUIT protocol to avoid underestimation of OXPHOS or electron transfer capacity.

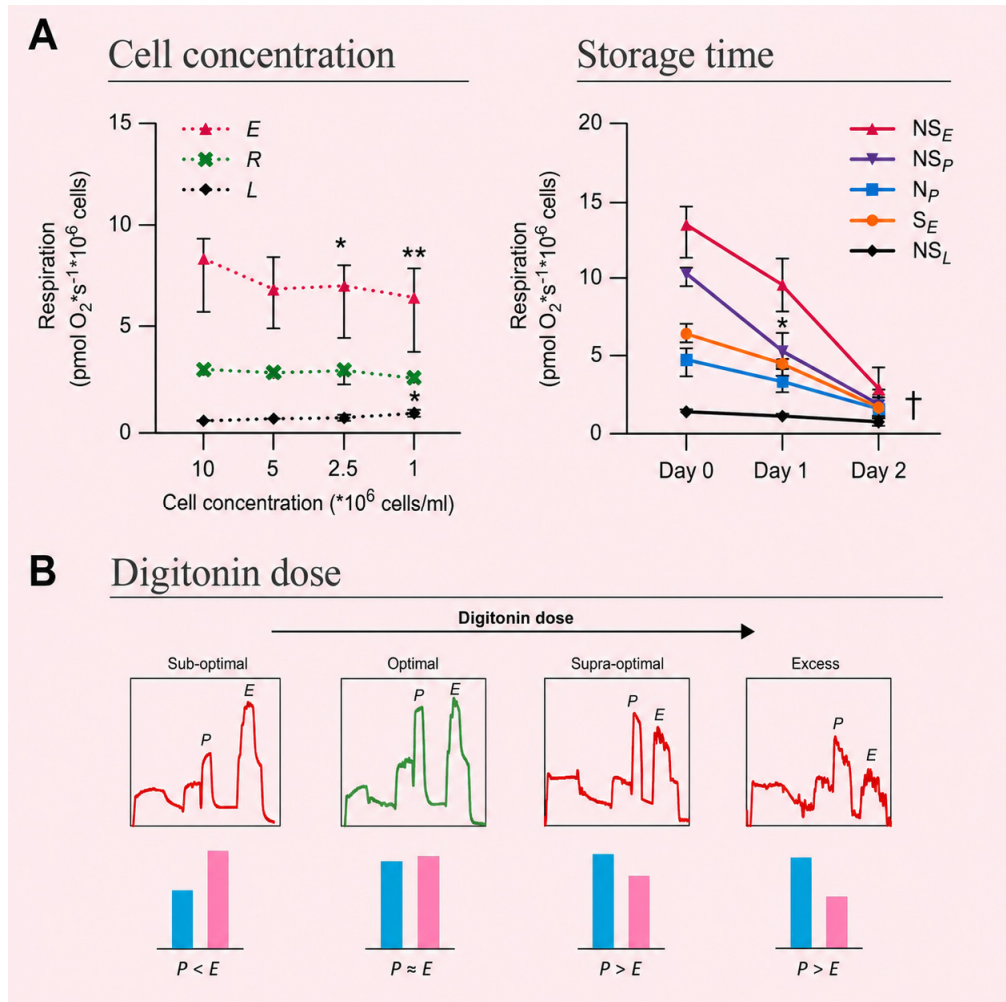


Figure 12. Influence of digitonin dose on respiratory measurements in permeabilized PBMCs. Schematic representation, based on representative Oroboros traces, of the relationship between OXPHOS capacity (P) and electron transfer capacity (E) at increasing digitonin doses. At a sub-optimal dose, incomplete plasma membrane permeabilization may limit substrate and ADP access and lead to an underestimation of P ($P < E$). At the optimal dose, P and E are comparable. At supra-optimal doses, E may be underestimated ($P > E$), while excessive digitonin exposure reduces both P and E. Therefore, digitonin dose should be evaluated within the complete SUIT protocol rather than selected solely from the maximal respiration observed during digitonin titration.

Discussion

Cell type specificity and clinical significance of antibiotic mitochondrial toxicity

This study found that multiple antibiotics at high clinical concentrations can significantly impair mitochondrial function of HEI-OC1 cells derived from the inner ear but have no effect on circulating immune cell PBMCs. This discovery has significant clinical implications: 1) it explains why certain patients (especially those carrying mtDNA mutations) exhibit extreme ototoxicity sensitivity to specific antibiotics (such as aminoglycosides), while other systems show no apparent damage. 2) The different responses observed in HEI-OC1 cells and PBMCs suggest that antibiotic-associated mitochondrial effects may vary between cell types. In this study, PBMCs were included as an accessible comparison cell type, rather than as a model for mitochondrial function in the inner ear or other tissues. 3) More importantly, through permeabilization experiments, this study has for the first time accurately located the mitochondrial toxicity sites of multiple antibiotics (especially carbapenems) upstream of the respiratory chain (which may involve substrate transport, pyruvate dehydrogenase activity, or TCA cycle intermediate supply), rather than directly inhibiting the complex itself. This provides new ideas for developing targeted protection strategies, such as supplementing specific substrates.

Comparing intact-cell and permeabilized-cell respiration

The difference between intact-cell and permeabilized-cell respiration in this study has important methodological implications. Intact-cell respiration reflects the integrated bioenergetic state of living cells, rather than the function of a single respiratory-chain complex[70]. It is influenced not only by the activity of the ETS, but also by substrate uptake, glycolysis, TCA cycle flux, mitochondrial substrate transport, ADP/ATP exchange, ATP demand, mitochondrial membrane potential and cellular stress responses[70]. Therefore, changes in OCR in intact cells indicate an altered cellular metabolic state, but they cannot by themselves identify the exact site of mitochondrial impairment.

In contrast, permeabilized-cell respiration allows mitochondrial respiratory pathways to be assessed under more controlled conditions. Selective permeabilization of the plasma membrane with digitonin allows exogenous substrates, ADP and inhibitors to reach the mitochondria, while mitochondrial structure can be largely preserved when the permeabilization conditions are appropriate[71, 72]. By using defined substrates such as pyruvate, malate, glutamate and succinate, CI-linked and CI+CII-linked respiration can be evaluated. Further additions of oligomycin, FCCP, rotenone and antimycin A allow the separation of OXPHOS capacity, ETS capacity and residual oxygen consumption [71, 73, 74].

Thus, intact-cell and permeabilized-cell experiments address different levels of mitochondrial bioenergetics. Intact-cell respiration is closer to the physiological state of living cells and captures the overall metabolic response to treatment. Permeabilized-cell respiration, by contrast, is more useful for testing whether defined respiratory pathways remain functional when substrate and ADP supply are controlled. When the two approaches give different results, this should not necessarily be interpreted as a contradiction. Instead, it may suggest that the primary effect occurs upstream of the respiratory complexes, for example at the level of substrate availability, glycolytic supply to mitochondria, pyruvate metabolism, TCA cycle substrate provision, mitochondrial substrate transport or cellular ATP demand[70, 71].

This distinction is particularly important for interpreting antibiotic-induced mitochondrial effects. In the present study, several antibiotics reduced respiration in intact HEI-OC1 cells, whereas most CI- and CI+CII-linked OXPHOS capacities were preserved after digitonin permeabilization. This pattern suggests that the reduction in intact-cell OCR may mainly reflect upstream metabolic limitation or altered cellular bioenergetic regulation, rather than a major direct inhibition of CI or CII under substrate-saturating conditions. However, this interpretation requires caution, because permeabilized-cell respiration is highly dependent on appropriate digitonin conditions. Insufficient permeabilization may limit substrate and ADP access, whereas excessive permeabilization may damage mitochondrial membranes and affect respiratory measurements. Therefore, permeabilization conditions need to be optimized for each cell type and experimental model[75].

Overall, combining intact-cell and permeabilized-cell HRR provides more information than either approach alone. Intact-cell respiration captures the integrated metabolic response of the cell, while permeabilized-cell respiration helps determine whether specific mitochondrial respiratory pathways remain functional under defined substrate conditions. This distinction is useful for interpreting the antibiotic study and supports the broader methodological focus of this thesis on cell-type specificity and context-dependent interpretation of mitochondrial respiration.

Mitochondrial mechanism of FLASH effect: an unseen perspective

An important finding of this study was that no statistically significant differences between CONV and UHDR irradiation were detected for mitochondrial respiration, ROS-related signals, ATP-related signals or mitochondrial membrane potential under the experimental conditions used. Both irradiation modalities affected several mitochondrial readouts, but the overall responses were similar. These findings do not support a distinct mitochondrial response to UHDR irradiation in the cell models and at the time points examined. However, they do not exclude a contribution of mitochondria to the FLASH effect in more complex biological settings. Factors that were not represented in this in vitro system, including transient changes in oxygen availability[76], interactions between tumour and stromal cells, immune responses, vascular effects and three-dimensional tissue organization[77], may be important. The transcriptomic results were consistent with this interpretation, as both irradiation modalities affected mitochondrial-related gene sets, while few differences were detected between CONV and UHDR irradiation.

LHON Pathophysiological Heterogeneity and the Prospects of Bypass Therapy

This study detected mild but statistically significant complex I dysfunction (decreased CI/CII ratio) in PBMCs of LHON patients, which has long been a controversial issue in this field[78]. Our results support the existence of systemic and detectable respiratory chain defects in LHON patients, but their severity is mild enough to be compensated by peripheral blood cells through metabolic reprogramming (such as upregulation of complex II activity).

NV189 and idebenone should not be regarded as interchangeable complex I-bypass treatments. NV189 is a cell-permeable succinate prodrug that releases succinate inside cells. By supporting electron entry through complex II, it can increase electron flow downstream of complex I when complex I function is impaired. In contrast, idebenone is a short-chain coenzyme Q analogue. After NQO1-dependent intracellular reduction, it can shuttle electrons directly to complex III, thereby bypassing complex I, and may also influence cellular redox balance[79]. Idebenone is approved in Europe as Raxone for visual impairment in LHON, and its clinical benefit has been evaluated mainly by visual outcomes rather than by ex vivo respiratory measurements[80, 81].

The more consistent acute increase in respiration observed with NV189 in the rotenone-inhibited PBMC model should therefore not be interpreted as evidence

that NV189 is more effective than idebenone in LHON. NV189 directly increases the availability of a complex II substrate, whereas the effects of idebenone depend on its intracellular reduction and the cellular redox environment. The reduction in oligomycin-sensitive and FCCP-stimulated respiration after idebenone in this model should be interpreted as a model-specific observation[82]. A more meaningful comparison would require longer exposure times and additional readouts, including ATP-related signals, ROS, redox status, and disease-relevant retinal ganglion cell models.

In this study we have shown that mitochondrial respiration is significantly increased in a proposed LHON model (PBMCs from healthy blood donors partially inhibited by rotenone) with addition of the succinate prodrug NV189, returning respiration to a level comparable to control cells (vehicle-treated PBMCs from blood donors). However, since no total decrease in respiration was seen in the LHON blood cells, partially inhibiting PBMCs with rotenone probably does not create a representative disease model for LHON blood cells. Still, since it is difficult to measure the respiration in the affected RGCs it is uncertain how the proposed LHON model compares to the affected cells. For PBMCs from healthy controls and LHON-patients, NV189 also significantly increased the respiration. Since the effect was still apparent after complete rotenone inhibition, creating cells where most of the respiration was dependent on complex II respiration, it is probable that the increased respiration comes from complex II activity.

Succinate prodrugs have previously been shown to increase respiration in patient samples and disease model with complex I dysfunction, both in genetic mitochondrial disease, such as Leigh's syndrome, and from toxicants affecting complex I. In conclusion, succinate prodrugs seem to be able to increase mitochondrial respiration in multiple models of complex I dysfunction.

In this study it was shown that LHON PBMCs rely more on complex II respiration than complex I respiration when compared to control PBMCs, indicating a small dysfunction in complex I, as evident by a low $\Delta N/\Delta S$ ratio. In other studies, a decrease in complex I coupled respiration has been observed in LHON cybrid fibroblasts in two of the pathogenic variants but not in m.11778G>A where instead an increase in respiration has been reported. Hence, it is difficult to conclude whether there is less complex I dysfunction in other cell types than RGCs in LHON patients or if RGCs have a lower capacity to compensate for the dysfunction[83, 84].

In this study, glutathione levels in blood from LHON patients were all within the reference interval. In the literature, it has been reported that mitochondrial diseases as a group have lowered GSH, increased GSSG[85], a decrease in total glutathione and a low ratio between GSH and GSSG in whole blood indicating increased levels of oxidative stress[4, 86]. For LHON specifically, however, a previous study also demonstrated normal levels of GSH and GSSG[87]. Possibly, the marginal

dysfunction in complex I is insufficient to affect the glutathione system to such a degree that it is visible in whole blood. It is also possible that a large increase in oxidative stress is only occurring during the acute vision loss or within the retina, also not having enough of a systemic effect to show in blood.

Idebenone does not seem to increase the oligomycin-sensitive respiration in the partially rotenone-inhibited LHON cell model[81]. To our knowledge there are no previous comparison between idebenone and succinate prodrugs in LHON patients or model[88]. Idebenone has been reported to partially compensate for complex I dysfunction by facilitating electron transfer[89], although reported effects on cellular respiration vary widely, with both increases and decreases described[78, 82].

Some possible improvements to this study have already been mentioned. For instance, the cohort size was limited, which is expected in a rare disease study. In addition, LHON shows incomplete penetrance and a marked male predominance, making balanced subgroup analyses difficult. A strength of this study is using primary patient cells rather than cultured cells, and an interesting future perspective would be direct measurements of ROS and ATP production in LHON patient blood cells and investigation of the effects of succinate prodrugs on these measures. Synergic effects of idebenone and succinate prodrugs could also be explored.

In conclusion, the mitochondrial respiration increases with succinate prodrug NV189 in partially rotenone-inhibited PBMCs and healthy control and LHON patient PBMCs. PBMCs from LHON patients display a limited complex I dysfunction, which does not affect the total respiration, and which was not seen in platelets. Glutathione levels in LHON patient blood are not altered.

Methodological requirements for reliable PBMC respirometry

This study addressed several methodological factors that may influence the reliability of PBMC respirometry. First, storage time and temperature affected respiratory measurements, supporting the use of clearly defined sample-processing procedures. Second, digitonin dose needed to be evaluated within the complete SUIT protocol, because both insufficient and excessive permeabilization altered respiratory measurements. Third, the study provided respiratory data and internal respiratory ratios from healthy individuals under the experimental conditions used[90]. These findings may improve the reproducibility of PBMC respirometry and facilitate its investigation in selected clinical and research contexts[91]. However, additional validation in larger and more diverse cohorts is required before reference intervals or diagnostic cut-offs can be established. Furthermore, blood-

cell respiration should not be interpreted as a general surrogate for mitochondrial function in other tissues.

PBMCs as an accessible sample into mitochondrial function

PBMCs and other peripheral blood cells are often considered a practical model for studying mitochondrial function in humans. However, peripheral blood cells should not be regarded as a metabolically uniform cell population. Chacko et al. showed that platelets, lymphocytes, monocytes and neutrophils display distinct bioenergetic profiles, with differences in respiration and in their responses to mitochondrial inhibition[92]. This suggests that blood-derived cells are active metabolic systems with cell-type-specific mitochondrial characteristics, rather than passive sampling material. Kramer et al. further proposed that platelets and leukocytes may serve as bioenergetic biomarkers of systemic metabolic and mitochondrial stress, while also emphasizing that different blood cell populations differ in their reliance on glycolysis and oxidative phosphorylation[48]. Therefore, PBMC-based mitochondrial assessment is valuable because of its accessibility and translational potential, but its results should be interpreted in relation to cell composition and study context, rather than being directly generalized to all tissues.

The value of PBMCs also relates to their immunological relevance. PBMCs include T cells, B cells, NK cells, monocytes, and a small proportion of dendritic cells, whose activation, proliferation, and effector functions are closely linked to their metabolic state. Resting and activated immune cells differ in their reliance on OXPHOS, glycolysis, fatty acid oxidation, and other substrate pathways. Therefore, PBMC mitochondrial respiration may reflect not only intrinsic mitochondrial function, but also changes in immune cell composition, activation status, and the systemic inflammatory or stress environment. This feature supports the potential use of PBMCs in the study of systemic diseases, drug responses, mitochondrial medicine, and clinical monitoring[93].

PBMCs may also be useful in the study of primary mitochondrial diseases. Because mtDNA- or nuclear gene-related mitochondrial disorders often have a systemic basis, peripheral blood cells can in some cases capture part of the mitochondrial phenotype. Previous studies have suggested that lymphocytes or PBMCs can be used to detect selected respiratory chain defects, particularly those involving complex I, complex IV, or ATP synthase, and that high-resolution respirometry provides a relatively rapid and minimally invasive functional assessment[94]. In the LHON study included in this thesis, PBMCs showed a mild reduction in the CI/CII respiratory ratio, whereas absolute respiration was not clearly decreased. This finding suggests that a complex I-related phenotype in LHON can be detected in

peripheral blood cells, although the effect appears modest and may be influenced by the metabolic plasticity and compensatory capacity of blood cells. In addition, NV189 enhanced respiration in both partially rotenone-inhibited PBMCs and LHON PBMCs, supporting the use of PBMCs as an accessible human cell model for the preliminary evaluation of CI-bypass strategies.

Evidence for the diagnostic use of blood-cell respirometry in primary mitochondrial disease remains limited. In a prospective study of 113 paediatric patients, Westerlund et al. found that a platelet respiratory ratio had high specificity but low sensitivity for detecting mitochondrial disease[95]. Combining several respiratory measures improved sensitivity but reduced specificity. Korandová et al. subsequently reported that cryopreserved PBMC respirometry identified disease-associated patterns in a proportion of patients with mitochondrial disease, although the small control group limited assessment of diagnostic accuracy[96]. Alonso et al. reported group-level differences in blood-cell respiration and mtDNA copy number between patients and controls, but substantial overlap between the groups may limit classification of individual patients[97]. Taken together, these studies suggest that blood-cell respirometry may provide supportive functional information, but it is not currently sufficient as a stand-alone diagnostic test for primary mitochondrial disease.

However, the accessibility of PBMCs does not mean that findings from PBMCs can be directly extrapolated to other tissues. PBMCs are a highly heterogeneous cell population, and inter-individual differences in the proportions of lymphocytes, monocytes, and NK cells, as well as blood collection, isolation, storage, cryopreservation and thawing procedures, and cellular activation status, can all influence the overall oxygen consumption signal. In other words, OCR measured by PBMC-HRR reflects mitochondrial functional information but is also affected by cell composition and pre-analytical conditions. Therefore, the interpretation of PBMC respiration requires careful consideration of sample handling, cell number, storage time, temperature, digitonin permeabilization conditions, and normalization strategies[98, 99].

More importantly, mitochondrial function is highly tissue specific. Cochlear cells, retinal ganglion cells, skeletal muscle, brain tissue, tumour cells, and immune cells differ substantially in their energy demands, substrate preferences, mitochondrial content, antioxidant capacity, and stress responses. Therefore, PBMCs should not be considered a universal surrogate for mitochondrial function in all tissues. Recent studies comparing mitochondrial respiration in blood cells and skeletal muscle also suggest that the correlation between PBMC or platelet respiration and skeletal muscle respiration is limited, and that blood cells may not accurately reflect mild changes in muscle mitochondrial function[100].

The findings of this thesis further support this view. In the antibiotic study, the same concentrations of antibiotics clearly affected mitochondrial respiration, ROS

production, ATP-related signals, and $\Delta\Psi_m$ in HEI-OC1 cells, whereas comparable changes were not observed in PBMCs. This indicates that PBMCs cannot simply represent the drug sensitivity of inner-ear-related cells, and suggests that antibiotic-induced mitochondrial stress is strongly cell-type specific. In contrast, in the LHON study, PBMCs showed a mild complex I-related phenotype and could be used to evaluate the respiration-enhancing effect of a succinate prodrug. The PBMC methodological study further demonstrated that, even within the same cell model, sample size, storage conditions, and permeabilization conditions can markedly influence HRR results. Taken together, PBMCs represent a valuable, accessible, and translationally relevant cell model, but their results must be interpreted with caution in relation to the specific disease context, cell composition, and experimental conditions.

From screening to mechanistic validation: Seahorse and Oroboros

During most of my PhD work, I mainly used the Oroboros O2k for high-resolution respirometry. More recently, I started preliminary work using the Seahorse XF platform to assess cellular metabolism in head and neck squamous cell carcinoma cell lines. These ongoing experiments are not presented as a separate completed study in this thesis, but they provided practical experience that helped me reflect on the different roles of the two platforms in experimental design.

Oroboros has clear advantages for detailed mechanistic analysis. It allows flexible protocols and is particularly useful when intact-cell respiration needs to be compared with permeabilized-cell respiration. With defined substrates, ADP and inhibitors, it is possible to examine CI-linked, CI+CII-linked OXPHOS capacity and ETS capacity in more detail. Therefore, Oroboros is well suited for follow-up experiments when a respiratory phenotype has already been identified and the next question is where the limitation may occur. Platform choice is also linked to cell preparation, because Seahorse is commonly used for attached cells, whereas Oroboros is often used for suspended cells or permeabilized preparations[101].

In contrast, Seahorse has an important advantage during the early optimization and screening phase. Because it uses multi-well plates, many conditions, concentrations and replicates can be tested in one experiment. This is especially useful for optimizing cell density, treatment time, drug concentration, FCCP titration and possibly initial permeabilization conditions. Seahorse-based assays are widely used for adherent cells and can provide parameters of mitochondrial respiration through the Cell Mito Stress Test[102]. In addition, Seahorse protocols can also be adapted for intact cells, permeabilized cells and isolated mitochondria, which makes it useful not only for screening but also for early optimization of assay conditions[103].

Compared with Oroboros, where each instrument has only two chambers, Seahorse can make this exploratory stage much more efficient.

If I were to repeat these experiments, I would use Seahorse earlier in the project to screen experimental conditions and identify the most informative treatment windows. Once clear differences are observed, I would then use Oroboros for more detailed validation, especially with permeabilized-cell protocols to determine whether specific respiratory pathway capacities are affected. In this way, Seahorse and Oroboros should not be viewed as competing methods, but as complementary platforms. Seahorse is useful for metabolic phenotype screening, whereas Oroboros provides higher-resolution mechanistic information. This is particularly relevant for tumour cell models, where mitochondrial respiration and glycolytic compensation may both contribute to the observed metabolic phenotype[104, 105].

At the same time, Seahorse data also require careful interpretation. The higher throughput does not remove the need for optimization, because OCR and ECAR values can be affected by cell number, cell attachment, FCCP concentration, edge effects, normalization strategy and statistical handling across wells and plates. For this reason, Seahorse screening may be strengthened by appropriate normalization and, when possible, by combining extracellular flux measurements with additional readouts such as cell number or mitochondrial content[106, 107].

This experience also changed the way I think about mitochondrial experiments. If I had learned Seahorse earlier during my PhD, I would probably have developed a more comparative and critical view of different respirometry platforms from the beginning. It also reinforces one of the main methodological messages of this thesis: mitochondrial data are shaped not only by the biological model, but also by the platform, assay design and experimental conditions used to generate them.

Conclusion

This thesis examined mitochondrial respiratory and redox responses in selected models of pharmacological, genetic and irradiation-related stress, together with methodological factors affecting PBMC respirometry.

First, several antibiotics impaired intact-cell respiration and altered ATP- and ROS-related measurements in HEI-OC1 cells, whereas comparable effects were not observed in PBMCs at the concentrations tested. Most respiratory pathway capacities were preserved after HEI-OC1 cells were permeabilized and supplied with defined substrates. This pattern is consistent with an effect on upstream metabolic processes or cellular bioenergetic regulation rather than major direct inhibition of respiratory complexes under substrate-saturating conditions. The different responses of HEI-OC1 cells and PBMCs also demonstrate that antibiotic-associated mitochondrial effects are cell-type dependent.

Second, PBMCs from patients with LHON showed a reduced complex I-to-complex II respiratory ratio, while total respiratory capacity was not reduced. This indicates a mild complex I-related phenotype in PBMCs rather than a severe systemic respiratory defect. NV189 increased respiration in partially rotenone-inhibited PBMCs and in PBMCs from patients with LHON, supporting further investigation of succinate-driven complex I bypass. However, these findings were obtained *ex vivo* and do not establish therapeutic efficacy in retinal ganglion cells or patients.

Third, CONV and UHDR irradiation affected mitochondrial oxygen consumption and other mitochondrial-related readouts in head and neck cancer cells and normal fibroblasts. However, no statistically significant differences between the two irradiation modalities were detected under the experimental conditions used. The transcriptomic analysis also showed broadly similar mitochondrial-related responses. These results do not support a distinct mitochondrial effect of UHDR irradiation in the cell models examined and indicate that tissue-level or microenvironmental factors may need to be considered when studying the FLASH effect.

Fourth, this thesis evaluated methodological factors affecting high-resolution respirometry in human PBMCs, including chamber cell concentration, storage time and temperature, and digitonin permeabilization. The study also provided respiratory data and internal respiratory ratios from healthy individuals under the experimental conditions used. These findings may improve the reproducibility of

PBMC respirometry, but the results remain specific to blood cells and should be interpreted in relation to cell composition, sample handling and the clinical or experimental context.

Overall, the findings of this thesis emphasize that mitochondrial respiratory measurements are strongly influenced by cell type, biological context and experimental design. Combining intact- and permeabilized-cell respirometry can improve mechanistic interpretation, while PBMCs provide an accessible but tissue-limited model for human mitochondrial research.

Perspectives on future development

The studies included in this thesis also identified several questions that could be addressed in future work. The following suggestions focus on limitations that emerged directly from the experiments and on experimental models that may help clarify the present findings.

Accurate prediction and protective strategies for antibiotic mitochondrial toxicity

The findings of this study suggest that antibiotic-associated mitochondrial effects are cell-type dependent and that the reduction in intact-cell respiration may involve processes upstream of the respiratory-chain complexes. This discovery opens new directions for precision medicine and toxicity protection in the future. Firstly, future studies could evaluate whether screening for established MT-RNR1 variants before aminoglycoside treatment can help identify individuals at increased risk of ototoxicity. In particular, the variants m.1555A>G, m.1494C>T, and m.1095T>C are associated with an increased risk of aminoglycoside-induced hearing loss[108]. Broader mtDNA haplotypes may influence the clinical expression of m.1555A>G in some families, but current evidence is not sufficient to use haplotypes alone for clinical risk prediction[109]. Because intact-cell respiration was reduced while most respiratory pathway capacities were preserved after permeabilization, future studies could investigate which upstream metabolic processes are affected. Measurements of substrate uptake, glycolytic activity, pyruvate oxidation and TCA-cycle metabolites could help distinguish between impaired substrate availability and altered cellular energy demand. If a specific metabolic limitation is identified, targeted rescue experiments could then be designed. These experiments should first be performed in inner-ear-related cell models and subsequently validated in more physiologically relevant systems, such as cochlear organoids or animal models.

Further investigation of mitochondrial responses to FLASH irradiation

The absence of statistically significant differences between CONV and UHDR irradiation in the present cell models suggests that future studies should examine factors that were not fully represented in this study. Measurements performed during or immediately after irradiation may help capture rapid changes in oxygen availability and ROS production[110]. Systematic variation of oxygen concentration, dose rate and pulse structure could clarify whether these parameters influence early mitochondrial or redox responses. More complex models, including three-dimensional cultures, endothelial or immune-cell co-cultures and animal models, may also help determine whether interactions between cells and the tissue microenvironment contribute to the FLASH effect[111]. These studies should relate early biochemical changes to biologically relevant outcomes such as DNA damage, cell survival and normal-tissue injury.

Gene therapy and metabolic intervention for LHON and other mitochondrial genetic diseases

In this study, the succinate prodrug NV189 increased mitochondrial respiration in PBMCs from patients with LHON, providing preliminary support for further investigation of complex I bypass strategies. However, substantial preclinical work is still needed before this approach can be considered for clinical application. Before considering clinical studies, succinate prodrugs should be evaluated in more disease-relevant preclinical models. In vitro, NV189 should be tested in LHON patient-derived retinal ganglion cells or retinal organoids to determine whether it improves not only respiration, but also ATP-related signals, ROS levels, axonal and cell survival[112]. Dose-response and longer exposure experiments would also be needed to assess potential toxicity and to compare NV189 directly with idebenone[113]. In animal models of mitochondrial optic neuropathy, further studies should examine whether succinate prodrugs reach the retina and optic nerve, preserve retinal ganglion cells and axons, and improve visual function. Such studies would provide stronger evidence that succinate-based complex I bypass is a relevant therapeutic strategy for LHON. Meanwhile, gene-editing approaches may provide a longer-term option for LHON and other mtDNA diseases. However, conventional CRISPR/Cas9 is not yet established for reliable mtDNA editing because delivery of guide RNA into mitochondria remains difficult[114]. TALE-based nucleases and CRISPR-free base editors can alter selected mtDNA variants or shift heteroplasmy in experimental systems[115], but their therapeutic use is still limited by target-site compatibility, editing efficiency, unintended edits, and safe delivery to retinal

ganglion cells in vivo[116]. Future studies should therefore first evaluate these approaches in patient-derived retinal ganglion cell models and animal models, with careful assessment of editing specificity, heteroplasmy shift, retinal delivery, and long-term safety.

Further evaluation of blood-cell respirometry in primary mitochondrial disease

Blood-cell respirometry requires further evaluation before it can be considered for routine use in the diagnostic work-up of primary mitochondrial disease. Existing studies indicate that respiratory abnormalities can be detected in platelets or PBMCs in some patients, but diagnostic performance remains uncertain[95-97]. Future prospective studies should include sufficiently large and well-characterized control groups, predefined diagnostic thresholds and independent validation cohorts. Sensitivity, specificity, predictive values and reproducibility should be assessed for individual respiratory measures and combinations of measures. Because primary mitochondrial diseases are rare, high specificity would be required to limit false-positive results, while adequate sensitivity would be necessary if the test were intended to help exclude disease. Clinical utility should also be evaluated by determining whether the result changes diagnostic decisions or patient management. At present, blood-cell respirometry should be regarded as a potentially supportive functional assessment rather than a stand-alone diagnostic test. Its value may ultimately lie in combination with clinical evaluation, genetic testing and other biochemical or functional investigations.

General methodological considerations

Across these studies, an important priority is to match the experimental model and measurement strategy to the biological question. Tissue-relevant models are needed when studying inner-ear toxicity or LHON, while blood cells remain useful for accessible human studies provided that their limitations are recognized. Likewise, combining screening methods with detailed respirometry may help distinguish an observed metabolic phenotype from a specific respiratory pathway defect. These considerations provide a practical basis for the next experimental steps arising from this thesis.

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About the author

Tianshi Liu completed his master's degree in otorhinolaryngology at Fudan University Shanghai Medical College, where he also received clinical training and obtained his medical licence. After gaining extensive experience in clinical practice, he moved to Sweden in 2022 to pursue doctoral studies and conduct mitochondrial research at Lund University. His doctoral work focuses on mitochondrial function.

