Mitochondria in chemical-induced toxicity

Stel, W. van der

Assessing mitochondria-related organ toxicity

using new approach methods in an adverse outcome pathway framework

Wanda van der Stel, Peter Bouwman, Bob van der Water, Erik Danen

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Over the last decades, mitochondrial-malfunctioning has been acknowledged as an important factor in the occurrence of chemical-induced organ toxicity. This implies that data concerning the relationship between mitochondrial perturbation and the development of organ failure need to be integrated in chemical risk assessment. The outcome of mitochondria related-toxicity depends on the severity of events and the concentration-time relationship between events. This can be captured using Adverse Outcome Pathways (AOPs) that bundle knowledge of (perturbed) biological processes in consecutive events, from chemical interaction to eventual adversity in organs. To allow the use of the AOP approach for chemical safety guidelines, it is important to select biologically relevant events and to move from qualitative descriptions to quantitative assessment. These events have to be properly specified and the AOP needs to be populate them with sufficient and robust quantitative data. In this review, we propose a core of mitochondrial key events that represent the major targets in mitochondrial toxicity and can be used to create AOP events. Furthermore, we review a set of methods for the evaluation of these mitochondrial key events, including the possibilities for integrating omics technologies. This set of methods is focused on *in vitro* assays to reduce the number of animal tests. Proper estimation of mitochondrial perturbation in the context of organ toxicity will support early-stage risk assessment of new and existing chemicals.

Keywords: Mitochondria, quantitative adverse outcome pathway, risk assessment, methods, omics

Introduction

In the last decades, mitochondrial involvement in the occurrence of chemical-induced organ toxicity has become increasingly clear. [Will 2014, Dykens 2007, Dykens 2007]. Mitochondria are multi-functional organelles, involved in supplying the cells with energy, creating building blocks like proteins and sugars and providing a storage compartment for ions. Perturbation of any of these mitochondrial functions can lead to cellular stress, cell death and eventually organ failure. To reduce the incidence of mitochondria-related organ failure upon use of pharmaceuticals and exposure to chemicals in general, it is important to improve early identification of mitochondrial toxicants.

Assessment of chemical-induced mitochondrial malfunctioning is nowadays included in the early phase of drug development but is less addressed during the design and production of other types of chemicals. To support and improve the integration of mitochondrial toxicity assessment in risk and hazard assessment of chemicals compounds, it is important to verify and create guidance concerning quality and usability of the mitochondrial toxicity data. One way to structure and link toxicity data is the use of adverse outcome pathways (AOPs) [Ankley 2010, Vinken 2013, Leist 2017]. AOPs describe, using a series of key events, the link between the occurrence of adverse effects and the initial chemical interaction with the cell. This structured representation of toxicological processes provides the opportunity to couple existing mitochondrial toxicity measurements to relevant biological events. The simplified presentation of biological processes provides a basis to clearly describe essential events in the process of cellular perturbation, organ failure and eventual human health issues.

In the past, AOPs have been used as a qualitative description of various key events leading to different types of organ toxicity based on existing data[3]. However, to allow implementation of the AOP framework into risk assessment for chemical-induced toxicity, it is essential to define and quantify the different events or event relationships. To be able to quantify the events, methods need to be selected that allow time and concentration dependent assessment of the changes within the biological processes belonging to the events. The use of quantitative AOPs (qAOPs) in toxicity assessments is promoted by the international Organization for Economic Co-operation and Development (OECD). The OECD already approved two qAOPs in the past and promotes the creation of new qAOPs by providing guidelines and various knowledge tools[4] [Foran 2019, Battistoni 2019].

In response to the OECD initiative, researchers have started to develop quantitative AOPs to assess an increasing number of human organ toxicities [Angrish 2017]. In particular mitochondrial toxicity assessment could benefit from the application of quantitative AOPs. Mitochondrial toxicity is characterized by series of thresholds which

need to be overcome before tissue injury occurs. Under normal conditions, these thresholds should protect the cell/organ from instant injury related to fluctuations in mitochondrial functioning. Injury originating from malfunctioning of mitochondrial processes occurs generally when most of the mitochondria of a cell are perturbed. One exception on this rule is mitochondrial stress which results in the release of mitochondrial DNA and with that causes an innate immune response [West 2015]. Cells can recover from a few malfunctioning mitochondria via the production of new mitochondria (biogenesis), exchange of functional proteins and/or genomic material between organelles (fission and fusion) and by discarding non-functional organelles (mitophagy). In this way, cells reduce the number of damaged mitochondria, while relying on the available pool of healthy mitochondria. However, when the number of damaged mitochondria is too large or the processes required to "cure" the mitochondrial pool are disturbed, cell death may be induced to remove defective cells from the tissue. Acute high levels or sustained presence of mitochondrial toxicants can eventually lead to organ toxicity.

This review will discuss the experimental assessment of mitochondrial toxicity and integrate this information in qAOPs for chemical risk assessment. The central part of this review will focus on the biologically relevant processes in mitochondrial toxicity and the range of assays available to measure their activity. Finally, we will briefly consider the use of mitochondrial toxicity assays in AOPs in a regulatory context.

Quantifiable mitochondrial key events

The framework of AOPs provides a powerful tool to link molecular events to cellular perturbations and consequent organ malfunctioning. In order to link single events to interconnected networks of AOPs, it is crucial that the key event descriptions are specific for clearly defined biological processes. AOPs created using the OECD format are stored in de AOP-wiki database (https://aopwiki.org/), which thus far contains 303 AOPs and 1368 key event entries. 17 of these 1368 key events are related to mitochondrial functioning and can be grouped into mitochondrial damage/dysfunction (6 out of 17), ATP/electron transport/OXPHOS impairment (4 out of 17), metabolism related (3 out of 17) and mitochondrial DNA related issues (4 out of 17). As discussed by Dreier *et al* the mitochondria-related key events are redundant, and not properly specified.

In general, key events are divided into: 1) initiation events (molecular initiation event = first key event in AOP), 2) the adverse outcome (final key event in AOP), and 3) the remaining "in between" events (not first or last event). Mitochondrial key events belong to the group of early events, including the initiating and early key events. Most mitochondrial processes are interconnected, and consequently perturbation

of one process will eventually result in malfunctioning of others. This means that the initiating event for one chemical could be an in between event for other chemicals.

The integration of key events into qAOPs requires the selection of specific and reproducible assays. The assay selection depends on multiple factors: 1) Assay type: To reduce the need for animal experiments in early-stage risk assessment, it is preferred to select new approach methodologies (NAMs), including both *in vitro* and *in silico* approaches. 2) Capacity: Assay capacity can be an issue when there is a need for large scale high throughput perturbation assessments. 3) Complexity: Methods with complex production, optimization and safety steps are only suitable for specialized labs and are therefore less likely to be integrated in regulatory approved mitochondrial toxicity assessments.

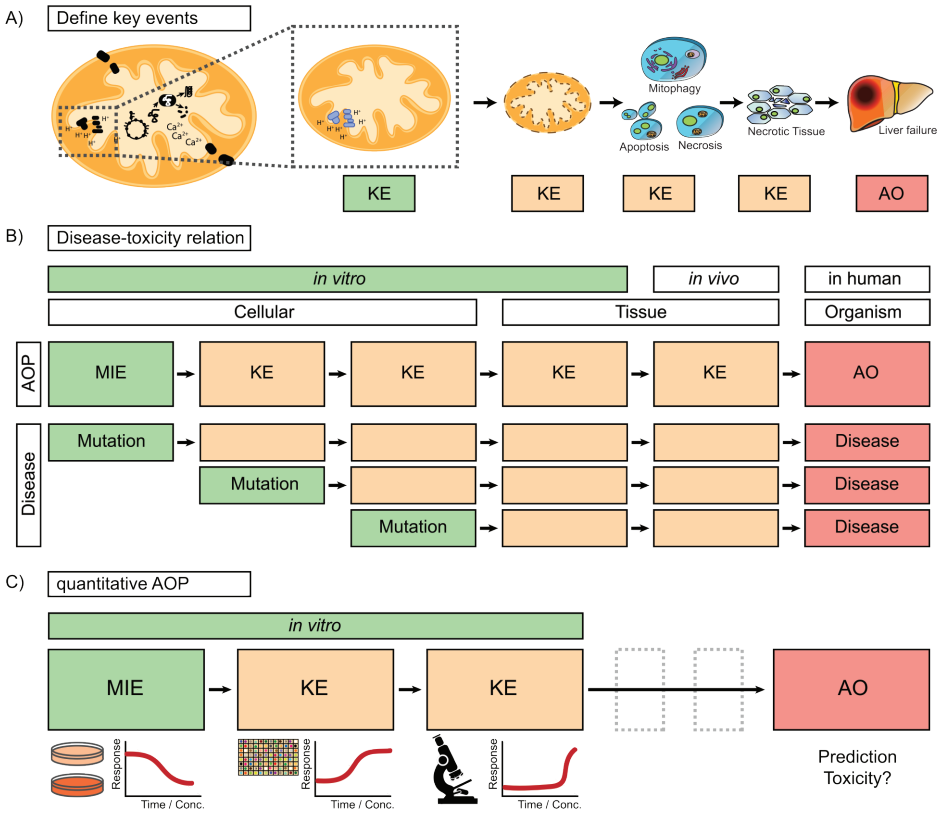


Figure 1: AOP approach to define mitochondrial key events
A) Illustration of the mitochondrial key event in an adverse outcome pathway (AOP) describing the relationship between mitochondrial perturbation and the occurrence of organ toxicity (in this case liver failure). **B)** Schematic representation of the key features in an AOP and the relationship to the occurrence of disease upon mutation in corresponding mitochondrial processes. **C)** Schematic representation of the steps in an AOP that can be quantified using *in vitro* methods to support risk assessment (prediction of toxicity).

In this review, we aim to subdivide the mitochondrial key events into six subgroups that can be used to support an AOP describing organ toxicity resulting from mitochondrial perturbation (figure 1A and figure 2): mitochondrial metabolism, oxidative phosphorylation (OXPHOS), mitochondrial dynamics, mitochondrial UPR, mitochondrial calcium homeostasis and mitochondrial degradation. To determine the relationship between mitochondrial process perturbation and adversity, we use available information from mitochondria-associated human diseases. It is well known that mutations of genes encoding mitochondrial proteins result in disease at various stages of life. This relationship between protein perturbation and disease is also likely to occur in case of chemical-induced perturbation of the same proteins (figure 1B). To enable integration of the defined key events into qAOPs and chemical risk assessment, we also address various established *in vitro* methodologies which can be linked to the defined events (figure 1C).

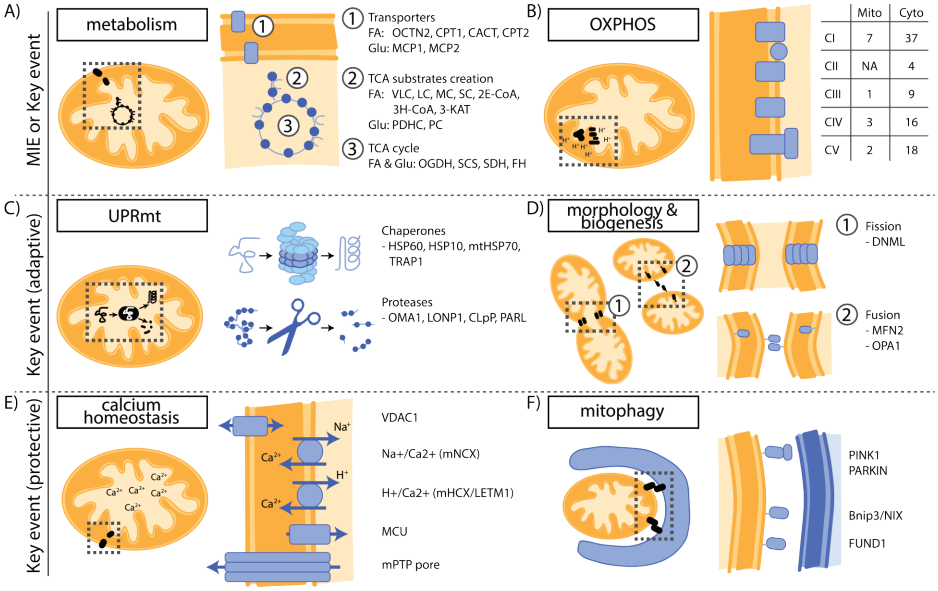


Figure 2: Mitochondrial key events

Illustrations of the six major mitochondrial key events: 1) metabolism, 2) OXPHOS, 3) UPRmt, 4) fission/fusion/biogenesis, 5) calcium homeostasis and 6) mitophagy. The individual illustrations per key event demonstrate perturbed components, which are frequently observed in diseases. The targets include for: A) *Mitochondrial metabolism*: Transporters and enzymes involved in TC substrate creation and the TCA cycle itself. B) *OXPHOS*: The different sub-proteins forming the proteins complexes I to V (CI-CV) which are coded by the mitochondrial or cellular genome. C) *UPRmt*: Chaperones and proteases. D) *Mitochondrial dynamics*: Proteins involved in the processes of fission and fusion. E) *Calcium homeostasis*: Various ion pumps and pores. F) *Mitophagy*: Proteins of 3 major pathways. The key events are structured based on the proposed type of key events and the location in the AOP (initiating event or subsequent key event). OXPHOS = oxidative phosphorylation, UPRmt = mitochondrial unfolded protein response.

Mitochondrial metabolism – citric acid cycle (TCA cycle)

Description

Cells require energy in the form of ATP, which is obtained from energy rich substrates including carbohydrates (e.g. glucose) and fatty acids [Smith 2017, Zhou 2018]. Acetyl-CoA, the starting material driving the TCA cycle in the matrix of the mitochondria, is generated, depending on available glucose levels, from pyruvate (itself derived from glucose via the glycolytic pathway) or from fatty acids (via beta-oxidation). The TCA cycle itself is a series of enzymatic reactions that result in the creation of NADH and $FADH_2$. Subsequently, under aerobic conditions NADH and $FADH_2$ are utilized to produce ATP in the oxidative phosphorylation. Notably, in low oxygen conditions cells will rely on the limited amount of ATP produced during the conversion of glucose to pyruvate in the glycolytic pathway. Cancer cells opt for this mechanism even when oxygen is not limited, referred to as the Warburg effect [DeBerardinis 2020].

Disease

Malfunctioning of the TCA cycle may ultimately result in mitochondrial encephalomyopathy. Mitochondrial encephalomyopathy manifest itself already in very young children and affects predominantly two tissue types both relying heavily on mitochondrial respiration, namely brain and muscle [DiMauro 1999, Merrit 2018]. Perturbations observed at the molecular level in these tissues are subdivided in three groups based on the perturbed biological process: substrate transport, preparation of substrates for the TCA cycle and the TCA cycle itself.

Transporters: Disorders or deficiencies caused by malfunctioning transport are in general autosomal recessive and the result of mutations in genes coding for the major transporter proteins of fatty acids (OCTN2, CPT1, CACT and CPT2) [Longo 2006] and pyruvate (MCP1 and MCP2[5,6,7] [Oonthonpan 2019]. Contrarily, increased expression of the fatty acid transporters (CPT/OCTN2), resulting in fatty acid accumulation, increases the risks for developing/ worsening other diseases, including infarcts and leukemia. Both diseases benefit from treatment with fatty acid transporter inhibitors, like ST1325 and R-(+)-Etomoxir [Liepinsh 2014, Abozguia 2006, Gugiatti 2018]. Decreased expression of the pyruvate transporters exacerbates cancer development by stimulating cell proliferation via a metabolic switch from mitochondrial respiration to glycolysis [Zhong 2015, Li 2017].

TCA substrate creation: Disturbance of the synthesis of acetyl-CoA, the substrate for the TCA cycle, causes a subset of metabolic deficiencies. Both pyruvate and fatty acid can be converted to acetyl-CoA. Pyruvate conversion occurs in the pyruvate dehydrogenase (PDH) complex or by pyruvate carboxylase (PC). Ineffective pyruvate conversion results in reliance on glycolysis for energy and excessive accumulation

of lactate (lactic acidosis)[8]. In fact, disruption of mitochondrial metabolism caused by inhibition of the PDH complex is a promising therapeutic strategy for patients suffering from bacterial infections [Zhou 2018]. Interestingly, PDH inhibition also demonstrated to be beneficial for pancreatic cancer patients illustrating the complex process of metabolic rewiring in cancer cells [Zacher 2011, Qin 2020, clinical trials: NCT03374852 and NCT01830322]. Fatty acid conversion into acetyl-CoA occurs in a stepwise process, removing 2 carbons in every cycle. This process is termed beta-oxidation. This process requires very long-, long-, medium- and short-chain acyl-CoA dehydrogenases (respectively VLC, LC, MC, SC). Other enzymes involved are the peroxisomal bifunctional protein 2-enoyl-CoA hydratase (2E-CoA)/ 3-hydroxyacyl-CoA (3H-CoA) dehydrogenase and 3-ketoacyl-CoA thiolase (3-KAT). [diMauro 1999, Merritt 2018]. Malfunctioning of any of these enzymes results in a combination of hypoglycemia, liver dysfunction, cardiomyopathy, skeletal myopathy and rhabdomyolysis. Conversely, stimulation of the switch from fatty acid to pyruvate metabolism by inhibiting the beta oxidation enzyme 3-KAT is used for the treatment of heart diseases [Sabbaha 2002].

TCA cycle: The TCA cycle consists of an interplay between nine enzymes. Metabolic disorders are mainly caused by recessive mutations in genes coding for α-Ketoglutarate Dehydrogenase (OGDH; conversion α-ketoglutarate to succinyl-CoA), succinyl-CoA synthetase (SCS; conversion of succinyl-CoA to succinate), succinate dehydrogenase (SDH; conversion succinate to fumarate) and fumarase (FH; conversion of fumarate to malate) [Rustin 1997]. Mutations in these genes manifest in central nervous system and muscles disorders, primarily in children up to 7 years. As described for the other enzymes involved in mitochondrial metabolism, TCA cycle enzymes are viewed as promising targets for cancer therapy. Various cancer types demonstrate mutations and/or overexpression of α-ketoglutarate dehydrogenase complex (KGDHC; OGDH is part of this complex) and/or isocitrate dehydrogenase (IDH2; conversion isocitrate to α-ketoglutarate) changing the TCA cycle usage. Inhibitors for both enzymes are currently being evaluated in phase II/III clinical trials (KGDHC inhibitor; CPI-613 and IDH2 inhibitors; AG221 and AG881) as treatment for multiple cancer types[9] [Anderson 2018].

Chemical toxicity

Chemical-induced perturbation of mitochondrial metabolism is observed upon exposure to a variety of chemicals and affects both fatty acid transport and beta-oxidation [Fromenty 2019]. For most mitochondrial toxicants, the molecular initiating event and the key events are unresolved. These include commonly used drugs such as acetaminophen, amiodarone and ibuprofen, which affect multiple steps in the mitochondrial metabolic pathways. However, the result of chemical-induced

mitochondrial toxicity is most often macrovascular and/or microvascular liver steatosis (development of an inflamed fatty liver).

Glucose metabolism and especially pyruvate conversion by the PDH complex is reported to be affected by psychoactive drugs [Sacks 1991]. Perturbation of the TCA cycle, more specifically inhibition of aconitase, by the chemotherapeutic agent fluorouracil, has been reported to result in neurotoxicity [Kwon 2010, Ki 2002, Pirzada 2000].

Methods

Assessment of mitochondrial metabolism is mostly focused on the activity of the enzymes involved and less on the substrate transport (table 1). Although transport protein deficiency can be assessed in patient materials using immunohistochemistry, this is not sufficient for the detection of chemical-induced protein malfunction. The assessment of TCA enzyme activity is achieved using radiolabeled substrates, or the spectral changes observed upon increased or decreased levels of NADH/COA. Radiolabeling of the TCA cycle is achieved by the addition of [$^{13/14}$C]-pyruvate or [^{14}C]-α-ketoglutarate followed by the detection of produced radio-labeled CO_2 or the other products of the TCA cycle. The use of radiolabeling is generally not desired and spectrometry measurements present an alternative. They rely on the differences in absorbance spectra between native and phosphorylated forms of substrates (NADHP/NADH) produced during metabolism or indirectly by addition of enzymes to stimulate the formation of NADHP from the available NADH. However, these spectrometry measurements demonstrate a lower signal-to-noise ratio and are more difficult to interpret than TCA cycle radiolabeling. Furthermore, the creation of NADH and some of the other metabolic intermediates is not limited to the mitochondria. Nonnenmacher *et al* optimized a method which only permeabilizes the outer cell membranes. The partially functional cells have easily accessible mitochondria and can be used for mitochondrial-compartment specific metabolic studies [Nonnenmacher 2017]. Finally, the specific process of beta-oxidation can be tracked based on the degradation of administered fatty acids. This can be achieved by using radiolabeled palmitic acid and assessing the introduction of [^{14}C] or [^{3}H] into downstream products. Another option is the assessment of fluorescence signal appearing upon oxidation of synthetic beta-oxidation substrates or the accumulation of fatty acids stained with fluorescent dyes into the various compartments of the cell.

Integration into qAOPs

In conclusion, perturbation of mitochondrial metabolism is observed in both disease and toxicity. The observed chemical-induced mutations in genes coding for metabolic components indicates that perturbation of mitochondrial metabolism is most likely an early key event or even molecular initiating event for both disease and toxicity.

Table 1: Mitochondrial key events - part 1

Table depicting mitochondrial key events and a subdivision of biological processes belonging to these specific key event. Per biological processes the table elaborates on: 1) methods available to assess related biological processes, 2) examples of the applied techniques, 3) specification of the technique mentioned in in the example, 4-11) columns assessing in detail the assay including the (dis)advantages that need to be considered when using these techniques in a AOP context. * Cells: Yes = Cellular assay/ No: Assay performed in tissue or with isolated enzymes; ** Life: Yes = Cellular assay without the need of lysation or permeabilsation of membranes; # Isol. Mito.: Yes = Assay can be performed in isolated mitochondria; $ Mod.: Yes = assay requires modification/transfection of used cell types; & Mito-target: Yes = Assay (for example tags) can be targeted specifically to the mitochondrial compartment. (Source numbers are provided at the end of the chapter).

KE	Process	Method		Examples	Specification	Cells *	Live **	Isol. Mito. #	Label-free	Mod. $	Mito-target &
Metabolism	TCA	Radio-labeling		PDHc, CS, KDHc, FH	Integration of radio-labeled atoms into TCA substeps	Yes	No	N / Y	No	No	No
		Optical spectrometry		PDHc, CS, aconitase, IDH, KDHc, SCS-A, SDH, FH, MDH	Color spectrum changes caused by substrate conversion	N / Y	No	No	Yes	No	No
	Fatty acid oxidation	Radio-labeling		[14C]- or [3H]-palmitic acid	Integration of radio-labeled atoms into fatty acids	Yes	No	No	No	No	No
		Fluorescent labels		Fluorescent fatty acid (FA)	Fluorescence upon metabolism of FP by FAO	Yes	Yes	No	No	No	No
				Nile red, lipidTox, NBD	Accumulation of fatty acids in cellular compartments	Yes	Yes	No	No	No	No
OXPHOS	Oxygen consumption or localisation	Elektrodes		Clark, microelektrodes	Oxygen concentration in liquid using a catalytic platinum surface	Yes	Yes	No	Yes	No	No
		Oxygen sensors	Soluble (natural)	Myoglobulin, PPIX, NADH	Fluorescent spectrum change upon available oxygen level	Yes	Yes	No	Yes	No	No
			Soluble (natural/ plasmid)	Myo-mCherry	Myoglobin subunit introduced in FRET sensor	Yes	Yes	No	No	Yes	Yes
			Soluble (synthetic)	Various probes	Fluorescent spectrum change upon available oxygen level	Yes	Yes	No	No	No	No
			Static (synthetic)	Seahorse Analyzer	Plates containing oxygen sensitive FPs	Yes	Yes	No	Yes	No	No
				Seahorse Analyzer (complex specific)	Seahorse plus membrane permeabilisation protocol	No	No	Yes	Yes	No	No
		Electron paramagnetic resonance (EPR)/ Magnetic resonance imaging (MRI)		Oxygen presence	EPR = free radicals using electron spin, MRI/NMR = presence of oxygen based on nuclear spin	No	No	No	No	No	No
		ImmunoFluorescent (IF) staining		Pimonidazole	Low O2 levels results in detactable reduced-pimonidazole	No	No	No	No	No	No
		Near infrared spectrometry (NIRS)		Hemoglobulin	Oxygen saturation of hemoglobulin in microvasculature	No	No	No	No	No	No
	Membrane potential	Potential sensitive dyes		TMRE, JC1, mitoNr, MitoOrange, Rho123	Potential dependent dyes accumulate in polarised mitochondria	Yes	Yes	No	No	No	Yes

Table 1: Mitochondrial key events - part 1 (continued)

KE	Process	Method		Examples	Advantage	Disadvantage	Source
Metabolism	TCA	Radio-labeling		PDHc, CS, KDHc, FH	substrate specific, very acquirate	measurement in supernatant, radio-labeling	1,2,3
		Optical spectrometry		PDHc, CS, aconitase, IDH, KDHc, SCS-A, SDH, FH, MDH	natural occuring	measurement in supernatant, isolation of enzymes for specificty	1, 4-8
	Fatty acid oxidation	Radio-labeling		[14C]- or [3H]-palmitic acid	substrate specific, very acquirate	radio-labeling, supernatant	9, 10
		Fluorescent labels		Fluorescent fatty acid (FA)	dynamic tracking, high through put options, complex cell systems, single cell		11
				Nile red, lipidTox, NBD	dynamic tracking, high through put options, complex cell systems, single cell	requires system with enough FA	12, 13
OXPHOS	Oxygen consumption or localisation	Elektrodes		Clark, microelektrodes	non-invasive	not fit for high throuhput	14-16
		Oxygen sensors	Soluble (natural)	Myoglobulin, PPIX, NADH	natural occuring	Noise-ratio high, because natural occuring	17-19
			Soluble (natural/plasmid)	Myo-mCherry	mcherry is stable and not toxic, FRET is very sensitive, complex cell systems, single cell	low signal-to-noise ratio, sensitive to environmental factors	20
			Soluble (synthetic)	Various probes	cheap, also plate reader assay, high throughput, single cell		21-27
			Static (synthetic)	Seahorse Analyzer	OCR and ECAR measurement; kinetic measurement, stress test, semi high through put, complex cell systems	specialist equipment	28-32
				Seahorse Analyzer (complex specific)	identification of complex specific inhibition	specialist equipment, not possible in complex cell systems	33
		Electron paramagnetic resonance (EPR)/ Magnetic resonance imaging (MRI)		Oxygen presence		only tissue, not suitable for studies assessing MIE/ early KE in animal free studies	34-41
		ImmunoFluorescent (IF) staining		Pimonidazole		only tissue, not suitable for studies assessing MIE/ early KE in animal free studies	42, 43
		Near infrared spectrometry (NIRS)		Hemoglobulin		only tissue, not suitable for studies assessing MIE/ early KE in animal free studies	44, 45
	Membrane potential	Potential sensitive dyes		TMRE, JC1, mitoNr, MitoOrange, Rho123	dynamic tracking, high through put	nuclear staining required fo cells tracking after complete depolarisation; active removal of dye; dye quenching	46-48

Table 1: Mitochondrial key events - part 2

Table depicting mitochondrial key events and a subdivision of biological processes belonging to these specific key event. Per biological processes the table elaborates on: 1) methods available to assess related biological processes, 2) examples of the applied techniques, 3) specification of the technique mentioned in in the example, 4-11) columns assessing in detail the assay including the (dis)advantages that need to be considered when using these techniques in a AOP context. * Cells: Yes = Cellular assay/ No: Assay performed in tissue or with isolated enzymes; ** Life: Yes = Cellular assay without the need of lysation or permeabilsation of membranes; # Isol. Mito.: Yes = Assay can be performed in isolated mitochondria; $ Mod.: Yes = assay requires modification/transfection of used cell types; & Mito-target: Yes = Assay (for example tags) can be targeted specifically to the mitochondrial compartment. (Source numbers are provided at the end of the chapter).

KE	Process	Method		Examples	Specification	Cells *	Live **	Isol. Mito. #	Label-free	Mod. $	Mito-target &
OXPHOS	ATP levels	Magnetization-transfer (MT) techniques		31P	Nuclear spin of P atoms	No	No	No	No	No	No
		HPLC		ADP/ATP ratio	Concentration of ATP or ADP effect the peak height of HPLC spectrum	Yes	No	No	No	No	No
		Firefly luciferase based-assays	Solutions	ATPlite (luciferase based)	ATP to AMP conversion by luciferase with light as by-product	Yes	No	Yes	No	No	No
			Plasmids	mtLuc, cytLuc, pmeLuc, nucLuc	Targeted luciferase plasmids (same principle as ATPlite)	Yes	Yes	No	No	Yes	Yes
			Biosensors	Carbon nanotube (SWNT)	nanotube-luciferase conjugate, produced oxyluciferin effects NIR fluorescence of tube	Yes	Yes	No	No	No	No
		Biosensors (ATP binding subunits)	Plasmids	FRET: Ateam, and many others (FoF1-ATP synthase e subunit)	ATP selective subunit used in FRET construct (2 FPs)	Yes	Yes	No	No	Yes	Yes
				QUEEN: 1FP (FoF1-ATP synthase e subunit)	ATP selective subunit used in FRET construct (1 FP)	Yes	Yes	No	No	Yes	Yes
				Perceval (GlnK1 subunit)	GLnk1 conformation change upon ATP binding results in confromation change of FP	Yes	Yes	No	No	Yes	Yes
			Probe	Aptamer	Oligonucleotide-ATP interaction results in conformation changes and with that fluorephore changes	Yes	Yes	No	No	No	No
		Staining with small molecules	Spectrum shift	1·2Zn (PPi specific molecule)	Change in fluorescence emission upon addition of PPi	Yes	Yes	No	No	No	No
			Signal upon interaction	ATP-red1, or based on eurpium, imidazolium	Fluorephore excitate light upon binding ATP (otherwise colorless)	Yes	Yes	No	No	No	No
	Complex activity	Optical Spectrometry		Complex I, II, III, IV or V activity	Color spectrum changes caused by substrate conversion	No	No	Yes	Yes	No	No
mtUPR	**Chaparone activity**	Western blot or fluorescent labeled proteins under natural promotor		Increase HSP60,HSP10, ClpP, ATF5 & no effect at BIP, HSP73, HSP72	Presence/expression of mitoUPR specific proteins	Yes	No	No	No	No	No
		Luciferase activity coupled to mtUPR TF		Coupled to CHOP, Mure 1, Mure 2	Luciferase activity upon mitoUPR specific TF activation	Yes	Yes	No	No	Yes	No

Table 1: Mitochondrial key events - part 2 (continued)

KE	Process	Method		Examples	Advantage	Disadvantage	Source
OXPHOS	ATP levels	Magnetization-transfer (MT) techniques		31P		only tissue	49-51
		HPLC		ADP/ATP ratio	cell system independent	Samples collection required (tissue, cells)	52, 53
		Firefly luciferase based-assays	Solutions	ATPlite (luciferase based)	plate reader assay, high throughput	measurement in lysate; effected by number of cells	54, 55
			Plasmids	mtLuc, cytLuc, pmeLuc, nucLuc	subcellular localisation, high throughput, single cell		56-58
			Biosensors	Carbon nanotube (SWNT)	high signal-to-noise ratio	not compartment specific	59
		Biosensors (ATP binding subunits)	Plasmids	FRET: Ateam, and many others (FoF1-ATP synthase e subunit)	can be compartment specific, single cell, high throuhput	low signal-to-noise ratio, sensitive to environmental factors	60, 61
				QUEEN: 1FP (FoF1-ATP synthase e subunit)	no effect of growth, can be compartment specific, single cell, high through put, higher signal-to-noise ratio (than 2FPs))		62
				Perceval (GlnK1 subunit)	ATP:ADP ratio, can be compartment specific, single cell, high throughput	sensitive to environmental factors	63
			Probe	Aptamer	single cell, high throughput	not compartment specific	63
		Staining with small molecules	Spectrum shift	1·2Zn (PPi specific molecule)	water soluble; pH insensitive, single cell, high throughput	not compartment specific	65
			Signal upon interaction	ATP-red1, or based on eurpium, imidazolium	single cell, high throughput	not compartment specific	66-68
	Complex activity	Optical Spectrometry		Complex I, II, III, IV or V activity	natural occuring	isolated mitochondria	69, 70
mtUPR	**Chaparone activity**	Western blot or fluorescent labeled proteins under natural promotor		Increase HSP60,HSP10, ClpP, ATF5 & no effect at BIP, HSP73, HSP72	possible for any cell model, easily to combine with other markers	seperation between ER/mito response not 100%	71
		Luciferase activity coupled to mtUPR TF		Coupled to CHOP, Mure 1, Mure 2	plate reader assay	seperation between ER/mito response not 100%	71

Table 1: Mitochondrial key events - part 3

Table depicting mitochondrial key events and a subdivision of biological processes belonging to these specific key event. Per biological processes the table elaborates on: 1) methods available to assess related biological processes, 2) examples of the applied techniques, 3) specification of the technique mentioned in in the example, 4-11) columns assessing in detail the assay including the (dis)advantages that need to be considered when using these techniques in a AOP context. * Cells: Yes = Cellular assay/ No: Assay performed in tissue or with isolated enzymes; ** Life: Yes = Cellular assay without the need of lysation or permeabilsation of membranes; # Isol. Mito.: Yes = Assay can be performed in isolated mitochondria; $ Mod.: Yes = assay requires modification/transfection of used cell types; & Mito-target: Yes = Assay (for example tags) can be targeted specifically to the mitochondrial compartment. (Source numbers are provided at the end of the chapter).

KE	Process	Method		Examples	Specification	Cells *	Live **	Isol. Mito. #	Label-free	Mod. $	Mito-target &
Dynamics	Fission and Fusion	Immunofluorescent (IF) staining		TOMM20, mtHSP70	Staining of mitochondrial specific proteins	Yes	Yes	No	No	No	Yes
		Mitochondrial compartment staining	Fluorescent dyes	Rho123, TMRM, mitoTracker	Mitochondrial dyes accumulate transient or stabile into mitochondria	Yes	Yes	No	No	No	Yes
			Plasmid (one FP)	mitoGFP, mitodsRED, mitoLoc etc…	Expression of mitochondrial targeted fluorphores	Yes	Yes	No	No	Yes	Yes
			Plasmid (photoactivatable)	mitoPAGFP	Photoactivation of a FP in invidual mitochondria to follow fusion	Yes	Yes	No	No	Yes	Yes
			2 Plasmids (one FP)	Combined mGFP, mdsRed, or mBFP	Two populations of mitochondria transfected with 2 or more FPs	Yes	Yes	Yes	No	Yes	Yes
		Cross link enzyme activity	2 Plasmids (enzymes)	Cells expressing N- or C-terminus of enzyme	Two populations of mitochondria transfected N-/T-terminus of enzyme	Yes	Yes	Yes	No	Yes	Yes
		Western blot	Protein localisation	Drp1 (cyto-mito)	Expression of mitochondrial specific proteins in mitochondrial fraction	Yes	No	No	No	No	Yes
			Protein cleavage	OPA1 (long-short)	Cleavage of OPA1 protein	Yes	No	No	No	No	No
	Biogenesis	TF activity measurment	Plasmid (one FP)	NRF1mitoGFP reporter plasmid	Nrf1 regulated expression of mito targeting plasmids	Yes	Yes	No	No	Yes	Yes
		mtDNA vs nDNA	qPCR, MMqPCR, duplex qPCR	mtDNA genes vs nDNA gene	Expression of mitochondrial and nuclear gene	Yes	No	No	No	No	No
			ELISA, colorimetric, WB, cytrometry	COX1 (mtDNA) vs SDH-A (nDNA)	Expression of mitochondrial and nuclear DNA coded protein	Yes	No	No	No	No	No
		Marker expression	Western blot, RTqPCR	PGC1A, NRF1, NRF2, TFAM	Expression of biogenesis specific proteins	Yes	No	No	No	No	No

Table 1: Mitochondrial key events - part 3 (continued)

KE	Process	Method		Examples	Advantage	Disadvantage	Source
Dynamics	Fission and Fusion	Immunofluorescent (IF) staining		TOMM20, mtHSP70	possible for any cell model, requires a lot of samples in conc, time dependent studies	one process, only useful when performed in living cells (fixation disrupts mitochondrial morphology)	72, 73
		Mitochondrial compartment staining	Fluorescent dyes	Rho123, TMRM, mitoTracker	assessment of both fission and fusion	dyes ar einfluenced by environemntal factors (MMP)	74
			Plasmid (one FP)	mitoGFP, mitodsRED, mitoLoc etc…	assessment of both fission and fusion, not effected by environmental factors		75
			Plasmid (photoactivatable)	mitoPAGFP	very high resolution (per mitochondria)	only fusion	76, 77
			2 Plasmids (one FP)	Combined mGFP, mdsRed, or mBFP	difference fusion/ biogenesis and difference close proximity/fusion	higher resolution required to separate populations	78
		Cross link enzyme activity	2 Plasmids (enzymes)	Cells expressing N- or C-terminus of enzyme	plate reader assay	enzyme activity can be effected by chemicals	79
		Western blot	Protein localisation	Drp1 (cyto-mito)	wb for specific celllular compartments	only fusion; measurement in lysate	80-82
			Protein cleavage	OPA1 (long-short)	post-translational changes are more dyamic than protien expression	only fission (conversion L-OPA1 -> S-OPA1); measurement in lysate	83
	Biogenesis	TF activity measurment	Plasmid (one FP)	NRF1mitoGFP reporter plasmid			84
		mtDNA vs nDNA	qPCR, MMqPCR, duplex qPCR	mtDNA genes vs nDNA gene	directly related to nuc/mtDNA copy number	measurement in lysate	85
			ELISA, colorimetric, WB, cytrometry	COX1 (mtDNA) vs SDH-A (nDNA)		measurement in lysate, protein expression depends on more than only the nuc/mtDNA copy number	86, 87
		Marker expression	Western blot, RTqPCR	PGC1A, NRF1, NRF2, TFAM	possible for any cell model, requires a lot of samples in conc, time dependent studies	measurement in lysate	88

Table 1: Mitochondrial key events - part 4

Table depicting mitochondrial key events and a subdivision of biological processes belonging to these specific key event. Per biological processes the table elaborates on: 1) methods available to assess related biological processes, 2) examples of the applied techniques, 3) specification of the technique mentioned in in the example, 4-11) columns assessing in detail the assay including the (dis)advantages that need to be considered when using these techniques in a AOP context. * Cells: Yes = Cellular assay/ No: Assay performed in tissue or with isolated enzymes; ** Life: Yes = Cellular assay without the need of lysation or permeabilsation of membranes; # Isol. Mito.: Yes = Assay can be performed in isolated mitochondria; $ Mod.: Yes = assay requires modification/transfection of used cell types; & Mito-target: Yes = Assay (for example tags) can be targeted specifically to the mitochondrial compartment. (Source numbers are provided at the end of the chapter).

KE	Process	Method		Examples	Specification	Cells *	Live **	Isol. Mito. #	Label-free	Mod. $	Mito-target &
Calcium homeostasis	Calcium flux	Calcium flux measurement (mitochondrial - targeted plasmids)	Luminescence	Aequorin, Berovin, Obelin, Phialidin, Mitrocomin, Mnemiopsin	Bio-luminescence upon binding to CA2+	Yes	Yes	No	No	Yes	Yes
			FRET	Cameleon, G-CaMP & Tropin C biosensor	Ca2+ selective subunit (CaM/Troponin) used in FRET construct (1/2 FPs)	Yes	Yes	No	No	Yes	Yes
			Fluorescence	Camagroo	Conformational change of FP upon CA2+ to CaM subunit	Yes	Yes	No	No	Yes	Yes
			Ratiometric	Pericam, GEM-GECO1	Similar principal as Camagroo, but with circular FP	Yes	Yes	No	No	Yes	Yes
				GECO1 fused to MICU1, Cvsue, ROMO1	Fluorescence measurement in mitochondrial subcompartments	Yes	Yes	No	No	Yes	Yes
		Calcium flux measurment (dyes)	Fluorescence	Rhod-1, Rhod-2, Fluo-1, Fluo-2, Fluo-3, Fluo-4, etc…	FP intensity change upon binding of Ca2+	Yes	Yes	No	No	No	No
			Ratiometric	Still-1, Still-2, Indo-1, Fura-1, Fura-2, Fura-3, etc...	FP intensity change upon binding of Ca2+ (dual emission/exitation)	Yes	Yes	No	No	No	No
Degradation	Mitophagy	pH detection (FP)		mt-Keima, Rosella	pH dependent shift of FP spectrum	Yes	Yes	No	No	Yes	Yes
		Co-localisation mitochondria/ lysosomes (IF or plasmid)		Mito: dyes, cytochrome-c & lysosome: LAMP1, LC3, MAP1LC3B, SQSTM1	Co-localisation of mitochondrial and lysosome labeled-proteins	Yes	Yes	No	No	Yes	No
		Co-localisation mitochondria/ mitophagy proteins		Mito: dyes, IF & mitophagy: Parkin2-FP	Detection of mitochondria tagged for degradation	Yes	Yes	No	No	Yes	No
		Electron microscopy		Mitochondrial and lysosome structures	Electrons as light source for high resolution images of cellular structures	Yes	Yes	No	Yes	No	No

Table 1: Mitochondrial key events - part 4 (continued)

KE	Process	Method		Examples	Advantage	Disadvantage	Source
Calcium homeostasis	Calcium flux	Calcium flux measurement (mitochondrial - targeted plasmids)	Luminescence	Aequorin, Berovin, Obelin, Phialidin, Mitrocomin, Mnemiopsin	plate reader assay	sensor is "utilized" upon usage, so requires constant expression, delayed dynamics	89-97
			FRET	Cameleon, G-CaMP & Tropin C biosensor		endogenous CaM expression can effect efficiency	96, 98-102
			Fluorescence	Camagroo		pH sensitive, endogenous CaM expression can effect efficiency	96, 102-104
			Ratiometric	Pericam, GEM-GECO1	higher affinity for Ca2+ and larger dynamic range, than Cameleon and Camagroo	pH sensitive	96, 102, 105-107
				GECO1 fused to MICU1, Cvsue, ROMO1	labeling of multiple mito compartments	complex analysis and interpretation	96, 102, 108, 109
		Calcium flux measurment (dyes)	Fluorescence	Rhod-1, Rhod-2, Fluo-1, Fluo-2, Fluo-3, Fluo-4, etc…		localisation (some are more mitochondrial than others), equal loading is difficult	96, 110-113
			Ratiometric	Still-1, Still-2, Indo-1, Fura-1, Fura-2, Fura-3, etc...	less issues with probe loading, bleaching, optical path length, illumination intensity (compared to single emission/excitation)	localisation (some are mote mitochondrial than others)	96, 114
Degradation	Mitophagy	pH detection (FP)		mt-Keima, Rosella	resistent to lysosomal degradation	double FP (rosella) are less stable than single FP (Keima based)	115, 116
		Co-localisation mitochondria/ lysosomes (IF or plasmid)		Mito: dyes, cytochrome-c & lysosome: LAMP1, LC3, MAP1LC3B, SQSTM1	single cell resolution	detailed analysis required to confirm overlap	117
		Co-localisation mitochondria/ mitophagy proteins		Mito: dyes, IF & mitophagy: Parkin2-FP	single cell resolution	detailed analysis required to confirm overlap	118-120
		Electron microscopy		Mitochondrial and lysosome structures	high resolution	low throughput	119, 121

TCA: Assessment of the TCA cycle enzyme activity using radiolabeling and spectrometry is possible for any cell model, because the metabolite/substrate assessment is performed in the supernatant. The use of radiolabeling requires special expertise and reduces the likelihood of integration into standard toolkit. Therefore, the use of reporter assays for TCA cycle enzyme activity would be more suitable for a standard qAOP approach. However, until now most reporter assays are not suitable for live cell assessments, precluding time-resolved analyses. The development of TCA cycle enzyme specific fluorescent ligands will provide the opportunity of real time monitoring and would also enhance enzyme specificity of read outs.

FA: Fatty acid oxidation is also measured using both radiolabeling and cell specific fluorescent dyes. Fluorescent dyes suitable for live measurements do support the assessment of fatty acid conversion and accumulation dynamics in cell systems with different complexities, including 2D, 3D, or co-cultures. A disadvantage of the use of fatty acid accumulation as a read out is that high amounts of fatty acid are required for the formation of detectable aggregates.

OXPHOS – oxidative phosphorylation (mitochondrial respiration)

Description

In the mitochondrial metabolic pathways, electrons are being pumped over various protein complexes in the mitochondrial inner membrane using flavin proteins. The electron flow supports the passage of protons over the same protein complexes, creating a proton gradient in the intermembrane space. Subsequently, the created surplus is channeled back into the matrix via the ATP synthase, which in turn uses the proton translocation to drive the conversion of more ADP into ATP [Alberts 2014].

Disease

Mitochondria contain a circular genome which encodes 14 proteins, 22 tRNAs and 2 rRNAs, all of which are part of the mitochondrial respiration machinery. The mitochondrial encoded proteins include 2 subunits of the ATP synthase (complex V), 3 subunits of complex IV, 1 subunit of complex III, 7 subunits of complex I and the protein humanin[10]. Mutations in the protein complex encoding genes, located in both the mitochondrial and the nuclear genome, will decrease OXPHOS capacity. The mutations identified in the mitochondrial genome can be both heteroplasmic and homoplasmic, and can be restricted to single tissues [Goodwin 2015, Wallace 2013]. Decreased OXPHOS capacity is associated with strokes, seizures, myopathy, impaired vision, liver failure or gastrointestinal abnormalities, which mostly manifest in childhood or early adolescence [Jacobs 2006, Merrit 2018]. The mitochondrial encoded micropeptide humanin was shown to be protective against starvation, oxidative stress and other injuries in *in vitro* systems [Yen 2012]. Reduction of functional

humanin, caused by mutations in the gene or age-related decreased expression, does not directly affect the OXPHOS capacity of the cell, but does result in the loss of its protective function which can worsen diseases such as Alzheimer, stroke, cardiovascular disease, metabolic diseases and cancer [Gong 2014].

Chemical toxicity

Inhibition of the mitochondrial respiration complexes has been reported for chemicals which also perturb fatty acid oxidation. This combined perturbation of OXPHOS and fatty acid oxidation is correlated with the transition of steatosis to steatohepatitis [Fromenty, 2019, Kaufmann 2005]. The chemicals which are known to interfere with mitochondrial respiration complexes are in many cases not inhibitors of just one protein complex. For instance, various liver toxicants demonstrated inhibition of multiple complexes *in vitro*, including Benzbromarone [Dykens 2008, Nadanaciva 2007], Diclofenac [Nadanaciva 2007, Acuna 2012, Ghosh 2016], Entacapone [Grünig 2017], Flutamine [Ball 2016], Metformin [Owen 2000, Nadanaciva 2007, Brunmair 2004], Nefazodone [Dykens 2008, Nadanaciva 2007] and Tolcapone [Grünig]. In addition, epidemiological studies indicate a relationship between neurological diseases (e.g., Parkinson) and exposure to pesticides designed to inhibit mitochondrial complexes [Dhillon 2008; Tanner 2011]. Mitochondrial complex inhibitors are widely used in pesticides, insecticides or fish poison[11] [Lümmen 1998, Bartlett 2001]. The lipophilic properties of for example the complex I targeting rotenone facilitate its uptake by gills and trachea and low concentrations can result in toxicity in fish in the form of cardiac and neurological failure and indicate a risk for human exposure [McKim 1991, Ling 2003].

Methods

Assessment of OXPHOS functioning can be based on mitochondrial membrane potential (1), oxygen homeostasis (2), ATP content (3) and protein complex activity (4) (table 1). 1) The driver of OXPHOS is the proton gradient over the mitochondrial inner membrane. This potential difference can be assessed using dyes that only accumulate in the mitochondria when there is a potential difference and are released into the cytoplasm during depolarization. 2) Oxygen consumption during ATP production can be monitored based on oxygen localization (in situ oxygenation) or oxygen consumption rates (OCR). Oxygen localization in various tissue types using electron paramagnetic resonance (EPR), immunofluorescence and spectrometry supports is assessed to study the local effects of a stroke, ischemia or cancer. In the context of chemical-induced perturbation the assessment of oxygen consumption is more informative for effects at the cellular level. Electrode measurements are based on amperometry, which means the detection of oxygen ions based on changes in an electric current. Alternatively, more sensitive oxygen sensors can be used, which rely on optical detection rather than electrochemical signals. The sensor can be soluble

or static. They are subdivided in endogenous (myoglobulin and NADH) or synthetic molecules that react with oxygen. 3) The end-product - ATP - of OXPHOS can be monitored using assays based on firefly luciferase, biosensors or small molecules. Firefly luciferase converts D-luciferin in the presence of ATP and Mg^{2+} to oxyluciferin AMP and light. The amount of emitted light correlates with the amount of ATP used in the reaction. In the last decades, various ATP sensors have been developed which combine an ATP binding region (being parts of complex V or nucleotide strings) with single or double fluorophores (FRET). Researchers also developed small molecules, which upon interaction with ATP become fluorescent or demonstrate a shift in their emission spectrum. 4) The direct interaction with any of the complexes involved in the OXPHOS can be assessed using enzyme activity assays or complex specific respiration in isolated mitochondria or permeabilized cells using complex specific substrates and inhibitors.

Integration into qAOP

To summarize, mutations in almost all OXPHOS related genes are reported to result in lethality or diseases. The use of chemical inhibition of the complexes in pest control is expected to result in acute human toxicity. Indeed, a correlation between neurological disorders in humans upon prolonged exposure to OXPHOS inhibitors has been observed among farmers [Tanner 2011, Wang 2011]. In combination, with the above described direct relationship between OXPHOS protein malfunctioning and disease, it suggests that OXPHOS perturbation is a molecular initiating in both disease and toxicity.

Oxygen consumption is an important readout for the assessment of OXPHOS activity. The first oxygen specific electrodes for *in vivo* measurements were developed in the fifties. The next generation of oxygen consumption assays consists of oxygen sensitive fluorophores, with seahorse being the most recent, providing a non-invasive method to assess the behavior of both adhesive and non-adhesive cells upon changes in their environment. However, these techniques are less suitable for high-throughput measurements and are not used in live cell monitoring. The addition of the complex specific OCR measurements requires a successive addition of buffers and ligands and supports the accurate identification of complexes effected upon chemical exposure. However, the protocol makes it impossible to perform real-time assessments, which could be required when toxicity is not acute (chemicals which become toxic upon metabolism or accumulation).

Other assay options are the various soluble (mainly synthetic) probes and sensors for oxygen levels, membrane potential and ATP levels. They provide the opportunity to follow the distribution and presence of these markers in a high- throughput microscopy

setting on single cell level. The use of stable integrated sensors like single/multi-fluorophore FRET has the added value of organelle specificity. For example, subtle deviations in specific mitochondrial ATP dynamics could be more predictive for the outcome than overall cellular ATP levels, which are in some cell systems mostly driven by glycolysis. Most probes and biosensors are suitable for more complex cell systems but do require more elaborate and cell type specific optimization. This includes the effects of: environmental factors like pH (FRET sensors are pH sensitive), transporter expression (probes can be actively excreted) or cell behavior (e.g., influence of cell growth on read outs). Eventually, the probe and biosensor read out per cell could be linked in the same setup to later key events like cell death.

Mitochondrial protein structure – UPR^{mt}

Description

Mitochondria are largely independent organelles with their own genome and enclosed by double membranes. The majority of the mitochondrial proteins, including 84 out of 97 respiration complex proteins, are produced in the cytosol and have to be transported into the organelle by a variety of target-specific translocases. Subsequently, these proteins need to arrange into various large multi-protein complexes. To properly coordinate and control the protein arrangements, the mitochondria have a dedicated quality control system focused on translation and protein folding [Münch 2018, Qureshi 2017]. After the detection of misfolded proteins, the mitochondria initiate different responses, including upregulation of chaperone and proteasome activity, induction of antioxidant responses, and downregulation of pre-RNA processing/translation. All of these responses are focused on reduction of the number of improperly folded and assembled proteins [Münch 2018].

Disease

Mitochondrial UPR related diseases are divided into disorders affecting chaperones or proteases. Important mitochondrial chaperones include HSP60, HSP10, mtHSP70, and TRAP1 [Voos 2002, Yi 2018]. Malfunctioning of the HSP60-HSP10 complex caused by both autosomal dominant and recessive mutations in chaperone genes can result in neurological disorders, but in general results in embryonic lethality [Bross 2018].

Mitochondrial specific proteases include OMA1, LONP1, CLpP and PARL. Hyper-activation of OMA1, caused by mutations in *AFG3L2*, results in autosomal dominant optic atrophy or spinocerebellar ataxia, depending on the location of the mutation [Baderna 2020, Tulli 2019]. Autosomal recessive mutations in *LONP* cause neurological disorders or symptoms similar to mitochondrial metabolic diseases like lactic acidosis and muscle weakness [Dikoglu 2015, Strauss 2015, Peter 2018]. Malfunctioning of CLpP, caused by autosomal recessive mutations, results in Perrault syndrome, which is

associated with hearing loss and in some cases neurological problems [Brodie 2018]. Reduced levels of PARL are observed in obesity and Parkinson disease, but so far no causal link has been discovered [Chan 2013]. Myopathy due to decreased capacity of the mitochondrial UPR has been associated with aging and forms of Alzheimer disease [Shpilka 2018].

Chemical induced toxicity

Thus far, human mitochondrial UPR perturbing agents have not been reported. However, an *in vivo* screen in *C. elegans* of 1200 FDA approved chemicals identified Auranofin, Chlorprothixene HCl, Methacycline HCl and Minocycline as inducers of the mitochondrial unfolded protein response (mtUPR), based on HSP60 expression [Rauthan 2015]. By contrast, Yumnamcha *et al* did not observe any change in the expression of the mtUPR markers, LonP, YME1L1, mtHSP40 or PDIA upon Auranofin exposure of human retinal cells [Yumnamcha 2019].

Methods

Activation of the UPR can be assessed based on the activity of involved chaperones and proteases (table 1) [Jovaisaite 2015]. When assessing the mitochondrial UPR it is important to distinguish the responses triggered in the mitochondria from those coming from the ER or the cytoplasmic compartment. To assess chaperone activity, one could monitor the expression of transcription factors involved in the induction of the chaperone responses or the presence/localization of the chaperones themselves (both at the mRNA and protein levels). To distinguish mitochondrial UPR from ER UPR, a combination of several components from these responses should be analyzed.

Integration into qAOPs

In conclusion, development of adversity in the form of disease has been observed upon malfunctioning of several mitochondrial UPR specific proteins. Contrarily, no direct correlation has been found between chemical-induced adversity and any of these proteins. This in combination with the goal of the mtUPR, degradation of malfunction proteins like the ones observed upon mutations of genes involved in OXPHOS or metabolism, could indicate a possible more secondary role of the mtUPR in chemical adversity.

Specific assessment of the mtUPR is challenging, because of the interconnection of involved proteins between the ER, the cytosol and the mitochondria. The expression of especially mitochondrial specific chaperones and proteases, rather than ER specific UPR components, could indicate mitochondrial specific damage, but the close contact between these organelles renders this extremely difficult [Rowland 2012].

Mitochondrial dynamics – fission/fusion and biogenesis

Description

Mitochondria are dynamic organelles that adjust their morphology based on the energy requirements of cell. The mitochondrial morphology changes are divided into fission and fusion. Fission being the process of splitting mitochondria in smaller compartment, which enables transport of mitochondria and clearance of damaged parts. Fusion describing the process of mitochondrial merging into longer and interconnected structures, which facilitates ATP production and the exchange of materials between organelles [Westermann 2010, Youle 2011, Hamacher-Brady 2016, Giacomello 2020]. In addition, the process of fission is involved in the growth of the mitochondrial mass in the cell. Only splitting and merging of mitochondria should not result in a change in total mitochondrial mass. However, the cell can enlarge its mitochondrial mass via the process of biogenesis. Various cellular factors stimulate the replication of the mtDNA. Subsequently the mitochondria can split into two functional daughter mitochondria through fission [Jornayvaz 2010, Hock 2009].

Disease

If the fragile balance between fission and fusion is disturbed for a prolonged period it can lead to a drop in energy production causing cell and ultimately tissue dysfunction. Indeed, mutations in genes encoding components of the machinery controlling fission and fusion are observed in cardiovascular disease, amyotrophic lateral sclerosis and neurodegenerative diseases [Suárez-Rivero 2016, Chen 2009]. Neurodegenerative diseases can be divided into disorders caused by mutations in either fission related genes, *MFN2* or *OPA1*, or fusion related genes, *DNM1L*. Mutations in *MFN2* effects sensory and motor neurons in Charcot-Marie-Tooth (CMT) disease [Züchner 2004]. CMT is observed as an autosomal dominant, recessive and even X-linked dominant pattern[12]. Mutations in *OPA1* are associated with autosomal dominant optic atrophy, a disease affecting the retinal ganglia cells (*OPA1* is an abbreviation from optic atrophy type 1) [Delettre 2000, Alexander 2000]. In case of *DNM1L*, the major factor involved in fission, autosomal recessive and dominant negative mutations are observed. The location of the mutation determines the severity of the disease, which can lead to early childhood lethality or epileptic phenotypes and neurological disorders [Fahrner 2016]. There is also evidence that altered mitochondrial dynamics are involved in the progression of Huntington's disease (HD). Pharmacological inhibition of excessive fission is feasible in *in vitro* systems and mice and offers potentials for HD treatment [Reddy 2014]. Finally, alterations in the process of biogenesis are observed in diabetic patients [Nisoli 2007, Heinonen 2015].

Chemical induced toxicity

Changes in the expression of fission and fusion factors, but also altered mitochondrial morphology are observed upon exposure to various toxicants [Meyer 2017]. Perturbation of mitochondrial biogenesis occurs upon interference with mtDNA replication or translation of the related mitochondrial proteins. Both types of perturbation will prevent the production of new mitochondria. Vice versa, upregulation of mitochondrial biogenesis is also observed during toxicity and may represent a compensatory mechanism to counteract other types of mitochondrial perturbations [Zhond 2017].

Methods

The process of fission and fusion can be evaluated by manual or automated inspection of mitochondrial morphology, visualized using mitochondrial specific dyes, immune-fluorescent staining of mitochondrial proteins or stable integration of mitochondrial targeted and labeled plasmids (table 1). The localization (mitochondrial-cytoplasmic) or activation (e.g. cleavage of OPA1) of proteins crucial in the machinery of fission and/or fusion can be assessed using Western blot. Changes in biogenesis are monitored by comparing effects on mitochondrial mass and cellular mass. This can be done at the mRNA level, i.e. mitochondrial mRNA vs nuclear mRNA or at protein level, comparing amounts of proteins originating from mtDNA versus proteins originating from nuclear DNA (e.g. COX1 vs SDH-A).

Integration into qAOP

To summarize, perturbation of proteins regulating mitochondrial morphology has been observed as causal for several neurological disorders. Direct interaction of chemicals with mitochondrial morphology proteins has not been reported so far. However, the observation of changes in expression of these proteins is an indication of at least the occurrence of secondary responses to possibly counteract effects caused by interference with for example OXPHOS and mitochondrial metabolic pathways. Monitoring the dynamics of fission and fusion could therefore be an indicator of the significance of the perturbation in earlier key events.

Mitochondrial dynamics has been studied based on tracking shape and number of mitochondria. Mitochondrial number related to mitochondrial mass (quantity of for example membrane proteins or mitochondrial DNA) can be integrated in pipelines using all sorts of cell lines and simple plate readers. The assessment of fission/fusion ratio's is more challenging and requires sophisticated microscopy and time-lapse imaging, which is not always possible in every cell model.

In general, it is important to keep in mind that the assessment of mitochondrial morphology should: 1) be based on the fission/fusion ratio in multiple mitochondria

to prevent conclusions based on malfunctioning of individual mitochondria, which happens also under healthy conditions, 2) keep in mind spatial resolution, because acute effects can be cell type specific and location specific and 3) keep in mind time dynamics, because changes in morphology are constantly happening and some changes can be considered as temporal/protective.

When assessing specifically mitochondrial biogenesis it is important to distinguish between the number of mitochondria and the number of mtDNA copies. It is therefore required to additionally assess specifically the expression of mtDNA genes/proteins or study the exact copy number of mtDNA.

Mitochondrial Ca^{2+} homeostasis

Description

Ca^{2+} ions are important players in cellular signaling as second messengers, which makes their distribution and storage important factors in the maintenance of cellular functioning. The major storage of the Ca^{2+} pool is in the ER. From the ER, Ca^{2+} ions constantly shuttle to the mitochondria for storage and support of mitochondrial function including OXPHOS and metabolism, and regulation of cell migration, ROS signaling and regulation of cell death [Ben-Hail 2014, Paupe 2018, Prudent 2017, Görlach 2015, Wan 1989]. Mitochondrial Ca^{2+} levels are regulated by 4 major transporters: VDAC1, the Na^{+}/Ca^{2+} exchanger (mNCX), H^{+}/Ca^{2+} exchanger (mHCX), the Ca^{2+} uniporter (MCU) and the mitochondrial permeability transition pore (mPTP) [Phillips, 2016, Ben-Hail 2014, Giorgi 2018]. Besides its role in the regulation of Ca^{2+} levels, mPTP is involved in transport of molecules smaller than 1.5kDa [Halestrap 2009].

Disease

Unbalanced distribution of Ca^{2+} can affect all processes relying on this second messenger. Furthermore, an overload of Ca^{2+} caused by irreversible opening of the Ca^{2+} pores depolarizes the mitochondria and triggers apoptotic or necrotic cell death [Rasola 2011]. Dysregulation of the localization of Ca^{2+}, because of malfunctioning mitochondria, is observed in various diseases including diabetes, cardiovascular diseases, neurodegenerative diseases and metabolic diseases [Giorgi 2012]. For example mitochondrial metabolic diseases caused by inhibition of the PDH complex can be a result of changes in the Ca^{2+} level [Lai 1988].

Mutations in the various mitochondrial transporter genes are rarely observed, with the exception of patients lacking *VDAC1* presenting with fatal mitochondrial encephalomyopathy [Huizing 1996]. Increased expression levels of transporters are correlated with development of cancer and neurological diseases. Overexpression of *MCU* correlates with increased proliferation of colorectal cancer [Liu 2020].

Overexpression of *VDAC1* supports anti-apoptotic activity in cancers, via interactions with hexokinases [Shoshan-Barmatz 2012]. In general, excessive Ca^{2+} release aggravates the progression of various neurological diseases (Alzheimer, Parkinson and Huntington) and cardiac ischemia reperfusion-injury. The excessive release of Ca^{2+} caused by changed transporter expression can be counteracted using pharmacological inhibition of the transporter [Dey 2020]. On the other hand, the involvement of the individual transporters in the development of a Ca^{2+} overload is controversial, for example the NCX pump can revert its transport mode and therefore support both an in and outward flow of Ca^{2+} [Samanta 2018].

Finally, perturbation of the mPTP protein complex or an occurrence of a Ca^{2+} overload triggers the opening of the mPTP. This results in the release of soluble mitochondrial molecules with a molecular weight below 1.5kDa into the cytoplasm. This is combined with water intake based on the associated change in osmotic pressure. Opening of the pore for a short period supports regulation of the mitochondrial-cytoplasmic ion gradients. Prolonged opening contributes to Ca^{2+} loss, a drop in of ATP synthesis and release of pro-apoptotic molecules, including cytochrome c, SMAC/DIABLO and Omi/HtrA2 [Garrido 2006]. Cytochrome C shuttles under normal condition between mitochondrial complex III and IV. However, upon release into the cytoplasm it stimulates, together with SMAC and Omi, the assembly of the apoptosome and the activation of the caspases responsible for the execution of apoptosis. The mPTP is therefore used as a target in cancer treatment [Olszewska 2013]. Furthermore, increased expression of mPTP components (CypD) is associated with neurodegenerative disease in *in* vitro and *in vivo* models [Rao 2014]. Although overexpressed mPTP may be targeted in neurological diseases, existing mPTP inhibitors are either too toxic or lack clinical effects.

Chemical toxicity

No toxicity has been identified thus far via direct interaction of chemicals with the Ca^{2+} transport proteins. Possible mechanisms of Ca^{2+} toxicity could include undesired formation of membrane pores. Some strains of bacteria produce pore-forming toxins that initiate a flux of Ca^{2+} from the mitochondrial matrix into the cytosol [Bouillot 2018]. Bacterial infections accompanied by this type of toxins target mostly the immune system of the host cell [Los 2013].

Methods

Assessment of Ca^{2+} homeostasis specifically in the mitochondria can be achieved using genetically encoded Ca^{2+} sensitive FRET-, fluorescent-, ratiometric constructs or bioluminescent proteins that can be targeted to mitochondria (table 1). In addition, there is a range of dyes capable of detecting Ca^{2+}. However, most of them are not

specific for the mitochondria and require a combined staining with a dye or antibody targeting the mitochondria.

Integration into qAOP

To conclude, perturbation of Ca^{2+} fluxes and storage are correlated with the severity of certain diseases. Excessive amounts of Ca^{2+} aggravate diseases and can eventually trigger cell death. No chemicals have been reported to cause adversity by directly targeting the Ca^{2+} distribution. This indicates that the perturbation of Ca^{2+} rather belongs to a later key event than the molecular initiation event.

Assessment of Ca^{2+} fluxes can be achieved using dyes and upon integration of Ca^{2+} specific biosensors. A wide variety of options exits, which provides the opportunity to choose and optimize the assay for the cell type of interest. However, it also illustrates the difficulty of selecting a common strategy that would suit multiple cell types and models with different levels of complexity.

Mitochondrial degradation - mitophagy

Description

Cells are equipped with mechanisms to eliminate a surplus of unnecessary and/or malfunctioning structures and organelles using autophagy. Autophagy is defined as the process during which parts of the cytoplasmic content including organelles are engulfed by vesicles and eliminated. This process of organelle elimination is also referred to as mitophagy when it is specifically focused on mitochondria [Yuole 2011, Hamacher-Brady 2016, Palikaras 2018]. Under normal conditions mitophagy is also used to support cellular differentiation and maturation. Certain cell types change their mitochondrial content during development, sperm-derived mitochondria are for instance degraded in fertilized oocytes and red blood cells do not require mitochondria to properly function [Palikars 2018]. Under stress conditions – for example chemical exposure – mitophagy can be triggered by loss of mitochondrial membrane potential, nitrogen-starvation and hypoxia. Which proteins are involved in the mitophagy process depends on the trigger. The most common mitophagy pathway is centered around PINK1 (*PINK1/PARK6*) stabilization and Parkin (*PARK2/PRKN*) recruitment to the mitochondria. But under hypoxic conditions, cells rely on Bnip3/NIX and FUNDC1 driven mitophagy processes.

Disease

Malfunctioning of the mitophagy system has been observed in several diseases. Decreased activity is for example linked to heart disease and Parkinson disease, while hyper-activation correlates with improper blood/immune cell maturation and the induction of apoptosis [Palikars 2018]. About 10% of the Parkinson disease cases are

inherited [Thomas 2007]. A significant number of these cases demonstrate an autosomal recessive or dominant-negative mutation in in two gene involved in mitophagy, *PINK1* and *PRKN* [Valente 2004, Puschmann 2017, Kitada 1998, Balestrino 2020].

Chemical induced toxicity

Undesired excessive induction of mitophagy is observed upon exposure to different classes of chemicals, including some SIRT1-agonists, p53-inhibitors and PARP1-inhibitors [Georgakopoulos, 2017]. In general, the activation of mitophagy is a result of membrane potential loss and/or ROS formation, both originating from perturbation of OXPHOS. Another reported trigger is NAD+ accumulation, which is the result of malfunctioning mitochondrial metabolism. Often, massive activation of mitophagy is not triggered directly by chemicals but arises from chemical-induced perturbation of the above described key events.

Methods

While monitoring mitophagy it is important to clearly distinguish the observed effects from general autophagy (table 1). This can be achieved by performing co-staining of lysosome or proteins specific for mitophagy and mitochondrial markers. Other options are the use of mitochondrial targeted plasmids, including pH sensitive constructs, which will be triggered upon fusion with the low pH lysosome during mitophagy. In addition, mitophagy can be distinguished from general autophagy at high resolution when using electron microscopy.

Integration into qAOP

To summarize, mitophagy or specifically degradation of perturbed mitochondria is an end-stage in the process of mitochondrial malfunctioning. Enhanced levels of mitophagy are observed both in diseases and upon exposure to selected chemicals. Integration of mitophagy into mitochondrial AOPs would help to quantify the threshold between still functional cells and cells that are switching to cell death protocols. Quantifying the number of mitophagy events in a cell could distinguish between situations/exposures that would still allow recovery/adaptation and the moment at which cell/tissue are not be able to coop anymore.

AOP wide quantification of biological events

In the last decades, the assessment of effects on mitochondrial function has been widely integrated in drug safety testing. Robust assessment of mitochondrial health can be based on biochemical readouts for the different mitochondrial-involved processes that can be coupled to key events, including respiration, energy-carrier production and byproduct formation.

Several additional sources of data can support these mechanism-based toxicity assessments. In the last decade various omics approaches, including metabolomics, transcriptomics and proteomics have successfully been used in addressing a variety of biological questions in all fields of biology and are recently also integrated into toxicity assessments [Brockmeier 2017, heijne 2005]. However, integration of these large-scale data sets is not yet common practice in a regulatory context. To support the use of omics techniques chemical safety regulation, it is important to enable integration of the dense information coming from omics technologies into toxicological pathways and relevant key events. This integration will make the obtained information more understandable and comprehensive, which is required for decision making [Hartung 2016].

The omics approaches can be used in an AOP context to: 1) monitor multiple components of pathways related to key events, 2) support identification of new key events, 3) perform cross-species extrapolation or to study preservation of responses between species and 4) feed information into biomarker identification programs. The use of expression patterns of a large unbiased set of transcripts, proteins and/or metabolites upon exposure to chemicals, specifically when they are chemically or biologically related, could support the identification of common markers and support prediction of toxicity [Kohonen 2017]. The identification of new biomarkers can help to specify existing AOPs.

Metabolomics

Metabolomics is the study of the metabolite profile of a cell system, tissue or body fluid and can serve as a measure for the effect of stressors upon any type of metabolism [Bouhifd 2013]. The assessment of metabolite levels can be an indicator for the adaptive responses initiated upon for instance chemically-induced shortage of certain substrates or a change in energy requirements.

Methods

Assessment of the metabolome is nowadays achieved using nuclear magnetic resonance (NMR) or mass spectrometry (MS). NMR detects molecules present in a sample based on the interaction of their nuclear spin with a magnetic field. MS uses the behavior of ions created from a metabolite in a magnetic field. Selection of the technology depends among others on the studied sample properties like polarity and abundance, the desired approach (targeted vs untargeted) and the available resources including money, time and space [Bouhifd 2013, Emwas 2015]. Metabolomics samples most often include the non-invasive collection of urine, blood and feces or the invasive extraction of a biopsy. Assessment of the metabolome *in vitro* is possible using cell lysates. The use of cell lysate requires the optimization of cell number to achieve enough signal for lowly abundant metabolites. In addition, the

in vitro culture conditions should be carefully selected because of the presence of a variety of anions and metabolites in growth media [Smith 2020]. In addition, when collecting metabolite profiles of different culture types (2D/3D cultured cell systems and non-adherent cells), sample preparation and cell environment affect the yield and purity and should be optimized per *in vitro* model. Extraction and collection methods affect specific metabolites varying in polarity, size and stability differently. Cell environments like gel materials used in 3D cultures require digestion before metabolites are available for analysis [Mushtaq 2014, Muschet 2016, Mathon 2019, Vuckovic 2012, Zukunft 2018].

Metabolomics studies can be designed to be targeted or untargeted. A targeted approach includes the collection and purification of a selected set of metabolites before the MS or NMR is used to quantify their abundance. The set of metabolites can be linked to metabolic pathways of interest, be predictive for certain toxicities/diseases or performance very well in species cross validation. Untargeted metabolomics is the relative assessment of all metabolites present in a sample compared to a control sample. It results in large amounts of data, which requires post-processing to distinguish noise from the real metabolites. This approach allows a relative quantification of the complete metabolic fingerprint; however, it is still challenging to identify individual metabolites. Recently, hybrid approaches combining targeted and untargeted mass spectrometry are under investigation, which could facilitate metabolite detection and identification [Chen 2020].

Integration into qAOP

Metabolites provide a fingerprint of the entire cellular response and therefore changes in their expression may provide a complete view on the studied toxicity. The obtained information could therefore be highly relevant in describing later key events and support even extrapolation to *in vivo* and human data collected in for example clinical settings. Metabolomic profiles support qAOP both when collected using targeted and untargeted approaches. The limited number of metabolites assessed with targeted approaches can be used to support key events describing specific metabolic pathways. Untargeted assessment of metabolites informs biomarker/key event identification, species/tissues/cell type comparisons and can be used to compare differences/similarities at a larger scale for chemicals with known and unknown mode of action in read across attempts [Van Ravenzwaay 2007, Van Ravenzwaay 2016].

Unfortunately, metabolomics studies are limited by the relatively few reference databases describing relationships between metabolic profiles and pathologies/toxicities. Changes in metabolic profiles are also observed upon physiological processes like growth, development and even feeding cycles. Reference profiles

should allow proper separation between desired and undesired changes. The Consortium for Metabolic Toxicology (COMET) and aBASF group company (in Germany based chemical industry cluster) started with the creation of metabolomics reference databases to describe metabolic variations in the context of chemical exposure [Lindon 2005, Bollard 2010, Spagou 2011, Van Ravenzwaay 2012]. Metabolic profiles are influenced by species and sex [Van Ravenzwaay 2012], indicating that these variables should be incorporated in reference panels to allow comparison studies and support extrapolation of responses in for example AOP approaches.

A few attempts to incorporate metabolomics data into AOP driven research have been reported concerning androgen receptor activation by spironolactone [Davis 2017], cross species extrapolation in plant species [Florez-Sarasa 2016] and nanoparticle toxicity [Bannuscher 2020].

Mitochondrial studies using metabolomics

In the field of mitochondrial research, metabolomics is already widely used, because of the central role of mitochondria in multiple metabolic pathways. Perturbation of mitochondrial metabolism will lead to changes in the detected metabolites, for instance when inhibition of mitochondrial respiration leads to a switch in cellular commitment from mitochondrial respiration to glycolysis [ref].

The human metabolome can be assessed in the entire cell or specifically in the mitochondria or cytoplasmic compartment. Around 10% of the known metabolites are unique for the mitochondria and 20% are unique for the cytoplasmic fraction [Pan 2018]. Variations in these unique sets could indicate compartment specific effects. These changes in metabolic profiles, compartment specific or not, should be properly mapped to allow identification of molecular initiating events or key events. An option to achieve these reference maps is the use of models representative for mitochondrial diseases, for instance by knocking out mitochondrial complexes in mice or *c. elegans* [Esterhuizen 2017, Pan 2018]. Various studies already demonstrated the possibilities for using metabolomics to identify mitochondrial involvement in diseases, e.g. in diabetic kidney disease [Li 2017, Buzkova 2018].

Transcriptomics

Transcriptomics studies changes in the expression of RNA transcripts in cells, tissues or organs [Lowe 2017]. The complete profile of RNA transcripts present at a specific moment in time informs about cellular modifications initiated in response to for example chemical induced stress. Most of the information can be derived from mRNAs. In addition, the study of other types of RNA, like non-coding RNA or miRNA, provides the opportunity to monitor quick modulation of the transcript profile caused by this subset of RNA. Changes in non-coding RNAs have been reported in case

of disease and upon exposure to chemicals [Dempsey 2017]. These changes could reflect the adaptation required in (early) responses during toxicity and are therefore considered as markers in regulatory toxicology and studied as possible therapeutic targets [Aigner 2016, Ratti 2020].

Methods

The transcriptome is generally studied using microarrays or RNA-sequencing. Microarrays assess a set of transcripts covert by the probes on the used chip. This set can be an interesting toxicity subset, the whole genome, a list of non-coding RNAs and could even contain various splicing variants as longs as it is possible to design transcript specific probes. The detection of the transcript abundance is based on the fluorescence signal coming from the hybridized transcripts-probes on predefined spots of the chip.

RNA-sequencing captures the exact sequence of every transcript without the need of transcript specific probes, which makes the technology excellent for detailed assessment of mutations, splice variants and "new" transcripts. The transcript detection is based on the high throughput sequencing of one nucleotide per round, where the four nucleotides have a unique fluorescent color [Lowe 2017]. A recent approach in the assessment of the transcriptome is TempO-seq. TempO-seq is a form of targeted sequencing, in which probes representing a subset of transcripts are used to select transcripts of interest and with that reduce the information density to be sequenced using RNA-sequencing technology [Mav 2018, Bushel 2018].

Samples suitable for the assessment of the transcriptome with any of these technologies only need to allow enough extraction of RNA [Lowe 2017]. Optimization of RNA extraction from any *in vitro* cell model includes selection of proper cell densities and lyses protocols. For instance, RNA-sequencing technologies require less RNA than microarrays. And similar to metabolomics, cell systems cultured in 3D-matrices require a more firm lysis protocol than 2D cultured or non-adherent cells.

Integration into AOPs

The transcripts are the tools of the cell to change protein expression (mRNA) or perform subtle changes to already transcribed mRNA (non-coding RNA) upon detection of malfunctioning. Understanding the cellular decisions, based on the induction of mRNA coding for proteins involved in adaptations, detoxification or cell death, will help to understand the toxic potential of a chemical and to indicate which events in de AOP are triggered with certain concentrations at specific timepoints.

In the last decades, transcriptomic data is increasingly used to obtain more mechanistic understanding in the occurrence and development of adversity. As discussed intensively by others, to enable the use of transcriptomics in regulatory chemical risk

assessment and regulatory decision making, it is necessary to standardize and evaluate data quality, storage and processing [Martens 2018, Buessen 2017]. Furthermore, to support integration of transcriptomics data into AOPs and biologically relevant key events, it is important to streamline data analysis into structured pipelines assessing not only individual genes, but also gene ontology enrichment, pathway (under/over) representations and co-regulated gene networks [Martens 2018, Nymark 2018, Serra 2020]. The integration of transcriptomics into AOP frameworks has been done in studies of various species and organs [Gust 2020, Gomes 2019, Lee 2019, Oki 2019]. Studies investigated the effects of substances like nanoparticles [Labib 20216] or followed development of chemical-induced diseases like cholestasis [Wolters 2016, Gijbels 2020].

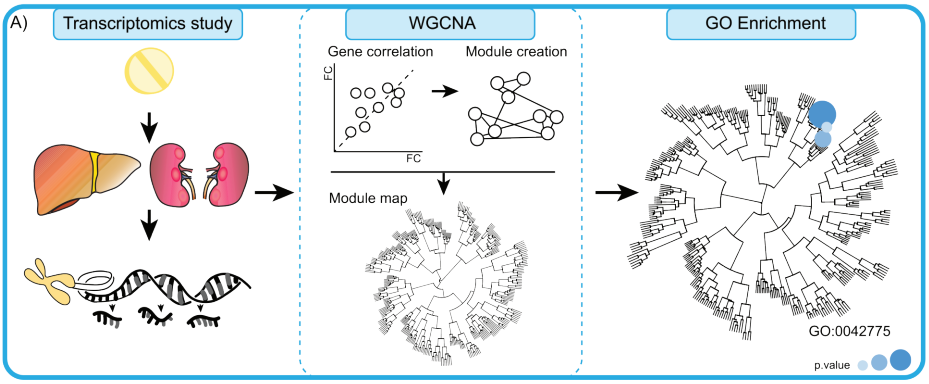
Mitochondrial studies using transcriptomics

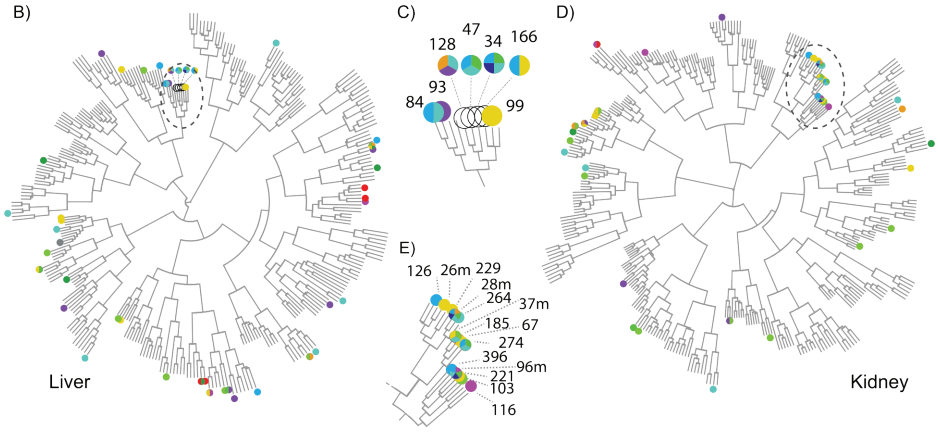
The use of transcriptome analysis upon chemical exposure could provide new insights for the assessment of mitochondrial toxicity. The early changes in transcript variations could reflect changes in cellular and mitochondrial biochemical reactions. Under normal and perturbed conditions there is a dynamic relationship between the nucleus and mitochondria [Quirós 2016]. Mitochondria and the nucleus are continuously interacting concerning the availability of energy, building blocks or storage places of ions, and the functional integrity of the mitochondria (for instance the mitochondrial membrane potential or mitochondrial DNA integrity). This constant communication initiated upon minimal changes in cellular-mitochondrial homeostasis could make it the perfect early marker for chemical toxicity. Monitoring this communication based on the mRNA transcriptome could provide valuable information concerning all these parallel processes and pick up the earliest signs for toxicity.

As discussed before, the expression of RNA transcripts originating from the mitochondrial genome can serve as indication of mitochondrial perturbation [Mercer 2011]. However, most of the mitochondrial proteins are encoded by the nuclear genome. Transcriptomic approaches monitoring the complete genome would provide information concerning all nuclear encoded mitochondrial proteins, and additionally give insights into the induction or inhibition of any other cellular process. For instance, the assessment of the cellular transcriptomic profile upon chemical exposure enabled the identification of toxic vs non-toxic concentrations of Manganese [Fernandes 2019] or chemicals that mimic disease states [Pearson 2016]. Furthermore, transcriptomic changes upon rotenone exposure helped to identify more detailed information concerning mode of action, recovery, and the threshold for cell death [Harris 2018].

Integration of transcriptomics profiles into chemical assessments

The integration of large-scale transcriptomic profiles into risk assessment requires more sophisticated data handling than the assessment of a small number of relevant





F)

Module	Hubgene	Full name	Function
34	UQCR10	Ubiquinol-Cytochrome C Reductase, Complex III SX	Mitochondrial subunit (complex III)
47	MRPS16	Mitochondrial Ribosomal Protein S16	Supports protein synthesis in the mitochondria
84	LOC301124	NA	Cristae formation mitochondria *
93	Bloc1s1	Biogenesis Of Lysosomal Organelles Complex 1	Synthesis organelles of endosomal-lysosomal system
99	MRPL34	Mitochondrial Ribosomal Protein L34	Supports protein synthesis in the mitochondria
128	TMEM126B	Transmembrane Protein 126B	Component of mitochondrial complex I
166	SMIM8	Small Integral Membrane Protein 8	*No clear function defined*

G)

Module	Hubgene	Full name	Function
26m	RGD1563941	Chromosome 11 Open Reading Frame 71	*No clear function defined*
28m	NDUFB7	NADH:Ubiquinone Oxidoreductase Subunit B7	Mitochondrial subunit (complex I)
37m	MRPL27	Mitochondrial Ribosomal Protein L27	Supports protein synthesis in the mitochondria
67	MRPS17	Mitochondrial Ribosomal Protein S17	Supports protein synthesis in the mitochondria
96m	NDUFA9	NADH:Ubiquinone Oxidoreductase Subunit A9	Mitochondrial subunit (complex I)
103	MTFP1	Mitochondrial Fission Process 1	Mitochondrial dynamics
116	SLC13A2	Solute Carrier Family 13 Member 2	Succinate transporter *
126	SMIM8	Small Integral Membrane Protein 8	*No clear function defined*
185	TIMM13	Translocase Of Inner Mitochondrial Membrane	Chaperone in import of proteins from cytoplasm
221	TFB2M	Transcription Factor B2, Mitochondrial	Mitochondrial specific transcription factor
229	PRELID3B	PRELI Domain Containing 3B	*No clear function defined*
264	NDUFA6	NADH:Ubiquinone Oxidoreductase Subunit A6	Mitochondrial subunit (complex I)
274	SMIM20	Small Integral Membrane Protein 20	Assembly Mitochondrial complex IV
396	DEPDC7	DEP Domain Containing 7	*No clear function defined*

Figure 3: Gene co-expression analysis
A) Schematic representation of the performance of gene ontology (GO) enrich using a module network created using weighted co-regulated gene-network analysis. 1) the WGCNA analysis performed by Callegro *et al* unpublished work is based on microarray data for … compounds in both liver and kidney rat tissue, 2) the WGCNA analysis results is captured in a map depicting the hierarchical clustering of all identified gene modules and 3) The created network map can be used to study clustering of relevant mitochondrial GO term like GO:0042775 which represents mitochondrial ATP synthesis coupled electron transport. The color tone and the size represent the p.value per module for the selected GO term (the larger and darker the circle the more significant). **B)** Annotation of GO terms per module in the *in vivo* rat liver module map including GO terms describing 11 terms in combination with mitochondria. **C)** Zoom in on a branch of the *in vivo* rat liver map including a high density of mitochondria related labeled modules. **D)** Annotation of GO terms per module in the *in vivo* rat kidney module map including GO terms describing 11 terms in combination with mitochondria. **E)** Zoom in on a branch of the *in vivo* rat kidney map including a high density of mitochondria related labeled modules. **F)** Table depicting for the modules in C the hubgene and its function. **G)** Table depicting for the modules in E the hubgene and its function.

markers. One way to summarize the information from thou3sands of gene changes is the search for enrichment of terms belonging to predefined classes like gene ontologies or components of specific biological pathways as described in for instance KEGG, Reactome or Wikipathways [Ashburner 2000, Carbon 2021, Kanehisa 2000, Jassal 2020, Martens 2021]. Both an increase and a decrease in related terms can help to identify mode of action, adaptive vs toxic concentrations or off target effects.

Another option is the use of machine learning strategies, which enables the clustering of transcripts into biologically relevant and understandable subgroups without using predefined classes [Tarca 2007]. The subgroups can be defined using unsupervised and supervised methods, which support different types of comparisons. The unsupervised methods support clustering of for instance chemicals based on similarities of their induced gene expression pattern without prior knowledge. This information can be used in read across cases to predict the outcome of a target chemical based on a biological/chemical analogue (also called the source chemical). Supervised methods can be used to label new chemicals as inhibitors of for instance mitochondrial metabolism or OXPHOS based on expression patterns induced by treatment with chemicals known to inhibit these key events.

A widely used method to perform gene clustering by the unsupervised approach is gene co-expression analysis. Gene co-expression analysis assesses correlations between gene expression of all included genes and creates subgroups of genes (modules), which demonstrate correlated changes upon exposure to for example a chemical. This has been done in the past based on the microarray expression patterns obtained upon exposure to 170 chemicals, 3 concentrations, 3 time points and 3 different modules (primary human hepatocytes, rat liver slices and rat kidney slices) (available in the TG-GATES database) (figure 3A) [Callegaro 2021, Sutherland 2018, Igarashi 2015]. The obtained gene modules can be studied for their enrichment in gene ontology terms, like mitochondrial related processes (figure 3B (*in vivo* rat liver data) and D (*in vivo* rat kidney data)). Clustering of mitochondrial gene ontology terms is observed in gene modules created with *in vivo* data of rat both in the liver and kidney (figures 3C & F (*in vivo* rat liver data) and 3E & G (*in vivo* rat kidney data)). The expression patterns of these module clusters could be

included in the assessment of chemical-induced mitochondrial perturbation. A more supervised assessment including the defined module tree could include the inclusion of well-known mitochondrial toxicants into the teaching set of chemicals (at the moment mainly consisting of drugs), to create a module expression pattern that represents the selected class of chemicals. This profile could be used as a basis to identify chemicals with a similar mode of action.

Proteomics

Study of the cellular proteome upon chemical exposure creates the opportunity to follow both expression and compartment specific localization of proteins [Aslam 2017]. The increase in newly translated proteins correlates with the presence of newly transcribed mRNA. The total protein pool is not one-to-one correlated to the mRNA levels in the cell. Differences in protein expression in mammalian cells could only be captured for 40% using mRNA expression data [Tian 2004]. Protein levels are subject to variance in synthesis and degradation rates. On top of that protein functioning is also dependent on post-translational modifications, which adds a lot of extra information with more than 300 possible modifications [Zhao 2009]. The study of the proteome plus the related post-translational modifications informs about the reason for the actual observed phenotypic response, be it adaptation or adversity.

Methods

High through-put quantification of changes in the proteome is based on the relative expression of certain peptides between a test and control sample. Peptides present in the sample can be labeled using different methods: ICAT, ICPL, iTRAQ or SILAC [Shiio 2006, Schmidt 2005, Gan 2007, Ong 2017, Geiger 2011]. All labeling methods are based on the incorporation of a light atom in one sample and a heavy atom in the other, which can be for instance C12 vs C13 or H0 vs H1. The difference between the two atoms can be identified using tandem MS (MS/MS). Here, two MS analyze samples in series resulting in better separation/quantification of atoms with very similar sizes [Mclafferty 1980]. ICAT/ICPL labels consist of 1) an affinity tag, which can be used for extraction and with that reduction of complexity before the MS analysis, 2) linker with the light/heavy atom and 3) group reactive towards cysteine groups for ICAT and toward lysine groups and N-termini for ICPL. ICPL tags are designed with more than two types of heavy atoms creating the possibility to study more than two samples in one MS run. iTRAQ is a variant from ICPL, which also labels N-termini and protein side chains. They differ in their starting masses, because iTRAQ probes (one sample can be a multiplex of up to 8 different labels) all have the same mass. This results in one peak in the first MS run. The first MS run is followed by a fragmentation round resulting in up to 8 labels with different mass, which will be separated in the second

MS round. This approach helps to reduce the complexity and improves interpretation of the output of the first MS round. Finally, samples can also be labeled with light and heavy atoms before collection by using medium with amino acids containing light or heavy atoms (methods = SILAC).

Sample collection for proteomic studies requires the integration of an identifiable tag, as described above, but also the type of extraction requires consideration based on the study aim. For instance, extraction of membrane protein requires the use of specific detergents to solubilize the protein [Lee 2018]. Different types of detergents can have various effects on protein structure, charge or protein-protein interactions. Furthermore, inclusion of for example post-translational modification requires specific enrichment for the studied modification.

Integration into AOPs

The presence, expression levels and functionality of proteins, like detoxifying enzymes, during the chemical-exposure determines if a cell will be able to survive chemical-induced malfunctioning and additionally if this survival will be with or without permanent damage.

In the 21st century, proteomics is increasingly used in toxicology assessment and so far applied on drugs, natural products, industrial chemicals, metals and nanoparticles [Rabilloud 2015]. The data is used for identification of new biomarkers that are predictive for the occurrence of certain type of toxicity and their severity [Wetmore 2004]. The study of individual protein levels, like chemical targets, in multiple tissues can also be used to predict susceptibility of these particular tissues towards toxicity or predict susceptibility of specific individuals towards certain chemical-induced adversities [Van Summeren 2012, Kennedy 2008]. Besides single protein assessments, proteomics data can (like the transcriptomics data) be used for functional assessments (protein-protein interactions and enrichment of particular groups or pathways) and network analysis unraveling new interactions that drive machine learning approaches [Titz 2014].

It is also important to keep the high level of proteome complexity in mind. Proteins can be studied at multiple levels beyond the identification of amino acid sequences, including protein folding, post-translational modifications and protein-protein interactions. Thus far, the use of proteomics in toxicology is still challenging, because the technique does not yet support detection of low abundant proteins, very large-scale analyses, and, although further developed than metabolomics, the available reference databases for protein annotations are not complete [Manzoni 2018].

Integration of proteomics into AOPs, although limited, has been reported for example for endocrine disrupting agents [Johansson 2020], and triclosan [Guo 2018]. In addition, there is the option to study specifically post-translational modifications of the proteome, which was reported in an AOP context by Smith *et al* [Smith 2018].

Mitochondrial studies using proteomics

Starting from the 21st century proteomic studies have been used for the assessment of effects caused by various chemical substances. Rabilloud *et al* noted that a large number of these studies reported changes in the expression of mitochondria-related proteins (10 out of 31 drugs studies, 7 out 17 natural products studies, 14 out 30 industrial chemicals studies and 2 out of 19 nanoparticles studies) [Rabilloud 2015]. Identification of mitochondrial protein targets helps to find the potential mode of action of chemicals. However, time and concentration dependency should not be overlooked, because mitochondrial perturbation can be both the initiating event and a later key event.

Assessment of the proteome upon mitochondrial perturbation is usually only done for the mitochondrial proteome. The mitoproteome is studied in isolated mitochondria and provides information on the proteins within the mitochondrial membrane and matrix. However, this provides very limited information concerning the status of the mitochondria. The use of the whole proteome, including the mitochondrial proteins encoded by nuclear DNA and the transcription factors involved in mitochondrial retrograde and anterograde signaling, will provide more detailed information about the mitochondria and its responses upon stress [Kühl 2017].

Combined omics approaches

The combination of transcriptomic, proteomics and metabolomics studies provides multi-level information concerning the response of the cell to a chemical insult. Linking mRNA variations to changes in protein levels and eventually to excreted metabolite levels provides the opportunity to study the effect of a chemical-induced increase in gene expression upon adaptation/survival of cells/tissues/organs. What is the required change in mRNA expression before significant changes in protein expression are observed? Are these thresholds similar or distinct in different tissues? Do enriched pathways determined by transcriptomics result in actual pathway activity? Can transcriptional responses and subsequent proteomic alterations be linked to a more easily accessible marker for mitochondrial toxicity such as metabolomics analysis in body fluids of test species or people? Progress in this area will aid the development of quantitative AOPs.

Integration of all omics techniques into the assessment of effects induced by single chemicals has been done for example for cyclosporin A [Wilmes 2013], cisplatin [Von Stechow, Puigvert 2013 2013, Wilmes 2015, Späth 2019] and methapyrilene [Craig 2006]. This approach provides the opportunity to study the link between certain phenotypes, environmental factors and the cellular factors [Williams 2016]. Combination of transcriptomics, proteomics and metabolomics to identify biomarkers in an AOP driven approach has been described [Rodrigues 2018].

Future perspective and conclusion

Nowadays chemical risk assessment faces the challenge of evaluating large numbers of chemicals that might affect human health. Over the years numerous assays have been developed to assess known risk factors in *in vitro* approaches, which should minimize the use of animal tests. Recently, more data-rich technologies like omics enter this arena of risk assessment. To make chemical risk assessment as efficient and coherent as possible, it is important to integrate these different methodologies in biologically relevant testing strategies. One way to do this is the use of AOPs. In this review, chemical-induced mitochondrial toxicity has been discussed using an AOP inspired approach. We highlight the importance of key event identification, the broad range of available key event specific assays and the integration of the methodologies into quantitative assessments.

The use of AOP relationships in risk assessments, and in particular in qAOPs, helps to link and quantify biological events observed during the development of adversity. The readout of one assay alone can easily miss specific types of toxicity, for instance toxicity resulting from concentration-/time-dependent accumulation or toxicity only occurring in specific cells/tissues. The combination of multiple related (as defined in a specific AOP) measurements will allow a broader readout and thereby help to create confidence about the identified chemical hazard.

The integration of omics technology is thus far not standard in regulatory chemical risk assessment. The technology is very data-rich and can provide a broad picture of cellular fitness, including interactions between signaling pathways and well-known toxicity markers. Simple assays are very valuable to study for instance target binding specificity and efficiency. Transcriptomics adds the opportunity to identify unexpected off-target effects which may otherwise only be discovered after running large sets of *in vitro* assays representing all facets of toxicology, which will eventually only lead to identification of known chemical-targets. In addition, the use of omics will also make it easier to detect (dis)similarities between chemicals, which can be used to categorize them into specific classes.

The AOP-driven biologically relevant combination of multiple targeted *in vitro* assays performed in cell types of different complexities and high throughput omics supports flagging of hazardous substances, that require further testing to study species/tissue specific metabolism, possible accumulation and tissue specific forms of toxicity. Omics technologies when applied in multiple cell systems also helps to add information concerning genetic diversity and the difference in overall cellular responses between multiple systems.

Although most of the established assay systems are very well suited for the identification of toxic and non-toxic conditions, their relevance for the human situation is not always clear. At the moment we still rely on animal-based approaches for final decision making, especially for chemicals which are not easily assigned toxic or non-toxic. To enable the use of AOP-driven combinations of target *in* vitro methodologies and omics technologies to their full potential, they require validation using large sets of diverse chemicals which will help to distinguish toxic from non-toxic conditions. This will improve flagging of the possible hazardous substances class and use the *in vitro* technologies to their full potential. Therefore, broadening our understanding of the opportunities and limitations of all available methodologies will support the assemble of biologically relevant testing strategies, and improve *in vitro* based chemical related (mitochondrial) risk-assessment.

Reference numbers

1= Reisch 2007, 2= Kerr 2012, 3 = Nonnemacher 2017, 4 = Murphey 1967, 5 = Else 1988, 6 = Park 2000, 7 = Schwab 2005, 8 = Jones 2013, 9 = Huynh 2014, 10 = Ma 2020, 11 = Uchinomiya 2020, 12 = Grandl 2010, 13 = Wiederschain 2011, 14 = Clark 1953, 15 = Diepart 2009, 16 = Wu 2010, 17 = Wittenberg 1970, 18 = Anderson 1999, 19 = Mik 2008, 20 = Penjweini 2018, 21= Rumsey 1988, 22= Hynes 2003, 23 = Hynes 2006, 24 = Takahashi 2006, 25 = Will 2007, 26 = Dimitriev 2012, 27 = Wang 2014, 28 = Ferrick 2008, 29 = Gerencser 2009, 30 = Ribeiro 2014, 31 = Vivakaruni 2014, 32 = Yepez 2018, 33 = Van der Stel 2020, 34 = Kida 2004, 35 = Bobko 2009, 36 = Liu 2009, 37 = Diepart 2009, 38 = Williams 2009, 39 = Halevy 2010, 40 = Christen 2014, 41 = Danhier 2014, 42 = Kennedy 1997, 43 = Rosenberger 2009, 44 = Liu 2009, 45 = Banaji 2010, 46 = Perry 200, 47 = Lemaster 2007, 48 = Vos 2007, 49 = Befroy 2012, 50 = From 2017, 51 = Chen 2018, 49 = Befroy 2012, 50 = From 2017, 51 = Chen 2018, 52 = Manfredi 2002, 53 = Bhatt 2012, 54 = Wilson 1998, 55 = Crouch 1993, 56 = Contag 1997, 57 = Di Virgilop 2006, 58 = Rahendran 2016, 59 = Kim 2010, 60 = Imamura 2009, 61 = Depaoli 2019, 62 = Yaginuma 2014, 63 = Ozalp 2010, 64 = Berg 2009, 65 = Lee 2004, 66 = Xu 2009, 67 = Liu 2013, 68 = Wang 2016, 69 = Birch-Machin 2001, 70 = Spinazzi 2012, 71 = Durand 2017, 72 = Giedt 2016, 73 = Ouellet 2017, 74 = Harwig 2020, 75 = Vowinckel 2015 (e.g. mitoloc), 76 = Berman 2008, 77 = Karbowski 2015, 78 = Meeusen 2007, 79 = Jourdain 2010, 80= Smirnova 2001, 81 = Harder 2004, 82 = Gawlowski 2012, 83 = Baker 2014, 84 = Nilsson 2015, 85 = Hsieh 2018, 86 = Memon 2017, 87 = Fazzini 201, 88 = Popov 2020, 89 = Rizzuto 1992, 90 = Inouye 1993, 91 = Markova 2002, 92 = Fonteriz 2010, 93 = Jafarian 2011, 94 = Aghamaali 2011, 95 = Markova 2012, 96 = Bonora 2013, 97 = Bakayan 2016, 98 = Miyawaki 1997, 99 = Miyawaki 1999, 100 = Heim 2004, 101 = Garaschuk 2007, 102 = Palmer 2016, 103 = Baird 1999, 104 = Griesbeck 2001, 105 = Nagai 2001, 106 = Fonteriz 2010, 107 = Akimzhanov 2011, 108 = Zhao 2011, 109 = Waldeck-Weiemair 2019, 110 = Minta 1998, 111 = Thomas 2000, 112 = Fonteriz 2010, 113 = McKenzie 2017, 114 = Grynkiewicz 1985, 115 = Katayma 2011, 116 = Um 2018, 117 = Sargsyan 2015, 118 = Dolman 2013, 119 = Patergnani 2015, 120 = Hammerling 2017, 121 = Eskelinen 2011