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Mass-spectrometric multi-omics linked to function – State-of-the-art investigations of mitochondria in systems medicine☆

Lisa Kappler [a], Rainer Lehmann [a, b, c, *]

[a] Institute for Clinical Chemistry and Pathobiochemistry, Department for Diagnostic Laboratory Medicine, University Hospital Tübingen, 72076, Tübingen, Germany
[b] Core Facility DZD Clinical Chemistry Laboratory, Department for Molecular Diabetology, Institute for Diabetes Research and Metabolic Diseases (IDM) of the Helmholtz Zentrum München at the University of Tuebingen, Tuebingen, Germany
[c] German Center for Diabetes Research (DZD), Tuebingen, Germany

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ABSTRACT

Multi-omics applications are essential tools in systems medicine and biology. Alterations in molecular fingerprints are closely related to changes in cellular and subcellular function, but a linkage of functional investigations and omics analyses is quite rare. In mitochondria, lipids, proteins and post-translational modifications thereof are directly linked to function but also to structure. However, the complementary combination of multi-omics analyses and functional investigations in mitochondrial research is challenging. Analytical chemists can implement this strategy in systems medicine projects, thereby providing comprehensive results as a base for correct data interpretation and a deeper understanding of the role of mitochondria in health and disease. In this review we will discuss state-of-the-art approaches of this multistep process focussing on analytical, but also pre-analytical aspects, like mitochondrial purification and sample quality control, as joint key for valid functional multi-omics investigations of mitochondria by complementary lipidomics, proteomics and respiratory analyses.

1. Introduction

Mitochondria, the cellular powerhouse, play a vital role in many aspects of biology and medicine [1]. From yeast to humans they are the site of the tricarboxylic acid cycle, β-oxidation of fatty acids, and the respiratory chain (electron transport chain; ETC). Numerous diseases like cancer, type 2 diabetes, neurological disorders etc. are directly linked to alterations in mitochondrial function [1], and mitochondrial diseases are one important aspect in newborn screening [2]. In tissues, mitochondria are tailored to different specific needs and demands [3]. Hence, an in depth understanding of pathologies would allow the development of personalized therapies and treatments. The molecular bases of mitochondrial alterations affecting proper physiological function are only partially understood. Lipids and proteins have been shown to be directly linked to mitochondrial structure and function [4–6]. Very likely a combined mitochondrial dysregulation of lipid and protein homeostasis is etiologically associated with the pathogenesis of many diseases, or is even the underlying pathomechanism. But up to now lipid and protein profiling had been detached and a combination of lipidomics and proteomics data is quite rare in general and particularly in mitochondrial studies [7].

Recently, comprehensive investigation by multi-omics strategies came more and more in the focus of analytical chemists [8]. By definition the intention of multi-omics strategies is to analyse the same samples with two or more omics-methods, to integrate the diverse omics data to find coherently matching relationships and reach a comprehensive, overall picture of biomedical processes [8]. Systems medicine and systems biology are the major fields of application for multi-omics. One of the keys in mitochondrial research could be analytical combinations of lipidomics and proteomics, including profiling of post-translational modifications (PTMs) of proteins. Combining a multi-omics approach with functional investigations like high-resolution respirometry in this context enables to cover not only mitochondrial molecular fingerprints of lipids and proteins, but also the impact of detected alterations for mitochondrial key functions.

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* Corresponding author. Institute for Clinical Chemistry and Pathobiochemistry, Department of Diagnostic Laboratory Medicine, University Hospital Tuebingen, Hoppe-Seyler-Str. 3, 72076, Tuebingen, Germany.
E-mail address: rainer.lehmann@med.uni-tuebingen.de (R. Lehmann).

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Abbreviations

2D	two-dimensional
ATP	adenosine-5′-triphosphate
CER	ceramide
CID	collision induced dissociation
CL	cardiolipin
CS	citrate synthase
DAG	diacylglycerol
DC	differential centrifugation
DDA	data-dependent acquisition
DIA	data-independent acquisition
DI-MS	direct infusion MS
ER	endoplasmic reticulum
ESI	electron spray ionization
ETC	electron transport chain
IMS	ion mobility spectrometry
IS	internal standard
HILIC	hydrophilic interaction chromatography
HR	high resolution
LC	liquid chromatography
MACS	magnetic cell isolation and separation
MRM	multiple reaction monitoring
MS	mass spectrometry
MS/MS	tandem mass spectrometry
MSI	mass spectrometric imaging
MTBE	methyl *tert*-butyl ether
NP	normal phase
nLC	nano-liquid chromatography
oxCLs	oxidized cardiolipins
PAGE	polyacrylamide gel electrophoresis
PC	phosphatidylcholine
PE	posphatidylethanolamine
PG	phosphatidylglycerol
PI	phosphatidylinositol
PL	phospholipid
PRM	parallel reaction monitoring
PS	phosphatidylserine
PTM	post-translational modification
ROS	reactive oxygen species
RP	reverse phase
SIMPLEX	simultaneous metabolite, protein, lipid extraction
SM	sphingomyelin
SRM	selected reaction monitoring
UC	ultracentrifugation

In this review we will discuss current state-of-the-art analytical, but also pre-analytical aspects for multi-omics profiling linked to mitochondrial function by complementary lipidomics, proteomics and respiratory analysis. Functional analysis will be described in very brief in chapter 5, detailed methodological information is provided in a recent review [9]. The coherent combination of molecular fingerprints and functional aspects is a prerequisite for a deeper understanding of the role of mitochondria in health and disease.

2. Mitochondrial isolation and sample preparation for multi-omics and functional analyses

2.1. Preanalytical aspects and pitfalls

A big difference to analytical approaches aiming at lipid or protein profiles of cells or tissues is the inclusion of an appropriate purification in the sample preparation process as prerequisite for subcellular analyses. Selecting the most suitable isolation procedure for mitochondria is quite challenging. Various procedures exist in terms of time investment and required equipment, but also to the resulting sample quality (i.e. mitochondrial purity and enrichment) [10,11]. Since the sample amount in comprehensive biomedical studies, e.g. human biopsies, is limited and often rather small, another challenge is to isolate sufficient amounts of adequatly pure mitochondria.

A pitfall with tremendous impact on the results of high-resolution multi-omics investigations and functional studies is scarce purity of mitochondrial extracts. Misleading data or misinterpretation of the results, as well as irreproducibility of the data are the consequences. In view of the fact that multi-step isolation procedures are prone to random errors, the sample purity, mitochondrial enrichment and functionality need to be controlled on a regular basis. Remaining intact contaminant organelles, debris thereof, or compounds of other cell compartments like the cytosol should be controlled in samples of purified mitochondria to avoid erroneous conclusions drawn from lipidomics or proteomics data, since rarely lipids and proteins are specific for mitochondria. Functional integrity of mitochondria can easily be checked for instance during respirometric measurements [11]. In the following paragraphs 2.2 and 2.3 overviews over mitochondria isolation, purity control, and sample preparation for multi-omics analysis are discussed (see also a schematic overview in Fig. 1).

2.2. Purification of mitochondria from cells and tissues

Differential centrifugation (DC) is a simple, fast and therefore frequently applied procedure to isolate intact and vital mitochondria from tissues or cell cultures (for methodological details see Refs. [10,11]). In brief, tissues or cells are minced and homogenised, commonly using a Dounce homogeniser. Other homogenisation procedures are for instance sonication or detergent-based cell disruption (e.g. by digitonin [12]). Subsequently, a slow (800−1000 g) followed by a high speed (8000 to 10,000 g) centrifugation step is performed. The first step sediments unbroken cells and cell debris. The second step, using the supernatant of step one, is to pellet mitochondria at high speed. DC is particularly suited for mitochondria isolates for functional investigations. For omics research the purity of samples generated by DC should be considered as inadequate for high resolution profiling analysis of lipids or proteins because of contaminating organelles or subcellular compounds [11]. One important exception is the use of DC for targeted LC-MS analyses of mitochondria-specific compounds like cardiolipins. In such a case either the quick and easy to perform DC is suitable or the direct use of tissue is feasible [13,14].

High purity, needed for high-resolution multi-omics analysis, can be achieved by a combination of DC followed by high-speed ultracentrifugation with a density gradient (UC; 30,000 to 80,000 g; e.g. percoll or sucrose gradient) (Fig. 1) [11]. One drawback of UC, besides the necessity to have access to an ultracentrifuge, is an unavoidable loss of up to 50% of mitochondria, which should be considered if e.g. the available tissue starting amount is low.

An alternative purification strategy is the application of a commercial procedure applying MAgnetic Cell isolation and Separation (MACS). The procedure was originally described by Hornig-Do et al. [15]. Homogenates of cells or tissues are incubated with mitochondria-specific anti-TOM22-antibodies, which are

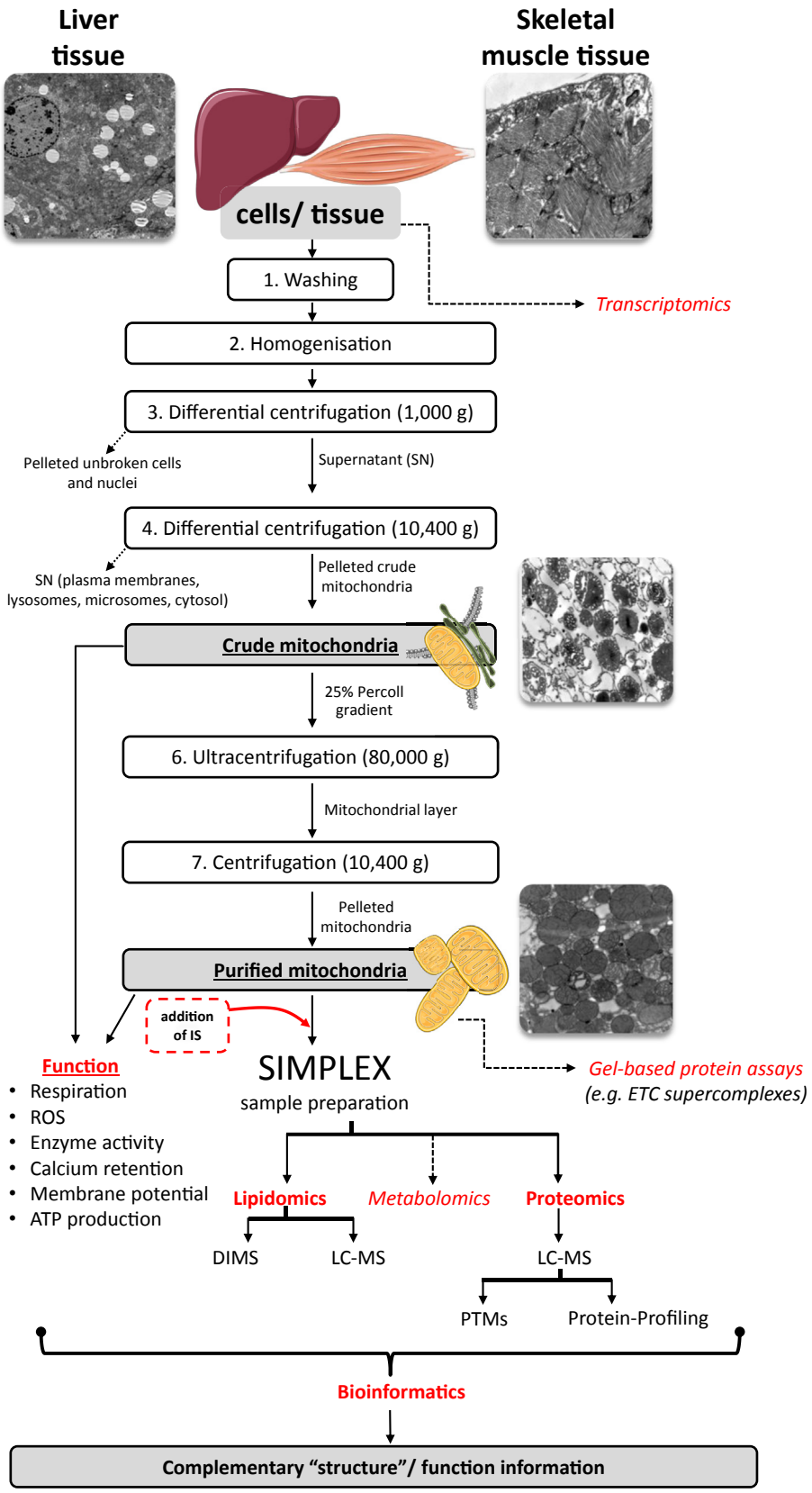

Fig. 1. Schematic overview of the workflow and analytical approaches of complementary multi-omics and functional state-of-the-art investigations of mitochondria in systems medicine. ATP, adenosine-5′-triphosphate; DI-MS, direct infusion mass spectrometry; ETC, electron transport chain; IS, internal standards; LC-MS, liquid chromatography-mass spectrometry; PTMs, post-translational modifications; ROS, reactive oxygen species; SN, supernatant. Electron microscopic illustrations were kindly provided by P. Fallier-Becker (Institute for Pathology, University Hospital of Tuebingen).

conjugated to paramagnetic beads. The bead/antibody/mitochondrion complex is then isolated in a magnetic field. Purification by MACS is more rapid and achieves higher yields of mitochondria from mouse tissue than a combination of DC and UC [15]. A comparison of liver mitochondria isolated by MACS or DC showed a better physiological function (respiration rate) and in a proteomics analysis less contaminations by other organelles in samples isolated by MACS [16]. However, we demonstrated recently in cell culture samples that isolation by DC + UC for subsequent lipidomics analysis was superior in comparison to MACS or DC alone with respect to contaminations by other organelles [11].

2.2.1. Purity control of mitochondria isolates

Specific mitochondrial lipids and proteins can be used to determine the mitochondrial content and enrichment in the isolates (see 2.2.2). Ideally, quantitative targeted MS analysis of distinct specific markers of contaminants and generation of abundance ratios should be integrated in multi-omics approaches to evaluate and rule out relevant impurities (see 2.2.3). Noteworthy, complete purity of mitochondria can be hardly achieved due to their direct physical contact sites with other organelles like endoplasmic reticulum (ER). Traces of compounds from other subcellular structures will always remain. A threshold for a tolerable percentage of contaminations from other cellular compounds should be defined. In our hands the amount of contaminations in mitochondria isolated by DC + UC lies around 10% of non-mitochondrial proteins according to a evaluation using a mitochondrial database (for details to the data bases see chapter 2.2.3).

2.2.2. Markers to determine mitochondrial content and enrichment

The prerequisite for mitochondrial markers is, that the selected lipids or proteins are unique and solely present in mitochondria. The only lipid class fulfilling this criterion are cardiolipins (CLs). In a study investigating skeletal muscle biopsies from humans, CLs are described to be excellent markers of mitochondrial content [17]. Of note, the CL content relative to the total tissue phospholipid content can vary tissue- and cell-type-dependently (e.g. low in liver and high in heart [14]). Aside from exact quantitative MS parameters, the determination of citrate synthase (CS) activity is a long-standing well accepted measure for the quantification of mitochondrial content. In a comparison of common markers Larsen et al. showed the strongest association of mitochondrial content (determined by the gold standard, i.e. transmission electron microscopy) with cardiolipin content, followed by CS activity and ETC content as well as complex activity [17]. No correlation to mitochondrial content was achieved for mtDNA [17].

2.2.3. Markers to detect impurities in mitochondria isolates

The origin and amount of impurities in samples of isolated mitochondria can also be quantified by characteristic subcellular marker proteins. Examples for such organelle-specific impurity markers are the chaperons GRP78/BIP and calnexin for ER contaminations, perilipin 2 for lipid droplets, giantin for the Golgi apparatus, lysosomal membrane glycoprotein 1 for lysosomes, and lamin B and fibrillarin for the nucleus, as well as glutamine fructose-6-phosphate aminotransferase for cytoplasm. A proteomics based comprehensive list of organelle specific marker ensembles was recently published [18]. Quantification of these markers by MS is the method of choice, but less sensitive and less specific immunological detection is also feasible. Targeted MS-based proteomics analysis for exact quantification even of low abundant markers is described in chapter 4.2.

It is important to note, that based on the (patho)physiological conditions organelles might tether in more or less close proximity to mitochondria, like for example mitochondrial-associated membranes (MAM). MAMs result from communication between ER and mitochondria and contain therefore not only mitochondrial proteins and lipids, but also from ER. Special procedures are described to specifically isolate these MAMs [19].

An alternative approach to detect impurities, described by Walheim et al., is the comparison of a list of all identified proteins (i.e. a list of UniProt or gene-IDs) with the entries in a mitochondrial database (e.g. MitoMiner v4.0) allowing the calculation of the share of proteins that are evidently localized in the mitochondria [20]. Preferably only proteins which were identified by $\geq$ 4–6 unique peptides and which passed a false discovery rate (FDR) test with a setting of $\leq$1% should be included in the alignment [20].

2.3. Sample pretreatment of purified mitochondria for multi-omics mass spectrometric analysis

After purification of mitochondria from cells or tissues, the next processing step is the extraction of all compounds of interest. Before starting extraction, a share of each sample is usually used to determine the protein amount of the isolated mitochondrial fraction (e.g. by Bradford or bicinchoninic acid assays). By this, in the final data evaluation the levels of detected lipids, proteins or other metabolites can be adjusted to the used mitochondrial protein amount. Furthermore, before the extraction carefully selected internal standards (IS) should be added to the samples. The selection of IS for valid analysis and quantification of complex lipid species was recently discussed in detail [21]. Importantly, in proteomics analysis aiming to cover also post-translational modifications, specific enzyme inhibitors should be added to all buffers throughout the sample processing, such as phosphatase inhibitors (e.g. PhosSTOP®, Sigma-Aldrich) for the investigation of the mitochondrial phosphoproteome. Moreover, prevention of lipid oxidation could be another relevant preananalytical consideration, recently studied in detail by the group of M. Giera in plasma samples [22].

An eminently suited sample preparation strategy for MS-driven multi-omics projects was recently developed by the group of R. Ahrends [23]. The method is entitled SImultaneous Metabolite, Protein, Lipid EXtraction (SIMPLEX) [23]. SIMPLEX leads simultaneously to sample extracts for lipidomics, metabolomics and proteomics analyses, including phosphoproteomics (Fig. 1) and is therefore the extraction method of choice for sample preparation in multi-omics projects in systems medicine studies. The extraction is methyl *tert*-butyl ether (MTBE)-based, performed in just one sample tube and is therefore suitable for very low sample amounts, like small pieces of human biopsies. A detailed description of all steps is provided in Ref. [23]. By saving valuable sample material (e.g. of human origin) compared to other extraction methods, SIMPLEX opens up perspectives for additional analyses such as subsequent targeted, quantitative MS-analysis, or further biochemical, molecular, or histological investigations.

3. Lipidomics analysis of mitochondrial lipids

Mitochondrial lipids are essential for membrane structure and fluidity, mitochondrial fusion and fission, electron transport chain (ETC) assemblage, as signalling molecules and much more [24]. These organelles are not only the site for acylchain degradation to gain energy via oxidation of fatty acids, but also sites for lipid remodelling, synthesis and inter-organellar lipid trafficking. The lipid profile of mitochondria is dominated by the following classes: phosphatidylcholines (PC) ~40 mol%, phosphatidylethanolamines (PE) ~35 mol%, cardiolipins (CL) ~10–15 mol%, phosphatidylinositols (PI) 10 mol%, and phosphatidylserines (PS) ~2 mol% and very few sterols and sphingolipids (note: some tissues contain also PC

and PE plasmalogens in the range of 5–30 mol% of total phospholipids) [24].

In 1994 Han and Gross were first demonstrating the application of MS for lipid profiling [25] and introduced in 2003 the concept and the term lipidomics [26]. Currently, various analytical strategies and techniques are applied to investigate lipid species in subcellular compartments, cells, tissues or living organisms [27]. But comprehensive lipid investigation is an ambitious task and analytically quite challenging, since lipids are very heterogeneous, structurally distinct and diverse molecules. Additionally, in particular in some types of esterified long-chain polyunsaturated fatty acid residues, modifications like peroxidation may occur *in vivo*, namely at C18:2, C18:3, C20:3, C20:4, C20:5, C22:5 and C22:6, which further increase the complexity of the lipidome. But, most of these lipid oxidation products are low abundant, and thus are not often analytically covered (0.03–3.0 mol% of total non-oxidized lipids [28]).

Since 2005, lipids are grouped into eight categories according to their chemical and biochemical properties, namely fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides [29]. In the LIPID MAPS database (http://www.lipidmaps.org) a daily increasing number, currently more than 43,500 lipid structures (as of August 2019), are listed. From an analytical point of view there are still numerous perspectives for new developments and method improvements in lipidomics [27]. A still remaining major bottleneck in multi-omics is the lack of sophisticated bioinformatic tools for combined handling, evaluation, pathway enrichment analysis as well as intelligible presentation of the very complex and big data. Recently initiatives started to tackle this severe limitation for lipidomics [30]. Additionally, the widespread application of lipidomics concomitant with big divergence in identification and quantification of lipids [31], emphasizes the need to follow standards how to report valid data [32] and which IS to select [21]. A comprehensive view on all these aspects is presented on the web page of the "Lipidomics Standards Initiative" (https://lipidomics-standards-initiative.org/).

In MS analysis of lipidomes the most common applied ionization techniques are electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) [27]. Generally ESI-MS can be performed either with an upstream chromatographic separation of lipids (typically liquid chromatography, LC), entitled LC-ESI-MS, or by Direct Infusion MS (DI-MS), meaning continuous injection of crude lipid extract mixtures via ESI into the MS for a defined time (also entitled as shotgun lipidomics; throughout this review we will use the technical term "DI-MS lipidomics"). MALDI is mainly used for Mass Spectrometric Imaging (MSI) of lipid structures. For details about MSI see Ref. [33].

3.1. Direct infusion-MS (DI-MS) lipidomics

DI-MS lipidomics is a simple and fast analytical strategy. Currently two different approaches are most commonly applied, namely tandem MS (MS/MS)- and high resolution (HR)-based DI-MS lipidomics (Fig. 2) [34]. The usual principle in MS/MS-based DI-MS lipidomics, typically performed on a triple quadrupole MS, is the generation of characteristic lipid class-specific head group fragments by collision- induced dissociation (CID). These fragments are common for all lipid species belonging to the same class. A detailed list of specific fragments can be found on the web page of the "Lipidomics Standards Initiative" (https://lipidomics-standards-initiative.org/resources/lipid-class-specific-fragments). Subsequent to scans of class-specific fragments, specific neutral loss (NL) or precursor ion (PI) scans are applied to detect typical fragments of acyl chains, which are then used to identify the chain composition and by that specific molecular species. One important prerequisite for MS/MS DI-MS lipidomics is the use of at least two internal standards spanning the range of chemical structures of the entire class of lipids [21]. These two or more IS are needed to correct the effects of differential fragment ion thermodynamics and fragmentation kinetics [21]. At low mass accuracy or resolution there is the risk of artificial ions, not belonging to the lipid class of interest, in the "class-specific" fragment scanning. Furthermore isobaric and isomeric lipids cannot be identified by this approach.

HR-based DI-MS lipidomics, mostly performed on quadrupole time-of-flight (qTOF), or Orbitrap MS, is suitable for broad qualitative and quantitative analyses. In contrast to MS/MS DI-MS lipidomics, false-positive identifications are largely reduced by the high mass resolution and the accurate determination of the mass-to-charge (m/z) ratio of fragment ions in selective windows. HR-MS can also be applied to perform data-independent analysis (DIA) [35] (Fig. 2). The advantage of DIA is a broader coverage range of identified lipids by scanning after fragmentation of the precursor ion, all fragments in isolation windows with defined m/z instead of recording a set number of most abundant fragments as in data-dependent analysis (DDA) [35] (Fig. 2). One variant of DIA using a hybrid quadrupole high-resolution time-of-flight (qTOF) with a 1 m/z isolation window entitled MS/MS^{all} was used for lipid profiling in biological samples [35]. DIA generates a huge amount of data for mining, including isomers and also the possibility to identify lipid species usually remaining undetected [36]. Other strategies to increase the coverage are multiplexing the extraction strategy, selective ionization of lipid classes with different electrical propensities [34], or the use of ion mobility spectrometry (IMS) [37] (Fig. 2). IMS means the separation and identification of ionized lipids based on their mobility in the gas phase of a carrier gas, which facilitates the identification with respect to isomers, conformers and enantiomers [37].

The biggest limitation of DI-MS lipidomics is a high susceptibility to sample matrix interference with lipid ionization. Cumbersome, additional sample pretreatments, such as solid phase extraction (SPE), may help to reduce these matrix effects, but may also lead to a loss of compounds of interest. A better choice for highly affected samples might be the application of LC-MS instead of DI-MS lipidomics.

3.2. LC-MS lipidomics

The general advantage of LC-MS lipidomics is the very efficient chromatographic separation prior to highly sensitive detection by MS. The preceding chromatographic step drastically reduces matrix effects as well as ion suppression, and can also be adapted to special analytical requirements. Isomers and isobars can be separated and highly sensitive targeted analysis can be performed easily and fully automatic [34]. It is feasible to detect also low abundant lipids, which are difficult using DI-MS. A recently reported nano-LC-nanoESI-MS approach further improves the sensitivity up to 3 orders of magnitude [38].

For the chromatographic separation of lipids, stationary phases with nonpolar as well as polar selectivity are applied. In reverse phase (RP)-LC, which is the most popular in lipidomics, lipid separation is based on the equilibrium between the hydrophobic bonded hydrocarbon chain(s) and the polar mobile phase. Hence, differences in acyl chain lengths and number of double bonds allow the separation of hundreds of lipids. A problem in RP-LC-MS could be that IS may elute at different retention times than the corresponding lipids, which could lead to differential ionization efficiency by differing solvent composition and/or matrix effects [39]. The use of stable isotope labelled IS, if available, dissipate this risk. In normal phase (NP)- or hydrophilic interaction chromatography

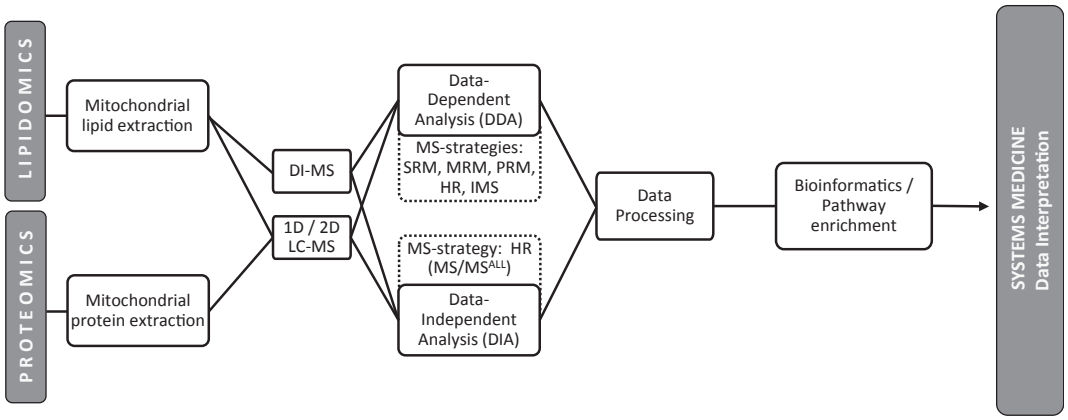

Fig. 2. Overview of mass spectrometry-based lipidomics and proteomics strategies in multi-omics approaches. DI, direct infusion; 1D, one-dimensional; 2D, two-dimensional; HR, high resolution; IMS, ion mobility spectrometry; LC, liquid chromatography; MRM, multiple reaction monitoring; MS, mass spectrometry; PRM, parallel reaction monitoring; SRM, selected reaction monitoring.

(HILIC) the separation of lipid classes is based on their polarity and here IS show similar retention as the compounds of interest [39].

The most common applied MS approaches in LC-MS lipidomics are selected or multiple reaction monitoring (SRM, MRM) with data-dependent analysis (Fig. 2). Up to now less common, but upcoming, are LC-MS DIA approaches (Fig. 2), opening up perspectives of an increase in coverage [40], as well as the implementation of IMS (reviewed in detail in Ref. [37]). Promising chromatographic approaches to increase the separation power like ultra-high-performance supercritical fluid chromatography (for methodological details please refer to Ref. [39]), as well as two-dimensional strategies like combining lipidomics and metabolomics analysis in one run and thereby saving valuable sample material in systems medicine approaches [41] were recently reported. LC-MS-driven lipidomics is also well-suited for the discovery and identification of novel lipids. The group of G. Xu developed an analytical strategy entitled "pseudotargeted lipidomics", which combines nontargeted and targeted lipidomics in one LC-MS run thereby covering 3377 targeted lipid ion pairs and >7000 lipid molecular structures [42]. To get deeper insights in mitochondrial lipid metabolism, stable isotope-assisted lipidomics approaches could be an additional analytical objective to study for example kinetics of mitochondrial lipid pathways, pathway crosstalks or metabolization of distinct lipids (for methodologic details to stable isotope-assisted lipidomics please refer to Refs. [43,44]).

3.3. *Cardiolipin, challenging analysis of the mitochondrial signature lipid*

This chapter will be centered on analytical aspects of one lipid class, namely the cardiolipins (CLs), since its presence is a special characteristic of mitochondrial membranes in distinction from all other eukaryotic (sub)cellular membranes [28]. For overviews of analytical applications of other mitochondrial lipid species by lipidomics as well as metabolomics by MS, please refer to a recent review by X. Liu et al. [7].

CLs are glycerophospholipids with a central glycerol connected by two phosphodiester linkages at C1 and C3 to two groups of diacylglycerol phosphatidic acid moieties, thus it has four acyl groups. CLs were first isolated in the 1940s from heart, which led to its designation. Cardiolipins represent 10–15 mol% of the lipid content of the inner mitochondrial membrane [24]. They interact with various mitochondrial proteins, and play important roles in the molecular organization of mitochondria, like cristae morphology, fission, integrity of respiratory chain complexes and function, as well as apoptosis, and signalling in ageing [45]. Noteworthy, CLs are sensitive to oxidation. The reasons are the close proximity to the major sites of ROS production in the inner mitochondrial membrane and their high unsaturation levels [28]. Dysregulation of CL composition and content or remodelling of CL acylchains is associated with mitochondrial dysfunction in many diseases [45].

Analytical coverage of the majority of CL species, including the hydrolytic metabolites mono-lyso-CLs and di-lyso-CLs can be achieved by RP- or HILIC-LC-MS approaches [28], as well as DI-MS lipidomics [35,46]. For the inclusion of oxidized CLs in the analytical profile three analytical challenges must be overcome: a) coverage of very low abundant compounds, b) potential overlapping of isobaric non-oxidized and oxidized CL species, and c) isotopic splitting of CL species [28]. Additionally, for the majority of CLs no standards are available which further complicates the unequivocal identification.

Successful strategies to minimize problems related to isobaric compounds are for instance to increase the chromatographic resolution power, as demonstrated by the group of C.L. Hoppel applying RP-ion pair chromatography with acidified triethylamine [14,47], or by introducing a second chromatographic dimension by combining HILIC- and RP-LC-MS to separate oxidized phospholipids from their non-oxidized counterparts before MS analysis [48]. Identification strategies for CLs and oxCLs, as well as descriptions of characteristic fragmentation pattern are summarized in detail in Refs. [14,28,47]. Recently a DIA MS/MS^{ALL} DI-MS strategy was applied describing in each of eight mouse tissues >150 non-oxidized mitochondrial cardiolipin species including mono-lysocardiolipin implementing $[M-2H]^{2-}$ +0.5 isotopic peaks and the MS/MS spectra for identification [35]. Noteworthy, as mentioned above, CLs are the only mitochondrial lipid species, which can also be analysed in homogenised tissues or cells without the need to isolate mitochondria, since CLs are uniquely mitochondria-specific lipids.

4. Proteomics analysis of mitochondrial samples

The estimated number for human mitochondrial proteins is over 1500 [1]. The complexity of the mitochondrial proteome is further increased by reversible and irreversible post-translational modifications (PTMs) [6]. Transient reversible phosphorylation (O-linked on Ser/Thr/Tyr amino acid residues) is the most widely studied PTM in mitochondria, since it regulates many major metabolic processes, such as ATP production. Besides phosphoproteomics, analytical chemists investigating PTMs also have a focus on

acetylations, O-linked glycosylations, succinylations, SUMOlyations, S-nitrosylations, ubiquitinations, as well as proteolytic cleavages [49]. However, low abundance and low stoichiometry of PTMs are hampering comprehensive detection, thus various sophisticated biochemical techniques and PTM-specific enrichment strategies preferably in combination with LC-MS analysis are applied (as recently reviewed in Ref. [49]). An additional analytical challenge in (phospho)proteomics is the wide dynamic range in protein abundance spanning several orders of magnitude and the extremely small phosphorylated share within distinct proteins [49].

4.1. Bottom-up and top-down proteomics approaches

Nowadays, mitochondrial proteome analyses are preferably performed on a gel-free base by various LC-MS strategies. The two common strategies are entitled “top-down” (i.e. analysis of intact proteins) and “bottom-up” (also entitled “shotgun” proteomics). In bottom-up proteomics analysis mitochondrial protein samples are first digested by an endoproteinase (usually trypsin, less frequent Lys-C or Glu-C). Subsequently, the resulting peptide mixtures are analysed by LC-MS/MS aiming to identify and quantify mitochondrial proteins according to their specific amino acid sequences by 4–6 top peptides per ion precursor [50]. Details about the less frequent applied “top down” analysis were recently reviewed [51]. For the evaluation of mitochondrial proteomics data the currently most comprehensive database is Mitominer v4.0 consisting of information from 12 different organisms including humans (http://mitominer.mrc-mbu.cam.ac.uk/release-4.0/begin.do).

Complex protein digests in bottom-up proteomics experiments contain several tens of thousands of peptides. Hence, 1D-chromatographic strategies may be limited with respect to separation power, dynamic range, selectivity, and sensitivity. Solutions are the introduction of a second, orthogonal chromatographic dimension (2D-LC-MS) or even multiple dimensions, entitled LC^n-MS, aiming to generate as much peak capacity as possible (for details please refer to Ref. [52]). An example for a new 2D bottom-up strategy, reported recently by Loroch and colleagues, covered in human cell culture lysates >7500 phosphorylation sites in >3000 proteins spanning a dynamic range of several orders of magnitude [53].

As described above for lipidomics also proteomics MS data can be acquired either in DDA or DIA mode (Fig. 2). In DDA, the top peptides (usually 10 to 20) are selected in a full mass MS1 scan, fragmented and then acquired in the MS/MS mode for subsequent identification by database search algorithms [54]. However, the stochastic precursor selection, leads to a bias towards the most abundant peptides by undersampling low abundant peptides. DIA leads to less missing values between replicates and provides a promising tool for comprehensive proteome coverage, since it is less affected by diverse dynamic ranges (as reviewed in detail in Ref. [50]).

Comprehensive quantification is another major prerequisite of state of the art proteomics, but also a challenge. Different stable isotope-assisted strategies have been established using either isotope-labelled synthetic standards, metabolic incorporation of isotopes (Stable Isotope Labelling by Amino acids in Cell culture, entitled SILAC [55]), or chemical reactions of a labelling reagents with a distinct functional group (isobaric Tags for Relative and Absolute Quantification, named iTRAQ [56]). Label-free protein quantification (LFQ) was also demonstrated, performing a comparison of precursor ion intensities or spectral counting [57]. A DIA based LFQ approach was applied to relatively quantify mitochondrial proteins in the brain of rats [58] and to comprehensively quantify the hepatic mitochondrial proteome of mice using Sequential Window Acquisition of all THeoretical Mass Spectra (SWATH-MS) [50].

4.2. Targeted MS-based approaches

Commonly used targeted MS-based technologies in quantitative proteomics are SRM, MRM and the recently introduced parallel reaction monitoring (PRM) (Fig. 2). Details of PRM are discussed in a recent review [59]. The advantage of targeted MS compared to DDA and DIA is an increase in sensitivity and linearity over a wide range, which facilitates exact quantification even of selected low abundant proteins in complex mixtures. SRM/MRM approaches are usually performed on a triple quadrupole and PRM on high-resolution hybrid MS like a quadrupole-Orbitrap. Absolute quantification of proteins of interest by SRM, MRM or PRM can be achieved by spiking in stable isotope labelled peptide standards [59]. An example of PRM application in mitochondrial research investigating mitochondrial ubiquitination and mitophagy was recently reported [60].

5. Functional analysis: mitochondrial respirometry

In multi-omics projects the important linkage between elucidated molecular fingerprints and alterations in mitochondrial function is rarely drawn. Function can be assessed e.g. by ATP production via bioluminescence, calcium retention capacity via fluorescence, measurement of membrane potential, and oxidant emission (for analytical details to these approaches please refer to Ref. [9]). But most frequently studied is respiration via oxygen consumption, a key readout of mitochondrial function [9], which we will briefly discuss here. Currently two instruments are mainly used to study mitochondrial respiration, the high-resolution Oxygraph-2k (Oroboros Instruments, Austria) and the high-throughput Seahorse XF (Agilent, USA) [61,62]. Both instruments record mitochondrial respiration in real time. Exemplarily, an Oxygraph-2k high-resolution respirometric analysis is performed with 100 μg purified mitochondria, or ~400,000 cells, or 2 mg liver or muscle tissue, or 60 mg white adipose tissue. Noteworthy, the achieved results should be normalized (e.g. to citrate synthase activity) and subtraction of non-mitochondrial oxygen consumption from the data is essential to balance out possible differences in contaminating protein amounts between samples (for details see Ref. [61]) and to rule out oxidative side reactions. One of the first examples linking multi-omics analyses of mitochondria with tissue-specific function was recently reported by L. Kappler et al. [3].

6. Conclusion

The combination of high-resolution multi-omics approaches, like lipidomics and proteomics, with functional investigations, as well as the integration of these complex data is a big challenge for analytical chemistry and applied informatics. But in the future, understanding the cause of functional alterations in mitochondria on a molecular base can be a central building block for the development of personalized treatments in many diseases. This complementary analytical strategy will play an important role for proper interpretation of global mitochondrial data in state-of-the-art systems medicine projects.

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