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A report of the TASK3-WG4 omics working group of the ILAE/AES joint translational TASK force

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SPECIAL REPORT

A companion to the preclinical common data elements for proteomics, lipidomics, and metabolomics data in rodent epilepsy models. A report of the TASK3-WG4 omics working group of the ILAE/AES joint translational TASK force

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Abstract

The International League Against Epilepsy/American Epilepsy Society (ILAE/AES) Joint Translational Task Force established the TASK3 working groups to

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create common data elements (CDEs) for various preclinical epilepsy research disciplines. This is the second in a two-part series of omics papers, with the other including genomics, transcriptomics, and epigenomics. The aim of the CDEs was to improve the standardization of experimental designs across a range of epilepsy research-related methods. We have generated CDE tables with key parameters and case report forms (CRFs) containing the essential contents of the study protocols for proteomics, lipidomics, and metabolomics of samples from rodent models and people with epilepsy. We discuss the important elements that need to be considered for the proteomics, lipidomics, and metabolomics methodologies, providing a rationale for the parameters that should be documented.

KEYWORDS

case report form, lipidome, metabolome, omic data processing, preclinical, proteome, rodent

1 | INTRODUCTION

Advancing the understanding of molecular mechanisms underlying epileptogenesis and its consequences is essential to identify: (a) new drug targets; (b) common and distinct molecular signaling pathways that underlie diverse epileptic phenotypes; (c) new markers of therapy response, and (d) new diagnostic and/or predictive biomarkers for early detection of epileptogenesis and for monitoring disease progression.¹ In this regard, generation and integration of multimolecular data by modern genomics, transcriptomics, proteomics, metabolomics, and lipidomics from tissue specimens of humans and animal model systems is pivotal. Recent advances in the omic technologies now allow an unparalleled amount of data to be generated with high sensitivity, speed, and precision from virtually any tissue type, even when only minute sample volumes are available for analysis. Application of omics technologies to tissue specimens from animal models of epilepsy or patients can point to molecular imaging marker candidates, which may help identify new drug targets, and inform development of novel disease-modifying therapies. Similarly, omics analysis of biological fluids (eg, plasma) from animal models and patients could uncover core signatures that can determine disease stage and predict further development, aid with diagnosis and responses to treatment, and allow a better discrimination of seizure types and phenotypes. In this regard, standardization of experimental design, data procurement and processing, and provision of a collective repository of instrument-specific data are essential to improve portability of methods across research laboratories and to ensure reliability of reference values for omics-data across experimental models and patients with epilepsy.

Key Points

1. This joint ILAE/AES initiative introduces common data elements (CDEs) related to measurement of proteomics, lipidomics, and metabolomics parameters in biological samples from adult rodents.
2. Case report forms (CRFs) and a companion paper discussing their use are provided for proteomics, lipidomics, and metabolomics methodologies.
3. Future use of these forms is aimed at helping standardize animal experiments to improve and facilitate future meta-analysis studies.

Mass spectrometry (MS) is a powerful tool for profiling proteomics, lipidomics, and metabolomics in a qualitative and quantitative manner. Developments in the MS-analytical workflows in each of the omic fields allow for high-throughput provision of an unparalleled amount of information on the structural heterogeneity, molecular identity, and quantity in each sample specimen.

Continuing the efforts of the TASK3-WG4 Omics Working Group of the ILAE/AES Joint Translational Task Force to improve standardization of the various epilepsy research methods, with the first article focusing on genomics, transcriptomics and epigenomics,² we report here the guidelines and rationale of experimental design, sampling and data procurement for proteomics, lipidomics, and metabolomics.

As also indicated in the first omics article,² omics-based studies are methodologically complex and require their own unique considerations, which must be

accounted for as early as possible during the planning stages of the study. The considerations include the following: (a) The need for suitable animal models; (b) Optimization of animal environment, housing and management; (c) Preferred analytical procedures for sample collection and storage; (d) Consideration of methodologies to be used (including quality control, sample preparation, and analytical workflows), often unique for a specific omics-approach; (e) Bioinformatics, which includes a sufficiently large sample size to achieve the desired statistical power when hundreds or thousands of different variables are compared, as well as expertise in handling and analyzing large data sets, data annotation and cross-omic integration; and (f) Specialized data management.

Due to the complexity of omics-based studies, harmonization of all stages of the analytical process and adoption of common practices will not only increase the scientific rigor and reproducibility, but also ensure that data can

be readily reused according to FAIR principles,³ shared and compared between laboratories, as well as integrated with other omics-based datasets to provide a systems level understanding of epilepsy. To this end, the TASK3-WG4 Omics Working Group sought to develop common data elements (CDEs), generate Case Report Forms (CRFs), and draft companion papers to assist with the CDE/CRF implementation.

2 | METHODS

The Omics Working Group consists of 12 (pre)clinical epilepsy researchers, selected on their relevant expertise who developed CDEs and CRFs for various omics modules. In this paper, we focus on four modules: (a) Sample collection and storage for proteomics, lipidomics, and metabolomics (Figure 1); (b) Methodologies for proteomics (Figure 2); (c) Methodologies for lipidomics

Omics Studies

Case Report Form: 1 CRF Module – Sample collection and storage for proteomics, lipidomics and metabolomics.docx

CRF module: Sample collection and storage for proteomics, lipidomics and metabolomics

Date that this CRF was filled out:

Name of person filling out CRF:

Project name/Identifier:

Animal ID:

CDE Name	Data Collected
Subject	
Species used	<input type="checkbox"/> Rat <input type="checkbox"/> Mouse <input type="checkbox"/> Other
If other, please specify	
Specify strain used	
Sex	<input type="checkbox"/> Male <input type="checkbox"/> Female <input type="checkbox"/> Unknown
Body weight (g)	
Date / Time of sample collection MM/DD/YYYY; hh:mm:ss	
Sample collected during fasting	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If collected during fasting, specify fasting time (hours)	
Sample collected during estrous cycle	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If collected during estrous cycle, specify day	<input type="checkbox"/> Proestrus <input type="checkbox"/> Estrus <input type="checkbox"/> Metestrus <input type="checkbox"/> Diestrus
Anesthesia	
Anesthesia	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If anesthesia was administered, specify type	<input type="checkbox"/> Isoflurane <input type="checkbox"/> Sevoflurane <input type="checkbox"/> Ketamine/Xylazine <input type="checkbox"/> Pentobarbitone <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	

FIGURE 1 Sample collection and storage form (CRF; see main text for details)

Route of administration	<input type="checkbox"/> Intramuscular <input type="checkbox"/> Intraperitoneal <input type="checkbox"/> Intravenous <input type="checkbox"/> Inhalational <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Isoflurane concentration induction %	
Isoflurane concentration maintenance %	
Sevoflurane concentration induction %	
Sevoflurane concentration maintenance %	
Ketamine dose mg/kg	
Xylazine dose mg/kg	
Pentobarbitone dose mg/kg	
Other anesthesia dose mg/kg	
Starting time of anesthesia in hours and minutes	
Total anesthesia duration in minutes	
Animal sacrifice (select all applicable)	<input type="checkbox"/> Decapitation <input type="checkbox"/> Overdose of anesthetic <input type="checkbox"/> Unknown <input type="checkbox"/> Not sacrificed
Blood collection	
Collection modality	<input type="checkbox"/> Trunk <input type="checkbox"/> Cardiac puncture <input type="checkbox"/> Lateral saphenous vein puncture <input type="checkbox"/> Tail vein puncture <input type="checkbox"/> Cannula collection <input type="checkbox"/> Orbital plexus <input type="checkbox"/> Retromandibular plexus <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
If cannula collection, specify blood vessel	
Blood volume collected in ml	
Blood collection container company	
Blood collection container type	
Volume of tube ml	
Anticoagulant used	<input type="checkbox"/> K ₂ EDTA ¹ <input type="checkbox"/> Heparin <input type="checkbox"/> Citrate <input type="checkbox"/> Unknown <input type="checkbox"/> Other

¹ EDTA, K₂ Ethylenediaminetetraacetic acid, is recommended for RNA analysis.
² Indicate final concentration in blood (recommended K₂EDTA about 5 mM)

FIGURE 1 (Continued)

and metabolomics (Figure 3); and (d) Data management (Figure 4). These modules are designed to be used together, with an investigator able to select appropriate options in the four modules. The modules are also designed to be used in conjunction with published CRFs including the “core animal characteristics CRF” described in Harte-Hargrove et al. that covers general animal husbandry⁴ and “individual experimental” CRFs covering EEG, physiologic data, pharmacological studies, and behavioral analysis CRFs.^{4–8}

The forms are an extension of the preclinical CDEs created by the TASK3 group of the ILAE/AES Joint Translational Task Force^{4–9} and are also analogous to the CDEs created by the National Institute of Neurological Disorders Stroke (NINDS) for traumatic brain injury research¹⁰ and the EPITARGET consortium (Targets and Biomarkers for Antiepileptogenesis).¹¹ The proposed recommendations originate mostly from previously published methods in rodent epilepsy model research. Within the

Omics Working Group, the modules were divided among the members according to their expertise. Each subgroup prepared a draft for the CDE/CRF forms and the accompanying part of the manuscript, presented this to the whole group during one of the 10 Working Group meetings, and the draft was assessed and adjusted until a consensus was reached. The final version includes feedback from members of the TASK3 group and the Task Force.

The CDEs presented here apply primarily to samples from adult rodents, rats or mice, and are not readily applicable to immature animals, which will need specific CDEs taking into account their size, developmental stage and ongoing acquisition of specific functions.¹²

3 | RESULTS

First, we describe the sample collection and storage options specific for proteomics, lipidomics and metabolomics,

If other, please specify	
If EDTA ^{1,2} , concentration in mM	
If Heparin ² , concentration in U/ml	
If Citrate ² , Concentration mM	
Plasma separation	
Number of centrifugation steps	<input type="checkbox"/> 1 <input type="checkbox"/> 2 <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Centrifugation speed step 1 in g	
Centrifugation time step 1 in minutes	
Centrifugation temperature step 1 in °C	
Centrifugation speed step 2 in g	
Centrifugation time step 2 in minutes	
Centrifugation temperature step 2 in °C	
Samples kept on ice before centrifugation?	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
Serum separation	
Clotting time in minutes	
Clotting temperature in °C	
Centrifugation speed in g	
Centrifugation time in minutes	
Centrifugation temperature in °C	
Cerebrospinal fluid collection	
Cerebrospinal fluid collection container company	
Cerebrospinal fluid collection container type	
Volume of tube in ml	
Volume collected in µl	
Faeces collection	
Faeces collection container company	
Faeces collection container type	
Volume of tube in ml	
Weight collected in mg	

FIGURE 1 (Continued)

followed by CDEs outlining analytical techniques and data management. We provide a rationale and an overview of the elements that are included in the CDEs and the corresponding CRFs. The CDE and CRF modules linked to this paper are also available as electronic files.

3.1 | Sample collection, preprocessing, and storage for proteomics, lipidomics, and metabolomics

Appendix S1 and S2

3.1.1 | Rationale

For all three omics approaches, the goals of sample collection, preprocessing, and storage are to preserve sample integrity and prevent ex vivo metabolism and degradation of proteins, lipids, and metabolites before downstream

testing. Standardization of each of the steps of the omic-workflows is pivotal both for preclinical and clinical samples, and with a carefully designed collection and aliquoting protocol, it may be possible to perform all three analyses in the same sample. For example, cells, tissues, and body fluids can be snap-frozen and later used for a variety of omics approaches. Typically, scarring or a shrunken, hard hippocampus such as in chronic epileptogenic brain tissue does not create unique problems or require a specific method.

3.1.2 | Measurements

Several sample types can be used for analysis, including whole blood, plasma, serum, urine, cerebrospinal fluid, saliva, sweat, tissue (eg, sections, punches, and dissected regions), cells, microdialysis samples, and feces. However, numerous factors during the preanalytical (or preexamination) phase can affect the quality and chemical

Tissue collection	
Collected tissue	<input type="checkbox"/> Brain tissue <input type="checkbox"/> Single cells <input type="checkbox"/> Selected cells <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If brain tissue, specify collected tissue	
If brain tissue, specify collected weight	
If single cells, specify collected cell type(s)	
If selected cells, specify collected cell type(s)	
If other, please specify	
Sample storage	
Storage temperature	<input type="checkbox"/> -20°C <input type="checkbox"/> -70°C <input type="checkbox"/> -80°C <input type="checkbox"/> Liquid nitrogen <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Volume per aliquot in µl	
Delay between collection and storage in minutes	
Storage time in days	
Number of freeze/thaw cycles	

Parameters

Date and time of sample collection (MM/DD/YYYY; hh:mm:ss)							
Time point after initial insult (days)							

Instructions: Please check boxes where applicable. If none of the predetermined options is appropriate use the default space to specify your answer. This form is to be filled in for one individual animal.

FIGURE 1 (Continued)

composition of the samples, resulting in spurious or inconsistent results. For example, a tube of whole blood left at room temperature for several hours will undergo significant changes in its chemical composition due to ex vivo enzymatic activities and/or spontaneous reactions, such as oxidation and isomerization. A sample collected during fasting conditions may have a very different metabolomic profile than a sample collected after a meal. Several metabolites and proteins exhibit ultradian (recurrent cycle repeated during 24-h period that is longer than 1 h), circadian (one cycle daily), infradian (cycle with periods longer than a day—eg, menstrual cycle) and multidien (multiday cycles), or circannual (yearly cycle) changes, making the timing of sample collection critical. The investigator needs to be aware of the preanalytical variables associated with their sample type(s) and test(s) of interest and take every measure to eliminate or at least reduce them. Table 1 lists some of the most common sources of preanalytical factors that are known to affect laboratory results. The reader is referred to the Tietz Textbook for a comprehensive discussion of the topic.¹³

Sampling of blood, urine, sweat, saliva, stool, and interstitial fluid (by microdialysis) can be performed repeatedly from the same animal to collect longitudinal information for omics studies. The researcher should pay attention to the maximum volumes of blood that can be collected. For laboratory animal volumes, see <https://www.nc3rs.org.uk/our-resources/blood-sampling>. For a detailed description of blood collection, see the genomics, transcriptomics, and epigenomics companion paper.² CSF collection in animals is often performed during a lethal experiment, but it can also be performed multiple times. To do so, a cannula can be implanted in the cisterna magna.¹⁴ Brain or other organs are usually collected during a terminal experiment when the animal is under anesthesia. To prevent ex vivo metabolism, the organ should be removed as quickly as possible and immediately snap frozen. Note that freezing can introduce morphological artifacts due to damage from ice crystals. An alternative to snap freezing is focused-beam microwave irradiation of the brain, which instantly stops all metabolic reactions and has been successfully used for brain tissue metabolomics studies.

Omics Studies**Case Report Form: 2 CRF – Proteomics.docx****CRF module: proteomics**

Date that this CRF was filled out:

Name of person filling out CRF:

Project name/Identifier:

Animal/ID:

CDE Name	Data Collected
Experiment type	
Omics level	<input type="checkbox"/> Proteomics
Input type	<input type="checkbox"/> Bulk primary tissue <input type="checkbox"/> Selected cell-types from primary tissue <input type="checkbox"/> Cell-cultures <input type="checkbox"/> Single-cell/single nuclei <input type="checkbox"/> Cerebrospinal fluid <input type="checkbox"/> Whole blood <input type="checkbox"/> Plasma <input type="checkbox"/> Serum <input type="checkbox"/> Saliva <input type="checkbox"/> Sweat <input type="checkbox"/> Urine <input type="checkbox"/> Feces <input type="checkbox"/> Other
If other, please specify	
If bulk primary tissue, specify tissue type	
If bulk primary tissue, specify tissue localization	
Sample preparation	

FIGURE 2 Proteomics case report form (CRF; see main text for details)

This technique was first described in 2009¹⁵ and is not frequently used; however, it is also described in a few recent studies, see example.^{16,17} While microwave irradiation is compatible with proteomics¹⁸ and possibly also transcriptomics studies,¹⁹ it cannot be used to study enzyme activity or transporter activity due to protein denaturation.¹⁵

3.1.3 | Equipment

For the collection of blood and CSF samples, needles or cannulas are needed, as well as specific collection tubes with and without a variety of additives depending on the scope of the omic study (eg, citrate, EDTA, and heparin). A bladder catheter is required for urine collection in animals, and a microdialysis catheter is needed for interstitial fluid collection. Feces can be collected by expression (ie, gently squeezing the perianal area with a clean glove) of a fecal pellet into sterile collection cups.

Collection of feces from the cage is not recommended due to potential contamination from the bedding and not knowing the source of the feces if multiple animals are housed in the same cage. To separate plasma or serum from whole blood, a centrifuge is needed, and for the collection of brain tissue, dissection tools are needed. Finally, a homogenizer is needed for extraction of proteins, metabolites and lipids from tissue and stool samples.

3.1.4 | Procedure

- Blood collection

For blood collection in rodents, we refer to the genomics, transcriptomics, and epigenomics companion paper.²

For omic analysis, whole blood can be separated into its plasma or serum components. For plasma separation, centrifuge the anticoagulated blood within 1 h of collection

Quality control date and time MM/DD/YYYY hh:mm:ss	
Quality assessment type	<input type="checkbox"/> Hemolytic <input type="checkbox"/> Icteric <input type="checkbox"/> Lipemic <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Sample preparation date and time MM/DD/YYYY hh:mm:ss	
Sample preparation	<input type="checkbox"/> Protein precipitation <input type="checkbox"/> Ultrafiltration <input type="checkbox"/> Solid phase extraction <input type="checkbox"/> Liquid-liquid extraction <input type="checkbox"/> Derivatization <input type="checkbox"/> Tissue homogenization <input type="checkbox"/> Tissue pulverization <input type="checkbox"/> Centrifugation <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Internal standards used	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If internal standards were used, specify name(s)	
If internal standards were used, specify vendor(s)	
If internal standards were used, specify cat no	
If internal standards were used, specify concentration(s)	
External standards (calibrators) used	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If external standards were used, specify name(s)	
If external standards were used, specify vendor(s)	
If external standards were used, specify cat no	
If external standards were used, specify concentration(s)	
Label-free approach used	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
Quality control samples used	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If quality control samples were used, specify name(s)	
If quality control samples were used, specify vendor(s)	

FIGURE 2 (Continued)

using the following parameters: 1300–1500 g for 10 min without a brake at 4°C. Remove the plasma carefully from the cells and transfer to a sterile polypropylene tube. For serum separation, let whole blood collected in a tube without additive sit at room temperature for 1 h, and centrifuge at 1300–1500 g for 10 min without a brake at 4°C. Remove the serum carefully from the cells and transfer to a sterile polypropylene tube. To prevent platelet contamination, a second centrifuge step is needed (3000 g, 10 min, 4°C) before freezing. Freeze samples on dry ice or in liquid nitrogen and store aliquots at –80°C. The essential difference between plasma and serum is the presence or absence of fibrinogen and clotting factors, respectively. When the molecules of interest are readily susceptible to enzymatic and/or chemical changes (eg, for signaling molecules), plasma processing is recommended within couple of minutes; either immediate, 1–2 min, or within minimum affordable processing time during sampling, with the blood

kept on ice prior to processing and/or with the addition of enzyme inhibitors or antioxidants.

- CSF collection

For CSF collection from rodents, we refer to the first omics paper.²

- Tissue Collection

We recommend that fresh brain tissue samples are preserved with snap-freezing in liquid nitrogen or on dry ice as soon as possible.²⁰ Animals are often decapitated for fresh tissue collections, and the delay between decapitation and tissue preservation should be monitored and reported. Focused-beam microwave irradiation of the brain has been increasingly used as an alternative to decapitation.¹⁵ Microwave irradiation is usually performed

If quality control samples were used, specify cat no	
If quality control samples were used, specify concentration(s)	
If quality control samples were used, specify matrix/solvent	
Sample preparation method	
Specify protein precipitation precipitant	
Specify volume of precipitant	
Specify volume of sample added to precipitant	
Specify ultrafiltration device	
Specify centrifugation speed	
Specify centrifugation duration	
Specify centrifugation temperature	
Specify solid phase extraction cartridge vendor	
Specify solid phase extraction cartridge cat no	
Specify liquid-liquid extraction kit vendor	
Specify liquid-liquid extraction kit cat no	
Specify derivatization kit vendor	
Specify derivatization kit cat no	
Specify homogenizer vendor	
Specify homogenizer cat no	
Specify sample tubes vendor	
Specify sample tube cat no	
Specify beads vendor	
Specify beads cat no	
Specify homogenizing solution vendor	
Specify homogenizing solution cat no	
Specify volume of homogenizing solution	
Specify tissue weight that was homogenized	
Liquid chromatography method	

FIGURE 2 (Continued)

in lightly anesthetized animals and allows for ultrarapid fixation of the brain without the use of chemicals. This method has been successfully used for studies of brain metabolites, lipids, and proteins; however, functional protein assays (eg, enzyme activity studies) are not possible due to the heat-induced denaturation of proteins.¹⁷

3.1.5 | Sample storage

Most analytes are stable for a long time (months to years) if stored at -80°C or in liquid nitrogen.²¹⁻²³ However, repeated freeze-thawing, extended bench top processing of samples and poor standardization of procedures within and between cohorts can impact analyte stability resulting in spuriously elevated or decreased concentrations. For example, an untargeted metabolomics study of 1026 biochemicals in human EDTA plasma samples found that incubation of thawed EDTA samples on ice for up to 6 h

resulted in $<1\%$ of biochemicals changing significantly, and three freeze-thaw cycles affected approximately 2% of the biochemicals.²⁴

3.1.6 | Analysis and interpretation

Mass spectrometry (MS) coupled with liquid chromatography (LC) or gas chromatography (GC) are the most widely used methods for analysis of the proteome, lipidome, and metabolome.²⁵⁻²⁷ In vivo or ex vivo magnetic resonance spectroscopy (MRS) are also used for metabolomics and lipidomics analyses but are generally less sensitive than MS and not as widely available.²⁵ Two main MS strategies for metabolomic, lipidomic, and proteomic profiling are used as follows: (a) untargeted omic profiling, where all ionizable molecular species in a sample extract are acquired and (b) targeted omic-profiling, where selected molecules are profiled.²⁸ Untargeted methods are used for

Instrument used, specify vendor	
Specify instrument used	
Specify instrument cat no	
Chromatography	<input type="checkbox"/> Reverse phase <input type="checkbox"/> HILIC <input type="checkbox"/> Ion exchange <input type="checkbox"/> Mixed-mode <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Column used, specify vendor	
Specify column used	
Specify column cat no	
Specify column stationary phase	
Specify column inner diameter	
Specify column length	
Mobile phase A used	
Mobile phase B used	
Gradient used	
Flow rate used	
Column temperature used	
Injected sample volume used	
Mass spectrometry method	
Instrument used, specify vendor	
Specify instrument used	
Specify instrument cat no	
Instrument type	<input type="checkbox"/> TOF <input type="checkbox"/> Q-TOF <input type="checkbox"/> Single quad <input type="checkbox"/> Triple quad <input type="checkbox"/> Ion trap <input type="checkbox"/> Fourier transform <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Proteomics mode	<input type="checkbox"/> Untargeted <input type="checkbox"/> Targeted <input type="checkbox"/> Unknown

FIGURE 2 (Continued)

comprehensive, in-depth screens of a sample, that is, discovery of known or candidate molecular markers, while targeted methods are used for investigation of selected, low-abundant molecules, whose detection is hindered in untargeted profiling by the mixture’s molecular complexity and dynamic range, but also to study subregions of the brain or single cells.²⁸ For quantification purposes, the use of internal standards such as isotope labeled or unnaturally occurring homologues are typically used for untargeted and targeted lipidomics and metabolomics as well as targeted proteomics, while for untargeted proteomics label-free approaches are commonly employed.²⁸ Targeted approaches can also be developed as fully quantitative methods (known as absolute quantification), whereas untargeted methods are usually semiquantitative.²⁸

Because of the large differences in molecular mass, charge, structure, and pKa within and between the three omics fields discussed here, numerous different MS-based protocols have been developed. However, there are several similarities among the different protocols (summarized in

Figure 5), and with careful planning, multiomics studies of the same sample may sometimes be possible, particularly for liquid samples (eg, serum, CSF) but also from tissue samples, if steps are taken to control for the inherent cellular heterogeneity within a tissue or organ. The latter can be achieved through tissue pulverization and/or homogenization, which will allow for different aliquots of the same sample to be analyzed by different omics protocols.

3.2 | Proteomics

Appendix S3 and S4

3.2.1 | Rationale

The rodent and human proteome has about 18 000–21 000 proteins,^{29,30} which generally function as biological catalysts (enzymes), structural proteins (eg, cytoskeletal

Ionization mode	<input type="checkbox"/> ES+; <input type="checkbox"/> ES-; <input type="checkbox"/> APCI+; <input type="checkbox"/> EI; <input type="checkbox"/> CI-; <input type="checkbox"/> CI+; <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
MS mode	<input type="checkbox"/> Full scan <input type="checkbox"/> MS ⁿ <input type="checkbox"/> MRM <input type="checkbox"/> SIM <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Specify peak detection and processing software	
Data annotation	
Peak/spectral identification	<input type="checkbox"/> Reference to known standards <input type="checkbox"/> Rule-based fragmentation annotation <input type="checkbox"/> Database search <input type="checkbox"/> Self-established spectral library <input type="checkbox"/> Unknown
Database used	<input type="checkbox"/> MASCOT <input type="checkbox"/> SEQUEST <input type="checkbox"/> Proteome Discoverer <input type="checkbox"/> Peaks DB <input type="checkbox"/> Unknown <input type="checkbox"/> Other
Database version	
If other, please specify	
If self-established spectral library, specify availability	
Manual data curation	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
Database used for pathway analysis	<input type="checkbox"/> Ingenuity Pathway <input type="checkbox"/> Wikipathways <input type="checkbox"/> Pathway Tools <input type="checkbox"/> PANTHER <input type="checkbox"/> STRING <input type="checkbox"/> KEGG <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Database version	
Data quantification	
Quantification strategy	<input type="checkbox"/> Relative quantification <input type="checkbox"/> Untargeted quantification <input type="checkbox"/> Absolute targeted quantification – label-free data <input type="checkbox"/> Labeled quantification (isotope tag-based, TMT) <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	

FIGURE 2 (Continued)

proteins), DNA/RNA binding proteins, cell membrane bound receptor proteins or transporter proteins, as well as adaptor or binding partners of other proteins to exert their regulatory effects. In addition, there are more than 40 posttranslational modifications (PTMs) identified and related to human diseases or disorders. In general, a disease state will often be reflected by changes in the proteome, thus differential proteomic profiling can be an extremely powerful technique to identify protein biomarkers and potential drug targets, as well as map out disease pathways using System Biology tools.³¹

3.2.2 | Measurements

Proteomic analysis is used for concurrent detection of a variety of proteins and permits examination of relationships between proteins under different pathological or pathophysiological conditions. The general categories are identification, characterization, and quantification of a set

of proteins of a biological sample at a specific point in time and under a specific condition or biologic state. MS is the commonly used method for proteomic analysis. Although several different methods of MS proteomics are available, in general, the proteins are analyzed either in a “top-down” approach where intact proteins are analyzed, or a “bottom-up” approach where proteins are first predigested with a protease (such as trypsin) into small fragments before being subjected to MS analysis. For example, the technique often used in neurological disorder-related samples is nano-liquid chromatography (nLC) coupled to tandem mass spectrometry (MS/MS). The nLC step prefractionates the tryptic digestion-treated biological samples chromatographically, followed by introducing the peptides in each fraction to the mass spectrometer and ion source for peptide mass and sequence identification. Many different mass spectrometers can be used. For a global approach, peptides are sequenced using data-dependent methods, while in a targeted approach, a set of preselected peptides are detected and quantified.

Class or subclass-specific protein internal standards	
Pooled samples	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
Internal standards	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
Candidate identification criteria	<input type="checkbox"/> p-value <input type="checkbox"/> Log2 (fold change) <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
If p-value, specify value used	
If Log2 (fold change), specify value used	
Data validation	
Proteome confirmation by second method	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If yes, specify method	
Independent biological replicates for validation	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If yes, specify number	
Data statistical processing	
Statistical tests	<input type="checkbox"/> PCA <input type="checkbox"/> T-test + FDR <input type="checkbox"/> ANOVA <input type="checkbox"/> Regression <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	

Instructions: Please check boxes where applicable. If none of the predetermined options is appropriate use the default space to specify your answer. This form is to be filled in for one individual animal.

Abbreviations: ANOVA, analysis of variance; APCI, atmospheric pressure chemical ionization; CI, chemical ionization; EI, electron ionization; ES, electrospray ionization; FDR, false discovery rate correction; HILIC, hydrophilic interaction liquid chromatography; KEGG, Kyoto Encyclopedia of Genes and Genomes; MRM, multiple reaction monitoring; MS, mass spectrometry; PCA, principal component analysis; Q-TOF, quadrupole time-of-flight; SIM, single ion monitoring; TOF, time-of-flight

FIGURE 2 (Continued)

3.2.3 | Equipment

The most used instrument platforms are (1) nano liquid chromatography (nLC) (with a C18 reverse phase column) coupled to either (a) tandem mass spectrometry (MS/MS) or to matrix associated laser desorption ionization (MALDI) combined with mass spectrometry. The MS usually operates on electrospray ionization (ESI). Tandem mass spectrometry (MS/MS or MS2) is an instrumental analysis where two or more mass analyzers are coupled to increase their abilities to analyze peptide samples (providing further fragmentation such as the parent peptide sequence can be identified). For example, the peptides in a fractionated sample are ionized and the first MS1 separates these ions by their mass-to-charge ratio (m/z). Ions of a particular m/z -ratio from MS1 can then be selected and subjected to collision-induced dissociation, ion-molecule reaction, or photo-dissociation into smaller fragment ions (daughter ions), before they are introduced into MS2, which in turn separates and reports the fragments by their m/z -ratio. Tandem MS can be constructed by either (a) tandem in space: two mass detectors separated in space or (b) tandem in time: a single mass analyzer with the MS steps separated in time. For tandem mass spectrometry in

space, the different elements are often noted in a shorthand for the mass selector used.^{32–34}

In tandem MS in space, the separation mass analyzer is physically distinct but connected under high vacuum. The mass analyzer can be electric or magnetic sectors (E, B), transmission quadrupole (Q), q—radio frequency collision quadrupole, or time-of-flight (TOF). For example, QqQ' is triple quadrupole mass spectrometer and QTOF—quadrupole time-of-flight mass spectrometer (also QqTOF). In tandem MS in time, ion trap MS is a good example for proteomic analysis. Here, the separation is accomplished with ions trapped in the same place, with multiple separation steps taking place over time. A quadrupole ion trap or Fourier transform ion cyclotron resonance (FTICR) instrument can be used for such an analysis. With the latter, commonly used member of the FTMS is the Orbitrap analyzer (Thermo Fisher), such as the LTQ Orbitrap that has linear ion-trap on the front end. This provides stability, field uniformity and very high mass accuracy and dynamic range of frequency measurements. To date, due to the rapid advancement of MSMS instrumentation, all the above instruments have been considered suitable for proteomics analysis.

Omics Studies**Case Report Form: 3 CRF Module – lipidomics and metabolomics.docx****CRF module: lipidomics and metabolomics**

Date that this CRF was filled out:

Name of person filling out CRF:

Project name/Identifier:

Animal/ID:

CDE Name	Data Collected
Experiment type	
Omics level	<input type="checkbox"/> Lipidomics <input type="checkbox"/> Metabolomics
Input type	<input type="checkbox"/> Bulk primary tissue <input type="checkbox"/> Selected cell-types from primary tissue <input type="checkbox"/> Cell-cultures <input type="checkbox"/> Single-cell/single nuclei <input type="checkbox"/> Cerebrospinal fluid <input type="checkbox"/> Whole blood <input type="checkbox"/> Plasma <input type="checkbox"/> Serum <input type="checkbox"/> Saliva <input type="checkbox"/> Sweat <input type="checkbox"/> Urine <input type="checkbox"/> Feces <input type="checkbox"/> Other
If other, please specify	
If bulk primary tissue, specify tissue type	
If bulk primary tissue, specify tissue localization	
Sample preparation	
Quality control date and time MM/DD/YYYY hh:mm:ss	

FIGURE 3 Lipidomics and metabolomics case report form (CRF; see main text for details)

3.2.4 | Approaches

Proteomic analysis can be categorized into identification, characterization, and quantification of a subset proteome in a set of biological samples. Identification is the primary discovery and the description of the presence or absence of such protein. It also covers differential MS proteomics by identifying if a certain protein is differentially up- or downregulated between samples. Characterization means if the protein is modified by posttranslational modification (eg, phosphorylation or proteolysis or methylation). This can also be a part of differential MS. Lastly, quantification (quantitative MS) is the most recent advance in MS-proteomics. For our purposes, we are most interested in differential proteomics (eg, distinguishing epileptic versus nonepileptic state) and quantitative proteomics.

There are also two main approaches in terms of MS/MS sample set up, acquiring data and identification of differential proteome profiles: label-free versus mass tag

label, such as the tandem mass tag (TMT) method, which are explained below.

For differential MS, the most popular method is the isobaric mass tagging method for the tryptic peptide—that is, each set of samples is labeled chemically to one mass tag, while the second is labeled with another mass tag that is several Dalton heavier. Examples are the Isobaric tags for relative and absolute quantitation (iTRAQ) labeling reagent and the Tandem Mass Tag (TMT) reagent. Up to 10–12 mass tags can be simultaneously used in one experiment, making them highly adaptive for experimental design with multiple comparison groups.^{35,36} One of the potential disadvantages of the labeled-based approach is that certain proteins might not be well-labeled due to amino acid compositions, thus can be missed by TMT. The other common proteomic approach is the label-free method: Protein samples are subjected to protease completion digestion, followed by reverse-phase chromatographic separation and fractions are subjected to MS/MS

Quality assessment type	<input type="checkbox"/> Hemolytic <input type="checkbox"/> Icteric <input type="checkbox"/> Lipemic <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Sample preparation date and time MM/DD/YYYY hh:mm:ss	
Sample preparation	<input type="checkbox"/> Protein precipitation <input type="checkbox"/> Ultrafiltration <input type="checkbox"/> Solid phase extraction <input type="checkbox"/> Liquid-liquid extraction <input type="checkbox"/> Derivatization <input type="checkbox"/> Tissue homogenization <input type="checkbox"/> Fiber absorption <input type="checkbox"/> Tissue pulverization <input type="checkbox"/> Centrifugation <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	If other, please specify
Internal standards used	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If internal standards were used, specify name(s)	
If internal standards were used, specify vendor(s)	
If internal standards were used, specify cat no	
If internal standards were used, specify concentration(s)	
External standards (calibrators) used	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If external standards were used, specify name(s)	
If external standards were used, specify vendor(s)	
If external standards were used, specify cat no	
If external standards were used, specify concentration(s)	
Quality control samples used	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If quality control samples were used, specify name(s)	
If commercially available quality control samples were used, specify vendor(s)	
If commercially quality control samples were used, specify cat no	

FIGURE 3 (Continued)

for peptide identification. This method is very powerful for descriptive proteome analysis (eg, which proteins are present) in a given sample, but less reliable in differential proteomic analysis, although amino acid sequence coverage for a particular protein in a sample is generally reflective of protein abundance. Here, selected proteins with at least two favorable tryptic peptide ions are identified and often synthesized with N15 or C13 labeled to serve as internal standards. The MS/MS is set to a target acquisition mode and the analysis is also targeted only to those peptide ions. Due to the combined targeted acquisition followed by targeted data analysis, the turnaround time per sample is a much faster data-dependent mode of MS/MS. In addition, targeted MS is ideally suited for multiplexing a set of protein targets, wherein up to 20–30 protein targets can be selected, and their major tryptic fragments selected and combined for targeted MS analysis. This approach is termed Quantitative Multiplexed Proteomics.^{37–39} This approach in fact shares some similarity to the mass-tag label

method. This targeted MS/MS approach can be highly quantitative. However, label-free targeted proteomics is by nature not a global or exhaustive approach.

3.2.5 | Procedure

The sample quality and quantity and the number of samples (replicates) per group are key factors.⁴⁰ Increasing the number of replicates will result in more precise estimates of the observed difference between groups.⁴⁰ Commonly, a minimum of n = 4 biological replicates per study group is used. For targeted-based proteomics, up to 10–12 conditions can be run simultaneously. Sample amount is also important. Generally, it would be ideal to harvest about 100 µg or more protein from each sample. If the sample is a biofluid, after centrifugation to remove debris, it can be used directly, or subjected to preresolution of abundant proteins (such as albumin) using multi-affinity spun columns.

If quality control samples were used, specify concentration(s)	
If quality control samples were used, specify matrix/solvent	
Sample preparation method	
Specify protein precipitation precipitant	
Specify volume of precipitant	
Specify volume of sample added to precipitant	
Specify ultrafiltration device	
Specify centrifugation speed	
Specify centrifugation duration	
Specify centrifugation temperature	
Specify solid phase extraction cartridge vendor	
Specify solid phase extraction cartridge cat no	
Specify liquid-liquid extraction kit vendor	
Specify liquid-liquid extraction kit cat no	
Specify liquid-liquid extraction method (not commercially available)	
Specify derivatization kit vendor	
Specify derivatization kit cat no	
Specify homogenizer vendor	
Specify homogenizer cat no	
Specify sample tubes vendor	
Specify sample tube cat no	
Specify beads vendor	
Specify beads cat no	
Specify commercially available homogenizing solution vendor	
Specify commercially available homogenizing solution cat no	
Specify in-house used homogenizing solution	

FIGURE 3 (Continued)

This extra step (eg, for serum, plasma) helps to minimize abundant protein-mediated target ion signal suppression. For complex samples such as brain tissue, usually, a dedicated commercial sample preparation kit with a MS-compatible detergent-based lysis buffer (eg, EasyPep™ Mini MS Sample Prep Ki, Thermo Fisher) is used to ensure sample preparation uniformity. In the workflow, it is often recommended that an equally weighed combined sample is generated and subjected to the same proteomic analysis in parallel with the distinct individual samples. This will serve as a means of signal standardization across different sample runs. The prepared total protein samples are usually purified and concentrated. Next, the samples are subjected to tryptic digestion (commercial MS grade) (eg, at 1/50 ratio) usually for 4–6 h to overnight to ensure complete protein digestion.

From this point on, for differential tag-based proteomics, the peptide samples are subjected to differential mass tag labeling, and a standard peptide can be spiked in as

internal control. For a targeted proteomic workflow, at least three dominant tryptic peptides for a subset of preselected proteins are first identified, and these peptides are synthetically made with a nonradioactive heavy isotope (N15, C13). A known amount of an equally weighed mixture of all heavy isotope reference peptides are then introduced into each sample. Using this comparative approach, the native peptide counterparts can be accurately quantified by inference, and absolute quantity of the selected target-proteins can also be calculated accurately.

In both cases, the samples (sometimes further concentrated) are then subjected to nLC for fractionation by a C18 reverse phase column eluted with a gradient of increasing acetonitrile concentration. Each fraction is then subjected to ionization and applied to tandem MS/MS for mass and peptide sequence identification. Lastly, to analyze such proteomic data can be a significant undertaking. Thus, one should utilize commonly used proteomic analysis software tools such as Proteome Discoverer.^{31,41}

Specify volume of homogenizing solution	
Specify tissue weight that was homogenized	
Specify fiber absorption vendor	
Specify fiber absorption cat no	
Liquid chromatography method	
Instrument used, specify vendor	
Specify instrument used	
Specify instrument cat no	
Chromatography	<input type="checkbox"/> Reverse phase <input type="checkbox"/> HILIC <input type="checkbox"/> Ion exchange <input type="checkbox"/> Mixed-mode <input type="checkbox"/> Chiral <input type="checkbox"/> Supercritical <input type="checkbox"/> 2-D LC <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
If other, please specify	
Column used, specify vendor	
Specify column used	
Specify column cat no	
Specify column stationary phase	
Specify column inner diameter	
Specify column length	
Mobile phase A used	
Mobile phase B used	
Gradient used	
Flow rate used	
Column temperature used	
Injected sample volume used	
Gas chromatography method	
Instrument used, specify vendor	
Specify instrument used	
Specify instrument cat no	

FIGURE 3 (Continued)

3.2.6 | Analysis and interpretation

For differential and quantitative MS, generally a threshold of differentiation is preset (eg, 50% increase/decrease or 1.5-fold/0.50-fold differences between experimental groups). Statistical analysis is used to establish p-values. The smaller the *P*-value generally means the more likely the observed difference is real. Low *P*-values can also reflect high sample-to-sample variation within a group. Thus, a combination of addressing the fold-change and p-value usually can inform about the top protein candidates that are robustly up- or downregulated in a particular disease state model. For multiple testing conditions or multitesting group analysis, there a series of fold change/*P*-values analyses could be use. These bioinformatic tools should be conducted within the context of “fit- for purpose” analysis (ie, what do one wants to compare). An important visualization tool is the volcano

plot that combines these two factors. In terms of the overall study result and data analysis, common biostatistical tools such as *t* tests, ANOVA, and nonparametric tests are often used. For proteomic network analysis, a number of systems biology tools, including commercial packages (eg, Ingenuity Pathway Studio and Pathway Tools) or freeware such as KEGG (Kyoto Encyclopedia of Genes and Genomes), Wikipathways, PANTHER, and STRING are used to identify not only individual protein changes, but also protein pathways/networks with the most dominant changes. For more information, see.⁴² Some of these tools are also capable of identifying hubs or key driver networks as candidate therapeutic or biomarker targets. Lastly, some packages can further combine other omic data with proteomic data, for example, Ingenuity can integrate micro-RNAomic with proteomic data, while Pathway Tools can integrate protein/gene data with metabolomic data.

Injection mode	<input type="checkbox"/> Headspace <input type="checkbox"/> Solution <input type="checkbox"/> Fiber <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Column used, specify vendor	Column used, specify vendor
Specify column used	Specify column used
Specify column cat no	Specify column cat no
Specify column particle size	Specify column particle size
Specify column inner diameter	Specify column inner diameter
Specify column length	Specify column length
Gas used	<input type="checkbox"/> Helium <input type="checkbox"/> Nitrogen <input type="checkbox"/> Hydrogen <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Column temperature used	
Flow rate used	
Mass spectrometry method	
Instrument used, specify vendor	
Specify instrument used	
Specify instrument cat no	
Instrument type	<input type="checkbox"/> TOF <input type="checkbox"/> Q-TOF <input type="checkbox"/> Single quad <input type="checkbox"/> Triple quad <input type="checkbox"/> Ion trap <input type="checkbox"/> Fourier transform <input type="checkbox"/> TIMS-PASEF <input type="checkbox"/> IMS <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Lipidomics/Metabolomics mode	<input type="checkbox"/> Untargeted <input type="checkbox"/> Targeted <input type="checkbox"/> Unknown
Ionization mode	<input type="checkbox"/> ESI+ <input type="checkbox"/> ESI- <input type="checkbox"/> APCI+ <input type="checkbox"/> EI <input type="checkbox"/> CI- <input type="checkbox"/> CI+ <input type="checkbox"/> MALDI <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
MS mode	<input type="checkbox"/> Full scan <input type="checkbox"/> MS* <input type="checkbox"/> MRM <input type="checkbox"/> SIM <input type="checkbox"/> PASEF <input type="checkbox"/> NLS <input type="checkbox"/> PIS <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Specify peak detection and processing software	
Nuclear magnetic resonance method	
Instrument used, specify vendor	

FIGURE 3 (Continued)

3.3 | Lipidomics

Appendix S5 and S6

3.3.1 | Rationale

Lipidome is a term coined in molecular biology to describe the entire repertoire of lipids in each cell, organism and/or biological fluid. The term “cellular lipidome” was first defined by Han and Gross in 2003 when the authors identified and quantified all the detectable lipids in a cellular extract using MS.⁴³ Since 2005, when the first European initiative on lipidome characterization was started,⁴⁴ lipidomics (the study of lipidome) has been recognized and implemented as one of the core omics fields. Lipids are highly dynamic under various physiological and pathological conditions, as well as in response to various environmental factors.⁴⁵ They exhibit a high structural diversity

and are currently classified in eight major lipid classes each with a subsequent subclassification hierarchy: fatty acyls (FA), glycerolipids (GL), glycerophospholipids (GPS), sphingolipids (SP), sterol lipids (S/), prenol lipids (PR), saccharolipids (SL), and polyketides (PK).⁴⁶ The lipidome can be divided into (a) the membrane lipidome that encompass constitutive membrane lipids and (b) the mediator lipidome, comprising lipids with signaling and mediator function. Mediator lipids are often produced from membrane structural lipids by a complex repertoire of interconnected enzymatic processes which are regulated under physiological and pathological conditions. While in lipidomics the aim is to identify and quantify the complete set of lipids in each cell or biological matrix, in practice it is virtually impossible to achieve this aim under a single experimental protocol. This is due to the high structural heterogeneity, dynamic range, and varying physio-chemical properties exhibited by the lipids—a complexity which can be only addressed by several, tailored experimental

Specify instrument used	
Specify instrument cat no	
Metabolomics mode	<input type="checkbox"/> Untargeted <input type="checkbox"/> Targeted <input type="checkbox"/> Unknown
Specify peak detection and processing software	
Data annotation	
Peak/spectral identification	<input type="checkbox"/> Reference to known standards <input type="checkbox"/> Rule-based fragmentation annotation <input type="checkbox"/> Database search <input type="checkbox"/> Self-established spectral library <input type="checkbox"/> Unknown
If database search, specify database for lipidomics/metabolomics annotation	
Database version	
If self-established spectral library, specify availability	
Manual data curation	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
Database used for pathway analysis	<input type="checkbox"/> LipidMaps <input type="checkbox"/> HMDB <input type="checkbox"/> KEGG <input type="checkbox"/> METLIN <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Database version	
Data quantification	
Quantification strategy	<input type="checkbox"/> Relative quantification <input type="checkbox"/> Absolute quantification untargeted data <input type="checkbox"/> Absolute quantification targeted data <input type="checkbox"/> Label-free quantification <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Class or subclass-specific lipid internal standards	
Pooled samples	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
Internal standards	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
Candidate identification criteria	<input type="checkbox"/> p-value <input type="checkbox"/> Log2 Fold Change <input type="checkbox"/> Unknown <input type="checkbox"/> Other

FIGURE 3 (Continued)

protocols. Hence, multiple lipidomic methods, which are tailored to a lipid class, or several lipid classes are required to achieve deep characterization of the lipidome in a biological matrix.^{47–50}

3.3.2 | Measurements

Changes to lipids in the presence of pathological, physiological and/or environmental factors between experimental models and controls occur either at the concentration level for individual lipids or at the structural level, such as occurring via (per)oxidation or cell/organ/biological fluid-specific enzymatic processes. Therefore, both lipidome compositional pattern and lipid level changes are investigated as relevant metrics for comparison between various experimental and control conditions.⁵¹ The structural elucidation of a given lipidome is highly challenged by the high number of lipids present in each biological system—in

plasma alone around 600 lipids have been characterized, while overall a large portion of the lipidome remains structurally unknown.⁵² This is compounded by the high level of isomerism, stemming for example from the position of double bond or cis and trans conformation within a fatty acyl chain, different substitution with FA of sn1 and sn2 position in glycerol chain of PLs, or different linkages and linkage position of monosaccharide units in saccharolipids, etc.⁵³ Investigation of the structural heterogeneity of a lipid mixture by mass spectrometry is achieved by inferring accurate mass measurement and fragmentation pattern of lipid ion species, along with chromatographic behavior when mass spectrometry is hyphenated with chromatography. For modern MS instrumentation encompassing ion mobility separation, a fourth dimension of structural identification is enabled by the measurement of collisional cross section of lipids which reflects the structural conformation of the species. Quantitative determination of individual lipids in a mixture relies on reference to

If other, please specify	
Data validation	
Lipids or Metabolite confirmation by second method	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If yes, specify method	
Independent biological replicates for validation	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
If yes, specify number	
Data statistical processing	
Statistical tests	<input type="checkbox"/> PCA <input type="checkbox"/> PLS-DA <input type="checkbox"/> T-test + FDR <input type="checkbox"/> ANOVA <input type="checkbox"/> Regression <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	

Instructions: Please check boxes where applicable. If none of the predetermined options is appropriate use the default space to specify your answer. This form is to be filled in for one individual animal.

Abbreviations: 2-D LC, two-dimensional liquid chromatography; ANOVA, analysis of variance; APCI, atmospheric pressure chemical ionization; CI, chemical ionization; EI, electron ionization; ES, electrospray ionization; FDR, false discovery rate correction; HILIC, hydrophilic interaction liquid chromatography; IMS, Ion mobility spectrometry; MALDI, matrix-assisted laser desorption/ionization; MRM, multiple reaction monitoring; MS, mass spectrometry; NLS, Neutral Loss Scan; PASEF, Parallel Accumulation Serial Fragmentation; PCA, principal component analysis; PLS-DA, Partial least squares discriminant analysis; PIS, Precursor Ion Scan; Q-TOF, quadrupole time-of-flight; SIM, single ion monitoring; TIMS, trapped ion mobility spectrometry; TOF, time-of-flight

FIGURE 3 (Continued)

internal standards, which are typically either deuterated lipid species or “unnaturally” occurring lipids, that are spiked in the sample at the initial stage of extraction, thus accounting for the major postsampling alteration of lipids due to chemical, enzymatic processes and analytical procedures. Certainly, linearity, matrix effects, extraction efficiency, limit of detection and of quantification, selectivity and reproducibility are essential parameters to be defined and considered for validation of quantitative methods and results. Depending on the lipidomic approach and mass spectrometric method used a one-point or multi-point calibration is implemented for quantification purposes.^{53,54} Validation of the lipid alteration with a given physiological, pathophysiological, or environmental factor is essential for the precise identification of biomarkers, and can be achieved by cross-platform validation and/or by cross-method validation. Often, unbiased lipidome analysis is used to generate a candidate list of lipid biomarkers which are then validated by absolute quantification assays using mass spectrometry and/or other lipid functional assays or biochemical tools. Cross-laboratories validation of results is increasingly recognized and implemented within national and international focus programs and collaborative research initiatives.

3.3.3 | Equipment

Lipidomics analysis is carried out typically using (a) liquid-chromatography electrospray mass spectrometry;

(b) chip-based mass spectrometry⁵⁵; (c) matrix assisted laser desorption mass spectrometry⁵⁶; (d) imaging mass spectrometry for spatial distribution analysis of lipids within a tissue,^{57,58} or (e) gas chromatography mass spectrometry.⁵⁹ In particular cases, thin-layer chromatography is used for immunoassays of lipids and/or for subfractionation of lipid classes prior to mass spectrometric analysis. Liquid chromatography is hyphenated either with (a) high-end mass spectrometers capable of high resolution, mass accuracy, precision, speed of analysis, high fragmentation efficiency of the lipidome, with or without ion mobility separation, for unbiased (sub)lipidome analysis, or with (b) multiple reaction monitoring for selective, targeted quantitative and qualitative profiling of lipids. The targeted method has the distinct advantage of filtering out a large portion of lipid matrix or overlapping isobaric and near isobaric lipid ions enabling detection and quantification of low abundant lipids, such as lipid mediators and signals. Imaging mass spectrometry is an excellent tool for investigation of spatial distribution and changes of lipids in a tissue slice and cell population, by enabling acquisition of all lipid ions generated by a laser beam across an entire tissue slice. Here, lipid data are coregistered to an optical image of the tissue, allowing identification of lipid composition in selected histological regions. Chip-based mass spectrometry is a modern platform for the so-called “shot-gun lipidomics,”^{55,60} which capitalizes on increased chip-based ionization efficiency and reproducibility and speed of analysis for high-throughput lipidomic analysis. Gas-chromatography mass spectrometry is more useful

Omics Studies
Case Report Form: 4 CRF - Data management
CRF module: Data management

Date that this CRF was filled out:

Name of person filling out CRF:

Project name/identifier:

Storage details	
Storage types	<input type="checkbox"/> Local <input type="checkbox"/> External hard-drive <input type="checkbox"/> Institute cluster <input type="checkbox"/> External cluster <input type="checkbox"/> Cloud-based <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Specify storage locations	1. _____ 2. _____ 3. _____
Security	<input type="checkbox"/> username and password <input type="checkbox"/> encryption
Contact for access	Name: _____ Telephone: _____ Email: _____
Data availability	
Data availability	<input type="checkbox"/> No <input type="checkbox"/> On request <input type="checkbox"/> Publicly available
If on request, please provide contact details	
If publicly available please specify data storage site	
If publicly available please specify accession number	
Data provided according to FAIR principles	<input type="checkbox"/> No <input type="checkbox"/> Yes <input type="checkbox"/> Unknown
Data details	

FIGURE 4 Data management report form (CRF; see main text for details)

for specific lipid classes, such as analysis of free fatty acyls, or sterols. Given the diverse physio-chemical properties of lipids, in particular the ionization properties, unbiased and targeted lipidomics often require analysis in both the positive and negative ion modes.

3.3.4 | Approaches

Unbiased lipidomics aims at global lipid profiling in each sample capitalizing on the capability of the high end-mass spectrometers to ionize, fragment, detect and (in certain instruments) to conformationally separate hundreds to thousands of lipids in a single experiment. Depending on the aim of the study, unbiased lipid analysis can be applied to a particular fraction of the lipidome (sublipidome),

whereby the extraction method and analytical protocol (chromatography, ionization conditions, fragmentation, and detection conditions) are tailored to a lipid class or subclass, such as for glycolipidome, cardiolipidome, etc. Without lipidome fractionation and/or tailoring of extraction method toward a particular lipid class or subclass, unbiased lipidomics of a lipid extract will typically enable profiling of major membrane lipidome and glycerides: phospholipids and lysophospholipids, sphingomyelins, tri- and di-glycerides, ceramides, and to a rather limited extend glycolipids. Unbiased lipidomics can be carried out by chip-based MS, MALDI-MS, or LC/MS. Chip-based MS can also be on-line hyphenated with LC, while MALDI MS can be only off-line combined with chromatography. The lipids are identified and structurally characterized based on the accurate m/z measurement of intact lipid

Ethics agreement allows for third-party research	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
Organism	<input type="checkbox"/> Mouse <input type="checkbox"/> Rat <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Total number of files	
Total file size	
Raw data	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
File type	<input type="checkbox"/> .fastq; <input type="checkbox"/> .idat; <input type="checkbox"/> .bam; <input type="checkbox"/> .sam; <input type="checkbox"/> .txt; <input type="checkbox"/> Unknown <input type="checkbox"/> Other
If other, please specify	
Specialised software needed to view data	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown
Attached sheet linking files to samples and phenotype	<input type="checkbox"/> Yes <input type="checkbox"/> No <input type="checkbox"/> Unknown

Instructions: Please check boxes where applicable. If none of the predetermined options is appropriate use the default space to specify your answer. This form is to be filled in for one individual animal.

FIGURE 4 (Continued)

ion species, fragmentation pattern of individual lipid ions (where class specific fragment ions are generated which are indicative of specific structural features, see www.lipidmaps.org), and quantified based on reference to spiked internal standards of known concentration. For MS instruments equipped with ion mobility, separation based on structural conformation is afforded, where the collisional cross section of lipids is determined and used for lipid identification by reference to existing data bases of ccs values, or by ion mobility MS profiling of specific lipid standards.⁶¹ Chromatographic separation can be carried out by reversed phase and/or hydrophilic chromatography. Reversed phase chromatography is typically employed as it also allows lipid separation based on finer structural differences, such as fatty acyl chain lengths, number of double bonds, and to a certain extent position of double bond, head groups. Hydrophilic chromatography can be used to separate lipid class in subcategories based on the hydrophilicity properties, such as to separate phospholipids according to the different head groups, or glycolipids according to the glycosidic chain length and structure.⁶²

Lipid annotation is carried out using lipid libraries and data base searches, as well as by using lipid type-specific fragmentation rules. Such tools are either embedded in the instrument software and/or available as open source.

Targeted lipid analysis aims at profiling and quantifying a predefined set of lipids. This method is applied either for cross-validation of findings from an untargeted lipidomic profiling, or for investigation of low abundant lipids. Using class-or subclass-specific lipid internal standards and calibration standards, the method allows for multiplex absolute quantification of lipids. Availability of lipid standards is, however, limited by the difficulties in chemical synthesis of lipids and/or isolation of single compounds from biological matrices. Therefore, often use of a lipid

standard for various classes or subclasses is employed. Typically, this method is carried out using multiple reaction monitoring on quadrupole ion trap or triple quadrupole instruments; recently, high end mass spectrometers with parallel reaction monitoring capabilities can provide a viable complementary tool for absolute lipid quantification. Targeted lipid analysis is typically employed for mediator lipids which are not readily detectable in an unbiased analysis. Examples include eicosanoids, sphingosine- and ceramide- phosphates, prenols, and free fatty acyls. Where the set of lipids exhibit distinct propensities for ionization in positive and negative ion modes, either distinct analysis in the two ionization modes or single analysis on instruments with capability for fast ion mode switching (such as QTRAP) must be applied. Lipids levels determined by MS can be normalized either to protein content for tissue and cell prepare, to tissue weight when weighing prior to extraction, and in case of biological fluid to volume of fluid used for extraction.

In general, one of the limitations in lipid quantification is the limited availability of lipid standards and internal standards, given major difficulties in chemical synthesis and isolation of lipids.

3.3.5 | Procedure

Lipids are extracted from a biological sample using organic solvents to take advantage of their hydrophobic properties. Liquid-liquid extraction based on chloroform/methanol/water (Bligh and Dyer or Folch methods) has been the gold standard for decades for lipid extraction and total lipid content determination in biological samples.⁶³⁻⁶⁵ More recently, methyl-tert-butyl-ether (MTBE)-based methods have been demonstrated to be more effective and

TABLE 1 Common preanalytical factors that can affect laboratory results

Biological	Examples of affected tests	Solutions
Age, sex, species, strain.	Hormones, enzymes, many other.	Limit measurements to homogenous biological groups or use statistics to control for confounding factors.
Time of sample collection and menstrual/estrous cycle.	Cortisol, glucose, melatonin, estrogen, progesterone, many other.	Collect at the same time of day and consider the effects of the menstrual/estrous cycle in females. Pool multiple samples or perform long-term collections (eg, 24-h urine) to eliminate ultradian/circadian/circannual oscillations.
Dietary status.	Glucose, amino acids, fatty acids, many other.	Use the same diet in experimental and control animals. Collect fasting samples or note the time of the last meal.
Hydration status.	Most relevant for urine samples, for example, urea, creatinine, electrolytes, drugs, metabolites, many other.	Use 24 h urine collections or normalize measurements in random urine samples with urine creatinine.
Stress.	Cortisol, catecholamines, glucose, many other.	Limit as far as possible animal stressors, for example, transportation, unfamiliar handler, single housing, housing with other species, restraint, painful procedures, and anesthetics.
Medications, anesthetics, dietary supplements.	Hormones, many other.	Avoid use of medications and anesthetics unless necessary or part of the experimental protocol. Discontinue biotin supplementation several days before sample collection if streptavidin-based immunoassays are used.
Technical		
Use of tourniquet.	Potassium, lactate.	Loosen tourniquet as soon as possible.
Venous vs. arterial vs. capillary blood.	Bicarbonate, pO ₂ , pCO ₂ , glucose, many other.	Use venous samples for most studies unless specific reasons require arterial or capillary collections.
Blood collection tube additives.	Glucose, electrolytes, coagulation factors, many other.	Chose the proper tube type(s) for the test(s) in question.
Hemolysis, icterus, lipemia.	Numerous tests using spectrophotometric or colorimetric methods.	Avoid hemolysis by proper draw technique. Lipemic samples can be cleared by ultracentrifugation. Establish acceptable cutoffs for hemolysis, icterus and lipemia when validating analytical methods. Use non-spectrophotometric methods (eg, mass spectrometry), if possible.
Ex vivo metabolism.	Amino acids, lactate, ammonia, many other.	Process and analyze samples as soon as possible after collection. Some samples should be collected on ice and kept cold during analysis. Rapid (snap) freezing as soon as possible after collection (note: freezing can damage tissue architecture and hemolyze whole blood unless special precautions are taken).
Long-term stability.	Amino acids, potassium, glucose, lipids, many other.	Separate compounds of interests (eg, cells, plasma, lipids) as soon as possible and store promptly at -80°C. If necessary, stability studies can be performed by measuring the same compound in the same sample over long periods of time.
Light and pH.	Urine porphyrins, catecholamines, citrate, cortisol, many other.	Adjust the urine pH if needed and protect against light by wrapping the sample container in foil.

easier to use, as in this case the upper phase is the organic phase containing the extracted lipids, hence readily recovered and more amenable to automatization. One step organic solvent extraction, such as methanol, isopropanol, ethanol, MTBE are also increasingly used.⁶⁶ It is important to note, though, that the extraction methods must be

tailored to the lipid classes of interest for investigation, since the extraction efficiency of the diverse lipids varies with different solvents used. In this regard, solid phase extraction can also be used to enrich certain lipid categories, such as eicosanoids, or deplete lipid classes such as phospholipids. Subfractionation of the lipidome, that is, by use

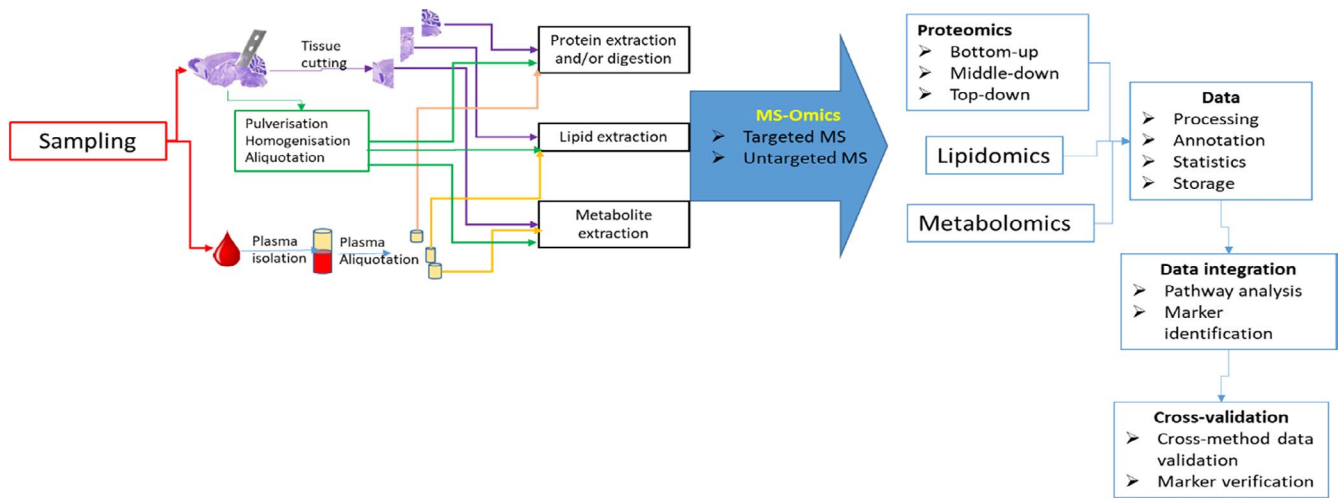


FIGURE 5 Overview of the mass-spectrometry-based analytical workflows for tissue and blood (or other body fluids) omic-profiling

of combined LLE, affinity and chromatographic fractionation, can also be employed for tailored extraction of lipid classes.

The addition of antioxidants and lipase inhibitors to ensure stability of the lipidome during extraction, as well as of internal standards to allow reliable lipid quantification, is required at the initial extraction step, typically together with the extraction solvent. Chemical and mechanical homogenization of tissues and cells is simultaneously carried after addition of extraction solvent and homogenization buffer. For tissues, a prior mechanical homogenization by pulverization of the frozen tissue can be applied to increase efficiency of the extraction of endogenous lipids. This procedure can also benefit splitting of the tissue for multi-omic analysis from the same sample as it precludes bias due to tissue heterogeneity. After homogenization, centrifugation is applied to separate phases and/or precipitate any remaining pellets. At this step, removal of proteins and water-soluble metabolites is enabled when using liquid–liquid extraction methods. The organic phase containing lipid extract is recovered, dried, and reconstituted in a MS or LC/MS compatible solvent for analysis. A direct injection into the MS of the lipid extract without subsequent drying and reconstitution in another solvent can also be opted for if the organic phase of the extraction is compatible with ionization of lipids and/or chromatographic method. Similar extraction strategies are applied for plasma and other biological fluids. For biological fluids, the homogenization step is replaced by vortexing.⁶⁷

For reliable comparative lipidome analysis, standardization across sample batches of the extraction time, temperature conditions, sample storage prior to analysis is essential. Carrying out all sample preparation steps at low temperature (4°C or 10°C, where the latter is safer to prevent low temperature lipid assembly) has proven

instrumental to diminish possible enzymatic alteration of the lipidome.⁶⁸

3.3.6 | Analysis and interpretation

The choice of analysis and analytical approach depends on the aim of the study or specifically on the lipid classes that are of interest.

Global profiling of a total lipid extract by unbiased lipidomic methods must be carried out in both positive and negative ion mode given that ionizability of lipids differ with their structure. For example, in positive ion mode tri- and diglycerides, sphingomyelins, phosphocholines, neutral glycolipids are readily ionizable, while in negative ion mode, the other phospholipid categories, along with free fatty acids, sialylated glycolipids, and cardiolipins are ionizable. Hence, two analyses per sample in positive and negative ion mode must be carried out to obtain a global lipid profile, unless the mass spectrometer has the capability for fast ion mode switching. While certain lipids can ionize in both positive and negative ion mode, no ionization mode will provide complete coverage of the lipids in a total lipid extract. Certainly, when a specific subfraction of the lipidome is prepared the choice of ionization mode, and chromatography is guided by the inherent properties of the sublipidome in this regard. For example, free fatty acyls, eicosanoids, sialylated glycolipids will be analyzed in negative ion mode unless otherwise derivatized to convert them into positively charged ions.

For selective quantitative and qualitative profiling of lipids by targeted mass spectrometry, for example, multiple reaction monitoring, the same rationale for the choice of chromatography and ionization mode applies. Specific to this approach is that prior knowledge of the MS-based descriptors of the target lipids is required: the m/z of the

precursor lipid and *m/z* of the specific fragment or of multiple fragments it generates in the MS collision cell. These lipid descriptors can either be inferred by manual analysis of the corresponding standard lipids or from literature and/or open-source lipid resources (www.lipidmaps.org). Retention time can also be set as a criterion to select the target lipid precursors in a multiple reaction monitoring (MRM) experiment.

For annotation of lipids detected in an unbiased lipidomic analysis various lipid libraries and data bases are embedded in the instrument provider software tool kit and several Web-based open-source resources are freely available. A collection of such resources is available on www.lipidmaps.org. Similarly, descriptors of lipids for use in mass spectrometry annotation such as molecular masses, lipid ion and lipid fragment masses and ion mobility can be retrieved from www.lipidmaps.org. Lipid concentration references in cells, tissues and plasma under pathological conditions are to some extent available in MarkerDB <https://markerdb.ca>; however, unfortunately not yet for epilepsies.

While the currently existing lipidomics studies of epilepsy models have provided valuable information of lipid changes with various epilepsy conditions,^{67,69–72} the expansion of lipid data in this context is necessary and expected soon. Lipid pathways and signaling regulated under epilepsy conditions can be inferred from lipidomics data using pathway analyses tools available at www.lipidmaps.org and the KEGG PATHWAY Database (www.genome.jp). Certainly, advances in omic investigations will aid in expediting lipid pathway identification and bridging of the lipidomics to other omic fields. The choice of statistical tools such as PCA, ANOVA, PLS-DA for lipid data analysis is guided by the experimental design and the biological question of the study. Machine learning and artificial intelligence tools are not yet part of standard toolkit in laboratories for lipid data processing; hence, it requires concerted collaborative efforts across the different expertise fields to valorize these capabilities for lipid data integration and pattern identification.

3.4 | Metabolomics

Appendix S5 and S6

3.4.1 | Rationale

The metabolome is a snapshot of the chemical state of a biological system and allows the scientist to distinguish between various normal or pathological states by comparing large amounts of biochemical data. Inherent in this technology is the assumption that no single metabolite is sufficient, and rather a panel of metabolites is required.

The metabolome is the entire collection of small molecules⁷³ (typically <1500 Da in size) in a biological system (i.e. organism, organ, tissue, and cell), and it has been estimated that several thousand compounds comprise the human metabolome (see the Human Metabolome Database for updated information [www.hmdb.ca]). The metabolome includes several classes of molecules such as amino acids, organic acids, carbohydrates, purines, pyrimidines, and lipids. The latter class is treated as a separate “lipidome” category (see previous section). The term “metabolomics” typically refers to the analytical techniques used to identify and quantify the different components of the metabolome. However, because the chemical properties of metabolites vary greatly, no single approach can identify and quantify all components of the metabolome. Instead, a combination of different extraction, separation, and detection steps that use a variety of instrumental and computational resources are necessary.

3.4.2 | Measurements

To compare changes in the metabolome between different experimental/study conditions, accurate and reproducible quantification of individual metabolites is an essential, but often extremely challenging task that requires attention to several key test performance characteristics specifically: (a) accuracy (trueness), (b) precision (reproducibility), (c) upper and lower limits of detection, (d) linearity, and (e) carryover.⁷⁴ Ideally, each metabolite should be validated for the above parameters, and each sample type should be validated to account for the effects of different sample matrices on the measurements. To facilitate accurate and reproducible quantification, each sample run should include several calibrator and quality control samples, and all samples should be spiked with stable isotope labeled internal standards (preferably ¹³C- and/or ¹⁵N-labeled samples). Levey-Jennings plots are strongly recommended to track the test performance over time and the use of Westgard Rules adds an additional layer of quality assurance.⁷⁴ However, due to the vast and distinct structural diversity of metabolites in various biological matrices, it is virtually impossible for a laboratory to validate the performance characteristics for the entire metabolome, even in a single sample type. A more manageable approach is to validate a smaller set of chemically similar metabolites, for example, amino acids, in a few sample types that are of interest to the lab, for example, brain tissue homogenates, CSF and/or serum, and then expand to other metabolites and sample types through collaborations or fee-for service arrangements with other metabolomics laboratories. An alternative approach is to start out with a less rigorously validated, semi-quantitative metabolomics screen and

follow up with a confirmatory study using a fully quantitative and rigorously validated approach.

3.4.3 | Equipment

The most used instrument platforms are (a) gas chromatography combined with mass spectrometry (GC–MS), (b) liquid chromatography combined with mass spectrometry (LC–MS), (c) matrix associated laser desorption ionization (MALDI)-mass spectrometry, and (d) nuclear magnetic resonance (NMR) spectroscopy. Less commonly used platforms include (e) supercritical chromatography combined with mass spectrometry, (f) desorption electrospray ionization (DESI) mass spectrometry, and (g) liquid chromatography combined with various detectors, and (h) gas chromatography with various detectors.¹³

3.4.4 | Approaches

Like the other omic fields, the metabolomic approaches can be classified as untargeted and targeted (reviewed in⁷⁵). Untargeted approaches screen for many compounds within a pre-defined mass range, for example, 50–1000 Da. The output is usually a list of “hits” that are each defined by a precursor (“parent”) mass/charge (m/z) ion ratio, by one or more fragment (“daughter”) m/z ions if collision induced fragmentation was performed, and by a retention time if GC or LC was performed. Library searches are then performed to determine the most likely chemical identity of the “hit,” and comparisons with pure standards run through the mass spectrometer at the same conditions as the sample are used to confirm the identity of the “hit.” Targeted approaches are pre-programmed to identify a set number of defined compounds, such as a selection of amino acids, organic acids, and acylcarnitines. While untargeted approaches are semiquantitative and usually not validated with respect to all the key performance characteristics (see above), targeted approaches can be fully quantitative and extensively validated. As mentioned in the preceding text, several instrument platforms can be used, most commonly LC and GC combined with MS, and NMR spectroscopy. Various software packages can be used for peak detection and quantification, for quality control, for library searches, and for statistical analyses.

3.4.5 | Procedure

Samples for metabolomic analysis must be deproteinized before analysis, and removal of lipids can help reduce ion suppression and thus increase analytical sensitivity; however, lipid removal is not necessary.⁷⁶ The above can be

done by adding a precipitating agent such as methanol or acetonitrile to serum, plasma, CSF, saliva, or tissue homogenate followed by vortexing and centrifugation. Stable isotope-labeled internal standards can be added to the precipitating solution. The supernatant is used for analysis. Microdialysis and urine samples do not usually require deproteinization. Tissue and feces must be homogenized before deproteinization, and this can be achieved using various approaches including ultrasonic homogenization or bead beating. Homogenization is often done in phosphate buffered saline (eg, Dulbecco's Phosphate Buffered Saline) at a 1:10 ratio tissue: buffer. Solid-phase extraction using commercial extraction cartridges and a vacuum or air pressure manifold, or liquid–liquid extraction, can be used as an alternative to deproteinization by precipitation. The advantage of the latter methods is that they can be used to concentrate the samples and remove unwanted compounds, such as lipids or molecules of certain charges or affinities. The disadvantages are that they are more expensive and labor intensive and that they remove compounds that may be of interest to the investigator. Sweat is usually prepared by heating the sweat collection pads in a sealed headspace vial together with a solid phase microextraction (SPME) fiber.⁷⁷

3.4.6 | Analysis and interpretation

The analysis depends on the instrumentation used. For LC separation, many stationary and mobile phases are available, allowing for separation of compounds such as amino acids, organic acids, sugars, and stereoisomers. However, some metabolites are not sufficiently separated by standard LC or MS (eg, isoleucine, leucine, and allo-isoleucine) and may require derivatization before LC–MS. Several types of GC columns and carrier gases allow for separation of volatile compounds, although many sample types will need derivatization for GC. A variety of instrument configurations can be used for MS with the most common types being tandem MS (MS/MS), ion trap, and time-of-flight (TOF). MS/MS and ion traps are often used for targeted quantification while TOF is typically used for untargeted studies.

3.5 | Multiomics

3.5.1 | Rationale

Multiple aberrant cellular, molecular, and metabolic processes may be simultaneously occurring in various biological compartments in complex diseases such as epilepsy. To capture these dynamic biological changes, it may be beneficial to utilize a multiomics approach rather than any

single modality. Overlaying multiple omics technologies has a clear advantage in enriching information at a molecular and biochemical level that no one omics method alone can deliver. The rationale for each individual omics platform is provided in the preceding sections. The combination of omics platforms has distinct advantages when small amounts of tissue is available from preclinical or human surgically resected tissue. Combining the platforms can be synergistic in providing more in-depth datasets, and allow one to determine whether metabolites are indeed altered in response to gene or protein expression changes. Multiomics studies can be highly complementary, particularly if they are applied to individual animals/patients. In the case of microbiome changes, adding blood or fecal metabolomics to fecal sequencing data can provide the identities of metabolites altered by changes in the gut flora.

Examples for the potential of multiomics studies of a particular epilepsy phenotype are application of a dual lipidomics-transcriptomics method in brain punches from mouse epilepsy models⁷⁸ or proteomics-transcriptomics in rodent models of epilepsy.^{79,80} Determining from the same tissue specimen both lipid or protein changes and mRNA expression of translated proteins/enzymes can shed a new light on the disease mechanism, such as new aspects of signaling pathways including regulation of enzyme and receptor activity and function in epilepsy models. For an overview of multi-omics studies in animal models of epilepsy or resected brain tissue from patients with temporal lobe epilepsy we refer to a recent review from Bruxel et al.⁸¹

3.5.2 | Measurements, equipment, approaches, and procedures

The description for each of these is provided in the preceding text for the individual omics platforms.

For dual lipidomics and transcriptomics, a combination of liquid–liquid extraction and commercially available RNA extraction kits has proven to render not only improved extraction efficiency of membrane and mediator lipids but also of mRNA. Essentially, adding antioxidants, lipase inhibitors, lipid internal standards and chloroform to the RNA extraction buffer, allow for excellent extraction and preservation of RNA integrity, as well as efficient lipid extraction from small tissue samples obtained from different brain regions. Subsequently, lipids can be analyzed by either of the MS-based methods, targeted and untargeted lipidomics, whereby the lipid extraction efficiency allows for multiple MS analyses to be carried out.^{68,78} mRNA can be subsequently investigated by either qPCR or RNA sequencing.

Lipidomic and proteomic investigation from the same sample specimen could also be facilitated by the very

nature of organic solvent extraction of lipids leaving the proteins in the precipitate or the aqueous phase when liquid–liquid extraction is used. Interest in integrating lipidomics and proteomics data for a pathophysiological condition steadily increases,⁸² where insights into the crosstalk between lipids and proteins can expedite the understanding of the affected pathways, as well as the joint functional role of lipids and proteins in a particular condition. Similarly, metabolomics and proteomics approaches can be and are increasingly applied in disease mechanism investigation, particularly to understand the metabolic dysregulation mechanisms of diseases.

3.5.3 | Analysis and interpretation

Analysis of each independent omics approach is discussed in the preceding sections.

For dual or multi-omic analysis, the key development and optimization step is the extraction procedure. In particular, defining the chemical milieu to both preserve the integrity of the different molecular categories and allow an efficient extraction of the molecular categories, while rendering the extracts suitable for individual omics analyses, from the same sample. While currently there are only very few studies reported on new methods for emerging multi-omic extraction and analysis from the same sample specimen, they undoubtedly prove the feasibility of the technique and provide the basis for future advances. It is obvious that multi-omic extraction conditions will need to be tailored to the sample type and amount.

The data collected from multi-omics can be used to annotate disease-associated transcriptomic signatures, identify common signaling networks, and generate cause-effect relationships between the modalities measured. In this way, combining omics approaches can provide a more accurate approach to understanding disease pathophysiology. The data from each platform can also independently provide validation of any biological finding.

3.6 | Data management

[Appendix S7](#) and [S8](#)

3.6.1 | Rationale

The biological sciences are in a midst of a data revolution. Over the past decades the development of high-throughput technologies in the fields of genomics, transcriptomics, and proteomics coupled with reduction in per sample costs has resulted in the generation of vast quantities of

data. Estimates by the European Bioinformatics Institute (EMBL-EBI) suggest that the amount of biological data will soon rival the amount produced through astronomical observations.⁸³ With vast quantities of data already produced an even a greater amount predicted to be produced moving forward, there are several considerations that must be considered, primarily storage of such data, along with data-security, and ease of data sharing. All these aspects should be included in a data storage plan. The development of a data storage plan is an essential aspect of any experiment, as such it is expected that every CRF from the other modules is also linked to a data storage CRF. This is to ensure that all data produced from experiments recorded in the CRFs is appropriately stored and accessible moving forward.

3.6.2 | Storage details

It is important to provide accurate details on where data is stored and how it can be accessed. In the past it was common practice to store data on a local computer or on external hard drives. As computers and external hard drives can be damaged resulting in data loss, or lost resulting in security breaches of sensitive information, this is not an appropriate permanent storage method unless proper safeguards are put in place. Appropriate backup methods include following the 3-2-1 back-up principle; three copies of the data, store two back-up copies on different storage media, with one located offsite. An alternative to these approaches is to store data on an institute cluster, external cluster or cloud-based storage system. Each of these storage systems should have disaster recovery and cybersecurity plans in place for important and sensitive data, meaning it is close to impossible to lose the stored data or have unauthorized persons access it.

Upon publication, it is now a compulsory requirement of many journals that all data used is uploaded to a public repository. This concept encourages open access to scientific research, facilitates further research efforts, and maximizes data use. It is highly recommended that the FAIR (Findable, Accessible, Interoperable, Reusable) principles for data management are used for such repositories.³ Both the National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov) and the EMBL-EBI (www.ebi.ac.uk) have public repositories for the storage and sharing of -omics datasets.

3.6.3 | Data details and data processing

It is important that any dataset is stored with appropriate documentation; this will ensure that the data can be

re-analyzed or easily used by third-party researchers. Information pertaining to the experiment type, the organism being analyzed, data format, and details linking each data-file to the appropriate phenotype are all necessary to facilitate reuse of data. Further, while it is preferred that all data is stored in its raw form, as this will allow other researchers to re-analyze the data as methods improve, if this is not possible, it is important that the processing pipeline that was applied to the data is included.

4 | DISCUSSION

The goal of the present CDEs, CRFs, and accompanying paper was to improve the standardization of experimental designs in proteomics, lipidomics, and metabolomics related to preclinical epilepsy research in laboratory models and humans. We have generated CDE tables with key parameters and CRFs containing the essential contents of the study protocols for the three omics approaches. Here, we discuss the strengths and weaknesses of these approaches, we suggest best practice strategies for designing and carrying out these types of studies, and we highlight new and promising directions in the field.

4.1 | Strengths

Compared with traditional research methods, omics technology displays higher sensitivity and resolution, which can explore proteome, lipidome, and metabolome more widely and efficiently. Furthermore, omics can provide insight into disease processes, as well as solutions for identification of biomarkers, molecular diagnosis, and drug treatment.

For proteomics, qualitative and quantitative detection of different proteins is possible. For lipidomics, the high accuracy, high coverage, and low sample consumption are advantages. For metabolomics, high sensitivity of mass spectrometry vs MRS allows for the detection of low abundance metabolites and the sample size can be smaller than for transcriptomics or proteomics.

By screening many molecules and using a big-data approach, new hypotheses can be formed. The integration of several parameters in multi-omics studies may give a more complete picture of alterations. By using unbiased or untargeted approaches, new and unknown compounds or targets may be revealed.

4.2 | Weaknesses

In general, a large amount of data is being generated when using omics technologies. This requires sophisticated data

analysis tools and proper statistical analysis, since these data are subject to false discovery/Bonferroni effects due to the large number of datapoints. Furthermore, standardization of methods across laboratories may be an issue. It requires a tremendous effort to validate and standardize such large numbers of compounds. While unbiased/untargeted approaches may lead to the discovery of novel molecules or targets, the identification and validation usually takes lots of time and effort.

For proteomics, absolute quantitation is sometimes not possible, and it is difficult to detect and quantify low-abundance protein, since the sensitivity may not be high enough.

For lipidomics, absolute quantification of some lipids is difficult to achieve, particularly of isomeric structures. Additionally, provision of lipid standards suitable for mass spectrometry is rather challenging and the number of currently available is not sufficient to encompass this structural diversity. Given the high structural diversity of lipids, inevitably multiple-analytical approaches are required for exhaustive qualitative and quantitative lipid profiling.

For metabolomics, metabolites can change rapidly, and stability is difficult to control, which may lead to false positives. Since metabolomics are extremely sensitive to endogenous and environmental changes, standardization, and controls are essential.

4.3 | Best practice strategies for designing and executing studies in proteomics, lipidomics, metabolomics, and multiomics

When designing omics studies, it is important to spend considerable time determining the most suitable study design and making sure conditions are standardized (eg, time of day, diet, and strain), to include proper controls, and to take care that the sample collection is done in a proper way (eg, type of collection tubes, proper preparation, and storage of samples). Traditionally, the different omic investigations are carried out on distinct sample specimens.⁸⁴ However, in recent years, advances in sample preparation and subsequent integrated-omic workflows have permitted multi-omic data to be collected from the same sample specimen, which prevents bias due to distinct heterogeneity and origin of the sample while improving the costs and procurement of samples.⁷⁸

However, integrated-omic approaches are essentially unexplored in epilepsy research. Since there are many different analytical platforms for omics, it is unlikely that a single laboratory can afford and manage all these platforms. Therefore, collaborations between laboratories are needed, so expertise and equipment can be shared. The

success of a multi-omic data analysis strategy depends largely on the adoption of adequate experimental designs, and on the quality of the measurements provided by the different omic platforms. However, the field lacks a comparative description of performance parameters across omic technologies and a formulation for experimental design in multi-omic data scenarios.⁸⁵ Often, analysis expectations are frustrated by underpowered experimental design, noisy measurements, and the lack of a realistic integration method.⁸⁵ Therefore, in a recent study, a set of harmonized Figures of Merit (FoM), performance metrics typically used in analytical chemistry to describe devices and methods, was proposed as quality descriptors applicable to different omic data types, and together with the MultiPower (an approach for power calculations) and MultitML (an R method to obtain the optimal sample size required by machine learning approaches) methods, the optimal sample size in a multi-omics experiment can be estimated and assessed.⁸⁵

4.4 | New developments and directions

Genomics, transcriptomics, proteomics, and metabolomics were developed in the 80's and 90's of the last century, followed by lipidomics at the beginning of this century and most recently, microbiomics. Although most omics approaches have been around for many years, the omics field is still rapidly developing and expanding. Novel developments in the field of proteomics, lipidomics, and metabolomics include refined methodologies for detection, identification, and data analysis.

For proteomics, improvements in mass spectrometer design and bioinformatics algorithms have resulted in the rediscovery and development of another sampling method: data-independent acquisition (DIA). DIA comprehensively and repeatedly samples every peptide in a protein digest, producing a complex set of mass spectra that is difficult to interpret without external spectral libraries.⁸⁶ Furthermore, both label-free targeted proteomic and TMT-labeled proteomics have greatly advanced in terms of increased multiplexing, as well as in sample preparation and throughput.⁸⁷ Lastly, advances in signaling and spatiotemporal proteomics, such as the detection of post-translational modifications, single-cell proteomics, imaging mass spectrometry and mass cytometry (or CyTOF, combining principles of mass spectrometry and flow cytometry to enable single-cell analysis of protein expression) are ongoing.^{87,88}

For lipidomics, recent advances in detection techniques (including NMR and MS) have greatly expanded the depth and breadth of lipid analysis. However, the extreme complexity in lipid structures is an insuperable

barrier for the achievement of high-throughput and high-coverage analysis of the complete lipidome. A realistic solution is to choose an appropriate analytical strategy tailored to the purpose of the study. The combination of high-resolution MS with other analytical facilities (eg, ion mobility spectroscopy) enables a supplementary separation dimension, facilitating discrimination between lipid isomers, and greatly improving the lipid coverage.⁴⁷

For metabolomics, developments in chromatographic methods have been effective in separating metabolites and increasing the number of metabolites detected. This has been paralleled by a shift in the field from the conventionally used relative metabolite concentration measurements to a more reliable absolute concentration determination. Furthermore, mass spectrometry imaging (MSI) is a novel powerful tool that enables untargeted investigations into the spatial distribution of metabolites in a variety of samples. It has the capability to image thousands of molecules, such as metabolites, lipids, peptides, proteins, and glycans, in a single experiment without labeling.⁸⁹ The most recent advances include metabolomics for ex vivo analysis, as well as in situ imaging of tissue specimens.⁹⁰

The largest hurdle for any omics dataset remains “making sense of the data.” In the era of “big data,” artificial intelligence has been playing more and more critical roles in data mining. Especially the machine learning and deep learning approaches show extreme power in processing and modeling omics data with huge and diverse volumes.⁹¹ Combining machine learning with omics has largely advanced both the biological insights and computational approaches, but there is still a long way to go.

5 | CONCLUSION

Proteomics, lipidomics, and metabolomics are important to identify novel disease biomarkers and therapeutic targets in epilepsy research. Further advances in the separate omics fields, as well as in multiomics approaches, will lead inevitably to increased data generation and the challenges associated with handling and storing this data, but ultimately promises better understanding of disease processes during epileptogenesis.

For preclinical and clinical research, sharing expertise and data can facilitate the discovery of biomarkers and novel therapeutic targets. For proper interpretation and comparison this data, standardization across laboratories and across techniques is critical. With this manuscript, we provide CRFs and CDEs that may aid future developments and improve the standardization of experimental designs for various omics approaches.

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CONFLICT OF INTEREST

M. Patel and Piero Perucca are Associate Editors for *Epilepsia Open*. None of the other authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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