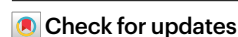


Consensus statement on mass spectrometry-based proteomic analysis of cardiac tissue

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Mass spectrometry-based cardiac proteomics provides direct molecular insight into cardiac physiology and disease. While plasma proteomics has advanced biomarker discovery, the analysis of cardiac tissue is essential for mechanistic understanding and therapeutic target identification; however, proteomic investigation of cardiac tissue faces unique challenges, including limited sample availability, regional heterogeneity, variability in collection and processing, and inconsistent reporting practices that hinder reproducibility and data integration. Here, we provide a practical framework for designing and conducting mass spectrometry-based proteomic studies of cardiac tissue and primary cardiac cells. We outline best practices and key considerations for sample handling, experimental design, data acquisition, quality control and statistical analysis. This guideline aims to support cardiac researchers in generating robust and reproducible proteomics datasets that advance our understanding of cardiac biology in both physiological and pathological contexts.

Understanding the molecular mechanisms underlying cardiac physiology requires detailed knowledge of the cellular and molecular components of the heart and how they are regulated in response to stimuli or in disease¹. Cardiac diseases are complex and multifactorial, arising from a combination of genetic, environmental and lifestyle factors. This complexity presents a challenge in addressing underlying disease processes and developing effective treatments. Proteomics, the large-scale study of proteins, bridges the gap between genotype

and phenotypic manifestations². The proteome reflects the functional summary of genetic predispositions dynamically shaped by environmental exposures and lifestyle factors and thereby serves as a tool to elucidate phenotypic traits and disease mechanisms (Fig. 1).

Advances in mass spectrometry (MS)-based proteomics now enable characterization of protein biology beyond relative abundance changes, encompassing post-translational modifications (PTMs), interaction networks and structural dynamics across spatial, cellular, and

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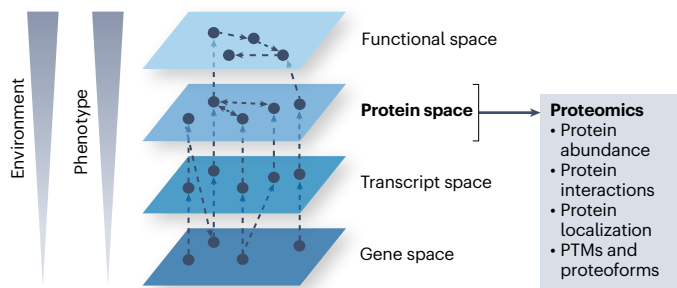


Fig. 1 | Cardiac proteomics as a bridge between genotype and phenotype in cardiac research. Understanding the molecular basis of cardiac diseases requires tools that capture the dynamic and functional state of cells and tissues. The proteome reflects the integrated influence of genetic predisposition, environmental exposures and lifestyle factors, making it a powerful resource for elucidating disease mechanisms and phenotypic traits. While plasma proteomics is more widespread than cardiac tissue proteomics in a clinical research context, a shift toward the latter is required for outlining disease mechanisms. Disease processes occur within affected tissues, and direct tissue analysis offers deeper mechanistic insights. ‘Gene space’ refers to DNA-based variation, ‘transcript space’ to mRNA expression, ‘protein space’ to the proteome measured by proteomics (including protein abundance, interactions, localization and PTMs), while ‘functional space’ reflects the resulting cellular activities and physiological processes. These layers are shaped by environmental influences and collectively determine the phenotype.

temporal dimensions down to the single-cell or subcellular level^{1,3–11}. Accordingly, cardiac proteomics presents a powerful strategy to investigate the molecular basis of cardiac physiology and disease.

In clinical research contexts, cardiac proteomics studies have predominantly centered on plasma proteome profiling using relative quantitative approaches, largely because plasma samples are readily accessible. Plasma-based analyses offer potential for biomarker discovery, aiding in diagnosis, treatment decisions, prognosis and therapeutic monitoring, while also providing systemic mechanistic insights, though with confounding contributions from noncardiac tissues; however, plasma studies often fail to capture, or even accurately reflect, the molecular events within cardiac tissue that drive disease pathology, as cardiac-derived proteins are diluted among abundant proteins derived from other organs. Thus, meaningful investigation of these tissue-level processes and the identification of new therapeutic targets necessitate direct proteomic analysis of cardiac tissue or cells isolated from the heart. By analyzing the proteomic landscape of cardiac tissue, researchers can directly observe the molecular alterations and pathways involved in the physiology, pathology or intervention of interest, enabling investigation of disease processes at the site of pathology.

However, realizing this potential requires addressing several distinct challenges. Foremost is the limited availability of human cardiac tissue samples, which restricts large-scale investigations and often necessitates the inclusion of specimens collected across multiple institutions and over time. The lack of harmonized protocols for sample collection, preservation and processing across sites introduces substantial variability and potential bias. Analytical inconsistencies further arise from regional and spatial heterogeneity within cardiac tissues, as well as from differences in protein extraction, enzymatic digestion, MS acquisition strategies and analysis. Compounding these issues is the absence of standardized reporting practices and metadata documentation, which impedes reproducibility, cross-study comparisons and data integration.

Critical aspects of study design, such as subject selection, cardiac sample collection, sample processing, data quality control and statistical assessment, are at times insufficiently addressed, undermining the

robustness of findings. These challenges are not restricted to cardiac proteomics, but are present in clinical proteomics more generally¹². Commonly encountered pitfalls include (1) control groups that are not matched with test groups on essential parameters; (2) inconsistent specimen collection conditions; and (3) sample handling or storage procedures incompatible with downstream quantitative analyses, as we will outline in detail in the section on study design. Addressing these limitations requires concerted efforts toward standardization across the entire workflow.

Herein, we outline essential considerations for designing and conducting cardiac proteomics studies using MS. The considerations are anchored in four steps, each of which influences the final experimental outcome (Box 1). Although the primary focus is on studies using human cardiac tissue, these recommendations are also broadly applicable to experimental investigations in cardiac cells and model organisms. This guideline provides a practical framework for conducting cardiac proteomics studies. Our aim is to support cardiac researchers and nonproteomics specialists in conducting cardiac proteomics investigations, with the goal of generating robust, reproducible data that enhance our understanding of cardiac biology in both physiological and pathological contexts.

Target information layers and cardiac sample requirements

MS-based proteomics enables comprehensive identification, quantification and characterization of proteins at scale. It has become a foundational technology in molecular biology owing to its high sensitivity, specificity and quantitative precision². The technology provides chemically precise data that allows unambiguous identification of protein isoforms and confident detection and characterization of reversible and irreversible PTMs on known proteins. With sufficient coverage, MS-based proteomics can also map coding single-nucleotide polymorphisms, novel splice variants and proteins of microbial or viral origin.

MS-based proteomics workflows can be classified as either top-down or bottom-up, depending on whether intact proteins are analyzed directly or enzymatically digested into peptides before analysis^{10,13}. Top-down approaches enable direct characterization of endogenous proteoforms in human heart tissue. Advantages of this approach include mapping of combinatorial PTMs³; however, shotgun (bottom-up) proteomics is the most widely used strategy and enables proteome-wide identification and quantification. In bottom-up proteomics, the peptides generated from enzymatic digestion are separated by liquid chromatography before MS analysis. Specific peptide ions (called precursors) are fragmented to generate tandem MS spectra (MS/MS spectra), which provide mass fingerprints characteristic of each peptide’s amino acid sequence. Informatics tools identify peptides by matching experimental fragmentation spectra to theoretical patterns derived from protein sequence databases. Peptides unique to a single protein or isoform (known as proteotypic peptides) allow unambiguous protein assignment. Peptides shared across multiple proteins owing to sequence conservation are assigned to ‘protein groups’. PTMs can also be detected and localized based on characteristic mass shifts, providing site-specific quantitative information.

Proteomic quantification is typically relative rather than absolute, necessitating study designs with comparisons between two or more groups. While relative quantification can be readily obtained from most MS datasets, the reliability and interpretability of quantitative comparisons depend on various factors emphasized in more detail below. Internal standards (proteins or peptides) can be added to samples to increase precision. These are often labeled with stable isotopes allowing them to be distinguished from endogenous counterparts while behaving nearly identically during chromatography and peptide ionization. Targeted approaches are important in clinical studies, where measuring a smaller number of proteins with high precision and reproducibility is critical.

BOX 1

Main steps of consideration in a cardiac proteomic experiment

Critical details for consideration include (1) what overarching approach is being prioritized; (2) experimental design details; (3) specific sample and experimental handling details; and (4) what infrastructure is being employed.

(1) Determine overall analytical strategy, defined by the biological question and target information layer of proteomics investigation

• Bulk proteomics

Global protein abundance profiling across entire tissue samples, providing an averaged proteome profile across all proteins in the tissue. Comparing profiles across conditions can identify protein abundance changes.

• PTMs

Detection and quantification of PTMs such as phosphorylation, acetylation, redox modifications, glycosylation, ubiquitination and other modification states, allowing insight into signaling dynamics and regulatory pathways.

• Interaction proteomics

Mapping of protein–protein interactions and analysis of changes in protein complexes and interaction networks.

• Single-cell proteomics

Resolves cell-type-specific protein expression in heterogeneous cardiac cell populations enabling analysis of cellular diversity and cell-type specific responses.

• Spatial proteomics

Protein profiling across anatomical regions of the heart, linking molecular expression to tissue architecture and localized disease processes, down to single-cell resolution.

(2) Study design and pre-analytical considerations

• Experimental study design

Quantitative proteomics primarily relies on relative quantification of protein abundance across samples, which is reflected in the typical experimental designs, such as case–control, matched pairs, longitudinal or population-based cohorts. Groups should be matched for confounding variables and limitations related to sample collection (for example postmortem tissue) should be carefully considered.

• Anatomical sampling and tissue composition

Anatomical sampling details and consistency across groups are critical for valid interpretation. Sampling locations should be precisely documented (for example atrial vs ventricular, left vs right, endo-, mid- or epicardial) and applied consistently across all samples. Note the predominant tissue type at each site, as myocardial, fibrotic, adipose or scar tissue, and whether these differ between groups. Ensure that selected locations are relevant for the biology studied.

• Sample handling, preservation and storage

Evaluate sample collection, preservation and storage procedures before the experiment. How was the sample handled and preserved after collection? What was the time from (1) death to sample collection (postmortem interval); (2) tissue excision to preservation (post-excision handling time; corresponding to ischemic time in surgical samples); and (3) storage to processing? These factors restrict the studies that can be pursued and are important covariates/confounders.

(3) Protein extraction and peptide processing strategies

• Sample homogenization and protein extraction

Effective tissue disruption, solubilization and extraction are critical for downstream data quality and reproducibility. Buffer composition, mechanical disruption and detergent or chaotrope choice must align with the experimental goals and the properties of cardiac tissue/sample studied.

• Peptide preparation workflows

Different quantification strategies require distinct peptide-handling approaches, including digestion methods, cleanup procedures, fractionation and possible labeling. These steps influence sensitivity, depth of coverage and quantification precision.

(4) MS measurement strategy

• MS selection

Different mass spectrometers have different advantages and can be applied with different purposes of study. Widely used proteomics instruments include Orbitrap and quadrupole time-of-flight (TOF)-based systems. Orbitrap analyzers offer high mass accuracy and resolution, making them particularly suited for applications such as detecting PTMs or multiplexed quantification strategies, whereas TOF analyzers provide rapid acquisition and broad mass range coverage. More recent hybrid platforms include timsTOF instruments (with trapped ion mobility spectrometry and parallel accumulation-serial fragmentation (PASEF) acquisition) and Orbitrap–Astral (integrating quadrupole, Orbitrap and Astral analyzers), enhancing analytical performance¹⁴. In addition to discovery-based approaches, targeted MS strategies such as selected/multiple reaction monitoring (SRM/MRM) and parallel reaction monitoring (PRM) allow hypothesis-driven validation with high sensitivity, reproducibility and quantitative precision.

• Data acquisition strategies

Data acquisition strategies are typically categorized into data-dependent acquisition (DDA) and data-independent acquisition (DIA). DDA selects the most abundant precursor ions from an initial survey scan for fragmentation and MS/MS analysis yielding high-quality spectra but suffers from limited reproducibility in which peptides are measured. DIA fragments all ions within predefined *m/z* windows, generating comprehensive MS/MS data across samples improving reproducibility and enhancing detection of low-abundance proteins⁹⁶.

• Quantification strategies

Two main quantification strategies are commonly employed. (1) Label-free quantification, which relies on MS signal intensity or spectral counts. This approach is cost-effective and scalable but can suffer from higher variability and missing values. (2) Multiplexed quantification, using isotopic or isobaric labeling, allows simultaneous analysis of multiple samples in a single run. This improves throughput and quantification precision but reduces coverage and introduces challenges such as ratio compression and more complex sample preparation.

Recent advances have expanded MS-based proteomics to spatially resolved, cell-type-specific and subcellular analyses, allowing the interrogation of tissue architecture and cellular heterogeneity at unprecedented resolution^{14–16}. A crucial step in designing a cardiac proteomics experiment is defining the target information layer, which is the specific molecular features one aims to measure. Each layer provides distinct biological insights and addresses different scientific questions, but imposes unique experimental

requirements. The analytical strategies used must therefore align with the study's objectives.

Bulk tissue proteomics

Bulk tissue proteomics remains a foundational approach for quantifying protein-level changes and identifying global trends in cardiac biology^{4,8,11,17,18}. These measurements are instrumental in uncovering broad insights into protein remodeling that takes place in an injured

or diseased heart that adapts and repairs and for investigating disease mechanisms², but are confounded by the input from multiple cell types, which can be present at different amounts in any tissue piece. Cardiac proteome investigations have been successfully applied to investigate pathophysiological mechanisms underlying various cardiac conditions, including arrhythmogenic¹⁹, ischemic²⁰, hypertrophic^{6,21,22}, dilated^{23,24} and diabetic^{7,25} cardiomyopathies of heart failure with reduced^{5,26,27} and preserved ejection fraction^{28,29}, of myocardial infarction³⁰ and to stratify patient groups^{17,31}.

PTM proteomics

While low microgram amounts of protein are generally sufficient for robust proteome profiling, global analysis of PTMs, such as protein phosphorylation ('phosphoproteomics'), often requires orders of magnitude more material. This is because robust PTM quantification typically necessitates enrichment of peptides harboring the modification, as PTMs occur at sub-stoichiometric levels and are subject to dynamic regulation³². Cardiac phosphoproteomics has been applied to elucidate signaling pathways activated in cardiac tissue and cardiomyocytes in response to stimuli such as β -adrenergic agonists^{9,33}, insulin³⁴ and GLP1 (ref. 35) as well as to outline signaling pathways involved in cardiac remodeling and repair^{36–44}. Although phosphorylation is the most extensively studied PTM, numerous other PTMs also play critical roles in regulating cardiac function and can be studied by proteomics approaches. The analysis of these modifications typically requires distinct enrichment or analytical strategies tailored to the chemical properties of each PTM class. Other PTMs that have been investigated in cardiac tissue include acetylation, ubiquitination, citrullination, glycosylation and glycation, among others.

Interaction proteomics

Interaction proteomics is a powerful application for studying protein complexes, typically performed in isolated cells or tissue samples. Interaction networks can be studied using affinity purification or immunoprecipitation followed by MS, as well as crosslinking MS to capture spatial constraints within protein complexes. In model organisms or cell culture systems, proximity labeling techniques such as proximity-dependent biotin identification (BioID)⁴⁵ or engineered ascorbate peroxidase (APEX)⁴⁶ enable the identification of proteins in the immediate vicinity of a tagged bait protein. Collectively, these approaches enable the identification of protein complexes, their regulation and subcellular localization. Proteomics can also quantify other dynamic processes, including the emergence of newly synthesized proteins, protein turnover⁴⁷ and structural changes⁴⁸ in cells or animal models.

Single-cell proteomics

Recent advances have enabled resolving cell-type-specific proteomic signatures. Traditional bulk tissue homogenate analysis provides an averaged view across all cell types included in a specific tissue, overshadowing signals from rare cell populations. For example, cardiac proteomics has profiled cardiomyocytes at the population level^{27,49,50}, but detecting proteins from rare cell types or low-abundance proteins remains challenging. In certain contexts, such as immune cell infiltration during cardiac inflammation or distinguishing between myocytes from the working myocardium and conduction system, single-cell resolution is essential. Recent improvements in MS sensitivity have enabled single-cell proteomics^{14,51,52}; however, current depth and quantitative precision remain limited compared to bulk approaches. Single-cell proteomics workflows can detect hundreds to a few thousand proteins per cell. This has also been achieved for human induced pluripotent stem cell-derived and adult cardiomyocytes. Current efforts in this area focus on generating cellular tissue atlases, with continuing improvements in depth and reproducibility¹⁶.

Spatial proteomics

Spatial proteomics couples protein identification and quantification with spatial information^{15,53–55}. This approach enables the study of rare or spatially distinct cell populations without tissue dissociation, preserving original tissue structure. Approaches include laser capture microdissection to isolate individual cells or defined micro-regions from tissue sections, followed by proteomic analysis. A notable innovation combines image-based segmentation with single-cell proteomics, enabling the investigation of complex cellular phenotypes while maintaining spatial resolution^{15,55}. This strategy is especially suitable for clinical and archival samples as it can collect target cells from fixed or frozen tissue. Imaging MS represents an alternative approach for studying spatial distributions of peptides in tissue sections by direct laser ablation and release of peptide ions from tissue sections^{53,54}.

Experimental and analytical demands across target layers

It is important to note that each of these experimental layers: proteome profiling, PTM analysis, interaction mapping, spatial proteomics and single-cell proteomics require specific sample preparation strategies and considerations. Each target layer comes with increasing complexity, both in the experimental design and in the subsequent data analysis (Fig. 2a). Although some cardiac proteomics experiments can be performed through direct submission of samples to a core facility, most studies benefit from early involvement of experts with specific expertise in cardiac biology and proteomics in the experimental design. The more complex the experimental design is, or the more specialized or demanding the analytical layer becomes, the more valuable such expert involvement becomes.

Bulk proteome measurements represent the most accessible type of analysis. Two example datasets: a bulk cardiac tissue proteome (Fig. 2b–d) and a bulk cardiomyocyte proteome (Fig. 2e,f) are illustrated in Fig. 2. These examples highlight challenges inherent to cardiac samples, particularly the dominance of myofibrillar proteins and the high mitochondrial content of cardiomyocytes. A considerable proportion of measurement time is spent on these abundant components creating a pronounced dynamic range that is especially challenging in cardiac specimens. As a result, even bulk proteomics can benefit from expert input. The abundance-rank plots also provide an immediate visual impression that certain cellular compartments, such as the myofibrils or the mitochondria, are easier to target and achieve higher coverage for than other cellular compartments; however, when methodological steps are optimized, including protein extraction, preprocessing and measurement strategy, bulk workflows can achieve sufficient depth to quantify less abundant but biologically informative proteins, such as plasma membrane proteins. This can be achieved even in single-shot measurements. The right optimization allows for many ion channels and receptor proteins to be covered in the data generated from bulk cardiomyocyte analysis (Fig. 2e,f).

Methodological considerations for each proteomics layer are summarized in Table 1. Designing a cardiac proteomics experiment involves defining inclusion criteria for study subjects, ensuring appropriate tissue sampling and preservation and selecting analytical strategies aligned with the biological questions. The choice of sample preparation and measurement platform affects the depth, accuracy and interpretability of cardiac proteomic datasets and must align with the specific biological questions and sample constraints of each study. In the following sections, we outline foundational principles for designing a cardiac proteomics experiment and a step-by-step guide to considerations throughout the experimental framework.

Study design considerations

Cardiac proteomics studies predominantly rely on relative quantification approaches, with intervention-based animal models and human case-control studies being the most frequently employed experimental

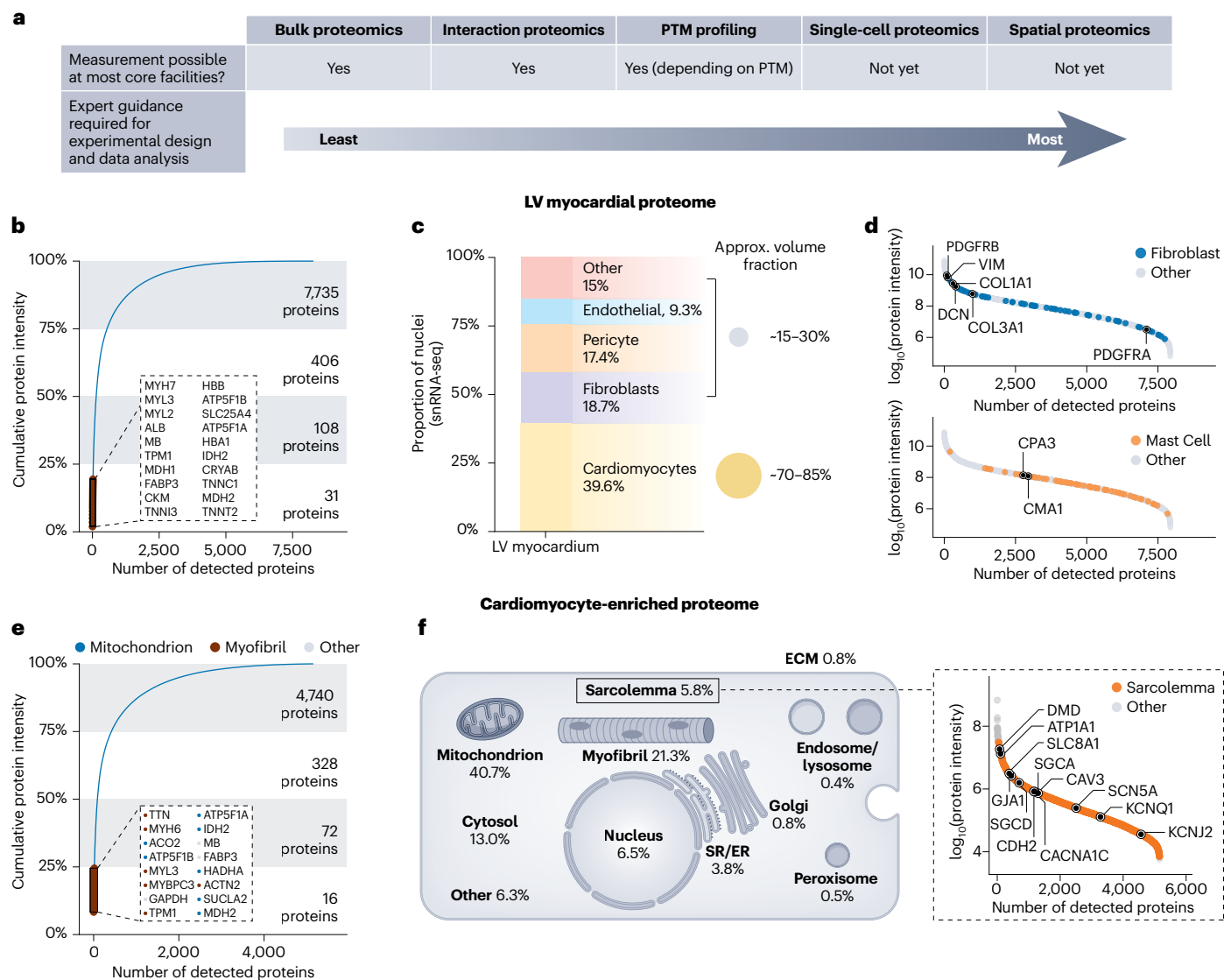


Fig. 2 | Consideration and challenges in cardiac proteomics. **a**, Overview of proteomics modalities and their relative accessibility and expertise requirements, ranging from bulk, interaction and PTM profiling to emerging single-cell and spatial approaches. **b**, Cumulative protein intensity profile from a bulk left ventricular (LV) human heart proteome. A small fraction of proteins, many of which are associated with cardiomyocytes, accounts for a disproportionate share of the total signal; the inset highlights dominant high-abundance proteins. **c**, Cell-type composition derived from single-nucleus RNA sequencing of the same tissue, showing cardiomyocyte predominance. Given that cardiomyocytes are several-fold larger than other cell types, they are estimated to occupy >75% of myocardial volume and therefore dominate the bulk myocardial proteome signal. **d**, Intensity-rank distributions of marker proteins for two less-abundant cardiac cell types (fibroblasts, top; mast cells, bottom), illustrating that proteins from less-abundant cell populations can also

be detected across the dynamic range when measurement depth is sufficient. Data are from Mulvey et al.⁹⁵. **e**, Cumulative protein intensity in isolated adult murine cardiomyocytes demonstrate that a small subset of highly abundant proteins dominates the proteome, with 16 proteins accounting for 25% of the total measured signal. Inset highlights these proteins, largely originating from myofilaments (red) and mitochondria (blue). **f**, Schematic of cardiomyocyte architecture summarizing the relative contributions of major organelles and compartments to total measured protein intensity. Despite extensive measurement time focused on myofibrils and mitochondria, proteins from other compartments can be quantified when sufficient depth is achieved. Intensity-rank distribution illustrates sarcolemmal protein abundances, highlighting several well-studied examples. Data are from Achter et al.³⁴. CM, cardiomyocyte; ECM, extracellular matrix; ER, endoplasmic reticulum; SR, sarcoplasmic reticulum.

designs. Drawing accurate biological conclusions requires careful selection of samples that are representative of both study groups. This consideration becomes even more critical given the typically limited availability of cardiac tissue specimens.

In case-control studies, participants or animals are designated based on the presence or absence of a defined cardiac condition or intervention. While the general principles of case-control design are well established in biomedical research, cardiac proteomics introduces additional practical complexities that are often underappreciated. A key challenge lies in ensuring consistency in sample

collection protocols, anatomical sampling sites and storage conditions across all study groups. Variability in any of these parameters can introduce substantial bias into proteomic analyses. In particular, the anatomical origin of the cardiac tissue must be standardized, as there are profound regional differences across the heart in terms of cellular composition and protein expression^{4,5,8}, which can confound results. Furthermore, the time interval between tissue excision and preservation, as well as in the case of human tissue from deceased donors, the postmortem interval before sample preservation, should be minimized and documented to ensure molecular integrity³⁶.

Table 1 | Purpose and sample requirements per proteomics target layer

Proteomics layer of focus	Purpose and application	Sample requirements and considerations
Proteome profiling	Quantification of global protein abundance across cardiac tissue samples. Used to identify protein-level changes and biological trends in health and disease.	- The typical input amount for robust analysis ranges from low micrograms to milligrams of proteins, depending on requirements such as labeling, fractionation and desired injection amount. Both fresh-frozen^{8,50} and FFPE tissue¹¹ are compatible with proteome profiling. - Potential biases should be considered, including differences in cellular compositions between conditions, for example fibrotic regions in diseased hearts. - Beyond whole tissue or cardiac cell proteomes, quantitative proteomics data can also be obtained from specific compartments, such as the cell surface or extracellular matrix, by using specialized strategies to enrich for these before MS measurement^{27,58,75}.
PTMs	Detection and quantification of dynamic modifications such as phosphorylation, redox, acetylation and ubiquitination. Used to identify signaling and regulatory mechanisms.	- Requires larger starting material than proteome profiling owing to low stoichiometry of PTMs. - Specifics of amounts required primarily depend on the target PTM and the enrichment strategy. Amounts can range from low micrograms to, more commonly, milligram-scale inputs. - Enrichment strategies (for example, TiO₂-enrichment for phosphopeptides and thiol-disulfide exchange for redox proteomics⁷⁶) are essential. - Immediate freezing post-collection is critical to preserve PTMs as is quenching enzymatic activity during sample handling, among others by including enzyme inhibitors in buffers, keeping sample cool and/or denaturing proteins. - Depending on the particular experimental conditions, it is possible to outline quantitative phosphorylation in cardiac tissue^{9,35,36} or in primary cardiomyocytes³⁴.
Interaction proteomics and structural proteomics	Identification of protein–protein interactions and macromolecular complexes. Provides insight into functional networks and regulatory assemblies.	- Techniques for interaction proteomics include (1) affinity purification (AP–MS) and (2) proximity labeling (BioID/APEX). AP–MS requires native lysates, whereas proximity labeling is compatible with denaturing conditions. - Methods for assessing protein structure and conformational changes include (1) solvent accessibility or (2) stability measurements. These include crosslinking MS, direct labeling of exposed amino acids (for example, hydrogen–deuterium exchange or covalent modification) as well as thermal proteome profiling and related techniques. - All approaches require technical and biological replicates, appropriate controls, and careful background correction. For examples of outlining protein interaction networks in cardiac tissue by AP–MS/MS, see refs. 77,78 and for proximity labeling, see refs. 78–80.
Single-cell proteomics	Proteome analysis at the individual cell level. Enables studies of cellular heterogeneity and identification of rare cell types.	- Requires isolation of single cells into microwells or reaction vessels. - Common protein content of a cell is ~200 pg. Because of the minuscule amount, sample preparations are not (yet) routine. - State-of-the-art measurement depths of single cells reach ~5,000 proteins for model cell lines, but for primary cells, commonly achieved measurement depths are still in the hundreds owing to a larger dynamic range of protein abundances. - Because of variations introduced in the workflow and the effects on reproducibility, quantitative studies remain challenging. At present, the efforts are mainly to generate atlases until depth and reproducibility are improved. Key references for experimental approach^{16,81,82}.
Spatial proteomics	Region- or cell-type specific proteome analysis while preserving tissue architecture. Enables study of specialized cardiac regions or rare cell populations.	- The starting point is a thin section of tissue mounted on a microscope slide, and then utilizing LCM to collect either a specific region or individual cells from a specific tissue area. The LCM instrument comprises a microscope coupled with a laser, which allows it to focus on the tissue, excise a region of interest, and transfer it to a sample preparation vessel. - LCM is compatible with both fresh-frozen and FFPE preserved tissues⁶⁰. Facilitates studies of specialized regions or even single cells within the native tissue architecture under both physiological and pathological conditions^{55,83–85}.

Preservation methods can also critically influence protein extraction efficiency and compatibility with downstream MS workflows and thus need to be consistent throughout.

In human studies, demographic matching across study groups based on variables such as age, sex and ethnicity is essential to reduce confounding and should also be considered in the data analysis⁵⁷; however, depending on the disease context, additional factors such as body mass index, comorbidities and medication history must be considered. Many of these variables are known to influence cardiac pathophysiology and may not be fully accounted for in standard matching procedures.

For some clinical conditions, endomyocardial biopsies are collected as part of diagnostic evaluation. These samples represent a valuable opportunity to examine cardiac remodeling in earlier disease phases than would otherwise be assessed using tissue from explanted hearts. While biopsies offer unique insights into disease mechanisms and patient-specific biology, their interpretation requires careful consideration of the relative nature of proteomics measurements. Appropriate control samples remain essential, and matched controls are required to distinguish disease-associated molecular signatures.

Identifying suitable control participants presents a particular challenge in cardiac proteomics. Ideally, controls should be individuals who would meet the inclusion criteria if they had the condition of interest but who are free from related exposures or outcomes. While rigorous demographic and clinical matching can reduce variability and increase statistical power, overly restrictive matching may also limit the generalizability of findings if the resulting cohort is not representative of the broader population. In practice, access to well-matched cardiac tissue from healthy individuals is rare¹⁹. Donor hearts are often the most feasible control source^{29,37,58,59}; however, their collection, handling and storage conditions can differ from those of case samples, thereby introducing technical artifacts. In particular, donor hearts intended for transplantation but subsequently rejected or not used have typically been preserved in specialized transplant solutions before rejection. Such preservation conditions can profoundly alter cellular signaling, metabolism and protein stability rendering these samples biologically distinct from freshly excised surgical tissue. Consequently, comparisons between samples subjected to different storage and handling protocols risk introducing technical and pre-analytical artifacts that may confound disease-related effects. These considerations underscore the importance of strict attention

Table 2 | Considerations for study design and cardiac samples for cardiac proteomics studies

Step	Check	Explanation and limitations
Cohort matching	Demographic and clinical variables	• Ensure matching of key variables across conditions tested. The most common variables to check for are age, sex and ethnicity. • Depending on the condition or disease evaluated there may be additional confounders to consider, such as body mass index, medication use, comorbidities and lifestyle factors that may also influence proteomic profiles. • Note limitations in the study's scope, when the case group consists of heart samples from late-stage disease presentations, such as heart transplant specimens.
Control selection	Appropriateness of control specimens, including demographic and clinical variables	• Ideally, controls should meet all inclusion criteria, but lack the condition of interest and related exposures. • Owing to limited sample availability, donor hearts often serve as controls in cardiac proteomics studies. • Ensure that control sample collection and handling mirror that of the case group to ensure comparability. • Note that donor hearts -including rejected ones- are often stored in transplant preservation solutions for several hours and that this will alter cellular signaling and metabolism. This makes these samples biologically distinct from fresh surgical tissue and may confound disease-related comparisons. The perfusion difference between donor hearts and biopsies also lead to differences in extent of blood in the samples, which will further skew analyses.
Sample origin	Collection context (for example, postmortem versus surgical)	• Postmortem samples may be suitable for protein identification but impose important limitations for any quantitative analyses. In particular, labile PTMs should not be analyzed in postmortem tissue. • Even if time between death and tissue collection is minimized, rapid and unspecific protein degradation occurs postmortem, distorting quantitative comparisons of protein abundance and confounding their interpretation. • Beware of differences in perfusion state (for example between tissue from rejected donor hearts and biopsies) blood content differences will have large impact on proteome profiles. • Fresh tissue from living individuals allows for broader proteomic analyses, including PTMs. • Especially PTMs are readily altered by differences in handling or prolonged storage, and if not addressed this can introduce systematic biases and complicate the interpretation of disease-related proteomic differences.
	Cardiac region of tissue sampling	• Are the specifics of the site of tissue collection known? The cardiac proteome varies substantially by anatomical location. Samples must be collected from the same anatomical region across all subjects. Otherwise, the results generated may represent regional variation instead of biological variation. Anatomical regions also contain different proportions of cell types and cellular subtypes. • Consider whether the anatomical site of collection is relevant for the condition being evaluated. • Only autopsy samples and explanted hearts allow free sampling, which pose a critical limitation. • Consider if the sample collection approach is matched between conditions to ensure content of sample is matched (for example, biopsies with biopsies, resections with resections).
Sample handling	Collection and preservation protocol	• Uniformity in sample handling is essential. Only compare samples collected using the same procedure, as differences in handling can introduce technical variation that may be misinterpreted as biological change. Determine whether samples were flash-frozen and stored at -80 °C, preserved in RNAlater, or fixed and embedded, and document the fixation method used. • Ensure standardized sampling handling across study groups to prevent technical artifacts.

Step-by-step guide to evaluate whether test and control specimens are appropriately matched and suitable for MS-based proteomics analysis.

to sample handling and storage, as well as the critical principle that cardiac proteomic data should only be compared across samples processed and stored under closely matched conditions. Limited access to appropriately matched control material remains the central constraint in most cardiac proteomics studies. As an alternative to comparisons with nominally healthy controls, some studies instead focus on comparative analyses between disease groups. While this strategy can mitigate certain technical confounders, it necessarily narrows the scope of biological questions that can be addressed.

An overview of considerations for the study design and the type of cardiac tissue samples collected from human sources are provided in Table 2.

Tissue sample collection and preservation considerations

Tissue handling and preservation for animal and human studies remain critical factors in MS-based proteomics, particularly for quantitative analyses and when investigating PTMs. The preservation strategy directly influences protein integrity and extraction efficiency, thereby determining the scope and reliability of downstream proteomic analyses. Moreover, the biological context in which cardiac tissue is collected greatly affects its suitability for specific applications. Postmortem samples may suffer from substantial protein degradation and are generally unsuitable for analyses of dynamic or labile PTMs as well as for quantitative protein abundance measurements⁵⁶. Postmortem samples can be used for qualitative proteomic applications, including protein identifications and proteome cataloging; however, nonspecific proteolysis and altered enzymatic activity occurring between death and sample collection can markedly

affect protein abundances and PTM patterns. Certain stable or irreversible modifications (for example citrullination and glycosylation) may represent exceptions, but reliable analysis of these PTMs requires a tightly controlled and well-characterized postmortem interval, along with empirical evaluation of postmortem effects on the target layer of interest. The relevant time scale over which protein integrity and stable PTMs are preserved is likely limited to minutes to a few hours, although the precise window is protein- and modification-specific and must be determined experimentally. Consequently, post-mortem tissue is generally more appropriate for qualitative rather than quantitative proteomic analyses. By contrast, tissue obtained from living individuals, such as during surgery or biopsy where processing time can be controlled, can often support a broader range of analyses, including PTM profiling, protein interaction mapping and single-cell proteomics, provided that preservation is immediate and consistent.

The time interval between tissue excision and preservation is another critical determinant of sample quality. Delays in tissue processing can lead to nonspecific protein degradation and loss or artificial induction of PTMs, thereby compromising the biological relevance of the data. In clinical settings, diagnostic biopsies may remain unprocessed for extended periods, rendering them unsuitable for PTM analyses. While global proteome profiles tend to remain relatively stable over handling times counted in minutes, consistency in time-to-storage across all samples is essential to avoid introducing bias in quantitative comparisons. Although this timing parameter is often difficult to control in practice, it should be explicitly considered when determining the types of analyses a given sample can reliably support.

Table 3 | Sample collection and tissue preservation considerations and implications for proteomics experiments

Step	Check	Explanation and limitations
Experimental scope	What types of analyses are feasible based on the collection strategy?	- The suitability of a cardiac tissue/cell sample for specific proteomic analyses depends on how it was collected. - Postmortem samples: may be appropriate for bulk proteome profiling and spatial proteomics. However, quantification warrants caution owing to unspecific protein degradation⁵⁶. In general, quantification cannot be pursued from postmortem tissue, unless the time from death to sample collection has been kept short (likely shorter than a few hours). The influence of the postmortem time interval needs to be benchmarked before using such samples for quantitative analyses; PTM quantification from autopsy samples is generally unreliable, especially for dynamically regulated PTMs that can change within seconds or minutes, the time interval from death to sample collection will influence the PTM decoration of proteins. - Surgically obtained samples from living individuals are generally preferred over autopsy samples: suitable for bulk proteomics, PTM analysis, protein–protein interaction mapping and single-cell proteomics (pending preservation method), and applicable for quantitative analyses.
Sample integrity	Time to preservation and storage conditions	- Delays between tissue excision and preservation can lead to protein degradation or artificially induced PTMs. Recording and control for time intervals are necessary to ensure consistency. - Delays in processing a tissue sample may affect the types of measurements for which it is suitable - In a clinical context, biopsies collected for diagnostic purposes can take hours before being handled rendering the biopsies unsuitable for PTM studies, as the remaining PTMs would not reflect the <i>in vivo</i> state. Proteome profiling could be less affected, as global remodeling would not occur immediately; however, as unspecific degradation takes place, the duration from collection to preservation and storage should be matched across conditions.
	Preservation method	- Fresh and flash-frozen samples stored at -80°C best preserve the <i>in vivo</i> molecular state, including PTMs and protein interactions. - FFPE samples are suitable for bulk proteome analysis but less reliable for PTM or interaction studies owing to prolonged fixation times and crosslinking artifacts. - Consider preservation method and effects on target layer. If in doubt, perform a benchmark experiment to evaluate whether preservation strategy affects target layer.
	Storage duration	- Storage conditions (for example, temperature and duration) should be equivalent across all samples. - Storage can affect protein integrity, especially for PTMs (and potentially protein complex and structure). For PTM mapping, immediate freezing post-collection and minimal storage duration are critical to maintain biological relevance. For proteome profiling from FFPE samples on the other hand, minimal remodeling occurs within a decade of sample storage⁶¹.
	Sample preparation	From a technical standpoint, it is advantageous to store samples until all samples for the project have been collected, as processing all samples simultaneously ensures uniform handling and minimizes technical variability.
	Anesthesia	- If samples were collected under anesthesia (for example, in animal studies), standardization is critical to minimize within-study variation. - Anesthesia alters dynamic PTMs, particularly phosphorylation. These changes may confound biologically relevant signals and should be carefully considered during study design.
Design of MS measurement strategy	Batches	What is the number of samples included in the study/will samples need to be processed in multiple batches? Allocate samples across batches in a manner minimizing technical bias (covariate balanced or randomized).
	Replicates and power analysis	- Adequate replication is essential to ensure statistical robustness and reproducibility. - Biological replicates capture inter-individual variability, whereas technical replicates assess measurement consistency. - A power analysis can be conducted to estimate the minimum number of samples required to detect a biologically meaningful effect size with statistical significance. This calculation depends on study design, expected effect size and both biological and technical variability. For example, using previous data from similar samples processed under consistent conditions, one can estimate sample size based on a Student's <i>t</i>-test with a type I error rate (α) of 0.05 and a desired fold change (for example, twofold) detectable in 80% of proteins. Required sample numbers can vary widely depending on these parameters⁶⁶. - Details of evaluating technical variability in quantitative proteomics experiments can be found in ref. 87.

The method of preservation also plays a pivotal role in maintaining molecular integrity. Snap freezing followed by storage at -80°C is considered the gold standard for preserving the *in vivo* state of proteins and PTMs. This approach is optimal for studies involving PTMs and protein–protein interactions if immediate measurements are not feasible. Formalin-fixed paraffin-embedded (FFPE) tissues, while more widely available in clinical contexts, are primarily suitable for bulk or spatial proteome analyses^{11,60,61}. Also specimen sizes must be taken into consideration, as the slow diffusion of formaldehyde through tissue can require several days for complete fixation of larger specimens, which will impact the protein profiles measured⁶². The fixation process introduces crosslinking and chemical modifications that limit the reliability of PTM and interaction studies. Therefore, the preservation method must be carefully considered in the context of the target layer of proteomics investigation.

Storage can impact protein stability and PTMs. For experiments focused on PTM mapping, it is essential that samples are frozen immediately after collection and stored under controlled conditions for

minimal durations. Extended storage may lead to loss or modification of PTMs, reducing the accuracy and interpretability of the results. Acceptable storage time will depend on the target layer. In general, the shorter the storage time the better. For phosphoproteomics experiments, proceeding with enrichments immediately is preferential, whereas months of storage even at -80°C will influence results. Table 3 outlines key considerations related to tissue collection and preservation, and how these factors influence the feasibility of various proteomic analyses.

Sample processing strategies

Sample preparation and processing are critical determinants of data quality, reproducibility and the overall success of an MS-based proteomics experiment. All preparation strategies introduce inherent biases, and no single approach enables comprehensive identification of all proteins across diverse cellular compartments, modification states and abundance levels. In general, proteomics workflows tend to favor soluble and highly abundant proteins, often at the expense

Table 4 | Sample preparation considerations

Step	Explanation and details
Tissue homogenization	Rationale: Cardiac tissue requires vigorous mechanical disruption to achieve reproducible homogenization owing to structural robustness and high cytoskeletal protein content. Key methods: Mechanical disruption techniques include bead beating, rotor-stator homogenization, Dounce homogenization and cryomilling for frozen samples. Temperature control: Keep samples at 4°C or frozen to minimize protein degradation and to preserve PTMs by minimizing activity of enzymes (for example, proteases and phosphatases) before cell lysis. Buffer additives: Include protease inhibitors in extraction buffers. For PTM analysis, specific enzyme inhibitors matching the modification may be added (for example, phosphatase inhibitors for phosphoproteomics, deacetylase inhibitors for acetylation studies or alkylating agents to protect redox-sensitive free thiols). Researchers should be mindful that specific inhibitors are rarely complete and should not be relied on exclusively (temperature-based enzymatic inhibition is more reliable). DNA management: High-viscosity lysates require DNA shearing when sample viscosity interferes with pipetting or downstream filtration/binding steps. Shear DNA via sonication (2–10 pulses at 30% amplitude with cooling between pulses) or with Benzonase treatment during lysis.
Cell lysis	Rationale: Broad proteome coverage requires effective solubilization of proteins before enzymatic digestion. Buffer selection: Lysis buffers can be tailored to increase extraction efficiency of different protein classes. For bulk proteomics or PTM studies of cardiac tissue, use strong detergents (for example, 1–4% SDS or sodium deoxycholate (SDC) in 50mM Tris-HCl, pH 8.5) or urea. For interaction proteomics, use mild detergent (for example, 0.5% NP-40 or 1% Triton X-100) to preserve protein complexes and avoid denaturation. Protein estimation: Protein extraction yield and concentration is estimated (for example, using a colorimetric method such as the commonly used bicinchoninic acid (BCA) assay) to determine how much enzyme is required for protein digestion and to normalize input amounts across samples. Ensure buffer components do not interfere with chosen protein assay.
Disulfide bond reduction and alkylation	Rationale: Reduction of disulfide bonds and alkylation of newly free thiols supports efficient enzymatic digestion and ensures predictable peptide fragments for detection by MS (as disulfide bonds link different parts of proteins). Some specialized workflows may opt to forego or modify these steps to minimize losses or where Cys-linked modifications are studied (for example redox proteomics). Chemistry selection: The choice of reduction and alkylation reagents can influence digestion performance, peptide identifications, and artifactual modifications. Common reductants such as dithiothreitol, TCEP and β-mercaptoethanol differ in stability, buffer compatibility and potential for unintended side reactions if reaction conditions are not well controlled. Among alkylating agents, iodine-based reagents such as iodoacetamide and iodoacetic acid provide efficient cysteine blocking but can introduce off-target modifications, particularly under elevated concentration, temperature or pH. Chloroacetamide is often considered to provide a more favorable balance, combining efficient cysteine modification with fewer side reactions under many workflows.
Enzymatic protein digestion	Rationale: Enzymatic digestion breaks large proteins into smaller peptides for MS analysis, which are more soluble and easier to separate chromatographically. Key methods: Numerous methods for protein digestion exist and should be chosen by evaluating additives and interfering substances in the buffer/sample to determine compatible digestion strategies, for example in-solution, on-bead, on-filter and in-gel digestion. Earlier methods relied on protein precipitation to remove detergents and contaminants from samples before digestion and subsequent measurement⁸⁸. Alternative strategies, such as suspension trapping⁶³, enable proteins to remain solubilized in strong detergents and be digested directly. Enzymes: Enzymatic digestion is usually performed with trypsin, and is optionally complemented with Lys-C, but can be adjusted according to biological questions. Determine protease as well as protease-to-protein ratio (typically 1:100 w/w). Other enzymes can be used, but in all cases, efficiency of digestion should be assessed and optimized using representative test samples.
Peptide cleanup	Rationale: Digestion is followed by peptide cleanup, which aims to concentrate and remove contaminants such as salts, residual reagents and detergents. Key methods: Common peptide cleanup strategies include solid-phase extraction, such as reversed-phase chromatography (C₁₈), which is widely used for desalting; however, reversed-phase materials are ineffective at detergent or polymer removal (as these contaminants bind and elute reversed-phase materials in a manner similar to peptides), and are also inefficient at retaining short, hydrophilic peptides. Styrene-divinylbenzene reversed-phase sulfonate (SDB-RPS) and hydrophilic-lipophilic balance (HLB) materials retain both hydrophobic and hydrophilic peptides; ion exchange resins such as SCX and SAX, are particularly useful for fractionation but require careful pH control; carboxylate-coated magnetic particles are suitable for removal of both detergents, polymers and salts^{86,89}, and suspension-trapping strategies allow direct peptide cleanup by integrating contamination removal and digestion in a single step⁶³. Regardless of strategy, there is considerable risk of sample loss and selective peptide depletion (for example, loss of small hydrophilic peptides) during peptide cleanup steps. Peptide estimation: After cleanup, total peptide amount is quantified to verify yield. Peptide quantification is especially important in labeled approaches, where equivalent amounts of peptides should be labeled, but the information is also used to normalize amounts analyzed by MS for quantitative comparisons.
Peptide labeling	Rationale: Peptide quantification can be broadly categorized into label-based and label-free methods. Labeling can improve throughput of proteomic methods and has important implications with respect to precision, accuracy of peptide and protein quantification. It also comes with higher cost, more complex sample preparation, and potential for interference from co-eluting peptides, ratio compression and constraints on samples-per-run. Key methods: Labeling strategies applicable to tissue samples are principally chemical labels, such as TMT^{90,91} or DiLeu⁹². Isotopic or isobaric labeling of peptides facilitate multiplexed measurements, allowing multiple samples to be analyzed in a single run. Advantages include increased throughput, reduced variability and improved quantification precision, up to the number of chemical tags. For enrichment approaches (for example, PTMs) multiplexing ensures consistent capture of modified species across samples. Limitations: Batch effects may arise when cohorts span multiple runs; these can be mitigated by including bridging samples. Labeling efficiency should be assessed, usually by including the label as a variable modification during database search. Multiplexing can reduce overall proteome coverage because a missing reporter ion in one batch compromises quantification across all samples analyzed together; however, when reporter ions are consistently detected, multiplexing provides more precise relative quantification than label-free MS. Label-free methods use simplified sample preparation workflows and pose no restrictions on the number of samples that can be analyzed but require more acquisition time. Label-free measurements quantify proteins based on their MS signal intensity or spectral counts without the use of labeling. Advantages: Cost-effective, straightforward sample preparation, and scalability. Limitations: Higher variability and potential for lower precision compared to labeled approaches, missing values. Needs regular quality control sample injections to assess instrument performance/drifts across runs, particularly in large studies, as these factors can affect quantification accuracy.
Fractionation	Rationale: Quantified depth of proteome coverage and protein sequence coverage can be improved by fractionating the peptide mixture before analysis. Alternatively, fractionation or subcellular enrichment can be carried out before protein homogenization. Key methods: Orthogonal fractionation methods offer the greatest capacity for increased depth of coverage, including high-pH reversed-phase chromatography (based on hydrophobicity), hydrophilic interaction chromatography and size-exclusion chromatography, using either HPLC or spin columns.

Table 5 | Measurement strategy considerations

Data acquisition strategy. DIA, DDA, PRM	DIA LC–MS/MS: Measures all ions within a specified <i>m/z</i> range, ensuring comprehensive MS/MS data for all detectable ions. Provides a complete dataset enabling more consistent quantification and improved identification of low-abundance proteins. Advantage: More comprehensive coverage, fewer missing features, improved reproducibility, and better quantification of low-abundance proteins. Limitations: Produces highly complex composite MS2 spectra, which increases analytical complexity and places greater demands on data deconvolution and FDR control, reliance on spectral libraries, larger data files and increased computational demands. The preferred strategy for label-free global proteomics experiments. DDA LC–MS/MS: Measures the most abundant precursor ions selected from a survey scan for fragmentation and MS/MS analysis. Provides high-quality spectra for selected ions, enabling detailed identification and quantification and thus, less likely to miss a rare peptide (or modified peptide) within the samples. Advantages: High-quality MS/MS spectra for selected ions, extensive spectral libraries, compatibility with MS2 multiplexing. Limitations: Bias toward abundant ions, potential for missing low-abundance proteins and limited reproducibility. PRM LC–MS/MS: Targets specific analyte ion–product ion pairs (transitions) for precise quantification of peptides or proteins of interest. Offers high resolution and mass accuracy for targeted proteomics and can assess stoichiometry of modified vs. unmodified peptides. Advantages: High-resolution analyzers enable parallel detection of all target ions; hybrid workflows possible (PRM+DDA in one run). Limitations: Requires accurate in silico prediction of transitions.
Sample randomization	Rationale: Optimize sample allocation before MS measurement to minimize the impact of potential systematic technical variation. Key methods: Randomization using a numerical routine, or preferentially by statistically balancing covariates across sample batches/plates to reduce technical sampling biases ⁹³ . Add concept of blocking for large studies running over a series of days/weeks. Include pooled quality control controls intermittently to evaluate systematic changes over time of acquisition queue.
MS instrument selection	Rationale: Choose MS instrument based on sample complexity and analytical goals. Different platforms offer varying sensitivity, resolution, and throughput. Modern proteomics primarily use Orbitrap-based systems, Orbitrap–Astral hybrids, Ascend Tribrid instruments and TOF analyzers, each optimized for different analytical priorities. Orbitrap platforms remain a standard for discovery proteomics and PTM mapping owing to their exceptional mass accuracy and resolving power, critical for distinguishing subtle mass shifts and complex peptide mixtures. They also integrate well with multiplexed quantification strategies (for example, TMT). Orbitrap–Astral hybrids combine Orbitrap precision with the fast acquisition speed of the Astral mass analyzer, enabling deep PTM profiling and improved sensitivity for low-abundance modifications without sacrificing accuracy. The Ascend Tribrid further enhances parallelization and ion transmission for complex workflows. TOF analyzers, including ion mobility-enabled systems such as timsTOF, deliver rapid acquisition and broad mass range coverage, making them ideal for DIA and large cohort studies. Advances in multi-reflecting TOF technologies have markedly improved resolution, expanding their utility for spatial and low-input proteomics.

of the detection of low-abundance or membrane-associated proteins. These limitations are particularly pronounced in cardiac tissue, where a small subset of extremely abundant proteins frequently dominate MS acquisition time, reducing the detectability of less-abundant cellular components. Even in high-resolution experiments, it is common for over half of the measurement time to be spent on peptides derived from just tens of proteins, as exemplified in Fig. 2. This technical challenge is rarely encountered in cell types other than cardiomyocytes or in whole-tissue samples outside the heart, and as a result, MS core facilities without specific expertise in cardiac proteomics may not anticipate it. Sample preparation techniques, such as fractionation or sarcomere-depletion, can improve the coverage but must be selected with an understanding of their strengths and weaknesses and how these will impact the research question at hand.

Best practices in sample handling contribute to data integrity. Where feasible, samples should be aliquoted and stored to avoid repeated freeze-thaw cycles, thereby limiting protein degradation. Consistent benchmarking with reference standards and regular quality control assessments are indispensable for ensuring protocol performance, detecting technical drift and maintaining data reliability.

Regardless of the biological question, effective homogenization and protein extraction are essential. Cardiac tissues and cardiomyocytes tend to be structurally robust and contain a high number of low-solubility proteins. These features require vigorous mechanical disruption, often in combination with strong detergents (for example, sodium dodecyl sulfate (SDS) or Triton X-100) and reducing agents to disrupt disulfide bonds and ensure effective solubilization of membrane and cytoskeletal proteins; however, the presence of such detergents can be incompatible with downstream MS analysis owing to their interference with MS-ionization and chromatographic procedures. Various strategies have been developed to overcome this challenge, including suspension trapping (S-Trap)⁶³, filter-aided sample preparation⁶⁴, single-pot solid-phase-enhanced sample preparation⁶⁵ and protein aggregation capture⁶⁶, allowing digestion of detergent-solubilized proteins (up to 10% SDS) without the need for individual sample precipitation, improving the recovery across protein classes. The use of MS-compatible surfactants has also been reported^{16,67}. Recent trends

in sample preparation emphasize the use of liquid handling robotics and automated platforms to minimize artifactual protein identification stemming from experimenter- or reagent-related sources. The automation strategy and miniaturization of workflows are particularly important for single-cell and spatial proteomics applications.

Following extraction and detergent removal, proteins are quantified to adjust protease digestion parameters and normalize input amounts across samples. Reduction and alkylation steps are performed to break disulfide bonds and block free cysteine residues from reforming them, aiding protein denaturation and preparing them for enzymatic digestion. While different proteolytic enzymes can be used, trypsin is the most common choice owing to its compatibility with denaturing solutions and its predictable cleavage at the C terminus of lysine and arginine residues, which facilitates peptide ionization and identification. For new samples, digestion conditions should be optimized to minimize missed cleavages and maximize protein coverage. Post-digestion, peptide cleanup is essential to concentrate analytes, remove salts, residual detergents and other contaminants that interfere with MS analysis. In workflows employing chemical labeling of peptides, quantification of peptide yield is particularly important, as equal peptide input is required for accurate multiplexing of isotopically or isobarically labeled samples, such as in tandem mass tags (TMT) or DiLeu labeling experiments. Multiplexing enables simultaneous analysis of multiple samples. These approaches offer improved throughput and quantification precision but come with higher cost, additional sample preparation steps and potential drawbacks such as ratio compression. Label-free methods, while more flexible and cost-effective, may suffer from increased variability and missing data. To increase proteome coverage and sequence depth, orthogonal peptide fractionation can be employed before MS analysis. Finally, the choice of mass spectrometer and data acquisition strategy must align with the study goals. An overview of considerations in the sample preparation steps and measurement strategies is provided in Tables 4 and 5, respectively.

Data evaluation, analysis and presentation

Robust data evaluation is essential to ensure the reliability and reproducibility of MS-based proteomics results. This process begins with

Table 6 | Basic quality control assessments of MS data and data analysis

Step	Checkpoint	Explanation and troubleshooting
Data evaluation	Inspect raw files	- Evaluate if all generated raw files are of similar size. - Examine total ion chromatograms for each run to assess signal stability and average intensity. - Check that sample signals are consistent in intensity and chromatographic shape. - Look for signs of sample contamination (for example, abundant +1 charge state peaks).
Data processing	Search engine selection	- There are several specialized search engines available for processing raw MS data. - For DDA data the most widely used platforms include MaxQuant, FragPipe and Proteome Discoverer. - DIA data are commonly analyzed using tools such as Spectronaut or DIA-NN. Note that Proteome Discoverer and Spectronaut are proprietary software suites, while MaxQuant, FragPipe and DIA-NN are free of charge for academic use. - When selecting a search engine, ensure compatibility with acquisition strategy and report the software version used. - There are always new search algorithms being developed with the goal of maximizing assignment of the MS spectra.
	Database selection	- Determine protein sequence FASTA file the data should be searched against (report source, version and number of entries). - Consider restricting search to reviewed amino acid sequences only, consider if the study design allows for evaluation of splice forms. - Consider how to handle common contaminants. - Determine and report details of spectral libraries for DIA.
	Peptide and protein identification	- Report number of peptides and proteins per sample using commonly accepted FDRs (for example, 1% FDR at peptide and protein levels based on decoy search). - Specify whether protein identifications are based on protein groups (peptides shared between two or more proteins) or proteotypic peptides (peptide unique to a single protein or isoform). - If an isoform is specifically identified (not only as part of a 'protein group'), it must be supported by a proteotypic peptide unique to that isoform. - Assess peptide distribution across proteins. Low identification rates may indicate poor digestion or instrument issues. - For PTMs, site localization probability should be assessed and reported (for example, % of modified peptides with a localization probability >0.75, also called 'class I').
Data quality control	Intensity profiles	Check consistency across samples. Review the number of MS1 (precursor) and MS2 (fragment) scans, ion intensities, and charge state distributions.
	Missing values	- Evaluate extent and pattern. - Imputation may be necessary for downstream analysis (especially for DDA data) but the imputation approach (which values are imputed and how) should be carefully evaluated, justified, and reported.
	Outlier detection	- Use principal-component analysis or clustering to identify outliers. - Investigate sample preparation or acquisition anomalies. - Removal of outliers should be adequately justified (for example, if clear technical abnormalities are identified).
	Digestion efficiency	Assess missed cleavage rates. High rates suggest suboptimal enzyme activity or buffer conditions. Can be evaluated from the percentage of peptides with missed cleavage sites for fully tryptic and semi-tryptic peptides. Check that you obtain consistent results across samples. If digestion is insufficient, adjust protease concentration or digestion conditions (for example, time).
	Labeling efficiency (for multiplexed samples)	- Check peptide labeling efficiency. Can be determined from the number of peptides identified without labeling. - Labeling efficiency should exceed 95%. Monitor unlabeled peptide counts. - Verify sample pH immediately before labeling.
Statistical analyses	Data distribution	- Assess data distribution and normality. - Apply appropriate normalization methods, such as median- or quantile-based normalization. - If samples were measured across multiple batches, implement batch correction, either as an ad hoc preprocessing step or by modeling batch effects within the statistical analysis. - Consider including relevant covariates (for example, age and sex) in the statistical model to account for biological and technical variability.
	Multiple testing correction	Compensate for multiple hypothesis testing. Use FDR-adjusted <i>P</i> values to control for false positives. Report hits based on suitable FDR (for example, 5%) and/or fold-change thresholds.
Supplementary data	Data tables	- Supplementary data table corresponding to all the proteins identified and quantified in the study, including those that show no significant difference between states, should be presented. - Columns should be clearly annotated and include a unique protein identifier, preferably based on UniProt or Swiss-Prot accession numbers. - If a protein is associated with multiple identifiers, the consensus or primary identifier should be reported to ensure clarity and avoid redundancy.
	Raw data sharing	- Deposit raw files in public repositories whenever ethically permissible. - Where possible, deposit de-identified patient sample proteomics data to access-controlled repository and specify data use policy. - If compliance with data protection regulations such as the General Data Protection Regulation (GDPR) is a concern, consider sharing aggregated data from pooled samples at the group level. - Ensure that all shared data are accompanied by comprehensive metadata to facilitate accessibility and reuse.
Reporting	Methodological transparency	- Describe instrument settings, acquisition parameters, and data processing workflows in detail. - Submit full datasets with the manuscript. - Refer to guidelines describing the minimum information that should be shared⁹⁴.

inspection of the raw data, followed by data processing, quality control assessments, protein identification and quantification. The choice of search engine, protein sequence database, normalization strategy and statistical methods influence the final dataset.

Raw MS data are processed using specialized search engines and quantification software that perform peptide identification, protein inference and quantification based on spectral intensity or ion counts. Key quality control metrics include missed cleavages and digestion

efficiency, consistency of intensity profiles across samples, the extent of missing values and outlier detection, peptide-to-protein distribution and overall identification depth.

Protein inference strategies determine whether reported proteins rely on unique (proteotypic) peptides or include razor/shared peptides. Specific claims about protein presence or behavior should be based on unique peptides whenever possible; however, some cardiac proteins share extensive sequence homology, which complicates inference and may lead to misinterpretation of absence or non-detectability. The choice of sequence database also affects identification: missing sequences cannot be identified, whereas overly redundant databases increase false positives. For common organisms, curated resources such as UniProtKB/Swiss-Prot⁶⁸ are recommended for consistency and comparability, whereas custom or related-species databases may be required for less-common models. Including isoforms and variant sequences requires careful consideration; however, inclusion of newly annotated short open reading frames can also lead to the discovery of new translational events⁶⁹. While many isoforms can be identified in MS data and their inclusion can improve spectra matching, it complicates protein inference because most isoforms share the majority of their peptides with their canonical counterpart^{70,71}. Additional safeguards, such as local false discovery rate (FDR) filters or open search comparisons, are recommended.

Before statistical testing, data distribution should be assessed to determine whether normalization is required. Common normalization methods include median centering and quantile normalization. Multiple hypothesis testing corrections, such as FDR adjustments, are essential to control for type I errors in both protein identification and quantitative comparisons.

Comprehensive reporting of both the acquired and the processed data is critical for transparency and reproducibility. The methods section must detail all aspects of the MS workflow, including instrument type, acquisition parameters and data processing settings. All identified and quantified proteins, including those not showing significant changes, should be included in supplementary tables, where each column should be clearly annotated. Data files should be deposited in public repositories such as Pride⁷² or MassIVE⁷³, where RAW files are particularly useful as they can be reprocessed by others as new tools or alternative analysis strategies emerge.

Main steps in this process are outlined in Table 6.

Conclusion and future directions

MS-based proteomics offers increasingly powerful tools for investigating cardiac biology and disease. While bulk proteomics provides deep coverage and robust quantification, single-cell, subcellular and spatial proteomics offer unique insights into cellular heterogeneity and tissue architecture; however, these emerging approaches currently lack the reproducibility and quantitative accuracy of bulk analyses. Technologies such as laser capture microdissection (LCM) combined with artificial intelligence (AI)-driven cell segmentation are already transforming fields like oncology and hold promise for cardiac research. Advances in ultra-low-input sample preparation and cell-sorting techniques are enabling unprecedented resolution of cellular microenvironments and disease mechanisms. As single-cell and spatial proteomics mature, they will complement bulk approaches to provide a more comprehensive understanding of molecular cardiology and cardiac disease. In a cardiac research context, these advancements enable detailed exploration of cardiac (patho)physiology, especially when integrating information across various data sources in a streamlined manner⁷⁴. Expanding proteomics-based investigations in both patient samples and animal models holds considerable promise for identifying molecular signatures of disease states and for informing therapeutic strategies based on the molecular profiles of individual hearts, toward personalized medicine. As emphasized throughout this

Consensus Statement, the insights that can be gained from cardiac proteomics are substantial, but the value of these datasets depends heavily on careful planning. The more deliberate the experimental design and the greater the attention to technical detail, the higher the impact and interpretability of the resulting data. Given that MS-based proteomics is a rapidly evolving field driven by continuous technological innovations, optimal outcomes are often achieved through close collaboration between clinical and basic cardiovascular scientists, ensuring that the necessary expertise is incorporated across all stages of the study.

Success in cardiac proteomics studies should not be judged by the sheer number of proteins identified, but by the quality and reliability of the quantification, as well as the orthogonal strategies used to confirm key findings. Proteomics is a powerful discovery tool, and its greatest impact comes when robust quantitative data are paired with mechanistic biological studies. With rigorous experimental design, execution and reporting, guided by this Consensus Statement, cardiac proteomics studies will generate reproducible datasets that illuminate disease mechanisms and accelerate therapeutic development. Adherence to best practices will enable cross-study comparisons, facilitate data integration and support the research community in advancing our understanding of cardiac biology in both physiological and pathological contexts.

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Author contributions

A.L., J.E.V.E. and M.M. conceived and discussed the content of the article and were the core writing group for the article. A.L. drafted the initial manuscript. J.S.A. generated the figures. All authors contributed to the writing, critical revision and final editing of the manuscript before submission.

Competing interests

R.L.G. is on the advisory board of ProtiFi and receives no compensation of any kind for this role. J.E.V.E. is on the scientific advisory boards of Seer and Chaparral. The remaining authors declare no competing interests.

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