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Chemical cross-linking and mass spectrometry enabled systems-level structural biology

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Abstract

Structural information on protein–protein interactions (PPIs) is essential for improved understanding of regulatory interactome networks that confer various physiological and pathological responses. Additionally, maladaptive PPIs constitute desirable therapeutic targets due to inherently high disease state specificity. Recent advances in chemical cross-linking strategies coupled with mass spectrometry (XL-MS) have positioned XL-MS as a promising technology to not only elucidate the molecular architecture of individual protein assemblies, but also to characterize proteome-wide PPI networks. Moreover, quantitative *in vivo* XL-MS provides a new capability for the visualization of cellular interactome dynamics elicited by drug treatments, disease states, or aging effects. The emerging field of XL-MS based complexomics enables unique insights on protein moonlighting and protein complex remodeling. These techniques provide complimentary information necessary for in-depth structural interactome studies to better comprehend how PPIs mediate function in living systems.

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Chemical cross-linking and mass spectrometry (XL-MS), Protein–protein interactions (PPIs), Native separation, Complexomics.

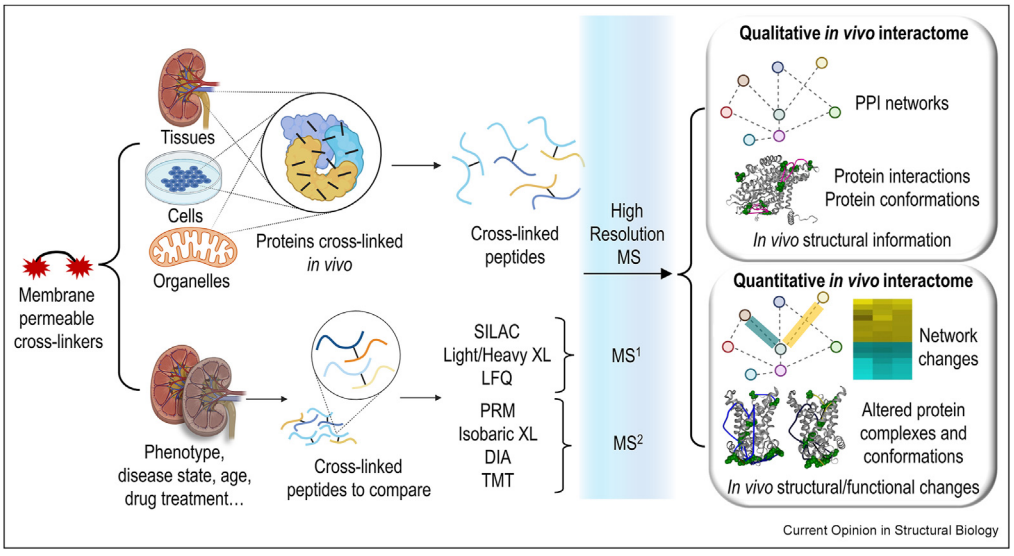
Developments in cross-linker design and methodology

Protein complexes and their dynamic modules underpin most biological function and mediate many diseases [1–5]. Spatial restraints generated from XL-MS analysis provide valuable medium resolution structural insights on protein assemblies. The high sensitivity, speed, and the ability to handle sample heterogeneity have made the XL-MS approach superior to traditional biophysical methods, especially in analyzing proteome-wide PPI networks [6–9]. In addition, quantification of cross-link products can elucidate novel conformations and interactions or shifts in structural equilibrium under different physiological or pathological states.

Development of *in vivo* XL-MS strategies is important as many PPIs are lost upon cell lysis (Figure 1) [10,11]. Many cross-linking technological advancements aim to increase numbers of detected cross-linked peptides using cell lysate cross-linking approaches. These advancements have potential to advance *in vivo* studies although many do not address challenges specific for *in vivo* cross-linking [12,13]. *In vivo* XL-MS poses particular challenges because of low efficiency of cross-link formation due to the high cellular protein concentration (200–300 mg/mL [14]) and high prevalence of Lysine residues (6% of all amino acids in human proteome [15]) relative to the achievable cross-linker concentration. Therefore, affinity enrichment features were incorporated even in early cleavable cross-linkers designed for *in vivo* applications [16].

Cross-link enrichment is an active area of development (Figure 2(**a**)). (3,5-bis(((2,5-dioxopyrrolidin-1-yl)oxy) carbonyl)phenyl)phosphonic acid (PhoX) is a cross-linker that can be utilized with immobilized metal ion affinity chromatography (IMAC) for the enrichment of cross-links formed in cell lysates [17]. However, the decreased membrane permeability of PhoX makes it less suitable for *in vivo* studies. Addition of *tert*-Butyl masking groups to PhoX (*t*Bu-Phox) improved its membrane permeability, enabling cross-linking in living cells [18]. Incorporation of MS cleavable glycosidic bonds into the cross-linker spacer arm is another strategy to increase

Figure 1

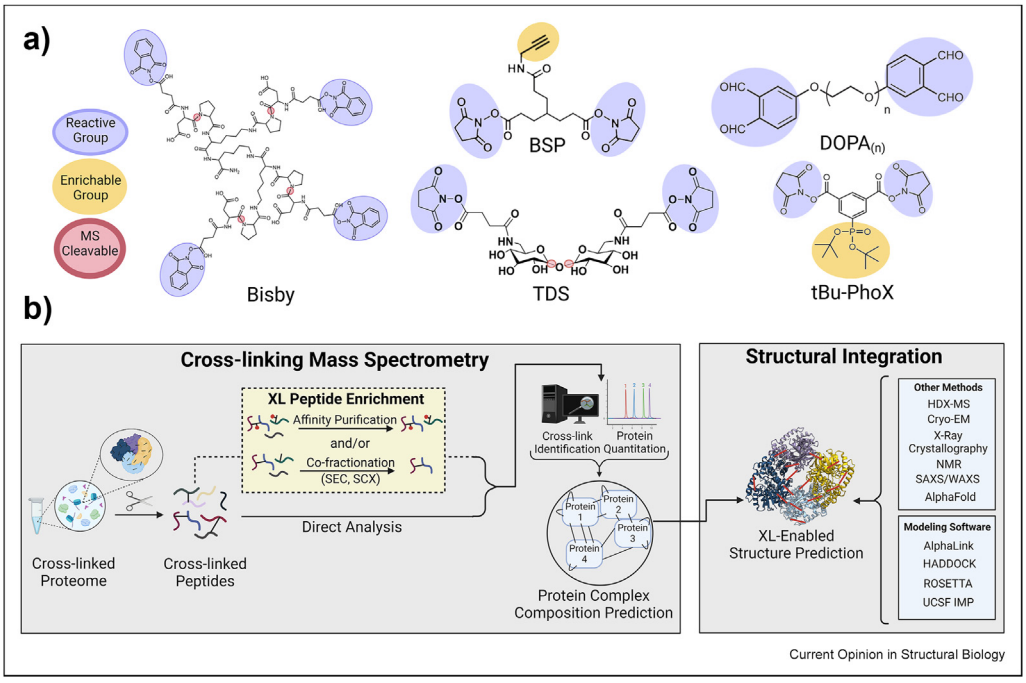


In vivo qualitative and quantitative cross-linking. In vivo cross-linking experiments utilize membrane permeable cross-linking reagents to capture native protein–protein interactions and conformations in intact cells, tissues and organelles. After the *in vivo* reaction is complete, proteins are extracted and digested, subjected to various methods to enrich cross-links, and analyzed on a high-resolution mass spectrometer. Mass spectrometry data is used to identify cross-links and obtain information on intra-protein (conformations) and inter-protein (PPIs) structural networks that exist in cells, tissues, or organelles. Quantitative *in vivo* XL can be used to compare phenotypes, disease states, age effects, drug treatments and other perturbations. Quantitation can be done on the MS1 level (LFQ, SILAC, isotope labeled light/heavy cross-linker) or on the MS2 level (PRM, DIA, TMT, isobaric cross-linker). Quantitative XL-MS allows detection of changes in PPI networks and protein conformations and complexes.

cross-linker permeability [19]. In addition to membrane permeability, *in vivo* cross-link formation is limited by the half-life of the activated ester groups commonly used for cross-linking reactions [12,13,18,20–23], measured to be 7.5 min at pH 8.0 [24]. The non-hydrolyzable, highly reactive di-ortho-phthalaldehyde (DOPA) cross-linkers provide an attractive solution [25]. Improved enrichment of *in vivo* cross-links achieved by protein-based click-chemistry conjugation with acid-cleavable linkers increased cross-linking coverage of low abundance proteins and detection of previously unreported PPIs [26,27]. Recently, a tetrameric cross-linker that can link up to four proteins extended XL-MS structural capabilities beyond binary protein interactions [28]. Bisby is based on a four-armed branched peptide backbone with cleavable features common to other PIR molecules. Identification of cross-linked species with four peptides indicates that all four reactive sites existed proximal to one another during cross-linker reaction. Detection of tetralinks was facilitated by novel real-time MS methods to provide structural insights on multi-protein complexes in intact organelles. Proteins and complexes exist within structural ensembles inside cells and the kinetics of cross-linker reactions can limit what cross-linked peptides may be observed. Therefore, quantitative measurements of cross-linked peptide levels are essential to visualize changes in phenotypes or in cells during drug treatment or other perturbations to help unravel complex or conformational dynamics. Alternatively, photo-cross-linking advancements offer significantly reduced reaction periods and may offer improved abilities for highly dynamic protein complexes [29]. However, the lower reaction specificity of photo-cross-linkers can generate a variety of cross-linked products yet each with lower abundance relative to cross-linkers with higher reaction specificity.

Methods that can enable quantitative XL-MS (qXL-MS) add another dimension to structural insights. Besides mapping PPI networks, qXL-MS elucidates changes in cross-linked levels in different samples. This capability informs how protein conformations, interactions, and/or modification levels are altered [30]. Similar to traditional quantitative proteomics methods, qXL-MS can utilize either MS^1-signals for XL quantitation such as label free quantitation (LFQ) [31], SILAC and isotope labeled cross-linkers [32–34], or utilize MS^n-signals for XL quantitation such as parallel reaction monitoring (PRM) [24], tandem mass tag (TMT) [35], Data Independent Acquisition (DIA) [36], as well as isobaric cross-linker approaches [37] (Figure 1). Implementation of traditional quantitation methods might require optimization for cross-linked samples. For example, identification of TMT-labeled cross-linked peptides benefits from increased normalized collision energy (NCE) compared to unlabeled cross-links [38]. Implementing a stepped NCE resulted

Figure 2



Novel cross-linkers and integration of cross-linking with other structural biology methods. **a**) Novel cross-linkers enable capture of higher order structural information (Bisby), enrichment of cross-linked peptides (BSP and *t*Bu-PhoX), enhanced detection with cleavability (Bisby and TDS), faster cross-link formation (DOPAn), and enhanced membrane permeability (*t*Bu-PhoX). **b)** Proteome cross-linking is performed to provide distance restraints of proximate residues within a protein or between subunits for protein complexes. Cross-linked peptides can be enriched by SEC, SCX, or affinity purification to facilitate identification. Identified cross-links, measured protein relative quantitation, and PPI network database can be used to predict the composition of protein complexes. XL-MS data can be integrated with other structural information either experimentally determined (HDX-MS, Cryo-EM, X-Ray crystallography, NMR, SAXS/WAXS) or computationally predicted (e.g. AlphaFold) to provide novel structural insights using tools such as AlphaLink, HADDOCK, ROSETTA, or UCSF IMP.

in higher reporter ion intensities, necessary for a reliable quantitation, with MS^2 based identification and quantitation outperforming MS^3. Recently, a hybrid strategy utilizing MS^3 for identification and subsequent selection of cross-links with targeted PRM quantitation was used with a commercial cross-linker [39]. LFQ, where cross-link abundance is estimated by integrating its precursor peak in MS^1 scans, was applied to compare respiratory supercomplex formation in WT and transgenic mice [40]. DIA-based quantitative proteome strategies have also supported qXL-MS [36].

An alternative quantitative approach involves modifying cross-linker molecules by inserting heavy isotopes at specific locations to produce two [37] or multiple isobaric molecules [41] to facilitate multiplexed quantitation of *in vivo* cross-linked peptide levels [20]. Isobaric cross-linkers such as isobaric quantitative Protein Interaction Reporter (iqPIR) reagents enable quantitation in MS^2 spectra using multiple peptide, fragment ions and replicate measurements to determine variance and establish statistical confidence filters on quantified ratios [42]. Apportionment algorithms are employed to the overlapping isotope envelopes of stump-containing peptide and fragment ions on the basis of the predicted relative peak intensities [42]. Encoding quantitative information into cross-linker molecules can reduce variability associated with sample processing since cross-linked proteins can be mixed pre-digestion.

Technological advancements in data acquisition have been adopted for cross-linked samples to improve depth of coverage for *in vivo* XL-MS. For instance, high-field Asymmetric Waveform Ion Mobility Mass Spectrometry (FAIMS) provides ion separation on the gas phase size/charge ratio, orthogonal to an upstream size exclusion chromatography (SEC) fractionation of analyte size. The combination of FAIMS and SEC, with FAIMS parameters optimized for each SEC fraction with machine learning, nearly doubled the number of observed cross-links [43]. Another instrumentation advance is Parallel Accumulation Serial Fragmentation (PASEF), which can partition cross-linked from linear and DE species for data dependent acquisition (DDA) [44,45]. Recently, a 4D-DIA method using PASEF for XL identification and quantitation from cellular and cell lysate samples showed an improvement in repeatable identifications of 83% compared to 38% with a DDA approach [46].

Furthermore, applying real-time library search (RTLS) with cross-linker specific diagnostic peaks can independently target either cross-links or a combination of cross-links and DE species, resulting in 14–35% increase in IDs [47].

The numbers of cross-linked species that can be detected and/or quantified in a given experiment are highly dependent upon the separation methods, mass spectrometry instrumentation, number of LC/MS/MS runs and many other parameters. However, current published datasets can reach tens of thousands of cross-linked peptides [48]. Moreover, each detected cross-link species holds intrinsic value as a protein or complex conformational probe that can enable future quantitative measurements to visualize systems-level conformational dynamics. This constitutes a tangible and actionable aspect of XL-MS data, not readily paralleled with many other structural biology techniques.

Informatics advances

Advancements in cross-linked peptide analysis software such as CRIMP 2.0 and MaxLynx have improved the automation of cross-linked peptide analysis and the reliability of their identifications [49,50], despite not yet integrating advancements in artificial intelligence (AI) based cross-linked peptide prediction tools that can predict retention times [51] and MS/MS spectra [52]. Future iterations of cross-linked peptide identification software capable of parallel analysis of different cross-linker chemistries and proteolytic cleavages that can be integrated into a combined output will be critical for the progress in complexomics.

Inference of protein conformations giving rise to acquired XL-MS data is a major informatics challenge (Figure 2(**b**)). Traditionally one looks for available structures where most cross-linked residues are within the expected distance range for the cross-linker. Sometimes however, no structures are yet known, or cross-links are formed in disordered regions or unsolved conformations of existing structures. AlphaFold2 harnesses the power of an artificial intelligence deep learning technique and co-evolving sequence variations to yield advanced protein structural prediction capabilities [53]. AlphaLink software, a modified version of AlphaFold2, incorporates experimental cross-link distance restraint information to complement its co-evolutionary relationships and can steer predictions towards sample specific protein conformations, including those that are state-specific [29]. This is an advance over existing ROSETTA [54] and HADDOCK [55] cross-link based protein docking. Use of qXL-MS data and Alphafold2 with shallow alignments revealed a non-canonical nucleotide transporter conformation was enriched in the mitochondrial interactomes in failing hearts [21].

Inter-protein cross-links give information about interactions between proteins. AlphaFold2-predicted structures can be leveraged to model pairs of interacting proteins using its FoldDock pipeline [56], which is important since the majority of protein interactions have not been structurally characterized. The interfaces of predicted high-confidence binary human protein complexes using this approach are enriched in post-translational modifications and disease-related mutations [1]. Moreover, the pairwise AlphaFold2 interactions are utilized in the CombFold software at the hierarchical and combinatorial assembly stage to model large protein complexes [57], as further expanded below.

Integration of experimental results, including cross-linking data, complements computational scoring and enhances the prediction accuracy of protein and complex structural models [55]. The IMProv software integrates the UCSF integrative Modeling Platform (IMP) with cryo-EM densities and crystallographic structures together with cross-link data, with the added option to use distance restraints conditioned by hydrogen–deuterium exchange MS [58]. Assembline is another software package that uses cross-link data together with low resolution in-cell cryo-ET maps to perform integrative structural modeling to generate model ensembles for large cellular complexes that preferentially satisfy cross-link distance constraints [59]. Cross-links can be mapped to protein complex structures predicted with AlphaFold Multimer [11] to assess them or used to guide protein complex prediction with IMP. However, at the scale of complexomics, the computational costs to predict the structure of all identified complexes and the labor costs to manually validate their legitimacy are too large. The integration of protein language models and geometric transformers into complexomics data analysis workflows holds promise for large-scale complex structure prediction and analysis. Recently, ESMFold a protein structure prediction algorithm that utilizes a protein language model with a geometric transformer has demonstrated much faster structure prediction than AlphaFold2 with similar accuracy, but it has not been directly trained on protein complexes [60]. Also, a deep learning model that predicts the quality of protein complex structures, DProQA, holds promise for the validation of large datasets of predicted protein complexes inferred by cross-linking mass spectrometry complexomics data [61].

A challenge for qXL-MS is to identify conformational causes of observed interactome differences in compared samples. Tüting et al., manually mapped cross-links on an impressive 146 ribosome structures to reveal conformational plasticity [62]. The XLinkDB public cross-link database and analysis toolset automatically incorporates all structures that exist in the protein databank (PDB) and maps observed cross-links on those with the highest homology and coverage [63]. XlinkDB

was recently further developed to support quantitation of dead-end (DE) peptides, cross-linker modified products with only one terminus attached to a protein, to help infer causes of observed cross-link abundance differences in two samples not due to protein level changes [64]. Whereas the abundance of both cross-links and their corresponding DE peptides are affected by conformational differences near the attached residues, including peptide PTMs, only cross-link levels are sensitive to solvent accessible distances between cross-linked residues. XLinkDB automatically integrates cross-link and DE peptide quantitation to generate possible hypotheses for observed cross-link abundance level differences. In this manner, DE peptides can play an important role in improved interpretation of XL peptide level changes and conformational differences between compared samples. Exploration of cross-link data as interaction networks and in context of structures is also facilitated by the xiView platform [65].

XL-MS enabled structural & cellular biology

Capturing PPIs and protein conformations in their native environments is of great utility to almost any biomedical research area. For instance, recent cross-linking experiments with intact human cytomegalovirus have revealed virion layer-specific organization of viral and host proteins, a critical target for the treatment of infectious diseases [66]. Additionally, coupling *in vivo* cross-linking with subsequent affinity enrichment of the bait protein allows capture of interactions of interest with much higher precision and resolution than conventional affinity purification mass spectrometry [67].

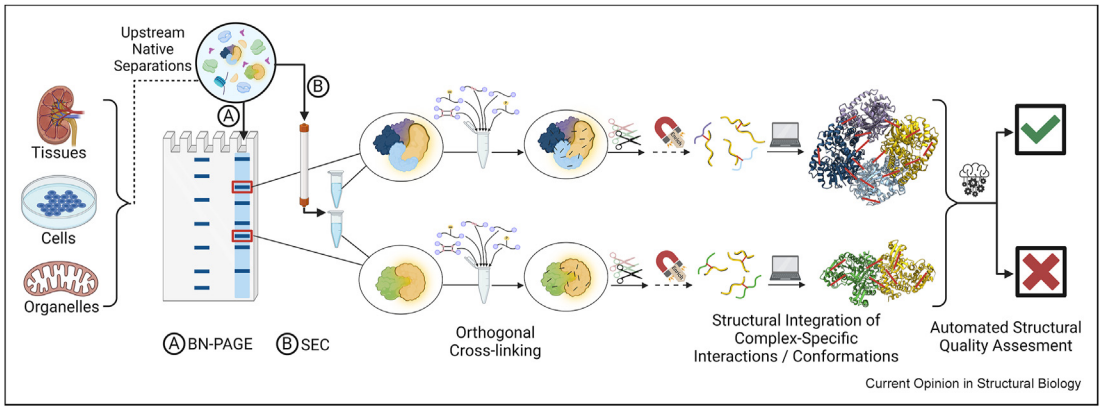
In vivo cross-linking is particularly advantageous in analyzing membrane proteins and their complexes, which usually require unperturbed membrane lipid environments for conformational stabilization. Unlike traditional methods, such as blue native PAGE (BN-PAGE) or complexome profiling, that require membrane extraction prior to analysis, cross-linking can be performed on intact cells, tissues or organelles, providing unique insights on protein organization within physiological environments. XL-MS has been applied to verify depletion of respiratory supercomplex assemblies upon subunit genetic manipulation and to study apoptotic pathways in relation to complex IV [40,68]. The ability of *in vivo* XL-MS to capture native mitochondrial environments has also been applied to study large non-membrane protein complexes, such as the 2-oxoglutarate dehydrogenase complex [69,70].

Furthermore, quantitative *in vivo* cross-linking enabled visualization of mitochondrial interactome changes within murine heart failure tissues and in age-related muscle function decline [21,22]. In both studies, molecular-level insight observed through interactome differences helped rationalize functional differences not regulated at the proteome level. Multiplexed quantitative cross-linking enabled visualization of conserved and unique interactome changes in cancer cells treated with multiple drugs that all target the molecular chaperone HSP90 for functional inhibition [20].

Although significant progress has been made in understanding PPI networks through *in vivo* XL-MS approaches, detection of low abundant cross-linked peptides remains challenging [71]. Additionally, identified cross-links often lack protein complex specificity. However, separation of protein complexes in their native state followed by cross-linking provides a complementary approach to reduce sample complexity and provide complex specificity for identified cross-links to inform the prediction of protein complex structures, especially since proteins often participate in several different complexes [72]. Separation of complexes from biological samples using native gel electrophoresis, such as BN-PAGE, or with SEC [73] has shown utility in complexome profiling [74,75], which seeks to identify complexes based on protein co-fractionation and abundance alone. Algorithms based on statistical clustering methods such as ComplexBrowser or AI algorithms trained on known complex databases, such as ComplexFinder, have been applied to enable automated identification of protein complexes in complexome profiling experiments, yet without XL-MS data [76,77]. Advancements in XL-MS and AI-enabled analysis are beginning to pave the way for the next generation of Complexomics, which seeks to identify and predict the structure of proteome-wide complexes using native separations and cross-linking (Figure 3). Most notably, a new cross-linking method that integrates native gel electrophoresis separation of protein complexes with in-gel cross-linking for the PPI analysis of fractionated protein complexes [72] could see wide applications, especially when combined with the developments discussed in this review. Similar approach utilizing SEC separations have been shown useful [78].

The future of XL-MS enabled structural biology holds many discoveries as cross-linker chemistries advance to enable better cross-link formation, quantitation, and enrichment, and as the detection and analysis of cross-links improves with new instrumentation and computational analysis methods. As XL-MS becomes widely utilized to tackle challenging biochemical and cell biology questions, the MS-based proteomics community has been actively engaged in developing guidelines and generating methodological and reporting standards from best practice in experimental design to data sharing and community benchmark exercise [79,80]. The proper adoption of a robust protocol in XL-MS workflow, and a rigorous assessment of error rate in data analysis will be critical for the routine application of XL-MS in MS-based proteomics core facilities. The development of community benchmarks in reagents and datasets will provide transparency in assessing methodological and software

Figure 3



The Future Workflow of Complexomics Integrates Complexome Profiling with Cross-linking MS and AI-enabled Informatics Pipelines. Upstream native separations such as size exclusion chromatography (SEC), native IEF liquid fractionation, or BN-PAGE can reduce sample complexity and facilitate cross-link identification. Orthogonal cross-linking with cross-linkers of various length, polarity, and reactivity could be used to broaden structural information sampling. Complex-specific cross-links derived from BN-PAGE in-gel cross-linking experiments or cross-linked SEC fractions combined with relative protein quantitation can be used to computationally predict protein complex composition and connectivity. Integration of XL-MS data with other structural information leads to novel structural insights as described in Figure 2(**b**). Structure analysis tools, such as DProQA, could be used to automatically evaluate the quality of the predicted structures.

advances for expert users. For the long-term sustainability of XL-MS as a standard structural method in near future, data access through public repositories will be essential for knowledge transfer to computational biologists and biology experimentalists to gain structural insights and develop testable hypotheses.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

No data were used for the research described in the article.

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