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Discovery to Human Disease Research: Proteo-Metabolomics Analysis

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Abstract : The advancement of high-throughput omics technologies and systems biology is essential for understanding complex biological mechanisms and diseases. The integration of proteomics and metabolomics provides comprehensive insights into cellular functions and disease pathology, driven by developments in mass spectrometry (MS) technologies, including electrospray ionization (ESI). These advancements are crucial for interpreting biological systems effectively. However, integrating these technologies poses challenges. Compared to genomic, proteomics and metabolomics have limitations in throughput, and data integration. This review examines developments in MS equipped electrospray ionization (ESI), and their importance in the effective interpretation of biological mechanisms. The review also discusses developments in sample preparation, such as Simultaneous Metabolite, Protein, Lipid Extraction (SIMPLEX), analytical techniques, and data analysis, highlighting the application of these technologies in the study of cancer or Huntington's disease, underscoring the potential for personalized medicine and diagnostic accuracy. Efforts by the Clinical Proteomic Tumor Analysis Consortium (CPTAC) and integrative data analysis methods such as O2PLS and OnPLS extract statistical similarities between metabolomic and proteomic data. System modeling techniques that mathematically explain and predict system responses are also covered. This practical application also shows significant improvements in cancer research, diagnostic accuracy and therapeutic targeting for diseases like pancreatic ductal adenocarcinoma, non-small cell lung cancer, and Huntington's disease. These approaches enable researchers to develop standardized protocols, and interoperable software and databases, expanding multi-omics research application in clinical practice.

Keywords : Proteo-metabolomics, mass spectrometry, integrative analysis, disease diagnosis

Introduction

High-throughput omics technology has been enabled by the growth rapidly of analysis field and informatics processing ability, becoming an essential and powerful tool in systems biology.[1-5] This technology integrates information from various biomolecular layers to provide a more comprehensive understanding of complex biological mechanisms, and can be applied to diseases caused by multiple factors (e.g., cancer, autoimmune diseases).[6-9] Multi-omics analysis facilitates the systematic study of dynamic molecular processes driving key cellular functions and has the potential to represent fundamental molecular logic, signal transduction, cellular metabolism, and phenotypic determination.[6,10] It is likely to make significant contributions to diagnostic and therapeutic information. Recent advances in high-resolution mass spectrometry (MS) have made the comprehensive study of biological molecules possible, especially proteins and metabolites. Proteomics and metabolomics technologies rely on MS platforms, offering reliable biomarker candidates of protein and metabolite for detecting disease-specific compounds. Proteomics is utilized to quantify the modification, and abundance with MS-based methods suitable for robust analysis of thousands of proteins in body fluids, tissues or cells.[11,12] Metabolomics analyzes diverse small molecule types such as amino acids, fatty acids, and drug reflecting metabolic function and can extract significant data for understanding pathological states and contributing to early disease diagnosis and treatment.[13,14] MS-based proteomics and metabolomics research

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enables nearly comprehensive measurement of proteins and metabolites but faces challenges due to experimental inefficiency, lack of standardized analytical workflows, and compatibility issues between different techniques.[15-17] The absence of bulk replication techniques like PCR limits the quantity and throughput of samples, placing these methods at a disadvantage compared to nucleic acid-based methods.[18-20] Despite the ability to measure expression levels, modification states, and functional associations of various molecular species, existing workflows for protein and metabolite data generation and analysis are often specialized and not compatible with each other. Efforts to overcome these limitations are ongoing. Furthermore, proteomics and metabolomics play a crucial role in understanding disease processes and in clinical practice. These technologies provide insights into disease processes by identifying biomarkers and biological pathways differences associated with diseases.[15,21,22] Also, integrating various omics data can reveal potential causal changes leading to diseases or therapeutic targets. The integrated omics approach, applied in clinical settings, contributes to predicting, preventing, detecting, and treating diseases more effectively.[23-26] This approach is already being used to improve patient care in clinical settings, aiming to utilize the complex list of omics data to offer the most appropriate treatment for patients. This review focuses on the future directions and methodological considerations of MS-based proteo-metabolomics research, emphasizing the importance of experimental design, sample preparation, and the production and integration of omics data. Current trends and future research directions aim to enhance the application of these technologies in disease research, deepening our understanding of human diseases. The development of data integration and analysis methods targets the application of an integrated omics approach in clinical practice, aiming to improve patient care in clinical settings and contribute significantly to disease prevention, early detection, and treatment.[27-29]

1. MS based proteo-metabolomics

Multi-omics approaches, utilizing MS2-based analysis, enhance the understanding of disease progression through phenotypic analysis, bridging the gap between numerous potential biomarkers discovered and the few approved for clinical use. High-quality experimental design is crucial, including sample preparation, MS operation, and data processing, alongside considerations for meta-information and bioinformatics capabilities. Despite challenges such as sample complexity and the limitations of sample biomass or access, efforts to overcome these include pooling and single-cell omics methods.[30] Sample preparation should reduce complexity and be compatible with both proteins and metabolites, including pre-fractionation steps to increase coverage.[31] Chromatography and MS considerations reveal that gas chromatography-MS and capillary electrophoresis-MS characterize a wide range of metabolites but have limited throughput.[32,33] High-performance liquid chromatography (HPLC)-MS is versatile but consumes large sample amounts, whereas nano LC (nLC)-MS offers adequate throughput and sensitivity for omics analysis, but nLC-MS columns are prone to clogging, affecting column performance.[31,34] High-throughput automation is essential to minimize human errors and improve reproducibility, especially in large-scale proteo-metabolomics.[10] Characterization of omics data and data set integration remain significant challenges, requiring input from various experts for quality assessment and data integration, with approaches including post-analysis integration, integrated data analysis, and system modeling techniques.[6,12,35] The lack of attention to data analysis requirements and the need

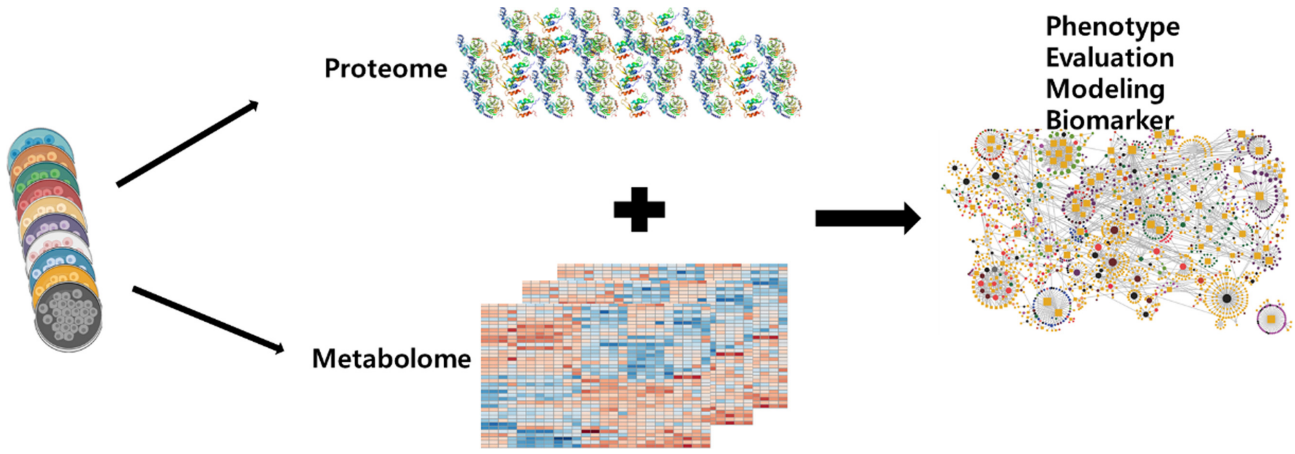

Figure 1. proteo-metabolomics analysis. Both proteomics and metabolomics are being studied on one platform of the mass spectrometer, and efforts are made to combine the omics data. Through this, through deeper understanding of phenotype and disease modelling.

for a generic analysis pipeline highlight the importance of planning analysis objectives beforehand. Finally, multi-omics research is often hindered by limited research staff and funding, with significant costs associated with accessing analytical instruments and multidisciplinary expertise. Funding disparities across omics research areas underscore the challenges in conducting comprehensive multi-omics studies.

2. Sample preparation

LC-MS-based proteome and metabolome analyses have traditionally been performed independently, with sample ionization and mass measurements vulnerable to interference from buffers, salts, polymers, and detergents, degrading MS system performance.[36-38] Recent techniques propose simultaneous molecular extraction from a single sample to minimize experimental variations and improve throughput, suggesting potential for automation and clinical applications.[39,40] Then, proteomics and metabolomics analyses, whether sequential or parallel, impact omics studies significantly, with preparation methods introducing biases and distortions.[27,41] The bottom-up approach in proteomics involves chemical or enzymatic digestion of proteins into peptides, employing methods like in-solution digestion, in-gel digestion, filter-assisted sample preparation, and suspension traps. Each method has its challenges, such as detergent interference or sample loss.[42-45] Metabolite sample preparation must navigate the diverse and complex chemical properties of molecules, often requiring separation from macromolecules and salts to reduce matrix effects.[46] Techniques like liquid-liquid extraction, solid phase extraction, protein precipitation, and ultrafiltration are common, with solvent choices tailored to the hydrophilic or lipophilic nature of the metabolites.[47,48] Simultaneous sample preparation methods for proteo-metabolomics, such as liquid-liquid extraction, aim to handle the limited sample quantities typical of clinical materials, addressing heterogeneity across different tissue sections.[49-51] Protocols like SIMPLEX (Simultaneous Metabolite, Protein, Lipid Extraction) for concurrent extraction of metabolites, proteins, and lipids from a single cell line have shown promise, offering a multi-molecule approach that enhances the understanding of diseases like Huntington's.[39] Similarly, techniques for the simultaneous extraction of DNA, RNA, proteins, and metabolites from a single sample have been developed, optimizing the analysis of plant tissues and cell lines for comprehensive multi-omics studies.[12,44,52,53] These advancements in sample preparation for MS-based proteomics and metabolomics underline the importance of integrating data from these analyses to gain deeper insights into biological and disease processes, emphasizing the need for innovative approaches to overcome the challenges of sample complexity and preparation biases.[29,54]

3. Data acquisition and feature identification

Data Acquisition by LC-MS

Despite improvements, the sensitivity and throughput of MS-based omics research lag behind genomic and transcript sequencing technologies, posing challenges for analyzing large sample sizes. Efforts like the Clinical Proteomic Tumor Analysis Consortium (CPTAC) are bridging gaps, but proteomics and metabolomics still strive to match the data generation and processing efficiency of nucleic acid profiling.[55,56] Enhancements in multiplexing, scan speeds, and automation are crucial for progress. Column optimization and the choice between different chromatography techniques, like HILIC for polar metabolites or C18 for broader metabolomics analysis, play a significant role in improving analytical performance.[49,57] Electrospray ionization (ESI), especially nano ESI (nESI), enhances sample transfer and ion detection due to smaller droplet size and higher ionization efficiency.[37,58] However, challenges remain in metabolomics studies, particularly in anionic ion mode, due to complexities in ESI in negative mode.[59] Retention time drift and peak intensity variability are notable issues, with replicated nLC-MS analysis showing significant variation in peak intensity. Internal standards can greatly reduce the coefficient of variation, enhancing the reliability of metabolomics assays.[60-62]

Raw Data Processing and Feature Identification

Processing raw MS data to identify proteins and metabolites involves different software tools for each omics field. In proteomics, database searches are constrained by known genomes and peptide chemistry, with tools like MaxQuant and various search engines facilitating peptide identification.[63] Metabolomics faces a broader identification search space due to structural diversity and lacks standardized fragmentation rules. Databases like PubChem, HMDB (Human Metabolome Database), and METLIN assist in compiling compound spectra, but the field suffers from a lack of standardized naming conventions for cross-resource transitions.[64-66] Identifying metabolites often relies on costly and time-consuming comparisons with reference standards, highlighting the need for meticulous experimental design and data analysis to ensure reliable metabolic function identification.[13,67] Various software tools support spectral filtering, peak detection, and data normalization, with XCMS and MetaboAnalyst being popular for their user-friendly interfaces and database integration, despite challenges in raw data processing and feature quantification.[68]

4. Integrative analysis of proteomics and metabolomics

The processed raw LC-MS data typically consists of a characteristic (i.e. protein, peptide or metabolite) and a matrix of biological samples and corresponding intensity

values. Proteomics and metabolomics potentially provide information about the activity of biological pathways, but generate large amounts of complementary data that require specialized mathematical, statistical and bioinformatics analysis strategies.[69,70] Currently, there are numerous omics data analysis and integration tools for this purpose, but each tool has several advantages and limitations. Data integration was characterized as a major bottleneck in all multiomics studies.[71,72] It's because data integration step requires proper input data and interpretation from a variety of scientists or experts as previously described. Some of these experts are needed to evaluate the quality and effectiveness of the study (experimental) design and the quality of the data collected from the device. Assuming that the data obtained is high quality and properly validated, you can follow a number of approaches to analyze and interpret multiomics data. This includes 1) integration after data analysis of omics, 2) integrated data analysis and 3) system modeling. Post-analysis data integration and integrative data analysis are mainly search tools or hypothesis generators to uncover new insights or provide a high level of mechanical understanding. System modeling techniques are primarily interpretation or hypothesis testing tools for mathematically explaining mechanical insights. System modeling can be used to predict comprehensive system responses or treatments (e.g. intervention identification). we briefly summarize these approaches for multi-omics data integration or the methodologies and software related with them.

Integration after data analysis of omics

In the post-analysis data integration approach, multiple omics data sets are first analyzed individually, and then the main models are combined or networked by synthesizing critical functions at joint nodes in the entire model path.[73, 74] This approach has been used in a wide range of studies, including the analysis of basic molecular biological systems, as well as the evaluation of biological waste water treatment systems, the search for microbial resistance of marine sediments after oil spills, and studies of permafrost microbial ecosystems.[75-77] This approach will require sufficient competence and experience in the field, as the subjective interpretation of the researcher can be entered in the integration process after analysis.

Integrated data analysis

The integrated data analysis approach uses special tools to merge multiple omics data sets before performing further data analysis and interpretation. This is not dependent on human interpretation or human prejudice, but each omics approach and platform. It makes it possible to derive statistical similarity between shares. It provides list presenting diverse tools that are available and can be used for integrated omics data analysis. For example, methods such as orthogonal bidirectional projection for latent structures (O2PLS) and orthogonal projections to latent structures (OnPLS**)**, the latter specifically designed for "multiple-block" analysis, enable the extraction of systematic changes common to two or more sets of individual omics data by aligning data structures across multiple blocks or datasets. This "multiple-block" approach in OnPLS allows for the simultaneous analysis of various omics layers, facilitating a comprehensive understanding of the underlying biological processes.[78-81] In clinical science, these techniques have been used to assess metabolic somatic and proteomic data in a xenograft model of human prostate cancer.[82,83] Also on interrogate biological interactions between six different mix data sets of asthma. Environmental science, the same technique has been used to characterize poplar different length work stress response or adaptation. Table 2 provides an overview of software tools for the analysis of integrated omics data. This table displays the domain, functionality, and other relevant attributes of each software tool. Particu-

Table 1. Potential limitations while designing proteo-metabolomics studies and possible strategies to overcome them.

Sample Type	Potential Limitations	Strategies to Overcome Limitation
Cell/Tissue	Limited biomass of sample Heterogeneity of cell type/ composition	Pooling Specific methods for small molecules Replication Homogenization
Serum/Plasma	clotting conditions and time, storage temperature, storage time, storage tube, freeze/thaw cycle and protease inhibitor high abundant protein	Aliquot and store with minimal thawing/re-freezing cycles in frozen liquid nitrogen Depletion
Urine	many metabolites but very few proteins	Choose appropriate target for analysis or appropriate omics analyses for target based on hypothesis
Cerebrospinal fluid	brain-specific proteins are complicated because most CSF proteins are derived from plasma, and very rich proteins tend to obscure less rich proteins.	Depletion of highly abundant proteins and sample pre-enrichment and desalting
Saliva	amylases, cystatins, and Igs	depletion of amylase and Igs prior to 2-DE analysis
other Body fluid	Abundance issue of protein and metabolites	Enrichment and nano flow injection, highly sensitive MS

Table 2. Overview of software tools for integrated omics data analysis, highlighting their domain, functionality, and applications in biomedical and environmental sciences. This table presents the several functionalities of integrated omics data analysis, including correlation network analysis, co-expression analysis, phenotype correlation, pathway enrichment, and gene ontology enrichment, essential for deciphering complex biological interactions and disease mechanisms.

Software Tool	Domain	Functionality
3Omics[72]	Medical (human)	- Correlation network analysis - Co-expression analysis - Phenotype generation - KEGG/HumanCyc pathway enrichment - GO enrichment - Name to ID conversion
BiofOmics[84]	Biofilm	- Experiment library - Data depository
BioCyc/MetaCyc[85]	Unspecified	- Predicted metabolic pathways in sequenced genomes - Online encyclopedia of metabolism - Metabolite database - Enzyme data set
CellML[86] (Open source XML language)	Unspecified	- Open-source language for biological cellular models
Cytoscape with MODAM[89], and; Cytoscape with OmicsAnalyzer[90]	Unspecified	- Multi-omic data miner and omicsAnalyzer were conceived as an accessible and plugin of Cytoscape that facilitates omics analysis - Compile all biological information regarding the system modelling through web link including multi-omics data - Model omics data
E-Cell[91]	Unspecified (Cells)	- Modeling, simulation, and analysis of complex, heterogeneous and multi-scale cellular systems
Escher[92]	Unspecified	- Web resource to visualize data on biological pathways - Design new pathway networks based on user data set and genome-scale models - Visualize data related to genes or proteins on the associated pathways - Identify trends in common genomic data types
LinkedOmics[93]	Medical (human)	- Data integration such as clinical data, miRNA, mutation - Gene level proteomics data analysis - Phospho-/glycol-proteomics
iPathwayGuide[94]	Medical (human)	- Gene analysis - Pathway analysis - GO analysis - Predicted miRNA analysis - Meta-analysis

larly, such tools can integrate the molecular profiles in genomics, proteomics, or metabolomics to facilitates comprehensive insights into disease mechanisms. Notably, 3Omics, the web-based platform, enables the integration of human transcript, proteomic and metabolic data.[72] In particular, 3Omics creates inter-omics correlation networks to support data visualization. In addition, this "one click" platform supports statistical analysis of integrated omics data and can perform pathway and gene ontology enrichment. Another popular web-based platform for integrated omics data analysis is "MetaboAnalyst".[95] This tool can integrate and analyze metabolomics data with whole body, proteomic and genomics data and can be used for data generated for a wide range of biological samples including humans, animals, plants and microorganisms. There are a lot of open access databases to aid with multi-omics integration, but a variety of open access tools are available to support with visualization of multi-omics data. These include tools that facilitate data quality checking, data normalization and data transformation (e.g. MetaboAnalyst and mixOmics).[96] It also includes software to support multivariate statistics, data clustering and data analysis. Many multi-omics integration tools include how to create and view interactive correlation or association maps (hairballs) as well as metabolic and signal pathways.

System modeling techniques

In addition to post-analysis data integration and inte-

grated data analysis techniques, a third approach to data integration, system modeling, is also available.[97,98] System modeling and simulation techniques are useful tools for predicting the complexity of biological systems. The model-based integration method relies on a well-defined understanding for the biological system under investigation to compare new experimental results to modeled predictions. This understanding is often based on having comprehensive and existing genomic, metabolic and / or metabolic data for the system under study. This modeling system can incorporate dynamic / dynamic models that solve differential or partial differential equation systems, and can include steady state models such as agent-based motion models or Petri-Net models or flux balance models.[99-102] Interestingly, almost all system modeling approaches are fixed with metabolic responses and extensive metabolic data. Some of impressive and frequentyl mentioned examples of multi-omics integration and the strongest success in systems biology have been achieved through system modeling methods. For example, in the 1990s, Palsson and colleagues modeled cell metabolism to quantitatively predict the growth and by-product secretion of E. coli and developed the concept of flux balance modeling.[99] Subsequently, the first successful attempt to kinematically model living cells was the E-cell project led by Tomama Masanori in 1999.[91] The project focused on dynamic modeling of metabolic pathways through gene expression of Mycoplasma genitalium and control of enzyme production. This single-cell model incorporates genomic, metabolic and proteomic data, and experimental metabolic somatic data. The E-cell concept was later extended to model human red blood cell metabolism.[103] The expansion from single-cell to multicellular and multi-organ systems began around 2013 with the advent of Recon 2, which is a community-based global reconstruction of human metabolism.[10] This integrated analysis model incorporates cell or tissue specific metabolomics data, protein data and gene expression data for the human body and various cell types. Recon 2 and later derivatives can model the effects of common drugs on human metabolism, predict the effects of disease gene mutations, and model disease states such as inflammatory bowel disease.[23,104-106,107]

As can be seen from the examples of system modeling for multi-omics integration above and many others, most multi-omics system models are based on some form of metabolic model or readings. It should be noted that quantitative proteins also meet this requirement. This fact highlights the main role that must be played in the multi-omics integration of MS-based proteomics and metabolomics, especially with regard to system modeling. The reason is that it can be quantitative because it plays an important role in system modeling and multi-omics integration. It is not possible to perform system modeling without an exact value or an exact concentration as an input, and similarly an system model cannot be easily identified without an accurate quantitative concentration as an output. Proteomics and metabolomics can provide both quantitative input and output data, which is very useful for system modelers.[91,108]

Current application

Proteomics and metabolomics, reflecting both environmental exposure and genetic coding, offer insights into disease phenotypes beyond what genome data can provide.[109,110] The dynamic nature of protein and metabolite levels allows for therapeutic targeting and a deeper understanding of biological systems through integrated bioinformation.[4,29] Recent efforts aim to integrate proteo-metabolomics data, promising for clinical use, especially in cancer research. Studies show that combining protein and metabolite biomarkers can enhance cancer diagnosis and treatment, with specific examples demonstrating significant improvements in diagnostic accuracy and predictive performance for diseases like pancreatic duct adenocarcinoma and non-small cell lung cancer.[82,111-114] Integrated approaches in research reveal cellular and molecular responses in conditions such as Ischemia and reperfusion injury (IRI), showing the impact on acute phase response, coagulation, complement pathways, and fatty acid signaling.[112] These studies utilize transcriptomics, proteomics, and metabolomics to understand protective stress responses and metabolic pathways, highlighting the potential of integrated omics in diagnosing and understanding diseases. Developments in data acquisition and feature selection to efficiently utilize this approach are also performed in recent research. A recent study reported advancements in proteomics and metabolomics concerning plants, notably introducing various bioinformatics tools and algorithms. It also facilitated the comparison of experimental data with existing genomic or metabolomic databases, and generated peptide spectrum match lists based on experimentally measured mass data.[115] Another study reported into the framework for data fusion specifically for biomarker discovery. This research proposed a framework for data acquisition and feature selection, which entails extracting relevant information from each analytical platform in the initial phase and integrating the derived latent variables for further analysis. After preprocessing, an explorative analysis is conducted to detect potential outliers and unexpected trends. The data fusion process, carried out in two steps, first extracts the most pertinent information from each data block, and then integrates this information in the second phase. This methodology facilitates biological interpretation and provides a systematic and integrated approach to acquiring and selecting features in proteo-metabolomics data.[116] Such endeavors in data integration aim to deepen our understanding of complex biological systems and are being continuously researched.

Conclusion

The integration of proteomic and metabolomic workflows provides a comprehensive view of biological sys-

tems, enhancing our understanding of dynamic responses. The use of integrated nLC-MS-based platforms facilitates consistent sample preparation, biomolecule identification, and complex biological sample analysis. Despite advancements, the field faces challenges in data analysis and interpretation, requiring the development of databases and tools for meaningful biological insights. Standardized protocols, quality control measures, and improved software/database interoperability are essential for reproducible and comparative multi-omics research. Increased funding and awareness for metabolomics, user-friendly software development, and advances in machine learning are critical for the integration and interpretation of multi-omics data. The integration journey of proteo-metabolomics, extending from precise sampling techniques to nuanced interpretation, harbors immense potential yet is fraught with critical limitations. Particularly, the variability inherent in biological samples and the challenge of effectively selecting features from vast datasets highlight a significant limitation. For instance, the ability to distinguish relevant biomarkers in the noise of comprehensive data sets remains a formidable challenge. This necessitates innovative solutions, such as machine learning models capable of identifying subtle patterns amidst complexity, thereby enhancing the reproducibility and fidelity of the analysis. Such endeavors not only propel forward our understanding of intricate biological systems but also open new avenues for personalized medicine, showcasing the creative potential to overcome current limitations and push the boundaries of what is scientifically achievable.

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Conflicts of Interest

The authors declare no conflict of interest. Minjoong Joo and Jong-Moon Park are employees of Basil Biotech. This paper reflects the views of the scientists and not the company.

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