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CHAPTER SEVEN

The Lipid Maps Initiative in Lipidomics

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Contents

Abstract

The Lipid Metabolites and Pathways Strategy (LIPID MAPS) initiative constitutes the first broad scale national exploration of lipidomics and is supported by a U.S. National Institute of General Medical Sciences Large Scale Collaborative "Glue" Grant. The emerging field of lipidomics faces many obstacles to become a true systems biology approach on par with the other "omics" disciplines. With a goal to overcome these hurdles, LIPID MAPS has been developing the necessary infrastructure and techniques to ensure success. This review introduces a few of the challenges and solutions implemented by LIPID MAPS. Among these solutions is the new comprehensive classification system for lipids, along with a recommended nomenclature and structural drawing representation. This classification system was developed by the International Lipids Classification and Nomenclature Committee (ILCNC) in collaboration with LIPID MAPS and representatives from Europe and Asia. The latest changes implemented by the committee are summarized. In addition, we discuss the adoption of mass spectrometry (MS) as the instrumental platform to investigate lipidomics. This platform has the versatility to quantify known individual lipid molecular species and search for novel lipids affecting biological systems.

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1. Introduction

Remarkable technological advances in the biological sciences have forged a new era of research in the field of systems biology. These comprehensive investigations of living organisms at the molecular system level can be classified into different fields: genomics, transcriptomics, proteomics, and metabolomics, otherwise known as the ''omics cascade.'' The integrative analysis of an organism's response to a perturbation on the ''omics'' levels will lead to a more complete understanding of the biochemical and biological mechanisms in complex systems.

An explosion of information has occurred in the fields of genomics, transcriptomics, and proteomics because each field was able to adopt a single instrumental methodology to analyze all of their respective components in a given sample. However, whereas genomics, transcriptomics, and proteomics have made significant strides in technological development, the tools for the comprehensive examination of the metabolome are still emerging. Although metabolomics is the endpoint of the ''omics cascade'' and the closest to phenotype, no single instrument can currently analyze all metabolites (Bino *et al.*, 2004).

Comprehensive investigation of the metabolome is hindered by its enormous complexity and dynamics. The metabolome represents a vast number of components that belong to a wide variety of compound classes, including nucleic acids, amino acids, sugars, and lipids. These compounds are diverse in their physical and chemical properties and occur in vastly different concentration ranges. Additionally, metabolite concentrations can vary spatially and temporally. Furthermore, diet- and/or nutrient-dependent biological variability confounds such analysis (Vigneau-Callahan *et al.*, 2001). For example, within the lipid classes, highly abundant compounds exist, such as fatty acids, triglycerides, and phospholipids, and trace level components, such as eicosanoids, which have important regulatory effects on homeostasis and disease states.

In addition to analyzing a vast number of metabolites, a comprehensive lipidomic investigation includes studying the metabolizing enzymes and lipid transporters. Together, these components play a role in cell signaling, metabolism, physiology, and disease, making them excellent targets for systematic measurements. The LIPID MAPS initiative is a result of our quest to characterize the mammalian lipidome to an extent similar to that of the genome and proteome (Dennis *et al.*, 2005). To accomplish this goal, we have formed a consortium of twelve independent laboratories from seven academic institutions and one company. We have six lipidomics cores that are charged with identifying, quantifying, and becoming experts in six major categories of lipids: fatty acyls, glycerolipids, glycerophospholipids,

sphingolipids, sterol lipids, and prenol lipids. This division of labor is essential, as there are hundreds of thousands of individual molecular species of metabolites in each category of lipids, presenting a challenge for routine quantification. Additionally, the Lipid Synthesis/Characterization Core is charged with generating quantitative lipid standards and the synthesis and characterization of novel lipids. We also have a Bioinformatics Core, a Cell Biology Core, and a MS Development Core. Finally, five independent sections explore more specific aspects of lipidomics as related to future core methodologies and the medical field.

The following text will discuss each of the LIPID MAPS contributions to lipidomics. The "Building Infrastructure in Lipidomics" section discusses the development of critical infrastructure for lipidomics to become a true systems biology approach. "Classification, Nomenclature, and Structural Representation of Lipids" describes the standardization of the lipid classification scheme, lipid nomenclature, and structural representation (Fahy *et al.*, 2005). "Mass Spectrometry as a Platform for Lipid Molecular Species" describes the adoption of MS as the major platform to investigate lipidomics and the challenges in quantifying the complex lipid metabolites in a biological system. This section addresses the complications associated with integrating the molecular species lipidomic data and the need for analyte standards. After the first 3 years of the project, we have implemented most of the infrastructure and have evolved our future plans to expand lipidomics, as described in the "Future Plans" section.

2. Building Infrastructure in Lipidomics

In 2003, the consortium identified several important challenges that any lipidomics effort would face. These obstacles included developing a robust and versatile platform to quantify the lipidome, specific reagents and experimental protocols, high throughput methods, data export standardization, computer algorithms for automated data analysis, visualization tools, libraries, and databases for integration of lipidomics with the other omic cascades.

Because no single quantization platform is capable of measuring the entire lipidome, the first goal of the initiative was to develop the requisite quantitation technology. In order for different laboratories to minimize instrument variability and to ensure interoperability and method sharing capabilities, LIPID MAPS chose for all cores to use the same ABI-4000 Q-trap mass spectrometers (Foster City, CA) for quantitative comparisons. We have and will continue to design stringent sets of isotopic-labeled reference compounds that will allow the accurate quantification of a wide range of lipid metabolites. The reference standards are intended to be commercially

available to all researchers worldwide, both within and beyond the lipid field, which will facilitate inter-laboratory comparison.

Once a requisite quantitation technology was established, we realized that, to successfully conduct bioinformatic analysis of data collected from six different laboratories, a significant level of reproducibility between experiments and between laboratories would be required. To achieve this reproducibility, we have employed a rigorously maintained set of common biological, biochemical, and analytical technologies in each of the consortium laboratories. To this end, we have tried to standardize all reagents. We purchased a large lot of fetal calf serum, which is being employed by all consortium laboratories for the duration of this project. We also prepared a large batch of RAW 264.7 cells obtained from ATCC (Manassas, VA) and froze enough stabs to supply fresh cells from this one batch to all laboratories for the duration of the project. Thus, all cells in these experiments were similarly passaged and treated. For example, to investigate changes associated with the binding of the Toll-like receptor 4 (TLR-4), we developed the methodology to prepare highly purified 3-deoxy-D-manno-octulosonic acid Kdo_2-Lipid A, and then evaluated it by electrospray ionization (ESI)-MS (ESI-MS), liquid chromatography (LC)-MS (LC-MS), and ^{1}H-NMR (nuclear magnetic resonance). Finally, we compared its bioactivity with laser plasma spectrometry (LPS) in RAW 264.7 cells and bone marrow macrophages from wild-type and TLR-4–deficient mice. Now, standardized Kdo_2-Lipid A ensures a crucial intra-laboratory quality control agonist for TLR-4 (Raetz *et al.*, 2006).

No universal classification system for lipids was suitable for modern informatics and experimental investigations. Unlike existing lipid legacy databases, such as Lipid Bank and Lipidat, our new system plans to integrate all the omic cascades associated with lipidomics. Integrating the MS data on lipidomics with the genomic data will lead to a more complete understanding of how complex lipidomic networks function, from biosynthesis to the removal of cellular lipids and the important roles of metabolites as second messengers. With thorough and extensive experimental planning, we are able to integrate and analyze large amounts of data that will be developed into ''road maps.''

3. Classification, Nomenclature, and Structural Representation of Lipids

With the emergence of lipidomics as a rapidly expanding field came an urgent need for an internationally accepted method of describing and classifying lipid molecules. The first step toward classification of lipids was

the establishment of an ontology that was extensible, flexible, and scalable. One must be able to classify, name, and represent these molecules in a logical manner that is amenable to databasing and computational manipulation. Lipids have been loosely defined as biological substances that are generally hydrophobic in nature and, in many cases, soluble in organic solvents (Smith, 2000). These chemical features are present in a broad range of molecules, such as fatty acids, phospholipids, sterols, sphingolipids, and terpenes (Christie, 2003). The LIPID MAPS consortium has taken a chemistry-based approach by defining lipids as hydrophobic or amphipathic small molecules that may originate entirely or in part by carbanion-based condensations of thioesters such as fatty acids and polyketides (Fig. 7.1A) and/or by carbocation-based condensations of isoprene units such as prenols and sterols (Fig. 7.1B) (Fahy *et al.*, 2005).

The classification scheme shown in Table 7.1 organizes lipids into well-defined categories that cover eukaryotic and prokaryotic lipid origins, and is equally applicable to archaea and synthetic (manufactured) lipids (Fahy *et al.*, 2005). Biosynthetically related compounds that are not technically lipids due to their water solubility are included for completeness in this classification scheme.

Lipids are divided into eight categories (fatty acyls, glycerolipids, glycerophospholipids, sphingolipids, sterol lipids, prenol lipids, saccharolipids, and polyketides) containing distinct classes and subclasses of molecules, and a 12-digit unique identifier is associated with each distinct lipid molecule.

For each lipid, the unique 12-character identifier based on this classification scheme is an important database field in the Lipids Database (Sud *et al.*, 2007). The format of the LIPID ID, outlined in Table 7.2, provides a systematic means of assigning a unique identification to each lipid molecule and allows for the addition of large numbers of new categories, classes, and subclasses in the future. The last four characters of the ID comprise a unique identifier within a particular subclass and are randomly assigned. Initially using numeric characters allows 9999 unique IDs per subclass; however, with the additional use of 26 uppercase alphabetic characters, a total of 1.68 million possible combinations can be generated, providing ample scalability within each subclass.

The classification system is under the guidance of the ILCNC, which meets periodically to propose changes and updates to classification, nomenclature, and structural representation. The ILCNC currently consists of Dr. Edward A. Dennis (chair), Dr. Robert C. Murphy, Dr. Masahiro Nishijima, Dr. Christian R.H. Raetz, Dr. Takao Shimizu, Dr. Friedrich Spener, Dr. Gerrit van Meer, and Dr. Michael Wakelam. The most recent meeting was on July 7, 2006 in La Jolla, CA, during which a number of recommendations were implemented to extend the original system (Fahy *et al.*, 2005). Some key changes can be seen in Table 7.3.

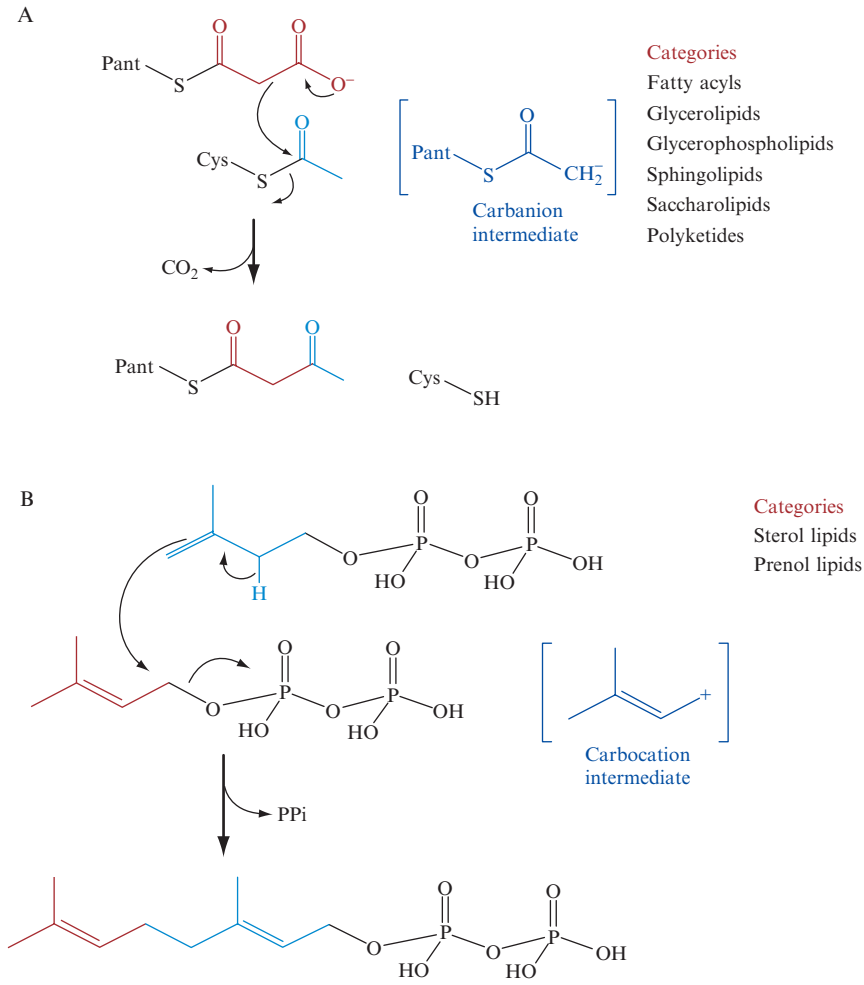

Figure 7.1 The LIPID MAPS chemistry-based approach defines lipids as molecules that may originate entirely or in part by carbanion-based condensations of (A) thioesters and/or by (B) carbocation-based condensations of isoprene units.

The ILCNC has been greatly assisted by many experts throughout the world who have given helpful advice and comments on enhancing the scope and utility of the lipid classification scheme. The scheme can be conveniently browsed on the LIPID MAPS website (http://www.lipidmaps.org), in which the various classes and subclasses are linked to the LIPID MAPS structure database (Fahy and Subramaniam, 2007).

The LIPID MAPS classification scheme has now gained widespread international acceptance and has recently been adopted by KEGG (Kyoto

Table 7.1 LIPID MAPS lipid categories and examples

Category	Abbreviation	Example
Fatty acyls	FA	Dodecanoic acid
Glycerolipids	GL	1-Hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycerol
Glycerophospholipids	GP	1-Hexadecanoyl-2-(9Z-octadecenoyl)-*sn*-glycero-3-phosphocholine
Sphingolipids	SP	N-(tetradecanoyl)-sphing-4-enine
Sterol lipids	ST	Cholest-5-en-3β-ol
Prenol lipids	PR	2E,6E-farnesol
Saccharolipids	SL	UDP-3-O-(3R-hydroxy-tetradecanoyl)- βD-N-acetylglucosamine
Polyketides	PK	Aflatoxin B1

Table 7.2 Format of 12-character LIPID ID

Characters	Description	Example
1–2	Fixed database designation	LM
3–4	Two-letter category code	FA
5–6	Two-digit class code	03
7–8	Two-digit subclass code	02
9–12	Unique four-character identifier within subclass	AG12

Encyclopedia of Genes and Genomes), where functional hierarchies involving lipids, reactions, and pathways have been constructed (http://www.genome.ad.jp/brite/). In addition, LIPID MAPS lipid structures are now available on NCBI's PubChem website (http://pubchem.ncbi.nlm.nih.gov/), where entries are hyperlinked to the LIPID MAPS classification system.

4. Mass Spectrometry as a Platform for Lipid Molecular Species

Mass spectrometry is an established and invaluable tool for the characterization of changes in lipidomics and lipid-mediated signaling processes resulting from disease, toxicant exposure, genetic modifications, or drug therapy

Table 7.3 Updates to LIPID MAPS classification system

1. Under the fatty acyls category, the hydroxyeicosatrienoic acids, hydroxyeicosatetraenoic acids, and hydroxyeicosapentaenoic acids subclasses have been expanded to include the corresponding hydroperoxy and keto analogues. The N-acyl amide and N-acyl ethanolamide subclasses have been renamed to N-acyl amines and N-acyl ethanolamines.
2. In order to improve representation of plant lipids in the classification scheme, the glycosylmonoradyl and glycosyldiradylglycerol classes were created within the glycerolipid category.
3. Several new bile acids (C22, C23, C25, and C29 bile acids, alcohols, and derivatives) and secosteroids (vitamin D4, D5, D6, and D7 and derivatives) have been added to the sterol lipids category.
4. It was decided not to rely on phylum- or species-based references in the classification scheme, but rather to use structural core units where possible. Accordingly, the phytosterol, marine sterol, and fungal sterol subclasses were removed from the sterol lipids category and replaced with a more extensive, structurally based list (ergosterols, stigmasterols, C24-propyl sterols, gorgosterols, furostanols, spirostanols, furospirostanols, calysterols, cardenolides, bufanolides, brassinolides, and solanidines/alkaloids) of sterols.
5. The retinoids subclass was added to the prenol lipids category. The hopanoids class was also moved to this category (from sterol lipids).
6. In the case of lipids with multiple functional groups (especially fatty acyls) where it may be difficult to objectively classify a structure, the Cahn-Ingold-Prelog rules are applied to place the lipid in the subclass with the highest Cahn-Ingold-Prelog "score." However, to ensure that lipids containing a particular functional group (e.g., a hydroxyl-fatty acid) can be located in a database that uses this classification system, an ontology-based system has been implemented, where a user may locate all lipids with the specified functionality, regardless of their subclass designation.
7. The LIPID MAPS glycerophospholipid abbreviations (GPCho, GPEtn, etc.) are used to refer to species with one or two radyl side-chains, where the structures of the side chains are indicated within parentheses, such as GPCho(16:0/18:1(9Z)).

(Watkins and German, 2002). MS provides a wide dynamic range to quantify lipids. In addition, it offers excellent selectivity for examining compounds using parameters such as ionization mode (positive/negative), mass selection, MS/MS characterization, and numerous MS^n techniques for structural elucidation. MS-based lipidomics can deliver detailed, quantitative information about the cellular lipidome constitution and provide insights into biochemical mechanisms of lipid metabolism, lipid–lipid, and lipid–protein interactions.

Traditionally, lipids have been analyzed using gas chromatographic (GC) separation, electron impact ionization MS (EI-MS), and flame ionization

detection (FID). GC is limiting because compounds must be thermally stable with high enough vapor pressure to volatilize during injection. Therefore, extensive sample manipulation is required on complex lipid samples to produce accurate data. Sample preparation can include pre-separation of lipid classes, hydrolysis, derivatization, or pyrolysis. Although highly quantitative, these labor-intensive preparations destroy structural information regarding lipid composition. One of the LIPID MAPS goals is to improve the ability to quantify discrete molecular species, utilizing MS.

Detection of the specific molecular species was made possible by the development of solid state ion sources (e.g., fast atom bombardment [FAB] or matrix-assisted laser desorption/ionization [MALDI]) and liquid-phase ion sources (e.g., electrospray ionization [ESI], atmospheric pressure chemical ionization [APCI], or atmospheric pressure photo ionization [APPI]) (Griffiths, 2003). Although MALDI and other laser-based ionization methods have great potential for nonpolar species, our initial work has focused on ESI ion sources, as they are efficient for a wide variety of metabolites and are easily mated to high performance liquid chromatography (HPLC). ESI in positive mode allows for the detection of phosphatidylcholine, lysophosphatidylcholine, phosphatidylethanolamine, and sphingomyelins. ESI in negative mode allows for the detection of phosphatidic acid, phosphatidylserine, phosphatidylinositol, phosphatidylglycerol, phosphotidylethanolamine, free fatty acids, and eicosanoids. Finally, compounds that are not readily ionized by ESI, such as neutral lipids (e.g., triacylglycerols), can be detected by examining ammonium, lithium, or sodium adducts with ESI in positive mode (Duffin *et al.*, 1991; Han and Gross, 2005; Hsu and Turk, 1999).

Ionized lipids are further studied by tandem MS (MS/MS) to identify lipid species within specific classes. For example, the specific headgroups of phospholipids produce characteristic fragmentation patterns that are used for their identification (Griffiths, 2003; Pulfer and Murphy, 2003; Merrill *et al.* 2005) used both neutral loss and precursor-ion methods to identify sphingolipids. Precursor-ion scans for m/z 184 in positive mode are used to identify sphingomyelin while scans for m/z 264 are used to identify ceramide. More complex lipids, such as gangliosides, require advanced MS^n experiments for elucidation.

Certain lipid classes lend themselves to automated computational lipidomic approaches because of structural similarities and differentiation only in their headgroups and acyl groups. Forrester *et al.* (2004) developed methods to automate spectral interpretation and quantification of multiple phospholipid species utilizing direct injection ESI-MS/MS analysis. Han and Gross (2003, 2005) have also attempted to eliminate the need for HPLC by developing a "shotgun lipidomic" approach. They utilized the traditional Bligh/Dyer extraction to produce tissue extracts, and then directly injected the sample onto the mass spectrometer in both positive and negative mode ESI. Hermansson *et al.* (2005), using LC-ESI-MS/MS, quantified

more than 100 polar lipids using a fully automated method from sample handling to computer algorithms analysis of the spectra produced. In most cases, only semiquantitative data were reported.

The current goal of LIPID MAPS focuses on the complete quantitative analysis of the exact molecular species. Accurate quantitative data require a known standard for each molecular species, as well as internal standards to mimic analytes in these biological samples. Internal standards are most commonly deuterated analogues or lipids containing odd chain fatty acids. LIPID MAPS has collaborated with suppliers who provide analytical, chemically pure standards for precise quantization. In some categories, such identification of discrete molecular species is extremely labor-intensive. While it would be labor- and cost-prohibitive to develop the standards for quantifying all of the known molecular species, we continue to develop as many standards as possible. Moreover, when unknown species are detected and characterized, their final identification requires chemically synthesized standards (Harkewicz *et al.*, 2007)

Progress to date in developing standards is shown in Table 7.4.

5. Future Plans

Signaling through lipid metabolism constitutes key cellular events that participate in innate immunity, inflammation, and a host of other physiological phenomena. The complexity of lipid metabolites in biological

Table 7.4 Mass spectrometry standards

Core	Library MS/MS SPECTRA[a]	Internal standard[b]	Primary standard[c]	LIPID MAPS standard[d]
Fatty acyls eicosanoids	74	16	16	0
Glycerolipids	21	15		15
Glycerophospholipids	182	24	40	24
Sphingolipids	5	15		15
Sterols	23	13	13	11
Prenols/other	13	1		7
Total	**318**	**84**	**69**	**72**

[a] MS/MS spectra of chemically pure analytes, which are located in the LIPID MAPS library on www.lipidmaps.org.
[b] Analyte analog (e.g., stable isotope, odd carbon fatty acid).
[c] Chemically pure, accurately quantitated analyte.
[d] Internal or primary standard that is a certified, chemically pure analyte, quantitated, and stability-tested for shelf life.

samples has presented a great challenge. As stated previously, the different subclasses of lipids are affected by various stimuli and produce both temporal and spatial metabolites in a biological system. Therefore, the true goal of ''lipidomics,'' namely the quantitative analysis of all lipid metabolites in a biological system, requires different tools to probe the lipidome for dynamic changes. Two complementary approaches are lipid fingerprinting and lipid profiling.

Lipid fingerprinting identifies patterns, or ''fingerprints,'' of lipid metabolites that change in response to various stimuli. These methods, which are not intended to quantify compounds, cast a wide net and generate and test hypotheses. Specifically, LIPID MAPS–collected lipid extracts will be subjected to a general lipid fingerprinting analysis using LC–time-of-flight (TOF)–MS. This technique is used to screen for differences in lipid expression among treatment groups and identify important lipid metabolites and potential biomarkers. The bioinformatics team will process the data and, when significant changes are detected, further efforts will be taken to identify and quantify these compounds. This approach allows for the discovery of novel lipids that change in response to a given stimulus as well as interpretation of the role in biochemical processes.

Lipid profiling uses analytical methods for quantifying metabolites in a pathway or for a class of compounds. LIPID MAPS has developed infrastructure for quantifying analytes in each lipid class using this approach. The quantitative lipid analysis information has allowed us to produce information that can be applied to known biochemical pathways and physiological interactions. Profiling methods are used to test specific hypotheses and investigate mechanisms of action within the biological systems. Individual analyte data comprise a platform-independent legacy database, while the collection of lipid profiles provide temporal snapshots that lead to identification of different response stages among the different various groups. In combination with fingerprinting, various biomarkers can potentially be discerned from each stage.

In future work, we will use both approaches to comprehensively investigate lipid changes resulting from a genetic modification, toxicant exposure, diet, disease, or drug therapy. LIPID MAPS plans to carry out systematic and quantitative measurements of lipid changes in an effort to reconstruct normal and pathological networks associated with inflammation. Our vehicle for these experiments will be primary macrophages and macrophage cell lines as well as mouse plasma and various tissues. We will continue to develop novel MS techniques, as well as methods for integrating lipidomic and genomic technologies. We anticipate that our efforts will provide the foundation for building a bridge to translational medicine and serve as a paradigm for interdisciplinary systems medicine projects.

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