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The Art and Practice of Systems Biology in Medicine: Mapping Patterns of Relationships

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Systems biology has developed in recent years from a technology-driven enterprise to a new strategic tool in Life Sciences, particularly for innovative drug discovery and drug development. Combining the ultimate in systems phenotyping with in-depth investigations of biomolecular mechanisms will enable a revolution in our understanding of disease pathology and will advance translational medicine, combination therapies, integrative medicine, and personalized medicine. A prerequisite for deriving the benefits of such a systems approach is a reliable and well-validated bioanalytical platform across complementary measurement modalities, especially transcriptomics, proteomics, and metabolomics, that operates in concert with a megavariate integrative biostatistical/bioinformatics platform. The applicable bioanalytical methodologies must undergo an intense development trajectory to reach an optimal level of reliable performance and quantitative reproducibility in daily practice. Moreover, to generate such enabling systems information, it is essential to design experiments based on an understanding of the complexity and statistical characteristics of the large data sets created. Novel insights into biology and system science can be obtained by evaluating the molecular connectivity within a system through correlation networks, by monitoring the dynamics of a system, or by measuring the system responses to perturbations such as drug administration or challenge tests. In addition, cross-compartment communication and control/feed-back mechanisms can be studied via correlation network analyses. All these data analyses depend critically upon the generation of high-quality bioanalytical platform data sets. The emphasis of this paper is on the characteristics of a bioanalytical platform that we have developed to generate such data sets. The broad applicability of Systems Biology in pharmaceutical research and development is discussed with examples in disease biomarker research, in pharmacology using system response monitoring, and in cross-compartment system toxicology assessment.

Keywords: systems biology • system thinking • proteomics • metabolomics • correlation networks

Introduction

A holistic view, or in contemporary words, *systems-based thinking*, has been an undercurrent through the existence of mankind. In science, however, a reductionist focus has been the Holy Grail from the time of Descartes and Newton. Furthermore, for political and/or religious reasons, it was convenient in the past to separate mind from matter, and a dominant mechanistic view of the world around us has developed on that basis.

By following reductionist principles, enormous scientific and technological achievements have certainly been made and, in the last century, the exploration of the subatomic world has been a mind-boggling example of the fruits of reductionism. Surprisingly, quantum physics, which appears from a distant perspective to be the ultimate form of reductionism, has stimulated systems thinking. Contextualization and connectivity have been key drivers for this change in approach, a change which is reflected in studies ranging from nonlocality in quantum physics to universal connections in cosmology.

The emergence of systems-based thinking across different scientific domains has occurred in the last century seemingly independently and often unnoticed by the mainstream. In the natural sciences, systems approaches emerged slowly but steadily in the last century with, among others, Bertalanffy[1] being recognized as an important initiator. From a broader perspective, Capra,[2] Laszlo,[3−5] and many others have contributed to a more comprehensive systems worldview. The desire

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to move to holism has, perhaps, always been an undercurrent in science and society throughout time. For example, Louis Bolk (1866−1930), who was a professor in anatomy at the University of Amsterdam, remarked "*How much broader our view of life would be if we could study it through reducing glasses*!" Furthermore, a remarkable systems view was beautifully outlined, as early as 1926, in the book *Holism and Evolution* by Jan Christiaan Smuts,[6] a statesman in South Africa and a great nature philosopher. The observations and insights in this book still serve as important inspiration for system research as implemented, for example, in the Biomatrix concept.[7,8]

In medicine, tremendous scientific advancements have been made in understanding diseases from a reductionistic viewpoint. The current mainstream understanding of diseases and drug treatments encompasses the "1 disease-1 target-1drug" and the "1-drug-fits-all" concepts which emerged as a result of successes in drug discovery during the 20th century.[9] In medicine and medical technology, many accomplishments have been realized from that basis but have led to the current situation whereby the success formula built on reductionism seems to have reached its limits and the pharmaceutical industry faces enormous challenges with productivity decreasing and development costs rapidly increasing.

How can the tide of decreasing productivity and increasing costs be turned and what might be the role of a systems approach to Health and Disease in the future? A better understanding of biology is obviously a first important step,[10,11] and systems biology offers such a possibility. Understanding biology requires knowledge of connectivity in systems and their self-organization.[4] Homeostasis, as a self-organization process, was emphasized some time ago by Claude Bernard, who stated that constancy of the internal environment is the condition for free life (1872). Walter Canon also described homeostasis as key principle of life in 1922. In today's clinical practice, however, the concept is largely used in a strict reductionistic mode.[12] Intervention is often based on regulating a single element such as cholesterol or glucose back into the statistical range found in "normal" people. Interdependence, nonlinearity, and multilevel dynamics, as basic principles from a systems regulatory viewpoint, have not yet penetrated medical practice. Moreover, an understanding that new properties emerge within a system at different levels of complexity, clearly underlines the need to study the behavior of an entire system, rather than focus on studying its components in isolation. As a result, in forward-looking medical systems science, connectivity[13] and network biology are becoming a focal point of research efforts. In network biology, important steps are made to better understand the structure and function of networks. However, the concept of scale-free networks and the importance of hubs (nodes with large number of edges) in disease and intervention are still the subjects of vigorous debate,[14,15] and more detailed examples need to be gained. In dynamic system studies, a shift in focus is needed from objects to relationships and from single quantities to the integration of quantities into a view on system quality. As a result, the need to map *patterns of relationship* is surfacing, as demonstrated in mammalian systems based on *de novo* measurements across transcriptomics, proteomics, and metabolomics.[16−18] To achieve this crucial understanding of complex relationships in an intact system, there is a great need for reliable, high-quality experimental data beyond the cellular level. From our point of view, correlation networks might be a useful approach to make progress in this area.

An understanding of organizing principles within a complex system opens up options for novel system-based intervention strategies. Systems theory[19] proposes that the scale and complexity of a problem (disease state) should be matched by the scale and complexity of the solution (intervention). This proposal, and the realization that biological processes are regulated via patterns of different molecules, lead naturally to the concept of combinatorial drug intervention for the most effective treatment of diseases.[20−22]

However, the design of such a combinatorial intervention is challenging. It entails multiple target selection in multiple compartments and matching different dynamics. In fact, the "target modulation" becomes the perturbation of biochemical pathways[23] or, more correctly, system regulation.[24] The current simplistic drive to get "the right drug to the right patient, in the right dose at the right time, via the right route" becomes more complex in such a multidimensional pharmacology setting. Recent progress in Medicinal Chemistry reveals such a trend toward multitarget drugs. Formerly known as "dirty drugs" with unknown off-target effects, these compounds have become of interest again in light of this new perspective.[25] Furthermore, novel drugs with activities at multiple targets are now being synthesized, and multiple drugs are being combined based on different target activities (polypharmacy). The latter case, although more complex from a regulatory perspective, provides opportunities to design combinations more precisely than might be possible via single compounds. New strategies of screening chemicals via multiple target readouts to obtain biological spectra[26,27] are a step toward a multidimensional pharmacology concept. However, in designing optimum combinations, the resulting drug target profiles are indicative of a compound's possibilities, but rather limiting, because synergetic effects cannot be detected via screening of single components in high-throughput target screening. Combinatorial screening in cellular assays is a step up the ladder, but still limits the discovery of the "best" compounds as system-wide synergies are not detectable. Reversed-pharmacology[28] on natural products[24] might become an attractive source for the discovery of synergetic effects and lead to a renaissance of the abandoned natural product research domain. Natural product research almost vanished when combinatorial chemistry was introduced into pharmaceutical discovery, but the latter approach is still challenged to deliver its hoped-for potential.

In forward-looking system science experiments, a change will be needed in experimental design whereby experiments will be performed using complex input perturbations followed by the measurement of system-wide responses. In addition, a desire to move to preventive health care and personalized medicine will create an opportunity for a systems approach. This might seem to be a great challenge in western societies, but the concept and practice have existed already for centuries in other oriental medical systems. Integration or fusion of different philosophies and practical approaches to healthcare across various medical systems is an attractive way for moving forward. Clinical trial designs need to be changed considerably to meet these goals and even moving toward $N = 1$ clinical trials in the future might become desirable.[29]

Critical for Systems Biology research is a sophisticated bioanalytical platform integrated with advanced biostatistical/bioinformatics methodologies. Reliability, combined with high-quantitative analytical quality (precision, accuracy, inter/intraday variability, etc.), is critical for success.[30] In general, to analyze complexity in systems biology research, high-resolution

Figure 1. Black skimmer (*Rhynchops niger*) fishing in the Pantanal, Brazil[32] by slicing through the water with the lower mandible of the open bill, illustrating an optimal systems biology experiment. Although at first sight it appears as a random fishing experiment, it is not. An optimal success discovery is obtained, by selecting the best fishing grounds based on prior ecosystem knowledge or information obtained during action.

separation methods combined with high-resolution mass spectrometric detection is a key factor. Moreover, sensitivity and comprehensiveness can be obtained via combined platforms with less peak capacity on each individual platform.

It is important to appreciate that translating materials and methods from a research environment to a production-focused, application environment requires considerable effort. The availability of validated commercial analytical components and instruments among others is often not recognized. The wealth of published bioanalytical papers dealing with cutting-edge research often obscures the lack of well-developed reliable and validated methodology. Without proper QA/QC procedures, which provide a basis for intra- and interlaboratory comparisons, it is impossible to compare data sets over time and between different laboratories, resulting in a considerable waste of human efforts and research budgets. It is encouraging to see new initiatives in the proteomics and metabolomics field toward such standardization goals. The same arguments hold for multivariate statistical approaches, for which proper validation of results is often lacking, and for experimental design, for which the implications of multivariate biostatistics as final data analysis methodology are not considered.

The fervent debate on the value of "random fishing" experiments, as suggested to be typical for systems biology, in contrast to hypothesis/target-driven strategies is fading away. Given the complexity of the biology and the limitations of bioanalytical systems to provide comprehensive analysis, an approach using as much prior knowledge as possible is a necessity to make decisions on sample types and the analytical technology. However, old dogmas should not restrict an attempt to discover new things. In addition, generating high-dimensionality data, for instance by transcriptomics, often results in zooming in only on the known gene-relations as a confirmatory tool of existing hypothesis or, often even worse, reporting new observations without examining false discovery rates.[31]

Nature is a magnificent teacher of optimization strategies. As illustrated in Figure 1, a sound systems-analytical strategy is illustrated by the black skimmer. In contrast to birds like kingfishers or terns, who have a targeted final fishing strategy, the black skimmer (*Rhynchops niger*) flies just above the water surface with the lower mandible of its open bill slicing through the water. The skimmer closes its bill as soon as a fish is hit.

From a distant perspective, the skimmer's strategy appears to be a random fishing experiment. However, studying the behavior more closely reveals that the "random fishing" part is either imbedded in a strategy based on prior knowledge of the best fishing grounds or is instantaneously optimized when successes by other birds such as terns is observed nearby. Conversely, the fishing behaviors of kingfishers and terns look very targeted, but they either select strategic positions and wait to see what passes or hover around to find positions to initiate the final targeted stage of fishing. In other words, an effective strategy combines a certain amount of discovery/exploratory behavior with prior knowledge on the ecosystem. The task of setting up systems-based experiments should follow a similar strategy and focus on increasing the chance of making new discoveries using prior knowledge without limiting new discoveries by following old paradigms. Optimal search strategies for nonreplenishable/nonrevisitable targets at unknown positions have been described[33,34] from an operations-research perspective. Such strategies range from those used to search for submarines, to Levy flights with fractal patterns, as those used by albatrosses searching for food. Although the boundaries for systems biology experiments are clearly different, in any probability-based research problem, adding information provides important values for conditional probabilities, emphasizing the detrimental impact of adding wrong information.

Systems-wide bioanalysis using mass spectrometric technologies combined with pattern recognition techniques has a considerable history. The system-characterization concept using pyrolysis−mass spectrometry (PyMS) and pattern recognition (PARC) proved to be powerful in the seventies,[35] but was limited to nonvolatile components (DNA, biopolymers such as carbohydrates, and proteins) and also by the pyrolysis process generating complex pyrograms. Direct chemical ionization,[36−38] in a special setup using temperature programming, combined with PARC was capable of capturing both

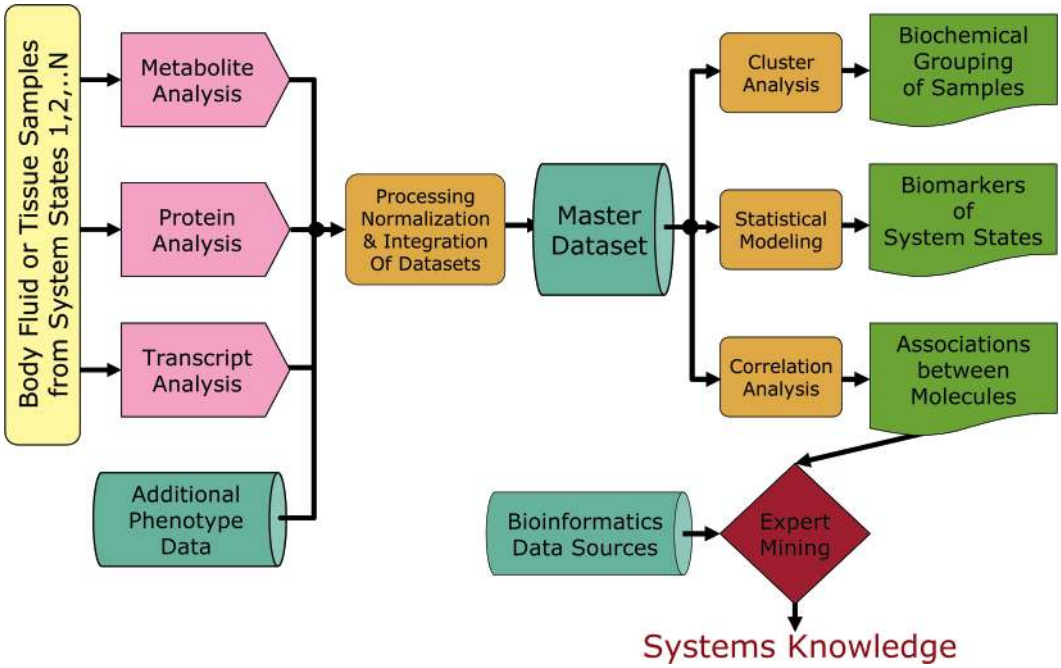


Figure 2. Systems biology workflow. Data are produced by different platforms (transcriptomics, proteomics, and metabolomics) followed by integration into a master data set. Different biostatistical strategies are pursued: clustering, modeling, and correlation analysis. Integration with extensive bioinformatics tools and expert biological knowledge is key to the creation of meaningful knowledge.

volatile and nonvolatile parts of systems as demonstrated by the molecular characterization of entire bacteria. The resulting information contained metabolite and various biopolymer levels. In fact, this approach was in concept similar to system biology today; however, the technology did not allow a high resolution at the protein/biopolymer level, making interpretation difficult, but its systems fingerprints were highly significant. In the past decade, the novel, "-Omics" technologies have opened-up a window to investigate unexplored biology.

In this paper, we describe the current status of a fully integrated and fully operational systems biology production platform. This platform has resulted from a 6-year effort which has incorporated multiple expertises over a wide scientific domain. Several improvements to the platform have been implemented based on practical experience during this period with internal research projects and pharmaceutical company collaborations involving up to hundreds of samples per project.

Some examples are presented to highlight the application of this platform to pharmaceutical research and development.

A Systems Biology Technology Platform

1. Systems Biology Workflow. The challenge of building a comprehensive systems biology platform is substantial as such a platform should address different sample types, especially blood (plasma, serum), cerebrospinal fluid, urine, and various tissues or organs. The platform should also be able to prepare and profile more unique samples, such as bile acid, saliva, synovial fluid, pleural fluid, pericardial fluid, peritoneal fluid, sweat, feces, nasal fluid, ocular fluid, intracellular fluid, intercellular fluid, lymph urine, liver cells, epithelial cells, endothelial cells, kidney cells, prostate cells, blood cells, lung cells, brain cells, adipose cells, tumor cells, and mammary cells. In addition, the platform must be capable of handling various sample numbers, amounts, and spatial- or time-resolved sampling.

Analytical sciences play a major role in the development of a standard routine and reliable platform, as will be outlined in separate sections below for proteomics and metabolomics. High-quality data generation, as well as the normalization and integration of data from different sources, is crucial. Furthermore, in addition to advanced statistical capabilities, the platform must encompass visualization tools and bioinformatics approaches in order to move from data sets to information and knowledge.

The general workflow used for systems biology experiments is depicted in Figure 2.

2. Proteomics Platform. The application of proteomics within a discovery-focused systems biology workflow places significant constraints on the type of approaches that are practical to implement. The discovery-focused proteomics method must support the analysis of hundreds of primary samples. This requires reliable methodology, reproducible measurements, and relative quantification coupled with throughput matched to the complementary measurement platforms. Finally, the results must be of a form that can be integrated with data from other omic as well as non-omic measurements/observations to enable a system-wide analysis.

The primary proteomics workflow we have adopted for plasma and tissue proteomics is based on a multidimensional liquid chromatography−MS/MS analysis of peptides labeled with the four-plex iTRAQ reagent (trademark of Applied Biosystems).[39] Advantages of this approach include the parallel relative quantification of up to four samples in a single experiment, an insensitivity of quantification to changing chromatographic behavior of the sample during a long series of sample runs in a single project, and the fact that every identified peptide component is quantified at the same time. This latter attribute, since not just "differential features" are identified, allows a very detailed quality control of the workflow: efficiency and reproducibility of protein extraction from the primary sample, alkylation, digestion, and labeling.

In the iTRAQ method, enzymatic digests from protein samples are treated with the reagent (an *N*-hydroxysuccinimide

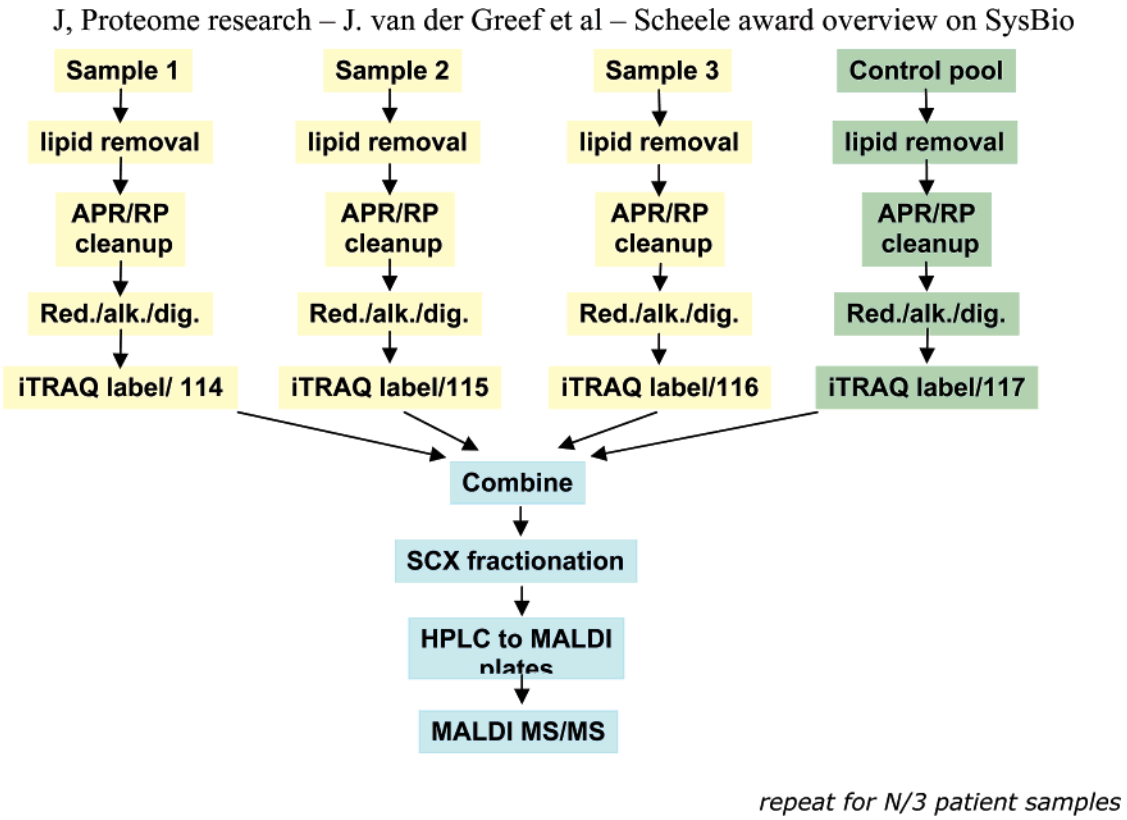


Figure 3. Multiplex plasma proteomic workflow. This workflow utilizes an isotope-coded reagent, iTRAQ, that enables the multiplexing of the workflow. Three study samples and one control sample are analyzed in a single MALDI MS and MS/MS experiment. The samples are processed in parallel until they are labeled with the coded iTRAQ reagent. Once labeled, the samples are mixed together, separated by two dimensions of liquid chromatography, spotted onto MALDI plates, and analyzed in the MS mode for molecular weight determination and in the MS/MS mode for quantification.

[NHS] ester) that derivatizes the free primary amino groups of peptides: the N-terminus (if not blocked) and the lysine residues. Four different varieties of the reagent are characterized by having the same mass but different positioning of the stable isotope labels. Therefore, in MS mode, where the ion signal reflects the molecular weight of the peptides, all four differently labeled peptides appear as a single component. In MS/MS mode, the subtle differences in the structure of the label become visible, and whereas the peptide backbone fragments (so-called a, b, and y) are still isobaric, four different reporter fragments are generated corresponding to m/z 114, 115, 116, and 117, respectively. Relative quantification of the peptides from four different samples is accomplished through the determination of relative intensities of these reporter ions.

In the systems pathology/pharmacology process, it is important to be able to compare any pair of samples. To achieve this goal for each project, a global proteomics reference sample is created, and a processed aliquot of this sample is a member of each iTRAQ mixture (arbitrarily the 117-labeled sample). Every primary sample is quantified against this global reference sample. To maximize analytical accuracy, the global reference sample is pooled from equal aliquots of each primary sample in the study and re-aliquoted into $N/3$ tubes where N is the number of primary samples. These reference aliquots are processed along with the other members of the same iTRAQ mix. The concept is illustrated in Figure 3.

One of the major challenges for a peptide-based proteomics approach (or shotgun proteomics) is to ensure the consistency of peptide and protein sets measured from sample to sample. As illustrated in Figure 4, there is a decreasing coverage of proteins with an increasing number of measured samples. This effect is observed in all discovery proteomics efforts independent of mode of ionization. To preserve a good statistical power for systems pathology/pharmacology projects, it is imperative to work against this unfavorable trend. On-line LC−Electrospray (ESI) MS/MS is not very well suited for this task because of the limited control over a real-time precursor selection process. Off-line LC−MALDI MS/MS has proven a reliable approach to exerting control over the precursor selection for identification and quantification.

The off-line nature of the LC−MS coupling combined with MALDI−MS and MS/MS coupling allows:

- Optimization of LC parameters independent of MS and MS/MS (for instance, use fast chromatography instead of long LC gradients);
- Analysis of entire (multidimensional) LC separations in MS mode before committing to MS/MS; and
- Optimization of MS/MS precursor selection from a (multidimensional) LC−MS peak list to facilitate nonredundant MS/MS analysis and the design of multipass MS/MS experiments over the same LC separation

In the broader scheme, inclusion and exclusion lists, respectively, containing peptides that are to be systematically measured or systematically skipped in MS/MS, play a key role. The inclusion list is populated by components suitable for quantification: fully alkylated, iTRAQ-labeled peptides from relevant proteins (i.e., not contaminants like trypsin or keratins). The exclusion list contains precursors not suitable for quantifica-

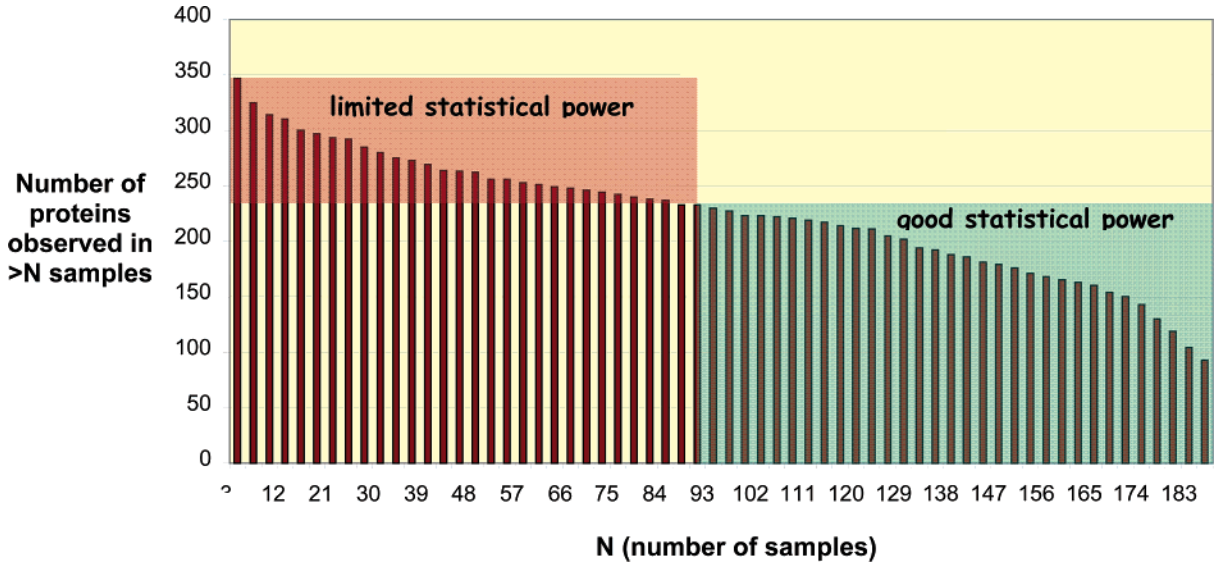


Figure 4. Experimentally observed protein coverage in a multiplex iTRAQ proteomics study. The number of observed proteins is plotted as a function of the number of samples (*N*) analyzed in the study. In this study, more than 300 proteins were detected in a single sample, approximately 230 are detected in 90 samples (approximately half of the total study size), and only 100 proteins are detected in every analysis. On the basis of the statistical power calculations, when a protein is detected in 50% or more of the samples, this will represent a good assignment in the study.

tion: incompletely labeled peptides, chymotryptic fragments, unidentified components, chemistry byproducts, and so forth. There is also a third class of precursors that has not been observed in previous iTRAQ mixes.

Implementing this approach to systems-wide proteomic projects starts with establishing a peptide catalog from a sample similar to the primary samples (extra aliquots of the global reference sample are good choice). From the analysis of this sample, initial inclusion and exclusion lists are created. The next iTRAQ mix in the analysis queue uses the initial inclusion/exclusion list and also allows the MS/MS of new (opportunistic) precursors. New identifications are sorted into the inclusion or exclusion sets, so these lists are dynamically updated before the next iTRAQ mix is analyzed. After data acquisition is completed for the last sample, a second pass MS/MS is designed to backfill precursors in iTRAQ mixes for which analysis preceded the instance the precursor was placed on the inclusion list.

In our platform, mass spectrometric data are generated on an AB4800 MALDI ToF/ToF platform. The instrument is very well suited to generate iTRAQ fragments with good ion statistics while producing high-quality backbone fragments for confident peptide identification. The data system, based on an Oracle architecture, lends itself well to integration into a pipelined environment supporting automated creation of LIMS entries, MS jobs, precursor selection, and MS/MS jobs, followed by a number of data processing steps leading to complete qualitative and quantitative results on the iTRAQ experiments.

For systems experiments it is important to have reliable processes in place to identify peptides, assign them to the correct proteins, and determine the correct relative expression value for the proteins. For identification, a three-pronged approach is used with the Mascot search engine (Matrix Science, Inc.), expert data curation, and MS/MS spectral matching procedures. The first pass is always a search with Mascot. In-house-developed acceptance criteria are used to autovalidate the matches returned by Mascot, considering ion scores, score differences, whether other peptides from the same protein were found, and whether the protein or peptide had been identified in previous experiments. To minimize or eliminate false positives and negatives, data from the entire project (many iTRAQ mixes) are utilized. First, all identifications that are sparse (as defined by statistical considerations) across the sample set are discarded. This set may contain numerous correct identifications; however, these are useless for statistical analysis. Most of the false positives are eliminated this way. False identifications consistently made throughout the project are discarded by expert curation, also relying on statistical tests of mass errors, chemical modifications, and so forth. False negatives can be rescued by MS/MS spectral matching: the best instance of the MS/MS spectrum of a peptide can be used as a template to find other measurements of the same peptide in different iTRAQ samples which may not have had the quality to pass autovalidation. Optionally, these spectra can be submitted into a second pass MS/MS job in an attempt to generate higher-quality data.

Once the peptide data set is complete for the project, an in-house tool is used to create the minimum data set of protein sequences which explains, or accounts for, all the peptides. Software packages, similar to this in-house tool, such as ProteinProphet,[40] are publicly available. An important consideration is that, based on quantitative behavior of the constituent peptides, decomposition of proteins into their differentially processed subforms might be necessary. In addition, it might be necessary to consider cases of peptides harboring polymorphic sites where the "host" protein would logically be resolved into three entities: the two polymorphic variant peptides and the rest of the protein. Other considerations to factor into the creation of the protein data set include inactive and active forms of plasma zymogens of the complement and coagulation systems.[41] With these considerations in mind, we seek to derive a minimum protein data set from the peptide data set, provided there is no quantitative evidence indicating otherwise.

Quantification of peptides is done by calculating the relative peak intensities with respect to the global reference sample (m/z 117). There are several alternative ways to roll up peptide

Table 1. QC Metrics in the iTRAQ Plasma Proteomic Workflow[a]

workflow step	QC metrics
Sample Acquisition	Incoming sample inspection with photographic capture
Lipid Removal	Protein content and recovery
Abundant Protein Removal	Protein content, column performance based on defined criteria
Digestion/iTRAQ Labeling	pH monitoring, reproducibility and efficiency of chemistry based on MS data
Peptide Separation (SCX)	Column performance based on defined criteria
LC−MS/MS Measurements	Column performance based on defined criteria, MS sensitivity
Peptide Ratios	Descriptive statistics of peptide ratios
Protein Ratios	Consistency of peptide ratios mapping to the same protein

[a] Several critical steps in the iTRAQ proteomic workflow are monitored to ensure the overall quality of the final results. There are several steps of digestions, labeling, and separation that must be repeated with a high degree of consistency to ensure good quantification and protein coverage within a study.

measurements into protein measurements, typically yielding very similar results. All distinct forms of a fully labeled peptides are used as independent estimates of the protein result, and finally, the median of all peptide estimates is accepted as the protein measurement.

2.1. Application to Plasma Proteomics. One of the key differences between the analysis of tissue and plasma samples is the enormous background represented by the abundant plasma proteins (albumin, immunoglobulins, etc.). To address this challenge, a number of depletion techniques can be used. In our platform, for human plasma samples, a chicken IgY antibody column is used to deplete the samples of 12 abundant proteins. The protein pool not retained on the antibody column is recovered on a reversed-phase column, and subsequently, this protein pool is reduced, alkylated, and digested by trypsin. The resulting peptide mixture is labeled with the iTRAQ reagent and combined with the three other samples designated for the same iTRAQ mix. The combined four-plex mixes are fractionated first on strong-cation exchange chromatography. The resulting fractions are analyzed further, after pooling some of the fractions, using HPLC-MALDI−MS/MS.

The experimental design implemented in our workflows has the following main objectives:

•The generation of a sample randomization scheme where different cohorts are unbiased in iTRAQ mixes and labels (114, 115, 116).

•The design of batches of sample preparation so each member of an iTRAQ mix is processed "same place, same time". For sustained operation, batches of 12 samples have worked well (9 primary samples and 3 reference samples).

•The optimization of separation and mass spectrometry strategies so that each single iTRAQ mix (2D LC−MS/MS experiment) can be run in 24 h or less on a sustained basis.

•The ability to follow data quality (QC reporting) as closely as possible to ensure that mass spectrometry results can be effectively fed back to the earlier stages in the workflow, and sample loss due to problems in sample preparation can be minimized.

The initial step in the design of the bioanalytical strategy is the sorting and distribution of the primary samples into four-plex iTRAQ mixes. As mentioned above, to compare the results derived from any of the primary samples, a common reference sample is generated and included in each iTRAQ mix. This sample is referred to as QCR and, ideally, is composed of a pool of material from the study samples. A secondary choice is a pool of samples or a single sample from the study, but not involved in the analysis, and a third choice is a pool of samples or a single sample of the same type obtained from another source/study. The result of this approach is that each iTRAQ mix contains three primary samples and one QCR sample. In our workflow, we use the iTRAQ labels with the 114−116 reporter fragments for the primary samples, and the 117 label is reserved for the QCR sample. The randomization of the primary samples ensures that in the individual iTRAQ mixes different sample cohorts are distributed in an unbiased way. Since each mix has one reference sample in it, this level of randomization is adequate.

Considering the large number of samples and length of time involved in the data generation phase of this discovery-focused proteomics workflow, it is critical to continuously monitor the performance of the overall process. Some of the QC steps focus on determining that the products of one step in the process are of sufficient quality to warrant advancing them to the next stage of processing; other QC steps are applied after the completion of the sample-analysis phase to assess the quality of the overall process. Several of the steps and variables that are monitored in these steps are listed in Table 1.

An example of one of the key steps that is monitored during the workflow is the extent of underacylation in the iTRAQ labeling step (Figure 5). The objective of this analysis is to ensure that incomplete labeling is limited to less than 10%. In the example shown in Figure 5, there are a total of 87 four-plex iTRAQ mixes, and a significant deviation in the labeling efficiency was observed at mix S46. This change in labeling resulted from cross-contamination of the sample by the glycine buffer used for the column elution step and led to a failed iTRAQ labeling. The samples that showed this characteristic were submitted to reanalysis.

Quantitative performance is assessed through the consistency of the ratios of peptides matching to the same protein. The relative standard deviations of these peptide measurements indicate the accuracy of the protein measurement (as an average of the matching peptide measurements). Medians of these relative standard deviations for each sample are plotted in Figure 6. During our studies, if a sample exhibits a high degree of quantification error, it is subjected to reanalysis. In the study highlighted in this figure, the primary source of problems is protein reduction. This level of QC enables us to update the workflow and improve the overall consistency of the results.

Table 2 summarizes the proteomic analysis results for a recent project that involved 189 primary samples and illustrates the extent of coverage of plasma proteins, as well as presents the coefficient of variation of quantification for this project.

3. Metabolomics Platform. The symbiosis and integration of chemometrics and metabolomics has been a crucial step and described from a historical perspective.[42] Comprehensive analysis of the metabolome requires analytical instruments and techniques that offer high sensitivity, resolving power, and dynamic range. Mass spectrometry is the technique of choice

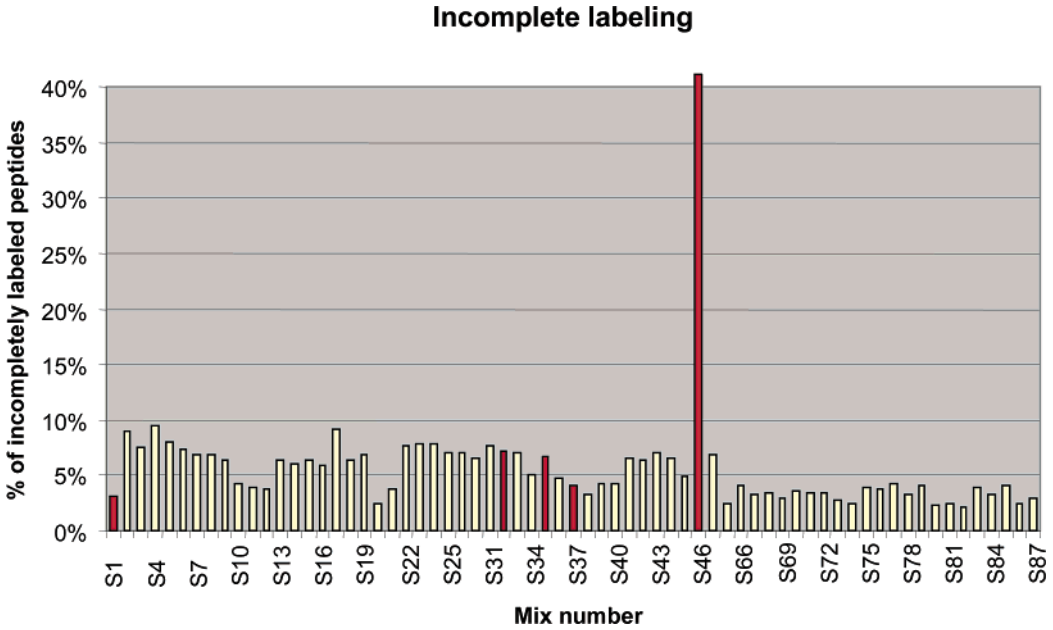


Figure 5. Percentage of incomplete iTRAQ labeling throughout a project. Red bars represent iTRAQ mixes where one or more member samples were re-run in the later phase of the project.

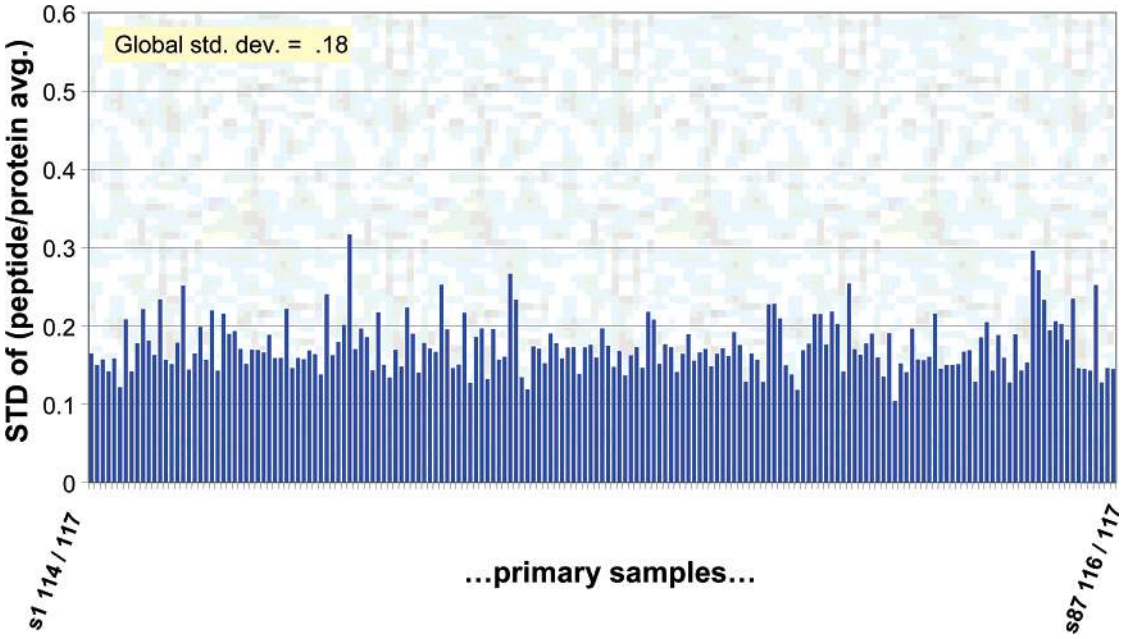


Figure 6. Distribution of "quantification errors" across the study samples. Samples where quantification failed for the first time have been replaced with their re-runs. The median of these error estimates is 0.16.

in most cases, as it combines all of these aspects. Resolution improves dramatically by using high-resolution systems based on ToF, but especially Fourier Transform (FT-ICR and Orbitrap) technology. The combination of mass spectrometry with separation methods, such as various modes of Liquid Chromatography, Gas Chromatography, and Capillary Electrophoresis, strengthens the dominant role of mass spectrometry in metabolomics as "hyphenation" (a colloquial term for linking of the separation method with the mass spectrometry method, e.g., LC−MS) increases the resolving power and dynamic range, and, in the correct setup, also increases sensitivity. Sensitivity is extremely important for two reasons: (1) for including low-abundance metabolites in a metabolic profile and (2) for working with limited sample volumes. Compared with NMR, hyphenated MS methods have, beyond their excellent coverage,

Table 2. Summary of Key Performance Metrics for a 189 Sample iTRAQ Plasma Proteomic Study[a]

Number of primary samples	189
Number of iTRAQ mixes	64
Total number of unique peptides	4050
Total number of protein classes	201
Total number of protein exemplars	241
Total number of protein nodes	347
Median CV peptide/protein ratio	16%

[a] A total of 347 protein nodes were assigned with a median ratio of 16%.

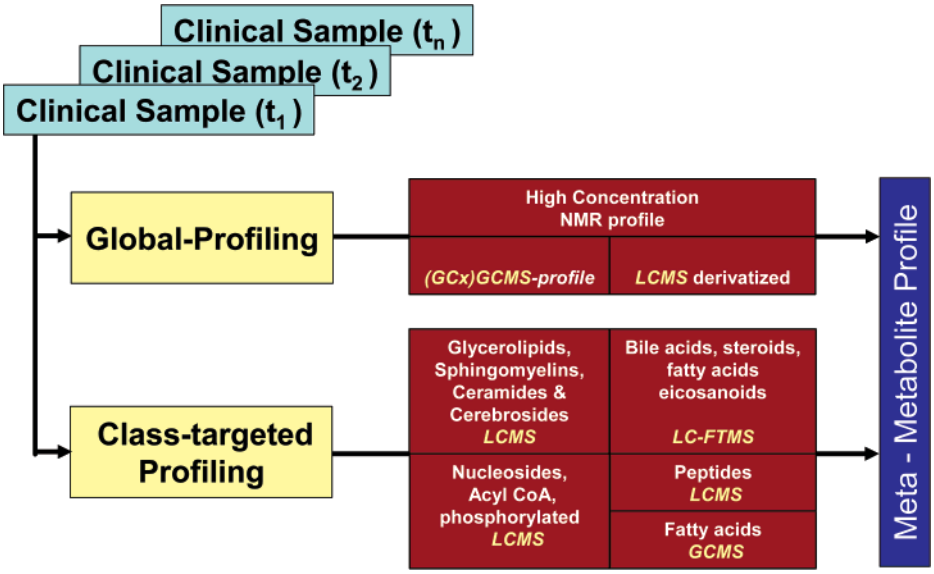


Figure 7. Schematic overview of a mixed global and targeted metabolomics platform using NMR, LC−MS, and GC−MS for comprehensive metabolic profiling.

two main challenges: (1) hyphenated MS does not provide universal detection and (2) quantification is not very straightforward.

3.1. Comprehensiveness. With regard to universal detection, any combination of separation conditions and ionization methods limits the number of metabolites detected and results only in submetabolome information. The chemical diversity of the total metabolome exceeds the span of any LC−MS or GC−MS method. This limitation is mainly caused by the separation method. Therefore, it is mandatory to combine a few diverse methods, each addressing a submetabolome, into an overall metabolomics platform to increase coverage.

Figure 7 illustrates our current metabolomics platform consisting of global profiling methods based on NMR, LC−MS, GC−MS, and a collection of class-targeted LC−MS and GC−MS methods including two-dimensional separation approaches (GC×GC). A number of these methods have been published previously[43−45] as well as the concept and performance of an earlier version of a metabolomics platform.[28]

A majority of these methods are being applied to metabolic profiling of the biological samples mentioned previously, for example, plasma, serum, urine, Cerebrospinal fluid (CSF), synovial fluid, and tissue homogenates. The selection of methods to apply is driven by prior knowledge and sample volume, time schedule, and budget constraints. The measurement by GC−MS global profile, a LC−MS global profile, lipid LC−MS, and bile acid LC−MS requires 100 μL of plasma (30, 10, 10, and 50 μL, respectively). This set of methods covers a wide range of metabolites, including organic acids, amino acids, many sugars, various lipid classes, fatty acids, bile acids, and steroids, and thus provides a very rich metabolic profile. The total number of distinct metabolites (not features) observed with this "standard" platform is in the order of 600−800. This broad-spectrum strategy is chosen to measure across many different classes at different concentrations versus in-depth analysis of a selected region of the metabolome. A focus on a selected region of the metabolome can be achieved by including more targeted approaches in the platform. This broad-spectrum strategy has advantages over straightforward profiling by NMR of a 100 μL of plasma sample, but in practice, NMR profiling (Carr−Purcell−Meiboom−Gill (CPMG) and diffusion edited (DE)) is typically used as well because it provides both complementary and confirmatory information. Total analysis time for this strategic approach is slightly longer than for NMR, mainly because GC−MS is relatively slow. LC−MS methods are performed on parallel instruments to reduce total analysis time, and currently, methods are transferred to Ultra Performance Liquid Chromatography (UPLC) technology to increase the LC−MS throughput.

Important aspects, which are crucial for any project, are a proper experimental design and data preprocessing; these aspects have been discussed in detail elsewhere and are therefore not addressed in this review.[46−49]

3.2. Quantitative Aspects of LC−MS and GC−MS. The major challenge of LC−MS and GC−MS in a comprehensive profiling setting is the achievement of quantitative performance. This in sharp contrast to target compound analysis, where the use of calibration standards and (stable-isotope-labeled) internal standards results in excellent precision and accuracy. Comprehensive profiling is, in essence, nothing more than recording the response of, often unidentified, substances in a set of samples. The response is a measure of concentration. However, the response is not only determined by the actual metabolite concentration, but also determined by the following factors:

• **Experimental Conditions, Mainly Instrument Related:** Intrabatch drifting and batch-to-batch differences in response are quite common in LC−MS and GC−MS and are caused by a variety of factors, including ion source contamination, GC injection device contamination, instrument maintenance between batches, and the use of multiple instruments in parallel with a set of samples. These factors lead to poor repeatability and reproducibility and have a major negative impact on data quality.

• **Chemical Structure:** Different compounds have different response characteristics, and especially in LC−MS, a minor difference in structure may have a considerable effect on the response, for example, factors of 2 or 3 orders of magnitude are not uncommon. This makes it impossible to quantify the metabolite in concentration units, which would require the

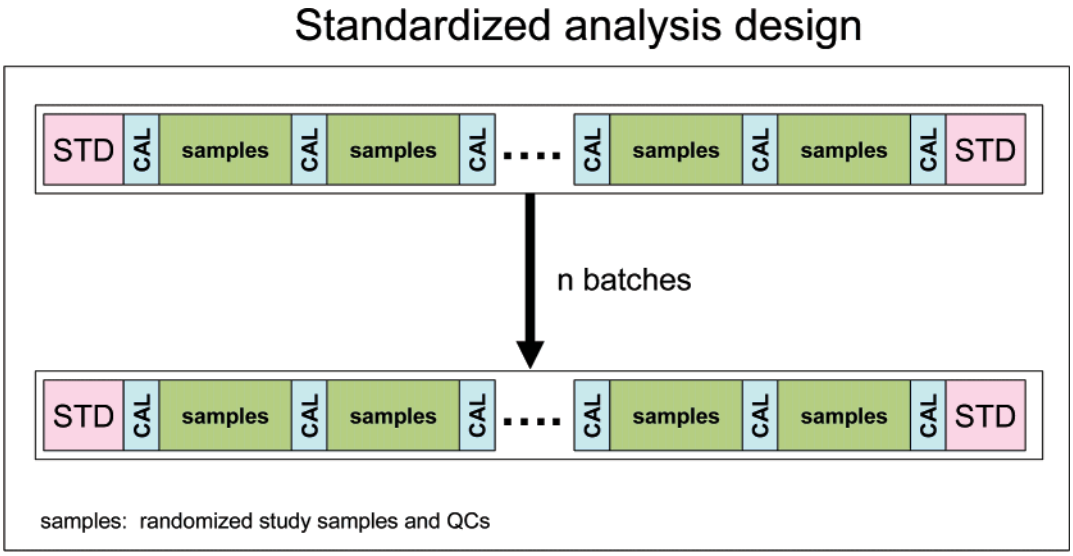


Figure 8. Standardized analysis design for metabolomics involving the analysis of calibration standards (STD) for quantification of specific metabolites, biological calibration samples (CAL) for correcting intrabatch temporal trends and interbatch differences, and randomized study samples. Mixed with the study sample are a number of independent quality control samples.

analysis of calibration standards. From a biomarker discovery perspective, this is not a real problem.

• **Sample Composition:** Large differences in sample composition influence the response of one or more metabolites, for example, ion suppression effects in ESI and impaired chromatographic performance in GC−MS (also leads to incorrect response). Variations in the concentration of high-abundance substances, such as glucose and urea, influence the responses of other compounds. For plasma and various tissues, this is typically not a major problem because the sample composition with respect to these highly abundant compounds is quite constant across subjects.

The magnitude of the first problem and its impact on data quality is proportional to the scale of a study. Small-scale studies, which employ only a single batch of a relatively small number of samples, only suffer from drift. If drift is small compared with random variation, it is often not even necessary to consider drift as an important negative factor for data quality. Large-scale studies, in which the number of samples exceeds the batch size limit, typically suffer from systematic differences between multiple consecutive batches. Achieving good reproducibility is even more challenging if:

• large time intervals (e.g., months to years in long-term studies) occur between the analyses of different batches of samples from the same study sample set;

• parallel instruments of the same type are used even in the same laboratory;

• instruments of the same type are employed in distinct laboratories;

• different instrument types are used; and

• all combinations of the above.

Metabolomics is rapidly engaging the challenges presented by large-scale applications,[46] but it only does so if platform reliability, encompassing repeatability and especially reproducibility, is excellent. Such reliability can be achieved in 2 ways. The first is to be fully in control of all the experimental conditions, but this is very difficult to achieve (if not impossible). The second way is calibration. It is important to point out that 'calibration', in the context of our platform, only refers to quantitative scaling of response and not quantification in SI units (but this is in fact only a very small step for identified compounds).

There are a number of ways to calibrate metabolomics data, but discussing all of them is beyond the scope of this paper. We have developed a calibration procedure for metabolomics data sets, similar in principle to the QCR strategy mentioned before for proteomics data sets, based on repeated analysis of a biological calibration sample (not a standard). The calibration sample(s) is(are) 'identical' to the real samples, either made by pooling (part of the) samples, or obtained from other sources. As mentioned above, the first type of calibration samples is preferred, but is sometimes not a viable option because of sample volume limitations.

These calibration samples have a number of favorable characteristics.

• Their biochemical diversity is very similar to the real samples.

• The metabolites are present at relevant concentrations.

• The calibration samples are very simple to make or obtain.

The calibration method requires a standardized sequence and batching design which is shown in Figure 8.

We employ this approach for all metabolomics methods, LC−MS (multiple methods), GC−MS, and also NMR. The number of CAL samples per analysis batch and their location in a measurement series is determined by the method performance for all metabolites. Critical aspects of the sample analyses that can influence this approach are drifting and precision. When more drifting is observed, more CAL samples are required to model and correct for it. The better the precision, the lower the number of CAL samples required. This standardization does not compromise optimal sample randomization schemes; that is, full flexibility in randomization is maintained (actually improved).

In summary, the calibration methods consist of the following 4 steps:

1. Selection of the best internal standard for all metabolites (statistical evaluation of CAL sample data and study sample replicates). This results in metabolite−IS pairs matching in physicochemical properties.

2. Scaling of all metabolites with the optimal internal standard.

3. Determination of the temporal trends in CAL sample data per analysis batch and the correction of these trends (curve fitting).

4. Removal of systematic batch-to-batch differences.

Figure 9 demonstrates the effects of this calibration procedure. The data were obtained with the derivatized LC−MS global method. Samples were derivatized in 2 sessions (lots), and the samples of each derivatization lot were measured in 4 analysis batches (LC−MS, in total 8 batches). The figure shows the PCA score plots of (a) the raw data, (b) the data after scaling each metabolite with its optimal internal standard, and (c) after the full procedure (optimal IS scaling, within batch trend removal and between batch offset removal). This example demonstrates that the calibration method effectively removes systematic differences in response due to variation of extraction/derivatization conditions and LC−MS instrumentation conditions, and results in very high quality data. A second example in Figure 10 demonstrates the effectiveness of this procedure by evaluating the correlation between plasma glucose levels determined with a validated reference method and plasma glucose levels determined with GC−MS.

The GC−MS data was obtained by the duplicate analysis of 366 human plasma samples in 17 batches. The calibration method results in a very good correlation between the 2 methods. Similar effects and performance were obtained for other metabolites.

The calibration method can also be applied to calibrate data obtained from 2 or more parallel instruments and data obtained with large time intervals between analyses. The latter can be achieved by storing ample CAL samples in a −70 °C freezer for future use (not shown here). Storage of the CAL samples also opens up the possibility of doing absolute quantification in concentration units by the standard addition method to determine the concentration of a particular metabolite in the CAL sample. With this concentration in hand, it is fairly simple to obtain the concentration of the metabolite in the project samples by single point calibration. This can only be done if the identity of the metabolite is known and if it is available as reference material.

In summary, the LC−MS and GC−MS bioanalysis methods of our platform generate very rich metabolomic data and can do so at a throughput very similar to NMR. The use of the calibration method described here results in reliable and reproducible quantitative data. The need for standardization, on an international level, for experimentation, validation, and reporting of metabolomics data[57] is recognized. Fortunately, initiatives are underway to achieve such standardization; otherwise, data generated from different sources will have little universal significance and will contribute little to our overall understanding of complex biological systems.

4. BioSystematics. Among the challenges inherent in data analysis and interpretation for systems biology in medical applications are the heterogeneity and volume of data which are generated by modern high-throughput methods in transcriptomics, proteomics and metabolomics, including those methods presented in the preceding sections. Such data present challenges from at least three perspectives: (i) statistical analyses, (ii) data integration, and (iii) bioinformatics.

From a statistical analysis perspective, the primary challenge is that the total number of measured analytes, N_a, is typically much larger than the number of available distinct samples, N_s. This regime of $N_a \gg N_s$ is an opposite extreme to that confronting statisticians analyzing results from, for example, large clinical trials in which a single or a few endpoints are assessed across hundreds or thousands of subjects. In typical systems biology data sets where $N_a \gg N_s$, the meaning of statistical significance as embodied in the alpha level or p-value of a result must be carefully considered, due to the issue of multiple hypothesis testing.[31] A given alpha value, while appropriate for comparisons of individual analyte levels, is not appropriate for the set of all N_a concurrent statistical comparisons in a systems biology analysis; at an alpha value of 5%, for example, the number of spurious, false positives in a systems biology data set consisting of 10 000 variables would be 500, which is often too large to be acceptable. To avoid a large number of false positives, approaches to adjusting alpha and p-values to account for the number of comparisons being performed have been developed. These include the classical Bonferroni correction, which is the most conservative, to the more recently developed False Discovery Rate[31] and q-value[50] methods.

In addition to the statistical false positive challenge, working in a regime of $N_a \gg N_s$ presents a number of difficulties and pitfalls in exploring the data for patterns and classifiers, such as would be done for a biomarker analysis. Among the most difficult challenges is avoidance of 'overfitting' in statistical models developed for analyzing multidimensional systems biology data sets. Overfitting can occur when, because the number of variables is much larger than the number of samples, it becomes trivial to separate two groups by any number of combinations of multivariate analyses. Ideally, to avoid overfitting, one would have at least two independent groups of samples to work with, and would use a separate one for fitting the statistical model and a distinct set(s) for testing the specificity and sensitivity of the result. In lieu of multiple, independent sample sets, methods such as cross-validation and bootstrapping can be used, but must be use with a high degree of care in order not to overestimate the performance of a statistical model in biomarker classification applications.[51]

The integration of data from large, multidimensional systems biology data sets also presents the challenge of data heterogeneity. For example, data sets from metabolomics, proteomics, and transcriptomics need to be standardized and combined for subsequent analyses. A particularly interesting aspect of these bioanalytical platforms is that the number of analytes profiled by each will not necessarily be similar; for example, while a transcript hybridization microarray may measure 10 000 or more gene transcripts, a mass spectrometry proteomic or metabolomic platform may profile a few hundred endogenous proteins or metabolites. Such asymmetry among platform yields not only presents statistical issues which need to be properly addressed, but also requires different approaches to deal with varying coefficients of variation, sources of variance, and other platform-specific parameters. The latter aspects of integrating disparate data sets are particularly important when using data from multiple platforms to construct statistical models for biomarker discovery and validation, in that high variability within one platform data set may unduly penalize all analytes within that platform at the expense of other data. An especially attractive approach to data integration is the consideration of correlation analyses, which will be discussed below.

Bioinformatics, in the context of systems biology, aims to interpret the large, disparate data sets and ultimately to provide

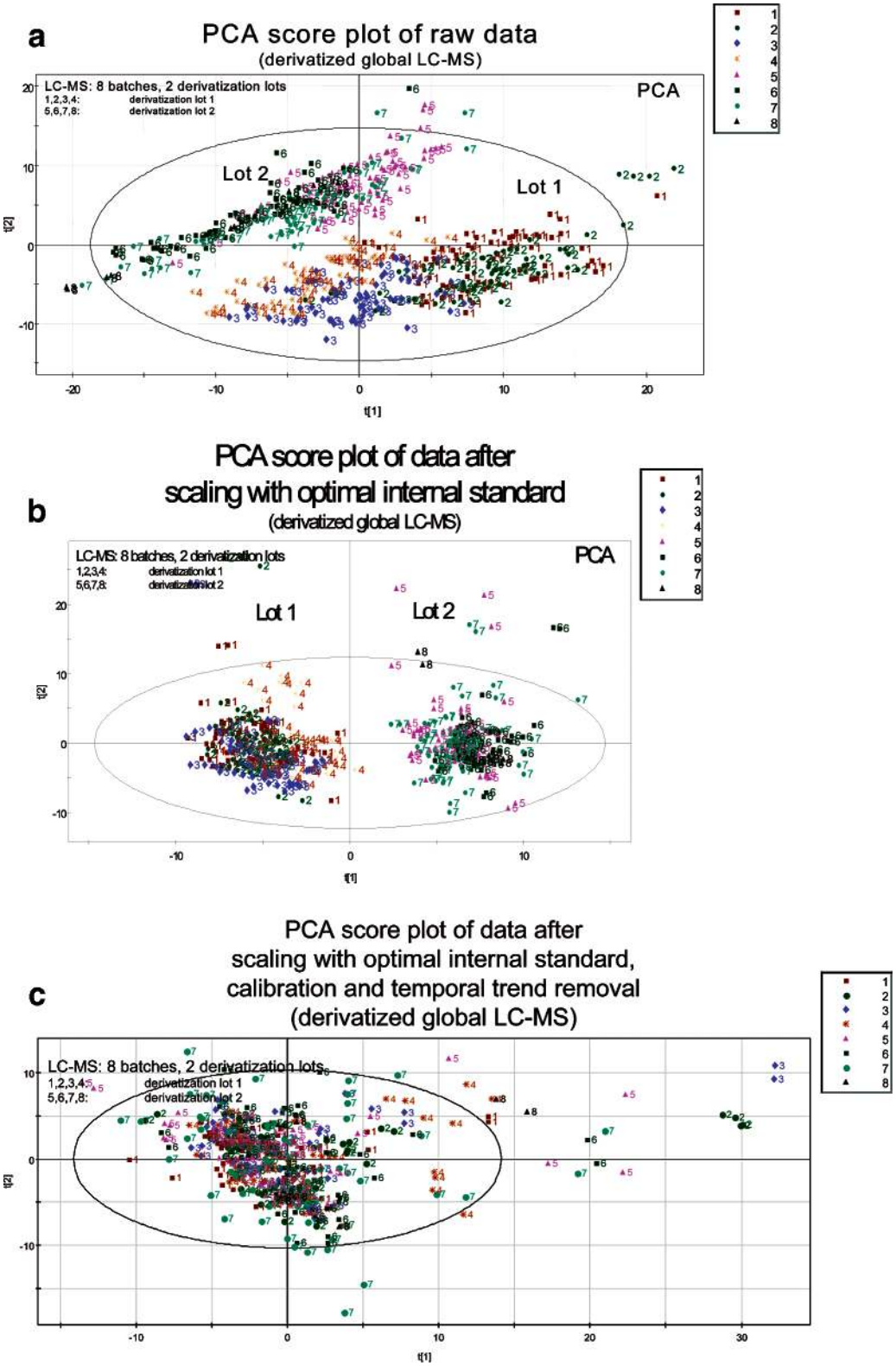


Figure 9. PCA score plots of LC−MS global data obtained by analysis of human plasma samples in 2 extraction/derivatization lots and 8 analysis batches: (a) raw data indicating systematic differences between derivatization lots, analysis batches, and also within batch drifting, (b) data after scaling each metabolite with the best matching internal standard, which removes some of the within and between batch differences, and (c) after correction of temporal trends and calibration which effectively removes all systematic error from the data, including major difference between the two derivatization lots.

insight into biological mechanisms which underpin the experimental observations. A classical bioinformatics approach is to begin by mapping experimental results onto *a priori* 'known' biochemical pathways, as curated by previous research and literature. A complementary activity is to analyze results at a more granular level, namely, at the level of biochemical

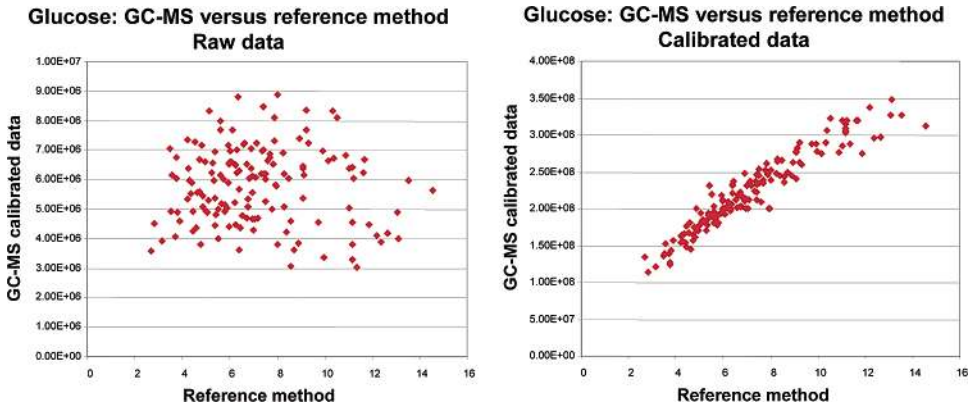


Figure 10. Correlation between glucose concentration obtained with GC−MS (arbitrary units) and a reference method. Data was obtained in 17 analysis batches, and the raw GC−MS data shows no correlation with the reference method as result of within and between batch differences. After calibration, a near perfect correlation between the two methods is obtained.

reactions, and to attempt to fit the observations into known reaction models. Yet a third approach is to process the data in an empirical, statistical manner and generate de novo hypotheses about the mechanistic origins of the observations. In general, all of these methods are applied by the practitioner and are relevant to our platform. The challenge of incomplete or inaccurate biochemical pathway data is compounded by the fact that much curation and literature is oriented primarily to genes and proteins, and databases for the roles of endogenous metabolites remain relatively scarce, particularly at the highly detailed level of metabolite molecular structure which methods such as mass spectrometry or NMR afford. Another challenge which bioinformatics faces is that system biology experiments often profile molecules which may have dramatically different temporal and spatial characteristics. For example, the time scale for the synthesis and degradation of proteins can be much different from the time scale for gene transcription or for metabolite synthesis, and these processes further have different spatial subcellular locations. The dynamics of biomolecular phenomena, and their localization, are contexts which are often difficult to discern in high-throughout profiling experiments, without a substantial effort in, for example, subcellular fractionation or temporally dense sample collection. Nevertheless, data obtained from systems biology studies offer a rich picture reflecting the underlying metabolism of the cell and the organism and is immensely valuable in understanding, at an unprecedented level of biomolecular specificity and coverage, the system under study.

A number of research groups, including our own, have found that the computation of correlations between pairs of analytes and the construction of correlation networks are useful approaches to examine the behavior of molecular systems.[52−55] Such an approach generates graphs that represent the associations between molecules without preconception of their involvement in biochemical pathways.[52−54] Figure 11 shows a typical example of a graphical representation of a correlation network. As illustrated in this case, the correlation network is displayed as a graphical representation of sets of pairwise mathematical correlations between intensity values of measured features. Measured features are represented by 'nodes', and correlations between pairs of analytes are represented by links, or 'edges', which connect the corresponding nodes. Graph edges represent the pairwise relationships between nodes. Each node is assigned a coordinate in the two-dimensional plane, such that the pairwise distances approximately reflect the similarity given by the correlation matrix; an edge is drawn between two nodes if their correlation exceeds a given quantitative threshold. Correlations can be derived for pairs of features measured either within or across tissues or biological compartments. Correlation networks in a systems biology setting can be very large, for instance, for ([20 000 genes + 1000 proteins + 500 metabolites] × 3 tissues × 3 methods × 3 states)$^2 \approx 3.37 \times 10^{11}$ correlations. Furthermore, calculations of correlation values for any two-analyte pair can be undesirably and trivially influenced by one or a few samples which have anomalous measurement values for one or both members of the analyte pair that are different from the rest of the measurements. To minimize such occurrences, each correlation calculation can be evaluated by a jack-knifing, cross-validation routine. Such a process is useful in identifying, for example, levels of correlation which are spuriously high because of a measurement error or the like. Interpretation of correlation networks needs to be undertaken with care[52,53] but provides a straightforward tool that can substantially enable disease diagnostic and intervention studies as outlined in the examples below.

Practical Illustrations of the Performance of the Systems Biology Platform

1. Systems Pathology. Systems pathology is based on biomarker pattern discovery, which in recent years has become an important focus within the pharmaceutical industry. It is recognized that biomarker research is complex and that validation of initial findings is a tedious process and needs a high-level of design of clinical trials. Typically, biomarker research is performed on readily available samples from clinical trials which are not designed for biomarker research in terms of available sample types, longitudinal sampling, or number of patients. Often, a single biochemical level is selected for biomarker discovery, typically proteomics or metabolomics, as blood (plasma, serum) or urine are most frequently available. Finding biomarkers is only the first step; understanding them is a major task. Moving from data via information toward system knowledge is typically only achieved by integration of different biological levels. Also, biomarker patterns describing disease pathology can often be more effective if data sets from proteomics and metabolomics are combined (see later). Of course, depending on a project's objective, the selection criteria can be optimized. An example is shown in Figure 12; in a small clinical study, the optimal combination of proteins and me-

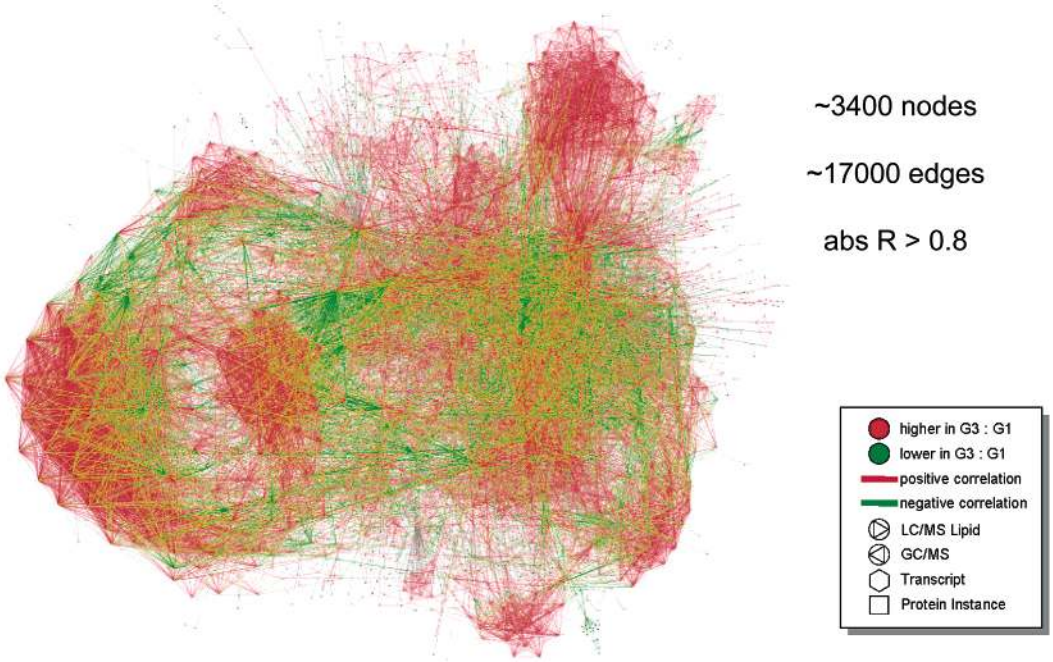


Figure 11. A correlation network created from an integrated tissue and body fluid data set comprising approximately 3400 analyte measurements (the nodes of the graph) and nearly 17 000 empirical correlations measured among these analyte measurements (solid lines). Analytes include proteins, endogenous metabolites, and gene transcripts. Clear, large-scale structure is evident in this two-dimensional network rendition, reflecting the underlying metabolic processes in the tissue and body fluid samples, and striking in its revelation of groups of analytes which exhibit high degree of correlation, and which are as such likely involved in distinct biochemical processes.

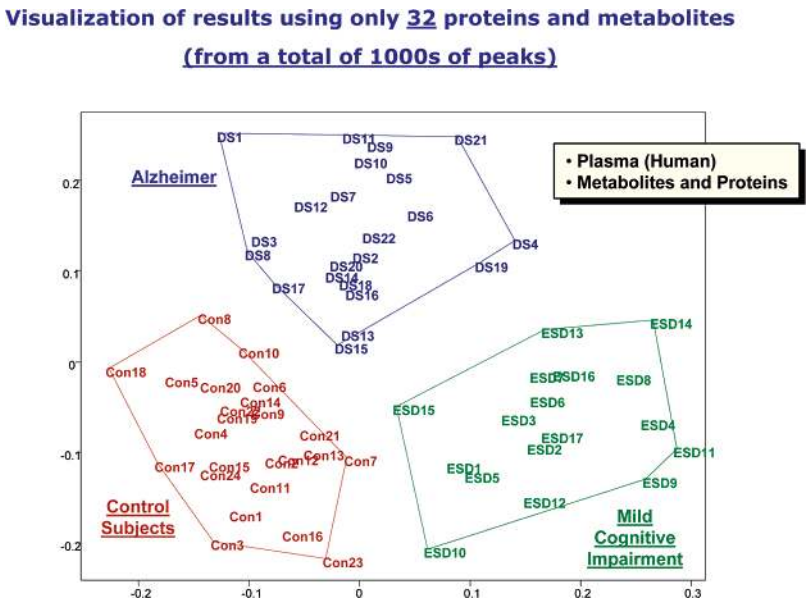
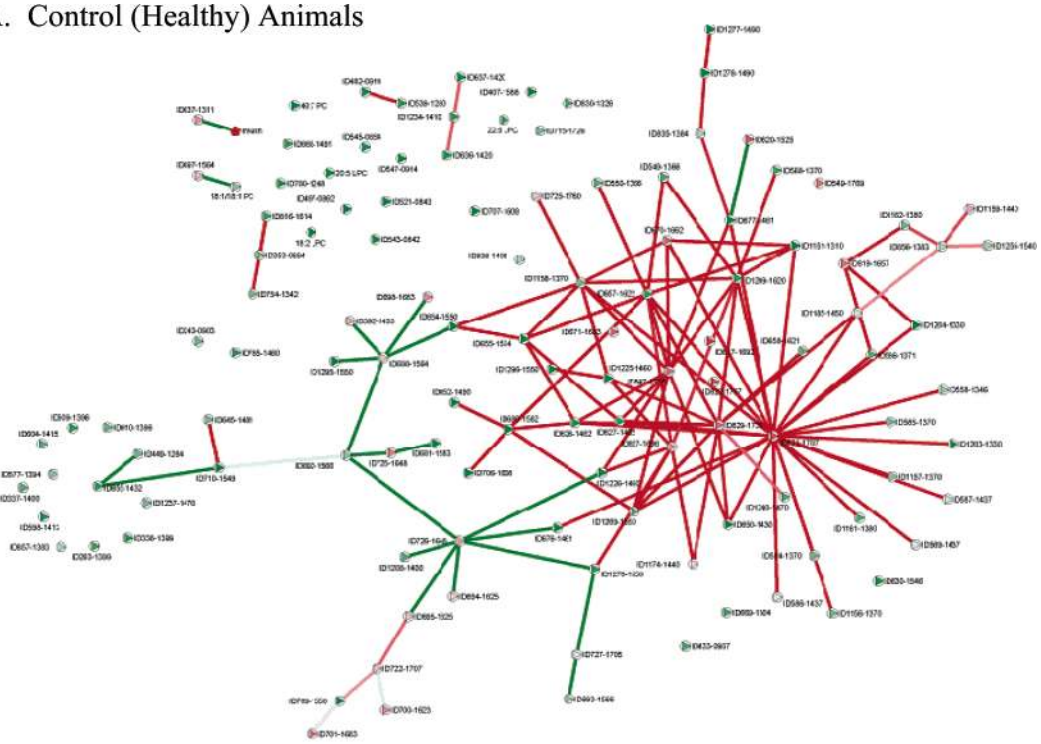


Figure 12. Principal components-discriminant analysis of plasma metabolomics and proteomics data from an Alzheimer's disease study. The abscissa represents the first principal component, and the ordinate represents the second principal component. Each annotated data point in this two-dimensional projection represents an individual human subject from the study. Three major groupings are seen to emerge from this multivariate projection, corresponding to physician-diagnosed controls, Alzheimer's disease patients, and patients diagnosed with mild cognitive impairment. Solid lines circumscribing groups are for visual aid only.

tabolites was derived using our platform from plasma samples to obtain a separation of 3 classes: control, mild cognitive impairment (MCI), and Alzheimer (AD). In fact, a set of 32 proteins and metabolites was found to be the optimal classifier. Further analyses, using correlation networks combined with the objective of a given project, are the next approach to reducing the number of components of a biomarker to the most efficient and practical number. In different development stages of a disease, different markers appear, some of which can be very indirect markers, and without understanding the role in the whole system, the value of such markers is hard to predict.

2. Systems Pharmacology. In systems pharmacology, responses are measured after perturbation with drugs, drug

A. Control (Healthy) Animals



B. Diseased Animals

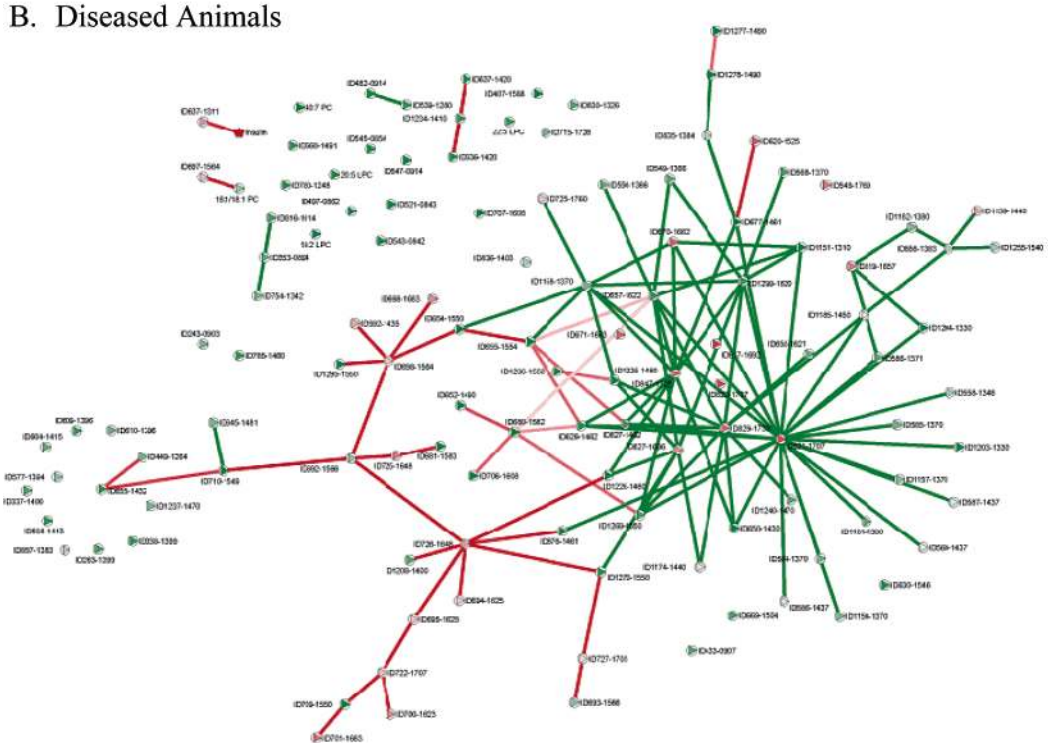


Figure 13. (Continued)

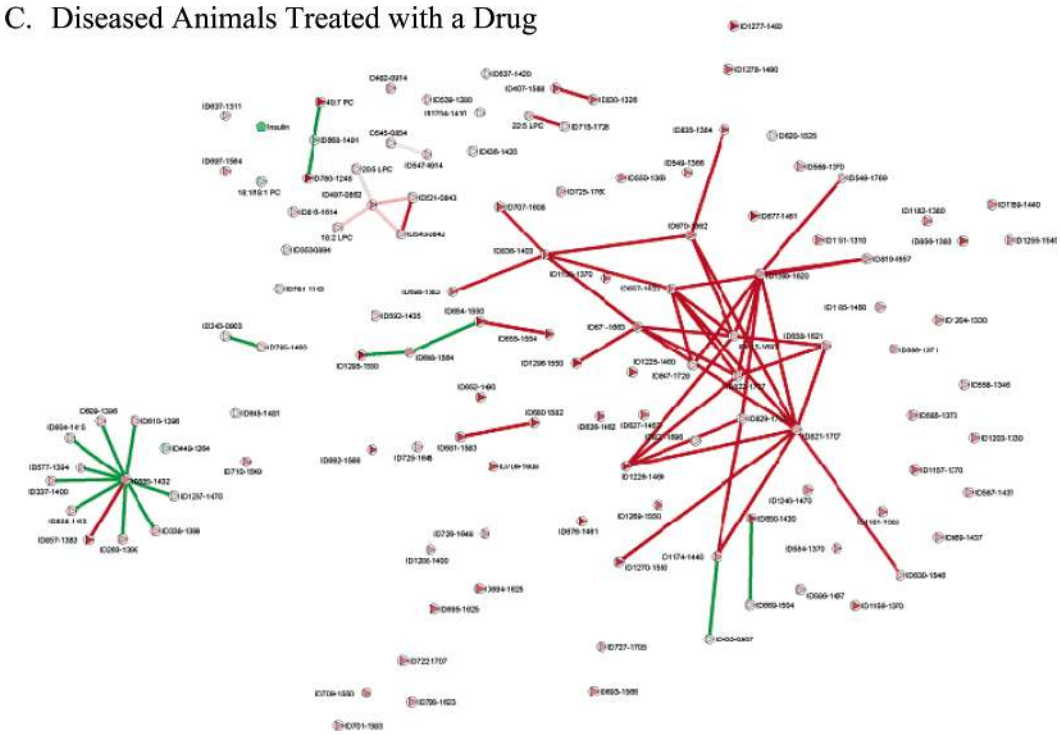


Figure 13. Correlation networks including multiple analytes in three distinct groups of animals: (A) control (healthy) phenotype, (B) disease phenotype, and (C) disease phenotype treated with a therapeutic compound (bottom). Each node in the graphs represents an LC−MS analyte as measured in post-necropsy adipose tissue. Red lines denote statistically significant positive pairwise correlations between analytes, and green lines denote statistically significant negative (inverse) pairwise correlations between analytes. The topological layout of the graphs is determined by an algorithm and is the same in the three panels; distance between analytes contains no information.

combinations, nutraceuticals, natural products, or extracts thereof. Although various other forms of systems response profiling can be used, in our platform, correlation networks function as the preferred readout to understand multitarget and network effects as well as to study spatial/temporal effects. In this way, drug−target effect monitoring is replaced by drug−system effect monitoring. Correlation networks can also serve as a tool for optimizing new generations of drugs, selecting the best in class, or generating new combinatorial options in drug rescue programs. Such a scheme has been described previously[54] and includes translation and reversed-translation between the preclinical and clinical phase of research based on systems comparisons. This scheme allows the selection of the best animal model and/or to determine which part of the animal disease correlation network best represents the human situation.

Understanding disease and drug action, using such a correlation networks strategy, is illustrated in Figure 13. The three boxes illustrate correlation networks for the control "healthy" animals, the "metabolic disorder" disease model animals, and the drug response in the disease model animals. In this example, adipose tissue was selected, and correlation networks between various molecules profiled with our systems biology platform are shown. A comparison of the network for the control animals with that for the disease model animals reveals that certain correlations still exist but that a change from positive to negative correlation has taken place. Changes in correlation might indeed be a sensitive tool for evaluating systems changes. The drug clearly restores part of the original correlation and illustrates the functional response in this compartment. It is also clear that an unmet biochemical need is still present, and a new correlation network is formed. The latter is a side effect from the disease perspective and should be further analyzed for possible toxicological implications.

With the use of such an approach, it would be possible to compare a series of drugs and to generate a system compartment activity profile for each and evaluate the possible effects of combination therapies on a system. In addition, cross-compartment links for various drugs can also be established; see below under systems toxicology.

3. Systems Toxicology. Figure 14 illustrates the integration, via a correlation network, of disparate types of data, namely, metabolomic, proteomic, and transcriptomic data, from both plasma and liver tissue. Drug-induced organ toxicity is an area of active investigation by the pharmaceutical and life sciences communities, and particularly useful are specific biomarkers of potential organ injury which are based in easily accessible biological fluids such as blood. The analytes represented in the correlation network figure interestingly are not the ones which show the largest 'mean fold-change' between treated and untreated groups, but rather are the ones which exhibit the largest correlations among themselves in tissue, and across the blood-to-tissue demarcation. Such correlation networks, which are generated in an unsupervised manner solely from empirical data, are useful in revealing important molecules acting in the organ tissue as well as potential circulating molecules which may be sensitive and specific surrogates for the drug-induced biochemistry occurring in the organ.

If correlation networks have a high node and edge count, generally above a few hundred of each, then they are examined for subnetworks or network motifs. This network motif analysis can focus on a few principles: (1) important *a priori* known

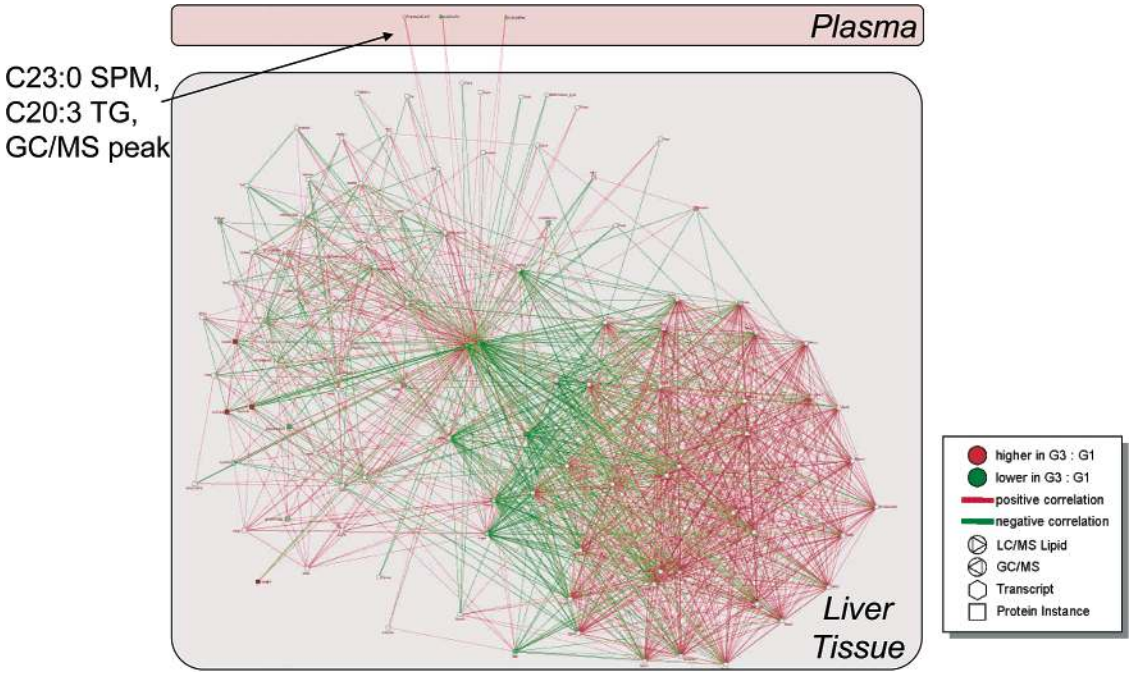


Figure 14. Correlation network of analytes across blood plasma (top of figure) and liver tissue (bottom of figure). Analytes include proteins, endogenous metabolites, and gene transcripts. Not only is structure evident among analytes profiled from liver tissue, but there are also a number of correlations to analytes profiled in plasma in this case. Such analytes can serve as useful circulating biomarkers for the tissue-based biochemical processes occurring in the organ.

analytes in the disease state and their neighboring nodes are areas of focus; (2) correlations which exhibit change upon disease or treatment (such "state-change" networks are of interest, because they may be revealing disease or drug processes); and, (3) highly interconnected nodes (e.g., those characterized by high node degree or high clustering coefficient) and their neighbors, both potential properties of "hubs" in scale-free networks, which are of interest as they may expose novel insights into disease or treatment mechanisms[55].

In the systems toxicology case illustrated here, administration of the toxic compound induced myriad changes which were detected across all bioanalytical platforms and in plasma and tissue. The correlation analysis serves to filter these findings to those for which the data provides evidence of a consistent relationship across plasma and organ tissue. The analytes and correlations depicted in the portion of the correlation network in the tissue compartment were subsequently mapped onto known biochemical pathways and reactions, and many were confirmed to correspond to drug metabolism and related biochemical processes in liver. However, many of the empirically observed relationships remained unexplained by existing pathway maps or literature, and, as such, offer the potential opportunity to explore novel biochemistry in this context.

Future Perspectives

Systems biology studies clearly are heavily dependent on the reliability of the technology platform. Extending the current platforms in more comprehensive formats can only be realized if reproducible and repeatable, novel protocols are being developed. Table 3 presents some data on the results of a recent system study using our platform of 190 primary plasma samples to illustrate the analyte coverage and data quality that is achievable.

Collaboration on a large scale, as initiated recently by funding agents, will be mandatory, but will only be successful if sufficient support for the analytical expertise for developing production-quality platforms appropriate for the applications is included in the funding programs.

Table 3. Number of Analytes Profiled in a Recent Human Plasma Study, Indicating the Number of Analytes Detected and Coefficient of Variation Metrics, by Bioanalytical Platform[a]

platform	number of analytes	coefficient of variation (CV) median (25−75th percentile)
Lipid LC−MS	219	7% (4−13%)
GC−MS	182	7% (4−12%)
Polar LC−MS	165	15% (9−23%)
Proteomics[a]	223	16% (12.5−20%)

[a] Proteomics represents multidimensional liquid chromatrography coupled with mass spectrometry approaches.

Although the direction toward a systems approach to healthcare is clear and the potential for systems biology[56] is also well-appreciated, the main issue remains how to successfully implement such a strategy in a highly regulated environment, such as that of the pharmaceutical industry. It has been argued that implementation, from the market side in terms of applications, is most likely to occur, for instance, by drug rescue programs or development of combinatorial approaches.[22] In addition, systems toxicology based on metabolomics as in the COMET initiative[57] in animal models or systems biology supported by the FDA Critical Path Initiative for drug evaluation might be important steps.

Systems pathology, pharmacology, and toxicology will all find different implementation paths within pharmaceutical industry, but given the complexity and the high reliability requirements for the platform technology, a more centralized function for in-depth studies is more likely with satellite applications using simplified platforms within different research domains. Translational medicine could become an important focal point to improve the preclinical to clinical transition, and systems

biology is ideally suited to evaluate animal models using system biomarker profiling and reveal the overlap with the human situation. New drugs can be optimized using such a translational and reversed-translational strategy.[54]

The most important step forward however is the paradigm shift needed to become a system thinking organization. The existence of Systems Biology as a scientific endeavor does not necessarily lead directly to a systems approach in healthcare. New intervention strategies need to be designed from this new perspective, and given the limitations of the technology, innovative routes need to be created. Such a paradigm shift will occur when a critical mass of global intelligence has embraced this concept of systems thinking.

An appreciation of complex connectivity is already making some impact on how "drug designers" think, and a shift from treating a target to treating pathways to treating an individual person has begun. For instance, connectivity in metabolic syndrome is moving toward understanding and modeling the gut-brain-pancreas-liver-adipose-etc. interaction and balancing.[58,59] The importance of integrating the study of gut flora has also been pointed out from a systems perspective.[60]

The acceptance of systems biology in the scientific domain has been very fast and is expected to grow even more rapidly. When the concept for our systems biology platform initiative was born in 1999 and effectuated in 2000 by establishing the first commercial Systems Biology entity to generate momentum in development of the concept, skepticism was high, but within 5 years, worldwide initiatives have been born and are underway in academia, industry, and via collaboration networks such as in the U.S., Europe, and China. The impact on global and personal health will become substantial as many people become aware that healthcare requires an integrative approach including psychology, lifestyle, nutrition, and therapeutic medicines.

Personalized medicine can benefit from a knowledge of the unique system characteristics of every person; systems biology suits this purpose well, and as has been described, innovative combination of the elements of intervention can provide such an approach, especially when, instead of disease management, the step to health promotion can be realized. Preventive medicine needs new diagnostic tools at the systems level, and as changes in the early phases of disease are hard to detect, changes in dynamics of the system, especially after a challenge test, might be the direction to enable a revolution in this area. The dynamical disease concept, as outlined from a nonlinear dynamics point of view, is illustrative.[61] Fusion of Eastern and Western medicine has been proposed on the basis of systems biology and is recommended in the 2029 project[62] by a group of very prominent scientists in the U.S. Our experiences also point to systems biology as an ideal bridge between the two cultural perspectives.[24] A solution-based study of biology starting with observed system improvements by perturbations with herbal medicine, followed by a reversed pharmacology strategy, might yield new insights in disease pathology as well as lead to the discovery of novel synergistic system targets. This approach is also used in theoretical modeling to grasp an all-encompassing model in cosmology that comprises nowadays both bottom-up and top-down approaches.[63]

A major next hurdle, but essential for the future, is the (re)-integration of the mind (psychology) into systems thinking. As outlined in the introduction, separation of mind and body in science was the beginning of reductionism. Realizing that it cannot be separated and that we are dealing with a mind–body system is crucial for a major step forward. Of course, the effects of stress are well-documented, and the effects of chronic low stress conditions are hardly understood, but for many diseases, stress plays an important if not dominant role. Neuropeptides are a beautiful illustration of system-wide action and reveal the importance of the connectivity between emotions and our physiology, explained by molecule–receptor-based research.[64]

The success of reductionistic and mechanistic biomedical research is clear, but relates primarily to acute and short-term problems. Systems approaches, by taking the myriad of connectivities into account, are more suited for addressing chronic, long-term improvements. This is a challenge for commercial activities aiming at short-term returns, but represents a major commercial opportunity for building sustainable models and addressing major challenges in health care. In many chronic diseases, such as type 2 diabetes, prior to the appearance of symptoms, the body system may have been out of a healthy state for over 10–20 years. Short-term improvements can be obtained by aiming at a particular subsystem related to the symptoms, but overall improvements can only be obtained by a systems approach which will act slowly, but in a way in which the system (human body) can follow the change and the self-healing properties, including homeostatic mechanisms, can take over. Instead of disease management, health promotion becomes the aim of the intervention.

Also, a systems analysis of the total healthcare system points to bottlenecks[19] and reveals new approaches to improve the current situation of the ever-increasing healthcare cost almost equivalent to the exponential cost trend in pharmaceutical Research and Development (R&D). In fact, a systems view on how we live will be crucial to make progress in the world we live in and will make an enormous impact on the future of medicine or, more importantly, global personal health.[65,4]

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