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Bioinformatics: Novel Insights from Genomic Information

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Abstract

While scientific methods have dominated research approaches in biology over the past decades, it is increasingly recognized that the complexity of biological systems must be addressed by a different approach, namely unbiased research involving the collection of large amounts of genome-wide information. To enable analysis of this information we and others are developing a variety of computational tools that allow bioinformaticists and wet laboratory biologists to extract novel patterns of data from these results and generate novel biological insights while generating new hypotheses for testing in the laboratory. There are two types of critical tools, databases to collate all information on biomolecules, especially interactions, and tools that reorganize information in a supervised (e.g. pathway analysis or gene ontology) or unsupervised (nonhierarchical clustering and network analysis) manner. Here we describe some of the tools we have developed and how we have used these to gain new ideas in the general area of infection and innate immunity/inflammation. In particular, it is illustrated how such analyses enable novel hypotheses about mechanisms associated with diseases and the mechanisms of action of immunomodulatory and other interventions, the definition of mechanism-based biomarkers/diagnostics, and prospective new interventions based on drug repurposing.

Introduction

As biology becomes more complex through the application of high-throughput approaches, it is critical to develop biologist-friendly tools to permit the integration of new information with existing knowledge. Postgenomic approaches to

functional analysis have tended to adopt the big science mode employing techniques dependent on high-throughput gathering of information. Current major methods include RNA-Seq (that has supplanted microarrays) for transcriptomic analysis, genome-wide association by comprehensive sequencing, CHiP-Seq for determining the binding site of transcription factors, CRISPR (clustered regularly interspaced short palindromic repeats) methods for rapid mutagenesis, which also help to generate mutant libraries, high-throughput mass spectrometry for proteomic analysis, and metabolomic methods. This overwhelming barrage of information requires the utilization of high-end sophisticated computational tools. These tools and their use, collectively termed bioinformatics, attempt to cluster/collate functional genomic information in either a supervised or unsupervised manner, in attempt to correlate global changes with the biological events that drove those changes. Thus, bioinformatics describes the two related analytical methods whereby 'bio' describes the intent to obtain biological insights while 'informatics' defines the computational nature of analyses that are necessary due to the shear amount of information being collected. Rather than comprehensively describing the vast field of informatics I will present a personal overview of our own philosophies regarding the analysis of high-throughput transcriptomic data, with illustrations from our own research. We provided a broader perspective, with specific reference to the immune system, some time ago [1], and the reader is directed to other resources for alternative perspectives [2–5].

New bioinformatic tools enable the supervised clustering of high-throughput transcriptomic data according to gene ontologies (functional descriptions), pathways, known interactors, and transcription factor binding sites (TFBS) upstream of dysregulated genes. They also enable unsupervised clustering according to global patterns of genes which are dysregulated (termed nonhierarchical clustering and often performed by creating heat maps). In addition, they also facilitate network analysis which attempts to interrelate dysregulated genes, where the proteins expressed by these genes are interconnected by their known tendency to interact within cells. This then enables one to determine key subnetworks and hubs/bottlenecks that define information flow within these networks. The supervised clustering methods are useful because they describe the dysregulated genes in terms of known biology. However, in our opinion, unsupervised clustering and network analysis methods are far more powerful because they do not make a priori assumptions that interpret new knowledge in terms of known events in cells but rather enable the determination of emergent properties, which are basically new biological insights into the processes driving the transcriptional differences observed. The results are always framed as hypotheses rather than knowledge per se and must be eventually tested in wet laboratory experiments

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but provide a powerful method of discovering new biology. I will illustrate how such analyses enable novel hypotheses about mechanisms associated with diseases or environmental factors and about the mechanisms of action of immunomodulatory and other interventions, and also allow to define mechanism-based biomarkers that can be used to diagnose disease syndromes and monitor/reveal the success of existing interventions. I will also describe strategies to define prospective new interventions based on drug repurposing.

Virtually any complex biological event can be investigated ranging from genetic disease to the influence of environment, nutrition, and interventions. Our own interests lie in innate immunity, which is a coordinated system of both specialized and nonspecialized immune cells, and serves as the body's first line of defense against pathogenic organisms. Unlike the adaptive arm of the immune system, innate immunity requires no previous exposure to threats, lacks true 'memory', and is an intrinsically hard-wired response. The innate immune system is essential for human survival, yet the outcome of an overly robust and/or inappropriate immune response can paradoxically result in harmful sequelae, including almost all known diseases such as cancer, atherosclerosis, ischemic heart disease, asthma, inflammatory bowel diseases, arthritis, and vasculitis. Similarly poor (or excessive) nutrition and various environmental factors can also cause immune dysfunction [5]. Regulatory networks that govern innate immune processes provide for the dynamic homeostatic control necessary to identify pathogens, amidst the normal host flora, and mount an appropriate response while minimizing host toxicity. These pathways interface to create a single system capable of selectively amplifying and integrating signals in a coordinated manner. As a consequence of its interconnectivity, dysfunctions in an innate immune pathway can destabilize the entire system, causing human inflammatory diseases that are acutely toxic or chronically debilitating. Importantly, while having inflammation as a common factor immune dysfunction shows substantial heterogeneity. For example, Goh et al. [6] used this framework to create a human 'diseasome' network, highlighting transcriptional level similarities, and differences, between disease types and also within tissue types. A similar type of meta-analysis was carried out by Jenner and Young [7], who identified a transcriptional program of 'common host response to infection' across 35 microarray experiments that –when combined – pooled the responses of 16 types of immune effector cells to various pathogenic stimuli. However, a meta-analysis need not be as broad, as demonstrated by Hampton et al. [8] by mining the data from 4 different cystic fibrosis (CF) microarray data sets to generate novel evidence that CF cells have an intrinsic defect in MHC processing and antigen presentation.

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Anti-inflammatory therapeutics often have proven clinical benefits during treatment (e.g. statins in atherosclerosis) but again a good anti-inflammatory treatment for one disease may not work for another implying substantial heterogeneity in immune/inflammatory dysfunctions. A large variety of proteins are involved in inflammation, and many of them can be affected by genetic mutations that impair or otherwise dysregulate the normal function or expression of that protein. The reason why inflammation can become chronic in some diseases is still open to debate, but might be different for each type of disease. We are thus developing strategies and tools to understand the complex nature and heterogeneity of the dysregulation of innate immunity/inflammation in humans [9–14]. We propose this will shed light on the most appropriate strategies to treat human diseases and syndromes, including inflammation, as well as provide mechanism-based biomarkers.

Systems Biology Tools

Our flagship program, InnateDB [9, 10], provides the basis for understanding biological connections in cells, according to known interactions between genetic elements (such as proteins). Collectively, these elements are termed the interactome and reflect a number of studies that demonstrate the physical, metabolic, or regulatory interactions of proteins. For example TRAF6 in humans is usually depicted as having a role in the major TLR4 to NF-κB pathway of innate immunity. However it has been experimentally documented to interact with 643 other proteins, with a further 99 predicted interactions in man (www.innatedb.com). This means that there is a massive potential for this protein to bridge multiple biological pathways and events when activated by innate immune stimuli (e.g. infection, disease, and inappropriate nutrition).

InnateDB is an open-source, publicly available database and systems biology analysis platform of all of the genes, proteins, molecular interactions, pathways, and signaling responses involved in human, mouse, and bovine innate immune responses. It is becoming an important tool in immunology as evidenced by the >6,000,000 hits from more than 55,000 visitors annually. While all known pathways (>3,500) and molecular interactions (316,000 in human) are present, the emphasis on innate immunity is achieved through the contextual review, curation, and annotation of molecular interactions and pathways involved in innate immunity. To date, the InnateDB curation team has reviewed more than 5,000 publications annotating >25,000 molecular interactions of >8,700 separate genes in rich detail, including annotation of the cell, cell line, and tissue type; the molecules involved; the species; the interaction

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detection method, and the publication source. By including interaction and pathway data relevant to all biological processes, a much broader perspective of innate immunity can be achieved, especially since an effective innate immune response requires the coordinated efforts of many disparate processes including the endocrine, circulatory, and nervous systems [1]. Additionally, one is able to investigate any biological signaling process of interest beyond the immune system.

InnateDB facilitates systems level analyses by enabling the integration, analysis, and visualization of user-supplied quantitative data, such as gene expression data, in the context of molecular interaction networks and pathways. This includes the statistically robust analysis of overrepresented pathways, interactomes, ontologies, TFBS, and networks. One can, for example, refine the network to show only molecular interactions between a list of differentially expressed genes (and their encoded products) or view all potential interactors regardless of whether they are differentially expressed. This can aid in the identification of important nodes that may not be regulated transcriptionally or which are expressed at an earlier or later time. Each network can be interactively visualized at the click of a button using the Cerebral plug-in for Cytoscape, which has been developed as part of the InnateDB project, and generates biologically intuitive, pathway-like layouts of networks [11]. Other visualization tools are also available. Network analysis is also available through other Cytoscape plug-ins as well as our new program NetworkAnalyst [12], which can then be easily utilized for further network analysis. Conversely, gene sets can be downloaded in many different forms for analysis using additional network analysis software (the R statistical package, for example, provides a number of graph/network algorithms).

While InnateDB is our most utilized program, we have recently built other tools. These include a platform for the meta-analysis of transcriptomic, proteomic, and metabolomic data, INMEX (www.inmex.ca [13]), an interactive customizable heat map visualization program, INVEX (www.invex.ca [14]), and the extremely fast network and hub analysis and visualization tool, NetworkAnalyst (www.networkanalyst.ca [12]) which now contains all 3 modules. In the following, we will illustrate how these tools can be applied.

Mechanistic Insights and Biomarkers

Salmonella enterica sv. Typhimurium normally causes self-limiting gastroenteritis and food poisoning in our society. In AIDS patients in Africa, it has been found to be associated with severe invasive disease, which is normally associated

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Table 1. Gene ontology (functional categorization) and pathway analysis of transcriptional responses attributable to iNTS in AIDS patients

Data sets compared	DE genes	Gene ontology (genes)	Pathways	Other DE genes of interest
Acute iNTS (n = 25) cf. HIV+ (n = 14)	1,214 upregulated	Cell cycle (n = 55) DNA replication (n = 25) DNA translation (n = 18) DNA repair (n = 26) Mitosis (n = 39) Cell division (n = 36)	Viral mRNA translation Viral replication Cell cycle Nucleotide excision repair	ARG1, CEACAM 6, complement, DICER, FCGR1A, IFNγ, MPO, MSR1, NFKBIB, PI3K, REL, SIGIRR, SOCS4, SOCS7, STAP2, TIRAP
Acute 'other' infections (n = 6) cf. HIV+	1,199 upregulated	Innate immune response (n = 51) Inflammatory response (n = 24)	IL-1 signaling IL-4 signaling Complement and coagulation Atypical NF-κB	IL10RB, IL18R1, IL18RAP, IFNGR1, CCR9, FAS, CARD17

Examined were differentially expressed (DE) genes when compared to patients with just AIDS (HIV+). Lack of a classical innate immune response and increased viral replication signatures were observed in contrast to differential responses attributable to 'other' *(E. coli and S. pneumoniae)* infections. Under other DE genes of interest, those with products associated with suppression of inflammatory responses are underlined.

with another species of *Salmonella* (*S. typhi*). Invasive nontyphoidal *Salmonella* (iNTS) is associated with rapid clinical deterioration in patients with HIV infections. We determined the transcriptional responses of 25 patients with underlying HIV infections complicated with iNTS, 14 patients with HIV infections without iNTS, and 6 HIV patients complicated with other acute bacterial infections (primarily *Escherichia coli*, a close relative of *S. enterica*, and *Streptococcus pneumoniae*) [15]. Around 1,200 genes were upregulated in both groups of infected patients compared to patients with HIV without a bacterial infection (table 1). However, supervised clustering revealed that there were profound differences. While upregulated genes from patients with acute infections were described as having ontologies and pathways typical of innate immune/inflammatory responses, this was not evident in patients with iNTS and could be explained by the upregulation, in these patients, of genes with products that are associated with suppression of inflammation (NFKBIB, PI3K, REL, SIGIRR, SOCS4, SOCS7). The poor prognosis in these patients could be explained by the lack of innate immune responses to control infection but also by the obvious viral signature, which was subsequently shown to reflect increased viral load [16].

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The manipulation of natural innate immunity represents a new therapeutic strategy against antibiotic-resistant infections [17]. Cationic host defense (antimicrobial) peptides, which are produced by virtually all organisms, defend against infections [18]. These peptides boost protective innate immunity while suppressing potentially harmful inflammation/sepsis. Using the principle of selective boosting of innate immunity, we have developed novel small innate defense regulator (IDR) peptides with no direct antibacterial activity, that are nevertheless able to protect against many different microbial infections and inflammatory diseases in animal models, including antibiotic-resistant infections, tuberculosis, and cerebral malaria, providing a new concept of anti-infective therapy. Given the complexity of innate immunity, we assumed that these IDR peptides would be similarly complex mechanistically. We found that the peptides entered cells and bound to intracellular receptors. To understand subsequent events, we analyzed transcriptional dysregulation in human monocytes [19, 20], including pathway overrepresentation, TFBS analysis of the upstream regions of dysregulated genes and network analysis. Thus, the prediction of pathways collectively implicated the involvement of 11 pathways, including the p38, Erk1/2, and JNK mitogen-activated protein (MAP) kinases, NF-κB, phosphatidylinositol-3-phosphate kinase, and two Src family kinases, and several of these were subsequently confirmed using biochemical methods especially involving pharmacological inhibitors and assessments of phosphorylation of pathway intermediates. Similarly, the TFBS analysis predicted that more than 15 transcription factors were involved, including NF-κB (most subunits), Creb, IRF4, AP-1, AP-2, Are, E2F1, SP1, Gre, Elk, PPAR-γ, and STAT3, and many of these were confirmed biochemically.

More globally, we obtained exciting leads regarding mechanisms using hub analysis. As mentioned, transcriptomic information can be used in conjunction with information regarding the known interactors of dysregulated proteins (contained within InnateDB) to construct a network of interacting molecules. These can then be probed mathematically to reveal proteins that interact with many other proteins that are termed hubs. Hubs are considered to be key molecules in signaling since they are highly interconnected; they are considered to receive and integrate multiple signals, and pass them on to downstream nodes. Tools exist for the extraction of key hubs from transcriptomic information such as the plug-in cytoHubba for Cytoscape (hub.iis.sinica.edu.tw/cytohubba/) and our new program NetworkAnalyst [12]. Figure 1 shows the top hubs extracted from studies where human monocytes were treated with IDR1 peptide. The top hubs (most interconnected proteins) were involved in the functioning of MAP kinases, induction of chemokines, and in anti-inflammatory pathways, particularly TGF-β and IFN-type responses. The first two correspond to known

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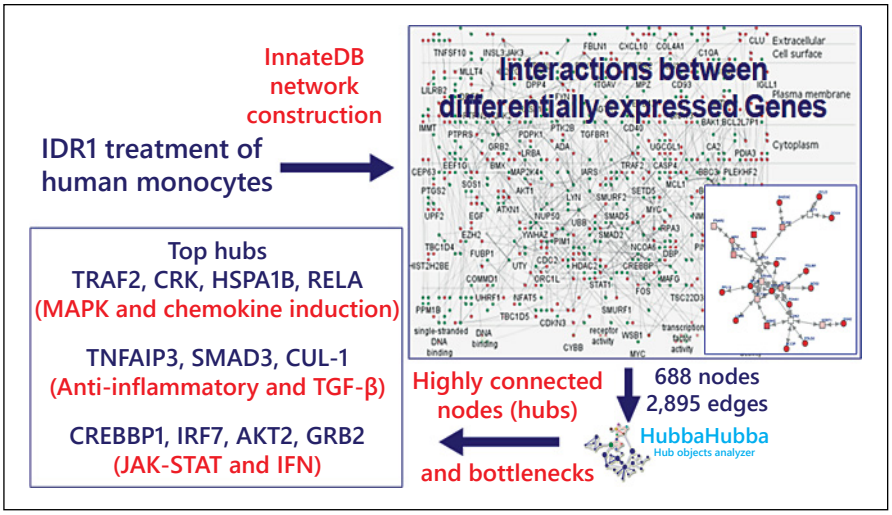

Fig. 1. Use of NetworkAnalyst to predict the key hubs in immune responses generated during IDR treatment of human monocytes. The network in the top right hand program was drawn using Cerebral and shows known (2,895) interactions (edges) between the 688 dysregulated genes (nodes: red upregulated, green downregulated). Hubs, identified using cytoHubba (Cytoscape plug-in version of HubbaHubba), are key proteins that interconnect with many other proteins and thus are predicted to be critical in the flow of information within biological signaling systems. By their nature, they are critical determinants of the mode of action and potential mechanistic-based biomarkers.

properties of the peptide, while the third is under active investigation in our laboratory with preliminary data showing that it is also involved. However, importantly, these hubs, being dysregulated and central to the network of transcriptional responses and thus biologically important, also represent excellent candidate biomarkers of the studied phenomenon and could potentially be utilized in diagnosing disease and/or testing response to treatment.

Drug Discovery and Repurposing

Another mechanistic insight was obtained in patients with CF. CF is the most common eventually fatal autosomal recessive genetic disease in our society. It is caused by mutations in the CF transmembrane regulator (CFTR), and severity and life expectancy is further influenced by modifier genes many of which impact on inflammation. Individuals with CF acquire chronic lung infections leading to hyperinflammatory lung disease, which causes progressive deterioration of lung function. Although suspected, no connectivity had been made between

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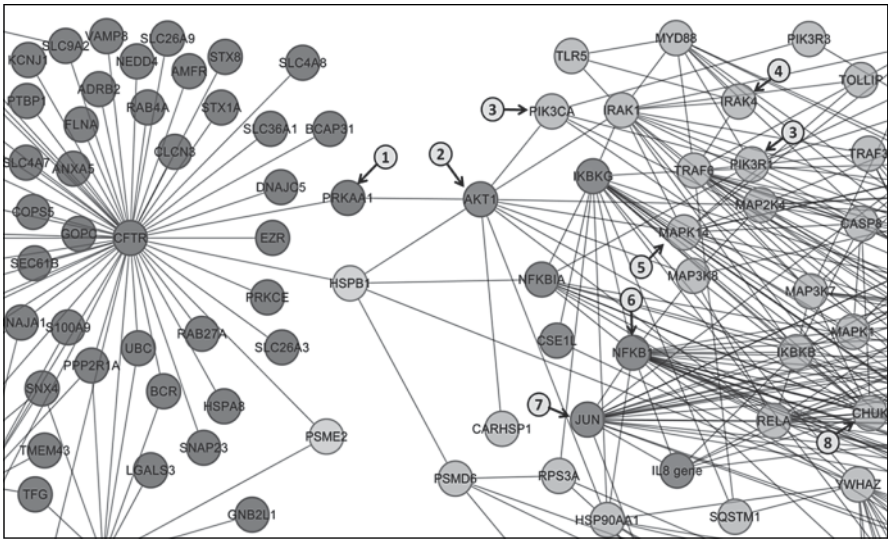

(1) PRKAA1:
Metformin, balanol analog 8, phosphonoserine, phenformine, phosphonothreonine, N-octane and 2 others

(2) AKT1:
Inositol, 1,3,4,5-tetrakisphosphate, arsenic trioxide and 1 other

(3) PIK3CA and PI3KR1
Isoproterenol, 1S,6BR,9AS,11R,11BR)-9A,11B-dimethyl-1-[(methyloxy)methyl]-3,6,9-trioxo-1,6,6B,7,8,9,9A,10,11,11B-decahydro-3H-furo[4,3,2-DE]indeno[4,5-H][2]benzopyran-11-yl acetate

(4) IRAK4:
1-(3-hydroxypropyl)-2-[(3-nitrobenzoyl)amino]-1H-benzimidazol-5-yl-pivalate

(5) MAPK14:
SB220025, 2-chlorophenol, 3-(benzyloxy)pyridin-2-amine, triazolopyridine and 47 others

(6) NFKB1:
Thalidomide and pranlukast

(7) JUN:
Irbesartan, arsenic trioxide and vinblastine

(8) CHUK:
Sulfasalazine, aminosalicylic acid and mesalazine

Fig. 2. Network analysis reveals potential repurposing of drugs. Analysis of transcriptomic data after challenge of CFTR mutant epithelial cells with flagellin (TLR5 agonist), cf. untreated cells, enabled the first demonstration of the linkage of the CFTR (left) and TLR5 (right) interaction networks (data from InnateDB visualized using the built-in network drawing program Cytoscape/Cerebral) and their bridging by at least 2 separate pathways (1) AKT via AMPK and (2) Hsp27. Superimposed on this are the results of the examination of these genes using the DrugBank database (see circled numbers with arrows pointing to the targeted genes), which provides all of the known pharmacophores for human gene products. Of note, metformin, a PRKAA1 (AMP kinase) activator, suppressed hyperinflammation in CF mutant cells. Reproduced from Mayer et al. [20] with permission from the American Association of Immunologists, Inc.

the CFTR status and hyperinflammation. We investigated the transcriptional responses of immortalized CFTR–/– epithelial cells compared to corrected variants with and without stimulus by flagellin, which had been shown to stimulate hyperinflammatory responses in CFTR–/– mutant cells [21]. The results, when submitted to InnateDB and a customized R-language-based assembly MetaGEX, revealed interconnectivity of the CFTR and innate immune networks through two pathways, PRKAA1 (AMP kinase)/AKT1 and HSPB1 (fig. 2). The

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database DrugBank [22] was then used to probe the fused network and predict inhibitors acting on the genes in this pathway that could potentially influence inflammation in CF (fig. 2). This then provided a set of repurposed drugs (many clinically approved) that could be used to influence inflammation in CF. To determine if the interconnected pathways influenced inflammation, we used the well-known approved drug metformin that activates AMP kinase and demonstrated that this suppressed inflammation by ~50%, even though AMP kinase and metformin had not previously been reported to influence inflammation.

Further, supervised mining of the genes differentially expressed in CF cells compared to corrected variants revealed the importance of stress, and, in particular, 54 genes related to the process of autophagy were dysregulated. Autophagy (also termed autophagocytosis), is a basic catabolic mechanism that involves cell degradation of unnecessary or dysfunctional cellular components through the action of lysosomes to allow the degradation and recycling of cellular components [23]. In the context of disease, autophagy is considered to be an adaptive response to stress that favors survival and in some cases is even involved in the resolution of infections, whereas in other cases it appears to promote cell death. With these bioinformatic cues about the potential involvement of autophagy in CF, we went on to show that CFTR mutant cells in fact demonstrated arrested autophagy that was not resolved, presumably converting this noninflammatory cell death mechanism into an inflammatory mechanism. We then demonstrated that the peptide IDR-1018 actually could resolve arrested autophagy and reduce inflammation.

Finally, we demonstrated, through pathway and network analysis, that there was a strong upregulation of endoplasmic reticulum stress and the unfolded protein response stress pathway, which we subsequently confirmed as a feature of CF cells [24]. This occurred through activation of IRE-1 rather than the PERK-eIF2a pathway and led us to show that salubrinal, a specific pharmacological inhibitor of the negative regulation of GADD34, upregulated this pathway and suppressed hyperinflammation.

Thus, informatic analysis not only resolved important and novel details of the nature of CF but also delivered 3 novel drugs and targets for resolving the life-threatening inflammation in CF.

The Future

These analyses only scratch the surface of what is possible using bioinformatics. I feel that the above-described examples clearly show that using unbiased experimental methods, such as whole genome transcriptome analysis in conjunction with incisive bioinformatic tools, one can go beyond the hypothesis-testing,

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so-called scientific method to generating fundamentally new hypotheses offering new biological insights. For us, the next frontier is (i) studying the variability of human inflammatory diseases and syndromes in order to appreciate how these various syndromes differ and how they might be individually and optimally treated and (ii) understanding the heterogeneity of any given disease. With regard to the latter, we are trying to ensure that all clinical studies collect 'metadata' that associate with each sample the parameters underlying the subject's condition, including relevant clinical data, treatments applied, and phenotypic data. With this information in hand, we are now developing new methods for multidimensional (hierarchical) clustering of metadata with transcriptional changes to develop signatures associated with each metadata parameter (age, temperature, therapies, and other defining characteristics). A prototype for this is provided by INMEX and INVEX [13, 14]. Critically, in all of our endeavors, we have tried to build informatic tools to be biologist friendly, since we believe expert biologists have the best potential to make the appropriate observations and discoveries of new relationships based on the analysis of data.

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Disclosure Statement

The author declares that no financial or other conflict of interest exists in relation to the contents of the chapter.

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