Published in final edited form as:

Int Immunopharmacol. 2007 December 20; 7(14): 1948–1960.

Applying genomics to organ transplantation medicine in both discovery and validation of biomarkers

Sunil Kurian, PhD¹, Yevgeniy Grigoryev, BS¹, Steve Head, MS², Daniel Campbell, BS², Tony Mondala, BS², and Daniel R. Salomon, MD¹

¹Department of Molecular & Experimental Medicine, The Scripps Research Institute, La Jolla, California, 92037

²The DNA Array Core Facility, The Scripps Research Institute, La Jolla, California, 92037

Introduction

The classical definition of a biomarker is a biological molecule whose presence can indicate a disease state or a high likelihood/risk of developing a disease or a specific disease phenotype. Thus, biomarkers are a very valuable and attractive tool for the accurate and specific diagnosis and prognostication of disease states as well as for determining and monitoring therapy. There are also rigorous criteria, which have to be assessed before a molecule can be considered a valid biomarker. Some of the important characteristics for a valid biomarker are the specificity of the “endpoint” measurement, ease of measurement and a link between the effect or endpoint and disease outcome. Not surprisingly, there have been a growing number of studies identifying possible biomarkers for the definition and diagnosis of disease states including cancer, cardiac diseases, diabetes, and organ transplantation, though at this point, very few have been validated and incorporated into routine medical practice. Recently a lot of work has focused on the integration of the latest technologies for systems biology including transcriptomics, proteomics, and metabolomics to hunt for biomarkers that can be profiled in cells, collections of cells, tissues and body fluids. For purposes of focus in this review, we will not discuss the potential application of genetics technologies such as identification of Single Nucleotide Polymorphisms (SNPs) associated with diseases, disease phenotypes or drug metabolism.

In effect, these new genomic technologies have changed the classical definition of a biomarker by expanding the concept to include suites of molecular measurements and complex patterns of interacting networks. Biomarkers that are defined by their functional association with the pathophysiology of a disease could range from RNA transcripts, cellular products, soluble cytokines, and proteins that localize in a cell or post-translational modifications of proteins that modify the cells or tissue responses to disease in such a way that they can diagnose, monitor therapy and determine outcomes. In contrast to functional biomarkers, there is another category of surrogate or disease-associated biomarkers that can still correlate with the disease by representing molecular bystanders or downstream consequences of complex scenarios rather than the primary mediators of the disease. Tremendous biological insight and therapeutic utility can be obtained by the identification and study of both these classes of biomarkers, functional and surrogate, especially when they can be converted into readily measurable, objective entities.

Corresponding Authors: Sunil Kurian, PhD smkurian@scripps.edu Daniel R. Salomon, MD dsalomon@scripps.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Brief history of biomarkers - discovery and current status

Historically biomarkers were primarily physiological indicators such as blood pressure, heart rate or body temperature levels, which could be measured and directly linked to the physiology of a disease condition. In the last century, the tools of chemistry added a large number of biomarkers to routine medical care that could be measured in blood, plasma and other body fluids and in some cases also tissues. These include blood glucose, cholesterol, creatinine, clotting factors and liver enzymes. Most recently, in the Era of the Human Genome, the term "biomarker" has become synonymous with a molecule or a gene product that can be a causative factor or a determinant of a disease but as already noted, could also be just a surrogate molecular marker. In 1999 an NIH expert group defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention." (1). One of the earliest molecular biomarkers was from the field of cancer biology where Dr. Joseph Gold found a substance in the blood of patients with colon cancer, which was normally found only in fetal tissues and named carcinoembryonic antigen (CEA) (2). This is a perfect example of a surrogate or disease-associated biomarker. In the following two decades many more molecular biomarkers were discovered. For example, prostate specific antigen (PSA), which was subsequently identified as kallikrein-3, has become a routine laboratory screen for prostate cancer and inflammation (3,4). PSA is a kallikrein-like protease present in seminal plasma and can be measured with highly sensitive immunoassays. Another new cancer biomarker is the ERBB2 gene also known as the HER-2/*neu* oncogene, which encodes a transmembrane tyrosine kinase receptor with extensive homology to the epidermal growth factor receptor. It has been known that ERBB2 amplification and/or over-expression occurs in 20% to 30% of breast cancers. ERBB2 expression appears to be associated with a more aggressive phenotype making it an attractive candidate for breast cancer (5,6). In fact, ERBB2, as a transforming cell growth factor, is a perfect example of a functional molecular biomarker. A controversial area in the field now is how to apply this biomarker to therapy choices with HER-2/*neu*-specific drugs in individual patients. Is it correct to limit the use of this new generation of drugs only to patients that are biomarker positive? We think that this process of shaping clinical practice through the use of biomarkers is going to be one of the major challenges in the next few decades.

At the same time biomarkers were being discovered in other diseases. Cardiac troponin T (cTn) was detected first in canine and rodent models as a reliable marker for cardiac injury (7). cTn was then established as a biomarker for the diagnosis of human cardiac disease, particularly cardiac ischemia and infarction (8) and also provided robust prognostic information (9,10). It is interesting to note that cTn is an example of convergent biomarker discovery, when work in relevant animal models is translated successfully into a human clinical setting.

With the discovery of more robust and reliable molecular biomarkers, it is natural that some of the older and classical biomarkers will become outdated. One example of this evolution is the measurement of creatine kinase MB, a cytosolic carrier protein for high-energy phosphates that went from routine use to take a backseat due to the availability of the more sensitive and accurate cTn assays (11). Indeed, another key point is how rapidly these biomarker choices are changing now. Thus, continuing with the example of cTn, there are a number of other biomarkers of cardiac injury and disease that are developing both commercially and experimentally that are showing significant promise including soluble CD40 ligand (12) myeloperoxidase (12), ischemia-modified albumin (12), pregnancy-associated plasma protein-A (12), placental growth factor (12), cystatin C (13-15) and fatty acid binding protein (16).

Early studies of biomarkers for kidney transplant rejection looked at parameters such as proteinuria (17), lymphocyturia (18) and urinary proteins such as beta 2-microglobulin (19,

20), though none of these were validated as robust enough for routine clinical use. Interestingly, using the latest technologies for proteomics, beta 2-microglobulin has been re-discovered as a possible biomarker for acute renal allograft rejection by using matrix-associated laser desorption ionization time-of-flight mass spectroscopy (MALDI-TOF MS) and Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF-MS) (21,22). This is an example where the utility of an older generation of biomarkers can be enhanced with the introduction of new technologies that significantly increase the sensitivity or specificity of measurements. Nonetheless, urinary excretion of beta 2-microglobulin is still a surrogate marker for kidney tubular cell injury that is many biological steps removed from the immune-mediated mechanisms of kidney transplant rejection.

The protein that is presently the primary biomarker for kidney transplant function is creatinine. Creatinine is a breakdown product of creatine, which is an important amino acid byproduct of muscle protein metabolism. The serum creatinine is inversely correlated with the glomerular filtration rate. Unfortunately, creatinine generation is also determined by the age, sex, weight and nutritional/catabolic state of the patient. Creatinine clearance was used to estimate kidney function as a metric for glomerular filtration rate (GFR) as early as the 1930's (23,24) and is still widely used now to estimate GFR using a number of formulae such as Cockcroft-Gault, Nankivell and MDRD (Modification of Diet in Renal Disease study) (25-27) designed to account for the factors noted above that affect creatinine generation and elimination. The obvious irony for kidney transplantation is that creatinine is still another surrogate biomarker for kidney function yet we are using it as the primary biomarker with which to manage complex immunosuppression regimes. Thus, it is evident that a new generation of biomarkers for transplantation that provide more direct evidence for the nature and intensity of the immune response and the adequacy of immunosuppression are needed.

As more sensitive molecular techniques have become available in the last 10 years, molecular markers of transplant rejection have been proposed that have the potential to be accurate, sensitive and easy to assay rapidly in a clinical laboratory setting. Some of the earliest studied markers in this category were granzyme B and perforin, which are transcripts expressed by cytotoxic T-lymphocytes (CTLs). It is known that CTLs promote allograft damage through the elaboration of these highly cytopathic molecules (28-30). Studies were initially done using kidney transplant biopsies and the challenge was to corroborate these results employing less invasive samples like peripheral blood or urine. Simon et al. successfully showed that recipients with acute rejection had higher levels of perforin and granzyme B transcripts in peripheral blood when compared to patients without rejection, suggesting that gene expression measurements by quantitative PCR (RT-qPCR) might serve as an early and non-invasive tool to predict kidney allograft rejection or test the adequacy of an immunosuppressive regime (31). A similar study with peripheral blood showed that perforin transcript expression was significantly higher in patients with acute rejection than in the control group, but there was no consistent pattern of granzyme B or FasL expression, another CTL marker (32). A third study of urine samples in 22 patients with acute rejection and 63 control subjects also demonstrated significant elevations of granzyme and perforin transcripts (33). Nonetheless, none of these have yet been validated to the point where any are available on a routine clinical basis.

Another gene of interest as a possible biomarker for predicting kidney transplant outcomes is FOXP3. FOXP3 is a member of the forkhead/winged-helix family of transcriptional regulators and is highly conserved in evolution from fruit flies to humans. Recent work has suggested that FOXP3 may be a master regulatory gene and a specific marker of T-regulatory cells. T-regulatory cells appear to be powerful inhibitors of antigen-driven immune responses and thus, have become a focus of work in both transplantation tolerance (34-39) and the regulation of autoimmunity (35). A recent study showed that urine FOXP3 mRNA levels could identify subjects at risk for kidney graft failure within six months after the incident episode of acute

rejection (40). Similar findings have also been mirrored in a study of human kidney transplant biopsies showing that infiltration of FOXP3(+) cells, presumably representing T-regulatory cells, occurs in acute cellular rejection (ACR) to a greater degree than in humoral (antibody-mediated) rejection (41). Interestingly, there was no correlation found in the ACR group with higher levels of FOXP3(+) and better outcomes. The authors suggested that T-regulatory cells concentrate in tubules, probably contributing to FOXP3 mRNA levels in urine and that could explain why urinary FOXP3 is a better predictor of transplant outcomes. However, it must be noted that this biomarker has not been validated in a large enough, prospective or therapy-controlled study to establish its clinical utility. Additionally, the evidence does not exclude the possibility that FOXP3 levels in urine are influenced by factors other than acute rejection (e.g., genetic variability and disease conditions) (42).

In conclusion, the development of noninvasive tests for disease detection has been one of the major goals of biomarker discovery. In any setting, an invasive approach like a biopsy is associated with significant risks and costs. In transplantation frequently repeated biopsies of the organ grafts are not practically applicable in clinical situations. This limitation is even more evident for cell transplants such as pancreatic islet cells that are injected into the liver via the portal vein. Therefore, developing minimally invasive (i.e. blood drawing) or noninvasive (i.e. urine) tools to monitor the function and immune status of the transplant and/or predict treatment outcomes would be extremely important. The premise of the remainder of this review, is that the application of new technologies developing in genomics, including gene, protein and metabolite expression profiling, promises to dramatically change our ability to discover and validate the next generation of biomarkers for medicine and organ transplantation.

DNA microarrays: transcriptomics as a tool for biomarker discovery and validation

Microarray technology is rapidly becoming an invaluable tool for establishing genomic signatures of disease. The power of this technology lies in the fact that it makes it possible to survey thousands of genes simultaneously, in fact, the whole human genome on a single chip. This feature makes it very appealing for drug discovery and pharmacogenomics. For oncology, the promise of microarrays is the classification of tumors according to their genomic signatures that would make diagnosis of certain cancers more efficient or establish very early the prognosis and even help select the best therapy. The application of genomics to immunology has already provided significant information about the workings of the immune system such as the transcriptional programs involved in T- cell, B-cell and innate immune biology (43-47). In transplantation, DNA microarrays have provided insights into immune responses that result in transplant rejection. Thus far, microarray analysis has been reported to look at gene expression in human kidney (48-51), liver (52-54), heart (55-57), and lung (58,59) transplants.

We recently conducted a study using high-density oligonucleotide DNA microarrays to define molecular signatures for acute allograft rejection using peripheral blood lymphocytes (PBL) as a means of minimally invasive diagnosis (48). We used Affymetrix HG-U95Av2 GeneChips to determine gene expression profiles for both kidney biopsies and PBL in four groups of transplant patients: normal donors, transplant recipients with well-functioning kidneys, patients with acute rejection and patients biopsied with acute kidney dysfunction that did not have acute rejection by histology. We successfully identified distinct gene expression profiles for both kidney biopsies as well as PBL that correlated and classified each of the four groups of patients. Our GeneChip results were validated using a set of 15 genes that were quantified by real-time quantitative PCR. This study established for the first time the successful application of high-density DNA microarrays to PBL as a minimally invasive diagnostic tool for transplantation. Similarly, the use of PBL gene expression profiling has been more recently demonstrated in heart transplant patients (56,60,61).

We performed gene expression profiling of protocol kidney transplant biopsies done two years post transplant in a randomized prospective trial comparing a calcineurin inhibitor-free immunosuppression, based on sirolimus (Rapamune®) and mycophenolate mofetil (CellCept®), to a regimen based on cyclosporine (Neoral®) and mycophenolate mofetil in kidney transplant biopsies (49). At the two-year post transplant time point, patients on the calcineurin inhibitor-free immunosuppression had better renal function and a significantly diminished prevalence of chronic allograft nephropathy as documented by histology. Gene expression profiles of kidneys that were graded with higher (more severe) Banff scores for chronic allograft nephropathy showed a marked up-regulation of genes that were involved in immune/inflammation and tissue injury/remodeling and fibrosis. We proposed that these results suggest that two mechanisms are at play in the pathogenesis of chronic allograft nephropathy: one that is dependent on ongoing immune/inflammatory tissue injury and one that determines the extent of tissue repair and remodeling. We believe that the balance of these two mechanisms determines the progression of chronic allograft injury and the long-term outcome of the transplant.

Another study was designed to identify genes whose expression during acute rejection is associated with progression to chronic allograft nephropathy (62). Chronic allograft nephropathy patients were compared to patients who had stable graft function over time. Microarray analysis and real-time quantitative PCR validations showed that transcript levels for surfactant protein-C, S100 calcium-binding protein A8 and A9, as well as beta-globin distinguished the two groups. The prognostic value of mRNA transcripts was tested in an independent cohort of rejection biopsies where they found that mRNA and protein expression of S100 A8 and S100 A9 in infiltrating cells was significantly higher in the control group compared with the chronic allograft nephropathy group.

A third study looked at gene expression profiles of kidney transplant biopsy samples with only chronic allograft nephropathy (63). Differential expression of profibrotic and growth factors like transforming growth factor-beta induced factor, thrombospondin 1, and platelet derived growth factor-C were up-regulated in chronic allograft nephropathy, whereas vascular endothelial growth factor (VEGF), epidermal growth factor, and fibroblast growth factors 1 and 9 were down-regulated. Selected differentially expressed genes were confirmed in microdissected samples by real-time quantitative PCR. Immunopathologic examination of biopsies revealed strong staining for TGF-beta but decreased glomerular VEGF expression.

In a similar study (64), markers identified first by microarray analysis of kidney transplant biopsies, including TGF-beta, epidermal growth factor receptor, and angiotensinogen, were tested by real-time quantitative PCR in urine and peripheral blood samples collected at the time of biopsy. Genes related mechanistically to fibrosis and extracellular matrix deposition (i.e., TGF-beta, laminin, gamma 2, metalloproteinases-9, and collagen type IX alpha 3) were up-regulated in chronic allograft nephropathy. Genes related to immunoglobulins, B cells, T-cell receptor, nuclear factor of activated T cells, and cytokine and chemokines receptors were also up-regulated.

A glimpse into the proteome box

While DNA microarray technology provides a wealth of information about the expression and roles of RNA transcripts in states of disease, it is critically important to associate the events at the level of transcription to the actual proteins that are being encoded, translated and modified. Using multidimensional gel electrophoresis, high-throughput mass spectroscopy, various low density arrays for protein-protein interactions or protein-specific antibody arrays, it is possible to study the proteomes of cells, tissues and body fluids in search of disease-linked proteins. At the molecular and cellular level, biological functions are carried out by proteins rather than

DNA or RNA (with the possible exception of ribozymes). Thus, information obtained by proteomic analysis greatly complements data obtained from DNA microarrays.

A major technical challenge for proteomics is the significant increase in the complexity of the proteome, representing several hundred thousand or more proteins, as compared to the RNA transcriptome, which represents about 35,000–50,000 genes total. One cause of this increased proteomic complexity is splice variants of genes that are manifest as different protein products. Another mechanism is that protein function and activity is regulated or restricted by post-translational and covalent modifications of protein structure (i.e. phosphorylation, sulfation, methylation, glycosylation), as well as other protein-protein interactions, or protein-small molecule interactions. Thus it is equally important to develop technologies to study the post-translational events of proteins that dictate the biological microenvironment of the cells and tissues, and thus, the entire organism. However, it is also important to emphasize here that another major challenge for proteomics is the current limitations of the technology in sensitivity and specificity that are also clearly related to the significantly greater complexity of the proteome. In this regard, the ongoing pace of developing new and better proteomic technologies is simply remarkable.

Proteomic analysis could have a wide application in the field of organ transplantation by providing unique information about cells and tissues in transplanted patients and eventually creating non-invasive tests to monitor biomarkers in body fluids, such as urine or blood, that would correlate with transplant rejection, function and immunosuppression. A proteomics application to monitor transplantation acceptance was reported by Pan et al. (65) using 2D PAGE and MALDI-TOF in a rat model of liver transplantation. The authors found that haptoglobin, which has been associated with inhibition of T-cell proliferation in studies of cancer patients and some *in vitro* culture assays, was up-regulated following liver transplantation. As additional proof, the level of RNA transcript expression and intracellular localization of haptoglobin correlated with the immune events in the liver, a good example of how proteomics can complement genomics. Another study using the same technology found over 100 proteins up-regulated in the heart during human cardiac transplant rejection (66). Two of these proteins alphaB-crystallin and tropomyosin were found to be cardiac-specific heat shock proteins and their levels were then measured post-transplant in serum from transplant recipients. Mean sera levels of alphaB-crystallin and tropomyosin were significantly higher in biopsies with acute rejection as compared to biopsies with no rejection.

In the field of kidney transplantation one of the earliest searches to identify potential biomarker candidates from the urine was performed with SELDI-TOF mass spectroscopy (67). Candidate proteins were identified by their molecular weight and ranked by their ability to distinguish between two classes of patients, acute rejection and no rejection, based on receiver operating characteristic (ROC) and Classification And Regression Trees (CART) analysis. The spectral data was able to correctly classify 91% of 34 urine specimens from the training set, giving a sensitivity of 83% and specificity of 100%. There was no validation set in this study.

A study in human kidney transplantation using the same technology, SELDI-TOF mass spectroscopy, profiled urinary protein spectra from five groups of subjects: acute rejection, acute tubular necrosis, recurrent or de novo glomerulopathy, stable transplant patients with excellent function and normal urine donor controls (68). Two distinct urine protein patterns were observed when comparing the normal controls and stable transplant groups to the acute rejection group. One urine protein profile (Rejection Pattern) had prominent peak clusters in three regions corresponding to Mass/Charge ratios (m/z values) of 5270 to 5550 (region I; 5 peaks), 7050 to 7360 (region II; 3 peaks), and 10530 to 11100 (region III; 5 peaks). These peaks always occurred together, whereas the urine protein profile for non-rejecting transplants and normal controls (Normal Pattern) had no peak clusters in the same m/z regions.

Another study using SELDI-TOF demonstrated that the identification of protein peaks by mass spectrometry from urine samples of renal transplant patients were all derived from non-tryptic cleaved forms of beta2-microglobulin (22). In vitro experiments showed that cleavage of intact beta2-microglobulin requires a urine pH less than 6 and the presence of aspartic proteases. However, patients with acute tubulointerstitial rejection had lower urine pH than stable transplants and healthy individuals and also start with higher amounts of aspartic proteases and intact beta2-microglobulin in urine. These factors ultimately lead to increased amounts of cleaved urinary beta2-microglobulin. They proposed that cleaved beta2-microglobulin, as an indicator of acute tubular injury, may be a useful tool for noninvasive monitoring of kidney allografts.

A more recent study looked at the differentiation of BK virus-associated nephropathy from acute allograft rejection in kidney transplant recipients (69). This is a major clinical problem because both classes of patients will present with acute kidney transplant dysfunction. Yet, the treatment of acute rejection with intensification of immunosuppressive therapy is the exact opposite strategy to the reduction in immunosuppression that is indicated for the patient with BK virus nephropathy. Using SELDI-TOF and bioinformatics they found that five SELDI peaks corresponding to Mass to Charge ratios (m/z values) of 5.872, 11.311, 11.929, 12.727, and 13.349 kD, were significantly higher in patients with BK virus-associated nephropathy. They concluded that proteomic analysis of urine might offer a noninvasive way to differentiate this condition from acute rejection.

Metabonomics

A major aspect of organismal biology is the metabolism and elimination of proteins, hormones and exogenous molecules including drugs. While we have already mentioned some studies of urine in transplantation, the metabolome or universe of molecular metabolites, can also be studied in other body fluids including blood, bile and saliva. It is also important to point out here that metabolomics and metabonomics are generally interchangeable terms. One focus of pharmacogenomics is the metabolism of drugs into detectable byproducts that could be novel biomarkers of greater value than measurements of intact molecule drug levels if they were associated with therapy responses to a specific disease state, for example, acute kidney transplant rejection. In fact, if a given drug therapy resulted in a set of molecular events that created a unique metabolome detected in blood plasma, for example, these metabolic biomarkers could be highly specific as metrics for therapeutic efficacy but actually not be comprised of any of the metabolites of the drug. In other settings, it is hoped that metabonomic profiles of drugs will also correlate with unwanted and dangerous side effects that could be used to enhance the safety of drug therapy.

Though we are not aware of publications at this time for NMR spectroscopy applied to transplant patients in any clinical setting, we provide two examples of its application to urine to suggest that transplantation and the host responses to tissue injury and immune rejection would be another important area to explore. Thus, NMR spectroscopy of urine and plasma samples was used to examine early graft dysfunction in a pig ischemia/reperfusion model (70). The aim of the study was to assess the ability of ^1H NMR spectroscopy of urine and plasma samples to predict early graft dysfunction after organ preservation in two commonly used preservation solutions, Euro-Collins (EC) and University of Wisconsin (UW). Kidneys were examined within 40 minutes after implantation and two weeks later. Fractional excretion of sodium and urine N-acetyl- β -d-glucosaminidase activity were improved but not significantly different in the experimental groups. Urinary concentrations of the alpha-class glutathione S-transferase were significantly greater in the EC group during the first week after transplantation. The most relevant ^1H NMR resonances for evaluating renal function after transplantation were those arising from citrate, dimethylamine (DMA), lactate, and acetate in

urine and trimethylamine-N-oxide (TMAO) in urine and plasma. These results suggest that graft dysfunction is associated with damage to the renal medulla reflected by TMAO released into the urine and plasma and that ^1H NMR resonances are sensitive enough to follow these changes in vivo.

Another study used ^1H -NMR spectroscopy in combination with pattern recognition tools to investigate the composition of organic compounds in urine from patients with multiple sclerosis, patients with other neurological diseases and healthy controls (71). Using the marmoset monkey model of experimental autoimmune encephalomyelitis (EAE), the relation of disease progression and alteration of the urine composition was investigated and compared to the measurements obtained with the human patient samples. We refer to this parallel study of human patients and a comparable animal model as “convergent genomics”. That this study obtained correlations between the metabonome of humans and animals with systemic autoimmune disease supports the potential of finding similar correlations in patients with kidney, liver and heart transplants.

A recent study has led to the development of a new statistical paradigm to co-analyze NMR and ultra performance liquid chromatography combined with orthogonal acceleration tof-MS (UPLC/oa-tof-MS) data (72,73). This method analyzes the intrinsic co-variance between signal intensities in the same and related molecules measured by the two techniques across different samples of urine. This makes it possible to establish a cross-correlation between the chemical shifts from NMR and the m/z fragmentation pattern determined by mass spectroscopy, which improves the efficiency of biomarker identification. This approach might also be of great value for exploring pathways in drug therapy for various metabolic states associated with disease. These papers are a perfect example of how the field is being advanced by the integration of multiple state-of-the-art technologies that are woven together by novel computing algorithms.

Finally, another source for metabonomic biomarkers is the low-molecular-weight range serum proteome, the peptidome, which may also contain disease-specific information (74). If so, it is an untapped resource of candidates for new and specific biomarkers, since it is comprised of a multitude of small protein fragments that present a “recording” or a “snapshot” of events taking place at the level of disease-associated microenvironments. Since intact tissue proteins are too large to passively diffuse through the cell and across the endothelial basement membranes into the circulation, the peptidome could provide an accessible portal to identify and quantify a wide range of protein changes that are taking place in all the cells and tissues.

Few or many: the eternal paradox of choices

Currently there are two approaches to biomarker discovery. The first one is a hypothesis-driven approach in which a candidate molecule provides the target for which a biomarker is developed. Typically, such hypothesis-driven research looks at individual candidate genes or proteins or at most a small molecular network. There are several pitfalls to this approach. First, any disease is the cell's or tissue's response to its microenvironment and this response is usually not governed by a single molecule, but rather a complex interplay of many pathways, cascades and networks of molecules. Second, cells or tissues interact with their surroundings in many ways. For example, via interactions with neighboring cells in direct physical contact or more remotely by chemical signals such as cytokines or growth factors that can trigger various cellular responses at a distant site. A single molecule or several in a single network cannot possibly direct and regulate these complex responses. Another possible confounder in a single biomarker approach is the very large variation or heterogeneity in patient populations. This heterogeneity can effectively negate the efficacy of a single biomarker, especially if it has not been validated in a large or diverse enough population. In fact, another important trend in biomarker development is essentially to accept this limitation of population heterogeneity and

focus biomarker validations on only specific subpopulations. At its most extreme implementation, one could imagine a future of “personalized medicine” where each patient essentially has a unique set of biomarkers. Frankly, we see this as a very distant future as it is rather impractical given the current technology and state of the art.

A different approach is looking for combinations or relatively large panels of biomarkers to diagnose disease conditions. We believe that significantly increasing the dimensionality of biomarker discovery will prove to be a more effective and accurate approach. Moreover, the ongoing development of genomic technologies is clearly enabling multidimensional, high through-put studies. These techniques allow an agnostic approach to biomarker discovery that eliminates the need to identify targets based a priori on specific biological knowledge of mechanisms and associations. They also enable the whole human genome to be screened for RNA transcripts simultaneously, the proteome by tandem mass spectroscopy, and the chemical signatures of cellular processes by metabonomics using nuclear magnetic resonance (NMR) spectroscopy combined with mass spectrometry. The readouts from these approaches include complicated patterns of gene expression and mass spectroscopic profiles or signatures, which consist of hundreds to thousands of candidate genes, proteins and metabolites that can serve as highly specific biomarker sets.

Biomarker validation and introduction into clinical practice

We need to establish the relationships between the pathogenetic mechanisms of disease and the universe of biomarkers that is discovered to be highly correlated. A key point is that a biomarker suitable for clinical use is defined by our ability to use its measurement as an indicator that a disease is present, that predicts a certain prognosis or disease phenotype or suggests a specific treatment intervention is indicated or effective. On the other hand, within the total set of biomarkers that might be correlated with a given disease, there will be a critical subset that are the actual determinants or what we would call “drivers” of pathophysiology. These “driver genes” could be expressed at only critical time points in the evolution of disease in which case a different set of driver genes might be identified and followed as a function of time during a disease's progression. Alternatively, driver genes might be expressed in only very specific tissue compartments. If these compartments are readily sampled, like urine and blood, then it could be relatively straightforward to incorporate them into clinical strategies. Therefore, in our studies of transplantation, we have focused on discovery of biomarkers in the transplant biopsies as well as in the peripheral blood, while others have focused on urine.

Once a set of correlative biomarkers has been identified, the next challenge is to validate these candidates. Validation of a set of biomarkers implies that they are early predictors of clinical disease or measures of risk, response to therapy and/or outcomes. A critical point is that biomarker validation must be done in real clinical settings. Generally, there are three basic steps that a candidate molecule must take before it can be considered a valid biomarker. The first step must experimentally optimize the assay conditions to increase sensitivity, reliability and specificity of the assay. The assay must also be reproducible and it must be capable of introduction into a standard clinical laboratory. For example, a tandem mass spectrometry proteomic assay for chronic transplant rejection that takes a full day to acquire the data for a single sample and another three days to analyze it is not going to work. The second step is biomarker characterization, which evaluates variability of a particular biomarker or set of biomarkers in human populations to determine relevant interactions and potential confounders. Many variables exist in real clinical patients, such as age, sex, ethnicity as well as diet, environment, drug and multiple disease interactions. The third step involves studies that aim to establish the possibility of a causal relationship between a biomarker candidate and a disease state. In our terms this would define discovery of a “driver gene” or functional biomarker.

The most common strategy for validation is the cohort approach, for example, studying patients with acute kidney transplant rejection defined by biopsy histology and kidney failure. In the context of specifically identifying the functional biomarkers within the set of all the correlative biomarkers found, the cohort approach avoids the problems of reverse causality that can occur when the downstream effects of the disease create the biomarkers (75). Urinary aflatoxin biomarkers, associated with the risk of hepatocellular carcinoma, have been validated in several studies using the cohort approach (76,77). However, at the end of the validation process, it is very important to remind the reader that the candidates that do not meet the criteria for functional biomarkers will be the vast majority of the starting set. Nonetheless, the entire biomarker set will be very valuable for clinical use even if only a small number of the full set is finally used in routine practice purely for pragmatic and technological reasons. Indeed, it is also highly likely that many functional biomarkers will not be identified for many years after their correlation with disease states and therapy have been established and this reflects the gaps in our understanding of systems biology.

Data that is generated using modern genomic technologies are becoming increasingly more complex and large data sets created with microarrays, proteomics and metabonomics need to be efficiently handled, processed, archived and searched. At the turn of the millennium, genomic information was largely absent from the investigational new drug (IND) submissions or new drug applications received by the FDA. Today that situation is rapidly changing and genomic data is being used to discover and validate new drug and biomarker candidates. A key question for the FDA is how suitable these new, high dimensionality technologies are for use in clinical trials of new drug therapies. If validated at this level, the hope of all of us doing clinical trials is that these new biomarker technologies will reduce the cost and time required to do a study and at the same time increase the safety and value.

The publication of studies with dissimilar or totally contradictory results, obtained using different DNA microarray platforms to analyze identical samples, has often raised concerns about the reliability of this technology. The MicroArray Quality Control (MAQC) project was developed in collaboration with the FDA to address these concerns. Performance and data analysis issues were studied to compare inter- and intra-platform reproducibility of gene expression measurements on most of the currently used commercial microarray platforms (78). The results showed high intra-platform consistency across test sites as well as a high level of inter-platform concordance in terms of genes identified as differentially expressed. These results represent an important first step toward establishing a framework of rules and criteria for the use of microarrays in a clinical setting.

A recent study compared the results of one- and two-color labeling on three different microarray platforms (79). In two-color designs, the control is co-hybridized with the experimental sample and only the differences identified. The data were evaluated for reproducibility, specificity, sensitivity and accuracy to determine if the two approaches provide comparable results. Cumulatively, these comparisons indicate that data quality is essentially equivalent between the one- and two-color approaches and strongly suggest that this variable is not a concern for decisions regarding experimental microarray design.

Another key study in the Microarray Quality Control Consortium involved ten voluntary data submissions that contained microarray data (80). They found that the results of microarray studies are heavily dependent on the quality of the starting material, the data analysis protocol and the statistical and biological pathway analysis tools subsequently available to interpret lists of statistically significant or differentially expressed genes.

Thus, it is important that we emphasize here that the approaches to the experiment as well as its data analysis are the most important variables, not the actual technology platforms. The

FDA now mandates that it is critical for investigators and sponsors of genomic data submissions to include a precise description of the steps involved before the actual array experiment, including the method of sample collection, storage, RNA extraction and labeling, as well as the data analysis protocol and biological pathway interpretation tools applied to these data. Another key step in introducing to clinical practice the validated genomic biomarkers obtained originally from DNA microarrays is moving the candidate genes onto a lower density platform, like real-time quantitative PCR, that will be more amenable to high-throughput and standardized clinical laboratory protocols. It is also important to note that these same issues for proteomics and metabonomics are just beginning to be addressed and that will be critically important if we are to see the integration of these different technologies. However, all the validation work described above for DNA microarrays was accomplished in only three or four years.

Conclusions

Until recently, the search for new biomarkers was excruciatingly slow. Often, it took years to associate some measurable parameter of physiology, such as blood cholesterol with the risk of heart disease. Understanding why a particular marker was associated with a disease could take decades longer, as clinicians waited for basic science to catch up with their medical observations. The field of biomarker research is much more promising today than it was 30 years ago. Currently, the new technologies for genomics developed in the Era of the Human Genome can identify hundreds and even thousands of biomarker candidates in a relatively high-throughput fashion. While such a large number of potential candidate biomarkers creates its own set of challenges for proper validation and integration into a knowledge of biological mechanisms, the field has embraced these challenges by developing new biostatistical and bioinformatics approaches. In this sense, one might suggest that the next phase will be the Era of Molecular Networks. We envision a future for biomarkers where these can be used to diagnose disease in its early stages, predict prognosis, suggest treatment options and then assist in the implementation of therapies. Of course, the reality is that there is still a lot of work to be done before this can become true. Because of the life and death nature of end stage organ failure that transplantation treats, the severe donor organ shortage, and the powerful and toxic drug therapies required for the lifetimes of transplant patients, the potential of new biomarkers for early and improved diagnosis, monitoring, and risk assessment are of great importance. We believe that harnessing the power of multiple technologies in parallel for gene and protein expression profiling and metabonomics is the best strategy to discover and then validate the next generation of biomarkers. At the same time, we believe that we can also go from biomarker discovery to novel understandings of the biology of health and disease. In this way, the road ahead takes two paths, one from biomarkers to diagnosis and therapy and the other to a new level of insight into the complex molecular networks that determine when a healthy cell or tissue becomes diseased and dysfunctional.

References

1. BDW. Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. *Clin Pharmacol Ther* 2001;69(3):89–95. [PubMed: 11240971]
2. Gold P, Freedman SO. Demonstration Of Tumor-Specific Antigens In Human Colonic Carcinomata By Immunological Tolerance And Absorption Techniques. *J Exp Med* 1965;121:439–62. [PubMed: 14270243]
3. Allhoff EP, Proppe KH, Chapman CM, Lin CW, Prout GR Jr. Evaluation of prostate specific acid phosphatase and prostate specific antigen in identification of prostatic cancer. *J Urol* 1983;129(2): 315–8. [PubMed: 6339742]
4. Kuriyama M, Wang MC, Lee CI, Papsidero LD, Killian CS, Inaji H, et al. Use of human prostate-specific antigen in monitoring prostate cancer. *Cancer Res* 1981;41(10):3874–6. [PubMed: 7284995]

5. Hayes DF, Thor AD. c-erbB-2 in breast cancer: development of a clinically useful marker. *Semin Oncol* 2002;29(3):231–45. [PubMed: 12063676]
6. Masood S, Bui MM. Prognostic and predictive value of HER2/neu oncogene in breast cancer. *Microsc Res Tech* 2002;59(2):102–8. [PubMed: 12373720]
7. O'Brien PJ, Dameron GW, Beck ML, Kang YJ, Erickson BK, Di Battista TH, et al. Cardiac troponin T is a sensitive, specific biomarker of cardiac injury in laboratory animals. *Lab Anim Sci* 1997;47(5):486–95. [PubMed: 9355091]
8. Alpert JS, Thygesen K, Antman E, Bassand JP. Myocardial infarction redefined--a consensus document of The Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction. *J Am Coll Cardiol* 2000;36(3):959–69. [PubMed: 10987628]
9. Antman EM, Tanasijevic MJ, Thompson B, Schactman M, McCabe CH, Cannon CP, et al. Cardiac-specific troponin I levels to predict the risk of mortality in patients with acute coronary syndromes. *N Engl J Med* 1996;335(18):1342–9. [PubMed: 8857017]
10. Galvani M, Ottani F, Ferrini D, Ladenson JH, Destro A, Baccos D, et al. Prognostic influence of elevated values of cardiac troponin I in patients with unstable angina. *Circulation* 1997;95(8):2053–9. [PubMed: 9133515]
11. Adams JE 3rd, Abendschein DR, Jaffe AS. Biochemical markers of myocardial injury. Is MB creatine kinase the choice for the 1990s? *Circulation* 1993;88(2):750–63. [PubMed: 8339435]
12. Apple FS, Murakami MM. Cardiac troponin and creatine kinase MB monitoring during in-hospital myocardial reinfarction. *Clin Chem* 2005;51(2):460–3. [PubMed: 15563477]
13. Jernberg T, Lindahl B, James S, Larsson A, Hansson LO, Wallentin L. Cystatin C: a novel predictor of outcome in suspected or confirmed non-ST-elevation acute coronary syndrome. *Circulation* 2004;110(16):2342–8. [PubMed: 15477399]
14. Sarnak MJ, Katz R, Stehman-Breen CO, Fried LF, Jenny NS, Psaty BM, et al. Cystatin C concentration as a risk factor for heart failure in older adults. *Ann Intern Med* 2005;142(7):497–505. [PubMed: 15809461]
15. Shlipak MG, Katz R, Fried LF, Jenny NS, Stehman-Breen CO, Newman AB, et al. Cystatin-C and mortality in elderly persons with heart failure. *J Am Coll Cardiol* 2005;45(2):268–71. [PubMed: 15653026]
16. Pelsers MM, Hermens WT, Glatz JF. Fatty acid-binding proteins as plasma markers of tissue injury. *Clin Chim Acta* 2005;352(1–2):15–35. [PubMed: 15653098]
17. Boesken WH, Schmidt M, Jontofsohn R, Heinze V. Proteinuria as diagnostic marker after human kidney transplantation. *Proc Eur Dial Transplant Assoc* 1975;11:333–42. [PubMed: 1105554]
18. Krishna GG, Fellner SK. Lymphocyturia: an important diagnostic and prognostic marker in renal allograft rejection. *Am J Nephrol* 1982;2(4):185–8. [PubMed: 6756146]
19. Maury CP, Teppo AM. Comparative study of serum amyloid-related protein SAA, C-reactive protein, and beta 2-microglobulin as markers of renal allograft rejection. *Clin Nephrol* 1984;22(6):284–92. [PubMed: 6441659]
20. Steinhauer HB, Wilms H, Schollmeyer P. Thromboxane B2 and beta 2-microglobulin as early indicators of renal allograft rejection. *Proc Eur Dial Transplant Assoc Eur Ren Assoc* 1985;21:1032–6. [PubMed: 3887370]
21. Oetting WS, Rogers TB, Krick TP, Matas AJ, Ibrahim HN. Urinary beta2-microglobulin is associated with acute renal allograft rejection. *Am J Kidney Dis* 2006;47(5):898–904. [PubMed: 16632030]
22. Schaub S, Wilkins JA, Antonovici M, Krokhin O, Weiler T, Rush D, et al. Proteomic-based identification of cleaved urinary beta2-microglobulin as a potential marker for acute tubular injury in renal allografts. *Am J Transplant* 2005;5(4 Pt 1):729–38. [PubMed: 15760396]
23. Hayman JM, Halsted JA, Seyler LE. A Comparison Of The Creatinine And Urea Clearance Tests Of Kidney Function. *J Clin Invest* 1933;12(5):861–75. [PubMed: 16694170]
24. Steinitz K, Turkand H. The Determination Of The Glomerular Filtration By The Endogenous Creatinine Clearance. *J Clin Invest* 1940;19(2):285–98. [PubMed: 16694745]
25. Buchler M, Bretagnol A, Desaldeleer C, Garreau P, Halimi JM, Al-Najjar A, et al. Estimation of the glomerular filtration rate through different methods in kidney transplant recipients: correlation with the creatinine clearance measurement. *Transplant Proc* 2006;38(7):2289–91. [PubMed: 16980067]

26. Lee BM, Yoon SN, Oh CK, Kim JH, Kim SJ, Kim H, et al. Fractional creatinine clearance of the donated kidney using Cockcroft-Gault formula as a predictor of graft function after living donor transplantation. *Transplant Proc* 2006;38(7):1974–6. [PubMed: 16979969]
27. Pitsavos C, Kurlaba G, Panagiotakos DB, Kogias Y, Mantas Y, Chrysoshoou C, et al. Association of creatinine clearance and in-hospital mortality in patients with acute coronary syndromes: the GRECCS study. *Circ J* 2007;71(1):9–14. [PubMed: 17186971]
28. Lipman ML, Stevens AC, Strom TB. Heightened intragraft CTL gene expression in acutely rejecting renal allografts. *J Immunol* 1994;152(10):5120–7. [PubMed: 8176228]
29. Strehlau J, Pavlakakis M, Lipman M, Maslinski W, Shapiro M, Strom TB. The intragraft gene activation of markers reflecting T-cell-activation and -cytotoxicity analyzed by quantitative RT-PCR in renal transplantation. *Clin Nephrol* 1996;46(1):30–3. [PubMed: 8832147]
30. Strehlau J, Pavlakakis M, Lipman M, Shapiro M, Vasconcellos L, Harmon W, et al. Quantitative detection of immune activation transcripts as a diagnostic tool in kidney transplantation. *Proc Natl Acad Sci U S A* 1997;94(2):695–700. [PubMed: 9012847]
31. Simon T, Opelz G, Wiesel M, Ott RC, Susal C. Serial peripheral blood perforin and granzyme B gene expression measurements for prediction of acute rejection in kidney graft recipients. *Am J Transplant* 2003;3(9):1121–7. [PubMed: 12919092]
32. Shin GT, Kim SJ, Lee TS, Oh CK, Kim H. Gene expression of perforin by peripheral blood lymphocytes as a marker of acute rejection. *Nephron Clin Pract* 2005;100(3):c63–70. [PubMed: 15824509]
33. Li B, Hartono C, Ding R, Sharma VK, Ramaswamy R, Qian B, et al. Noninvasive diagnosis of renal-allograft rejection by measurement of messenger RNA for perforin and granzyme B in urine. *N Engl J Med* 2001;344(13):947–54. [PubMed: 11274620]
34. Afzali B, Lombardi G, Lechler RI, Lord GM. The role of T helper 17 (Th17) and regulatory T cells (Treg) in human organ transplantation and autoimmune disease. *Clin Exp Immunol*. 2007
35. Hori S, Nomura T, Sakaguchi S. Control of regulatory T cell development by the transcription factor Foxp3. *Science* 2003;299(5609):1057–61. [PubMed: 12522256]
36. Khattri R, Kasprovicz D, Cox T, Mortrud M, Appleby MW, Brunkow ME, et al. The amount of scurf protein determines peripheral T cell number and responsiveness. *J Immunol* 2001;167(11):6312–20. [PubMed: 11714795]
37. Stock P, Akbari O, Berry G, Freeman GJ, Dekruyff RH, Umetsu DT. Induction of T helper type 1-like regulatory cells that express Foxp3 and protect against airway hyper-reactivity. *Nat Immunol* 2004;5(11):1149–56. [PubMed: 15448689]
38. Waldmann H, Graca L, Cobbold S, Adams E, Tone M, Tone Y. Regulatory T cells and organ transplantation. *Semin Immunol* 2004;16(2):119–26. [PubMed: 15036235]
39. Wood KJ, Luo S, Akl A. Regulatory T cells: potential in organ transplantation. *Transplantation* 2004;77(1 Suppl):S6–8. [PubMed: 14726760]
40. Muthukumar T, Dadhania D, Ding R, Snopkowski C, Naqvi R, Lee JB, et al. Messenger RNA for FOXP3 in the urine of renal-allograft recipients. *N Engl J Med* 2005;353(22):2342–51. [PubMed: 16319383]
41. Veronese F, Rotman S, Smith RN, Pelle TD, Farrell ML, Kawai T, et al. Pathological and Clinical Correlates of FOXP3 Cells in Renal Allografts during Acute Rejection. *Am J Transplant*. 2007
42. Kamoun M, Boyd JC. Urinary FOXP3 messenger RNA and renal-allograft rejection. *N Engl J Med* 2006;354(21):2291–3. [PubMed: 16723625]author reply 2291–3
43. Aujame L, Burdin N, Vicari M. How microarrays can improve our understanding of immune responses and vaccine development. *Ann N Y Acad Sci* 2002;975:1–23. [PubMed: 12538150]
44. Chtanova T, Kemp RA, Sutherland AP, Ronchese F, Mackay CR. Gene microarrays reveal extensive differential gene expression in both CD4(+) and CD8(+) type 1 and type 2 T cells. *J Immunol* 2001;167(6):3057–63. [PubMed: 11544289]
45. Glynne RJ, Watson SR. The immune system and gene expression microarrays--new answers to old questions. *J Pathol* 2001;195(1):20–30. [PubMed: 11568888]
46. Haddad R, Guardiola P, Izac B, Thibault C, Radich J, Delezoide AL, et al. Molecular characterization of early human T/NK and B-lymphoid progenitor cells in umbilical cord blood. *Blood* 2004;104(13):3918–26. [PubMed: 15331438]

47. Ishii KJ, Coban C, Kato H, Takahashi K, Torii Y, Takeshita F, et al. A Toll-like receptor-independent antiviral response induced by double-stranded B-form DNA. *Nat Immunol* 2006;7(1):40–8. [PubMed: 16286919]
48. Flechner SM, Kurian SM, Head SR, Sharp SM, Whisenant TC, Zhang J, et al. Kidney transplant rejection and tissue injury by gene profiling of biopsies and peripheral blood lymphocytes. *Am J Transplant* 2004;4(9):1475–89. [PubMed: 15307835]
49. Flechner SM, Kurian SM, Solez K, Cook DJ, Burke JT, Rollin H, et al. De novo kidney transplantation without use of calcineurin inhibitors preserves renal structure and function at two years. *Am J Transplant* 2004;4(11):1776–85. [PubMed: 15476476]
50. Sarwal M, Chua MS, Kambham N, Hsieh SC, Satterwhite T, Masek M, et al. Molecular heterogeneity in acute renal allograft rejection identified by DNA microarray profiling. *N Engl J Med* 2003;349(2):125–38. [PubMed: 12853585]
51. Stegall M, Park W, Kim D, Kremers W. Gene expression during acute allograft rejection: novel statistical analysis of microarray data. *Am J Transplant* 2002;2(10):913–25. [PubMed: 12482143]
52. Borožan I, Chen L, Sun J, Tannis LL, Guindi M, Rotstein OD, et al. Gene expression profiling of acute liver stress during living donor liver transplantation. *Am J Transplant* 2006;6(4):806–24. [PubMed: 16539639]
53. Flohe S, Speidel N, Flach R, Lange R, Erhard J, Schade FU. Expression of HSP 70 as a potential prognostic marker for acute rejection in human liver transplantation. *Transpl Int* 1998;11(2):89–94. [PubMed: 9561674]
54. Yagi T, Iwagaki H, Urushihara N, Kobashi K, Nakao A, Matsukawa H, et al. Participation of IL-18 in human cholestatic cirrhosis and acute rejection: analysis in living donor liver transplantation. *Transplant Proc* 2001;33(1–2):421–5. [PubMed: 11266892]
55. Alpert S, Lewis NP, Ross H, Fowler M, Valentine HA. The relationship of granzyme A and perforin expression to cardiac allograft rejection and dysfunction. *Transplantation* 1995;60(12):1478–85. [PubMed: 8545878]
56. Horwitz PA, Tsai EJ, Putt ME, Gilmore JM, Lepore JJ, Parmacek MS, et al. Detection of cardiac allograft rejection and response to immunosuppressive therapy with peripheral blood gene expression. *Circulation* 2004;110(25):3815–21. [PubMed: 15583081]
57. Mehra MR, Uber PA, Walther D, Vesely M, Wohlgemuth JG, Prentice J, et al. Gene expression profiles and B-type natriuretic peptide elevation in heart transplantation: more than a hemodynamic marker. *Circulation* 2006;114(1 Suppl):I21–6. [PubMed: 16820574]
58. Gimino VJ, Lande JD, Berryman TR, King RA, Hertz MI. Gene expression profiling of bronchoalveolar lavage cells in acute lung rejection. *Am J Respir Crit Care Med* 2003;168(10):1237–42. [PubMed: 12958056]
59. Lande JD, Patil J, Li N, Berryman TR, King RA, Hertz MI. Novel insights into lung transplant rejection by microarray analysis. *Proc Am Thorac Soc* 2007;4(1):44–51. [PubMed: 17202291]
60. Deng MC, Eisen HJ, Mehra MR. Methodological challenges of genomic research--the CARGO study. *Am J Transplant* 2006;6(5 Pt 1):1086–7. [PubMed: 16611349]
61. Deng MC, Eisen HJ, Mehra MR, Billingham M, Marboe CC, Berry G, et al. Noninvasive discrimination of rejection in cardiac allograft recipients using gene expression profiling. *Am J Transplant* 2006;6(1):150–60. [PubMed: 16433769]
62. Eikmans M, Roos-van Groningen MC, Sijpkens YW, Ehrchen J, Roth J, Baelde HJ, et al. Expression of surfactant protein-C, S100A8, S100A9, and B cell markers in renal allografts: investigation of the prognostic value. *J Am Soc Nephrol* 2005;16(12):3771–86. [PubMed: 16251238]
63. Hotchkiss H, Chu TT, Hancock WW, Schroppel B, Kretzler M, Schmid H, et al. Differential expression of profibrotic and growth factors in chronic allograft nephropathy. *Transplantation* 2006;81(3):342–9. [PubMed: 16477218]
64. Mas V, Maluf D, Archer K, Yanek K, Mas L, King A, et al. Establishing the Molecular Pathways Involved in Chronic Allograft Nephropathy for Testing New Noninvasive Diagnostic Markers. *Transplantation* 2007;83(4):448–457. [PubMed: 17318078]
65. Pan TL, Wang PW, Huang CC, Goto S, Chen CL. Expression, by functional proteomics, of spontaneous tolerance in rat orthotopic liver transplantation. *Immunology* 2004;113(1):57–64. [PubMed: 15312136]

66. Borozdenkova S, Westbrook JA, Patel V, Wait R, Bolad I, Burke MM, et al. Use of proteomics to discover novel markers of cardiac allograft rejection. *J Proteome Res* 2004;3(2):282–8. [PubMed: 15113105]
67. Clarke W, Silverman BC, Zhang Z, Chan DW, Klein AS, Molmenti EP. Characterization of renal allograft rejection by urinary proteomic analysis. *Ann Surg* 2003;237(5):660–4. 664–5. [PubMed: 12724632]discussion
68. Schaub S, Rush D, Wilkins J, Gibson IW, Weiler T, Sangster K, et al. Proteomic-based detection of urine proteins associated with acute renal allograft rejection. *J Am Soc Nephrol* 2004;15(1):219–27. [PubMed: 14694176]
69. Jahnukainen T, Malehorn D, Sun M, Lyons-Weiler J, Bigbee W, Gupta G, et al. Proteomic analysis of urine in kidney transplant patients with BK virus nephropathy. *J Am Soc Nephrol* 2006;17(11):3248–56. [PubMed: 17035609]
70. Hauet T, Baumert H, Gibelin H, Godart C, Carretier M, Eugene M. Citrate, acetate and renal medullary osmolyte excretion in urine as predictor of renal changes after cold ischaemia and transplantation. *Clin Chem Lab Med* 2000;38(11):1093–8. [PubMed: 11156334]
71. Hart BA, Vogels JT, Spijksma G, Brok HP, Polman C, van der Greef J. 1H-NMR spectroscopy combined with pattern recognition analysis reveals characteristic chemical patterns in urines of MS patients and non-human primates with MS-like disease. *J Neurol Sci* 2003;212(1–2):21–30. [PubMed: 12809995]
72. Crockford DJ, Holmes E, Lindon JC, Plumb RS, Zirah S, Bruce SJ, et al. Statistical heterospectroscopy, an approach to the integrated analysis of NMR and UPLC-MS data sets: application in metabonomic toxicology studies. *Anal Chem* 2006;78(2):363–71. [PubMed: 16408915]
73. Williams RE, Lenz EM, Evans JA, Wilson ID, Granger JH, Plumb RS, et al. A combined (1)H NMR and HPLC-MS-based metabonomic study of urine from obese (fa/fa) Zucker and normal Wistar-derived rats. *J Pharm Biomed Anal* 2005;38(3):465–71. [PubMed: 15925248]
74. Liotta L, Petricoin E. A Revolutionary Approach to Biomarker Discovery. *The Scientist* 2006;20(11):32–9.
75. Munoz A, Gange SJ. Methodological issues for biomarkers and intermediate outcomes in cohort studies. *Epidemiol Rev* 1998;20(1):29–42. [PubMed: 9762507]
76. Ross RK, Yuan JM, Yu MC, Wogan GN, Qian GS, Tu JT, et al. Urinary aflatoxin biomarkers and risk of hepatocellular carcinoma. *Lancet* 1992;339(8799):943–6. [PubMed: 1348796]
77. Wang LD, Zhou Q, Hong JY, Qiu SL, Yang CS. p53 protein accumulation and gene mutations in multifocal esophageal precancerous lesions from symptom free subjects in a high incidence area for esophageal carcinoma in Henan, China. *Cancer* 1996;77(7):1244–9. [PubMed: 8608498]
78. Shi L, Reid LH, Jones WD, Shippy R, Warrington JA, Baker SC, et al. The MicroArray Quality Control (MAQC) project shows inter- and intraplatform reproducibility of gene expression measurements. *Nat Biotechnol* 2006;24(9):1151–61. [PubMed: 16964229]
79. Patterson TA, Lobenhofer EK, Fulmer-Smentek SB, Collins PJ, Chu TM, Bao W, et al. Performance comparison of one-color and two-color platforms within the MicroArray Quality Control (MAQC) project. *Nat Biotechnol* 2006;24(9):1140–50. [PubMed: 16964228]
80. Frueh FW. Impact of microarray data quality on genomic data submissions to the FDA. *Nat Biotechnol* 2006;24(9):1105–7. [PubMed: 16964222]

Figure 1.