PROTEOMICS ON THE DIAGNOSTIC HORIZON – LESSONS FROM RHEUMATOLOGY

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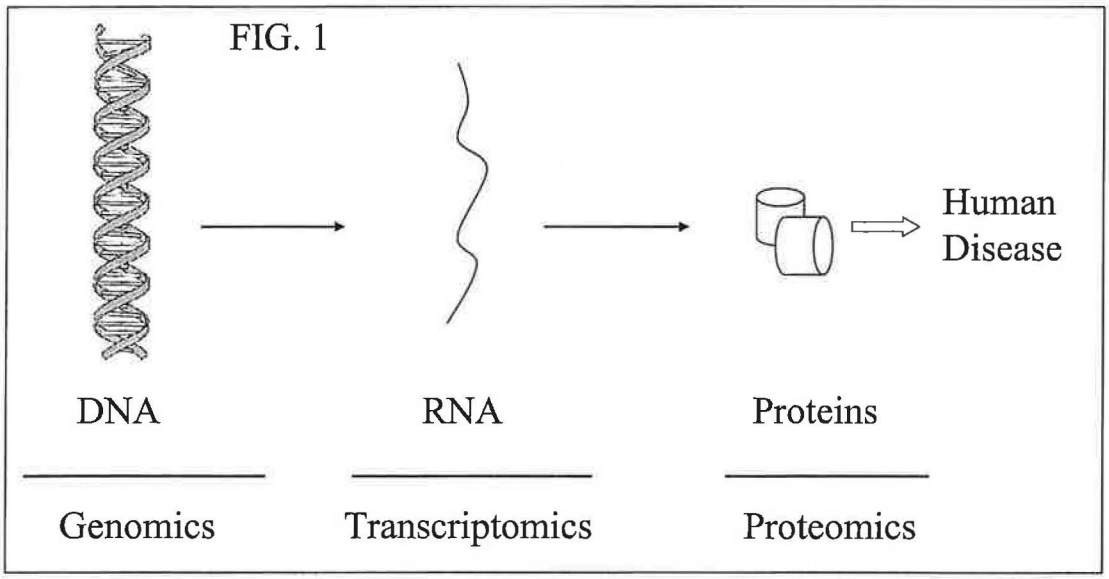
"DNA is not the true bottom line: .....Proteins embody the active life of cells, while nucleic acids represent only the plans" Norman G. Anderson, 1998.

The completion of the Human Genome Project in 2003 has laid before us the sequence of the human genome. Encoded in this blueprint are the genes that drive all biology and also dictate disease. Although the "blueprint" of human diseases may be encoded in the genome, it is clear that the execution of the disease occurs through altered protein function. Hence the wave of Genomics has been closely followed by a subsequent wave focused on studying RNA transcripts (Fig. 1).



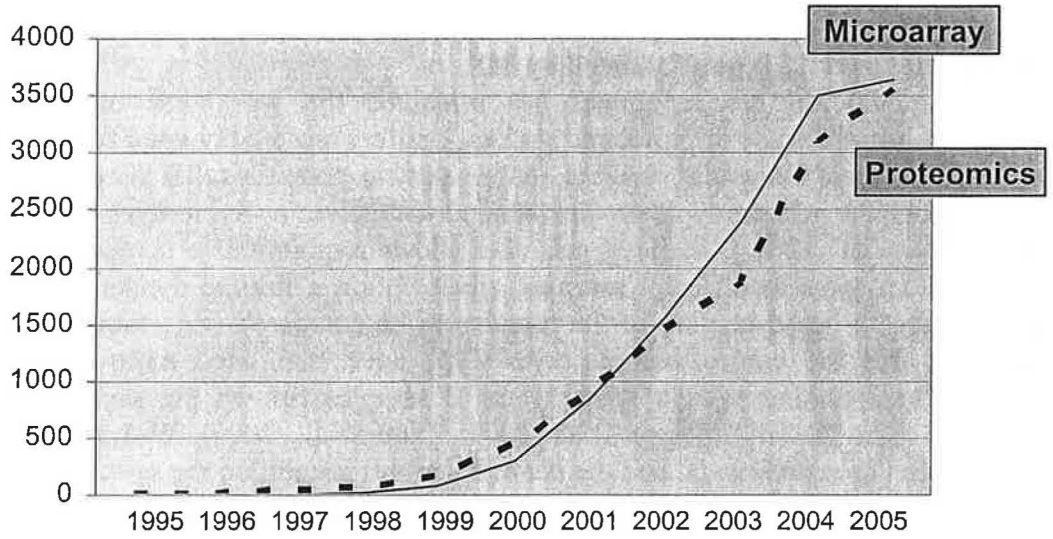
The development of DNA microhybridization has powered the functional genomics phase in which scientists have succeeded in scanning all of the messages encoded by the genome, termed the "transcriptome". Currently available DNA microarrays allow one to gauge the levels of expression of the different genes in the genome (at the RNA level) fairly comprehensively with a reasonable degree of accuracy and reproducibility. Over the past decade there has been an exponential rise in DNA microarray studies pervading all branches of Biomedicine (Fig. 2). This "functional genomics" or "transcriptomics" phase is rapidly giving way to the next "omics" wave, focusing on the expressed proteins (Fig. 1).

The US NHGRI (National Human Genome Research Institute) has identified the capturing of "an accurate census of the proteins present in particular cell types under different physiological conditions" as being one of the grand challenges facing us in the coming years (Collins et al., 2003). This in essence embodies the science of proteomics. Given that DNA microarrays already capture expressed messages (i.e., RNA) fairly comprehensively, one may ask if there is any added benefit in attempting to gauge the level of expression of the different genes at the protein level across the genome. There are at least 2 compelling reasons why proteomics may offer additional insights.

First, there is good evidence that although there is a general trend for protein concentration to rise with mRNA levels, the actual correlation is weak, and protein concentrations can vary by more than 2 orders of magnitude for any given mRNA level (Greenbaum 2003; Gygi et al., 1999). It is clear that whereas the levels of some proteins are controlled at the mRNA level, others are controlled at the translational and post-translational levels. Second, we now appreciate that although the human genome may be comprised of about 30,000 genes, the number of protein products encoded by these genes may range up to 100,000 or more due to post-translational modifications, alternate splicing etc. Since biological (and disease) phenotypes are ultimately dictated by proteins, a proteomic analysis rather than a transcriptomic analysis appears to have greater potential to shed light on the underlying biological/disease processes.

Although it is well accepted that a comprehensive analysis of the proteome is likely to be highly insightful, it is equally apparent that none of the currently available protein profiling technologies approach the capacity of the DNA microarrays in their ability to scan the *entire* proteome. Despite this limitation, several advances have been made over the past decade that allow scientists to capture snapshots of the proteome in a fairly reproducible fashion. The number of proteomic studies have been expanding at a near exponential rate

Fig. 2. The number of publications focusing on microarrays and proteomics over the past decade (based on PubMed search)



over the past decade (Fig. 2), with the growth being spear-headed by the studies in the field of cancer. Several of these studies have already yielded novel insights into the underlying disease processes, and in some instances have also uncovered potential biomarkers for the disease under investigation.

Applications in Rheumatology:

Given that current diagnosis and prognostication of systemic rheumatic diseases are still based on relatively complex criteria and since the prediction of flares or relapses is still an

inexact science, there is a clear need for better biomarkers in these diseases. Several of the proteomic approaches discussed in this overview have recently been applied in various rheumatic diseases (Fathman et al., 2005). The hope is that theses novel approaches will yield additional biomarkers that may assist the clinician in better managing patients with systemic rheumatic diseases – to identify RA patients who at risk for developing erosive, disabling disease, or to identify lupus patients who are at risk for developing end-stage renal disease, for example.

This review is organized as follows:

UNBIASED PROTEOMICS:
1A. 2Dgel → MS
1B. LC → MS
1C. SELDI → MS
1D. CE → MS

TARGETED PROTEOMICS
2A. Autoantibody discovery
2B. Analyte discovery

In general, the currently available methodologies can be classified into 2 broad categories – unbiased proteomic approaches and targeted (immuno-) proteomics. Unbiased proteomic approaches essentially encompass methodologies where no prior assumption is made about the nature of the proteins to be uncovered – hence, these approaches have the potential to highlight *any* subset of proteins in the entire proteome as being differentially expressed in a given disease state. Unbiased approaches may be gel-based (section 1A) or non-gel based (sections 1B-1D), as discussed further below. In contrast, the targeted or focused proteomic approaches restrict their study universe to a limited set of proteins – typically dictated by the available antigens or antibodies, as detailed further in sections 2A-2B. Reviewed below are the lessons we have learned (particularly with respect to rheumatic diseases) using these different proteomic approaches.

1A. Unbiased Proteomics: 2-D gels followed by MS

Two-dimensional (2-D) gel electrophoresis has remained the workhorse of protein separation over the initial decade of proteomic studies. It offers reasonably good separation of proteins based on their molecular weights and isoelectric potentials. To visualize the discrete protein spots, the gels are typically first stained with silver or Sypro dyes (offering a detection sensitivity of 1-2 ng). Staining gels also allows a quantitative comparison of corresponding protein spots in different samples, albeit within a limited dynamic range. More recent fluorescent based tagging of the proteins to be compared (e.g., labeling one sample with Cy3 and the control sample with Cy5) have facilitated more accurate comparisons of corresponding protein spots in the 2 samples run on the same gel, a technique referred to as "DIGE" (Tonge et al., 2001; Zhou et al., 2002). With the latter approach, since both the experimental and the control samples are run on the same gel, this allows both images to be perfectly overlaid without "warping". Typically these approaches have the potential to resolve about 1000 protein spots from complex tissue samples, with a detection sensitivity of 1-2 ng, based on the protein-labeling strategy used. Since the fluorescence based DIGE approach has a larger dynamic range of about 10^4, this also permits detection of less abundant proteins. Whereas 2-D gels serve to separate out the individual proteins into discrete spots, mass spectrometry (MS) serves to identify these spots. Once again, a variety of MS approaches are available, with MALDI-TOF (matrix-assisted laser desorption ionization-time of flight) constituting a commonly adopted approach.

In general, the 2-D gel based protein separation approach is conceptually simple and relatively inexpensive. On the other hand, 2-D gel based approaches have traditionally been inefficient at resolving proteins with extreme pI or molecular weights, as well as low-abundant and hydrophobic proteins. The effective resolution of this approach can generally be amplified by first pre-fractionating specific classes of proteins (e.g., membrane proteins, phosphoproteins, etc) before resolving them on the 2-D gels.

Applications in Rheumatology

This approach has been applied to almost all branches of Biomedicine, including Rheumatology. Interestingly, S100A9, a small calcium-binding protein, was highlighted as a potential candidate marker for rheumatoid arthritis by comparing the protein profiles of synovial fluid from rheumatoid arthritis and osteoarthritis patients (Sinz et al., 2002), using the 2D-gel/MS approach. This has subsequently been confirmed by another proteomic technique (see below).

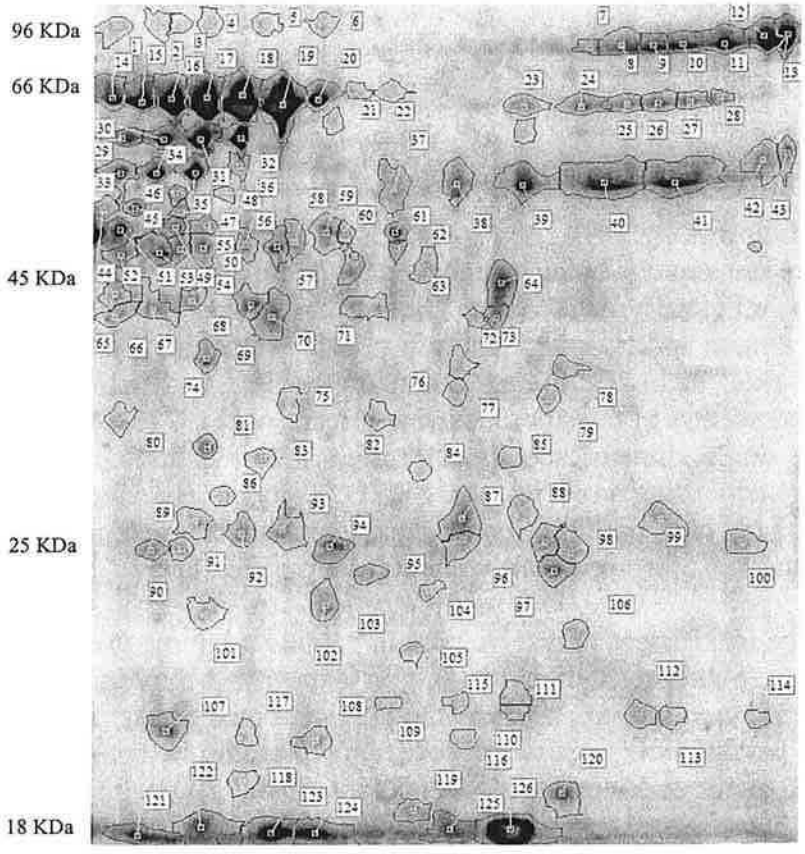
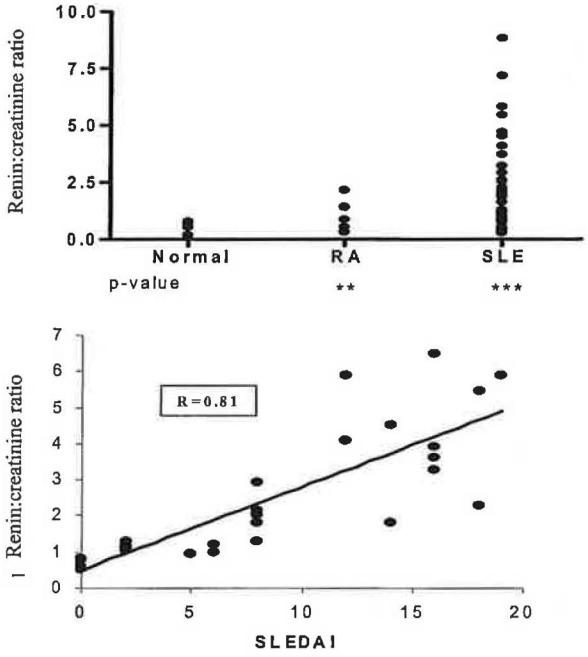


Fig. 3A. Urine samples from mice with immune/lupus nephritis were resolved on 2D-gels. A total of 126 protein spots that were significantly elevated in the urine from nephritic mice were identified by MS (Wu et al., 2005). Among these, 4 particular proteins, including renin (Fig. 3B) were noted to be elevated in the urine from patients with SLE (Wu et al., 2005).

Likewise, we have completed a comprehensive analysis of the proteins excreted in the urine during the course of immune/lupus nephritis using 2D-gels and MS analyses. A total of 126 voided proteins that were elevated in the urine from nephritic subjects were MS-identified (fig. 3A), of which 4 have been confirmed to elevated in larger sets of lupus nephritis patients, using an orthogonal method, as exemplified in Fig. 3B. Importantly, the urinary levels of these 4 molecules correlated well with disease severity and renal involvement (Fig. 3B, and Wu et al., 2005). Longitudinal studies are in progress to verify the utility of these 4 urinary molecules as potential biomarkers in lupus nephritis.

Fig. 3B. A total of four protein spots were identified as being elevated in the urine both in mice and patients with lupus nephritis, using 2D-gels (Fig. 3A, Wu et al., 2005), including renin. Importantly, the urinary levels of all 4 molecules correlated well with disease activity, as illustrated for renin.



1B. Unbiased Proteomics: LC followed by MS

Gel-based proteomic approaches are rapidly being supplanted by methods that involve peptide separation using high efficiency nanocolumn liquid chromatography (LC) separation techniques linked to a mass spectrometer. The use of LC overcomes many of the limitations inherent in 2-D gels, including the exclusion of proteins with extreme hydrophobicity or pI. In the initial version of this approach, one-dimensional LC (1-D LC) was performed. Typically, the single dimension adopted rested upon separating the peptides based on their hydrophobicity using a reveres-phase HPLC column. By coupling a 1-D LC system to a tandem MS, it is possible to distinguish individual proteins in complex mixtures containing more than 50 components without prior purification.

However, 1-D LC MS/MS may be insufficient to resolve protein/peptide mixtures that are more complex. More recently, a higher-resolution and higher-capacity 2-dimensional liquid chromatography technique coupled to tandem mass spectrometry has been introduced, which promises to be far more robust at identifying proteins in complex mixtures (Link et al., 1999; Delahunty & Yates, 2004; Delahunty, et al., 2005). In this novel approach dubbed as multi-dimensional protein identification technology (MudPIT) proteins are separated based on two physical properties – their charge (using a cation exchange column) as well as hydrophobicity (using a reveres phase column). The power of the 2D-LC MS/MS MudPIT approach in protein identification has been demonstrated in a variety of complex protein mixtures from a variety of sources, as reviewed (Delahunty, et al., 2005). This approach has also been reported to be relatively unbiased in that it has the capacity to resolve proteins with extreme pIs and sizes equally well. Moreover, it appears to be capable of resolving low-abundant proteins, membrane proteins and phosphoproteins fairly well (Delahunty & Yates, 2004; Delahunty, et al., 2005).

One prominent bottleneck of the MudPIT approach appears to be data-handling of the resulting MS spectra and computational protein identification. The challenges ahead are to further improve the dimensionality of the approach, further enhance its capability to detect low-abundant proteins, and to streamline the post-procedure data crunching steps so that reliable protein identification can be speeded up. An additional drawback of MS analysis has been the relatively non-quantitative nature of this methodology. It is heartening to note, however, that methods for stable isotope labeling of the sample proteins are rapidly paving the way for quantitative proteomics, as exemplified by the advent of ICAT (Gygi et al., 1999; Washburn et al., 2002).

Applications in Rheumatology

Pang et al (2002) have employed a variety of techniques including 2-D LC-MS to uncover inflammatory signatures in the urine. In that study they have verified the increase in a number of proteins, one of which was orosomucoid, which had previously been linked to inflammation. More recently, Liao et al (2004) have adopted a global proteomic approach to characterize the proteins profiles of synovial fluid samples from 10 RA patients with erosive versus non-erosive arthritis using LC/LC-MS/MS, after pre-fractionation of the synovial fluid to get rid of the more abundant proteins. The authors uncovered 30 different molecules that were elevated in the synovial fluid of RA patients with erosive arthritis compared to joint fluid of patients with non-erosive arthritis (N = 5 patients each), including various serum molecules (including CRP), metabolic enzymes and signaling molecules of the S100 family (Fig. 4). Among the novel molecules uncovered were 3 particular calcium binding proteins of the S100 family including S100A8 (calgranulin A), S100A9 (calganulin B), and S100A12 (calgranulin C), confirming previous findings obtained using the 2D-gel/MS approach (Sinz et al., 2002). The authors have also confirmed that these molecules were also elevated in the sera of RA patients. These S100 family molecules are presently being evaluated further for their potential to serve as disease markers in RA.

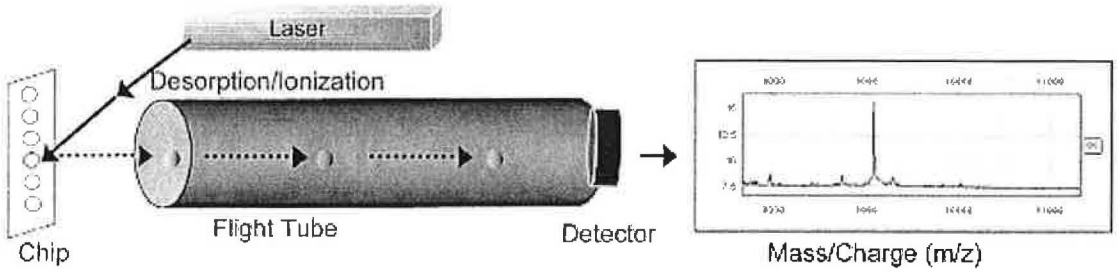
Fig. 4 Protein marker concentrations determined for serum pools from healthy controls, patients with nonerosive rheumatoid arthritis (RA), and patients with erosive RA, and the calculated concentration ratio between different samples[4]

	Serum concentration, ng/ml					
	S100A8	S100A9	S100A12	S100A4	S100A11	S100P
Healthy controls	8.4	8.7	5.3	43.8	10.7	9.4
Nonerosive RA	5.9	6.3	9.5	6.1	6.7	4.5
Erosive RA	28.4	123.0	80.1	6.1	8.7	6.4
Ratio, erosive RA/nonerosive RA	4.8	19.5	8.4	1.0	1.3	1.4
Ratio, erosive RA/healthy controls	3.4	14.1	15.1	0.1	0.8	0.7

1C. Unbiased Proteomics: SELDI-MS

One concern with the above approaches has been the high complexity of biological fluids and the "dynamic range problem" discussed further below. Furthermore, suitable software for interpretation of a vast quantity of MS spectra also posed a challenge. One approach to this dilemma was to reduce data complexity by collecting data on only a limited set of polypeptides (in a biological fluid) and also to reduce the effective resolution of the instrument. In effect, both of these were accomplished using the surface-enhanced laser desorption/ionization (SELDI) technology, which represents a classical solid-phase extraction chromatography coupled with laser desorption/ionization MS detection. In this approach, a complex solution of analytes (e.g., sera) is allowed to interact with an active solid surface (ion-exchange, hydrophobic surface, etc), laser bombarded/ionized and subsequently "identified" using MS (Fig. 5). The technical ease with which biological fluids could be compared using SELDI chips, the requirement for very small sample volumes without any pre-treatment, the high sensitivity (in the picomolar to femtomolar

Fig. 5. A brief description of the SELDI approach (adapted from Miyamae et al., 2005).
The samples to be studied are applied to active solid-surface chips pre-coated with different chemistries. The chip is next exposed to an ultraviolet nitrogen laser beam that dislodges the captured protein (desorption), transforming it into a gaseous ion (i.e., ionization) by protonization. The gaseous ions are accelerated in an electric field and "fly" to hit a detector. It is the variation in the time-of-flight (TOF) that allows for the separation of the different protein components originally captured on the chip surface. Hence, the mass/charge ratio of each peptide in the mixture translates to different TOF. These differences are captured as a proteomic "fingerprint" for each sample. By comparing the "fingerprints" of disease samples with that of controls, researchers are able to identify unique disease-associated signatures. The immediate challenge is to identify the actual peptide(s) that gives rise to the proteomic fingerprint(s).



range), the commercial availability of SELDI chips (and the corresponding software) and the relative simplicity of data interpretation has given birth to a large number of SELDI-based proteomic studies over the past 5 years, as reviewed (Coombes et al., 2005; Vidal et al., 2005). In particular, the SELDI approach has been useful in identifying proteins that are <10-20 kDa.

However, the SELDI based approach has been criticized for several reasons – low robustness, poor reproducibility, severely restricted in dynamic range, questionable sensitivity and specificity, inability to distinguish between experiment or technology specific variations from true biological variation, etc (Coombes et al., 2005; Diamandis et al., 2004; Naggerly et al., 2004, 2005). These critics have noted that mass spectrometry can be exquisitely sensitive to subtle differences in sample processing which could potentially impact the proteome being studied. MS can also be "temperamental", and small changes in the operating conditions can be amplified to produce large differences in the mass spectra, and these limitations appear to be more prevalent with the SELDI-based approach,

compared to the somewhat more robust traditional MALDI-based approach (Coombes et al., 2005). Improvement of the technology, removing any confounding factors in experimental design, and refining the peak-finding algorithms may augment the utility of this approach in the coming years.

This approach has been applied to the study of various disease states, with the cancer biologists taking the lead. In particular, its use in breast cancer, prostate cancer and ovarian cancer has helped uncover proteomic signatures that are uniquely expressed in the sera or other body fluids of disease patients (Tolson et al., 2004; Vlahou et al., 2003, 2004; Grizzle et al., 2004; Wadsworth et al., 2004; Petricoin et al., 2002; Li et al, 2002; Laronga et al., 2003; Adam et al., 2002; Xiao et al., 2001). Although the vast majority of these studies have simply identified differentially expressed spectral patterns, a couple have indeed succeeded in decoding the actual protein biomarker that is differentially expressed in disease. A good example of this is the identification of Hemoglobin as a serum biomarker that is elevated in ovarian cancer (Fig. 5, Christoph et al., 2001; Woong-Shick et al., 2005). These authors have reported that the combined monitoring of the biomarker they have uncovered (i.e., Hb) together with CA125 that was already in use, offered far superior sensitivity and specificity compared to the use of either marker alone (Woong-Shick et al., 2005). Similar ideas have also been put forward with respect to the use of multiple proteomic biomarkers in the diagnosis and clinical management of other malignancies (Li et al., 2002; Vlahou et al., 2001; Ludwig and Weinstein, 2005). The use of these SELDI chips has also extended to include several other biomedical fields (Calvo et al., 2005).

Applications in Rheumatology

More recently the SELDI approach has also been applied to a couple of rheumatic diseases. Miyamae and colleagues (2005) studied the serum protein profiles in a limited number of patients with systemic juvenile idiopathic arthritis using SELDI-MS. They noted that the patients harbored a particular protein peak prior to treatment (Fig. 6), and subsequently proceeded to identify this peak as serum amyloid A. These findings await independent confirmation.

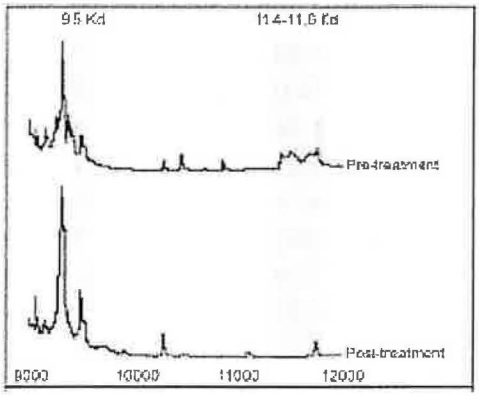


Fig. 6. Compared to post-treatment sera (bottom), Miyamae et al (2005) noted protein peaks at about 11.5 kD in SJIA patients, pre-treatment (highlighted), using SELDI-MS. This was subsequently identified to be serum amyloid A.

Likewise, Tomosugi and colleagues (2005) have reported distinctive proteomic profiles in the tears of patients with Sjogren's syndrome (Fig. 7). The authors have reported that the SELDI spectral patterns observed in SS patients offered 87% sensitivity and 100% specificity in discriminating the patients from the controls. These findings await independent confirmation. The specific proteins responsible for these differential spectral patterns currently remain unknown. SELDI-MS analyses of sera from RA and SLE patients are currently in progress in various laboratories across the country.

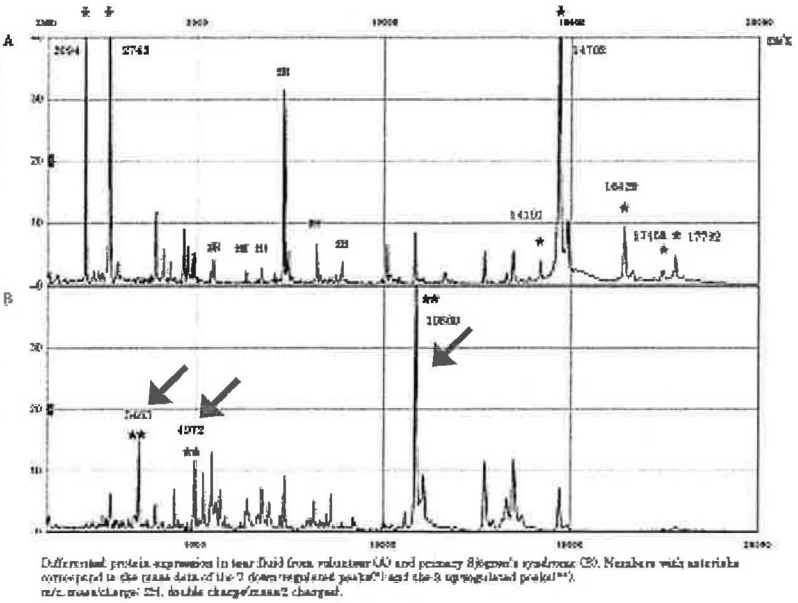


Fig. 7. The tears of normal subjects (Top) differed from the tears of patients with Sjogren's syndrome (bottom) significantly, based on their SELDI-MS spectra. The proteins that were hyper-expressed in Sjogren's tears await identification (Tomosugi et al., 2005).

1D. Unbiased Proteomics: Capillary electrophoresis - MS

Another recent player on the horizon is capillary electrophoresis (CE) TOF MS. CE is a very powerful tool in separation science, allowing proteins to be separated based on their charge relative to their hydrodynamic size (which closely approximates the mass of the molecule). CE has been touted to be superior to the proteomic approaches described above in terms of its resolution, ease of use and speed (Fliser et al., 2005). CE also has the incredible ability to separate similarly structured compounds. Given that it is particularly effective in resolving smaller proteins/peptides (< 20 kDa), it complements 2-D gel-based studies reasonably well. Despite these advantages, it is quite surprising that CE is not more widely used. The small sample volume that CE accommodates has been cited as one possible reason for this (Fliser et al., 2005).

Several studies have employed CE-MS to establish the proteomic fingerprints in the urine during disease (Kaiser et al., 2003; Wittke et al., 2003). A recent application of this technique has proven useful in differentiating diabetic nephropathy from normal control urine, and to further stratify diabetic nephropathy cases into those manifesting albuminuria from those with normal urinary albumin levels (Mischak et al., 2004). Thus far, there have been no reports of use of CE-MS in Rheumatology.

2. Targeted proteomics

Targeted proteomic studies have been executed in at least 2 different fashions, focusing in both cases on a limited number of proteins. On the one hand, microarrays coated with proteins have been used to determine the autoantibody specificities in systemic autoimmune diseases (Fig. 8, discussed in section 2A). On the other hand, microarrays coated with defined antibodies have been used to determine the levels of various analytes that the antibodies are specific for, in various body fluids, as discussed in section 2B. In

both cases, the study is limited by the availability of antigens or specific antibodies. Both these targeted approaches rest upon the generation of protein arrays. Protein microarrays are printed using the same methodology used for printing DNA microarrays. Essentially, various proteins/antigens (or antibodies) of interest are transferred from a microtiter plate onto a high binding capacity glass slide, as outlined in Fig. 8.

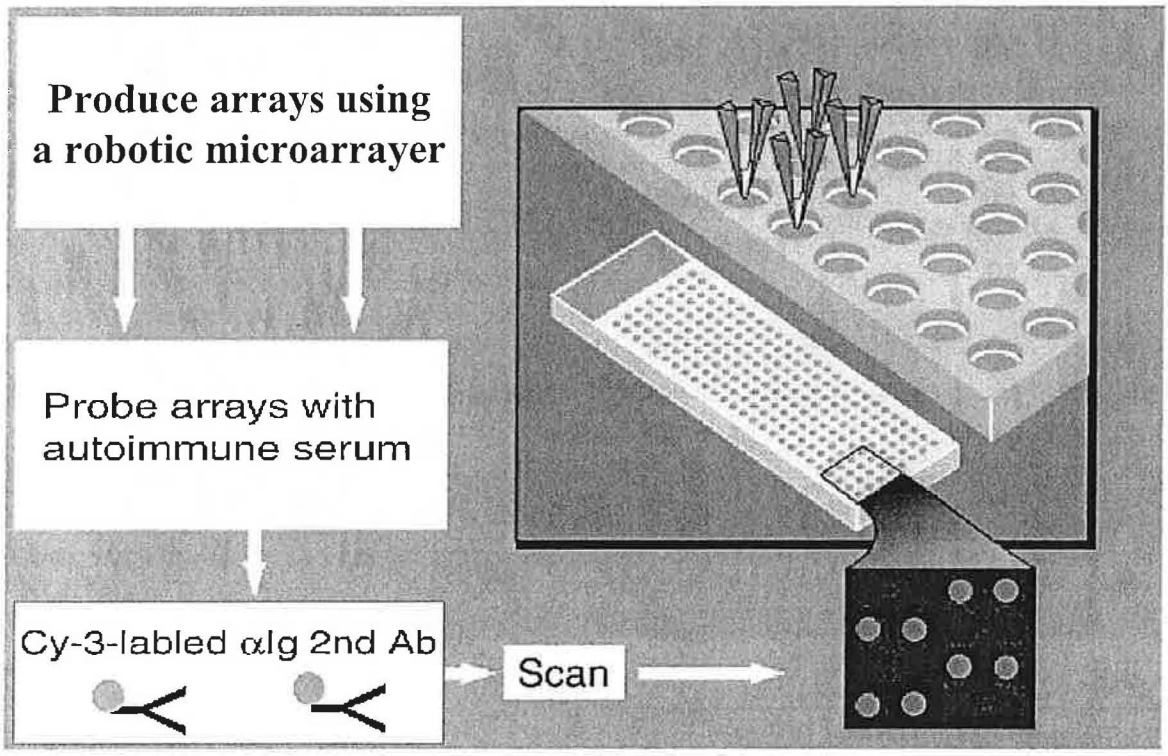
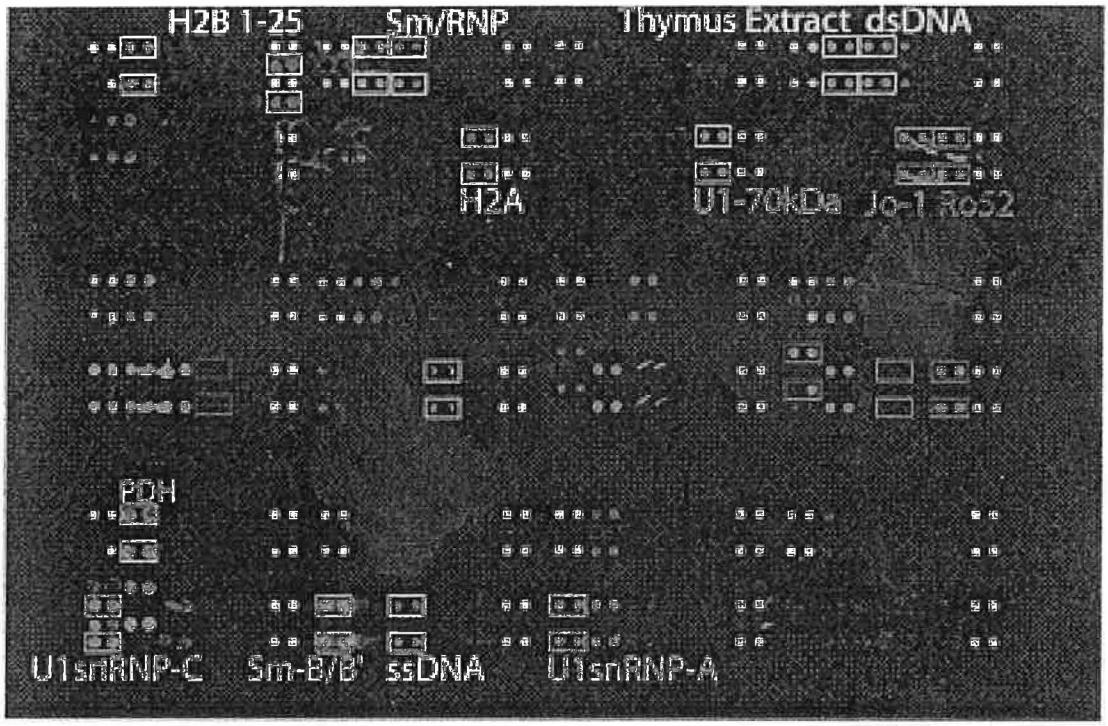


Fig. 8. Arrays coated with different antigens can be used to screen patient sera for the presence of various autoantibodies in a multiplexed fashion, as detailed in section 2A (Figure was contributed by Dr. P. Utz, Stanford).

Unlike DNA microarrays, the design of protein microarrays poses a significant set of technical problems. One obstacle is the vast range of analyte concentrations to be detected. Protein concentrations exist over a broad dynamic range (by up to a factor of 10^{10}); in most biological specimens low-abundance proteins co-exist with widely prevalent proteins. A second concern is that PCR-like amplification methods do not exist for proteins. Third, whereas DNA microarrays have been facilitated by the fact that hybridization probes can be manufactured for all known genes (or transcripts) in the genome with predictable affinity and specificity, similar probes (e.g., antibodies) exist only for a minor fraction of the proteome. Several of these concerns and possible remedies have been reviewed elsewhere (Liotta et al., 2003). In particular, a major goal of the HUPO (Human Proteome Organization) is the production and quantification of comprehensive antibody libraries that will be made available to the scientific community (Hanash, 2003). This would significantly augment the scope of targeted proteomics.

2A. Targeted proteomics –assaying autoantibodies



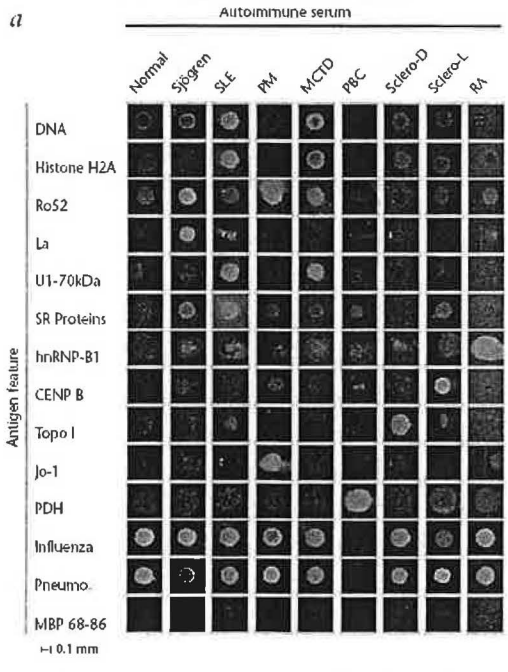


Fig. 9A. Robinson et al (2002) have fabricated glass slide arrays bearing about 200 autoantigens, which can be used to screen sera from patients with various autoimmune diseases, including systemic rheumatic diseases. Following the addition of sera to these antigen coated slides, the degree of autoantibody reactivity can be gauged using fluorescence labeled second-step antibodies. The specificities of these arrays were verified for several different antigen – antibody pairs (**Fig. 9B,** Right). The clinical utility of these arrays are being tested in many different laboratories.

One application of targeted proteomics has been in the study of autoantibody specificities in various rheumatic diseases. Robinson et al (2002) have pioneered an "autoantigen microarry" bearing about 200 distinct biomolecules representing major autoantigens targeted by autoantibodies in various autoimmune diseases. These authors have used sera and defined control antibodies to demonstrate the specificity and sensitivity of these arrays in detecting various autoantibodies prevalent in systemic rheumatic diseases, including

antibodies to DNA, histones, Sm/RNP, Ro, La, etc (Fig. 9A, 9B). These authors have also demonstrated how these parallel immunoassays surpass conventional ELISA assays in terms of specificity and sensitivity. Using similar approaches, several laboratories are currently working on using similar autoantigen arrays to define the autoantibody specificities that best characterize different rheumatic diseases and best forebode flares.

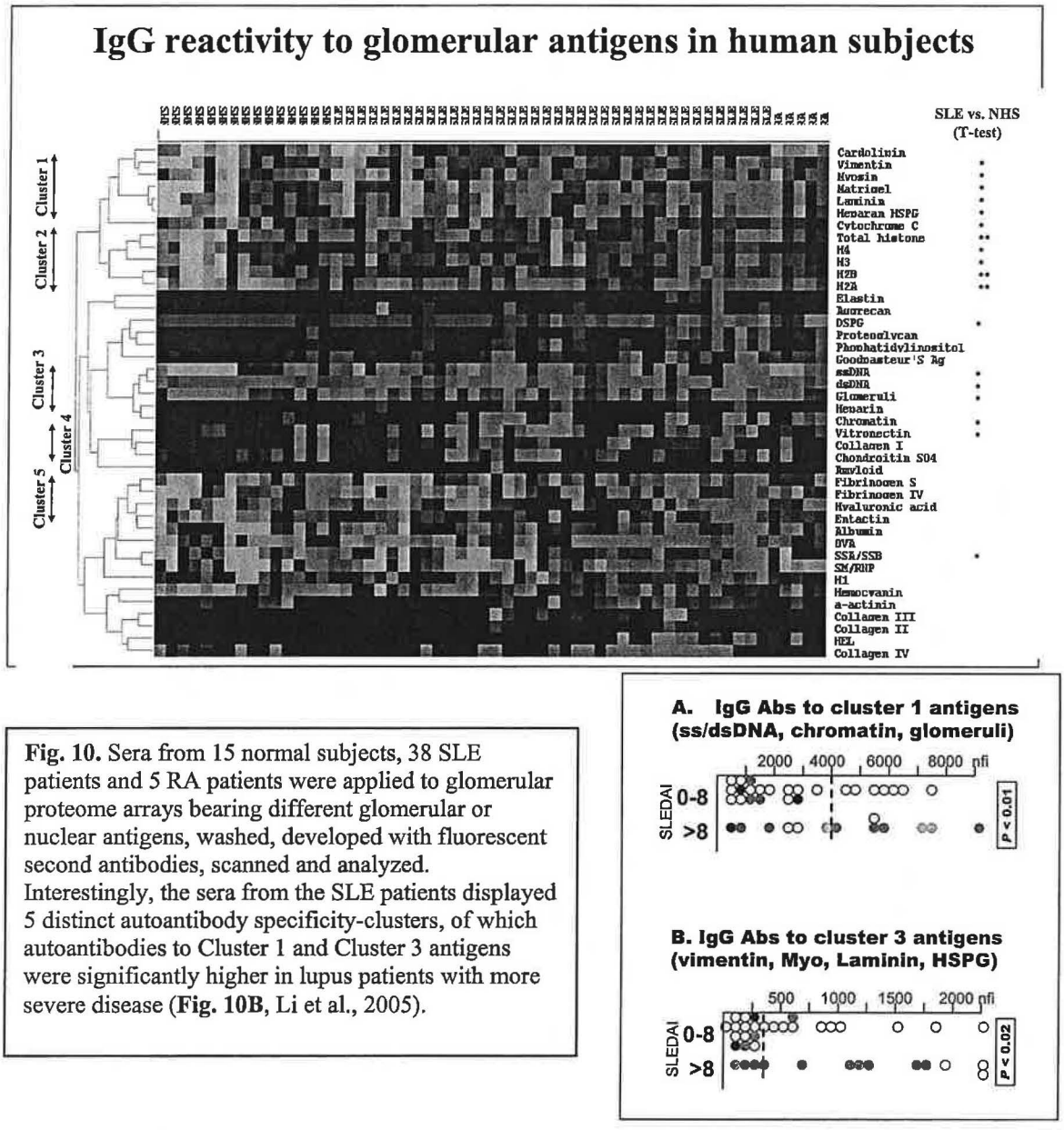


Fig. 10. Sera from 15 normal subjects, 38 SLE patients and 5 RA patients were applied to glomerular proteome arrays bearing different glomerular or nuclear antigens, washed, developed with fluorescent second antibodies, scanned and analyzed. Interestingly, the sera from the SLE patients displayed 5 distinct autoantibody specificity-clusters, of which autoantibodies to Cluster 1 and Cluster 3 antigens were significantly higher in lupus patients with more severe disease (**Fig. 10B**, Li et al., 2005).

For instance, in our laboratory at UT Southwestern, a more targeted "glomerular" proteome array has been fabricated by spotting about 30 different known glomerular antigens in order to define the autoantibody specificities that may best associate with disease (Li et al, 2005). This is an important exercise given that it has previously been demonstrated glomerular-binding autoantibodies are strongly associated with severe lupus

nephritis (Mohan et al., 1999; Liang et al., 2004). To study this further, sera from 38 SLE patients with varying degrees of disease severity and renal involvement were spotted onto the glomerular proteome arrays to decipher the patterns of autoantibodies in these patients. It was interesting to observe that SLE patients exhibited at least 5 distinct clusters of autoantibodies (Fig. 10A) of which reactivity to "Cluster 1" (i.e., reactivity to DNA, chromatin and total glomerular sonicate) and reactivity to "Cluster 3" antigens (i.e., reactivity to myosin, laminin, heparin sulphate and vimentin) was particularly associated with more severe disease, as marked by higher SLEDAI scores and worse renal disease (Fig. 10B, Li et al., 2005). Ongoing studies are aimed at directly testing the pathogenic potential of these autoantibody specificities and to track the emergence of these specificities in a longitudinal cohort of lupus patients.

2B. Targeted proteomics – assaying analytes in biological fluids

A second application of targeted proteomics is to gauge the levels of different analytes in different body fluids in different disease states, based on an autoantibody sandwich assays. A recent study has used antibody microarrays to simultaneously analyze the concentration of 78 cytokines, growth factors and soluble receptors in serum samples from patients with Crohn's disease and ulcerative colitis (Kader et al, 2003). Four cytokines were elevated in patients in clinical remission compared with patients with active disease (Kader et al, 2003). Among these factors was TGF-β, a cytokine that inhibits inflammatory activity and enhances T-regulatory function. Although these findings need to be confirmed, it's worth noting that this represents the very first proteomic study focusing on free analytes in body fluids in an autoimmune disease.

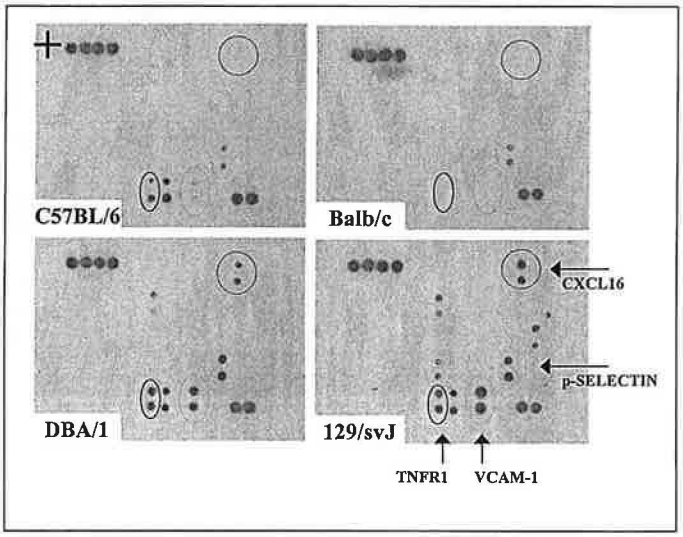
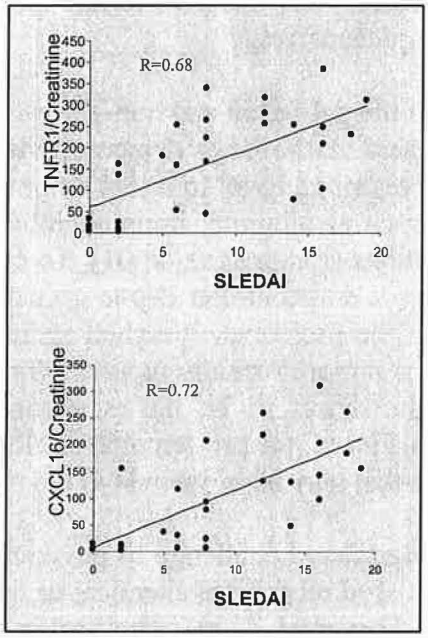


Fig. 11. Urine from strains with immune nephritis (e.g., DBA/1, and 129/svJ) and urine from mice with minimal disease (B6 and Balb/c strains) were applied to commercial arrays to detect the presence of hyper-expressed analytes. Both the disease strains shown as well as mice with spontaneous lupus nephritis (not displayed) exhibited significantly higher levels of 4 molecules in their urine (highlighted), correlating well with disease (Wu et al., 2005).

More recently, a similar approach has been undertaken in immune-mediated lupus nephritis. Essentially, urine from immune/lupus nephritis was applied to commercially available antibody-coated arrays. Interestingly, mouse strains with immune/lupus nephritis exhibited increased levels of VCAM-1, sTNFR-1, CXCL16 and p-selectin in their urine (Fig. 11) correlating well with renal disease (Wu et al., 2005). Importantly, these 4 molecules were also noted to be elevated in the urine of lupus patients (Wu et al., 2005), correlating well with disease severity (Fig. 12). Longitudinal studies are in progress to

determine the predictive value of these 4 molecules for use as potential biomarkers in lupus nephritis.

Fig. 12. Urinary levels of the 4 mediators are increased in lupus nephritis, and correlate well with SLEDAI (data for 2 of the 4 molecules are shown).



2C. Targeted proteomics – study of signaling pathways

Reverse phase cell-lysate microarrays constitute a third category of targeted protein arrays, as reviewed (Liotta et al., 2003; Chan et al. 2004; Fathman et al., 2005). These arrays are particularly powerful in uncovering the signaling pathways that are activated in various cell types in different disease states. Although this approach has been applied in the setting of tumors, they have yet to be applied to autoimmune and rheumatic diseases. Nevertheless, this novel technology has the potential to uncover signaling pathways that become activated during systemic autoimmunity and may potentially highlight molecular targets for therapy.

A look into the future

In so far as proteins are the final effectors and mediators that dictate disease pathogenesis, it is imperative that we decipher the proteomic basis of disease. From this perspective, one may view proteomics as a necessary component to move forward disease diagnostics and prognostication. On the other hand, Proteomics as currently practiced is far from being optimal. Several challenges need to be addressed before Proteomics can be integrated into mainstream Clinical Medicine, as reviewed elsewhere (Liotta et al., 2003). It is clear that Proteomics is still a very young Science that is likely to evolve rapidly over the coming years in several different ways, as it struggles to meet these challenges.

First, we recognize that we are really visualizing only a minute fraction of the expressed proteome, and there is a growing sense that the proteins that we are currently oblivious of may hold the secrets to disease pathogenesis (Patterson, 2004). Contrary to the "one gene – one protein" tenet, it is evident that the expressed proteome is far more complex than that suggested by the size of our genome, owing to post-translational modifications, splice variants, compartmentalization, translocalization, etc. Hence, we are only seeing the tip of the iceberg using current technologies. One important challenge is to broaden the fraction of the proteome that is rendered "visible" to us, and to enhance the resolution accordingly.

In this respect the 2-D gel approach may have reached its limits and may only attain qualitative enhancement in overall performance through scaling down, as exemplified by the "lab-on-a-chip" technology (Constans, 2005). In contrast, with the ongoing improvements in mass accuracy and resolution, the non gel-based LC-MS approaches hold great promise for the future. Hence, it appears almost certain that gel based approaches will give way to non-gel based LC-based methodologies. Currently, the multi-dimensional LC-MS (MudPIT) approach is rapidly gaining momentum. Future enhancements of this technology in terms of the augmented dimensionality, far superior methods for processing mass spectra data, and the ability to handle massive amounts of spectral data computationally are likely to render this approach a key player in studying the proteome more comprehensively.

With both the gel-based and non-gel based proteomic approaches, one of the challenges of studying sera is the large dynamic range of the proteins inherent in sera, ranging from proteins present at ng/ml to ug/ml range on the one hand, co-existing with highly abundant proteins such as albumin, immunoglobulin and haptoglobin, which are present at mg/ml concentrations (Liotta et al., 2001). To circumvent this problem, several investigators have adopted a pre-fractionation step to get rid of the highly abundant proteins before examining the rest of the proteome. Weighed against the more focused dynamic range that this step results in is a more recently reported drawback. Mehta et al. (2003) have noted that serum albumin turns out to be an excellent carrier molecule for several smaller bioactive molecules. Hence, the pre-removal of albumin could potentially carry away with it the very molecules that may be of interest to the researchers!

Another significant challenge in the coming years is quantitative proteomics. The present approach based on the use chemical or isotopic labeling, as exemplified by ICAT (Gygi et al., 1999; Han et al., 2001; Cutillas et al., 2004), holds great promise for the future of quantitative proteomics. A further challenge that lies ahead is to boost current computational capacity and speed so that reliable "information" can be extracted from reams of spectral data within a reasonable time frame.

A final challenge is to transition the current studies in "discovery proteomics" to "clinical proteomics" where novel biomarkers can be rapidly integrated into day-to-day clinical diagnostics and prognostication. It appears likely that the studies in "discovery proteomics" may help identify a handful of potential biomarkers, which could then be added to an existing panel of diagnostic tests, either in the form of miniaturized protein arrays (that serve to monitor the expression of a specific subset of the proteome of relevance to the disease) or integrated into traditional immunoassays (e.g., multiplexed ELISA assays). Attaining the ability to scan the entire proteome to decipher each patient's personal proteomic "barcode" will not only revolutionize our understanding of how disease evolves, it will also empower us to tailor personalized therapies for each individual, targeting specific biomolecules.

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