Proteomic strategies in the search of new biomarkers in atherothrombosis

Proteomics and biomarkers in atherothrombosis

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ABSTRACT

Extensive research has focused on the identification of novel plasma biomarkers to improve our ability to predict cardiovascular events in atherothrombosis. However, classical techniques can only assess a limited number of proteins at a time. Given that plasma contains more than 900,000 proteins, this approach will be extremely time-consuming.

Novel proteomic approaches make it possible to compare the expression of hundreds of proteins in several samples in a single experiment. The classical approach consists of separation of proteins on a two-dimensional gel followed by protein identification with mass spectrometry, although new complementary gel-free techniques are emerging. We can thus compare protein expression in an atherosclerotic plaque with that in a normal artery or study plasma proteins in patients with atherothrombosis as compared to healthy subjects. For such approaches, it is not necessary to study the literature to select potential biomarkers. However, as the number of patients that can be studied with most of these techniques is limited, what is really important is the design of the studies, assessing carefully what kind of patients should be included in order to obtain valid conclusions. Clinicians should thus play a key role in this design along with the basic scientist. In this paper, we review several proteomic strategies carried out by our group and others, and we make a call for collaboration between clinicians and experts in proteomics. This collaboration could increase greatly the likelihood of identifying new prognostic biomarker panels in atherothrombosis and other cardiovascular disorders.

KEY WORDS:

Atherothrombosis, biomarkers, proteomics, mass spectrometry

ABBREVIATIONS LIST

CVE: Cardiovascular Events

2DE: two-dimensional electrophoresis

HDL: high-density lipoprotein

HSP: heat-shock protein

LC: liquid chromatography

MALDI-TOF: matrix-assisted laser desorption ionization time of flight

MS: mass spectrometry

NSTEACS: non-ST elevation acute coronary syndrome

SELDI-TOF: Surface-enhanced laser desorption/ionization time of flight-MS

VSMC: Vascular smooth muscle cells

Identifying subjects at risk of developing an acute ischemic event remains one of the great challenges of cardiovascular medicine. Classical approaches, such as the presence of cardiovascular risk factors, are unable to accurately predict CVE. In recent years, plasma biomarkers have been the focus of extensive study. Although many potential molecules have been described, the results have not been consistent enough (1) and most of them are not used in the clinical practice.

Plasma contains more than 900,000 proteins, (2). Given that it takes approximately ten years from biomarker discovery to the development of a commercial kit (2), testing each of these proteins individually by traditional techniques may take an eternity. Moreover, when several studies about new potential biomarkers with negative results are published, investigators are discouraged about the usefulness of biomarkers. However, given the large number of proteins present in the plasma, reporting negative results for a few potential biomarkers does not invalidate this approach. Moreover, these studies are usually based on individual biomarkers, whereas the use of a panel of biomarkers reporting information of several mechanisms involved in this disorder may be more effective. We therefore need new methods to screen for novel biomarkers in atherothrombosis.

NEW PROTEOMIC APPROACHES

The standard techniques used for the assessment of proteins in biological specimens, such as enzyme-linked immunosorbent assay, determine only the levels of individual proteins. Proteomic approaches combine 2DE and MS, allowing hundreds of proteins in a given sample tissue to be assayed simultaneously (3)(Figure 1). In 2DE proteins are first separated according to their charge by isoelectric focusing in one dimension. Then, they are separated further in the second dimension according to molecular mass (4). After staining, gels of different samples are analyzed using computer software to detect differentially expressed protein spots. Finally, MS determines the molecular masses of the proteins identifying them (5). This technique requires the conversion of the proteins into gas-phase ions, using various procedures. The ions are separated according to the mass/electrical charge ratio (m/z) using a mass analyzer and analyzed with highly sensitive detectors (5).

Basically, two types of MS are used. In MALDI-TOF, ionization is achieved by mixing the sample with organic compounds which crystallize to form a matrix. A laser pulse vaporizes the peptides, which are accelerated in an electrical field and are sent to a flight tube, at the end of which the detector is located. For a given electrical acceleration voltage, the time of flight (TOF) to the detector is proportional to m/z. Small molecules fly faster than large ones. To identify a protein, the group of peptide masses obtained from its digestion is compared with the theoretical masses of the peptides that would be produced upon digestion of the proteins present in the databases (6). The second type of spectrometer vaporizes the sample directly from the liquid phase by electrospray ionization (ESI) or nebulizer (7) with an electrical field to disperse the sample. For this technique, a LC separation step is usually employed before detection to provide a much more reliable protein identification—even with impure protein preparations—than MALDI-TOF. A peptide can then be selected and broken up in a collision chamber. The resulting fragments are sent to the detector and their masses are obtained. The sequence of the peptide or a short sequence tag is determined by analysis of the fragmentation spectrum. These sequences are then used for database searching. Fragmentation spectra are therefore highly

informative and can be powerful tools for characterizing post-translational modifications and for de novo sequencing of unknown proteins.

In addition to 2DE/MS, other platforms have been developed. Gel-free "shotgun" proteomic techniques use LC separation procedures with automated tandem MS, and are being applied for the analysis of complex proteomic samples, where a whole proteome is digested with or without prior protein separation. The typical approach is called MudPIT (multidimensional protein identification technology) (Figure 2). This technique identifies proteins in complex mixtures, including basic, highly hydrophobic or extreme molecular weight proteins, which are difficult to resolve on 2DE gels. It has greater resolution than gel-based approaches, but requires rigorous statistical methods given the large amount of data analyzed.

Quantitative analysis can be performed using LC-MS/MS after differential isotopic or isobaric labeling of the proteins or peptides from two cell extracts, which are simultaneously quantified and identified (8,9). These approaches can be applied in different steps along the separation process, and include stable isotope labeling by amino acids in cell culture (SILAC), isotope-coded affinity tags (ICAT), tandem mass tags (TMT), and more recently, isobaric tags for relative and absolute quantification (iTRAQ). For example, in the iTRAQ method tagging is carried out on primary amines, eliminating the dependence on cysteine containing peptides, as in ICAT labeling, yielding complementary results to ICAT. The ICAT method identifies a higher proportion of signaling proteins, while iTRAQ detects a larger percentage of ribosomal proteins and transcription factors (9). These methods are compatible with sample fractionation to reduce protein complexity, allowing the measurement of low-abundance proteins. Also, they could potentially be used as the basis for automated, quantitative and global proteome analysis. However, most label-based quantification approaches have important limitations, mainly complex sample preparation and handling, increased sample concentration, incomplete labeling or reduced protein coverage. Therefore, classic label-free quantification is currently being improved to overcome some of these issues for quantifying complex protein mixtures in LC-MS-based strategies (10,11). These methods use direct comparison of peptide peak areas between LC-MS runs without any isotopic labeling. As a result, they do not require costly reagents and have the advantage of simplicity in sample preparation (10).

Alternative approaches have been used in recent years. In Array technologies, multiple binding antibodies are placed on a platform. However, it allows us to search only for pre-specified proteins for which antibodies are available. Moreover, the space available on the platform limits the number of antibodies that can be used. This approach may be useful to confirm the data obtained with the techniques described previously. With SELDI-TOF, we may identify a pattern of MS peaks (known as "proteomic fingerprint") in a given disorder. This approach is suitable for completing characterization of a proteome, although it cannot directly identify differentially expressing proteins. Finally, MALDI imaging uses MALDI for recording the spatial distribution of proteins and peptides within tissue sections mounted together with a MALDI matrix, and shows selected masses as color images. Figure 3 shows an overview of proteomic approaches.

A thorough description of proteomic techniques is beyond the scope of this paper and may be found in references 2,3,11-13. On the other hand, we will focus on the design of studies using proteomic approaches. Clinicians should be familiar with this field, as they have the

potential to collaborate with proteomic specialists to improve the design of future research making it to answer clinical problems of relevance to patients with atherothrombosis.

PROTEOMICS VERSUS GENOMICS

The importance of studying proteins resides in that they reflect what is happening in the organism. Humans have 30,000-40,000 genes (14), only about twice the number of less complex organisms, such as worms. However, due to alternative splicing of transcripts, and protein postranslational changes, a single gene may yield different proteins. Thus, while the genome is stable and gives information about the potential of an organism, the proteome is dynamic and reflects the biological processes that are taking place in that organism. Both approaches are complementary, and along with transcriptomics and metabolomics (which study the transcripts and the metabolites, respectively) integrate the so-called "omic" sciences (Figure 4).

PROTEOMIC STRATEGIES IN THE DEVELOPMENT OF BIOMARKERS IN ATHEROTHROMBOSIS (Figure 5)

IN VITRO STUDIES

Cells involved in atherothrombosis can be cultured and stimulated with proatherothrombotic factors, and their protein extracts can undergo proteomic analysis. Following this strategy, Fach et al (15) compared the effects of stimulation of monocytic cells with oxidized and native low-density lipoprotein. They used multidimensional LC and tandem MS, identifying 59 overexpressed proteins, and 17 underexpressed ones. Within the overexpressed proteins there were fatty-acid binding proteins, chitinase-like enzymes, cyclophilins, cathepsins, proteoglycans, urokinase-type plasminogen activator receptor and macrophage scavenger receptor. Coppinger et al (16) showed by shotgun proteomics that platelets release more than 300 proteins after being activated by thrombin, many of which were not previously attributed to these cells. Among these were secretogranin III, a monocyte chemoattractant precursor, cyclophilin A, a VSMC growth factor, and calumenin, an inhibitor of the vitamin K epoxide reductase-warfarin interaction. Given that platelets are anucleated cells and contain very small amounts of mRNA, proteomics is better than genomics and transcriptomics for assessing their status.

However, primary cell culture itself induces phenotypic changes. The proteome of cultured cells will therefore yield information on the potential of these cells to respond to atherogenic stimuli rather than on the exact proteome expressed by them within the arterial wall. The ideal approach to explore the cell proteome in atherosclerosis would be to separate different cell types from atheroma using laser microdissection.

Other in vitro strategies may help us to characterize potential biomarkers. For example, low HDL is associated with a higher incidence of atherosclerosis. However, torcetrapib, a drug that increases HDL plasma levels, enhances mortality (17). This lead to a debate about what HDL components should be measured. Recently, shotgun proteomics has shown that HDL is composed of complement regulatory proteins, protease inhibitors and acute-phase response

proteins, among others (18). Furthermore, HDL3 from patients with coronary artery disease was selectively enriched in apolipoprotein E, suggesting that HDL composition may be different in this disorder. Similarly, HSP27 has anti-inflammatory and antiapoptotic effects and could be a candidate biomarker. Using 2DE, Trott et al (19) found that phosphorylated HSP27, but not the hypophosphorylated form, decreased expression of cycling proteins and ubiquitination enzymes in endothelial and VSMC. This suggests that phosphorylated HSP27 may be an important regulator of vascular cell proliferation, and could be a good candidate biomarker.

PROTEOMIC ANALYSIS OF ATHEROSCLEROTIC TISSUE

Whole Tissue

Atheroma can be explored by proteomics. However, it is very heterogeneous, and the results may vary according to whether we analyze the lipid core or the fibrous cap. Laser Capture Microdissection (LCM), allows extraction of specific tissue sections, reducing sample heterogeneity. Nevertheless, the subsequent proteomic analysis is limited to techniques with extremely high analytical sensitivity due to the reduced amount of protein obtained (less than 10,000 cells are usually collected). We can compare atheroma with normal arterial wall and we can also explore the effect of different therapies. Also, in human atheroma we can search for differences between the proteome of those who develop CVE and that of those who remain stable during follow-up. With this approach, Pasterkampf et al (20) identified osteopontin as a candidate biomarker. After 3 years of follow-up in a validation cohort, they confirmed that high osteopontin expression was associated to the incidence of CVE. Such an approach may help identify new proteins with prognostic value, helping us to select patients for more intensive therapies. Additional studies are required to confirm whether plasma levels of the proteins discovered by this method are related to prognosis. This approach would be useful for the whole population and not just for those with endarterectomy.

Imaging MS is another emergent technology for the study of whole tissue. MS is applied to thin-tissue cryostat sections deposited onto MALDI plaques or protein chip surfaces (SELDI), evidencing the spatial distribution of proteins in tissue sections (Figure 5). Using this thecnique, we have shown the presence of high amounts of non-esterified fatty acids and vitamin E around intimal areas with high cholesterol accumulation in human atheroma (21).

Secretome

Another problem working with whole tissue is that many constitutive proteins could mask others that have altered expression and which could play an important role in this disease. An alternative strategy is culturing atherosclerotic plaques and analyzing the supernatant, obtaining the proteins secreted by the cells, that is, the secretome. In this way we can detect candidate biomarkers released from the vascular wall into the blood, providing information about the processes taking place in the vascular tree.

Combining this approach with 2DE/MS we found that complex human carotid atherosclerotic plaques released 202 proteins to the supernatant, non-complex plaques secreted 152, and healthy arteries released only 42 (22). The supernatant of cultured atheroma showed a

decrease in HSP27 levels as compared to that of normal arteries (23). We confirmed that the levels of this antiapoptotic and anti-inflammatory protein were lower in the plasma of patients with carotid atherosclerosis than in healthy subjects. However, in healthy women we found that HSP27 plasma levels were not related to the incidence of CVE (24).

Applying SELDI-TOF to this strategy we also detected lower levels of sTWEAK (soluble tumor necrosis factor-like weak inducer of apoptosis) (25), a protein involved in apoptosis, proliferation and inflammation. Surprisingly, sTWEAK plasma levels were lower in patients with carotid atherosclerosis than in healthy subjects and showed a negative correlation with carotid intima-media thickness. The coexistence of abnormal sTWEAK levels and an inflammatory environment predicted mortality in patients in hemodialysis (26).

Finally, we can also add drugs to the medium to assess their effect on the secretome. Adding atorvastatin to cultured complicated atherosclerotic plaques, reverted 66% of the proteins whose expression was altered to control values (27).

THE STUDY OF BLOOD BY PROTEOMIC APPROACHES

Blood Cells

In this setting, early treatment of the sample is necessary to avoid protein degradation. We studied the proteome of circulating monocytes in patients with NSTEACS (28). Using 2DE/MS, we detected seventeen proteins whose expression was altered as compared to expression in subjects with stable coronary artery disease. The number of proteins with abnormal expression decreased with time. At six months, the proteome of the circulating monocytes was similar to that of subjects with stable coronary artery disease, suggesting that, by this time, the processes that triggered NSTEACS have finished. Among the proteins showing abnormal expression, we found decreased levels of antiatherogenic proteins, such as paraoxonase I and HSP70, and anti-inflammatory proteins, such as protein disulfide isomerase. In contrast, there was overexpression of mature cathepsin D, whith proatherogenic effects, and enolase I, involved in macrophage transformation into foam cells.

Using a similar approach we shown that atorvastatin 80 mg/d affected the expression of twenty proteins in NSTEACS patients as compared to moderate statin therapy (29). Among them, there was a normalization of the decreased expression of HSP70, paraoxonase I, annexin I, which has anti-inflammatory properties, and annexin II, involved in spontaneous fibrinolysis.

While the study of circulating cells may uncover new proteins involved in atherothrombosis, cell isolation and protein extraction are time-consuming. Also, samples cannot be stored for more than 4 hours due to protein degradation. Studying plasma levels of the described proteins may therefore be more appropriate, as plasma may be obtained by simple centrifugation of the blood sample and stored until processing.

Plasma

Study of the plasma with proteomic tools faces certain problems. Only 9 proteins represent 90% of the protein mass in plasma. Therefore, it is necessary to improve the techniques to separate these high-abundance proteins that may mask low-abundance proteins.

Another pending issue is the enhancement of the resolution of the techniques. Until present, by combining the results from different proteomic platforms, more than 3,000 proteins have been detected in plasma (2,30)—far less than the 900,000 proteins hypothesized to be present. Nevertheless, many of these proteins are different forms of IgG produced throughout the person's history of immune events. These proteins hold a high similarity in sequence and most of them are unlikely to be biomarkers of atherothrombosis, being possible to deplete them using an affinity-based method. Thus, the number of potential biomarkers will be well below this number, probably around several thousand. Another relevant question is whether to use serum or plasma samples. Although it is possible to study serum, plasma contains the proteins of the coagulation cascade. Given the leading role of this system in triggering acute ischemic events, plasma would be the preferred type of sample provided we can adequately isolate the proteins of interest from the more abundant proteins.

Using 2DE/MALDI-TOF, Brea et al (31) found high levels of plasma haptoglobin and serum amyloid A to be associated with atherothrombotic rather than with cardioembolic stroke. Although these data need to be confirmed in larger populations, they may be useful in the management of these patients, as cardioembolic stroke should be treated with anticoagulants. Using the same approach, Mateos-Caceres et al (32) found a reduction in the concentration of several isoforms of α_1-antitrypsin and apolipoprotein A-I, and an increase in heavy chains of fibrinogen and γ-immunoglobulin in the plasma of patients with acute coronary syndrome.

DESIGNING PROTEOMIC RESEARCH: ROLE OF THE CLINICIAN

As we have seen, new proteomic approaches provide the researchers with a powerful tool in the search for new biomarkers. However, the clinician has a key role to play to avoid spending time and money conducting irrelevant studies with flawed approaches. For instance, studying plasma proteins in patients during an acute ischemic event may reveal new proteins implicated in atherogenesis that could be potential biomarkers. However, clinical cardiologists know that some of the proteins detected may simply be the consequence of myocardial necrosis and not play a causative role in plaque thrombosis. It would therefore probably be more interesting to focus on NSTEACS patients rather than in those with ST-elevation myocardial infarction, as necrosis is more severe in this condition.

Probably, the best approach in the future will be to study the plasma from patients with atherothrombosis and to follow them for a period of time, comparing the proteome of those who develop recurrences of CVE with that of patients who remain stable during follow-up. Given that proteomic techniques are costly (Table 1) and time-consuming, studying large numbers of patients is not possible. In this setting, matching of cases and controls by relevant clinical variables not limited to age and sex is essential to avoid bias leading to confounding results. Also, the cardiologist should carefully choose the end-points of the study. For instance, while heart failure is a dreaded event for these patients, its development may be the result of myocardial damage secondary to a previous infarction, rather than reflecting the progression of atherothrombosis. On the other hand, once the experiment is completed, it is possible to find numerous proteins differing between stable patients and those with recurrences. In order to select a panel with the minimal group of proteins retaining the maximal discriminative power for use in clinical practice, we can use several criteria. First, we must take into account the

strength of statistical significance by choosing those with lower "p" values. Second, we must focus on proteins whose function is potentially related to the disorder studied. For this purpose, collaborative efforts between clinicians, scientists and bioinformatics experts are warranted. Third, selecting proteins with stable plasma levels is also important. The clinician may help in designing new experiments to test the stability of the plasma levels of the proteins detected by proteomics.

Once the proteomic approach has yielded a candidate biomarker panel in studies with a limited number of patients (exploration cohort), the next step consists of testing this panel in a validation cohort using conventional methods. In these studies, larger populations may be included, being again of prime importance to control for clinical variables that could influence the outcome. Validation studies will confirm whether the selected biomarker panel really adds prognostic value to the clinical variables used routinely in the clinical practice.

CONCLUSIONS

Identifying biomarkers by conventional methods is a time-consuming task. Proteomics allows us to explore the expression of hundreds of proteins involved in atherosclerosis using atheroma specimens, circulating blood cells, plasma or serum. We may use strategies comparing samples from patients with healthy controls, from patients receiving different therapies or, more importantly, from subjects developing CVE with those remaining stable at follow-up. Given the large number of proteins present in the plasma and atheroma, this may be the only effective way to select a group of them that may improve our prediction of the occurrence of CVE. The proteomic approach does not require previous knowledge of the proteins to be assessed. Rather, patient selection, the strategy to follow and the kind of samples to be analyzed are of critical to obtain the maximal yield from this technique. Moreover, the number of patient samples to be analyzed is limited by the complexity and high-cost of this approach. In this setting, matching of the clinical characteristics of the populations to be compared is of great importance to avoid drawing wrong conclusions. Clinicians should then be encouraged to collaborate in multidisciplinary studies with proteomic experts in this task, to enhance the ability of cardiovascular medicine to predict which populations are at high risk of atherothrombotic events. Prevention programs could then focus on these high risk populations, using the most intensive therapies to decrease the incidence of CVE.

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FIGURE LEGENDS.-

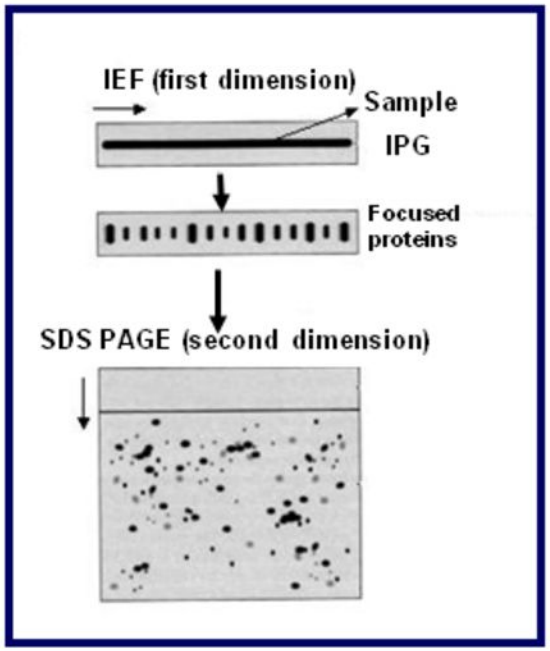
Figure 1. Classical proteomic approach. Left, protein separation by 2DE. Right, protein identification by MS. **IEF:** Isoelectrofocus. Adapted from reference 13, with permission.

Figure 2. Nanoscale LC-MS/MS setup. MudPIT column: a reverse phase (RP) and a strong cation exchange (SCX)-pre-column are followed by an RP-separation phase in the emitter tip. High voltage is applied in front of the column. Samples move into the first phase (1) and are trapped on RP-pre-column during the loading process. (2) Peptides elute onto the SCX-pre-column while contaminants are washed away. (3) Trapped peptides elute from the SCX-pre-column onto RP-separation column in a single salt application step. (4) Peptides are separated on the RP-phase according to their hydrophobicity and elute in an acetonitrile gradient directly into a mass spectrometer (MS), which ionizes the peptides, deflects them, and detects the ions. Data are delivered to a computer for analysis.

Figure 3. Overview of proteomics strategies. Four major proteomics approaches are outlined. **DIGE** (differential in-gel electrophoresis) represents an improvement in comparative 2DE. Samples of proteins from two experimental conditions are labeled with two fluorescent dyes, mixed together and run on a single 2DE gel, reducing variability, improving the sensitivity and the reproducibility of the 2DE process. See text for abbreviations

Figure 4. The "Omic" sciences. Genomics investigates the whole genome (DNA) and its functional relations. From DNA, RNA is transcripted. Transcriptomics studies messenger RNA (mRNA). From mRNA transcripts, proteins are translated. Proteomics analyzes the protein expression profile. Endogenous synthesized metabolites are examined by metabolomics.

Figure 5. Proteomic strategies for the study of atherothrombosis. See text and other figure legends for abbreviations.



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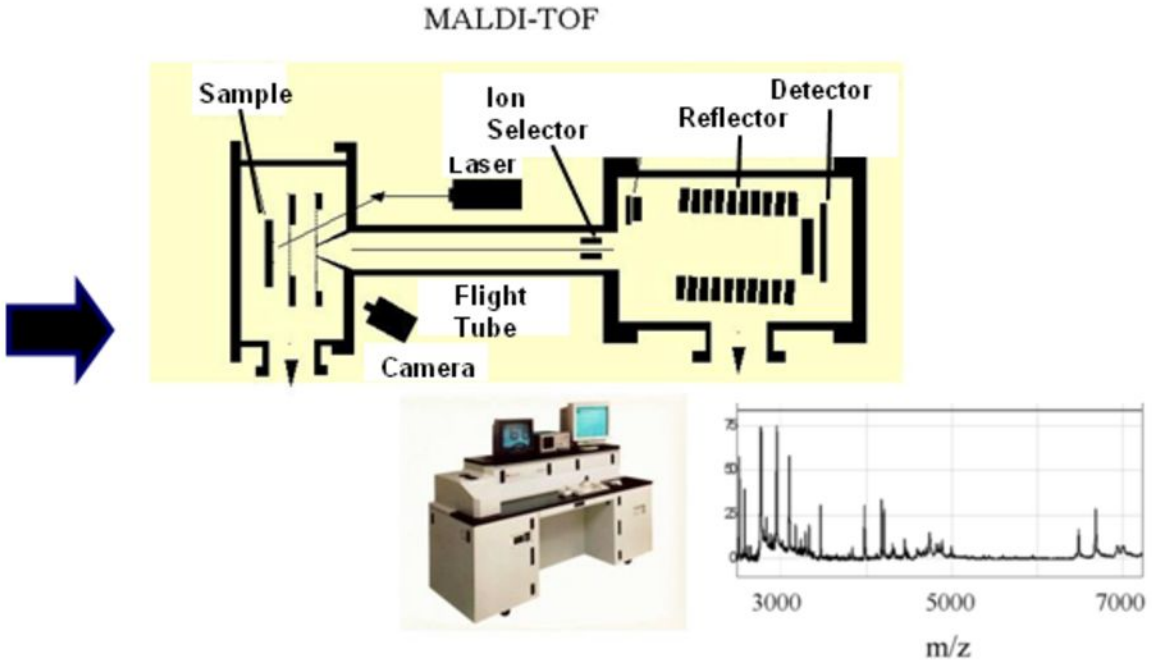

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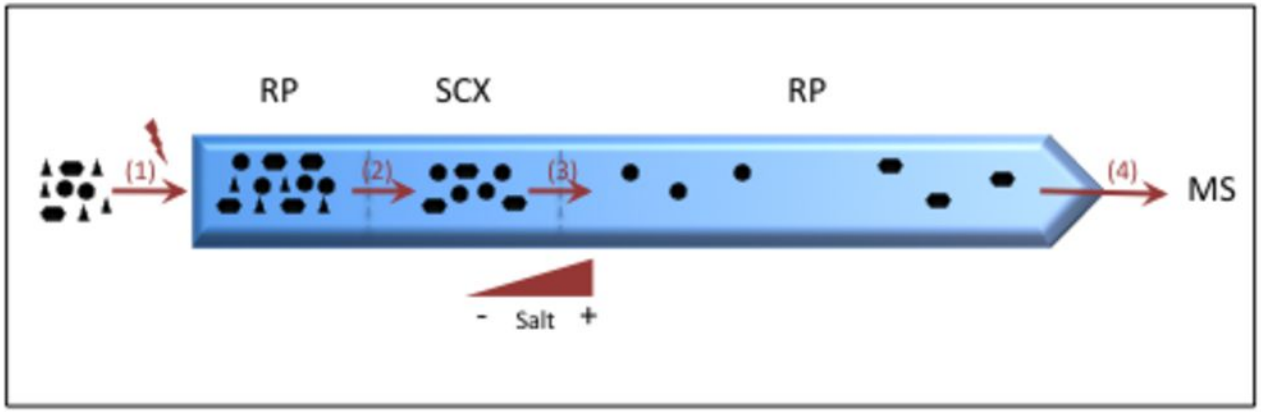
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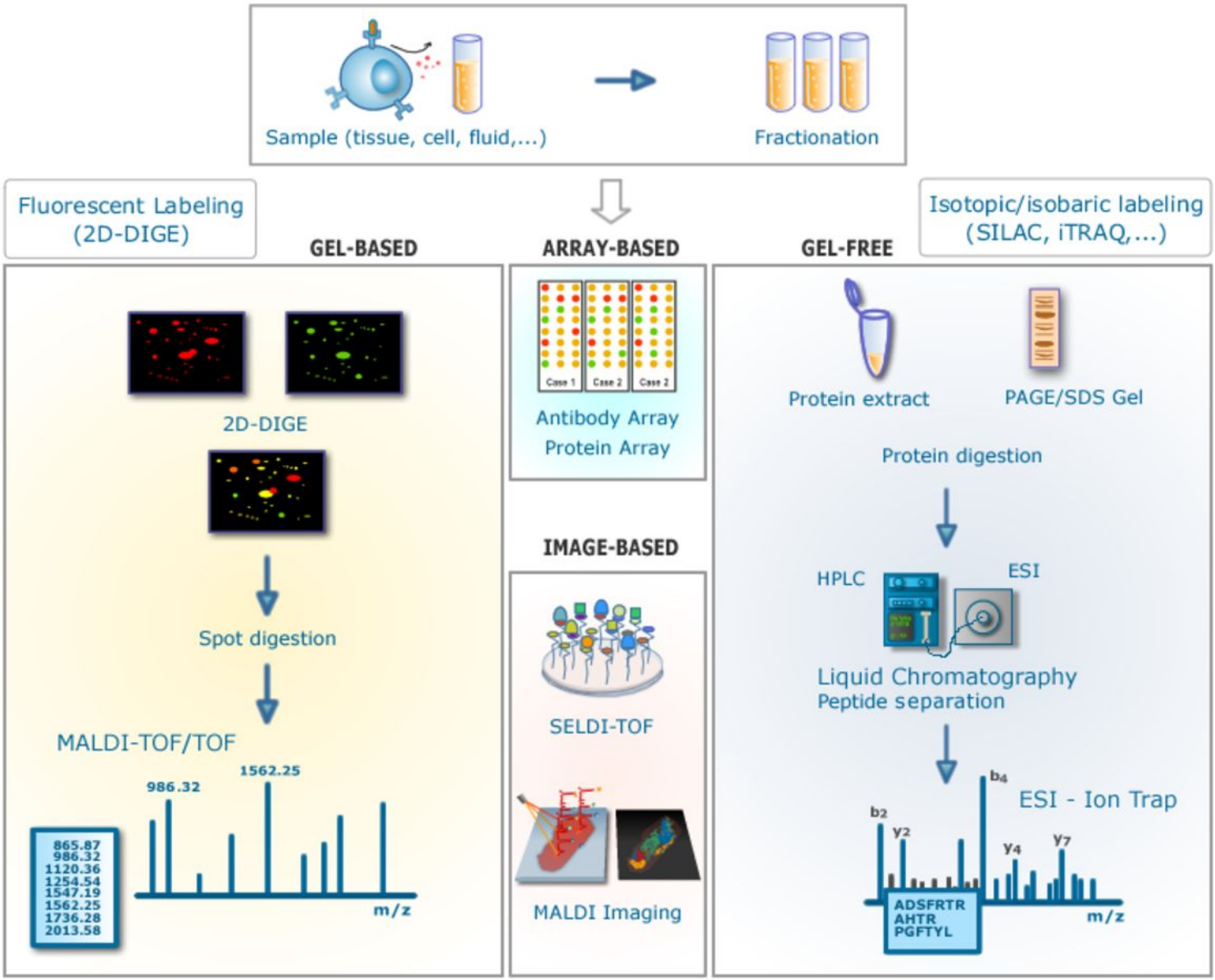
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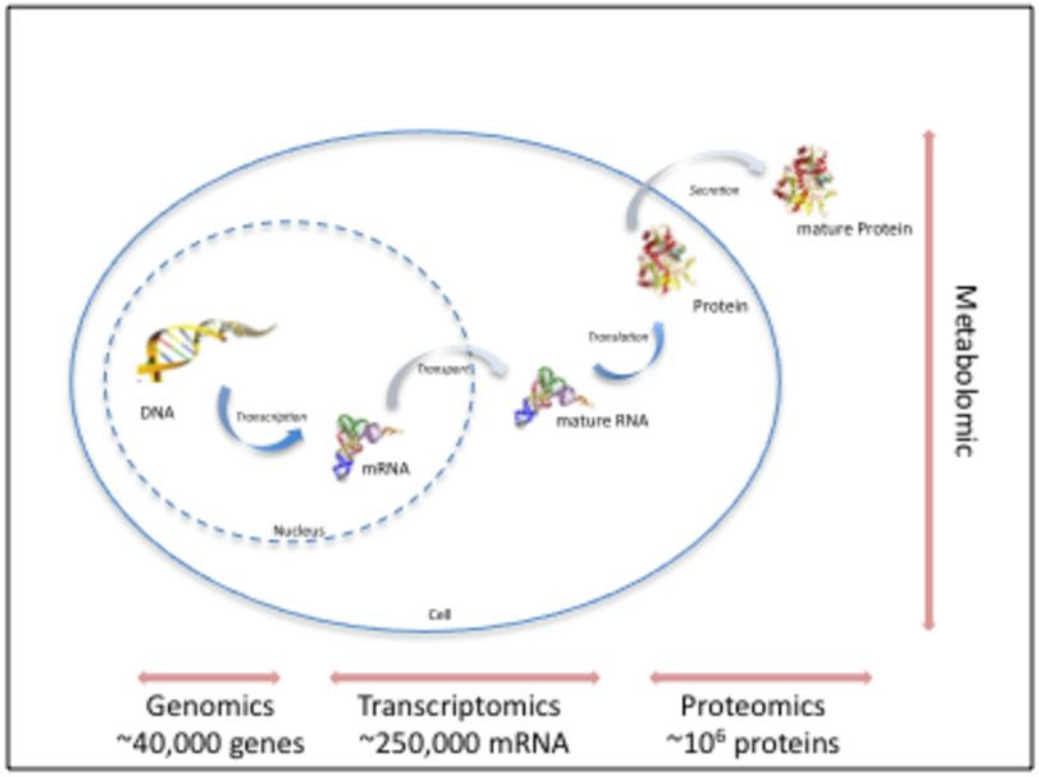
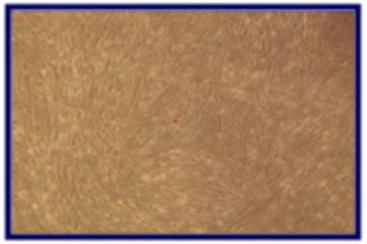
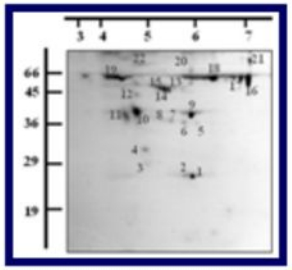
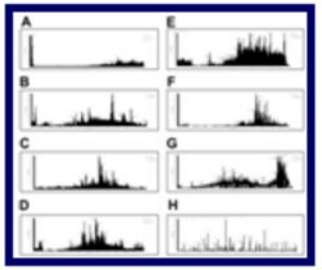
ARRAY-BASED
GEL-FREE
2D-DIGE
Spot digestion
MALDI-TOF/TOF
986.32
1562.25
865.87
986.32
1120.36
1254.54
1547.19
1562.25
1736.28
2013.58
m/z
Case 1
Case 2
Case 3
Antibody Array
Protein Array
IMAGE-BASED
SELDI-TOF
MALDI Imaging
Protein extract
PAGE/SDS Gel
Protein digestion
HPLC
ESI
Liquid Chromatography
Peptide separation
ESI - Ion Trap
b2
y2
b4
y4
y7
ADSFRTR
AHTR
PGFTYL
m/z

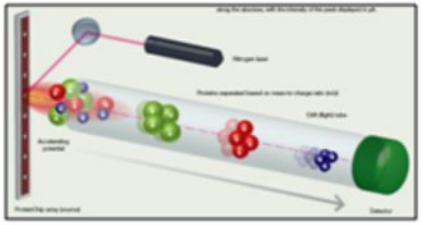

Secretion
mature Protein
Protein
Translation
Transport
mature RNA
DNA
Transcription
mRNA
Nucleus
Cell
Metabolomic
Genomics
~40,000 genes
Transcriptomics
~250,000 mRNA
Proteomics
~10^6 proteins



Cells



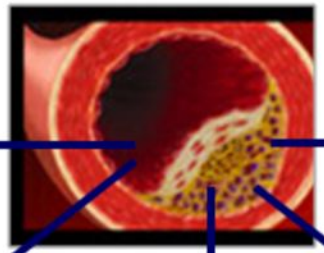




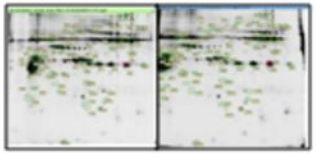
SELDI

Plasma

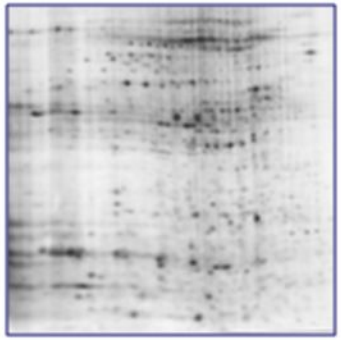
Serum



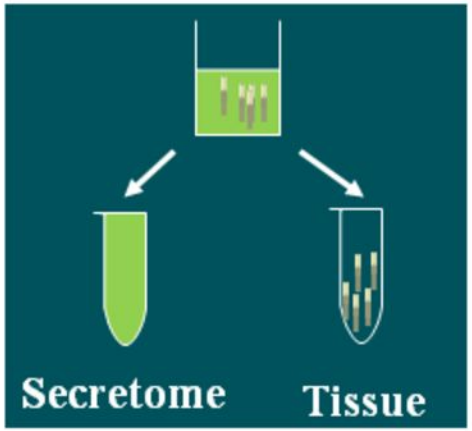
Whole Tissue

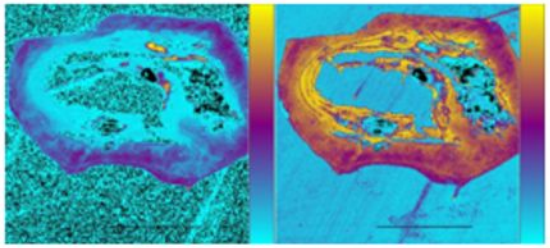


2DE 2D-DIGE



Circulating blood cells





MALDI-Imaging

Table 1.- Approximate costs of some basic proteomic techniques

TECHNIQUE	COST
2DE*	150-240 $
DIGE†	1,200 $
Protein Identification (MALDI-TOF) ‡	60-150 $
Protein Identification nanoLC-MS/MS §	600 $
MudPIT ‖	2,000 $

MALDI-TOF costs are displayed per spot identified. For the remaining techniques, costs are shown per sample analyzed except for DIGE (two samples). These prices are orientative and may vary in different laboratories.

***2DE:** Two-dimensional electrophoresis

†DIGE: Differential in-gel electrophoresis

‡MALDI-TOF: matrix-assisted laser desorption ionization time of flight

§LC-MS/MS: liquid chromatography-Mass spectrometry/Mass spectrometry

‖**MudPIT:** Multi-dimensional Protein Identification Technology