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Urinary Proteomics and Renal Transplantation

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1. Introduction

Nearly 60 years after the first successful organ transplantation in humans, it has been an exponential increase in our understanding of the immunological processes involved in organ transplantation. This knowledge has resulted in the identification of immunogenic drug targets and improvement on the management of patient's surveillance. The past 5 decades have also seen an explosive evolution in the fields of molecular biology, chemistry and informatics that have enabled increased data throughput, permitting the study of complete sets of molecules with increasing speed and accuracy using the omics techniques such as, genomics (DNA), transcriptomics (RNA), proteomics (proteins) and metabolomics (metabolites) (Bañón-Maneus et al 2007)

Despite overall improvements in immunosuppression regimens, chronic allograft dysfunction (CAD) continues to have a negative impact on graft and patient survival, even with the use of appropriate doses of immunosuppressive drugs to prevent acute rejection. Successful management requires an early detection along with adequate treatment. (Hariharan et al 2000 and Meier-Kriesche et al 2004)

Available diagnostic methods include clinical presentation, biochemical parameters and biopsies. Currently, the only non-invasive biomarkers for follow up the kidney graft are serum creatinine, glomerular filtration rate (GFR) and proteinuria but neither is particularly sensitive or specific and may not reflect early changes. At present, biopsy allograft is regarded as the gold standard for the diagnosis of CAD allowing its early detection; however, this is a costly procedure which is associated with clinical complications (Ojo et al 2000 and Nankivell et al 2003). The patient's management would be facilitated if there were appropriate biomarkers enabling the diagnosis or prognosis of different states throughout the post transplant course. At that point the proteomics have emerged as a really useful technology for biomarker discovery.

2. Urine as a source of biomarkers

The Biomarkers Definitions Working Group, defined Biomarker as a molecule that it is a characteristic, objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacological responses (Biomarkers Definitions Working Group, 2001). The ideal biological sample for detecting biomarkers is the so-called "proximal fluid", the bio fluid in closest contact with the site of disease (Decramer et al 2008). Then, in the context of kidney transplantation the closest biological sample, besides

the biopsy, is the urine which represent a fairly simple, non-invasive and inexpensive method for obtaining suitable samples for biomarker analyses, and presents a huge advantage as it can be performed regularly and frequently if necessary.

The human kidney (Fig. 1) is composed of 1 million nephrons, which can be divided in two functional parts: the glomerulous, which filters the plasma yielding the "primitive" urine, and the renal tubule, which reabsorbs most of the primitive urine. However, more than 99% of this primitive urine is reabsorbed. The remainder (the urine) exits the kidney via the urethra into the bladder (Fig. 1) (Decramer et al 2008). Therefore urine may contain

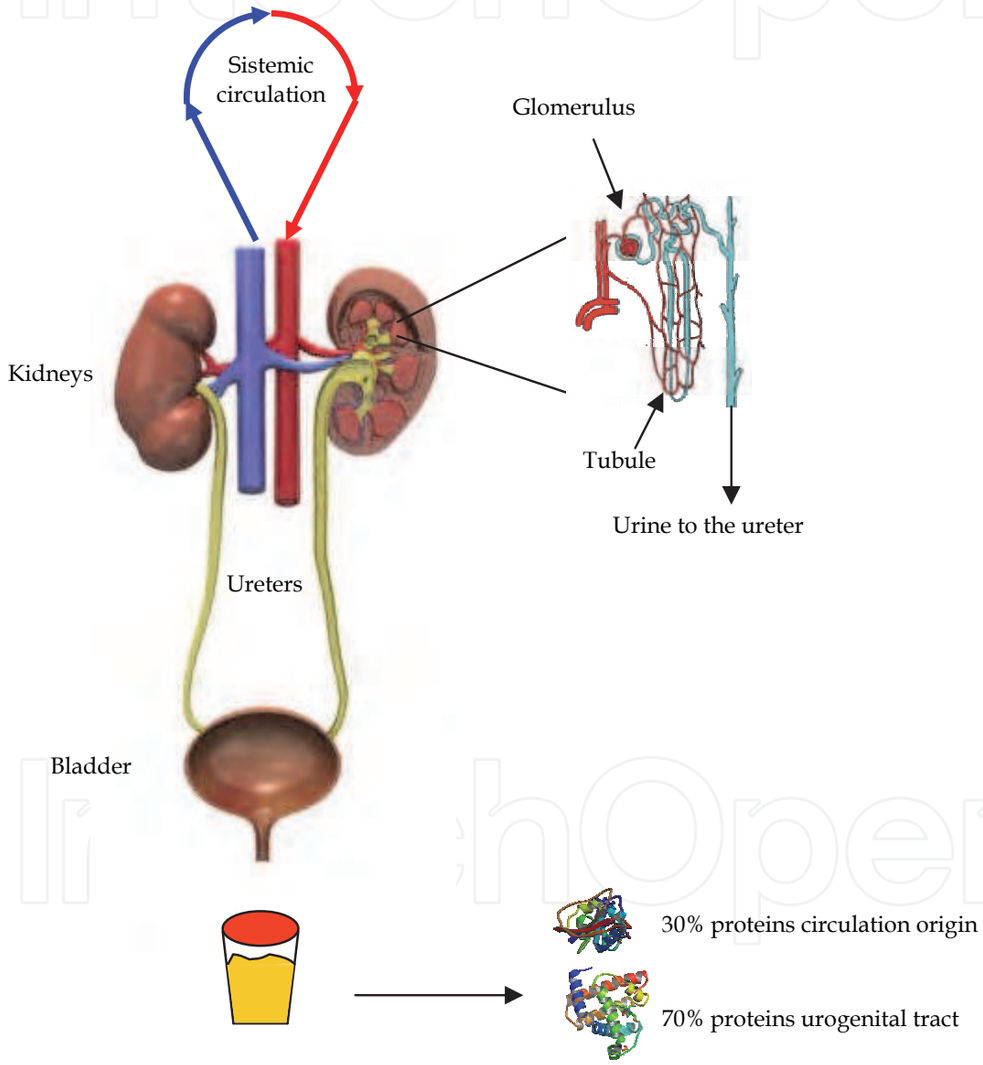


Fig. 1. Urinary proteins origin. Human kidney it is constituted by nephrons. The plasma filtration occurs into the glomerulus and the reabsorption into the tubule. The urine generated exits the kidney via urethra into the bladder. (Adapted Decramer et al 2008) (images from www. turbosquid.com and www.ratical.org)

information not only from the kidney and the urinary tract but also from more distant organs via plasma obtained by glomerular filtration. In healthy individuals, 70% of the urinary proteome originates from the kidney and the urinary tract, whereas the remaining 30% represents proteins filtered by the glomerulus (Thongboonkerd and Malasit 2005).
For these reasons urine is defined as "fluid biopsy" of the kidney and urogenital tract, and many of the changes in kidney and urogenital tract can be detected in the urinary proteome. Furthermore, as blood filtering, urine contains protein components that are similar to those found in the blood. Thus, pathological changes occurring in other organs can be detected in blood plasma and, therefore, can be detected in the urinary proteome. NHGRI (National Human Genome Research Institute) has said that the study of proteomics of fluids is one of the most promising tools for the development of noninvasive tools for early detection of human diseases. Human urine is a fluid that contains immediately accessible useful biomarkers, it is easy to obtain, non invasive, could be stored for long time and other advantages described into Table 1. But the urine has some disadvantages too.

ADVANTAGES	DISADVANTAGES
- can be obtained in large quantities using non-invasive procedures - easy assessment of reproducibility or improvement in sample preparation protocols - urinary peptides and lower molecular mass proteins are generally soluble. (Solubilization of these low molecular weight proteins and peptides is a process with a major influence on the proteomics analysis) - > 30 KDa compounds can be analyzed in a mass spectrometer without additional manipulation - urinary protein is relatively stable probably due to the fact that urine "stagnates" for hours in the bladder - can be stored for several years at -80 °C without significant alteration of its proteome - not only the changes in the kidney and genitourinary tract are reflected by changes in the urinary proteome but also changes at more distant sites	- it widely varies in protein and peptide concentrations mostly because of differences in the daily intake of fluid - standardization based on creatinine or peptides generally present in urine - definition of disease-specific biomarkers in urine, is complicated by significant changes in the proteome during the day - changes are likely caused by variations in the diet, metabolic or catabolic processes, circadian rhythms, and exercise as well as circulatory levels of various hormones

Table 1. Advantages and disadvantages of urine as source of biomarkers (Decramer et al 2008, Fliser et al 2005, Kolch et al 2005, Omenn et al 2005, Schaub et al 2004, Schiffer et al 2006, and Theodorescu et al 2006)

Clinical proteomics is growing significantly in recent years due to the prospect of identifying new targets for treatment and therapeutic intervention and biomarkers for diagnosis, prognosis, and therapeutic efficacy using technologies that allow us to compare proteomic profiles between different conditions pathophysiology. The measurement of protein in the urine has been used for many years for the diagnosis and monitoring of many kidney diseases.

3. Omics strategies and single molecule approaches

The major differences between omics strategies and single molecule approaches lies in throughput (hundreds of thousands versus one or a few), but also in experimental design. Classic single molecule bio medical research is based on hypothesis testing, building new experiments based on prior observations and theory, such that classic scientific research tends towards a reductionist approach in understanding disease and disease processes, owing to the limitations of most technologies and the complex nature of pathological systems. After a first omics hypothesis generating approach, classic single molecule experiments should ensue, in order to delve further into the mechanisms of the newly generated hypotheses. (Naesens and Sarwal, 2010)

The development of genomics and transcriptomics notwithstanding, gene polymorphisms and transcript levels correlate incompletely with the expression level of the functionally active proteins, which more accurately reflect actual cellular events. This poor correlation between genotype, gene expression and the localization or activity of the proteins is caused by the complex regulation of the transcription and the post translational modifications that change the properties of proteins. Proteins therefore provide a better picture of events that occur inside an organism and provide ideal biomarkers for disease conditions. (Abbott 1999, Quintana et al 2010 and Righetti and Boschetti 2007)

4. Proteomics technology

Proteomics methods are also increasingly being used in the field of organ transplantation. Because urine is the ideal non invasive specimen for renal diseases, the number of proteomic studies of urine has surged, and urine proteomics are a promising tool for the non invasive diagnosis of acute rejection and chronic allograft histological damage.

New tools and new applications of chemical technologies have revolutionized proteomics and peptidomics last years. Proteomics tools include gel electrophoresis (like one dimensional gel electrophoresis (1D); two dimensional gel electrophoresis (2D)) and gel free methods using mass spectrometry (like matrix-assisted laser ionization (MALDI); liquid chromatography mass spectrometry (LC-MS); surface-enhanced laser ionizationwith time of flight mass spectrometry (SELDI-TOF-MS)) (Naesens and Sarwal, 2010).

Proteome analysis of urine requires fractionation to reduce complexity of the sample. Fractionation can be obtained by different techniques. These fractions are subsequently analyzed by a mass spectrometer (MS) where the relative abundance of the different proteins and peptides is determined. Bioinformatics treatment of the protein data in combination with the fractionation parameters yields protein profiles representing the partial protein content of samples (Fig. 2)

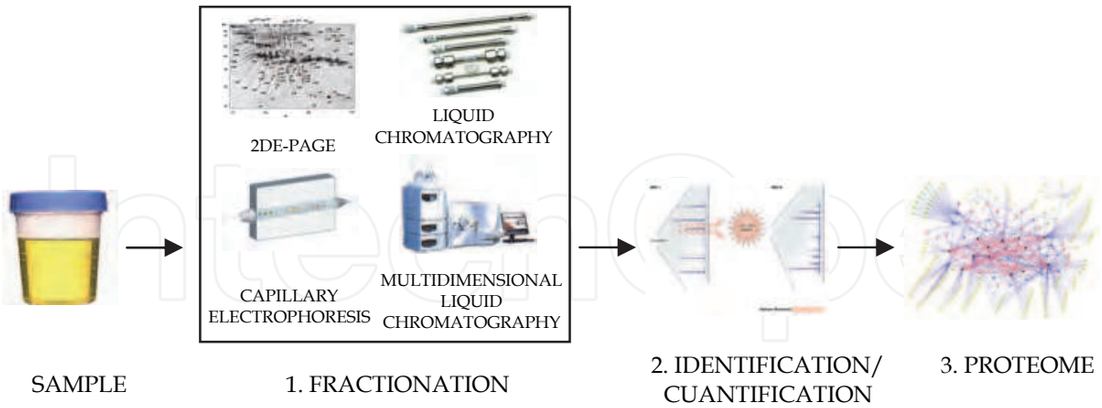


Fig. 2. Proteome analysis of urine requires fractionation to reduce complexity of the sample. Fractionation can be obtained by different chromatographic techniques or 2DE-PAGE. 2 These fractions are subsequently analyzed by a mass spectrometer (MS) 3 Bioinformatics treatment of the obtained data give us the sample proteome.

4.1 Urine sample: Collection, storage and preparation

Sample collection, storage and preparation it is a crucial issue, and many of the techniques described later requires different preparation methods. (Lee et al 2008)

Collection

The volume, protein concentration and composition of urine show considerable variation among the day. There is no ideal time of day to collect urine specimens for proteomics, but it is preferable to obtain samples at the same time of the day to minimize variations. The first morning void could be contaminated by cells from the lower urinary tract and bacteria harbored in the urinary tract. 24 hour samples have the problem of protein degradation. The second void of the morning could be one of the best samples that could be used, because it is easy to obtain when patients come to hospital and could be quickly processed. (O'Riordan et al 2006)

Some intra-patient variability in the urine proteome has been previously observed, but other investigations have identified only minor variations in samples collected for up to a year. Individual fluctuations seem to be minimally affected by diet and exercise. The relative influence of exogenous and endogenous factors needs further exploration, but should be considered when planning protocols and in interpretation of data (Akkina et al 2009).

Storage

The addition of protease inhibitors, filtration, centrifugation before and after freezing to reduce contamination by proteins leaking cellular debris and bacteria it is necessary. It is necessary to do some test to confirm the absence of red blood cells and leukocytes to avoid possible contamination of the samples.

Repeated freezing and thawing is known to fragment certain proteins, such as IgG and α-1-antitrypsin, and should be avoided. Urine stored at 37 °C can exhibit specific protease activity. An advantage of urinary proteomics is that the analytical reproducibility of the

urine proteomic profile is unaffected by long term freezing, remaining stable for several years, even when stored at -20ºC (O'Riordan et al 2006).

Preparation

The presence of several excessively abundant urinary proteins, among them albumin and uromodulin (uromodulin is most abundant in normal urine, whereas albumin predominates in urine from diseased kidneys), can interfere with analyses. Depletion of these abundant proteins can increase the relative concentration and odds of detecting lower abundance proteins, but with the depletion we can lose some not abundant proteins (Hewitt et al 2004). Isolating or concentrating urinary proteins may be essential in low concentration specimens, particularly for gel-based studies. Numerous methods have been compared including precipitation with organic solvents, centrifugal filtration, lyophilization and ultrafiltration but with varying results. Precipitation with Trichloroacetic Acid gave good qualitative yield. Reverse phase extraction has also been shown to be effective in specimen concentration as well as for desalting urinary peptides and segregating lower-molecular weight proteins (Bañón-Maneus et al 2007 and 2011).

4.2 Separation of proteins

Mono-and two-dimensional electrophoresis (2D-PAGE)

Two-dimensional electrophoresis was first described by O'Farrell over 30 years ago. The technology used for separation of proteins is polyacrylamide gel electrophoresis. For many proteomic applications, electrophoresis in one dimension is the method of choice. The proteins are separated according to their mass and how the proteins are solubilized in sodium dodecyl sulfate (SDS) there are generally no problems of solubilization. It is a simple, reproducible and allows the separation of proteins of 10-300 kDa. The 2D-PAGE two-dimensional electrophoresis allows separate thousands of proteins in a single experiment, and is currently the most efficient method for separating very complex protein mixtures. It is based on a separation of proteins according to the isoelectric point, followed by separation of proteins according to their molecular mass (Fig. 3). The first dimension separation is performed by isoelectric focusing, during which proteins are separated in a pH gradient until reaching a position where its net charge is zero, its isoelectric point (Fig. 4A). In a second dimension, proteins are separated by molecular weight in polyacrylamide gels (Fig. 4B). The high resolution of the technique is that the two separations are based on independent parameters. The key innovation for the 2D-PAGE was the development of gels with immobilized pH gradient (IPG). For detection of proteins has traditionally been using the radioactive labeling or staining with Coomassie blue or silver, for greater sensitivity. It also has developed a silver staining method compatible with surface protein digestion and mass spectrometry (Fig. 3) (Bañon-Maneus et al 2010, Oh et al 2004 and Thongboonkerd 2002). A recent development of 2D-PAGE technology is DIGE (Difference in Gel Electrophoresis), explained later.

The 2D-PAGE also has limitations: It is a very demanding technique, is time consuming, and difficult to automate, is limited by the number and type of proteins to solve; the very large or hydrophobic proteins do not enter the gel during the first dimension while proteins very acidic or very basic not well resolved, and in the presence of abundant proteins are difficult to detect low abundance proteins. Some of these problems can be resolved by fractionation,

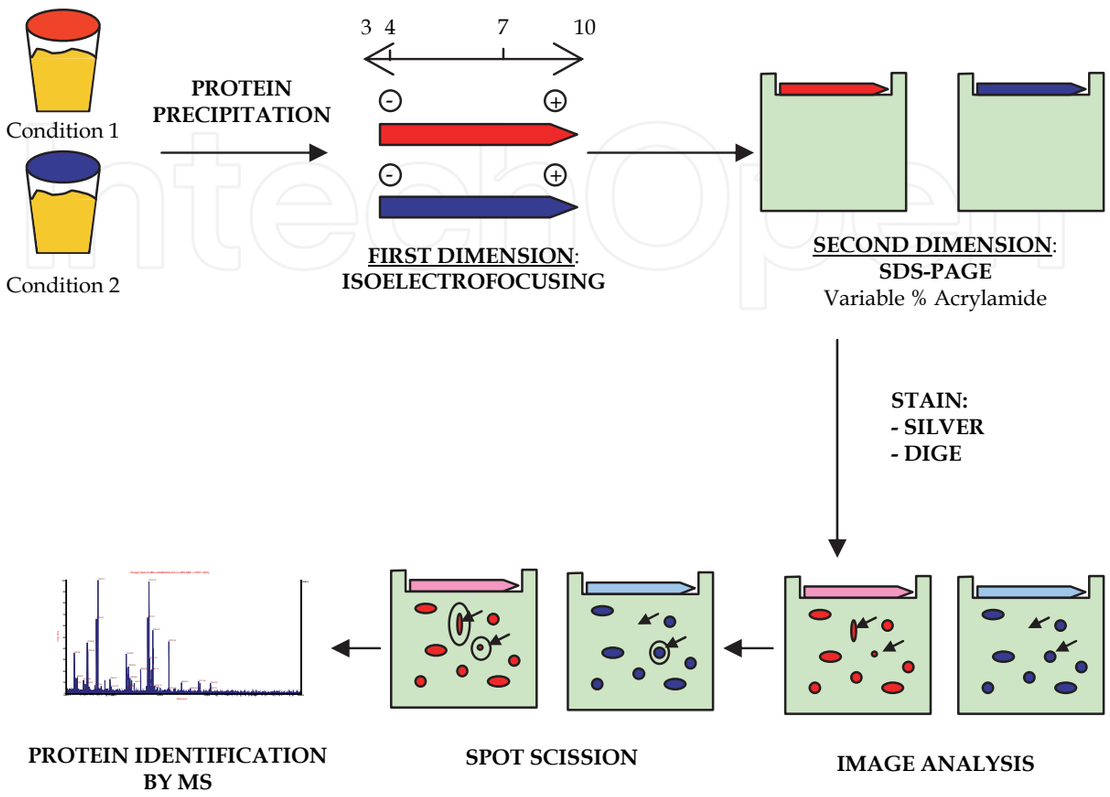


Fig. 3. 2D-PAGE workflow. After the protein extraction of the samples proteins were firs separated by the isoelectric point and after that by molecular weight. After the 2DE-PAGE staining the images were analyzed by finding of differential spots, that after the scission were identified by distinct Mass spectrometry approaches.

the use of certain solubilization conditions and the use of IPGs with different pH ranges. (Table 2) (Yoshida et al 2005)

Liquid chromatography

LC is a physical method of separation based on the distribution of the various components of a mixture into two immiscible phases one stationary and the other mobile. The mobile phase involves a liquid that flows through a column containing the steady phase. Classic LC is carried out in a column which is generally made of glass and filled with the steady phase. The steady phase may be a solid with different chemical properties which give rise to different types of chromatography – ion exchange chromatography, reverse-phase chromatography, and others. The mobile phase may be a pure solvent or mixture of solvents. After placing the sample on the upper part, the mobile phase flows through the column as a result of gravity. To improve the efficiency of separations, the size of steady phase particles was gradually diminished down to microns, and this called for high pressures to ensure mobile phase flow. (Table 2) (Cutillas et al 2003 and Mann et al 2002)

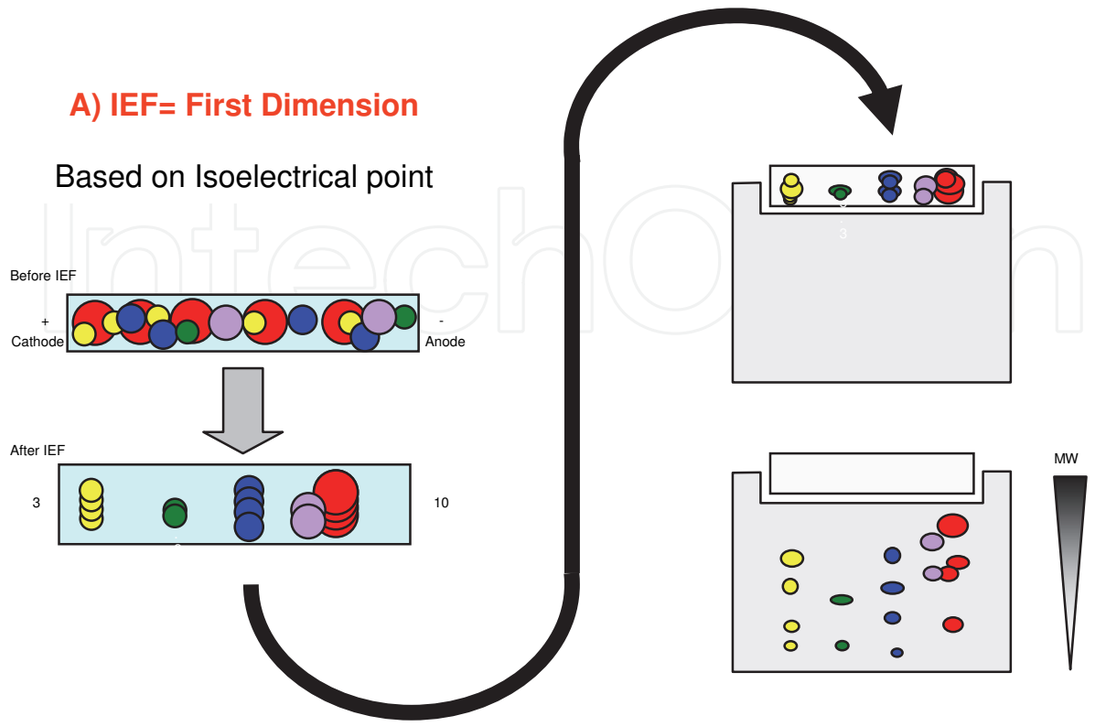


Fig. 4. A) IEF Samples were loaded into a dry polyacrylamide gel strips with an immobilized pH gradient for the separation by isoelectric point B) Second dimension, strips were loaded on the top of polyacrylamide gel and proteins were separated by molecular weight

Technology	ADVANTAGES	DISADVANTAGES
2DE-MS	For large molecules, sequencing of biomarkers easy to perform from spots	Restricted to selected IP, difficult to automate, time consuming, small molecules not detected (<10 kDa)
SELDI-TOF	High throughput, easy-to-use, automation, low sample volume	Restricted to selected proteins, low resolution MS, lack of comparability.
LC-MS	Automation, multidimensional, high sensitivity, used for detection of large molecules (>20 kDa) after tryptic digest, sequence determination of biomarkers provided by MS/MS	Reassembly of tryptic peptides into their precursor molecule can be problematic, time consuming, relatively sensitive toward interfering compounds, medium throughput
CE-MS	Automation, high sensitivity, fast, low sample volume, multidimensional	Generally not suited for larger molecules (>20 kDa)

2DE–MS, Two-dimensional gel-electrophoresis followed by mass spectrometry; LC–MS, liquid chromatography coupled to mass spectrometry, SELDI–TOF, surface-enhanced laser desorption/ionization coupled to mass spectrometry; CE–MS, capillary electrophoresis coupled to mass spectrometry

Table 2. Advantages and disadvantages of proteomic techniques for use in urinary biomarker discovery

High Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) involves dissolving the protein mixture in buffer and pumping it through a series of columns. The columns are composed of materials with various physical, chemical and immunological properties, which bind different proteins with varying degrees of affinity depending on the complementary protein properties. The proteins can then be eluted from the columns. The properties on which separation can be based are numerous; the elements most frequently applied to urine are size exclusion (based on size), reverse phase (based on hydrophobicity), strong and weak cation binding, and affinity binding (i.e. an immunoglobulin adsorbing to protein of interest). (Bañón-Maneus et al 2007)

Capillary electrophoresis

In a silica capillary, proteins or peptides are separated as a function of charge at a desired pH by an electric field in which the capillary is housed. Like HPLC, this method can be applied to intact proteins, as well as digests. Although a powerful separation technique, capillary electrophoresis (CE) does not yield reliable quantitative information. (Table 2) (Schiffer et al 2006)

Mass spectrometry (MS): Identification and characterization of proteins

Proteins can be identified by various means, among which include the sequencing of the N-terminal specific antibody detection, amino acid composition, co-migration with known proteins and over-expression and depletion of genes. All these methods are generally slow, laborious or expensive and therefore not suitable for use as large-scale strategies. However, the MS, because of its rapidity and high sensitivity, has become the preferred method for identifying large-scale protein and the first step to study the proteome of different organisms. It also allows the characterization of post-translational modifications that have physiological relevance, such as glycosylation and phosphorylation. To analyze proteins by mass spectrometry these must be converted into peptides through proteolysis, usually with trypsin (Mauri et al 2009). This so robust technique involves (Fig. 5):

- Conversion of peptides into gas phase ions using soft ionization techniques such as ionization-assisted laser desorption matrix (MALDI) from a sample in solid form, or by electrospray ionization (ESI) of a sample solution.
- Separation of ions according to m/z (mass / charge) in a mass analyzer (e.g. type analyzer TOF (Time Of Flight), quadrupole, ion trap, etc.). Optional Fragmentation of selected peptide ions through goal decomposition stable (or PSD technique: post source decay) or by collision-induced dissociation (CID) conducted in a tandem mass spectrometer combining two different analyzers.
- Measurement of the masses in a detector obtaining a mass spectrum that reflects the abundance of ions versus their value m / z

For protein identification it has been developed two strategies:

- Identification by peptide fingerprint (PMF: peptide mass fingerprinting) or peptide mapping using MALDI-TOF spectrometer type.
- Identification of peptides obtained by fragmentation of whole or partial sequence of amino acids (sequence tag) using a tandem mass spectrometer.

Peptide mass fingerprinting

Peptide mapping is a technique used routinely to identify proteins quickly, usually from SDS-PAGE gels or 2D-PAGE and that is normally performed in a mass spectrometer type

MALDITOF. In this approach the protein is digested with an enzyme, usually trypsin. The sample is incorporated into a metal plate with a matrix and crystals formed upon evaporation. Subsequently, the sample is irradiated with laser to ionize the molecules. The ions are accelerated by an electric field towards a detector, the value m / z of each ion is determined by the flight time from the source reaching the detector.

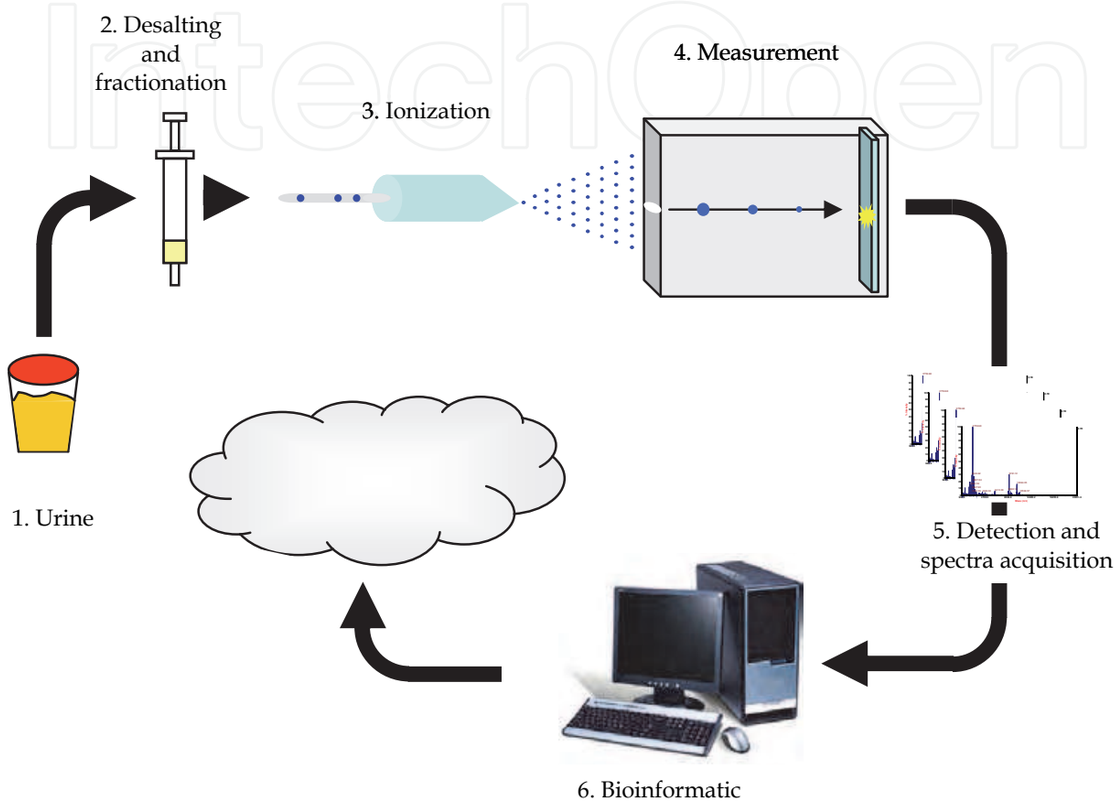


Fig 6. Urine samples were concentrated and separated from organic salts by solid phase. Each sample was applied and dried on an uncoated MALDI target plate using the sandwich technique.

The peptide fingerprint (PMF) of a particular protein is a set of peptides generated by digestion of a specific protease. These experimental peptide masses are compared with theoretical peptide masses of proteins present in databases by developing various algorithms available on the network. For the correct identification of the protein mass requires a large number of peptides matching the theoretical masses of peptides, covering part of the protein sequence database. The limitations of mass spectrometry are that the ionization of peptides is selective and not quantitative. In an equimolar set of peptides derived from digestion of a protein, some peptides may not be detected and the rest of them can be a large variation in signal intensity. If the amount of protein in the gel is small, the number of peptides observed can be small and therefore the protein can not be identified with certainty. The MALDI-TOF MS is of little use to analyze protein mixtures. Very clear protein spots from 2D gels can contain several proteins (Bañón-Maneus et al 2007 and Gazzana and Borlak 2007)

Peptide sequence

It is a strategy to identify proteins not annotated in databases or for the ambiguous identification by MALDI-TOF. The tandem mass spectrometer MS / MS can also determine the amino acid sequence. Ion is selected by a mass spectrometer and fragmented first collision with a gas and the fragments are analyzed in a second spectrometer. Peptide sequence can be done with MALDI ionization source type or ESI. (Gazzana and Borlak 2007)

Surface-enhanced laser desorption/ionization (SELDI)

SELDI–MS incorporates chromatographic and MS principles in a single platform. An activated surface on a 'chip' binds proteins on the basis of their chemical and physical properties; unbound proteins are washed off. A subset of the proteome is thus selected and the chip plugs directly into the mass spectrometer for analysis. This is a high-throughput screening technique that facilitates relative abundance profiling of individual proteins from different samples. Although the approach can be extremely useful for screening peptide/protein samples for recognition of biomarker ions, it does not enable the protein origin of these ions to be reliably discerned. Other disadvantages are use of relatively low-resolution MS, the fact that only a subset of the proteome can be studied on any particular surface, and that the performance varies between different machines (as does performance of a single machine over time). (O´Riordan et al 2006)

4.3 Expression level quantitative techniques

The main application of proteomics is the study of protein expression profile. There are two strategies that enhance the study of differential protein expression between different samples, the gel based and the free gel technologies.

Gel based quantitative proteomics: DIGE

Also recently described an approach based on the labeling of proteins with different fluorophores and the separation of samples by 2D-PAGE in the same gel. This methodology, called DIGE (Differential Gel Electrophoresis), minimizes the variability of the gels decreased analysis time and allows quantification of very specific expression profile. Briefly, two samples are differentially labeled with two different fluorescence CyDyes (p.e, Cy3 and Cy5), mixed, and then resolved simultaneously within the same 2DE gel. The introduction of a pooled internal standard labeled with a third dye (p.e. Cy2) improves the accuracy of protein quantification between samples from different gels allowing detection of small changes in protein levels. Differentially expressed proteins could be identified using protein fingerprinting MS methods as modern matrix-assisted laser desorption/ionization-time of-flight MS (MALDI-TOF-MS) instrumentation. (Alban et al 2003, Shaw et al 2003, Unlu et al 1997 and Wu 2006)

Gel Free quantitative proteomics: ICAT, iTRAQ, SILAC

The ICAT (isotope-coded affinity tags), which can determine the relative amount of protein between two samples. The two protein samples are labeled with the ICAT reagent light or heavy (as hydrogen or deuterium leads). This reagent binds to cystein and contains biotin to facilitate purification. Subsequently, the two samples are mixed and digested with trypsin. The peptides marked with ICAT reagent are separated in an affinity column and analyzed by MS. The relative intensity of the peptides identical in

each sample (differ in a mass of 8 Da) are abundant protein from which they came. The fragmentation of the peptide by MS / MS led to the identification of the protein (Sobhani 2010).

An improved approach analogous to ICAT has been developed called iTRAQ (Applied Biosystems). The technique is based upon chemically tagging the N-terminus of peptides generated from protein digests that have been isolated from cells, tissues, biological fluids in two different states (Chen et al 2010). The two labeled samples are then combined, fractionated by nanoLC and analyzed by tandem mass spectrometry. Database searching of peptides data fragmentation provides the identification of the labeled peptides and hence the corresponding proteins. Fragmentation of the tag attached to the peptides generates a low molecular mass reporter ion that is unique to the tag used to label each of the digests. Measurement of the intensity of these reporter ions, enables relative quantification of the peptides in each digest and hence the proteins from where they originate. There are four tags available enabling four different conditions to be multiplexed together in one experiment. (Gigy et al 1999)

Stable isotope labeling with amino acids in cell culture (SILAC) is a simple and straightforward approach for in vivo incorporation of a label into proteins for mass spectrometry (MS)-based quantitative proteomics. SILAC relies on metabolic incorporation of a given 'light' or 'heavy' form of the amino acid into the proteins. The method relies on the incorporation of amino acids with substituted stable isotopic nuclei (e.g. deuterium, 13C, 15N). Thus in an experiment, two cell populations are grown in culture media that are identical except that one of them contains a 'light' and the other a 'heavy' form of a particular amino acid (e.g. 12C and 13C labeled L-lysine, respectively). When the labeled analog of an amino acid is supplied to cells in culture instead of the natural amino acid, it is incorporated into all newly synthesized proteins. After a number of cell divisions, each instance of this particular amino acid will be replaced by its isotope labeled analog. Since there is hardly any chemical difference between the labeled amino acid and the natural amino acid isotopes, the cells behave exactly like the control cell population grown in the presence of normal amino acid. It is efficient and reproducible as the incorporation of the isotope label is 100%. This technology it is not yet available for human urine proteomics but it could be used in experimental models by the administration of pellet food with the isotope enhanced. (Quan et al 2011)

Protein Arrays

Protein arrays are rapidly being developed for the characterization of activities and for detecting protein-protein interactions on a large scale. Like DNA arrays, protein arrays will be essential for basic research and more applied research to drug discovery and development of diagnostic methods. In a pioneering work done by the group of Snyder, we developed a chip with 6,000 yeast proteins to identify new proteins that interact with calmodulin or phospholipids. The proteins were obtained by cloning the corresponding ORFs and each protein was expressed fused to GST (glutathione S-transferase) and a histidine tag. This important work showed that it is possible to prepare microarrays with thousands of proteins and used to study interactions. However, although significant progress has been made for the preparation of the arrays, we still need to face several technological challenges to allow allowing the use of this tool to many researchers. (Zhu et al, 2000)

5. Protein biomarkers for kidney transplantation

Currently follow up of renal transplant recipients is done by the physicians checking serum creatinine and glomerular filtration rate (GFR), but neither is particularly sensitive or specific and may not reflect early alterations (Paul 2009 and Nankivell 2003). At present, biopsy allograft is regarded as the gold standard for the diagnosis of kidney diseases allowing its early detection; however, this is a costly procedure that is associated with clinical complications (Beckingham et al 1994).

5.1 Acute renal allograft rejection

One of the major problems in renal transplantation is acute renal allograft rejection. Acute rejection is one of the key factors that determine long term graft function and survival in renal transplant patients. This fatal complication is inevitable if the diagnosis is delayed.

Mainly for groups reported urinary proteomic approach for acute rejection. Interestingly, each group found a different pattern of protein biomarkers that were associated with allograft rejection. These differences are not surprising, as each study had differences in disease definition, sample collection and handling, protocol for protein and data analysis. (Rush and Nickerson 2011)

Clarke et al reported the comparison between 17 urines from rejecting patients to urines from 15 stable (not biopsied) controls. Proteomic analysis of the urine was done using SELDI15 and ProteinChip Arrays with immobilized metal affinity (IMAC-3) and hydrophobic (H4) surface. The best candidate biomarkers were four proteins of molecular around 7 kd and one of 13.4 kd. A separate analysis using the CART algorithm in the Ciphergen Biomarker Pattern Software using two different proteins of 3.4 kd and 10 kd, respectively, correctly classified 91% of the 34 urine specimens in the training set, producing a sensitivity of 83% and a specificity of 100%. (Clarke et al 2003*)*

O'Riordan *et al* reported on the urine proteome in 23 renal transplant patients with biopsy-proven acute rejection, 22 recipients with stable graft function (characterized by serum creatinine) and 20 healthy volunteers (27). The urine was preadsorbed on four different chip surfaces, and was analyzed by SELDI-TOF. Protein masses that were useful in the construction of the classification algorithms were of approximately 2.0, 2.8, 4.8, 5.8 7.0, 19.0 and 25.6 kd. Patients that had experienced acute rejection could be distinguished from stable patients with a sensitivity of 90.5% to 91.3% and a specificity of 77.2% to 83.3%, depending on the classifier used. (O'Riordan et al 2004)

The main drawback with this two studies is that control samples (stable renal transplant recipients) where characterized by a serum creatinine and no biopsies were done at the time of urine collection.

Wittke et al reported the analysis done by CE-MS from 19 patients with subclinical or clinical rejection, 10 patients with urinary tract infection but without rejection, and 29 patients without acute rejection or urinary tract infection (28). These patients were from a centre that performs protocol biopsies, and the urine samples were obtained at the time of protocol biopsy. An additional cohort of 66 healthy controls was studied. The authors were able to discriminate the rejecting patients from those without rejection in 16 of 19 patients using combinations of 16 polypeptides. (Wittke et al 2005)

Finally, Schaub et al sought to determine whether such candidate proteins can be detected in urine using mass spectrometry. Four patient groups were defined on the basis of allograft function, clinical course, and biopsy result. Four groups where analyzed: acute clinical

rejection, stable transplant, acute tubular necrosis, and recurrent (or de novo) glomerulopathy. Urines were collected the day of the allograft biopsy. As a normal control group, urines from healthy individuals were analyzed, as well as 5 urines from non-transplanted patients with lower urinary tract infection. Three prominent peak clusters were found in 94% of the patients with acute rejection episodes, but only in 18% of patients without clinical and histologic evidence for rejection and in any of normal controls. In addition, the presence or absence of these peak clusters correlated with the clinicopathologic course in most patients. Acute tubular necrosis, glomerulopathies, lower urinary tract infection, and cytomegalovirus viremia were not confounding variables. (Schaub et al 2004)
In conclusion, proteomic technology together with stringent definition of patient groups can detect urine proteins associated with acute renal allograft rejection. Identification of these proteins may prove useful as non-invasive diagnostic markers for rejection and the development of novel therapeutic agents.

5.2 BKV renal allograft nephropathy

BKV renal allograft nephropathy (BKVAN have an important role in development of renal allograft dysfunction (Fishman 2002). About 6-10% of these patients develop BKVAN, and the reported graft loss rate in this group has been as high as 50% (6,7). BKVAN can resemble acute allograft rejection (AR) and differentiation between them can be challenging both at histological and molecular levels (Fishman 2002). The discrimination is important because the treatment is diametrically opposite for the two conditions. In general, immunosuppression needs to be reduced in patients with BKVAN, whereas it is increased in AR. Currently, these two clinical conditions cannot be differentiated in a reliable way on the basis of clinical and laboratory findings and a definitive diagnosis of BKVAN requires allograft biopsy. Even the histological differentiation of BKVAN from AR can be difficult unless viral inclusions are seen on allograft biopsy (Fishman 2002).
Jahnukainen et al used Surface-enhanced laser desorption/ionization (SELDI) time of flight mass spectrometry to compare the urinary of patients with BKVAN, AR and stable graft function. They were able to detect several peaks that were differentially expressed in the BKVAN group compared with both the AR and stable function groups. Peaks that corresponded to m/z values of 5.872, 11.311, 11.929, 12.727, and 13.349 kD were significantly higher in patients with BKVAN. As Mannon *et al* showed significant similarity of transcriptional expression of molecules associated with inflammation and fibrosis between BKVAN and AR (Mannon et al 2005). This probably is due to the similarity of the inflammatory response and leakage of inflammation related small molecular weight proteins into urine in both conditions. The limitations of this study are that all of their analyses were based on a limited sample size, and their results on the sensitivity and the specificity of the various algorithms should be interpreted with caution. A true assessment of sensitivity and specificity of the SELDI technique and the various models tested in this report cannot be determined until an independent validation set that is derived from another set of patients is assessed. Proteomic marker(s) profiles, together with plasma and urine BKV PCR and clinical information, may help in making differentiation of BKVAN from AR in a non-invasive manner. Histological verification of BKVAN probably will continue to be required for the foreseeable future, but it is likely that proteomic biomarkers could be used in deciding when a biopsy is necessary. Further studies on a larger number of patients are needed to validate these findings and to detect the identity of the significantly different peaks to develop robust, non-invasive methods for BKVAN diagnostics. (Fishman 2002).

5.3 Chronic allograft rejection

The survival half-life for kidneys from deceased donors is approximately 11 yr, and the pathogenesis of chronic allograft rejection (CAD) is multifactorial (Mauiyyedi et al, 2001). Analyses of graft histology reflected in the revised Banff criteria indicate CAD can be subcategorized, in part, on the basis of evidence of local inflammation and the presence or absence of interstitial fibrosis and tubular atrophy (IF/TA) (Solez et al, 2008). Although specific inciting factors are difficult to define in each situation, distinct histopathologic entities often correlate with likely causes. For example, calcineurin inhibitor toxicity frequently manifests as IF/TA without inflammation; ongoing cellular alloimmunity presents histologically with tubulitis with or without IF/TA; C4d staining suggests transplant glomerulopathy with or without IF/TA; and detectable, donor-specific serum antibodies underlie antibody-mediated allograft injury (Solez et al, 2008).

Because only a subset of patients develop CAD and at present physicians do not have the ability to reverse chronic fibrotic kidney damage, it is essential that the transplant community develop reliable and noninvasive approaches to predict which patients are most likely to develop graft failure so that appropriate interventions can be instituted before graft failure becomes clinically apparent (Mauiyyedi et al, 2001).

Urine proteomic profiling of CAD has been investigated in a few studies to date. Using SELDI as screening methodology and liquid chromatography coupled to mass spectrometry (LCMS) to obtain protein ID information, O'Riordan et al studied the urinary proteome of 75 renal transplant recipients and 20 healthy volunteers. Patients could be classified into subgroups with normal histology and Banff CAN grades 2-3 with 86% sensitivity and 92% specificity. Several urinary proteins associated with advanced CAN were identified including a1-microglobulin, b2-microglobulin, prealbumin, and endorepellin, the antiangiogenic C-terminal fragment of perlecan. Increased urinary endorepellin was confirmed by ELISA and increased tissue expression of the endorepellin/perlecan ratio by immunofluoresence analysis of renal biopsies (O'Riordan 2008).

Our group is also investigating the utility of proteomic analysis of urinary samples as a non-invasive method to detect and evaluate CAD. We did the two main proteomic approaches, gel based and gel free approach. Proteomics based on two-dimensional electrophoresis (2-DE) has been optimized with the development of Difference Gel Electrophoresis (DIGE). A proteome map of stable renal patients as a reference protein database, to validate the utility of 2D-DIGE technology in finding new candidates as CAD urinary biomarkers were established. Morning spot urine of kidney transplant patients with a biopsy two years after transplantation with CI/CT score 0-I-II/III (n=8/group) was collected. 2D silver stained and mass spectrometry (MS) analyses were used to establish the proteome map and 2D-DIGE and MS were used to identify proteins exhibiting differential abundance. In this work not only the urinary proteome of renal stable patients was established but we were able to identify 11 proteins with elevated levels on advanced CAD: β-2 microglobulin, MASP-2, α-1-β-glycoprotein, leucine-rich α-2-glycoprotein 1, α-1-antitrypsin, Gelsolin precursor, AIF-like mitchondrion-associated inducer of death, heparan sulfate proteoglycan, anti-TNF-α antibody light-chain, immunoglobulin lambda light chain and dimethylarginine-dimethylaminohydrolase 2 and wnt-1. Eight of these proteins, α-1-antitrypsin, angiotensinogen, β-2-microglobulin, dimethyl-arginine dimethylaminohydrolase-2, immunoglobulin lambda light chain, transferrin, trypsin precursor, and Zn-β-2-glycoprotein, have been described in other renal injuries thus reducing their validity as biomarkers of, but we identified wnt-1, a protein from wnt/β-catenin pathway that has been

described as a pathway really involved in fibrosis in other organs such as lung (Bañón-Maneus et al 2010).

Proteomic analysis using solid phase extraction as protein purification method and Protein profiling by MALDI-TOF was also performed. This is a relatively simple proteomic approach that allows rapid differential diagnosis of patients and information transfer between the laboratory and the clinical context. Fifty individuals: 32 patients with chronic allograft dysfunction (14 with pure interstitial fibrosis and tubular atrophy, and 18 with chronic active antibody-mediated rejection) and 18 controls (8 stable recipients and 10 healthy controls) were studied. Unsupervised hierarchical clustering showed good segregation of samples in groups corresponding mainly to the four biomedical conditions. Moreover, the composition of the proteome of the pure interstitial fibrosis and tubular atrophy group differed from that of the chronic active antibody-mediated rejection group, and an independent validation set confirmed the results from the training set (Quintana et al 2009). With the gel free approach we detected (by LC-M/MS) and quantified (by LCMS) 6000 polypeptide ions in undigested urine specimens across 39 CAD patients and 32 control individuals. Although unsupervised hierarchical clustering differentiated between the groups when including all the identified peptides, specific peptides derived from uromodulin and kininogen were found to be significantly more abundant in control than in CAD patients and correctly identified the two groups. These peptides are therefore potential biomarkers that might be used for the diagnosis of CAD. In addition, ions at m/z 645.59 and m/z 642.61 were able to differentiate between patients with different forms of CAD with specificities and sensitivities of 90% in a training set and, significantly, of 70% in an independent validation set of samples. Interestingly low expression of uromodulin at m/z 638.03 coupled with high expression of m/z 642.61 diagnosed CAD in virtually all cases (Quintana et al 2009).

This suggests that urinary proteome analysis can be used for the non-invasive monitoring of renal transplant patients, although it awaits validation in larger cohorts.

6. Conclusion

In summary, the application of proteomics in the field of renal and organ transplantation opens important options diagnostic and prognostic purposes. At present, urinary proteomics allows us a more early and accurate diagnosis of acute rejection by the experimental determination of peptides in the urine. The differential detection of peptides at diverse stages of the NCT in acute rejection allows us a better way to understand rejection and tolerance. The combined information derived from genomics and proteomics will lead us to consistently reduce the risk factors for graft failure (acute rejection, ischemia-reperfusion, immunosuppression, NCT), with increased life of the organs and improved patient's quality of life, because proteomics is one of the fields that can help to establish a connection between genomic sequences and biological behavior, constituting an important tool in functional analysis of genes of unknown function. The main advantage of a urine proteomic study as a source of markers for the detection of renal disease in the transplant is that urine is a fluid readily available and non-invasive. There are multiple proteomic techniques although it is noteworthy that, even though the results that each technique offers are very consistent, none is sufficient by itself to obtain and complete proteome and is advisable to combine several of the techniques described. Therefore, the primary application of proteomics is the search for markers that could be the basis for the realization of a microarray of proteins with diagnostic potential.

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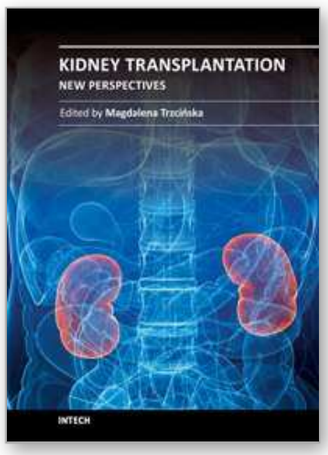
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Kidney Transplantation - New Perspectives

Edited by Dr Magdalena Trzcinska

ISBN 978-953-307-684-3
Hard cover, 334 pages
Publisher InTech
Published online 23, August, 2011
Published in print edition August, 2011

Although many years have passed since the first successful kidney transplantation, the method, although no longer considered a medical experiment, is still perceived as controversial and, as such, it triggers many emotions and thatâ€™s why conscious educational efforts are still needed for kidney transplantation, for many people being the only chance for an active lifestyle and improved quality of life, to win common social acceptance and stop triggering negative connotations. Apart from transplantation controversies piling up over years transplantologists also have to face many other medical difficulties. The chapters selected for this book are of high level of content, and the fact that their authors come from many different countries, and sometimes even cultures, has facilitated a comprehensive and interesting approach to the problem of kidney transplantation. The authors cover a wide spectrum of transplant-related topics.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Elisenda Banon-Maneus, Luis F Quintana and Josep M Campistol (2011). Urinary Proteomics and Renal Transplantation, Kidney Transplantation - New Perspectives, Dr Magdalena Trzcinska (Ed.), ISBN: 978-953-307-684-3, InTech, Available from: http://www.intechopen.com/books/kidney-transplantation-new-perspectives/urinary-proteomics-and-renal-transplantation

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