Proteomics - A Powerful Tool to Deepen the Molecular Mechanisms of Aortic Stenosis Disease

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1. Introduction

Valvular aortic stenosis (AS) produces a slowly progressive obstruction in left ventricular outflow track. Aortic valve replacement is warranted when symptoms or left ventricular dysfunction develop, which happen when the valvular stenosis is hemodynamically significant. Among the three main causes of valvular AS (congenital, rheumatic or degenerative), degenerative AS is the most prevalent in the older population, and due to the aging population it is the most frequent cause of valve replacement in western countries. Currently it is responsible for most valve replacements performed in Europe, and there is a steady increase of this disease and its social significance (Iun *et al.*, 2003).

Degenerative or calcific aortic valve disease is characterized by aortic valve leaflet thickening and calcification of the aortic valve, usually in an anatomically normal trileaflet valve. Several studies have demonstrated an association between degenerative AS and cardiovascular risk factors for atherosclerosis, such as hypercholesterolemia, hypertension, tobacco, the metabolic syndrome, etc, and as a consequence a similar pathobiologic process has been suggested for degenerative AS and atherosclerosis (Stewart *et al.*, 1997; Agmon *et al.*, 2001). But although there are many similarities between these two diseases, the differences are also significant.

The pathobiology of aortic valve calcification comprises three main processes: lipid accumulation, inflammation and calcification. Accumulation and oxidation of LDL-cholesterol particles, T-lymphocytes, macrophages, and production of inflammatory mediators such as interleukin-1-beta and transforming growth factors beta-1 have been observed at tissue level in the aortic leaflets of patients with degenerative AS. Angiotensin converting enzyme production has also been detected at valvular level in these patients. In

later stages of the disease, active cartilage and bone formation have also been reported (Mohler *et al.*, 2001; Freeman *et al.*, 2005). All these findings suggest that degenerative AS is an active process instead of a passive "wear and tear" phenomenon as it has been considered for decades.

Although hypercholesterolemia has been pointed out as a risk factor for degenerative AS, its relationship with this disease is far from being clear. The similarities between the pathobiology of degenerative AS and atherosclerosis suggested that statins could decrease the progression of degenerative AS and help avoiding its clinical consequences as has been demonstrated in coronary artery disease. However, the last trials that analysed moderate-to-severe degenerative AS have failed to demonstrate any benefit of statins in the clinical evolution of this disease (Cowell *et al.*, 2005; Chan *et al.*, 2010), despite having demonstrated, in the SEAS trial (Rossed *et al.*, 2008), a reduction of some coronary events with the reduction in LDL cholesterol. Therefore, once significant degenerative AS has progressed cholesterol does not appear to be longer related with the process, which is opposed to what has been observed in atherosclerosis. This has also been suggested by the only trial performed in patients with mild degenerative AS, the RAAVE trial, in which rosuvastatin showed a mild positive impact of cholesterol reduction on the progression of degenerative AS as assessed by echocardiography (Moura *et al.*, 2001). Taken together, this suggests that some risk factors can play a role only in the early stages of the disease, but once the calcification process starts to develop, the role played by the classical risk factors appears to wane, although the information so far available is somewhat contradictory (Walter *et al.*, 2010).

The conflicting information available makes it necessary to further study the pathophysiologic process of degenerative AS in order to unveil the basic mechanisms of the disease, a needed step in the process of developing reliable biomarkers of the disease as well as preventive therapies for it. Proteomics, a science aimed at discovering the proteins involved in the pathogenic process of diseases in an unbiased way, appears especially suited for studying the pathogenic processes involved in the development of degenerative AS. This chapter provides information about the different proteomics techniques for the AS study. We have begun to apply these methodologies to examine the physiological changes that accompany this pathology in a comprehensive manner. The techniques described in this chapter were optimized in our laboratory for the analysis of different kind of human samples. Data reported by our lab as well as by other authors will be presented to give the reader a wide perspective of the information available about the pathogenesis of this prevalent and clinically significant disease.

2. AS plasma proteome

At present, it is possible to perform a differential proteomic approach on a variety of biological samples, including cells, tissues or biological fluids (plasma, urine). In the context of biomarker discovery, biological fluids such as plasma or urine, represent the most logical compartment for investigation.

Plasma is the best clinical sample in terms of diagnosis and prognosis, due to several advantages including its low cost, not invasive and easy access (Veenstra TD *et al.*, 2005). Human plasma is a rich source of proteins and other metabolites which reflect the physiological or clinical status of patients. Since blood circulates throughout every organ and tissue of the body, it is expected to contain valuable information about the real physiological state of the organism. The protein concentration in plasma is tightly controlled

and, thus, variations can be considered as an indicator of the current state of health. Numerous biomedical/clinical proteomic studies have demonstrated that plasma protein levels reflect human physiological and pathological states and can be used for disease diagnosis and prognosis (Anderson NL *et al.*, 2002).
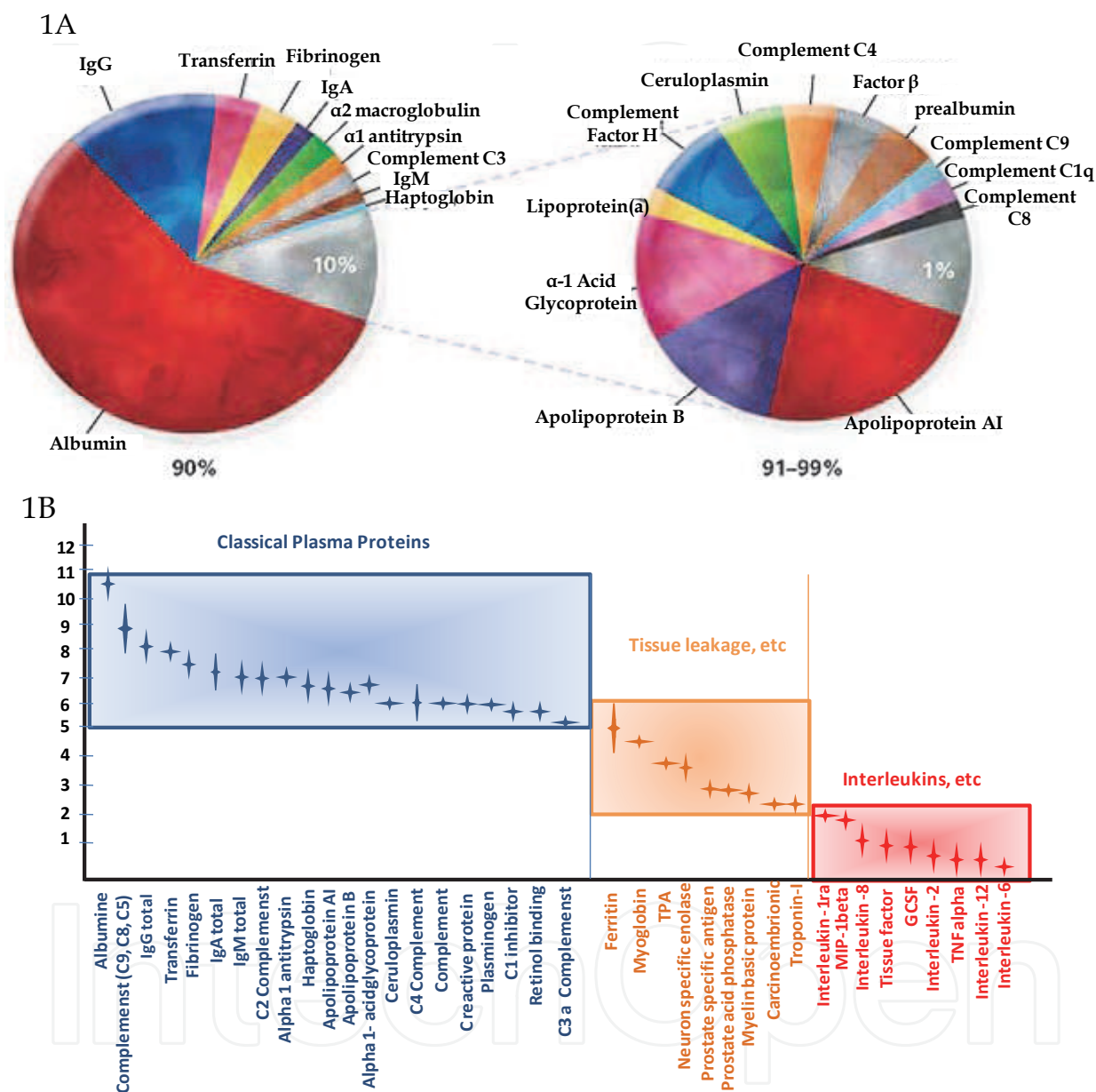

Fig. 1. Human plasma proteome. A, The large dinamic range of protein concentrations in the human proteome represents a significant experimental challenge as technologies must be sensitive across nearly 12 orders of magnitude (a 1 trillionfold range) for comprehensive analysis and the development of biomarkers. B, Estimated concentration of plasma proteins (based in Anderson and Anderson, 2002 Molecular and cellular proteomics)

However, the plasma proteome is very complex and presents a wide dynamic range of proteins (more than 10 magnitude orders) what makes its proteomic analysis very challenging, because high-abundance proteins tend to mask those of lower abundance

(Anderson NL *et al.*, 2005). In other words, only 20 major proteins comprise 99% of the plasma proteome and the rest of the proteins making up 1% of the plasma content (Figure 1). Potential disease biomarkers could be present in low concentrations, and not be detected by the current proteomic techniques (Darde VM *et al.*, 2010). Hence, it is essential to perform a pre-fractionation method. Cibacron Blue Dye and protein A/G columns have been used to effectively remove serum albumin and immunoglobulins from serum, allowing detection of proteins present at very low concentrations. Nowadays, the most common technique is the immunodepletion, which has been extensively used for the specific removal of the most abundant proteins, based on the action of specific antibodies (Zolotarjova N. *et al.*, 2005 and Wang YY *et al.*, 2003). Among these, Multiple Affinity Removal Columns (MARC) is the most effective method because it simultaneously removes multiple abundant proteins (Bjorhall K, *et al.*, 2005 and Seam N *et al.*, 2007).

There are several affinity columns as MARS-6 or MARS-14 (Agilent Tecnologies) to deplete the 6 or 14 more abundant proteins and even to deplete the 20 more abundant proteins with the Top-20 column (Sigma). In any case, there has been concerned regarding whether less abundant plasma proteins are removed jointly with albumin and other commonly depleted proteins by "nonspecific" binding (albuminome) (Yocum AK, *et al.*, 2005 and Granger J *et al.*, 2005). To save this problem, the combinatorial peptide ligand libraries (CPLL) have been proposed as an alternative method (Righetti PG *et al.*, 2006 and Sihlbom C *et al.*, 2008). This methodology was designed for sample equalization and it is used to analyze the "low-abundance proteome" in association with mass spectrometry (MS). CPLL have been recently used in plasma analysis to reduce their complexity, hence the most abundant plasma proteins would saturate all its corresponding hexapeptides in peptide library and the excess will be eliminated, while the less abundant plasma proteins will be more represented and more accessible to the differential analysis of protein expression (Righetti P G *et al.*, 2006).

Until now, the main approximation to plasma analysis has been the two-dimensional gel electrophoresis (2-DE), although it presents several limitations, since highly acidic or hydrophobic proteins and those with the highest or the smallest molecular weight in the sample may not be represented. However, 2-DE allows detecting changes in specific isoforms and, in general, approximately 1000-2000 protein spots can be visualized on a gel (Tannu NS and Hemby SE. 2006). Gel based approaches have enabled important advances in measurement of protein expression alterations in normal and diseases states. Moreover, new more sensitive fluorescence probes (Sipro Ruby, Cy2, Cy3 and Cy5) have been designed to improve spots detection and identification from 2-DE gels, improving hugely the "classical" silver stain. A methodological advance in 2-DE has been the 2-D Fluorescence Difference Gel Electrophoresis (2D-DIGE) (GE Healthcare). 2D-DIGE is based on direct labeling of lysine groups on proteins with cyanine CyDye DIGE Fluor minimal dyes before isoelectric focusing, enabling the labeling of 2-3 samples per gel with different dyes. This capability minimizes spot pattern variability and the number of gels by experiment providing a simple, accurate and reproducible spot matching. This is achieved by inclusion of an internal standard for every spot in every 2-DE gel, providing with experimental reproducibility and the highest accuracy in protein ratio measurements. In this way, the individual protein data from the control and diseased samples (Cy3 or Cy5) are normalized against the internal standard sample (Cy2 dye-labeled), Cy5:Cy2 and Cy3:Cy2.

To avoid 2-DE limitations, new alternatives have been developed, the most successful is based on multidimensional liquid chromatography coupled to MS (LC-MS/MS).
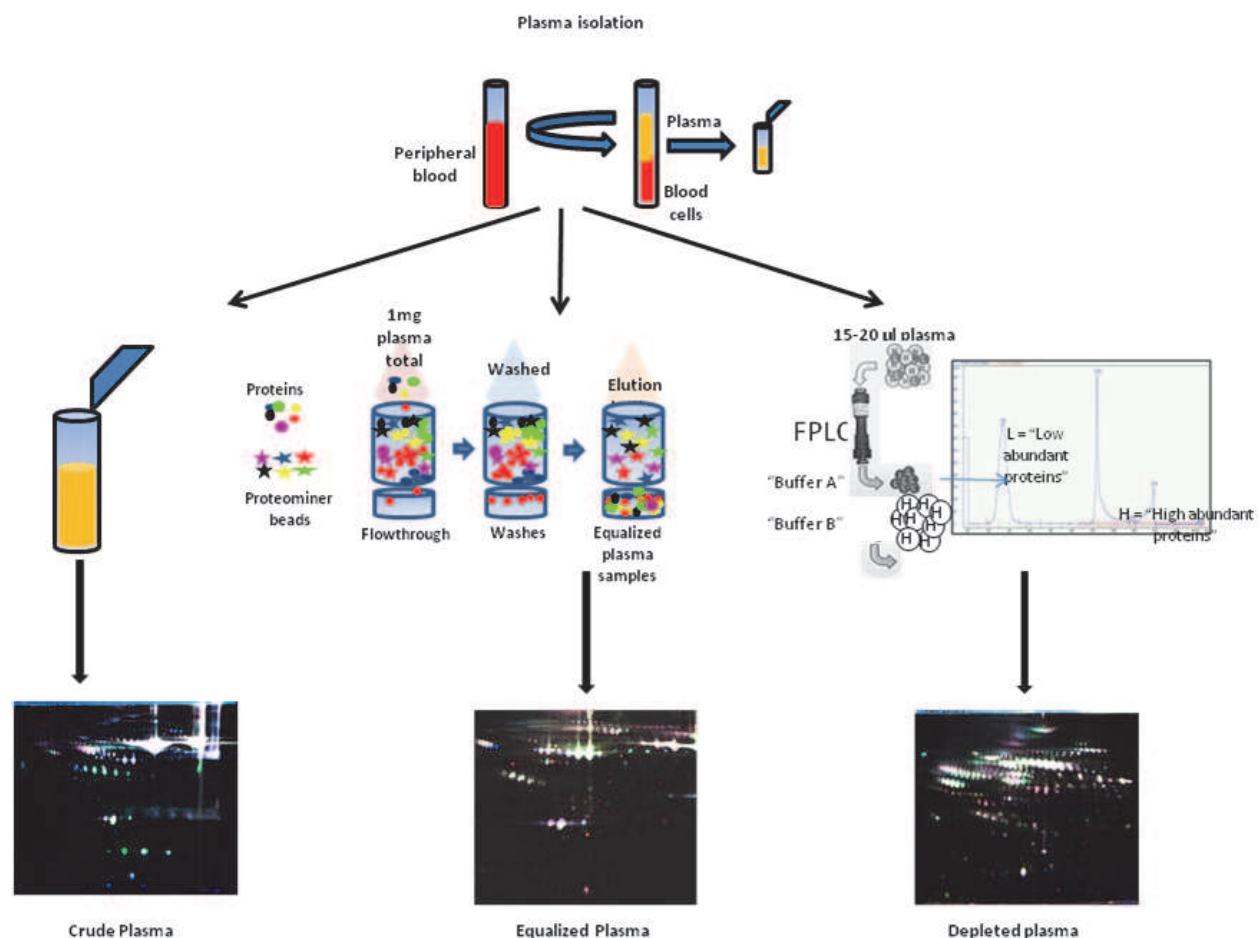
Fig. 2. Schematic workflow in 2D-DIGE plasma analysis. Isolation and processing of plasma samples 2D-DIGE analysis (left). 2D-DIGE of processed plasma samples using the combinatorial peptide ligand libraries (CPLL) (proteominer) (center) and depletion of most abundant plasma protein with affinity columns (right).

Electrospray ionization (ESI) development and recent advances in mass spectrometry (MS) provided the second-generation of proteomics technology based on LC-MS/MS. This approach is nowadays referred to as shotgun proteomics or MudPIT (Chen EI *et al.*, 2006 and MacCoss MJ *et al.*, 2002). To perform differential expression analysis , several probes have been designed for labeling both at protein and peptide levels (ICAT (isotope-coded affinity tagging), SILAC (stable isotope-labeling with amino acids in cell culture) or iTRAQ (stable isotope-tagged amine-reactive Reagents)) (Wu WW et al., 2006 and Graumann J *et al.*, 2008).

iTRAQ is an LC-based methodology which is gradually gaining in popularity. iTRAQ reagents have permitted relative expression measurements of large sets of proteins with a high grade of automation. The isobaric nature of the tags allows the peptide samples to be pooled postlabeling, without increasing the complexity of MS analysis. Identical peptides labeled with the different iTRAQ reagents exhibit the same parent ion in MS. Upon MS/MS fragmentation of parent ion, unique signature ions are generated which distinguish the individual samples and, hence, the relative abundance among the samples can be determined (Figure 3).
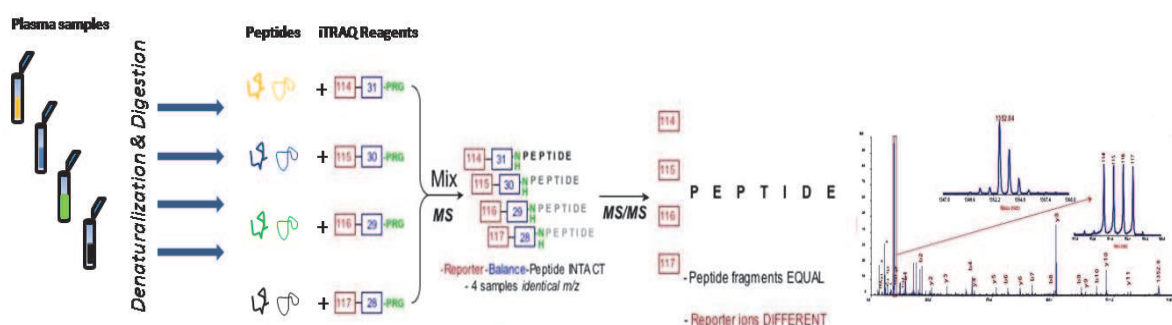
Fig. 3. Schematic workflow of iTRAQ labeling: After denaturation, reduction, alkylation, and digestion, each protein sample is modified with a distinct iTRAQ ® reagent. The iTRAQ ®-modified samples are then mixed to be analyzed by mass spectrometry. The MS/MS spectra of the individual peptides show signals reflecting amino acid sequences and also show reporter ions reflecting the protein contents of the samples.

In a different approach, the low molecular weight fraction can be investigated (plasma peptidome) by LC-MS/MS or by Matrix-Assisted Laser Desorption/Ionization-mass Spectrometry (MALDI-MS). The peptidome has been described as all peptides that have been expressed in any cell, tissue, or biofluid at any given time (Schulte I *et al.*, 2005 and Schrader M and Schulz-Knappe P, 2001). Peptidomics holds considerable promise for the discovery of new bioactive molecules and for elucidating biochemical regulatory networks. Endogenous peptides have already been established as messengers, hormones or cytokines in many physiological processes. Alterations in peptide levels under disease conditions implicate this class of molecules as potential biomarkers.

Ultrafiltrates of plasma have been recognized for several years to contain complex mixtures of thousands of peptides that are smaller than apolipoprotein C-I, the smallest major plasma protein. The complete set of small peptides from plasma has been termed the plasma peptidome, or the fragmentome (Tirumalai R S *et al.*, 2003) because it has been recognized that most small peptide components of plasma are derived from proteolytic degradation of larger proteins. Peptide fragments of many major plasma proteins, such as fibrinogen, apolipoproteins, transthyretin, and complement factors, have been detected (Adkins JN et al., 2002, Tirumalai RS et al., 2003). To obtain the plasma peptidome some authors have applied molecular weight filters (ranging 10-3,5kDa) to isolate small proteins and whole peptidome. With the focus on peptides rather than proteins, a hydrophobic-based capture technique, which removes salts and other hydrophilic compounds in a single step, is more compatible with direct LC-MS analysis (Zhou H *et al.*, 2006 and Tian R *et al.*, 2007).

Some biochemical studies in AS plasma samples using the ELISA technique have reported alterations in their proteins levels. Most of these studies were focused in proteins implicated in cardiovascular diseases outstanding the natriuretic peptide (Yandle TG *et al.*, 1986; Yandle TG *et al.*, 1993 and Hunt PJ. *et al.*, 1997) whose levels are prognostic of cardiovascular outcomes independently (26, Tsutamoto T. *et al* 1997). Recently, Qi W *et al.*, 2001, Prasad N *et al.*, 1997 and Talwar S et al., 2001. published that plasma natriuretic peptide levels were related to disease severity in AS. In addition, they also reported that cardiothrophin-1 (CT-1) levels were increased in AS patients and these results correlated with the trans-valvular aortic pressure gradient (TVPG) and could potentially be used to monitor progression of disease in a non-invasively manner. Ferrari G. et al have recently shown that individuals with severe AS exhibited higher plasma levels of NT-proBNP, BNP fragment, and

osteopontin compared with controls. Fetuin-A levels were lower in individuals with AS than in healthy controls. Asymmetric dimethylarginine (ADMA) were lower while homocysteine levels were higher in theAS patients.

Despite the potential offered by proteomics, plasma from AS patients has not been studied, until now. Taking into account the studies performed with one protein or with a small number of proteins, a proteomic plasma analysis, without bias, could give us a more complete sight at molecular level of this pathology and its possible relation with atherosclerosis. The HUPO Plasma Proteome consortium has identified a group of 345 cardiovascular-related proteins that could constitute a baseline proteomic blueprint for the future development of biosignatures for cardiovascular diseases (Vivanco, F. *et al*. 2007). Hence, new studies focusing in plasma from AS patients will be necessary to deepen in this pathology, in order to find new biomarkers and therapies.

3. Tissue, isolated cells and secretome

Protein tissue biomarkers offer a great promise to provide a clinical diagnosis and prognosis information that cannot be obtained from genomics or serum biomarkers. In the past, the discovery phase has been delayed by several hurdles including tissue heterogeneity and the lack of sensitive technology to identify and measure protein in small volumes of human biopsy tissue (Espina *et al.*, 2009). These hurdles have been largely overcome by the wide availability of laser microdissection technology, protein microarrays, and advances in mass spectrometry (Emmert-Buck *et al.*, 1996; Petricoin *et al.*, 2005). Under such considerations, the tools and methodology of proteomics are becoming increasingly important and, we could postulate that tissue proteomics studies provide us two main information data sets: 1) potential biomarkers of disease directly released from tissue lesions, which should be validated in wider cohorts before being applied to clinics; 2) discovery of action mechanisms and pathways involved in the formation and development of AS by direct "in-situ" proteomics (Alvarez-Llamas et al., 2007).

3.1 Stability in tissue: pre-analitycal variability

Today, there is an important need to develop standardized protocols and novel technologies that can be used in the routine clinical settings for seamless collection and immediate preservation of tissue in the search for biomarker proteins. In this sense, both clinicians and researcher are implicated. The fidelity of the data obtained from a diagnostic assay applied to the tissue must be monitored and quality controlled. Under the current standard of care, AS tissue is procured by AS replacement in a hospital-based operating room. The instant a tissue biopsy is removed from a patient, the cells within the tissue react and adapt to the absence of vascular perfusion, ischemia, hypoxia, acidosis, accumulation of cellular waste, absence of electrolytes and temperature changes (Espina *et al.*, 2008). Normally tissue is frozen in dry ice or liquid nitrogen or fixed to avoid degradation and to preserve for later proteomics analysis. Otherwise, the tissue is maintained in physiological serum if we want to develop secretome analysis. A multitude of known and unknown variables can influence the stability of AS tissue molecules: temperature, pH, hypoxia, dehydration, RNAses activity, proteinases activity, *ex-vivo* stress (Espina *et al.*, 2008) etc. For these reasons the protocol to follow must be the strictest as possible: The recommended maximum elapsed time ranges between 20 minutes to 2 hours from surgery to stabilization (freezing, tissue culture, fixation, etc.). Finally, the study of tissue can be addressed according to three main

sample sources: a) whole tissue proteomics, by which total proteins extracts obtained from areas of interest are studied; b) tissue sub-proteomes, i.e. specific aortic valve layers and cells isolated directly from tissue (apart from those studies based on cultured cells); c) tissue secretome, which is a media enriched in proteins that derive from diseased tissue and, therefore, one of the best sources for biomarker discovery.

3.2 Whole tissue

Different techniques were applied to human samples from shotgun proteomics or 2-DE to antibody arrays and MS Imaging. In the case of AS valves tissue proteomics analysis, the main tools employed until now are the 2-DE and the 2D-DIGE. Focus on the aim of maximizing the number of extracted proteins, AS valves must be processed following a "strong" and sequential extraction protocol (Gil-Dones *et al.*, 2010). The first step relies on a strong lysis buffer in which most of the soluble proteins are extracted, and the second was designed to extract the membrane and hydrophobic proteins. It is important to note that one of the most important problems associated with proteomic analyses of AS valves is the high concentration of calcium that interferes with further analyses. For this reason the extracts must be desalting (Gil- Dones *et al.*, 2010). Our group found that approximately 500 spots were well resolved in IPG strip of pH4-7 when compared with the 350 spots evident from the pH 3-10 strip, by 2-DE. Attending to 2D-DIGE, 1346 spots were detected. Significantly, the protein extraction protocol was compatible with both methods. The improvement in sensitivity associated with the 2D-DIGE technology with respect to 2-DE was clearly evident. We conclude that the use of fluorescent labeling and Decyder analysis software considerably increases the number of protein spots that can be detected in the analysis when compared to more traditional methods (Gil-Dones *et al.,* 2010).

In this sense, Matt P et al, 2007 studied aortic aneurysm associated with bicuspid (BAV) and tricuspid aortic valve (TAV) using 2-DE and mass spectrometry (MS). Few proteins showed significant differences, among these a phosphorilated form of HSP27 with significantly lower expression in BAV compared to TAV aortic samples. The phosphoprotein tracing revealed four different phosphoproteins including Rho GDP dissociation inhibitor, calponin 3, myosin regulatory light chain 2 and 4 phosphorilated forms of HSP27. Western blot analyses were made to validate the results obtained by proteomics analysis.

Following the same approach, mitral valves of chronic rheumatic heart disease (RHD) patients were studied by Fae KC et al., 2008, identifying three proteins recognized by heart infiltrating and peripheral T cells as protein disulfide isomerase ER-60 precursor, 78kDa glucose-regulated protein precursor and vimentin. These proteins were recognized in a proliferation assay by peripheral and heart infiltrating T cells from RHD patiens suggesting that they may involved in the autoimmune (Fae *et al.* 2008).

In a different approach, antibody array technology has experienced an important advance in the latest times. Commercially pre-arrayed platforms in diverse research areas are nowadays available, but only one study has been recently reported applying commercial antibody arrays to atherome plaque extracts (Slevin *et al.*, 2006).

3.3 Aortic stenosis sub-proteomes

Within the stenotic aortic valve coexists several cell types, mainly valvular fibroblasts or interstitial cells (VICs), endothelial cells (ECs), macrophages/foam cells and T-lymphocytes. Aortic valve tissue studies constitute a very valuable tool in the search for alterations and
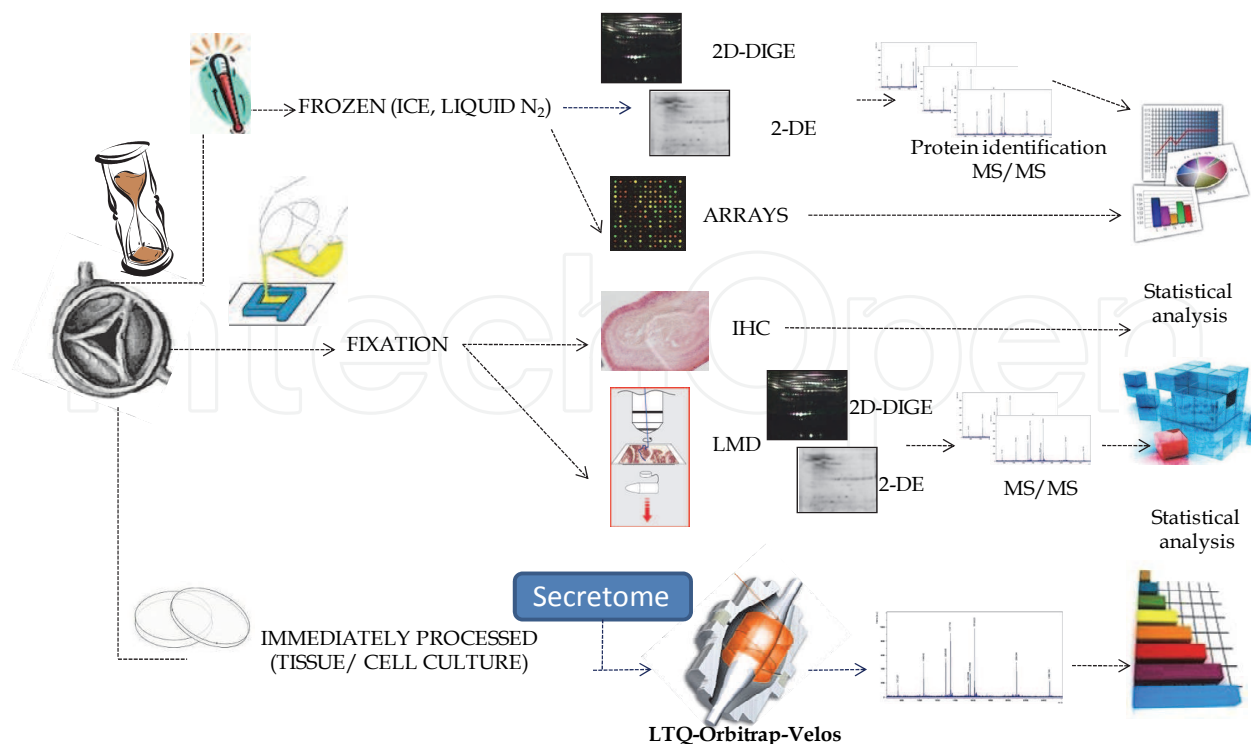
Fig. 4. Schematic diagram illustrating the methodology used for the proteomics analysis of aortic valve tissue.

disease specific biomarkers which would shed light on the understanding of AS and contribute to its early diagnosis. However, sub-fractionation of the aortic valve tissue in its structures or cellular components seems to be a complementary strategy to whole tissue analysis, in order to determine the specific contribution of those cells/structures in the pathogenesis of AS. Concretely, in the field of Proteomics, protein alterations observed in whole tissue samples may benefit from complementary assays involving localization of these proteins in the tissue, such as immunostaining techniques. Although the proteomic analysis of the different sub-fractions from the aortic valve tissue may represent a noteworthy strategy for AS research, this field remains almost unexplored. Strategies involving the combination of aortic valve tissue sub-fractionation and proteomic analysis of these sub-proteomes will be therefore discussed.

3.3.1 Laser microdissection

The different structures from a histology section of a tissue can be isolated by means of microscopical dissection. In this case, manual microdissection may be applied with the use of a microscopical needle under an optical microscope. However, laser microdissection (LMD), first described in 1996 (Emmert-Buck *et al.*, 1996), is a more accurate technique for the isolation of tissue regions, cells or even subcellular fractions. This first method, called laser capture microdissection (LCM), involved irradiation with a laser beam of a thermoplastic membrane in contact with the tissue. Since the heating of the tissue during microdisecction contributes to protein degradation, newly developed laser cutting techniques may be more adequate to implement in a proteomic workflow.

Even though laser microdissection is an outcoming methodology which has been applied in several cardiovascular studies, involving cardiac (De Souza *et al.*, 2004; Chimenti *et al.*, 2004;

Kuhn *et al.*, 2006, 2007; Roy *et al.* 2006; Grube *et al.*, 2006; Pan *et al.*, 2008) and arterial tissue (Stolle *et al.*, 2004; Chimenti *et al.*, 2010; Kwapiszewska *et al.*, 2005; Bagnato *et al.*, 2007; Ciervo *et al.*, 2008; Okami *et al.*, 2009; De la Cuesta *et al.*, 2009, 2011), it has been only used to date for the analysis of valvular tissue in a rat model of pharmacologically-induced valvulopathy (Elangbam *et al.*, 2008). Furthermore, its combination with proteomic methodologies may yield interesting results, in the same way that has been proven in other cardiovascular tissue samples (De Souza *et al.*, 2004; Bagnato *et al.*, 2007; De la Cuesta *et al.*, 2009, 2011). The principal challenge in the combination of LMD with Proteomics, is the scarce amount of protein that can be obtained from laser-microdissected tissue, since no long microdissection periods should be applied in order to avoid protein degradation. Nowadays, mass spectrometers can deal with scarce amounts of sample due to an exponential improvement in their sensitivity, therefore facilitating the analysis by LC-MS/MS of laser-microdissected tissue. On the other hand, the use of fluorescent labels such as the CyDyes from the *DIGE Fluor Labeling Kit for Scarce Samples* (GE Healthcare) allowed analyzing protein extracts from LMD-isolated structures/cells by means of two-dimensional electrophoresis. These dyes label through saturation the cystein residues from the proteins in the mixture and allow running 2-DE gels with less than 5μg of total protein (Sitek *et al.*, 2005). Since protein identification by MS of the spots in such gels is only possible with most abundant spots, these identifications can be performed using pooled samples from LMD or a reference proteome, which may be the one from the whole tissue microdissected (Kondo & Hirohashi, 2007).

The application of LMD to the analysis of the aortic valve tissue could provide specific data from the different layers/structures in the tissue, as well as from the behavior of the different cells in the stenotic milieu. In addition, its combination with a proteomic workflow may constitute a step forward in the understanding of the role played by the different cells involved in AS pathogenesis and in the search for novel tissue biomarkers of this pathology.

3.3.2 Living cells isolation

An alternative methodology to simplify tissue complexity and to study its cellular components is to enzymatically digest the tissue and separate the cells for subsequent analysis. This can be done by cell sorting methodologies or by specific explant culturing. In the first one, specific cell populations can be separated in a flow-cytometer and directly analyzed by proteomic techniques. The main problem in this workflow would be the high amount of tissue necessary to obtain enough cells for a proteomic analysis, which sometimes is impossible to extract, especially when dealing with biopsy material. Culturing sorted cells is an option, but constitutes a more complicated approach than explants culturing, since sorted cells may be damaged in the cytometer, which involves lesser sub-culturing capacity. In contrast, non-sorted cell suspensions should be cultured under specific conditions to favor a certain cell type, which sometimes may imply contamination by other cells. The main drawback of sub-culturing methodologies is that the cells may lose its *in vivo* phenotype within the culture, since the environmental conditions are completely altered in the *in vitro* situation. Nevertheless, cell cultures allow setting very strictly experimental parameters in order to diminish variation between replicates, and analyzing cells responses to different stimuli, which turns them into a very useful tool for proteomic analysis. For such purposes, the methodology called *stable isotope labeling with aminoacids in cell culture* (SILAC) (Ong *et al.*, 2002), constitutes a valuable approach to analyze both proteome and secretome of the different subset of aortic valve cells by means of differential LC-MS/MS.
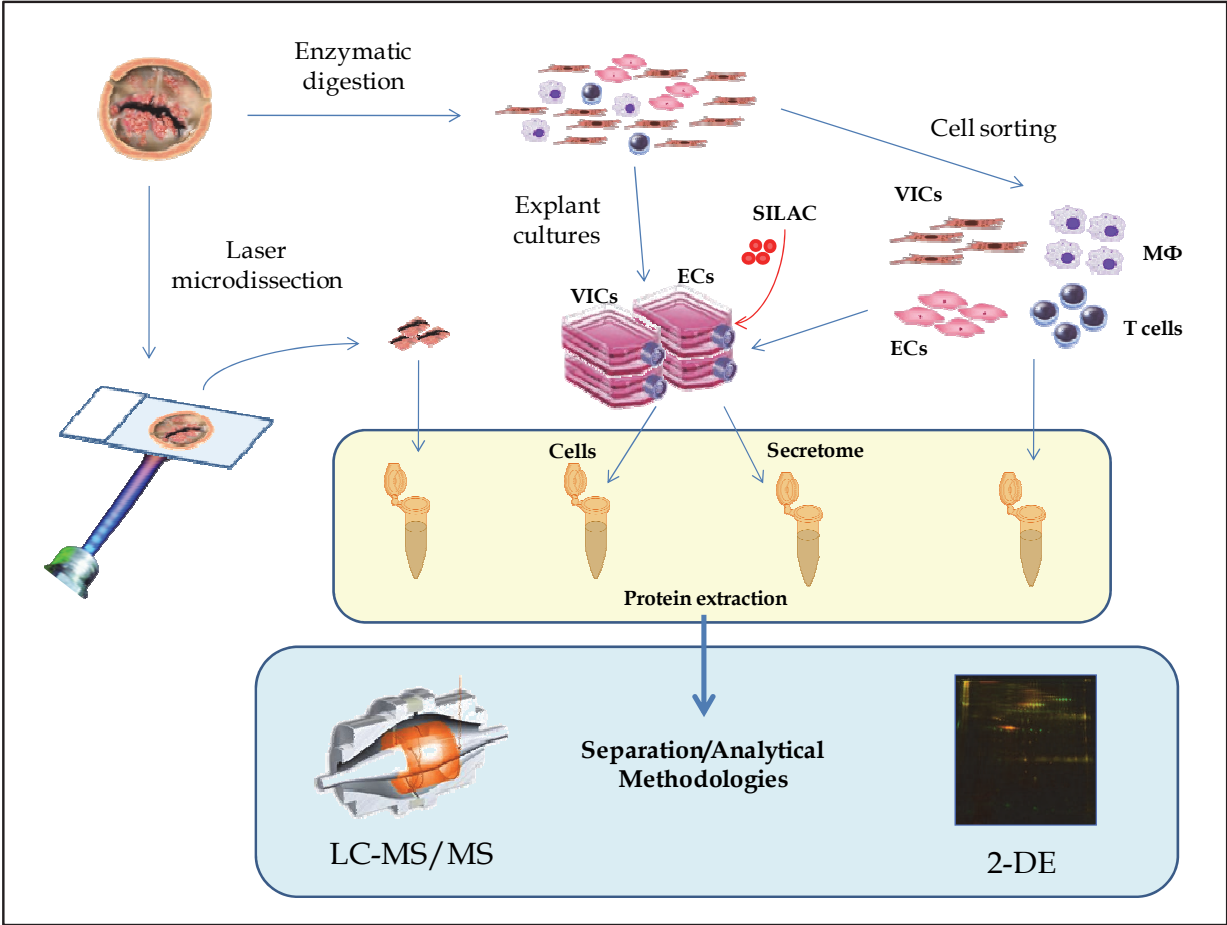
Fig. 5. Scheme of a proteomic workflow for the analysis of AS valve tissue sub-proteomes.

An additional step in the sub-fractionation of the tissue would be to isolate cell structures/organelles. Conventional ultracentrifugation can be applied to separate membranes, nuclei and cytosolic fractions for subsequent proteomic analysis (Huber *et al.* 2003). Using this approach, membrane and cytosolic proteins from Pro-calcific VICs after lipopolysaccharide (LPS) treatment were compared to non-treated ones by 2-DE (Bertacco *et al.*, 2010), in which constitutes the first proteomic study performed with cells isolated from aortic valves. On the other hand, cellular organelles and vesicles, like mithocondrias, Golgi stacks and vesicles or endosomes, are efficiently separated by density gradient centrifugation (Huber *et al.* 2003). Concerning secreted vesicles (microparticles, exosomes…), ultracentrifugation, either under density gradient or not (Van der Pol *et al.*, 2010), constitutes the easiest methodology to set up and to combine with proteomic analysis.

As far as we step forward in sub-fractionating cells proteome, the further the decrease on protein recovery will go. For this reason, while designing the strategy of a proteomic study, we should take into account the balance between the number of sub-fractionation steps and the amount of protein recovered.

3.3.3 Secretome

The term secretome comprises the sub-set of proteins that are actively released by cells or tissue in the extracellular compartment as consequence of the normal metabolism or in response to some stimuli. As such, it is a powerful source of key molecules involved in

pathogenesis development, individual response to pharmacological intervention or recovery status. Secretome studies are increasing in the last years as it provides an accurate model of the *in vivo* situation and it represents a sub-proteome of serum/plasma, showing a much narrower proteins concentrations dynamic range which enormously facilitates detection of minor proteins whose identification is otherwise obscured by high-abundance plasma proteins. Plasma, serum and urine are traditional sample sources for biomarker discovery, as they are easily and commonly obtained in clinical practice; however, secretome-based research will favor the detection of novel proteins or, at least, known molecules with new implications in the disease under study. Most studies are focused on the secretome obtained from in vitro cell cultures, assuming that such cells' behavior well simulates the *in vivo* condition *(Roelofsen et al., 2009)*. In particular, the cancer secretome, namely the whole collection of proteins secreted by cancer cells through various secretory pathways, has recently been shown to have significant potential, as secreted proteins might represent putative tumor biomarkers or therapeutic targets (Karagiannis *et al.,* 2010). The most perfect approximation to the real situation is the study of the *in vivo* secretome. In this research line, one of the few proteomics studies was carried out by implanting capillary ultrafiltration probes into tumor masses induced in mice (Alvarez-Llamas *et al.*, 2007; Hocking et al., 2010). However, this is not always feasible and the use of tissue explants (*ex vivo* approach) represents a compromise solution which gives information about secretory molecules coming from all tissue components as result of cross-talk between them and approaches the physiological situation better than the cell culture. Adipose tissue secretome was investigated as a tissue model representing not only energy storage depot but, more importantly, a key organ for the regulation of energy metabolism through secretion of a variety of adipokines involved in the regulation of energy metabolism (Roelofsen *et al.*, 2009; Hocking *et al.*, 2010). Two key points to consider when working directly with tissue in culture are: a) the need to ensure that all detected proteins are truly coming from the tissue and not "contaminants" derived from plasma and b) validation of identified proteins as secreted. A metabolic labelling approach allows differentiation between proteins synthesized by the tissue (labelled) and contaminating proteins from blood which remain unlabelled (Alvarez-Llamas *et al.*, 2007). In this sense, an optimized culture protocol should be developed to maximize label incorporation into proteins including a series of medium changes during the initial hours of culture, followed by an extended step of tissue culture. In any case, label incorporation is influenced by the rate of synthesis of each particular protein, which may condition the number of labelled proteins that can be detected at a particular time point. Incorporation of the label by a protein validates tissue origin but does not necessarily imply "intentional" secretion. Once synthesized by the tissue, the release of a protein into the media could be attributed to damage-induced tissue leakage and results in detection of intracellular proteins. One can assume that it is very challenging to totally avoid the presence of intracellular proteins, as cell lysis always takes place during cell/tissue culture. However, optimum culture conditions may favor secreted proteins enrichment. Classification of identified proteins as secreted ones is usually done by computational methods: classically secreted proteins (via the ER-Golgi pathway) can be predicted as containing a signal peptide by SignalP (Bensten JD, *et al.*, 2004) or being classified as extracellular via Gene Ontology analysis; non-classically secreted proteins, which do not contain N-terminal signal peptide, can be predicted by SecretomeP (Bendsten JD, *et al.*, 2004) and more recently by SecretP (Yu et al., 2010).

The aortic valve secretome is therefore an attractive target to further understand the AS pathogenic process, while revealing mechanisms in common to atherosclerosis. The study of atherosclerotic plaque secretome in the search of potential biomarkers of atherosclerosis has been reported (Duran *et al.*, 2007; Duran *et al.*, 2007), finding that HSP-27 release was decreased in atherosclerotic plaques and barely detectable in complicated plaques supernatants. Further validation and feasible detection in biological fluids should follow prior to the use of discovered proteins as markers of diagnosis or prognosis. In this case, circulating concentrations of HSP27 were found to be decreased in subjects with atherosclerosis compared to healthy subjects, which confirms the hypothesis that plasma content can reflect arterial wall secretion (Martin-Ventura *et al.*, 2004). Effects of atorvastatin treatment on atherosclerotic plaque secretion was also investigated, finding that 66% of the proteins differentially released by atherosclerotic plaques reverted to control values after administration, in particular, cathepsin D which becomes a potential target for therapeutical treatment (Duran *et al.*, 2007). Recent studies carried out in our laboratory point to a sub-set of proteins which are differentially released by healthy arteries in comparison to atherosclerotic coronary ones [submitted for publication] and whose role in AS development is currently ongoing. In Figure 4 a proposal for AS secretome obtention and proteomic analysis is shown.
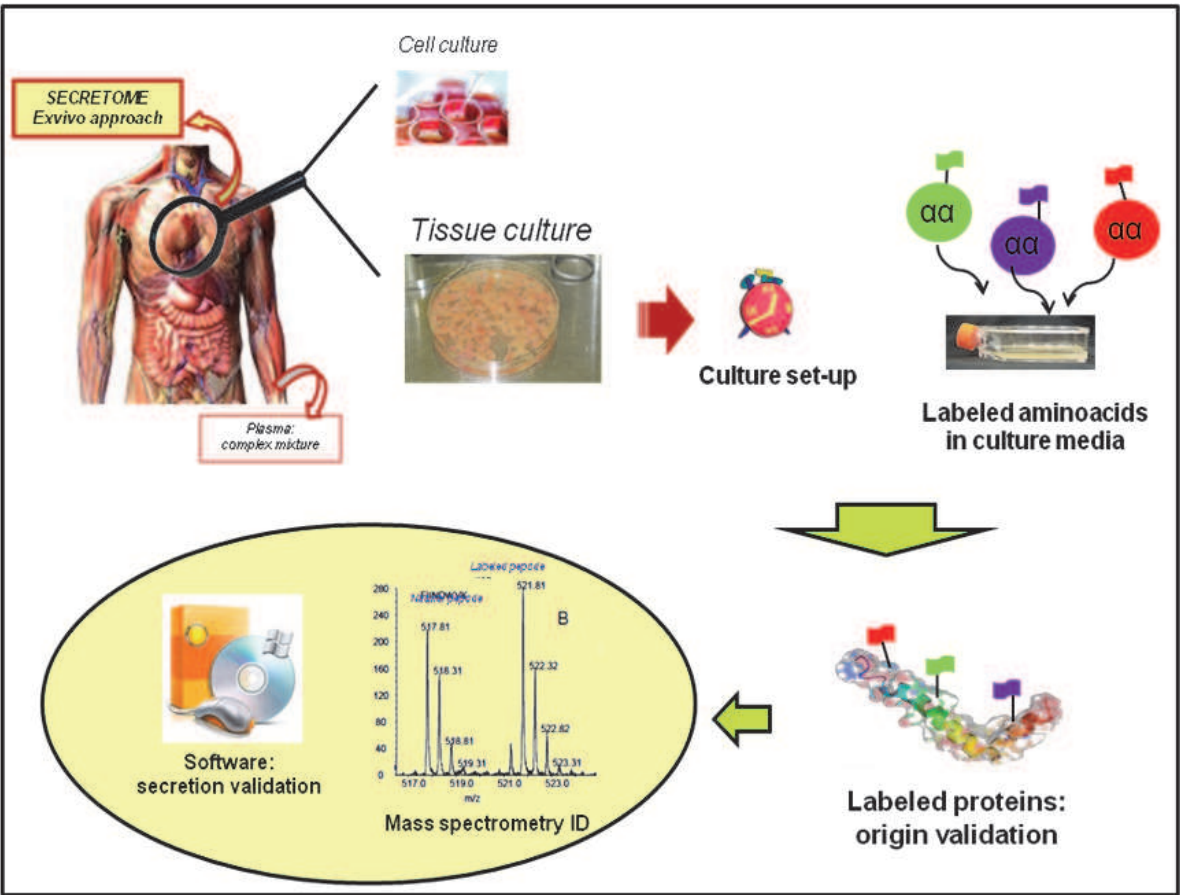

Fig. 6. Secretome from tissue explants is an ex-vivo approach with enormous potential as biomarkers source. It is key to validate proteins origin by i.e. label inclusion at protein level and secretion patterns by available softwares.

4. Conclusions

Recently, cardiovascular proteomics has experienced an impressive development. At present, hundreds of proteins have been associated with cardiovascular diseases using proteomic approaches and different kinds of biological samples. The proteomic approaches allow to explore the expression of multiple proteins at once, giving us the opportunity to know which proteins are involved in pathophysiologic process of degenerative aortic stenosis in order to unveil the basic mechanisms of this disease. Most probably, these novel approaches will facilitate the study of this pathology and disclose diagnostic markers and multiple potential targets for the design of more powerful and personalized therapies.

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6. References

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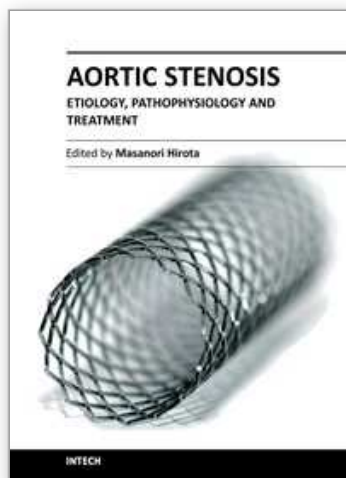
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Currently, aortic stenosis (AS) is the most prevalent valvular disease in developed countries. Pathological and molecular mechanisms of AS have been investigated in many aspects. And new therapeutic devices such as transcatheter aortic valve implantation have been developed as a less invasive treatment for high-risk patients. Due to advanced prevalent age of AS, further discovery and technology are required to treat elderly patients for longer life expectancy. This book is an effort to present an up-to-date account of existing knowledge, involving recent development in this field. Various opinion leaders described details of established knowledge or newly recognized advances associated with diagnosis, treatment and mechanism. Thus, this book will enable close intercommunication to another field and collaboration technology for new devices. We hope that it will be an important source, not only for clinicians, but also for general practitioners, contributing to development of better therapeutic adjuncts in the future.

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