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Chapter 6

Proteomics and Metabolomics in Aortic Stenosis: Studying Healthy Valves for a Better Understanding of the Disease

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Aortic stenosis

Aortic stenosis is characterized by the abnormal narrowing of the aortic valve (AV) opening, producing a blockage of the blood flow from the left ventricle into the aorta. Two different types of aortic stenosis can be distinguished. In congenital aortic stenosis an inherited abnormal formation of the AV exists. Otherwise, in acquired aortic stenosis external causes such as rheumatic fever or valve degeneration occur. Calcific aortic stenosis (AS) is the most common valvular disease in elder population and remain the main cause of aortic valve replacement in developed countries [1].

AS progresses from a primary stage of aortic sclerosis, with thickening and stiffness of the AV, to severe calcific stenosis. Its most common symptoms are dyspnea, angina and syncope, which predict the rapid deterioration of left ventricular function, the development of heart failure and, if the pathology progresses, the patient's death. Therefore, the appearance of any of these symptoms is considered as an indication for the treatment of this pathology. In these cases, surgery is recommended to replace the AV since there is not treatment to delay the progression of the disease.

Classically, this disease has been considered as a consequence of the aging process of the valve. However, recent studies have provided evidences that inflammation plays a key role in the physiopathology of AS as well as classical cardiovascular risk factors such as hypertension, hypercholesterolemia, diabetes, smoking, age or sex [2]. Degeneration of the valve begins with

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an endothelial dysfunction in the aortic side as a result of the abovementioned risk factors. Low density lipoproteins (LDLs) are accumulated in the subendothelial space of the valve, where they are oxidized, resulting in the activation of the endothelial cells. These cells express adhesion and chemotactic molecules, which attract inflammatory cells such as monocytes and T lymphocytes. Monocytes extravasate to the fibrous layer and differentiate into macrophages, which capture oxidized lipoproteins and become foam cells. Proinflammatory cytokines released by both cell types induce phenotypic differentiation of a subset of myofibroblasts to osteoblasts, which leads to the subsequent formation of calcium nodules [3]. These numerous similarities suggest a common relationship between atherosclerosis and AS.

1.2. Proteomic and metabolomic study of healthy valves

A complete knowledge of the structures involved in a disease it is important to understand its development. Previously, healthy tissue such as vasculature and myocardial have been succesfully studied to better understand the molecular mechanisms involved in vascular develepment and angiogenesis as well as biochemical changes that occur during physiological ageing [4, 5].

Histologically, AV consists of three layers: fibrosa, spongiosa and ventricularis. The fibrosa is located in the aortic side of the leaflets and it is mainly composed of fibroblasts and collagen fibers. The spongiosa is located below and it is formed by fibroblasts, mesenchymal cells and a polysaccharides-rich matrix. The ventricularis, found in the ventricular side of the leaflet, is made up of elastic fibers radially distributed. The AV is externally covered by several layers of endothelial cells. Collagen is responsible for the mechanical integrity of the valve, the spongiosa serves as a shock absorber and elastic fibers contract cusps during systole [3, 6].

However, it is also essential to perform studies at the molecular level of the tissue, looking for the discovery of tissue- and disease-specific markers. For this purpose, proteomic and metabolomic analyses can be ideally used since they allow the unbias analysis of hundreds or thousands of molecules at a time, detecting and identifying which molecules are present. In contrast to genomics and transcriptomics, proteomics and metabolomics study dynamic protein products, low molecular weight compounds and their interactions, which have a direct effect on the phenotype of the tissue (Figure 1).

Descriptive proteomics is a methodology that enables unbiased large-scale study of the set of all proteins in a biologic system at any given time. Thus, the expression, localization, interaction, structural domains and activity of these proteins, including splice isoforms and post-translational modifications (PTMs), can be studied [7]. Metabolomics is the study of a complete metabolome or a single group of particular metabolites, which are small molecules that participate in general metabolic reactions and that are required for the maintenance, growth and normal function of a cell [8]. The study of healthy valves through proteomic and metabolomic approaches and the subsequent integration of data, can provide molecular level information of the metabolic pathways that are more active in that tissue and will help to understand the mechanisms of physiological/pathological processes in aortic stenosis valves. This makes it easier the search for potential markers for early diagnosis of the disease, thus being able to predict which people may develop aortic stenosis in the future.

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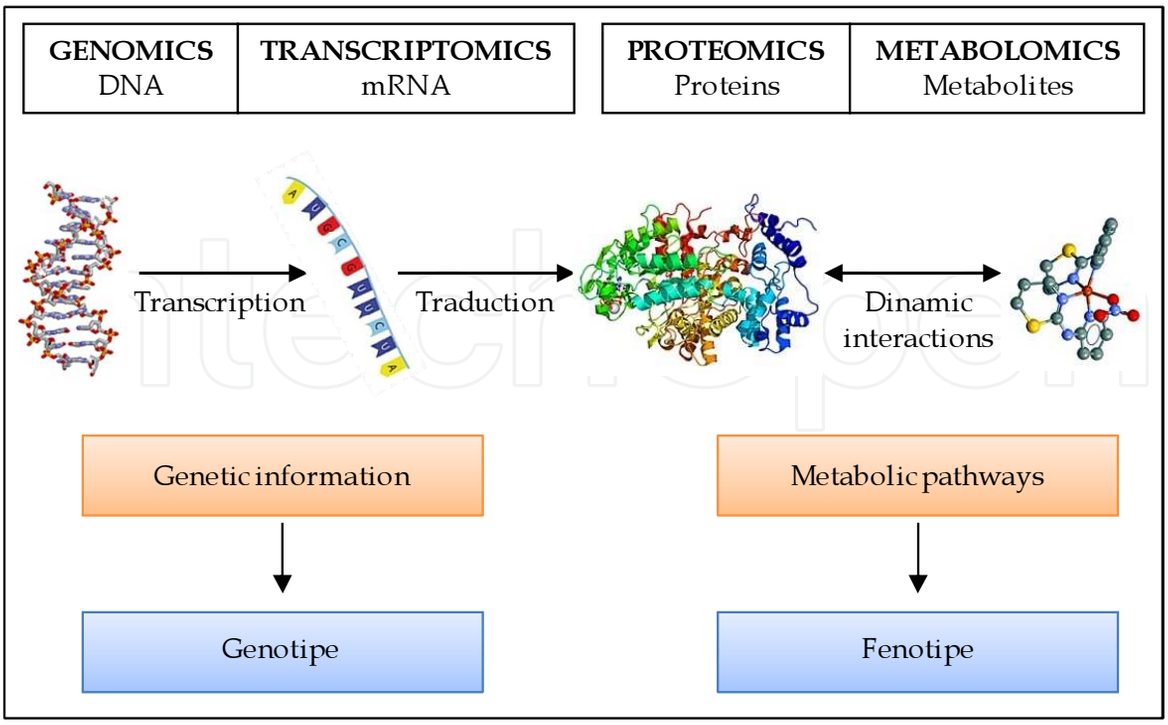

Figure 1. Dynamic study of the physiophatology of a disease through "-omics" technologies.

2. Proteomics

Proteomics is the large-scale study of the proteins content in a given sample (ie. biofluid or tissue) [9]. Since proteins are the final product of genes expression, proteomics constitutes a powerful tool for the study of biological systems thanks to proteome reflects the current state of the organism and it varies according to its functional situation [10]. These studies can be performed through a wide variety of techniques and methodologies, depending on the sample and the experimental design. In the case of descriptive proteomics, in which the most usually is the study of very complex samples, it is essential to perform certain steps to facilitate the study: 1) sample preparation, 2) protein separation and 3) analysis by mass spectrometry.

2.1. Sample preparation

Preparation of the samples prior to the analysis using proteomic techniques is an essential step for obtaining robust and reproducible data. Between the large number of standardized protocols, the selected one must be carefully chosen to suit the sample to be analyzed, as well as for the proteins of interest. In the case of the AV an effective and suitable protocol has been previously described [11] (Figure 2). Briefly, within 2 hours after surgery, valves were washed in PBS and then ground into a powder in liquid N2 with a mortar. 0.2 g of this powder was resuspended in 400 µl of protein extraction buffer (Tris 10 mM [pH 7.5], 500 mM NaCl, 0.1% Triton x-100, 1% β-mercaptoethanol, 1 mM PMSF) and then centrifuged to precipitate membranes and tissue debris [12]. Supernatant containing most of the soluble proteins was collected

and pellet was solubilized in 7M urea, 2M thiourea, 4% CHAPS prior to another centrifugation [13, 14]. This second supernatant, which was rich in hydrophobic proteins, was also collected and stored at -20°. Depending on which is the fraction of interest, supernatants can be analyzed together or separately. Because of the nature of the extraction buffers, sample must be processed to eliminate interference substances with the downstream analyses. Samples must be filtered by centrifugation and dialyzed against Tris 2mM. Before proteomic analyses it is also recommended the use of the commercial kit 2D-Clean-Up (GE Healthcare), which precipitates proteins while leaving interfering substances, such as detergents, salts, lipids, phenolics, and nucleic acids, in solution. The pellet can then be solubilized in a proper buffer for further analyses.

Instead of solubilized the proteins of whole tissue, histology sections or specific cell types can be used for isolate different structures of the tissue as regions, cells or even subcellular fractions by means of laser microdissection (LMD) [15]. It consists in cutting microscopically selected tissue by laser using different systems [16]: 1) selected regions are transferred onto a film or to a special cap; 2) selected regions can be catapulted into a collection tube and 3) cut samples fall down into the lid of a collection tube by gravitation. The collected tissue has to be lysed and prepared for downstream analyses. The main disadvantage of this methodology in combination with proteomics is the scarce amount of protein that can be obtain, since the laser cannot be applied for a long time in order to avoid protein degradation. However, mass spectrometers have exponentially improved their sensitivity, so there are several interesting studies combining LMD and Proteomics in other cardiovascular tissues samples [17-20]. The application of LMD to the analysis of the AV 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 tissue.

2.2. Protein separation

For complex mixtures, it is important to separate proteins according to their different characteristic to increase the efficiency of the study. For this purpose, the most usually used techniques are 2-dimensional electrophoresis (2-DE), off-gel fractionation, and 2-dimensional liquid chromatography (LC) [21-23] (Figure 3).

2.2.1. 2-Dimensional electrophoresis

This method stands out for its high applicability to a wide range of biological samples. In this case, a good protein separation can be reached through two simple steps: isoelectric focusing (IEF) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) [24, 25]. During the first phase, the protein mixture is separated on a pH gradient according to their isoelectric point (pI) using commercial strips covered by an acrylamide gel. To perform this separation, proteins are completely solubilized in a special buffer containing urea, a nonionic detergent (CHAPS) to prevent clustering, a reducing agent (DTT) to break the disulfide bonds and a mixture of ampholytes to minimize aggregations by charge interaction. In the second step, proteins are separated in a second dimension depending on their molecular mass and a two-dimensional protein spot map is obtained that can be visualized through different staining

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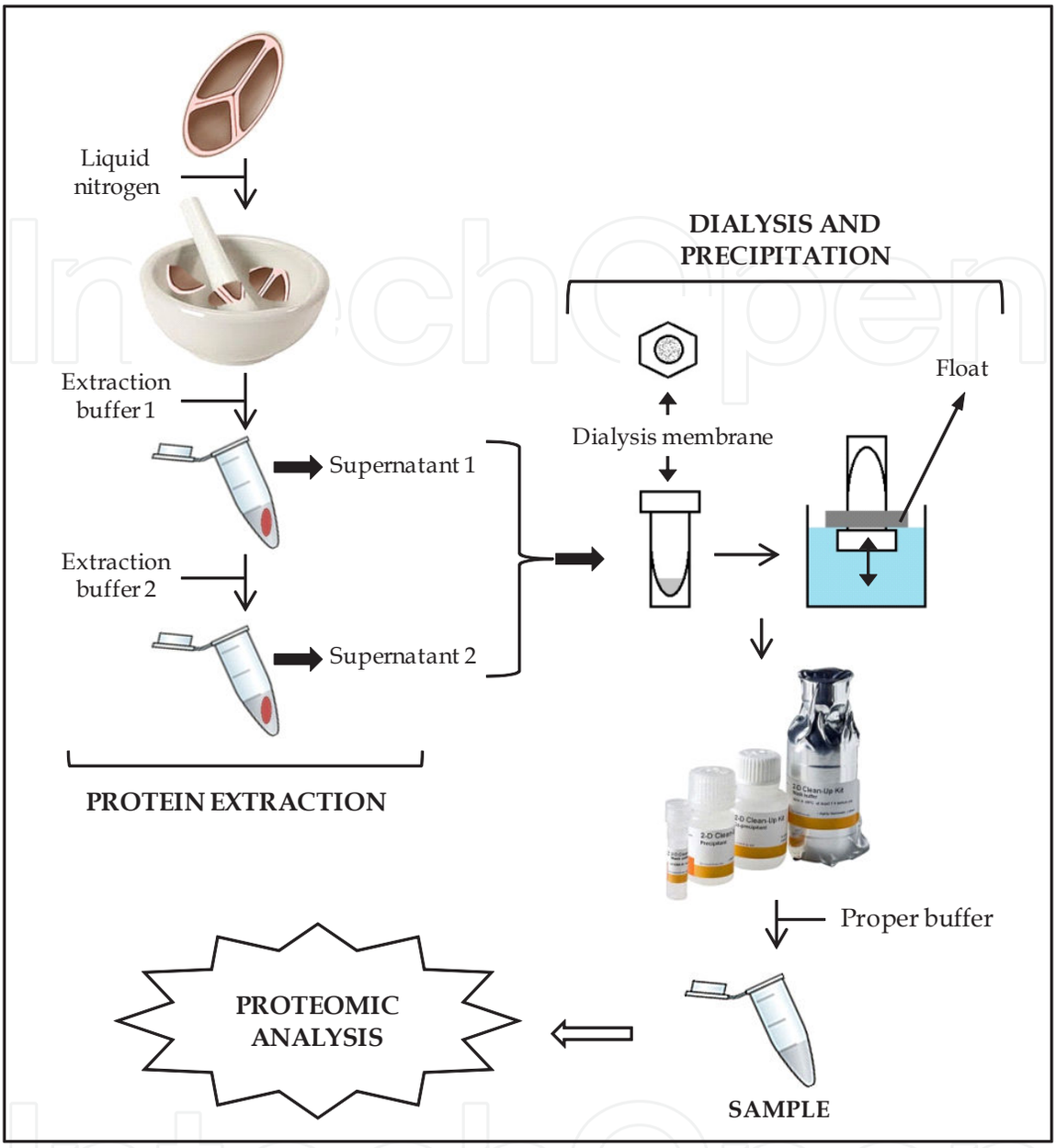

Figure 2. Protocol of aortic valve preparation for proteomic analyses

techniques as Coomassie blue, silver staining and Sypro Ruby [26]. Stained gels are scanned and digitalized using different computer programs to obtain an image of the gel and spots of interest will be identified by mass spectrometry techniques. There are three protein groups which are problematic: highly alkaline, extremely high and low molecular sizes and membrane-bound proteins. However, improving protein solubilization, pre-fractionation of protein groups of interest prior to 2DE, or adjustments to the 2-DE regime enhance separation of these more difficult-to-resolve proteins. The main advantage of 2-DE is that it delivers a map of intact proteins, which reflects changes in protein expression level, isoforms or post-translational modifications [27].

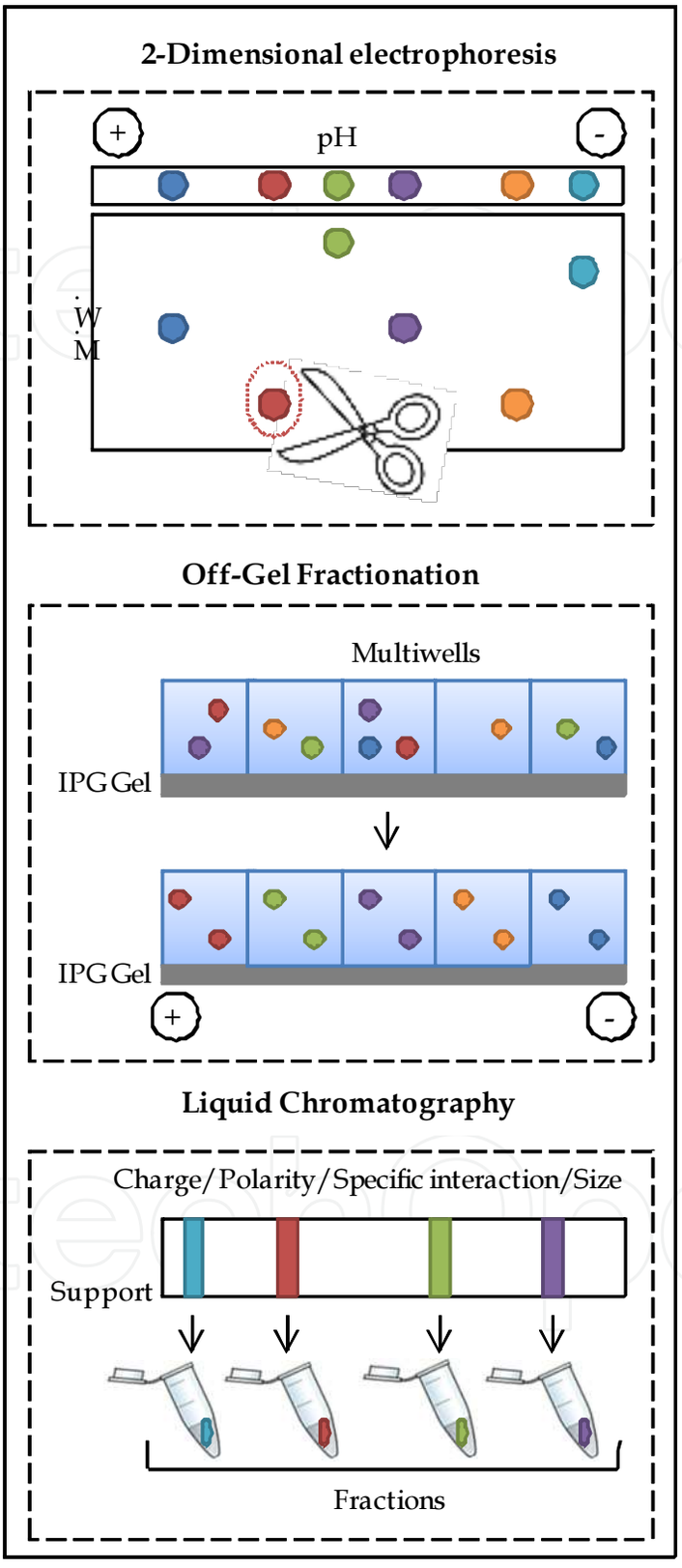

Figure 3. Most common used protein separation techniques

2.2.2. Off-gel fractionation

There is another protein fractionation technique which is based on off-gel IEF, where the proteins are separated according to their pI in a multiwell device. This separation is based on immobilized pH gradient (IPG) strips and permits to separate peptides and proteins according to their pI, but is realized in solution without the need of carrier ampholytes or buffers [28, 29]. The main advantage of this technique is that the fractions can be directly recovered in solution for further analysis and directly digested if necessary [28, 30]

2.2.3. Liquid chromatography

Liquid chromatography (LC) is a fractionation technique that can be applied to separate a wide range of molecules such as proteins or peptides according to their physical and/or chemical properties. Different types of chromatographic support can be distinguished: ion exchange (separation based on protein or peptide charge), reverse phase (according to their polarity), affinity (based on a highly specific interaction such as that between antigen and antibody, enzyme and substrate, or receptor and ligand) and molecular exclusion (depending on size). LC can be used before or after 2-DE [31-33] and can be directly coupled to a mass spectrometer for further identification [34, 35]. To increase the resolution of proteomics analyses, it is recommended the use several consecutive chromatographic methods. In this sense a combination of ion exchange and reverse phase chromatography allows a two-dimensional separation with the advantage that the second column can be directly coupled to MS, which enhances the automatization of the proteomic methodology. With this idea, multidimensional protein identification technology (MudPIT) has been developed. It consists of a column with two chromatographic supports in tandem: a cation exchange support in the proximal area followed by a reverse phase in the distal one. This method enables the two-dimensional LC using high-performance LC (HPLC) coupled to a mass spectrometer with electrospray ionization (ESI) source in an automated manner [36, 37]. This approach is more sensitivity and reproducibility than gel-based methodologies and allows the analyses of smaller quantities each time because of the development of new equipments which are able to work with sample volumes of the order of microliters and nanoliters. LC together with mass spectrometry has been successfully employed in a previous work in which the proteome of the coronary artery was described [17].

2.3. Protein analyses by mass spectrometry

Once our sample is separated, these fractions or spots must be analyzed to identify the different proteins. Since the development of matrix-assisted laser desorption ionization (MALDI) and ESI, two ionization methods that allows the analysis of proteins and peptides using MS, this technique have become indispensable for protein identification [38-40]. Mass spectrometers consist of three essential elements: ionization source, mass analyzer and detector (Figure 4). The ionization source ionizes and vaporizes the sample. Different ionization sources are MALDI, ESI or surface-enhanced laser desorption/ionization (SELDI) [41-43]. ESI allows the identification of many volatile and thermolabile compounds of a wide range of molecular weight with high sensitivity. MALDI and SELDI are very similar methods but they are used for low and high complex mixtures, respectively [44]. The mass analyzer separates the ions

according to their relation m/z. There are also different types of mass analyzer, such as quadrupole (Q), time of flight (TOF), ion trap (IT), Fourier transform ion cyclotron resonance (FT-ICR) and Orbitrap. The ion stream reaches to the detector and it is transformed into an electrical signal. These signals are then integrated by a computer system which generates a mass spectrum which represents the abundance of the different generated ions.

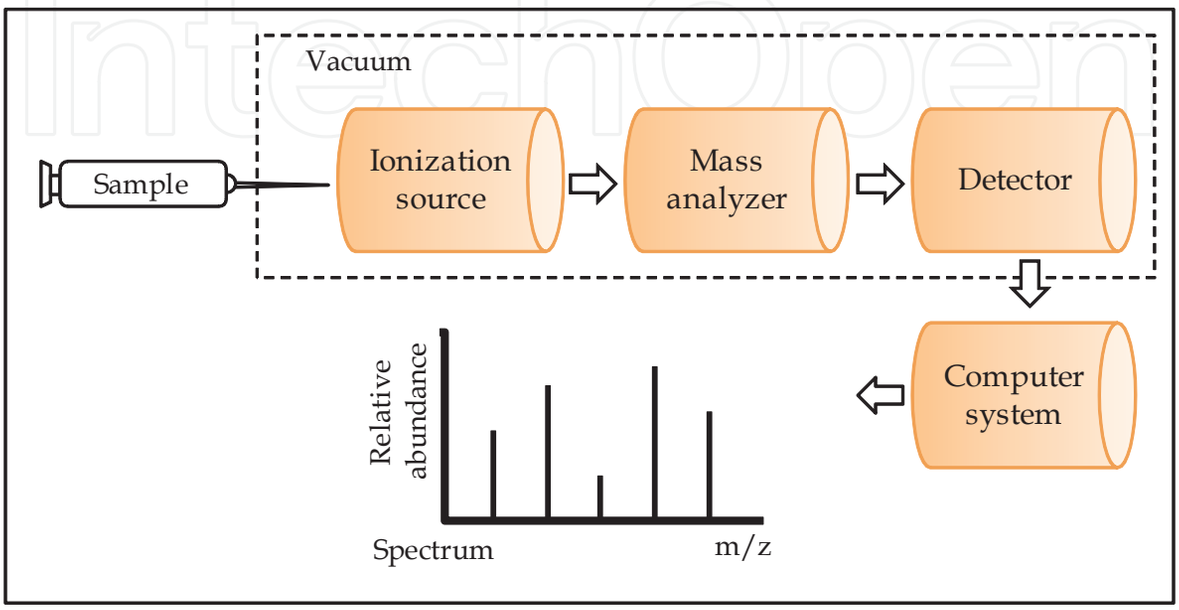

Figure 4. Different elements of a mass spectrometer

There are two different ways to identify the molecules included in a complex mix using MS: peptide mass fingerprinting (PMF) and tandem mass spectrometry (MS/MS or MS2). Briefly, identification based on PMF identifies proteins according to the peptides generated after digestion with specific endoproteases, usually trypsine. Experimentally obtained peptide masses are compared to theoretical peptide masses of proteins stored in databases through mass search engines such as MASCOT (Matrix Science Ltd.) [45], MS-Fit [46] or Profound [47, 48]. These samples usually come from gel bands, 2-DE spots or LC fractions with low complexity and are analyzed through MALDI-TOF or ESI-TOF [48, 49]. In the case of MS/MS, two mass analyzers are used in tandem. The first one allows the measurement of the m/z values of the peptides and the second one, after the fragmentation of some peptides, allows the measurement of the m/z values of these fragments. Thus, a partial or even total sequence of the peptide can be obtained. Most usually used mass spectrometer for this purpose are MALDI-TOF-TOF (for low complex mixtures) and ESI coupled to different analyzers (Q3, Q-TOF, IT, Q-Q-LIT, FT y Orbitrap).

2.4. Tissue sections analysis: MALDI imaging mass spectrometry

MALDI imaging mass spectrometry (IMS) is a powerful tool for investigating the distribution of proteins and small molecules present in thin tissue sections. This technique generates molecular profiles and two-dimensional ion density maps of peptide and protein signals

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directly from the surface of these sections [50]. For this analyses, it is indispensable a correct sample preparation in terms of chemical and structural integrity [51]. Preparation methods must avoid delocalization and degradation of the analytes so it is important to take into account several parameters such as treatment of tissue immediately after sample procurement, sectioning, sample transfer to the MALDI target plate, matrix application, and tissue storage after sectioning [52]. It is advisable the used of fresh tissue sections though sometimes it is necessary embedding the sample in gelatine or agarose to facilitate its manipulation [53-55]. One of the important aspects of tissue profiling is the comparison of histological features obtained from stained sections using light microscopy with molecular images obtained by mass spectrometry [56]. Previously, this was accomplished using two separate adjacent sections, one for histology and one for MALDI-IMS [57, 58]. However, visual registration between both sections it is difficult because of differences in tissue architecture. Ideally, histology and protein profiling should be performed on the same tissue section. For this reason, several commonly used histological dyes have been tested for compatibility with MS analysis. It was found that hematoxilin and eosin interfered with MS but, on the other hand, cresyl violet and methylene blue, between others, do not compromise mass spectra quality [56]. This methodology allows the study of the entire tissue, maintaining its structure, so MS analysis can be focused on specific morphological regions of interest.

3. Metabolomics

Metabolomics is the study of the set of final products and by-products of many metabolic pathways, called metabolites, which exist in humans and other living systems [8]. In order to perform a descriptive analysis of healthy valves, an untargeted approach, not focused on a specific group of metabolites, is the most recommended methodology. This method, also called metabolomic fingerprinting, permits to detect the largest number of metabolites optimizing different experimental conditions such as sample preparation and chromatographic and MS parameters. This approach usually compromises the sensitivity and specificity for identification of individual metabolites [59, 60]. Additionally, metabolomic fingerprinting involves less up-front method development when compared with targeted approaches but requires an exhaustive analysis due to the large number of identified metabolites. As well as in proteomics analyses, there are three main steps when performing a metabolomic study: [1] sample preparation; [2] metabolite detection and [3] data analysis.

3.1. Sample preparation

Optimization of sample-preparation protocol is extremely important in metabolomic studies because it affects both metabolite profile and quality data, leading to possible erroneous conclusions [61, 62]. An ideal sample-preparation method for metabolomic fingerprinting should have the following characteristics [1] non-selective to ensure adequate metabolite coverage; [2] simple and fast, with the minimum number of steps possible to minimize metabolite loss and/or degradation of the sample and enable high-throughput; [3] reproducible and [4] incorporate a metabolism-quenching step (low temperatures, addition of acid, or

fast heating) to represent true metabolome composition at the time of sampling (Figure 5) [63]. For tissue metabolomics, the protocol includes a previous step of manual homogenization at low temperatures follow by a quenching step in liquid nitrogen [64]. Although the tissue disruption technique can altered the precision and metabolite coverage, the selection of the extraction solvent have a stronger effect on the number of extracted metabolites [65]. Several aqueous and organic solvents can be used, such us methanol [66, 67], methanol/water [68], isopropanol/acetonitrile/water [69], acetonitrile/methanol/water [70], acidic acetonitrile/water [71] or methanol/acetonitrile/acetone [72] between others. However, there is no an established protocol for aortic valve so it is important the development of an adequate method before metabolic fingerprinting.

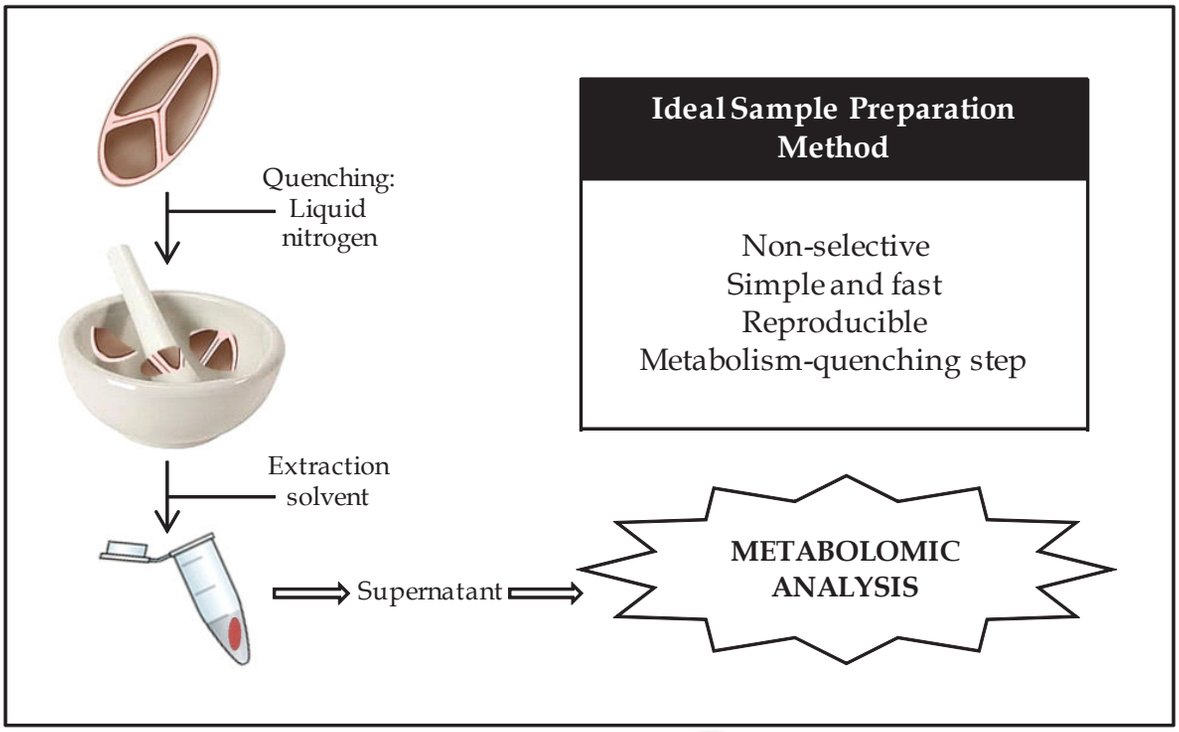

Figure 5. Protocol of tissue preparation for metabolomic analyses.

3.2. Metabolite detection

There is a great variability of metabolites in terms of polarity, solubility, and volatility with a wide dynamic range of concentration in biological samples. Therefore it is necessary to combine different chromatographic platforms for covering the largest range of metabolite possible. The two main platforms used in metabolomics analysis are nuclear magnetic resonance (NMR) and MS, usually coupled with a separation method for metabolites (LC or gas chromatography (GC)-MS) [73, 74]. The platform of choice will depend on the physico-chemical characteristics of the metabolites of interest. As it is explained below, CG-MS is a high-quality technique for analyzing hydrophobic compounds while LC-MS permits to determine a larger number of metabolites. Finally, NMR shows superior capability for

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determining the structure of unknown metabolites. Therefore, each platform has inherent advantages and disadvantages for the metabolomic analysis of different compounds and only through their combined use the best understanding of the physiopathology of AV will be achieved (Table 1).

Advantages	PLATFORM	Disadvantages
Minimal sample preparation Non-destructive Reproducible Structural information	NMR	Sensitivity Spectral resolution
No derivatization required Variety of metabolites	LC-MS	No comprehensive spectral libraries
Sensitivity Robust Reproducible Comprehensive spectral libraries	CG-MS	Derivatization required Limited mass range

Table 1. Comparison of the most common analytical methods used for metabolomics

3.2.1. Nuclear magnetic resonance

NMR is a spectroscopic analysis technique that exploits the specific magnetic spin or resonance frequency of the protons within atomic nuclei of specific molecules. When nuclei in a magnetic field are exposed to a radiofrequency pulse their protons temporarily move to a higher energy state, and then release a characteristic radiowave when they return to their normal energy state [75]. The resulting NMR spectrum is a collection of characteristic peaks and intensities of each compound that allow its identification [76]. NMR requires minimal sample preparation, it is a nondestructive and very reproducible technique and provides structural information of metabolites. These particular advantages confers NMR superior ability for the identification of unknown metabolites and constitutes a valuable approach for identification of unknown metabolites [77]. However, NMR is limited in terms of sensitivity and spectral resolution so it is not a good technology to identify metabolites that are found in low concentration [78].

NMR also allows the analyses of intact tissue using a technique called high-resolution magic angle spinning (HRMAS) NMR. The sample is spun at high speed about an axis at an angle of 54°44′(the so-called "magic angle") [79]. This rapid spinning at this precise angle has the effect of reducing dipolar coupling effects and narrowing of the broad lines found in this tissue. HRMAS-NMR has been applied successfully to analyze different intact cells and tissues [80-85], so it seems to be a powerful tool for the analyses of aortic valve tissue.

3.2.2. Mass spectrometry

As well as in proteomic studies, MS uses m/z value to identify metabolites. When coupled with chromatographic methods, MS can analyze a wide number of metabolites with enhanced

sensitivity. The two main strategies for metabolite separation are LC (for nonvolatile compounds in solution) or GC (for volatile samples or when the expected compounds can be easily made volatile by derivatization). LC-MS using ESI as ionization source is the most currently used platform for metabolomic studies since it permits the analysis of a wider variety of metabolites than GC-MS [86, 87]. Also, derivatization step prior the analysis is no necessary reducing losses of compounds during the sample preparation. Single quadrupole, ion trap and TOF analyzers are the most commonly used mass analyzers. GC-MS is a first-rate choice for the analysis of volatile compounds such as fatty acids and organic acids [88]. In most cases, it is necessary a prior step for chemical derivatization of non-volatile and thermally stable metabolites. The two more used derivatization reagents include BSTFA (N, O-Bis (trimethylsilyl) trifluoroacetamide) and MSTFA (N-Methyl-N-(trimethylsilyl) trifluoroacetamide) [89-91]. Advantages of GC include higher resolution and more robust and reproducible retention times than LC, besides the existence of mass spectral libraries, which facilitates the identification process.

In the course of the study of the physiopathology of AS the better comprehension of implicated and altered biochemical pathways will be acquired through the metabolomic study of biological samples, specially plasma and tissue. For this purpose NMR and MS represent the two most valuable techniques for metabolite identification thus the information that both techniques provide will suppose a cornerstone for the study of this disease.

3.3. Identification and data analysis

The data processing challenges in metabolomics are quite unique and often require specialized (and/or expensive) data analysis software and a complete knowledge of cheminformatics, bioinformatics and statistics for a correct interpretation of data. Ideally, data analysis softwares must be able to remove noise from the spectra, properly identify which metabolite generates every chromatographic peak and make a correct alignment of the peaks corresponding to the same compound in several successive samples [92, 93].

There are several commercial softwares available free such as MSFacts, MetAlign or MetaboAnalyst which automatically import, reformat, align, correct the baseline, and export large chromatographic data sets to allow more rapid visualization of metabolomics data [94, 95]. However, most companies usually generate data that can be only read with own softwares which use mass spectral libraries for peak detection, identification and integration [96].

4. Towards a global understanding of physiology

The large number of genomes, including human, that have been mapped and the knowledge of the genetic code, have increased the interest in other –OMICS technologies, such as proteomics and metabolomics. Both proteomics and metabolomics differ from genomics in terms of complexity and dynamic variability. Proteome and metabolome are constantly changing according to the genome and the environment, therefore the ultimate phenotype of cell, organ, and organism is reflected in proteomic and metabolomic profiles. This variability,

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enhanced by the using of different methodologies and equipments, usually impedes obtaining reliable and reproducible results when comparing patients and healthy controls. The study of a large number of healthy valves using a standardized protocol may provide useful information about the heterogeneity of its profiles. Thus, different profiles could be assigned to different states such as sex, age or risk factors. This way, the creation of a protein atlas unmasking the expression and localization of proteins coupled to metabolomic results would function as a global knowledgebase with valuable information about normal cellular function [97, 98]. All these information can be storage in conventional web-based databases, in order to obtain a reference material to be used in further studies, when studying different valve diseases, helping to deepen the understanding of the beginning and development of the disease. It would also be possible to find some variation involving metabolic predisposition to develop the disease in the future. This will exponentially increase the possibilities to discover potential therapeutic targets and will open the door to develop a personalized medicine in this disease.

The major challenge of generating a complete database of AV tissue is obtaining enough biological material to be described, since it is not an easily accessible specimen. It is necessary a complex coordination of basic research activities, facilities and infrastructures, as well as the creation of an integrated and multidisciplinary environment with the participation of several different specialists in cardiovascular diseases, i.e. basic researchers, cardiologists, surgeons, pathologists, epidemiologists, patients, patient advocacy groups, funding agencies and industrial partners. Issues related to sample collection, handling and storage, standardization of protocols, common references, number of patients, availability of normal controls, access to bio-banks, tissue arrays, clinical information, follow-up clinical data, computational and statistical analysis, as well as ethical considerations are critical, and must be carefully considered and dealt with from the beginning [99]. With such a big collaborating work, there will be a more effective translation of basic discoveries into clinical applications.

No matter how much ambitious and complete the descriptive study is, it is essential integration of the results through a system biology approach. This consists of placing proteins and molecules from experimental analysis in the context of a network of biological interactions, such as gene–gene, gene–protein or protein–protein interactions, followed by different 'guilt-by-association' analyses [100]. Usually, these networks are deduced from previously published interactions or from computational prediction models. Different tools exist to perform these analyses, most of them based on Cytoscape Web, a freely available network visualization tool for integrating biomolecular interaction networks with high-throughput expression data and other molecular states into a unified conceptual framework [101-103]. Using an integrative approach, we can obtain a more holistic picture of the molecular mechanisms that occurs in normal aortic valves.

5. Conclusion

The study of healthy valves through proteomic and metabolomic approaches and the subsequent integration of data can provide molecular level information of the metabolic pathways

that are more active in AV. The characterization of physiological proteins and metabolites in this tissue and the creation of a complete database with the results from the descriptive studies, may serve as a reference material for further studies. This would facilitate the searching for potential markers for early diagnosis of the disease, thus being able to predict which people may develop aortic stenosis in the future.

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