

## Review Article

# Metabolomic Profiling for Identification of Novel Potential Biomarkers in Cardiovascular Diseases

**Maria G. Barderas,<sup>1</sup> Carlos M. Laborde,<sup>1</sup> Maria Posada,<sup>2</sup> Fernando de la Cuesta,<sup>2</sup> Irene Zubiri,<sup>2</sup> Fernando Vivanco,<sup>2,3</sup> and Gloria Alvarez-Llamas<sup>2</sup>**

<sup>1</sup>Department of Vascular Physiopathology, Hospital Nacional de Parapléjicos, SESCAM, 45071 Toledo, Spain

<sup>2</sup>Department of Immunology, IIS-Fundación Jiménez Díaz, 28040 Madrid, Spain

<sup>3</sup>Department of Biochemistry and Molecular Biology I, Universidad Complutense, 28040 Madrid, Spain

Correspondence should be addressed to Fernando Vivanco, [fvivanco@fjd.es](mailto:fvivanco@fjd.es)

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Metabolomics involves the identification and quantification of metabolites present in a biological system. Three different approaches can be used: metabolomic fingerprinting, metabolic profiling, and metabolic footprinting, in order to evaluate the clinical course of a disease, patient recovery, changes in response to surgical intervention or pharmacological treatment, as well as other associated features. Characteristic patterns of metabolites can be revealed that broaden our understanding of a particular disorder. In the present paper, common strategies and analytical techniques used in metabolomic studies are reviewed, particularly with reference to the cardiovascular field.

## 1. Introduction

Cardiovascular disease (CVD) is the most prevalent cause of death in developed nations and it is increasing in prevalence in developing countries. While many factors contribute to the development of this disease in adults, such as, smoking, male gender, blood pressure, elevated cholesterol, diabetes, and renal failure, the mechanisms underlying CVD are still not fully understood [1–4]. One of the main problems in clinical practice is that the symptoms become evident late in the course of the disease. In fact, asymptomatic processes, that are associated with plaque formation, develop causing silent yet progressive tissue damage. If atheroma plaques finally rupture, highly thrombogenic material is released and an atherothrombotic event occurs. In this context, there is an urgent need to find out novel biomarkers of practical value for clinical intervention which, alone or combined with existing ones, allow cardiovascular risk prediction at individual level. Currently, controversy exists regarding contribution of biomarkers to the information derived from conventional risk factors. When novel markers utility for predicting CVD was investigated in a wide cohort

of more than 5000 individuals without CVD, the gains over conventional factors resulted to be minimal [5]. However, positive outcomes are expected when high-risk populations are investigated; thus, the risk level of selected patients, the chosen biomarkers to be investigated and other factors, such as, statistics, highly influence expected results. Combination of multiple biomarkers in assessment of individual responses adds only moderately to standard risk factors [6]. Therefore, there is a substantial interest in the discovery and use of newer biomarkers, to complement the best existing ones and to identify persons who are at risk for the development of cardiovascular disease and who could be targeted for preventive measures. In particular, finding biomarkers that predict the risk of rupture will provide the opportunity to institute a preventive life style and permit timely pharmacological treatment. Currently, the improvements in outpatient and inpatient care, diagnosis and biomarker discovery have reshaped the landscape of CVD. It is important to note that the new diagnostic methods currently available are based on noninvasive techniques that, although they present a number of benefits, may be limited in terms of specificity, sensitivity, availability and cost. The progress in “-omics”

technologies has provided sensitive, fast and robust tools to analyze biomarkers in CVD.

Metabolites are small molecules that participate in general metabolic reactions and that are required for the maintenance, growth and normal function of a cell. The term metabolome, derived from the word genome, refers to the complete set of metabolites in an organism and its organelles [7, 8] or the total complement of metabolites in a cell [9]. In this way, metabolomics and metabonomics refer to the use of analytical methods to identify and quantify all metabolites in a biological system, as well as the monitoring of changes in the metabolome of a biofluid, cell culture or tissue sample following perturbation [8, 10].

In parallel with genomics, transcriptomics and proteomics, application of metabolomic technologies to the study of CVD will increase our understanding of the pathophysiological processes involved and this should help us to identify potential biomarkers to develop new therapeutic strategies [11, 12]. Indeed, the identification and quantification of these low molecular weight molecules (e.g., lipids, amino acids, and sugars) will define the phenotype of these diseases [13]. From a clinical perspective, the study of metabolic changes that occur in response to different physiological processes will help establish the mechanisms underlying the disease. In terms of personalized medicine, pharmacometabonomic approaches can serve to predict the action of specific drugs in a particular individual based on the predose urinary metabolite profile. Furthermore, the gut bacterial fauna influences drug efficacy, which could be deliberately modified to optimize the benefits and minimize adverse effects of a given treatment [14]. In addition, this approach will help understanding how drugs act during patients' recovery or how they influence outcome.

## 2. Metabolomic Strategies, Analytical Approaches and Variability

There are several analytical strategies that can be used to analyse the metabolome [15], such as nuclear magnetic resonance (NMR) [16], Fourier transformation infrared spectroscopy (FT-IR) [17, 18], and mass spectrometry (MS) coupled to separation techniques such as high performance liquid chromatography (HPLC), gas chromatography (GC), or capillary electrophoresis (CE). The combination of these different analytical techniques offers important advantages when analyzing the complete metabolome. High field  $^1\text{H}$  NMR is one of the preferred platforms for urine and plasma analysis [19, 20], as it is a nondestructive technique that does not require prior separation of the analytes and it provides detailed information on molecular structure. For example, the capacity to predict the occurrence of exercise-induced ischemia in patients with suspected CAD was investigated by NMR blood analysis, demonstrating lactate, glucose, lipids, and long-chain fatty acids to be the main metabolites involved [21]. Xanthine and ascorbate were proposed as possible markers of plaque formation in an atherosclerotic mouse model [22] and lipoprotein subclasses can now be analyzed by a commercial NMR-based protocol called NMR

LipoProfile [23, 24]. However, one of the main limitations of NMR is the poor sensitivity, although this can be improved enormously when it is combined with mass spectrometry.

Coupled to a separation technique, MS has recently been introduced into the metabolomics field and its use in such studies will constitute the main focus of this paper. Indeed, gas chromatography/mass spectrometry (GC-MS), liquid chromatography/mass spectrometry (LC-MS) and capillary electrophoresis/mass spectrometry (CE-MS) are the most powerful techniques for metabolite separation and analysis. GC-MS provides an extraordinary resolution, permitting the separation of structurally similar compounds that would otherwise be very difficult to separate by HPLC. However, this technique requires the analyte to be volatile and thermally stable. In some cases, a chemical derivatization step is required prior to the chromatographic separation in order to render polar metabolites volatile. Some of the metabolites best suited for GC-MS include fatty acids, organic acids, steroids, di-glycerides, sugars and sugar alcohols.

For those metabolites that are not volatile and which cannot be derivatized, LC is the separation technique of choice. Thus, LC-MS can analyze a much wider range of chemical species (polar and nonpolar metabolites) with ample selectivity and sensitivity. Apart from reversed phase chromatography (RP-LC), which is widely used in metabolomics applications, hydrophilic interaction chromatography (HILIC) is a complementary approach suitable for very polar metabolites (nonvolatile). Indeed, the metabolites suited to analysis by GC or HPLC can be represented according to their polarity (see scheme in Figure 1). Similarly, capillary electrophoresis (CE) can be coupled to a mass spectrometer (MS), with the particular advantage of improving the resolution of separation as narrower peaks than with LC are obtained. Accordingly, different approaches have been described in combination with ion trap (IT), triple quadrupole (QQQ), time of flight (TOF), and Q-TOF instruments. The main advantage of QQQ and Q-TOF instruments is that they provide the possibility of identifying the compounds by tandem MS/MS analyses. In order to obtain a full overview of the detectable molecules, electrospray ionization (ESI) should be performed in both positive and negative modes on the same sample.

Irrespective of the analytical approach used in metabolomics, particular care has to be taken in preparing the sample. Bearing in mind that the typical half-lives of metabolic reactions in an organism are less than 1 s, it is important not to monitor metabolic changes extrinsic to the pathology or drug effect under study, producing misleading interpretations of the situation. Evidence-based epidemiological studies have led to the discovery of well-established biomarkers. These studies now tend to be complemented by control-case investigation using a different methodology, based on two main stages: the discovery phase, resulting in a set of novel biomarkers candidates and the validation phase, where discovered potential biomarkers are further validated in a different cohort of samples. In this context, biological variation would be expected to be higher than the analytical variability and thus, it is essential to pay particular attention to: (a) the precise definition of

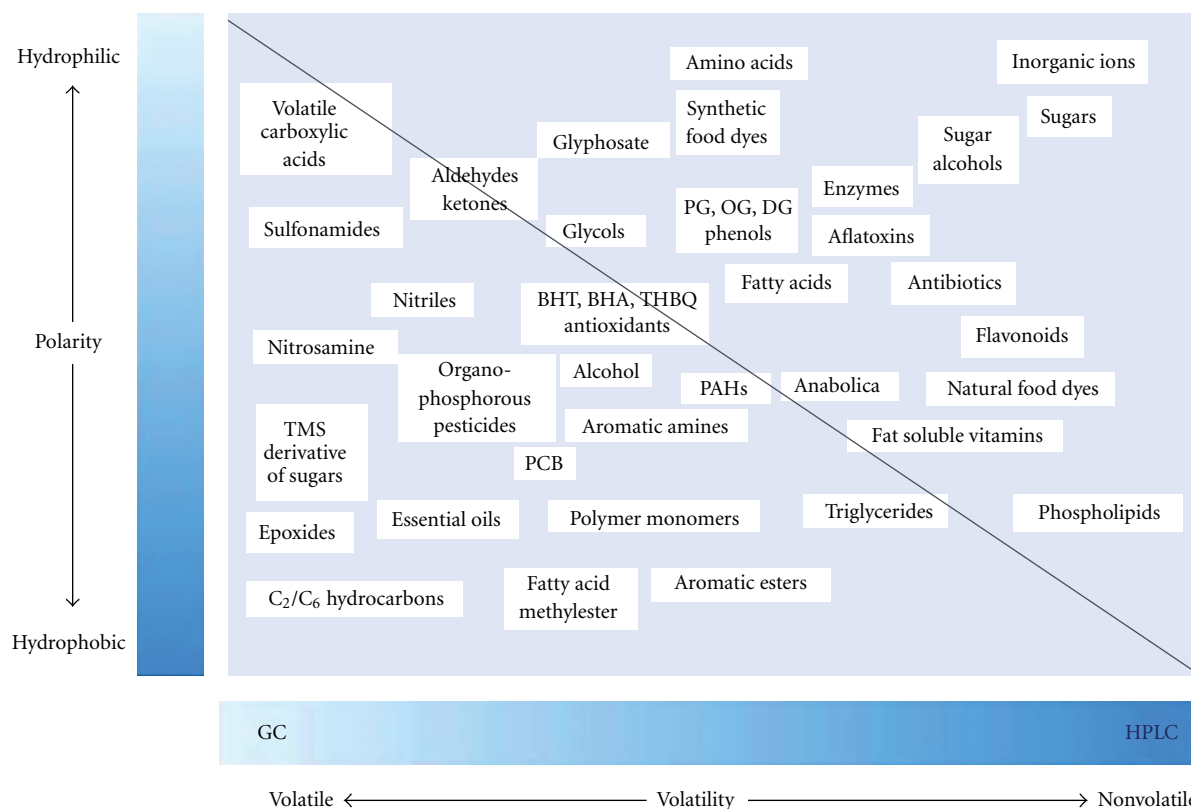


FIGURE 1: Suitability of gas and liquid chromatography for metabolomic analysis based on metabolite polarity. Courtesy of Agilent Technologies [53].

a clinical phenotype (in this sense, network-based analysis on associations among genes, proteins, metabolites, and environmental factors would be encouraged to increase sensitivity and selectivity of the diagnosis) [25] and (b) group matching in terms of sex, age, lifestyle, diet, or pharmacological treatment, otherwise attempts may fail in terms of disease prediction [26]. For instance, gender and statins treatment strongly influence the findings in studies of CVD and when individuals with normal coronary arteries were compared with CVD patients, a >99% confidence limit was only obtained for 6% of the predictions in the treated groups [27]. Technical reproducibility and sampling time are also critical to minimize external factors that will influence the results and their clinical relevance. Ideally, snapshots of different conditions should be taken so that they can be quantitatively compared. If all these considerations are kept in mind, metabolomic research can set out to identify characteristic patterns that can be used for diagnostic purposes and risk prediction, substituting traditional, more expensive clinical approaches (e.g., angiography).

In principle, metabolites can be measured in several body fluids or tissues, although plasma and urine are the most commonly used biological matrices in cardiovascular research due to their availability and clinical relevance as a source of potential biomarkers. Almost all cells in the body communicate with the plasma, either directly or through different tissues and biological fluids, releasing at least part of their intracellular content [28]. By contrast, urine is

produced by renal filtration of the plasma and it is widely considered as one of the most important samples for diagnosis as it contains not only many plasma components but also the catabolic products of different metabolic pathways. Sample pretreatment varies depending on the analytical platform chosen (see the common strategies employed in Figure 2). Metabolites from frozen tissue samples can be extracted and simultaneously fractionated by treating the ground tissue with mixtures of organic solvents, such that molecules are extracted in different fractions according to their polarity. If a biological fluid is the starting material (urine, serum, plasma), metabolite fractions are usually obtained after proteins are removed by precipitation. The crude or diluted sample can then be injected directly, although matrix effects causing ion suppression should be expected. If analyzed by LC-MS(/MS), it may be desirable to preconcentrate (e.g., by lyophilisation) or fractionate the sample prior to chromatographic separation. In case of GC-MS(/MS), preconcentration can be performed by solid phase microextraction (SPME) with or without head space (HS) procedures, which are particularly useful when analysing volatile organic compounds (VOCs). For CE analysis, the salt content should be minimized in the sample.

In general, three complementary approaches are used for metabolic research (see Figure 3): metabolic fingerprinting, metabolic profiling, and metabolic footprinting [29]. In the first case, and like proteomics strategies, an unbiased analysis is performed that is oriented towards defining

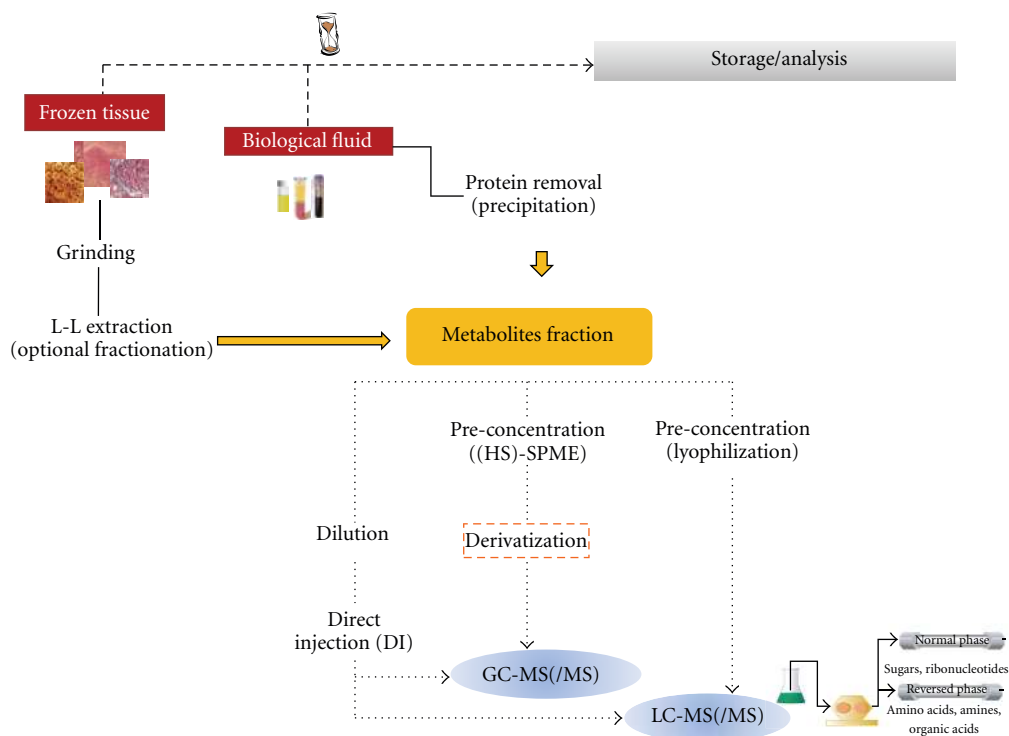


FIGURE 2: Schematic view of the sample pretreatment for metabolomic analysis of frozen tissue or biological fluid prior to GC-MS or LC-MS analysis.

clinically relevant differences rather than identifying all the molecules present in a sample [30]. Alternatively, metabolic profiling involves a preselection of a set of metabolites, or a specific class of compounds, that might participate in a targeted pathway. Metabolic footprinting does not rely on the measurement of intracellular metabolites but rather, on monitoring those that are secreted or fail to be taken up by a cell or tissue [31, 32]. Below, we will discuss relevant findings from these approaches in CVD (a compilation of the main studies is shown in Table 1).

### 3. Metabolic Fingerprinting in CVD

Metabolic fingerprinting does not aim to identify the entire set of metabolites but rather to compare patterns or fingerprints of metabolites that change in response to a disease state, pharmacological therapies or environmental alterations, for example. A wide variety of biological matrices can be used for metabolic fingerprinting, such as urine, plasma/serum, tissues/cells and saliva. This approach can be used as a diagnostic tool to evaluate the disease state by comparing healthy controls and disease subjects, or to assay the success of a particular treatment (prognosis/recovery) [33]. However, if we want to understand the mechanisms underlying a disease, qualitative and quantitative analyses are required. Once a differential pattern is discovered, which provides information that can be considered as the pathological phenotype [34], further steps to identify the participating compounds (qualitative) and to determine the

absolute amounts of metabolites that participate in the processes studied (quantitative) must be followed. This is not a trivial issue and prior to embarking on the task of discovering metabolic biomarkers, sufficiently sensitive and selective instruments and extensive compound libraries for metabolite identification should be available [35], while certain expertise in data analysis and interpretation will be necessary.

One of the few metabolomic studies in the field of CVD involved a comparison of the metabolomic fingerprint obtained by GC-MS of plasma samples from non-ST-segment elevation acute coronary syndrome (NSTEMACS) patients, stable atherosclerosis patients and healthy patients [36]. Citric acid, 4-hydroxyproline (4OH-Pro), aspartic acid and fructose were found to decrease in NSTEMACS patients, whereas lactate, urea, glucose, and valine increased. Both lactate and glucose are also involved in prediction of exercise-induced ischemia in patients with suspected CAD [21]. The decreased in 4OH-Pro was especially interesting because circulating 4OH-Pro is thought to prevent the binding of LDL to lipoprotein previously deposited in the vascular wall, as well as releasing already deposited LDL from the atherosclerotic lesions. It is also a component of collagen, which confers stability to the atherosclerotic plaque.

The high resolution of CE-MS makes it a powerful technique to separate and analyse charged metabolites, although only a few metabolomic applications have been published to date. The isolation of polypeptide fraction from urine or plasma was analyzed by CE-MS and used to discriminate between coronary artery disease (CAD)

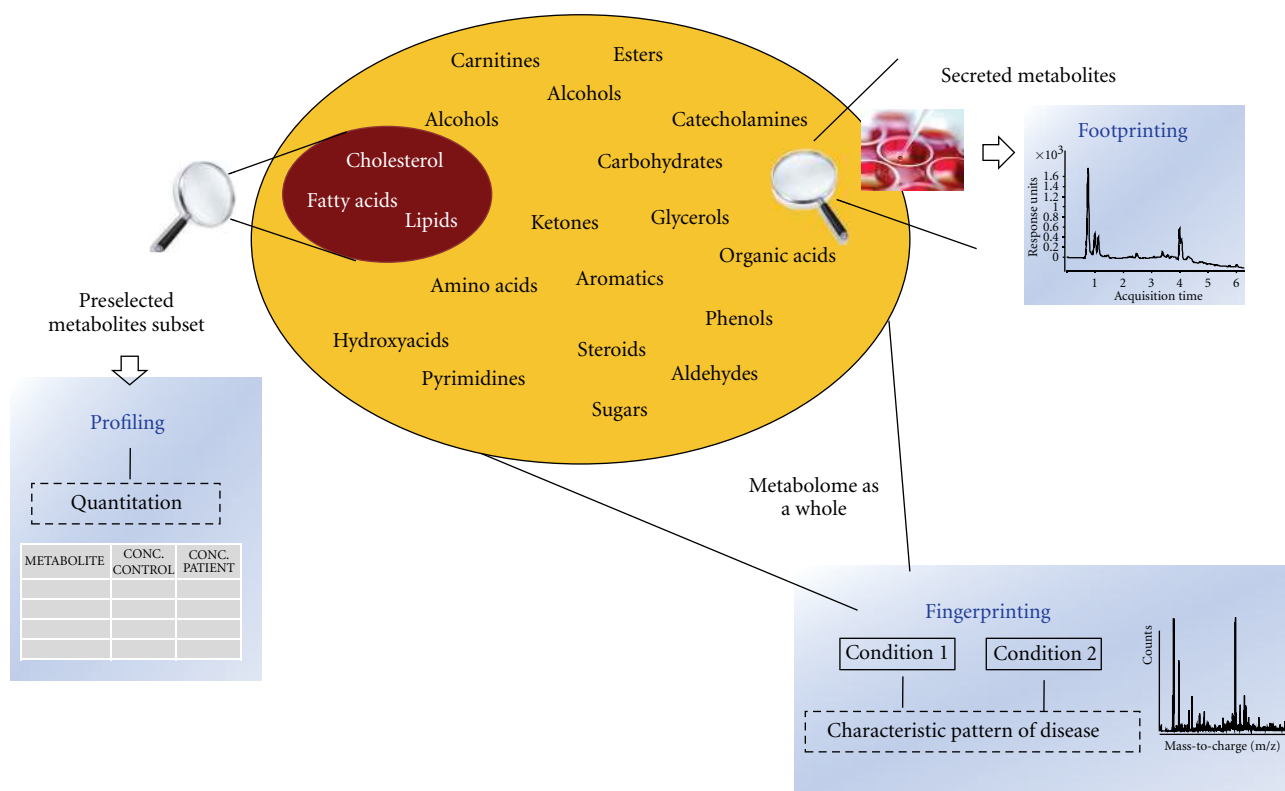


FIGURE 3: Common strategies in metabolomics: fingerprinting, profiling and footprinting.

and non-CAD patients with clinical symptoms and who had been subjected to coronary angiography [37]. The stability of urine samples and their resistance to oxidation or precipitation reflect the advantages of this biological fluid for proteomic analysis. Polypeptide profiling in urine is more reproducible than in plasma, with no significant loss of polypeptides over time when performing consecutive analyses over a 24-hour period, which also demonstrates the reproducibility of the CE-MS. In total, 200 of the most abundant polypeptides were detected and a set of 17 urinary polypeptides permitted CAD and non-CAD patients to be distinguished. Among them, collagen  $\alpha$ -1 (I and III) was augmented in CAD samples, which was corroborated by their increased expression found in atherosclerotic plaques. This increase points to an important role for collagen in the development of atherosclerosis.

#### 4. Metabolic Profiling in CVD

Metabolite profiling focuses on the analysis of a group of metabolites related to a specific metabolic pathway [38, 39]. In this approach, target metabolites are selected beforehand and they are assessed using specific analytical methods. Technological advances have increased the number of metabolites that can be quantified simultaneously. Moreover, the results of metabolic profiling are quantitatively independent of the technology used for data acquisition [40].

Metabolite profiling has been applied to CVD in order to identify and quantify metabolites that might serve as

new biomarkers. A metabolite profile of peripheral blood from individuals undergoing planned myocardial infarction (PMI) has been established [41]. Serial blood from 36 patients were obtained before and at various intervals after PMI, and the changes in circulating levels of metabolites were identified by mass spectrometry-based metabolite profiling. Most alterations produced by PMI were observed in the tricarboxylic acid cycle, in purine and pyrimidine catabolism, and in the pentose phosphate pathway. Indeed, 7 metabolites were significantly affected immediately 10 minutes after the onset of myocardial injury ( $P < .005$ ): alanine, aminoisobutyric acid, hypoxanthine, isoleucine/leucine, malonic acid, threonine, and trimethylamine N-oxide (TMNO). All these alterations were especially interesting as they were observed before any significant rise in the clinically available biomarkers in plasma (CKMB and troponin T). After 60 minutes, six new metabolites had also changed significantly ( $P < .005$ ): 1-methylhistamine, choline, inosine, serine, proline, and xanthine, with the later being a candidate of a marker for plaque formation in an atherosclerotic mouse model [22]. The anatomic origin of the early metabolic changes observed was further explored in a subgroup of 13 patients by simultaneously comparing the metabolite levels obtained in samples from peripheral blood and from a catheter placed in the coronary sinus. A further 8 metabolites were transmurally enriched at least 1.3-fold 10 minutes after PMI (taurine, ribose-5-phosphate, DCMP, lactic acid, AMP, malic acid, glutamine and glutamic acid) and once 60 minutes had passed, six additional metabolites augmented

TABLE 1: Compilation of the metabolomic studies in cardiovascular field, including candidate biomarkers.

Pathology (Patients no.)	Metabolite	Body fluid/tissue	Analytical technique	Replication	Reference
NSTACS (9), stable atherosclerosis (10), healthy (10)	4-hydroxyproline	Plasma	GC-MS	—	[36]
CAD (15), no CAD (14)	17 polypeptides (CAD pattern) Collagen $\alpha 1$ (I,III)	Urine, Plasma	CE-MS	Test set: CAD (26), no CAD (12)	[37]
PMI (20), control (16) SMI (12), control (9)	Aconitic acid, hypoxanthine, trimethylamine N-oxide, threonine	Plasma	LC-MS	Validation: PMI (16)	[41]
High-fat diet (9), common-diet (9) rats	12 altered in plasma, 8 altered in urine (atherosclerotic rats)	Urine, Plasma	LC-MS	—	[42]
Atherosclerosis (9), healthy (10)	24 altered metabolites (insulin resistance)	Plasma	NMR, GC-MS	—	[45]
CAD (12), LVD (10), control (17)	Acetylcarnitine, 3-hydroxybutyrylcarnitine	Plasma	FI-MS	—	[46]
Initial: CAD (174), control (174) Replication: CAD (140), control (140)	Dicarboxyl acylcarnitines	Plasma	GC-MS	Event replication: CAD (63), control (66)	[49]
Inducible ischemia (18), control (18)	Citric acid pathway	Plasma	LC-MS	—	[52]
Persistent AF: AF (8), SR (8) Post-operative AF: SR-AF (18), SR (19)	$\beta$ -hydroxybutyrate, ketogenic amino acids, glycine	Atrial tissue	NMR	—	[50]
Apo E <sup>-/-</sup> mice captroil treated (8), untreated (8)	Allantoin (drug treatment) Xanthine, ascorbate (plaque formation)	Urine	NMR	—	[22]

CAD: coronary artery disease, PMI: planned myocardial infarction, SMI: spontaneous myocardial infarction, LVD: left ventricular dysfunction, FI: flow injection, AF: atrial fibrillation, SR: sinus rhythm. Numbers in brackets correspond to number of assayed individuals (or animals if so specified).

(glycerol-3-phosphate, orotic acid, succinic acid, glycerate-2-phosphate, taurine and malic acid).

Plasma and urine samples from atherosclerotic and control rats have been compared by ultra fast liquid chromatography coupled to ion trap-time of flight (IT-TOF) mass spectrometry (UFLC/MS-IT-TOF) [42]. Accordingly, 12 metabolites were identified as potential biomarkers in rat plasma and 8 metabolites in rat urine. The concentration of leucine, phenylalanine, tryptophan, acetylcarnitine, butyrylcarnitine, propionylcarnitine and spermine decreased in plasma, and 3-O-methyl-dopa, ethyl N2-acetyl-L-argininate, leucylproline, glucuronate, N(6)-(N-threonylcarbonyl)-adenosine and methyl-hippuric acid were diminished in the urine of atherosclerosis rats. Conversely, ursodeoxycholic acid, chenodeoxycholic acid, LPC (C16:0), LPC (C18:0) and LPC (C18:1) increased in plasma and hippuric acid augmented in the urine from atherosclerosis rats. The alterations to these metabolites reflected the abnormal metabolism of phenylalanine, tryptophan, bile acids and amino acids. Lysophosphatidylcholine (LPC) plays an important role in inflammation and cell proliferation, highlighting the relationship between LPC with the progress of atherosclerosis and other inflammatory diseases.

The lipidomic profile of mice liver homogenates from cholesterol-free, low cholesterol and high cholesterol diets demonstrated the influence of dietary cholesterol intake and atherosclerosis [43]. To obtain individual metabolite fingerprints, nearly 300 metabolites were measured in plasma

samples by LC-MS/MS, including di- and tri-glycerides, phosphatidylcholines, lysophosphatidylcholines and cholesterol esters. When dietary cholesterol intake increased, the liver compensated for the elevation in plasma cholesterol by adjusting metabolic and transport processes related to lipid metabolism, which leads to an inflammatory, pro-atherosclerotic state. A cholesterol-free diet did not induce early atherosclerosis, while the low cholesterol diet only mildly induced early atherosclerosis. By contrast, intense early atherosclerosis was induced by the high cholesterol diet, in association with proinflammatory gene expression. Indeed, a relationship appears to exist between cholesterol intake (measured as cholesterol plasma levels) and atherosclerotic lesion size.

The lipidome of cell membranes and tissues has been studied by measuring the plasmalogens contained in rabbit and rat myocardial nuclei by ESI-MS [44]. Plasmalogen is an ether lipid where the first position of glycerol binds a vinyl residue with the double bond next to the ether bond. The second carbon has a typical ester-linked fatty acid and the third carbon usually has a phospholipid head group, which can protect cells against the damaging effect of singlet oxygen. This seems to be the reason for the strong enrichment of plasmalogens found in the membrane of myocardial cells.

Metabolic changes associated to atherosclerosis have also been investigated through NMR and GC-MS metabolite profiling [45]. There are clear biochemical explanations to

these findings, and the alterations to these metabolites, which cause the final atherosclerotic lesion, can be related to different disorders. For instance, insulin resistance in diabetic patients increases the activity of transaminases, which are critical enzymes in amino acid metabolic pathways. Hence, if the insulin response is deficient, these amino acid pathways will be altered, and many others metabolites will be affected such as glutamate, ketoglutarate, succinyl-CoA, 4-OH-L-proline (4OHPro), 2-hydroxybutyrate, creatinine, pyruvate, oxaloacetate, malate, glycolate and 2,3,4-trihydroxybutirate. These effects could indicate damage to tissue at the intima artery walls.

The myocardial metabolic response has been investigated in CAD and left ventricular dysfunction (LVD) patients, both at baseline and following ischemia-reperfusion (I/R) [46]. Accordingly, glucose, lactate, free fatty acids, total ketones, 3-hydroxybutyrate, pyruvate, leucine/isoleucine and glutamate are present at lower concentrations in a preischemia state in the coronary sinus (CS) than in arterial samples (reflecting myocardial uptake). By contrast, the alanine concentration is higher (reflecting release). A principal components analysis (PCA) shows several potentially important postoperative metabolic changes during the clinical course of the disease. Ventricle dysfunction are associated with the global suppression of metabolic fuel uptake, and limited myocardial metabolic reserves and flexibility following global I/R stress is associated with cardiac surgery.

Some citric acid metabolites are depressed in acute ischemia and acute myocardial disease [47]. The citric acid cycle plays an important role in oxidative phosphorylation and ATP production in cardiomyocytes, and citric acid cycle intermediates are supplied by glycolysis and  $\beta$ -oxidation of fatty acids. Metabolomic profiling based on quantitative mass spectrometry was also used to study the heritability of premature coronary disease in 117 individuals unaffected by CAD but with a family member affected [48]. There was a strong heritability of amino acid levels such as arginine, ornithine, alanine, proline, leucine/isoleucine, valine, glutamate/glutamine, phenylalanine and glycine, free fatty acids such as arachidonic, palmitic, linoleic and acylcarnitines. Hence, it was concluded that metabolic changes associated with CAD can be inherited and they are strongly related to age. This would indicate that metabolic processes could be controlled genetically, implying a correlation between genotype and phenotype in families with CAD. More recently, a subset of 69 metabolites was shown to have diagnostic value, such that some derived factors showed discriminative capability for CAD after PCA. Moreover, a signature composed of dicarboxyacylcarnitines was predictive of further cardiovascular events in those patients and most significant differences persisted after adjustment for CAD risk factors [49].

Metabolic changes in human atrial fibrillation (AF) have been investigated by NMR, performing a quantitative analysis of 24 previously selected metabolites. Significant differences were found for beta-hydroxybutyrate, ketogenic amino acids and glycine, all of which augmented in AF patients when compared to control subjects, suggesting a pathological role for ketone bodies. Metabolic profiles enable

more than 80% of patients at risk of AF at the time of coronary artery bypass grafting to be classified, as a discordant regulation of energy metabolites was found to precede post-operative AF [50]. The effect of drug treatment on apoE<sup>-/-</sup> mice was investigated by NMR analysis of metabolites in urine, showing allantoin to act as a marker for drug treatment, and xanthine and ascorbate as possible markers of plaque formation (both were elevated in untreated mice) [22].

## 5. Conclusion

The application of metabolic analysis to cardiovascular diseases is an emerging field [51], and at this incipient stage it is not possible to clearly define a metabolic picture which is responsible for CVD prediction and progression. Further metabolomic investigation promises to improve researchers and clinicians knowledge of these diseases in three critical ways. Firstly, a complete description of the metabolites altered in a disease will better define the pathophysiology of the disease. Secondly, metabolic profiling will enhance the feasibility of high-throughput patient screening to diagnose the disease state or risk evaluation [52]. Indeed, the identification of clinically relevant changes in circulating metabolites that may be considered as potential new biomarkers will also help with the evaluation of prognosis and will contribute to the development of new therapeutic strategies. Thirdly, metabolite profiling will enable the effects of pharmacological treatments to be monitored, in particular, assessing the individual's response to a particular drug. In contrast to genomics, metabolomics defines dynamic states that reflect the actual status of an organism, which requires the control of many variables (from an individual's status to metabolite degradation following sample collection). Failure to do so may lead to the production of erroneous results and misleading conclusions. Minimal protocol-specific differences can produce inconsistent findings, which must be clearly overcome prior to proposing the use of a biomarker to the scientific community. Similarly, the results must be confirmed in a validation cohort composed by a different set of samples than that used in the discovery phase. Adequate follow-up studies must corroborate earlier predictions, and adjustment for conventional risk factors to assess significant contribution of a discovered metabolite to current knowledge should be included. To date, there have been considerable efforts in improving instrumentation (e.g., mass spectrometry) and the analytical methods suitable to complement these approaches (e.g., based on NMR), resulting in an expansion of the metabolites with potential roles in the development of atherosclerosis that can be quantified. However, further research is still needed prior to proposing an ideal platform for metabolite analysis that can replace conventional CVD diagnosis in clinical practice. With the growth of public metabolite databases, further improvements in the sensitivity and selectivity of analytical techniques and the development and routine use of novel platforms of demonstrated potential, novel targets are expected to be discovered in the near future.

## Acknowledgments

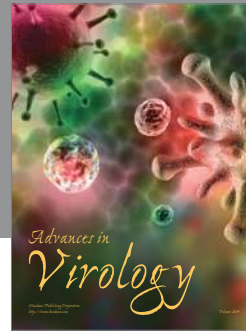
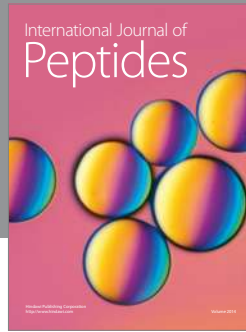
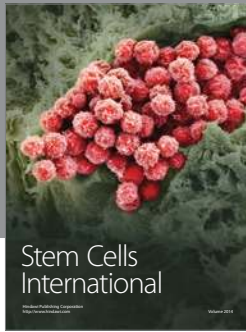
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