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Review

Metabolomics in early detection and prognosis of acute coronary syndrome

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ABSTRACT

Acute coronary syndrome (ACS) is one of the most dangerous types of coronary heart disease (CHD) and contributes to significant mortality and morbidity worldwide. Outcomes in these patients remain a challenge despite improvements in diagnosis and treatment. Risk stratification continues to be problematic and the identification of novel predictors is crucial for improved outcomes. As such, there is a strong need for the development of novel analytical methods as well as the characterization of better predictive and prognostic biomarkers to enable more personalized treatment. Metabolite profile analysis may greatly assist in interpreting altered pathway dynamics, especially when combined with other 'omics' technologies such as transcriptomics and proteomics. In this review, we describe ACS pathophysiology and recent advances in the role of metabolomics in the diagnosis and the molecular pathogenesis of ACS. We briefly describe key technologies used in metabolomics research and statistical approaches for data reduction and pathway analysis and discuss their application to CHD.

1. Introduction

Cardiovascular disease (CVD) is the most common cause of death in developed countries and now is increasing in developing countries too [1,2]. Coronary artery disease [3], is responsible for approximately 8.14 million deaths in the world [4]. Based on clinical signs, extensive arterial blockage, and myocardial injury, CAD is divided into several groups: non-obstructive coronary atherosclerosis (NOCA), stable angina (SA) and ACS. ACS is a common complication of CHD, which affects more than a million people each year, and one-third of them are affected before the age of 70. This complication is the main cause of mortality and disability in developed countries [5]. Clinically, the symptoms include high levels of lipoproteins containing cholesterol and triglyceride and high and low levels of HDL-type lipoprotein [6]. The most common method for diagnosing of ACS is angiography, which is an invasive imaging method [7]. Investigating and evaluating signs and symptoms based on risk factors is a key factor in classifying and preventing death in triage. Increasing knowledge in this field can lead to accelerated treatment by removing the blood clot, reducing the size of the necrotic area and in ultimately reducing mortality [8,9]. Although several risk factors, such as diabetes, smoking, hypertension, and hyperlipidemia are involved in the onset of this disease, the precise molecular mechanism is unknown. One of the main problems in disease diagnosis in the clinic is the late appearance of disease symptoms. In fact, asymptomatic processes associated with the formation of atherosclerotic plaques unknowingly lead to tissue damage. Therefore, early diagnosis of the disease is necessary to prevent the rupturing of atherosclerotic plaque and the formation of clots, which is important for urgent proceedings, as well as for reducing mortality and disability in patients. In this case, the identification of a new set of biomarkers that are clinically valuable can help in the early diagnosis and treatment of the disease. Diagnosis of CAD is based on clinical symptoms, electrocardiogram (ECG), cardiac markers, stress tests, and coronary angiography [10–12]. Among these methods, invasive coronary angiography is the gold standard diagnostic method [13]. However, the proprietary technology and high cost of this technique have limited its access for public use [10]. Furthermore, it was shown that a significant number of patients undergoing invasive angiography have normal

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coronary arteries [14]. One of the strategies recently used to identify the molecular mechanisms involved in CVD is the metabolomics approach. Determining the metabolome profile of patients and finding possible biomarkers can help in early diagnosis of the diseases and a more tailored personal treatment.

1.1. Pathogenesis of ACS

Experimental models of atherogenesis have been provided a lot of information about the molecular mechanisms of plaque development. However, the change from the stable coronary artery to the unstable coronary artery is less understood, due to the lack of animal models that reflect human disease [15]. The sudden clinical manifestation of ACS is strong evidence of discontinuity in the natural history of atherothrombosis. When the primary diagnosis of atherosclerosis fails, the progression of coronary atherosclerosis can remain clinically hidden for many years, even decades or throughout the patient life. This subject has been shown in dying individuals due to non-coronary causes with the high prevalence of coronary atherosclerosis. In contrast, some patients develop ACS at a particular time in their lives, which can last for a few years. These simple clinical observations suggest that the mechanisms responsible for the development and instability of plaques are different and the causes and mechanisms of plaque instability can vary. Additionally, samples indicating a type of coronary plaque, as a cause of instability, are not sufficiently suited to finding of postmortem studies [16,17]. In fact, plaque fissure has often been asymptomatic and gradually, turning into a sudden coronary obstruction discreetly.

ACS is the most common type of CVD and is usually accelerated by rupture or erosion of atherosclerosis plaque [3]. It has been suggested that atherosclerotic plaque is often created as a result of the gradual increase in cholesterol, macrophages, and fibrin tissues in the arterial walls over many years [18]. Therefore, symptoms are only apparent when the disease is in the advanced stage. Biochemical necrotic markers, especially troponin I and T, play an essential role in the diagnosis, classification, and management of ACS patients [19–21]. However, the new high-sensitivity troponin generation has low specificity for ACS diagnosis at early stages. [22]. These limitations in the early diagnosis of ACS highlights the need for identification of new biomarkers with acceptable specificity. [23]. Generally, advanced obstruction leads to narrowing of the atherosclerotic coronary artery, as a small platelet thrombosis (clot) which can completely block the arteries. Thus, occlusion of the arteries by thrombosis leads to a high degree of stenosis and will cause obstruction of blood flow in vessels, which causes ST-segment elevation myocardial infarction (STEMI). Non–ST-segment elevation ACS (NSTE-ACS) is the result of an incomplete or transient obstruction of blood vessels at the site of stenosis [24]. A fibrous cap is located in the center of the lipid-rich plaque, which is known as the necrotic core (Fig. 1). This fibrous cap is located between the blood flow chamber with its coagulation factors and the lipid center of the plaque that is filled with clotting material. The morphometric study has identified the characteristics of ruptured plaques that led to myocardial infarction. Such plaques often, but not always, have thin fibrous caps (50 to 65 μm) [25]. Ruptured plaques also tend to have large lipid core and high inflammatory cells. Fibrous cap thickness of less than 55 μm was identified as a morphological indicator of plaques causing fatal ruptures. More than 30% of these plaques had luminal stenosis with less than 75%. On the other hand, sites of plaque rupture provoke fatal coronary events that contain a few numbers of smooth muscle cells (SMC) [26].

1.2. Molecular mechanism of ACS

The molecular mechanism of the onset atherosclerosis is as follow: the risk factors such as high blood cholesterol levels, high blood pressure, diabetes, and cigarette smoking [27–29], cause damage to endothelial blood vessels and cause its abnormal function. Abnormal endothelial function, which is characterized by reduced bioavailability to endothelial nitric oxide (eNOS) and high endothelin-1 (ET-1) production, leads to abnormalities in vascular hemostasis and increased expression of adhesion molecules. It also increases the production of platelets and activates them in the bloodstream through the secretion of local active substrates [30,31]. The progression of the atherosclerosis process is due to the transport of low-density lipoprotein [32] within the endothelium to the arterial wall. Probably, this process occurs at the site of endothelial damage created by oxidized low-density lipoprotein (Ox-LDL) and physical, chemical forces or infection. Endothelial cells, SMCs, and macrophages are sources of oxidants to oxidative alteration of phospholipids. These cells convert LDL into Ox-LDL by active oxygen species. Ox-LDL can damage endothelial cells and induce expression of adhesion molecules such as P-selectin and chemotactic factors such as monocyte chemoattractant protein-1 (MCP-1) and macrophage colony stimulating factor (CSF). Chemoattractant molecules cause the migration of monocytes and leukocytes into sub-endothelial space (intima). This process leads to the activation, and attachment of monocytes and T lymphocytes to endothelial cells. Monocytes and T cells enter the intima, then monocytes digest lipoproteins and differentiate into macrophages [33]. Dendritic cells (DCs) which are known as antigen-presenting cells can active T helper 1 cell producing IFN-γ which active macrophages [34]. Macrophages produce reactive oxygen species (ROS) and convert Ox-LDL to highly oxidized LDL. This kind of LDL molecules is absorbed by macrophages and converts them into foam cells. Foam cells combine with leukocytes to develop the fatty streak. This process continuously produces foam cells. The proliferation of SMC with the continuous influx of monocytes and macrophages produce fatty streak and lead to more advanced damage and ultimately will protrude into the arterial lumen. In the next step, calcification occurs, and fibrosis continues and leads to produce a fibrous cap that surrounds the lipid-rich core. In ACSs such as myocardial infarction, when fibrous plaques rupture (Fig. 1), the formation and release of substances within the atherosclerotic plaque causes a thrombus and may ultimately cause blockages of the vessels [33].

1.3. Inflammation, collagen synthesis, plaque rupture, and thrombogenesis

Macrophages also produce a matrix metalloproteinase, an enzyme that digests the extracellular matrix, which results in rupture of plaque [27]. The ratio between SMC and macrophages has an important role in plaque vulnerability and a tendency to rupture. Although plaque rupturing may cause ACS, it is often clinically silent or uncomplicated in 99% of cases [35]. The degree of progression of atherosclerosis lesions is variable, non-linear, and unpredictable [36]. Extensive research on the fibrous cap of plaque has focused due to its importance in most fatal AMI. The fibrous cap structure, which prevents plaque rupture, has the flexibility similar to interstitial forms. The association between the thinning of the fibrous cap and the fatal rupture of the plaque has led to the hypothesis that the defect in collagen metabolism of plaque plays an important role in the excretion of this extracellular matrix protein as well as in strengthening the fibrous cap [37]. These considerations have created great interest in the role of molecular mediators of collagen metabolism, which may operate during atherogenesis. Inflammatory cells accumulate in the site of ruptured plaques and inflammatory biomarkers precipitate ACS [24]. Rupture of collagen inside the plaque may compromise the integrity of the plaque and consequently lead to ACS [24].

A study on collagen biosynthesis control by human SMC indicated that exposure to IFN-γ, a substance that is produced from activated T cells, strongly inhibited the ability of SMC to produce new collagen [38]. In SMC stimulated by the transforming growth factor beta (TGF-β) for the production of interstitial collagen, IFN-γ reduces collagen synthesis to basal or lower levels. Another study showed that there is an inverse relationship between T-cell accumulation in human atherosclerotic plaques and mRNA level of interstitial collagen. This study

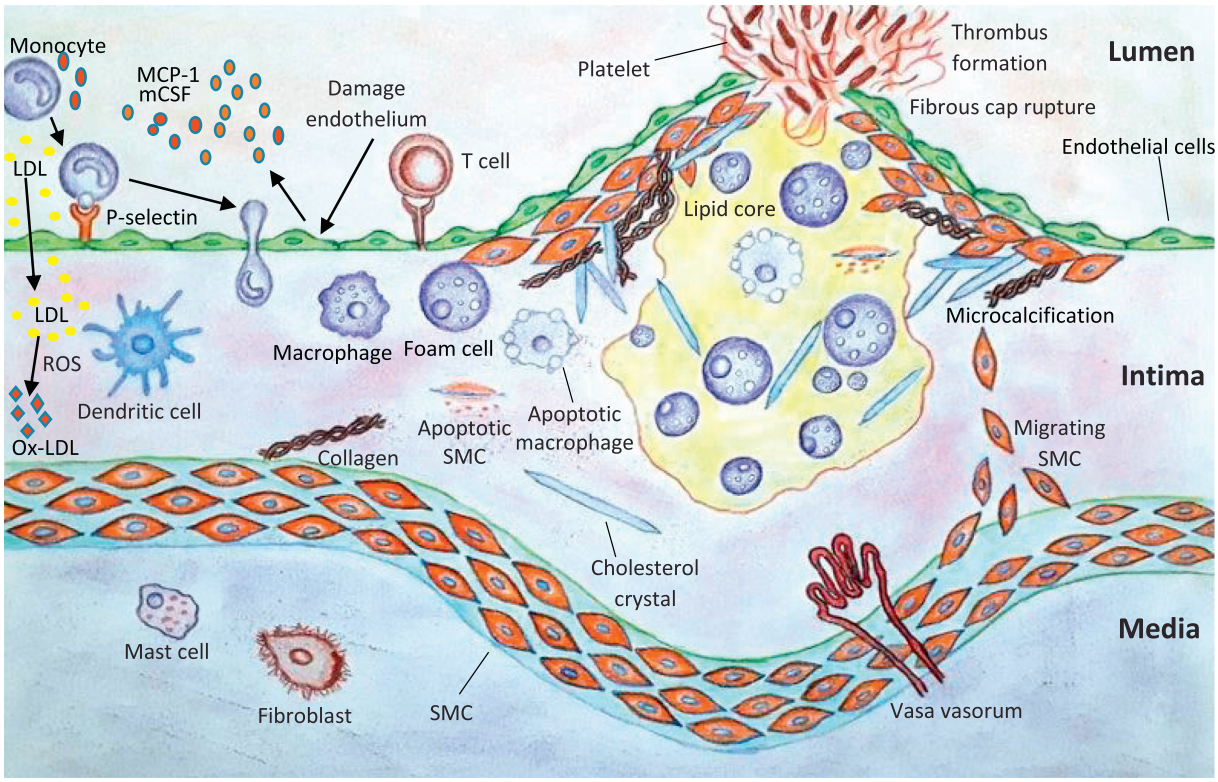

Fig. 1. Atherosclerosis plaque formation. ROS produced by endothelial cells, SMCs and macrophages in intima and in endothelial damage sites, leading to the onset of events the formation of plaque.

strongly supports the inhibition of collagen synthesis by T-cell derived mediators [39]. The amount of each macromolecule depends not only on the amount of its synthesis but also on the degree of its degradation. Interstitial collagen is usually very stable and is resistant to degradation by proteolytic enzymes. Only a few numbers of human proteases that have collagenase activity are able to catalyze the fibrillar collagens. These enzymes belong to the matrix metalloproteinases (MMP) family. Macrophage is a type of immune cell, which is abundant in wounds that cause fatal thrombosis, produces all three kinds of MMP collagenases (MMP-1, MMP-8, and MMP-13) in plaques [40]. The regulation of MMPs production by human macrophages has shown that T-cell-derived CD40-ligand (CD40L/CD145) increases the production of interstitial collagenase by human macrophages [41]. Therefore, cross-talks between acquired immune cells (T cells) and a large number of effector innate immune cells (macrophages) prevents the synthesis and degradation of interstitial collagen. Recent studies have shown that systemic inflammatory response to AMI, resulting in increased protease activity in the site of plaque that in turn deteriorate inflammation reactions [42].

1.4. Oxidative stress, atherosclerosis, and vascular disease

There is much evidence that chronic and acute excessive production of ROS under pathophysiological conditions has critical role in the development of CVD. ROS can be formed by various enzymatic electron transport systems, such as: nicotinamide adenine dinucleotide (phosphate) oxidase, xanthine oxidase, lipoxygenase, mitochondria, and uncoupled nitric oxide synthase in vascular cells. Different animal models of oxidative stress reinforce the notion that ROS play a central role in atherosclerosis and other CVD [31]. In response to growth factors and cytokines, and during natural metabolic events such as respiration and phagocytosis, eukaryotic cells produce oxidants. To compensate for this, cells use both enzymatic and non-enzymatic mechanisms to protect against the toxic effects of oxidants. Enzymatic mechanisms include the activity of enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase. Non-enzymatic antioxidants include glutathione, ascorbate, and α-tocopherol. However, in pathophysiologic conditions, excessive oxidants decrease the ability of cellular antioxidant systems, as a result of which oxidative stress can damage lipids, cell membranes, proteins, and DNA [33].

1.5. Metabolomics

Metabolomics is a term used to describe the measurement of small molecules in a biological sample. This technology enables identification, quantification, and characterization of small molecule metabolites of less than 1500 Da found in a cell, organ or in a specific organism. In human metabolomics studies, biological samples are body fluids (e.g. urine, blood, saliva etc.), tissues, biopsies, or even respiratory secretions. Metabolomics is a relatively new approach to the omics research filed, which is related to genomics, transcriptomics, and proteomics, and its origins are in the venerable discipline of analytical biochemistry. The benefits of recent technology advancements have enabled us to highlight metabolite profiling in a large number of biological samples with increasing applications in the study of human diseases like CVD. Abnormal metabolism is also a sign of CAD. Metabolic changes in the heart are a result of changes in the metabolome of biological fluids [43]. A combination of small metabolites may have tremendous diagnostic values [13]. Advances in OMICS-based technologies have created a sensitive, fast and powerful tool for analysis of biomarkers in CVD [44]. The application of genomics, transcriptomics, and proteomics technologies, as well as metabolomics approaches to study of metabolites in biological samples, also has expanded our understanding of pathophysiologic processes involved in CVD and helped us to identify biomarkers for therapeutic strategies [45].

Metabolomics has the ability to determine a large number of metabolites simultaneously compared to the classical laboratory techniques, thus enabling extensive coverage of biological processes and

metabolic pathways [46]. An attractive aspect of metabolite profiling is that relatively few human endogenous metabolites (estimated about 7000) are associated with predictable genes (25,000), transcripts (100,000) and proteins (1 000,000) [47]. Although, the metabolome, which human cells interact with is far more complex, i.e. in terms of dietary, environmental, pollutant and gut flora as a source. Since the metabolome is closely related to the genotype of an organism, metabolomics provides a unique opportunity to observe genotype-phenotype relationships, as well as genotype and environment interactions. Metabolites exist in a wide range of concentrations and exhibit significant chemical diversity. Therefore, there is no single analytical tool to be able to reliably identify all of these metabolites in human samples within a single analysis [47].

Metabolomics is a research field rapidly developing in biological systems in which metabolite changes are measured in response to disease progression [48]. Metabolites are described as reporter molecules related to disease or phenotype, and their high amounts in biological samples are often directly related to pathogenic mechanisms [49]. Hence, metabolomics is widely being used in a range of health applications including pharmacology, pre-clinical trials, toxicology, transplantation monitoring, neonatal screening, and clinical chemistry [50].

1.6. Analytical techniques used in metabolomics

Metabolome analysis strategies [51] are usually carried out using nuclear magnetic resonance (NMR) [52], and mass spectroscopy (MS) coupled with separation techniques such as high-performance liquid chromatography (HPLC), gas chromatography (GC), or capillary electrophoresis (CE). Combining these different analytical techniques will provide important benefits when fully analyzing the metabolome. Liquid chromatography-mass spectrometry (LC-MS) is the most common technique used for identifying small molecules metabolites with high reliability and sensitivity [53,54].

1.6.1. Nuclear magnetic resonance (NMR)

The proton NMR (^{1}H NMR) is one of the highly used technique for analysis of urine and plasma samples [55–59]. In NMR based metabolomics, the individual analytes within the sample are separated by their magnetic resonance shift, resulting in a spectral profile. Chemical shift, the separation of resonance frequencies from an arbitrarily chosen reference frequency, usually is expressed in terms of the dimensionless units of parts per million. The resonance frequency of a given nucleus is modified by its molecular chemical environment allowing identification of different molecules (usually using reference libraries or databases). The parameters that characterize each peak include its resonance frequency, its multiplicity, and the area under the peak yield a relative measurement of the concentration of nuclei (or a metabolite). An internal standard is often added to the samples for chemical shift calibration and quantification [47]. Some of NMR advantages include a non-destructive method of the sample and high reproducibility [60], which does not require chemical manipulation of samples and provides accurate information about the molecular structure and concentration of metabolites, and detecting more than 100 metabolites depending on biological samples [61]. For example, by NMR analyzing of the blood, the presence of lactate, glucose, lipids, and heavy chain amino acids was shown to be the main metabolite involved in the onset of ischemia induced by exercise in patients with CAD [62]. It is thought that xanthine and ascorbate are potential markers of plaque formation in an atherosclerotic mouse model [63]. Lipoprotein lipids can also be analyzed by commercial-NMR protocols, called NMR lipoProfile test [64,65]. This procedure began more than 20 years ago [66] and progressed rapidly and was used to determine the components of lipoproteins [67]. The methodology combines two molecular windows that contain the majority of the metabolic information available by ^{1}H NMR from native serum, e.g. serum lipids, lipoprotein particles as well as various low-molecular-weight metabolites [68]. In summary, NMR provides structural information and quantitative determination of metabolites in complex mixtures and often does not require preparation of the sample. This technology exploits the physical properties of certain atomic nuclei (^{1}H, ^{13}C, and ^{31}P) in a metabolic mixture [60]. However, NMR compared with mass spectrometry is an insensitive technique and can mainly detect metabolites in mM and uM concentrations.

1.6.2. Mass spectrometry (MS)

Currently, gas chromatography–MS (GC–MS) and liquid chromatography-MS (LC-MS) are the most common instruments for metabolomics. Due to its greater sensitivity compared with NMR, MS is increasingly used in the metabolites assessment of biological samples. MS measures the ratio of mass to charge of ions formed by molecules, which is usually separated by chromatography techniques. Electrospray ionization is the most common technique used in organic MS [69], which allows the measurement of almost any compound that can be dissolved [70]. However, ionization of metabolites can be affected by what is known as ion suppression, also known as matrix effect. The suppression of ions occurs when certain analytes of interest compete for ionization efficiency with other compounds in the matrix, that are “preferably” more ionized than the less polar metabolites of interest in the complex mixture. Therefore, quantitative changes may be misrepresented as a result of matrix effects, causing either suppression (underestimation) or enhancement (overestimation) of the target analyte response [70]. In modern instruments, the ion suppression often handles better compared to an older instrument.

GC–MS is mainly used in the analysis of volatile non-polar metabolites. In GC–MS, the sample is evaporated into the gas phase and transported through a GC chromatography column. In principle, biological fluids such as plasma and urine can enter MS directly after a few pre-treatments procedures, such as protein precipitation. The GC–MS offers the extraordinary solution that allows the separation of similar structural compounds that are very difficult to isolate with HPLC. However, this technique requires that the analyte is in a volatile state and at the same time stable in high temperatures. In some cases, a chemical derivatization step, a process that increases or decreases the volatility of the compound, is carried out to make polar metabolites volatile. Some of the metabolites that are suitable for GC–MS include fatty acids, organic acids, steroids, diglycerides, sugars, and sugar alcohols [44].

For those metabolites that are not volatile and cannot be derivatized, the LC technique is a selective separation method. LC-MS can analyze a wide range of chemical species (polar and non-polar metabolites) with high selectivity and sensitivity. Similarly, capillary electrophoresis (CE) can be coupled to a mass spectrometer (MS), with the particular advantage of improving the resolution of separation [44]. Separation techniques can also be extended to two-dimensional chromatography, prior to MS, which has shown to significantly increase the detection of metabolites [71].

1.7. Targeted and untargeted approach for metabolic profiling

There are two approaches to determine the metabolic profile, targeted and non-targeted [72,73]. In the targeted approach, specific metabolites with a known identity, chemical characteristic, or biochemical property are profiled. In MS targeted metabolic profiling, this topic often involves the addition of reference compounds at various concentrations or the use of stable isotope-labelled standards to biological samples before extraction and the derivatization steps [74]. The targeted analysis is often done in a “modular” format, in which the sample is divided into aliquots that are extracted using various solvents or subjected to different derivatization steps tailored for the chemical properties of a cluster of analytes of interest. In this approach, when applied with the appropriate internal standards, it has the advantage of being a more quantitative method. A major limitation is a narrow list of analyte detected often several hundreds of metabolites belonging to 6 to

7 chemical classes. Nevertheless, targeted methods can provide a thorough assessment of the selected group of metabolites belonging to certain metabolic pathways, including elements of mitochondrial metabolism.

Untargeted metabolic profiling involves the use of NMR (less sensitive method) or MS (high sensitive) to simultaneously measure a large number of potential metabolites or analytes in biological samples [75,76]. This approach is generally used to compare two different biological or clinical conditions (case-control studies) and to report the difference between the two states and more as a hypothesis-driven approach. Often, the chemical identity for the vast majority of the peaks are not known and subsequent chemical/spectral analysis should be performed to confidently to identify molecules of interest. Although untargeted metabolic profiling can provide more true coverage of the metabolome, it is often hampered with the low number of identified compounds and many of the signals do not originate from a metabolite [77]. Untargeted analysis ideally should be carried out to generate a new hypothesis or at the early stages of studies to identify new metabolic pathways or candidates, which further need to investigated and validated by targeted methods to achieve a meaningful biological understanding of phenotypes [78]. Untargeted analysis requires long-term collaboration with a metabolomics lab and is quite a time-consuming approach to achieve results and require subsequent validations. Similar to genome-wide association studies (GWAS), no prior information about relevant metabolites is required when designing an untargeted method. On the other hand, targeted approaches, similar to disease gene relationships studies are focused on the predefined set of genes (or in this case, metabolites) and focus on a number of specific pathways. The benefits of this type of approach include higher degrees of selectivity and hence sensitivity to untargeted approaches for absolute measurements (rather than relative) of metabolites and easy of compound identification [72]. Untargeted techniques are the closer and true picture of metabolome with the caveat of a large number of unknown chemical identities. The so-called “unknowns” analytes are important in biomedical research, as many of these “unknown” features could lead to potential metabolites of interest that can be strongly associated with clinical phenotypes [79,80].

1.8. Statistical approaches and metabolite identification

There are many types of statistical methods used in the analysis of metabolomics data most common are: a) univariate and b) multivariate statistical methods. Application of univariate statistical methods such as *t*-test or two way ANOVA for analysis of high-throughput metabolomics approach has been used to reduce the number of measured analytes to a smaller subset of discriminatory metabolites [81,82]. Univariate results require multiple testing corrections such as the Bonferroni and the FDR (False Discovery Rate) corrections [83]. However, univariate methods can be less effective in discrimination between groups if the level of one/multi metabolite(s) in question is quite low [84]. There are also strong relationships between metabolite (e.g. metabolic pathways or chemical similarities) that are often neglected in such analysis.

Multivariate methods to an extent can overcome this problem. One of the most common multivariate approaches is unsupervised principal component analysis (PCA). The PCA method is a regression approach, reducing the data dimension into a new space defined by principal components. Each principal components are the linear combination of original variables and are orthogonal to one another. In the case of metabolomics data, original space is defined by metabolites and its dimension is proportional to the number of metabolites which have been detected. The PCA analysis is composed of two matrices termed the score matrix (S) and loading matrix (L). Scores plot show the position of each sample in the new coordination axis (PCs) and loadings show the weight of each variable (metabolites) in relation to the score plots [84].

The second group of clustering methods is a supervised clustering method. One of the most commonly used approaches in metabolomics is Partial least squares Discriminant Analysis (PLS-DA), a supervised method that uses information about sample class for discriminating them [85,86]. There are other supervised methods such as orthogonal PLS (OPLS), support vector machine (SVM), self-organizing map (SOM) [87,88], hierarchical cluster analysis (HCA) [89] and k-means clustering methods [90,91] which have been commonly used in the analysis of metabolomics data.

There are several online databases used for the identification and assignment of metabolomics data. Some of these databases are HMDB [92], LipidMaps [93] and Metlin [94] for a more complete list of resource see e.g. http://www.metabolomicssociety.org/database.

The Human Metabolism Database (HMDB) (http://www.hmdb.ca/) is a comprehensive and open source available to the Web, which includes detailed information on human metabolism [95]. The HMDB used to identify the complete collection of small molecules in the human body including peptides, lipids, amino acids, nucleic acids, carbohydrates, organic acids, biogenic amines, vitamins, minerals, food additives, drugs, cosmetics, contaminants, pollutants [96]. HMDB 1.0 launched with 2180 human metabolites in 2007 [97] and after that introduced new versions of HMDB 2.0 with 6408 human metabolites in 2009, and HMDB 3.0 with 40,153 metabolites in 2013 [92,97,98]. The last version of HMDB 4.0 includes 18,192 metabolic reactions that contain 114,100 metabolites with new information about the effects of drugs and SNP variations on the levels of metabolites in various biofluids [95]. LIPID MAPS (Lipid Metabolites and Pathway Strategy) structure database (http://www.lipidmaps.org/) contains more than 43,000 unique lipid structures. The LIPID MAPS Consortium is an online tool for performing tasks such as drawing and predicting lipid structures using mass spectrometry (MS) data [99]. Most recently, the use of endogenous metabolites has grown largely due to their use in the discovery of diagnostic and pharmacological biomarkers. METLIN (http://metlin.scripps.edu), a free database available which has been established to help a wide range of metabolites research and to facilitate the identification of metabolites through mass analysis [94]. METLIN was introduced as a database for identifying known metabolites and is currently expanding to identify known and unknown metabolites and other chemical compounds [100]. For a more detailed review see, Refs: [101, 102].

1.9. Effective role of metabolomics in the diagnosis and prognosis of CVD

Perturbation in heart energy metabolism plays an important role in a number of cardiovascular pathologies. In addition, the comorbidity of other conditions with CVD can change the systemic and myocardial metabolism, which often helps to worsening heart function. Myocardial energy metabolism plays an important role in regulating cardiac function, and its contribution has been acknowledged in recent decades. As the heart is the most metabolically demanding member of the body, it is not surprising that disturbances in the energy metabolism of the heart play an important role in a number of cardiovascular pathologies. Where these conditions can cause major systemic metabolic disturbances that have an enormous impact on various organs including the myocardium. However, even in the absence of obesity and diabetes, changes in the basic metabolism of multiple organs, resulting from the onset of CVD, can contribute to changes in the metabolic profile of a patient. With advances in utilizing the “omics” platforms, were are getting closer to a better understanding of the molecular, cellular and functional changes that exist in CVD, as well as prediction of how these changes may affect the intermediary metabolism [50]. The major problem in diagnosing CVDs, such as ACS, is the late appearance of symptoms in the disease. In fact, asymptomatic processes associated with the formation of plaques overtime cause tissue damage [44]. Therefore, the early diagnosis of ACS is essential for preventing rupture of atherosclerotic plaque and the formation of thrombotic clots for immediate action and reducing mortality and disability. Identifying

potential candidate biomarkers, in this case, can be helpful. It is important to note that new diagnostic methods are better based on non-invasive techniques. For precise medical care, we need to accelerate the discovery of the new specific biomarkers of disease, pharmacodynamics of the drugs, and the metabolic profile of the association with the risk factors of the disease.

1.10. Metabolomics studies in CHDs

Here we review examples of the application of metabolomics in CHDs. In one of early 2002 studies, metabolomics was used to compare the serum of 36 patients with severe CAD compared to 30 patients with normal angiography using ^{1}H NMR with the results shown that accurate diagnosis of CHD and determining its severity was possible [103]. However, some of the results were later disputed citing that many variables included in the major lipid regions of the spectra, were affected by gender and drug treatment confounding the results [104]. In another independent study, 1010 patients plasma with heart disease were analyzed using the LC/ESI/MS-MS method showing a strong association between arginine and its downstream metabolites, such as ornithine and citrulline with CAD, incidence of major adverse cardiovascular events (MACE = death, myocardial infarction, stroke) over a 3-year follow-up in subjects undergoing elective cardiac catheterization [105]. In a separate study, 35 patients with non–ST-segment elevation (NSTE) ACS, with 35 healthy subjects, were analyzed using GC–MS and LC-MS/MS analysis, identifying 68 metabolites in healthy subjects and NSTEMI patients [106]. After statistical analysis, the presence of 18 metabolites in the patient was confirmed including alanine, 2- OH butyric acid, 3- OH butyric acid, 2-keto-3-methyl valeric acid, serine, leucine, threonine, glycine, 2- OH benzoic acid, 5- OH tryptophan, palmitic acid, linoleic acid, cis-vaccine acid, lactate, alpha-valeric acid, citrate, 5-oxoproline, stearic acid. Of these, 10 metabolites include 2-OH butyric acid, 3- OH butyric acid, 2- OH benzoic acid, palmitic acid, citrate, cis-vaccenic acid, lactate, alpha-OH valeric acid, 5-oxo-proline, stearic acid in NSTEMI patients increased while 8 metabolites; alanine, 2-keto-3-methylvaleric acid, serine, leucine, threonine, glycine, 5-OH-tryptophan and linoleic acid decreased revealing a potential panel of biomarkers for early diagnosis in ACS, however, due to small size of the study further validation was required [106].

A metabolic study was conducted on 9 samples of NSTEACS and compared with 10 samples of stable atherosclerosis and 10 samples of healthy subjects using GC–MS. The values of metabolites such as citric acid, 4-hydroxyproline, aspartic acid, and fructose decreased while lactate, urea, glucose, and valine increased in NSTEACS versus healthy people [107]. Evidence suggests that lactate glucose, lipids, and long-chain fatty acids are also involved in predicting ischemia induced by exercise in CAD patients [62]. Reduction in the amount of 4-hydroxyproline was interesting because the 4-hydroxyproline turnover is thought to hinder the binding of LDL to the lipoprotein that already precipitated in the vessel wall and to prevent the release of LDL sedimentation in atherosclerotic lesions [44]. 4-Hydroxyproline is also a collagen-forming compound that causes arteriosclerotic plaque stability [44].

The metabolic profiling of peripheral blood from patients with planned myocardial infarction (PMI) was examined from 36 patients, before and at various intervals after PMI using MS technique [108]. Metabolic changes identified as the result of PMI were from the trichloroacetic acid cycle, purine catabolism, and pyrimidine and in the pentose phosphate pathways. Levels of alanine, amino-isobutyric, hypoxanthine, isoleucine/leucine, malonic acid, threonine, and trimethylamine-n-oxide acid were immediately affected by the myocardial infarction after 10 min. All of these changes were especially interesting when metabolites appeared before increasing of plasma levels of clinic biomarkers such as CKMB and troponin T. Also after 60 min, six additional metabolites were significantly affected were 1-methylhistamine, choline, inosine, serine, proline, and xanthine [63].

Metabolic changes in human arterial fibrillation (AF) were studied by NMR for quantitative analysis of 24 selected metabolites from previous studies [109]. In this study, significant differences were found between β-hydroxybutyrate, ketone and glycine amino acids in AF patients compared to the controls, which indicated the physiological role of the ketone bodies in the metabolic profiling and were able to identify more than 80% of patients at risk for AF [110]. The effects of drug treatment on apoE −/− mouse were analyzed by NMR analysis of metabolites in urine and showed that allantoin increased as a biomarker in treated mice with captopril, and also xanthine and ascorbate increased as potential biomarkers for plaque formation (both in untreated mice) [63].

Cheng et al. studied the metabolome profile of 27 patients with unstable angina (UA) and 15 healthy subjects and identified a total of 91 metabolites. In this study, ultimately 5 metabolites selected that could identify UA with the precision prediction of 80%, and including, D-ribofuranose, 1-deoxyglucose, hexadecanoic acid, octadecanoic acid, and 4 (1H)-pyridinone. The expression of the above metabolites decreases in UA. Ribofuranose is a five-carbon sugar and a common energy source in the human body and presents in the structure of the ATP molecule, which is the main constituent of energy production in the cells [111]. D-Ribofuranose is an effective compound in the treatment of fibromyalgia, a type of skeletal muscle disorder and chronic fatigue syndrome [112]. Also, its sugar unit may play an active role in the molecular mechanism of single-stranded DNA breaks induced by low-energy electrons [113]. 1-Deoxyglucose also decreased in patients with UA as well as its plasma concentration in diabetic patients [114]. In non-diabetic patients, plasma levels of 1-Deoxyglucose may be used as a marker for screening for postprandial (after a meal) high blood sugar (hyperglycemia) associated with cardiovascular risk [115].

Hexadecanoic acid is one of the most commonly found saturated fatty acids in animals and plants. This metabolite is used to produce hormone-like materials and regulates a range of functions including blood pressure, blood clotting, blood lipid levels, immune response, and inflammatory response to infection. Hexadecanoic acid is the first fatty acid produced from longer fatty acids during lipogenesis (fatty acid biosynthesis). According to the World Health Organization, there is convincing evidence that a diet containing hexadecanoic acid can increase the risk of CVD [116]. Octadecanoic acid is used to produce margarine, a variety of solid and cream fats. Although this metabolite is a saturated fatty acid, it has little effect on blood cholesterol. 4 (1H)-pyridinone is also an analgesic metabolite, which may have anti-inflammatory activity [117]. It has been shown that its effects are comparable to aspirin [118]. It was also shown that the analgesic activities of this metabolite are higher than acetylsalicylic acid and also have a high anti-inflammatory effect than indomethacin [119]. Decreasing the expression of these 5 metabolites in UA may be associated with blockage of energy and inflammatory response [111].

Zhao et al. used swine models, nine disease and eight controls as well as 27 patients with UA compared to 15 controls to understand the molecular mechanism of UA. Authors identified 21 metabolites in plasma samples of the swine model and 20 metabolites in the samples of patients with UA, which were significantly different from the controls. Among these metabolites, 8 metabolites were shared between swine specimens with myocardial infarction and patients with UA. These metabolites include 4, 1-benzene dicarboxylic acid, 5, 1-n-hydrogliocytol, 2-keto-de-gluconic acid, azelaic acid, heptanoic acid, pentanoic acid, ribitol, and serine. Authors suggested that such metabolites could be considered as a bridge between the molecular mechanism of myocardial infarction in swine model and those patients with UA. However, more studies were needed to confirm this hypothesis. Decreased plasma concentrations of 1, 5-anhydroglucitol have been confirmed in diabetic patients hence diabetic patients were excluded. A reduction (low expression levels) of 1, 5-anhydroglucitol was observed in patients with UA and the swine model with myocardial infarction. In addition to the noted metabolite, seven other metabolites

were also decreased. Therefore, it was concluded that UA may be associated with the blockage of energy and inflammatory response [120]. The same research team also found that glucose and fatty acids in patients with UA were decreased as well [111].

Sun et al, investigated on 45 patients with UA and 43 with atherosclerosis as controls [121]. This study was performed using LC-MS, which led to the identification of 16 metabolites. In patients with UA, 12 metabolites were significantly higher and 4 metabolites were lower than the atherosclerosis control group. The researchers further found that 16 metabolites could be used to distinguish accurately patients with UA from atherosclerosis. Of the 16 metabolites identified, three phospholipids, including phytosphingosine, phosphatidylcholine, and phosphatidylglycerol were significantly disrupted in the patients with UA. Phytosphingosine and phosphatidylcholine were up-regulated in the patients with UA in comparison to the atherosclerosis control group, while phosphatidylglycerol, was down-regulated in patients with UA compared to atherosclerosis control. The investigators have suggested that phospholipid metabolism is potentially disrupted in patients with UA due to increased choline levels, which is a product of phospholipid metabolism [121]. Phospholipids are an integral part of all cell membranes and are involved in various cellular processes, including cell-cell interaction, cell differentiation, cell proliferation and apoptosis [122]. Phospholipid metabolism plays an important role in the pathogenesis of metabolic syndrome and hepatic steatosis, which in turn leads to the progressive development of CVD [123], cardiovascular pathophysiology, including necrotic nucleus formation, erosion, and plaque rupture and platelet accumulation [124]. In addition, Shi et al., and Qi et al., respectively found that choline and phytosphingosine are significantly increased in ACS [125,126], which is similar to findings of Sun and colleagues. Therefore, it seems that metabolism disorders of phospholipids play an important role in the development of UA [121].

Li et al., studied urine samples from 27 patients with UA and compared with 20 healthy controls using NMR spectroscopy and identified 20 metabolites that could distinguish UA patients from the controls. These metabolites include creatinine, cis-aconitate, methylmalonate, and 3-hydroxybutyrate, amino acids such as glutamine, citrulline, tryptophan, phenylalanine, *t*-methylhistidine, lysine, and aspartate, and the other metabolism molecules TMA, choline, carnosine, hippurate, trigonelline *N*-methyl nicotinamide, and indol-3-acetate. Authors showed that by using a combination of 18 metabolites were able to predict UA. The sensitivity of the method was 92.6% with a specificity of 90% and an accuracy of 89.1%. The altered pathways were 7 including aminoacyl tRNA biosynthesis, arginine and proline metabolism, histidine metabolism, alanine metabolism, aspartate, glutamate, and phenylalanine metabolism, lysine degradation, and tryptophan metabolism, which in patients with UA were affected. [127]. Creatinine is derived from creatine and phosphocreatine with the phosphagen system being very important for cellular energy transfer and body homeostasis [128]. Under normal conditions, most creatine molecules are converted to phosphocreatine that ultimately produces ATP by a reversible enzymatic reaction. Urinary creatinine levels were decreased in patients with UA, suggesting that these patients were not able to produce ATP hence more creatine is needed to produce ATP [129]. *cis*-Aconitate is an important intermediate compound in the Krebs cycle and its reduction may lead to the breakdown of the Krebs cycle. Perturbation in energy metabolism may result from abnormal heart function and inadequate oxygen uptake in the body. Methylmalonate can eventually be converted to succinyl-CoA by the action of a series of Krebs cycle enzymes. Seok et al. found that there is an imbalance in methylmalonate metabolism in patients with ischemic heart failure. Also, changes in methylmalonate concentrations indirectly show some kind of turbulence in the Krebs cycle [130]. Glutamine is a type of glucogenic amino acid and an important fuel in gluconeogenesis. Glutamine has a central role in the tricarboxylic acid cycle and plays an important role in many metabolic pathways, especially the maintenance of homeostasis of amino acids [127].

Fan et al., conducted a comprehensive metabolomics study on 2324 patients from different groups of CAD, collected from four different centers. The study groups included patients with normal coronary artery disease, non-obstructive coronary atherosclerosis, SA, UA, and AMI. Overall, 89 metabolites were identified and altered metabolic pathways were investigated, including decreased phospholipid catabolism, increased amino acid metabolism, increased acylcarnitine with the short-chain fatty acid, reduced tricarboxylic acid cycle and decreased bile acid biosynthesis. The researchers concluded in their study that determining the metabolic profile of plasma samples is a powerful method for identifying metabolic disorders in coronary artery patients [131]. In this study, all patients with no significant CAD (type NOCA) had less than 50% of obstruction. Also, it has been shown that 63.2% of SA, 61.8% of UA, and 71.4% of AMI patients had blockage more than 70%. In contrast, only a small portion of patients with UA or AMI had a blockage of less than 50%. Compared to patients with normal coronary arteries, patients with non-obstructive coronary atherosclerosis, lysophosphatidylcholine, lysophosphatidylethanolamine, and phosphatidylethanolamine were down-regulated and phosphatidyl sphingosine was up-regulated [131].

A large portion of lysophosphatidylcholines and lysophosphatidylethanolamines in plasma are produced from phosphatidylcholines and phosphatidylethanolamines by the activity of lecithin cholesterol acyltransferase. Reduced activity of lecithin cholesterol acyltransferase has been linked to CAD [132,133]. It is known that an increase in the levels of sphingolipids is a feature of obesity and important cardiovascular risk factors [134]. The role of the gut microbiome in the development of chronic cardiovascular disease has also been addressed. For example, using non-target metabolomics, high levels of trimethylamine n-oxide (TMAO), choline, and betaine were observed in people with cardiovascular disease [78]. The gut microbiota metabolism of phosphatidylcholine produces trimethylamine, which is further metabolized to a proatherogenic species, choline, TMAO. This reduction of phosphatidylcholine has consequences for CAD [135].

Phosphatidylinositol, an intracellular Ca^{2+} activator, was up-regulated in SA. Increased phosphatidylinositol showed severe vascular calcification in patients with SA [136]. Compared to SA, creatine, 2-hydroxylauric acid, tryptophan, isobutyryl-carnitine, propionyl-carnitine, and acetyl-carnitine were up-regulated in UA while aspartic acid, phosphocholine, lysophosphatidylcholine 16:0, and lysophosphatidylcholine 18:1 were down-regulated. Increased in creatine levels are associated with a decrease in the activity of the creatine kinase system. The creatine kinase system protects the cardiovascular system against ischemia and increases contractility [137]. 2-Hydroxylicauric acid is a medium chain fatty acid that is associated with metabolic abnormalities of fatty acids during plaque rupture [138]. Tryptophan is closely interconnected with the activity of the immune system and inflammation [139]. By detecting tryptophan, the levels of inflammation and immune activity were assumed to be high in UA compared with SA [140]. The increased level of short-chain acylcarnitines explains active fatty acid metabolism in UA [140]. Reduction of aspartic acid is associated with a high risk of myocardial damage in UA [141]. Phosphocholine was decreased in most UA patients compared to the SA patients [131]. Tryptophan, arginine, and leucine had high levels in AMI patients. Activated biosynthesis of amino acids as an indicator of AMI has been reported previously [108]. The common biochemical pathways in these studies are the pathway for biosynthesis of carbohydrates, ATP synthesis, lipogenesis (fatty acid synthesis), the pathway for biosynthesis of amino acids and the tricarboxylic acid cycle. An MS-based metabolomic approach was used to study the alterations in the metabolic phenotype between 20 ACS patients and 20 non-ACS patients. Overall 23 modified metabolites were identified including 7 lysophosphatidylcholine, 2 lysophosphatidylethanol amines, 3 glycerophosphocholines, 3 sphingomyelins, carnitine, L-tryptophan, caffeine, and paraxanthin [23].

Table 1
Compilation of the metabolomics studies in the cardiovascular field, including candidate biomarkers.

Pathology	Sample	Analytical technique	Altered Metabolites	Replication	Ref.
ACS (20), Non-ACS (20)	Plasma	LC-MS	7 lysoPCs, 2 lysoPEs, 2 glycerophosphocholines, 3 SM, L-tryptophan, glucose, caffeine, paraxanthine, palmitoleic acid, indoleacrylic acid and oleic acid		[23]
ApoE receptor-deficient mice model	Urine	NMR	Allantoin, xanthine and ascorbate		[63]
1010 subjects (without CAD, patients with CAD)	Plasma	LC-MS/MS	Arginine, ornithine and citrulline		[105]
NSTEACS (35), HS (35	Plasma	GC-MC	Alanine, 2-OH-butyric acid, 3-OH-butyric acid, 2-keto-3-methylvaleric acid, serine, leucine, threonine, glycine, 2-OH-benzoic acid, 5-OH-tryptophan, palmitic acid, linoleic acid, cis-vaccenic acid, lactate, alpha-OH-valeric acid, citrate, 5-oxo-proline and stearic acid	GC–MS	[106]
ACS (9), SA (10), HS (10)	Plasma	GC–MS	Citric acid, 4-hydroxyproline, aspartic acid, and fructose, lactate, urea, glucose,		[107]
PMI (20), control (16), SMI (12), control (9)	Plasma	LC-MS	Aconitic acid, hypoxanthine, trimethylamine N-oxide, threonine		[108]
27 UA patients and 15 HC	Plasma	GC	D-ribofuranose, 1-deoxyglucose, Hexadecanoic acid, octadecanoic acid and 4(1H)-pyridinone		[111]
mini swine models with MI and patients with UA	Plasma	GC–MS	1,4-Benzenedicarboxylic acid, 1,5-Anhydroglucitol, 2-Keto-dgluconic acid, Azelaic acid, Heptanedioic acid, Pentanedioic acid, Ribitol, Serine		[120]
45 UA and 43 AS	Plasma	RRLC-QTOF/MS	Phytosphingosine, phosphatidylcholine and phosphatidylglycerol		[121]
27 urine samples from UAP patients and 20 HC	Urine	NMR	Lysine, indol-3-acetate, hippurate, and aspartate, 3-hydroxybutyrate, methylmalonate, proline, glutamine, TMA, creatinine, cis-aconitate, citrulline, histidine, choline, tryptophan, phenylalanine, *t*-methylhistidine, carnosine, *N*-methylnicotinamide, and trigonelline		[127]
UA (101), HC (132)	Serum	H NMR	Lactate, myo-inositol, lipid, VLDL, 3-hydroxy butyric acid, and LDL		[142]

NSTEACS: ACS: Acute coronary syndrome, CAD: Coronary artery disease, NSTEACS: Non–ST-segment elevation ACS, HS: Healthy subjects, SA: Stable angina, HC: healthy controls, MI: Myocardial infarction, AS: Atherosclerosis, UAP: UA pectoris, PMI: Planned myocardial infarction, SMI: Spontaneous myocardial infarction. Numbers in brackets correspond to number of assayed individuals (or animals if so specified).

A study on unstable angina pectoris (UAP) was also performed by Yao et al., which led to the identification of 18 altered metabolites. In this study, 22 patients with UAP and 22 healthy controls were used and ^{1}H NMR spectroscopy was obtained for each patient group. The results of study have been shown that lactic acid, myo-inositol, lipid, VLDL, 3-hydroxybutyric acid, TMAO, and LDL were up-regulated in UAP group in comparison to the healthy control group, while threonine, Creatine, cholesterol, phosphocholine/glycerophosphoryl choline, glutamic acid, glutamine, lysine, tricarboxylic acid, isoleucine, leucine, and valine were down-regulated in relation to the controls. So they concluded that phospholipid and amino acid metabolism were probably impaired in UA patients [142].

2. Conclusion

Metabolomics on biofluids is a powerful approach for characterizing metabolic disturbances related to various heart diseases, see Table 1. Differences in small-molecule metabolites may reflect underlying ACS mechanism progression and serve as biomarkers discovery for evaluating ACS progression, defining pathological mechanism and identifying therapeutic targets. Further investigations related to plaque development and instability and evaluation of molecular panels identified in various studies (validation of the results) are needed. Without reported acute events but high cardiovascular risk, metabolomics would help in defining specific biomarkers for disease progression and risk stratification and would cover a gap between atherosclerosis development and irreversible acute damage. In the near future, well-designed and prospective studies are needed to show how the integrated “omics” data can be translated to clinical practice to achieve greater power in diagnosis and management of CHD.

Conflicts of interest

The authors declare no conflicts of interest.

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