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Metabolomics in atherosclerosis

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ABSTRACT

It is well established that atherosclerotic cardiovascular disease (ACD) is a leading cause of death in the West. There are several predisposing factors for ACD, which can be divided into two groups: firstly modifiable risk factors, including hypertension, dyslipidaemia, type 2 diabetes mellitus, obesity, smoking and a sedentary lifestyle and secondly the unmodifiable risk factors such as age, gender and heredity. Since single biomarkers are unable to provide sufficient information about the biochemical pathways responsible for the disease, there is a need for a holistic approach technology, e.g., metabolomics, that provide sufficiently detailed information about the metabolic status and assay results will be able to guide food, drug and lifestyle optimisation. Rather than investigating a single pathway, metabolomics deals with the integrated identification of biological and pathological molecular pathways. Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the two most commonly used techniques for metabolite profiling. This detailed review concluded that metabolomics investigations seem to have great potential in identifying small groups of disturbed metabolites, which if put together should draw various metabolic routes that lead to the common track pathophysiology. The current evidence in using metabolomics in atherosclerotic cardiovascular disease is also limited, and more well-designed studies remain to be established, which might significantly improve the comprehension of atherosclerosis pathophysiology and consequently management.

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1. Introduction

It is well established that atherosclerotic cardiovascular disease (ACD) is a leading cause of death in the West [1]. It is a chronic inflammatory disorder [2] that begins in early childhood with fatty streak formation in the coronary arteries [3] and remains silent until late adulthood, when lipids start to accumulate in the intima forming atherosclerotic plaques [4]. There are several predisposing factors for ACD, which can be divided into two groups: first, modifiable risk factors, including hypertension, dyslipidaemia, type 2 diabetes mellitus, obesity, smoking and a sedentary lifestyle and, second, the unmodifiable risk factors such as age, gender and heredity [5]. Ageing is the predominant risk factor for ACD, and it renders many of the modifiable risk factors more prevalent and more severe [6]. Thus, the aetiology of atheroma formation is multifactorial with a synergistic effect of many risk factors. At present, cardiologists remain using conventional risk factors, derived from the Framingham studies [7]. Although helpful, those risk factors do not detect all instances of CAD, and indeed a significant percentage of patients with myocardial infarction has no conventional risk factors [8]. Other markers such as coronary calcification have been found to improve risk stratification but still fall a long way short of 100% prediction [8]. Conventional coronary angiography is invasive, time consuming and expensive and subjects the patient to radiation, while computed tomography coronary angiography (CTCA) also suffers from many of the same limitations.

On the other hand, single disease biomarkers are always desired to identify risk factors or people affected by a disease to evaluate progress or to monitor an intervention in treating the disease. This principle has been shown useful for, e.g., bacterial infections, where a specific molecule or group of molecules are characteristic of the disease state and largely distinctive within the matrix being sampled. The commonly investigated biomarker in ACD is cholesterol. However, serum cholesterol levels may fail to identify the exact pathway to their abnormal levels since sources of increased cholesterol levels are not only food but also endogenous biosynthesis or slow conversion to bile acids [9]. Since single biomarkers are unable to provide sufficient information about the biochemical pathways responsible for the disease, there is a need for a holistic approach technology, e.g., metabolomics, that provide sufficiently detailed information about the metabolic status and assay results will be able to guide food, drug and lifestyle optimisation [9].

The metabolome refers to the complete set of small molecule (low molecular weight (<1500 Da)) metabolites in a cell, tissue, organ or organism [10], while metabolomics is the comprehensive analysis and quantification of these small molecules based on biofluids and tissue.
analysis [11,12]. Rather than investigating a single pathway, metabolomics deal with the integrated identification of biological and pathological molecular pathways [13].

Roger William together with colleagues were the first pioneers who introduced the concept, in late 1940s, that individuals might have a metabolomics profile, which could be reflected in the structure of the biological fluids. By utilising paper chromatogram, William examined taste threshold and the excretion profiles from alcoholics and schizophrenics. He linked each of these disorder with a specific metabolite profile [14]. In 1960s and 1970s, gas chromatography and liquid chromatography further advanced the technique, which made them more available [15].

The term metabolite profile was first introduced by Hornings et al. in early 1970s [16]. They suggested that metabolite profiles may be valuable for characterising both normal and disease state. The "omics" approach includes genomics, transcriptomics, proteomics and the rapidly emerging metabolomics [17].

Mass spectrometry (MS) and nuclear magnetic resonance (NMR) spectroscopy are the two most commonly used techniques for metabolite profiling [18]. Each of these techniques has important strengths and weaknesses. For example, MS is innately more sensitive than NMR but requires a prior separation of metabolites using chromatography (liquid or gas chromatography) or capillary electrophoresis (CE) [19]. Further, the ionisation or ion suppression effect could impair the analytic quantification. The MS can, with standard techniques, detect smaller metabolites than NMR (picomolars for MS and micromolars for NMR), and those metabolites detected with NMR need to contain a hydrogen atom. By using NMR sample, recovery is non-destructive, and the sample is analysed in only one measurement while for MS only a small amount of sample is used that eventually will be destructed [20].

Both techniques can be used to characterise metabolic data in a targeted or non-targeted aspect. For the targeted approach, the investigator focuses on a limited set of metabolites of known identity. Untargeted approach deals with the investigation of as many peaks as possible, with unknown underlying identities of the species. Therefore, untargeted analyses are considered more sensitive and more likely to discover new biomarkers, while target analyses are used in biomarker validation [21].

Because of data complexity, NMR can assign definitive metabolite identities to only a subset of peaks arising from the sample. Although MS can generate sometimes thousands of peak metabolites from the biofluids, chromatographic extraction time and mass to charge ratio are often insufficient to confidently assign peak intensities [21]. The organisation of the human metabolome database (HMDB) is a comprehensive online database that gathers small molecule metabolites found within the human body. The HMDB second version, produced in 2009, was able to identify 6500 metabolites [22] and the latest version available from 2013 has significantly expanded to identify more than 40,000 metabolites [23].

During the last decade, there has been growing interest in the application of metabolomics for early disease prevention and to potentiate drug development and therapy monitoring [8,9]. In the setting of coronary atherosclerosis, metabolomics has been shown to identify a number of disordered biomarkers, some of which may be susceptible to modification. The development of a set of metabolites which could aid prediction of ACD would certainly be welcomed. Furthermore, it is possible that urine or salivary samples may be used for metabolic analysis, which even avoids the need for phlebotomy. In this review, we will discuss the latest metabolomic approaches within the field of ACD and its related risk factors.

2. Atherosclerosis

2.1. Animal studies

Lyso-phosphatidylcholine (LPC) is a class of phospholipids that are intermediates in the metabolism of lipids. LPC is generated by an enzyme found on oxidised LDL called lipoprotein-associated phospholipase A2 from phosphatidylcholine [24]. Increased levels of this enzyme have been associated with higher risk of developing ACD [25]. LPC is involved with early pro-inflammatory events occurring in the initial state of a plaque formation by increasing adhesion of monocytes on endothelial cells, as well as activating inflammatory genes (NF-KB) during fatty streak development. This leads to the activation of apoptosis, necrosis and formation of cholesterol crystals which causes plaque vulnerability [26]. Clish et al. [27] found that using highly performing liquid chromatography–mass spectrometry (HPLC-MS), lyso-PC was elevated in the liver of the apolipoprotein E3 (Apo E3) Leiden transgenic mouse (susceptible to development of ACD) in contrast to controls.

In another study by Kleemann et al. [28], ApoE Leiden mice were fed a low vs. high cholesterol diet. The amount of dietary cholesterol positively correlated with development of atherosclerosis. With increasing dietary cholesterol intake, the liver switched from a mainly resilient to a predominantly inflammatory state, which is associated with early lesion formation. The high cholesterol evoked changes involving specific transcriptional master regulators, some of which are established, others newly identified, several of these regulators control both lipid metabolism and inflammation and thereby link the two processes. The liver and plasma were analysed with HPLC/MC identified disturbance in di-and triglycerides, phosphatidylcholines, lysophosphatidylcholines and cholesterol esters.

The effect of different diets on atheroma formation has been also studied. Martin et al. investigated the effect of several diets fed to hyperlipidaemic hamsters and observed that aortic cholesteryl ester, assessed by NMR spectroscopy, was an early accumulator in atherogenic plaques. The lowest atherogenicity was obtained with the plant-oil cheese diet, followed by the dairy fat cheese diet, while the greatest atherogenicity was observed with the butter diet. Aortic cholesteryl ester was positively correlated with very low density lipoprotein (VLDL), cholesterol and N-acetylglycoproteins and negatively correlated with trimethylamine-N-oxide (TMAO) and albumin lysyl [29].

Likewise, Jove et al. observed that a high-fat diet caused an increase in ceramide and docosahexaenoic acid (DHA) in plasma and a tissue sample from the aorta. Free cholesterol in the aorta was positively correlated with taurocholic acid, suggesting that it could be a biomarker for early atherogenesis [30]. Furthermore, a high-fat cholesterol cholate (HFFC) diet has been shown to alter plasma and urinary metabolism in LDL-receptor-deficient mice using H-NMR spectroscopy. The HFFC diet caused a significant perturbation in choline metabolism, notably the choline oxidation pathway a significant reduction in the urinary excretion of taurine, betaine and dimethylglycine [31].

In addition to these studies, recent ones showed that not only diet and liver have a role in the development of atherosclerosis but also gut floras have demonstrated great importance. It has been suggested that microbiome, i.e., the collective genomes of the microorganisms that reside in the gut, can increase cardiovascular risk either via metabolism of l-carnitine [32] or phosphatidylcholine (Wang at al. 2011). Mice were given either a control diet or a diet rich in choline. The gut microflora was suppressed in half of the mice given broad-spectrum antibiotics [33]. Upon analyzing the plasma, TMAO was suppressed to a non-detectable level. The development of the aortic root lesion was increased 3-fold in mice not treated with antibiotics, and hence with preserved gut microflora, and in those fed a choline-enhanced diet. Thus, Wang et al. supposed that dietary supplements of choline, TMAO and betaine (which are all metabolites of phosphatidylcholine) could enhance the development of atherosclerosis. Furthermore, it was proposed that these metabolites are involved in the upregulation of scavenger receptors on macrophages hence the formation of foam cells.

In another study, Stöhrl et al. [34] also linked atherosclerosis to the metabolism of carnitine. By using a targeted metabolomic approach, the group studied the plasma of genetically modified mice susceptible to atherosclerosis. Interestingly, they observed a decrease in the blood concentration of free carnitine, acetylcarcintine and glutarylcarcintine/3-hydroxy-hexanoylcarnitine.
2.2. Human studies

Brindle et al. [35] were the first to investigate the use of metabolomics for the diagnosis of ACD. They could not only confirm the presence of ACD but also distinguish its severity by using H-NMR spectroscopy on human sera. A supervised partial least squares discriminant analysis to orthogonal signal-corrected data sets allowed >90% of subjects with stenosis of all three major coronary vessels to be distinguished from subjects with angiographically normal coronary arteries, with a specificity of >90%. In general, the regression coefficients, or loadings, most influential for the triple vessel disease (TVD) samples lie around ρ0.86 (due mainly to CH3 groups from fatty acid side chains in lipids, in particular, LDL and VLDL) and ρ1.26, 1.3 and 1.34 (due mainly to (CH2)n groups from fatty acid side chains in lipids, in particular VLDL and LDL). The loadings most influential for the normal coronary artery (NCA) samples lie around ρ1.22 (due mainly to (CH2)n groups from fatty acid side chains in lipids, in particular, HDL) and ρ3.22 (due to choline-N(CH3)3 + ).

A lipidomic approach was used by Sun et al. to study the plasma metabolome of patients with unstable angina and atherosclerosis controls and suggested that 16 potential biomarkers could aid in diagnosis of unstable angina. Phytosphingosine and phosphorylcholine were elevated, and phosphatidylglycerol was reduced relative to the atherosclerosis control [36].

Similarly, Stübiger et al. [37] used targeted lipidomics to study the plasma in young patients with familial hyperlipidemia that are at higher risk to develop ACD. Significant alteration of sphingomyelin (SM)/phosphatidylcholine (PC) and phosphorylcholine (PC)/lyso-phosphatidylcholine (LPC) and positive correlation of SM with LDL-C and LPC with VLDL-C were found in the familiar hyperlipidaemic group in contrast to normolipidaemics. Further, a positive correlation of oxidised PC with IMT and HDL-C but negative correlation with oxidised LDL was observed.

Using quantitative MS-based metabolic profiling in 117 Caucasians with strong family history of premature ACD, Shah et al. [38] showed significantly increased ketone bodies (β-hydroxybutyrate), several amino acids, e.g., glutamate and glutamine, free fatty acids (arachidonic acid) and most notably acylcarnitine. Acylcarnitine facilitates the entry of long-chain fatty acids into the mitochondrion via the carnitine shuttle, which is critical for its use by the myocardium for β-oxidation. Where there are fatty acid oxidation defects, acylcarnitine species accumulate and are released into the circulation. In a follow-up study, the authors distinguished the metabolic profile of 314 ACD patients from controls based on differences in branched-chain amino acids and acylcarnitines between patients and controls. Interestingly, the dicarboxylicarnitine was associated with the incidence of CV events [39].

Comparative use of techniques has also been tested by Teul et al. [40], who investigated the plasma of 9 patients with stable carotid atherosclerosis and 10 healthy individuals. They showed that a combination of both gas chromatography–mass spectrometry (GC-MS) and NMR resulted in a broader collection of derived metabolites than using either method alone. They also reported alteration of many metabolomics pathways, such as amino acid metabolism, decrease in metabolites of Krebs cycle and pyruvate and an elevation of ketone bodies and 2,3,4 trihydroxybutyrate (THD). Most of the changes can be associated with alterations of the metabolism characteristics of insulin resistance that can be strongly related to the metabolic syndrome.

To investigate the effect of age on the progression of ACD, Rizza et al. [41] using MS were able to profile 49 metabolites in elderly with a high rate of previous ACD. They suggested that the metabolic profile in elderly was associated with mitochondrial dysfunction and damage and those specific metabolites could aid in the prediction of major CV events.

Zheng et al. [42] investigated the variability on human serum metabolic profile in communities at risk of atherosclerosis. They calculated intraclass correlation coefficients (ICC) for 178 metabolites detected by untargeted method in 60 patients. They observed that pathways of lipid and amino acid metabolism had a relatively high ICCs and that metabolites in carbohydrate pathway showed relatively low ICCs.

2.3. Lipidomics and atherosclerosis

The predictive value of metabolomics has also been studied. Studies of lipid profiles indicate that individuals with smaller particle LDL have a greater CV risk compared to those with larger particle LDL. McMahan et al. [4] studied the LDL subclasses using H-NMR spectroscopy with carotid intimal-medial thickness (IMT) detected by ultrasound and found that both forms of LDL were significantly associated with IMT, but the large and small LDL particles were inversely correlated. Furthermore, Shah et al. [43], using MS in over 2000 patients undergoing cardiac catheterisation, identified five metabolites associated with a higher mortality, namely, the medium chain acylcarnitines, short- and long-chain dicarboxylicarnitines, branched-chain amino acids and fatty acids.

In 4309 healthy sera of individuals, Wurtz et al. observed using NMR that increasing concentrations of VLDL, intermediate-density lipoprotein (IDL) and LDL subclasses and low concentrations of HDL were associated with phenotypes that were at the greatest risk for atherosclerosis, findings that mirror biochemistry [44]. Likewise, the urine profile of 4630 patients from four populations groups (Japan, China, UK, USA) showed that alanine correlated positively while hippurate correlated inversely with blood pressure [45]. The same technique has been shown to differentiate between high- versus low-risk individuals based on conventional atherosclerosis risk factors (cholesterol, triglycerides, LDL and HDL). Low-risk individuals had high 3-hydroxybutyrate and low levels of threonine, whereas higher risk individuals were associated with low level of a ketoglutarate and dimethylglycine [46]. The HDL in human plasma has recently been shown to play a central role in atheroprotection.

Lipidomic approach revealed that the abundance of PC, LPC PS and PA was elevated in small, dense in contrast to large, light HDL; the inverse occurred for sphingomyelin and ceramide. Interestingly, several components of HDL were strongly correlated with antioxidative, antithrombotic, anti-inflammatory and antiapoptotic activity [47]. Intraplaque lipids have also been analysed by Stegemann et al. [48], who showed a higher concentration of polyunsaturated cholesteryl esters with long-chain fatty acids and certain sphingomyelin.

The effect of inducible myocardial ischaemia was also tested in 36 patients using MS, and 23 metabolites were significantly altered in the ischemic group but not in controls, while six metabolites were related to the Krebs cycle, including citric acid with a high degree of accuracy. Oxaloacetate and citruline were significantly reduced in the ischemic group and correlated with the severity of ischemia [49].

2.4. Proteomics and atherosclerosis

Proteomics have also been studied in individuals and patients with atherosclerosis. In 359 urine samples from individuals with severe ACD using capillary electrophoresis coupled to ESI-TOF-MS, Zimmerli et al. [50] identified 15 metabolites, which discriminated between patients and healthy controls with a 98% sensitivity and 83% specificity as well as with the level of exercise after coronary intervention. Subsequently, the same authors reported 238 discriminatory biomarkers in another sample of ACD patients with a sensitivity of 79% and specificity of 88%. Among these markers were fragments of α1-antitrypsin, collagen type 1 and 3, grain-like neuroendocrine peptide precursors, membrane-associated progesterone receptor component 11, sodium potassium ATPase gamma chain and fibrinogen alpha chain [51].

Early proteomic markers of ACD have also been studied by Delles et al. using (CE-MS) in ApoE-deficient mice fed a high-fat diet compared to a low fat diet. Polypeptide fragments of alpha1-antitrypsin, epidermal-like growth factor and collagen allowed identification of atherosclerosis with a sensitivity of 90% and 100% specificity. Furthermore, using immunohistochemistry, α1-antitrypsin, EGF and collagen type I were shown to be
highly expressed in atherosclerotic plaques [52]. As well as the coronary artery, metabolomics studies have also been carried out on the aorta. Mayr et al. [53] using NMR spectroscopy analysed progressive aortic atherosclerotic lesions of Apo E(−/−) mice and reported fibrinogen, transferrin, haemopexin and immunoglobulins. ApoA1 (a component of HDL) and reduced 1-Cys-peroxiredoxin, an antioxidant, were significantly decreased, while the antioxidant 1-Cys-peroxiredoxin was elevated.

In addition to urine and tissue samples, proteomic approaches showed great promise in plasma analysis. Donahue et al. [54] in an attempt to determine the plasma proteome of 53 patients with angiographically diagnosed ACD and controls, which were analysed by LC-MS. As many as 95 proteins (involved with immune defense, inflammation, growth and coagulation) were discerned in patients vs controls. Even in patients with acute coronary syndrome, Dardé et al. [55] identified similar altered proteins at day 0, 4, 60 and 180 using 2D gel electrophoresis.

2.5. Association of metabolites with atherosclerosis risk factors

Spijkers et al. studied the plasma metabolome and isolated arterial tissue from spontaneous hypertensive rats (SPR) and Wistar–Kyoto (WKY) normotensive rats and found altered sphingolipid metabolism in the former group. They also observed endothelium-dependent contraction in the arteries of SHR but not in WKY upon administration of a sphingosine kinase inhibitor (SK1) or sphingomyelinase. These contractions were mediated by ceramide, which was elevated in the plasma and correlated with severity of hypertension [56]. Ceramide is known to cause apoptosis [57] and vascular dysfunction [58].

In humans, plasma lipidomics were studied in 19 hypertensive and 51 normotensive males. Graessler et al. [59] observed a significant reduction of ether phosphatidylinolines and ether phosphatidylethanolamides and suggested that these ether lipids could contribute to development of hypertension. More specifically those reduced ether lipids constituted arachidonic acid. In addition, among the obese subjects, there was a significantly increased level of saturated triacylglycerides (TAG) and diacylglycerol (DAG).

Furthermore, testing of Mexican Americans at risk of dyslipidaemia and insulin resistance demonstrated that higher systolic blood pressure was significantly associated with DAG and that the diastolic blood pressure was associated with elevated levels of monohexosylceramide, phosphatidylcholine and DAG [60]. Considering that DAG acts on TRPC6 channel mediating vasoconstriction [61], it was proposed as a potential marker for hypertension. Also, a comparative study between African Americans and Caucasians showed significant reduction in palmitic, oleic, palmitoleic, arachidonic and linoleic free fatty acids in the latter compared to the former [62].

In a recent study, Zheng et al. [63] investigated the serum of patients with incident hypertension. Upon using gas chromatography–mass spectrometry and liquid chromatography, they found a significant association between six steroids and the risk of incident hypertension (highest versus lowest quintile hazard ratio, 1.72; 95% confidence interval, 1.05–2.82; P for trend, 0.03), in both men and women.

3. Conclusion

The current state of understanding atherosclerosis pathophysiology and its relationship to risk factors is not entirely comprehensive neither satisfactory, particularly in various groups of patients who do not fit within the one big box. Metabolomics investigations seem to have great potential in identifying small groups of disturbed metabolites, which if put together should draw various metabolic roots that lead to the common track pathophysiology. Nevertheless, this association does not necessarily imply that these disturbed metabolites have caused the atherosclerosis; other potential explanations include the atherosclerosis causing the disturbed metabolites or that the metabolites are simply markers for the disease. The current evidence in using metabolomics in atherosclerotic cardiovascular disease is limited and more well designed studies remain to be established which might significantly improve our comprehension of the disease process and particularly before investigation of pharmaceutical agents.

Conflicts of interest

The authors report no relationships that could be construed as a conflict of interest.

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