Research Article

Plasma metabolomics reveals biomarkers of the atherosclerosis

Atherosclerotic cardiovascular disease remains the leading cause of morbidity and mortality in industrialized societies. The lack of metabolite biomarkers has impeded the clinical diagnosis of atherosclerosis so far. In this study, stable atherosclerosis patients (n = 16) and age- and sex-matched non-atherosclerosis healthy subjects (n = 28) were recruited from the local community (Harbin, P. R. China). The plasma was collected from each study subject and was subjected to metabolomics analysis by GC/MS. Pattern recognition analyses (principal components analysis, orthogonal partial least-squares discriminate analysis, and hierarchical clustering analysis) commonly demonstrated plasma metabolome, which was significantly different from atherosclerotic and non-atherosclerotic subjects. The development of atherosclerosis-induced metabolic perturbations of fatty acids, such as palmitate, stearate, and 1-monolinoleoylglycerol, was confirmed consistent with previous publication, showing that palmitate significantly contributes to atherosclerosis development via targeting apoptosis and inflammation pathways. Altogether, this study demonstrated that the development of atherosclerosis directly perturbed fatty acid metabolism, especially that of palmitate, which was confirmed as a phenotypic biomarker for clinical diagnosis of atherosclerosis.

Keywords: Atherosclerosis / Biomarkers / GC/MS / Plasma metabolomics / Palmitate

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1 Introduction

Atherosclerotic cardiovascular disease remains the leading cause of morbidity and mortality in industrialized societies [1]. Atherosclerosis (also known as arteriosclerotic vascular disease) is a condition characterized by thickening of the arterial wall resulting from build-up of fatty materials such as cholesterol. It is a syndrome affecting arterial blood vessels, a chronic inflammatory response in the walls of arteries, in large part due to the accumulation of macrophage white blood cells, promoted by low-density lipoproteins. The increased vascular wall thickness in atherosclerosis is also due to the accumulation of the cells and extracellular matrix between the endothelium and the smooth muscle cell wall [2]. Epidemiological evidence reveals a cause and relationship between chronic inflammation and atherosclerosis [3]. Microvascular barrier dysfunction is implicated in the initiation and progress of inflammation in atherosclerosis [4], leading to chronic infection and inflammation associated with the development of atherosclerosis [5].

An important connection between inflammation and disease may be attributed to the secretion of highly reactive oxygen and nitrogen species by macrophages and neutrophils, including hypohalous acids, nitrous anhydride, and nitrosoperoxycarbonate [4].

The lipid fraction of blood plasma has a profound effect on the development of cardiovascular disease, an evidence that the aqueous metabolites in vivo also have a role in following the effects of myocardial infarction and monitoring the development of atherosclerosis (reviewed by Waterman et al. [6]).

Metabolites reveal the dynamic processes underlying cellular homeostasis. Recent advances in analytical chemistry and molecular biology have set the stage for metabolite profiling to help us understand complex molecular processes and physiology [7]. Metabolomics is the comparative analysis of metabolite flux and how it relates to biological pathways and physiology [7]. Metabolomics is the comparative analysis of metabolite flux and how it relates to biological phenotypes [7]. As an intermediate phenotype, metabolite signatures capture a unique aspect of cellular dynamics that is not typically interrogated, providing a distinct perspective on cellular homeostasis [7].
Atherosclerosis is a complicated and multifactorial disease, induced not only by genotype, but also, even more importantly, by environmental factors. The study on the metabolic perturbation of endogenous compounds may offer a deeper insight into the development of atherosclerosis [8]. As for this point, the metabolomics provides the promise to improve our understanding of the complex process of atherosclerosis development [9]. A body of data demonstrated that metabolomics technology was applied to study metabolites profiling modification and phenotype development of atherosclerosis-based human and animal models. The analytical techniques adopted from mass spectrometry (LC/MS or GC/MS) to NMR, and main plasma or urine were applied in these studies. Further, these studies revealed the metabolic perturbations of phenylalanine, tryptophan, bile acids, amino acids, fatty acids, etc. involved in the development and progress of atherosclerosis [10–12]. However, these studies mostly focused on clarifying variations in the metabolites pool and the fingerprint of urine and plasma, to phenotype metabolic syndrome during the development of atherosclerosis. Low specific attention was paid to the discovery of metabolite biomarkers used for the clinical diagnosis of atherosclerosis.

The application of metabolomics in the discovery of diagnostic disease-specific biomarkers has made some progress [13–15]. The main purpose of this study was to discover specific biomarkers for use in the clinical diagnosis of atherosclerosis. We adopted a GC/MS-based approach of analyzing the plasma metabolites from healthy subjects and patients with stable atherosclerosis.

2 Materials and methods

2.1 Chemicals and reagents

Acetonitrile (HPLC grade) was purchased from Sigma (USA); methoxyamine hydrochloride and triethylamine (in heptanes) were purchased from Sigma. High-purity water (LC-MS grade) was purchased from Fisher Scientific (USA).

2.2 Study subjects and sampling

Stable atherosclerosis patients (n = 16) and age- and sex-matched non-atherosclerosis healthy subjects (n = 28) were recruited from the local community (Harbin, P. R. China). Subjects provided written informed consent approved by the Clinic Institutional Review of the Heilongjiang University of Chinese Medicine Board and in accordance with the Declaration of Helsinki. All study subjects were screened with detailed medical history, physical examination, and hematological and biochemical profile. A standard dinner was given to each study subjects throughout seven days before sampling blood. Five milliliters of arterialized venous blood was obtained from a catheterized hand vein before breakfast on the eighth day. Plasma was isolated and kept in liquid nitrogen until GC/MS analysis.

2.3 Sample preparation

In this study, plasma samples were prepared by using a simple protocol recorded in [16]. Briefly, the thawed plasma samples were vortex-mixed with acetonitrile. The supernatant was separated and dried out under a stream of N2 gas. Finally, the precipitation was derivatized by methoximation and trimethylsilylation for GC/MS analysis.

2.4 Plasma metabolic profiling

The GC/EI MS system consisted of an Agilent 6890/5973 GC/MS system, G1701DA workstation, NIST02 MS library, and an SE54 capillary column (30 m × 0.25 mm × 0.25 μm) (Agilent, USA). The injection port temperature was 260°C. Injection into the GC-MS system was performed in the pulsed splitless mode with a pulse pressure of 207 kPa and a pulse time of 1.5 min. The GC oven was programmed with an initial temperature of 120°C, which was held for 2 min and then increased to 270°C at 5°C/min and held at the final temperature for 2 min. High-purity helium (99.999%) was used for the carrier gas at a flow rate of 1 mL/min. The GC-MS interface temperature was set at 280°C. The EI source temperature was set at 230°C. The energy was 70 eV. The split ratio was 1:20. The selected mass range was 30–650 Da and the selected scan speed was 3.2 scans per second. The pretreated plasma samples were profiled based on the above GC/MS method. Identification of GC/MS detected peaks was performed by comparing their mass spectra and retention index in the NIST02 MS library. For overlapping peaks, an AMDIS of deconvolution software was adopted to identify all the peaks in the chromatogram before NIST02 MS library searching [17].

2.5 Data processing

By metabolic profiling analysis of Section 2.4, the raw data of TIC metabolic profiling of the plasma samples from the healthy subjects and the patients with stable atherosclerosis will be achieved. The Agilent Mass Hunter Workstation software (Demo Version) (Agilent) was used to generate a 3D data matrix constructed with the sample ID as observations, the metabolites ID as the response variables, and peak area of metabolites as abundance. Then, the 3D data matrix was imported into Ezinfo software incorporated in MakerLynxV4.1 SCNS714 for pattern recognition analysis (Waters Company, USA). By pattern recognition analysis of raw data, the metabolic profiling modification will be clarified. In this study, we adopted a pattern recognition analysis that included principal components analysis (PCA), orthogonal partial least-squares discriminate analysis.
(OPLS-DA) and hierarchical clustering analysis (HCA) [13, 18–21].

3 Results and discussion

Efforts to reduce cardiovascular events in “high-risk” individuals or recurrent events in patients with established atherosclerotic cardiovascular disease emphasize broad-based implementation of guideline-directed therapeutic lifestyle changes and pharmacotherapy. Despite successful implementation of these evidence-based strategies, many individuals are not properly identified before their first event or they continue to experience cardiovascular events despite “optimal” levels of biomarkers [9]. The key cause is the lack of clinical metabolite biomarkers that could be used for the clinical diagnosis of atherosclerosis. In this study, we mainly focused on discovering a plasma biomarker for atherosclerosis, which can be validated to distinguish patients and healthy subjects using a GC/MS-based metabolomics analysis.

The typical MS TIC plots of the plasma samples are shown in Fig. 1, which only indicated some macro-difference of the fingerprint regions between the healthy subjects and the patients with stable atherosclerosis. The direct observation of the metabolic profiling of TIC MS does not characterize and clarify the subtle and essential metabolic modifications of small molecular metabolites in plasma.

Figure 1. Typical MS TIC plots of plasma analysis from (A) healthy subject and (B) patient with atherosclerosis.
between the healthy subjects and the patients with stable atherosclerosis.

These complex and subtle modifications could significantly contribute to profound biochemical interpretation of the pathophysiological events in biological body [13, 15]. This subtle and profound metabolome modification can only be achieved by the pattern recognition analysis of raw data of metabolic profiles. This is achieved by importing raw data or pre-processed data into pattern recognition related software.

In this study, a total of 240 metabolites were detected and profiled by analysis of plasma samples of healthy subjects and patients with stable atherosclerosis using the Agilent Mass Hunter Workstation Software (Fig. 2), the mass range of most of the metabolites is below 200 Da, and almost concentrated, distributed within 30 min of running time. Meanwhile, Agilent Mass Hunter Workstation Software constructed the 3D data matrix with the sample IDs as observations, the metabolites ID as the response variables, and the peak area of metabolites as abundance. Then, the Ezinfo software-based PCA was performed based on this 3D data matrix. Obvious groups classification was characterized by scores plot resulting from PCA, suggesting that the plasma metabolites profiles of the patients with atherosclerosis were significantly different from those of the healthy subjects. These are in accordance with the previous studies [10–12], which confirm that the development of atherosclerosis results in the obvious metabolic modification of metabolites pool in human plasma due to modifications and perturbations of related metabolic pathways.

The scores plot resulting from OPLS-DA and the hierarchical clustering plot furthermore confirmed this result (Fig. 3B and C). The similarity value is only 0.10 in HCA analysis; two separated clustering groups were validated, and except few overlaid samples, suggesting that the highly significant difference of plasma metabolomes between the healthy subjects and the patients with stable atherosclerosis can be attributed to effect of atherosclerosis on metabolites pool of human body [10–12].

By observing S-plot resulting from OPLS-DA, three metabolites were found that contributed the most, toward groups’ classification of plasma metabolome of healthy subjects and patients with stable atherosclerosis, which was characterized by the score plots resulting from PCA and OPLS-DA. The chemical structure of these three metabolites was identified by combining targeted peak deconvolution of AMDIS software and NIST02 MS library searching as palmitate, stearate, and 1-monolinoleoylglycerol (Fig. 4B–D), which are all fatty acids. The palmitate and stearate are long-chain saturated fatty acids (SFAs). It has been shown earlier that the presence of the long-chain SFAs in parenteral formulations may have harmful effects on the vascular system [22].

Inflammation and insulin resistance associated with visceral obesity are important risk factors for the development of atherosclerosis, and the metabolic syndrome [23]. The dyslipidemia of insulin resistance, with high levels of albumin-bound fatty acids, is a strong cardiovascular disease risk [24]. However, overproduction of plasma fatty acid is one of the main causes inducing inflammation and insulin resistance [25–27]. The urokinase plasminogen activator system with its receptor uPAR contributes to the migratory potential of macrophages; a key event in atherosclerosis; the free fatty acid (FFA) is proved to differentially stimulate the uPAR expression in human monocytes/macrophages [28]. Especially, the long-chain SFA induce proinflammatory cytokines in human macrophages via pathways involving de novo ceramide synthesis, contributing to the activation of macrophages in atherosclerotic plaques [29].

Our results from this study revealed that the metabolic disorder of fatty acids is the most obvious phenotype in the development of atherosclerosis indicating that fatty acids may be used as biomarkers for clinical diagnosis of atherosclerosis.

Palmitate is the most abundant fatty acid in vivo, accounting for approximately 26% of the total plasma fatty acids [30]. Palmitate can induce apoptosis via a p38 MAPK-dependent pathway and a reduction in IB in hCAECs independent of p38 MAPK activity [31]. Though apoptosis is directly involved in the development and progression of atherosclerotic lesions, the protein kinase C signalling is of importance in atherosclerosis as well as apoptosis. Protein kinase C iota is activated by saturated fatty acids and mediates lipid-induced apoptosis of HCAEC [31]. This suggests that palmitate induces apoptosis and inflammation in hCAECs via distinctly different mechanisms and p38 MAPK may have exerted a key role in the FFA-induced coronary endothelial injury and atherosclerosis [31]. Because inflammatory cytokines are the potent activators of p38 MAPK, which plays a very important role in modulating inflammation, most likely p38 MAPK is also involved in the FFA-mediated inflammation in the vascular endothelium [31]. In this study, our results showed that palmitate was markedly increased by 8-fold in the plasma of patients with

![Figure 2](https://example.com/figure2.png)  
**Figure 2.** Coefficient plot of retention time, mass and abundance of peak area, and distribution. This plot can phenotype the number of metabolites after deconvolution of peaks.
stable atherosclerosis, when compared to the healthy subjects (Fig. 5). This suggests that the development of atherosclerosis can induce overproduction of palmitate. The palmitate accelerates atherosclerosis development by inducing apoptosis and inflammation via special p38 MAPK pathways.

Stearate is also a type of saturated fatty acid (C18:0), the structure of which is very similar to palmitate (C16:0). Stearate’s proinflammatory response was confirmed by the phosphorylation of IkappaB-alpha and NF-kappaB in a dose-dependent manner [25], and stearate has also been shown to inhibit invasion and proliferation and induce apoptosis in various human cell types [32, 33]. In this study, stearate was significantly increased by 3-fold in plasma of patients with stable atherosclerosis compared with healthy subjects, suggesting that stearate also contributes to the development of atherosclerosis via inducing apoptosis and inflammation pathways. However, 1-monolinoleoylglycerol is a type of unsaturated fatty acid (C18:2) with two double bonds. The expression levels were also significantly increased by 3-fold in the plasma of patients with stable atherosclerosis compared with the healthy subjects.

To date there is no data to support 1-monolinoleoylglycerol’s mechanisms of action on atherosclerosis development. Therefore, it is questionable to select 1-monolinoleoylglycerol as biomarker to diagnose atherosclerosis in clinical practice. It requires further confirmation and support from biochemical data to make such a conclusion.

In summary, we confirm that fatty acids are the most direct-targeted metabolites pool during the development of atherosclerosis. Though the published data [25, 32, 33] may support the potential effect relationship between stearate and atherosclerosis development, the proofs are obviously inferior to palmitate. The palmitate is the ideal candidate of plasma biomarker for the clinical
diagnosis of atherosclerosis [22–31], and supported by the data from our study. It also supports the concept that a single biomarker is efficient to diagnose specific diseases rather than a pool of metabolites, as previously shown sarcosine was selected to diagnose prostate cancer progression [13].
4 Concluding remarks

In this study, the biomarkers discovery-based metabolomics technology was applied to characterize plasma metabolic profiling modification during the atherosclerosis development by comparing with healthy subjects by GC/MS. Subsequently, multiple tools of pattern recognition analyses (PCA, OPLS-DA, and HCA) were introduced to characterize plasma metabolome change between the healthy subjects and the patients with atherosclerosis, which revealed that key metabolites significantly contributed to the above plasma metabolomes classification. Finally, three fatty acids: palmitate, stearate, and 1-monolinoleoylglycerol were identified as probable potential biomarkers used for the clinical diagnosis of atherosclerosis. Moreover, by carefully reviewing the previously published data, and the results from our study, palmitate was evidenced as a biomarker for the clinical diagnosis of atherosclerosis.

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The authors have declared no conflict of interest.

References


