Mini Review

Metabolomics in Alcoholic Liver Disease

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Alcoholic liver disease (ALD) is a major cause of morbidity and mortality worldwide among the people with alcohol abuse worldwide. Diagnosis of ALD might be clinically challenging as there is no reliable diagnostic "biomarker" that predict and confirm patients at the risk of ALD. As a new promising "omics" in systems biology, metabolomics provides a useful tool for discovering novel molecular signature and understanding biochemical pathways to improve disease diagnosis, prognosis and therapy. Thus, the aim of the present review is to summarize the metabolic biomarkers and metabolic pathways associated with ALD described in recent metabolomic studies.

INTRODUCTION

The sustained and excessive alcohol consumption leads to alcoholic liver disease (ALD), ranging from the simple hepatic steatosis to steatohepatitis, progressive fibrosis, end-stage cirrhosis and superimposed hepatocellular carcinoma.[1] ALD has been widely considered to be a major cause of morbidity and mortality among the people with alcohol abuse worldwide.[2,3] According to estimate by the World Health Organization, approximately 3.3 million deaths and 5.1% of the global burden of disease and injury were attributable to alcohol consumption.[3] The alcohol directly exerts toxic effects on multiple organs, especially the liver where the alcohol is primarily metabolized.[4] Hepatic steatosis, characterized by lipid accumulation inside hepatocytes, is the most common and early response to excessive alcohol drinking, which is reversible with abstinence from alcohol.[5] If alcohol consumption continues, hepatic steatosis is vulnerable to progressing to irreversible states, including hepatitis, progressive fibrosis, cirrhosis and hepatocellular carcinoma, particularly in the presence of co-factors, such as the persistent hepatitis B or C virus infections.[6,7] High level of alcohol drinking is also associated with an increased risk of malignancies, chronic pancreatitis, cardiomyopathy, chronic gastritis, diabetes type 2 and the disorders of the nervous system.[1,8] Both acute and chronic drinking delivers unique pathological consequences that affect liver injury and disease, and the molecular mechanisms by how ethanol impairs on the liver have been extensively studied, but not fully understood. Understanding these mechanisms is of significant importance for clinical treatment guidelines and therapeutic interventions.

To date, the diagnosis of ALD is largely relied on a history of habitual alcohol intake, physical signs and laboratorial abnormalities of liver injury after excluding other etiologies for chronic liver disease.[9,10] Patients often deny or minimize their alcohol abuse, and patients with early stages of ALD, such as alcoholic hepatic steatosis and hepatitis, are completely asymptomatic or have no clinical signs.[11] The diagnosis of alcohol abuse is based on self-reporting, but collection of objective data is advisable. Laboratory tests, including aminotransferase (AST) and alanine aminotransferase (ALT) have been used to diagnose ALD.[12] However, an increasing evidence has demonstrated that absolute level of the circulating aminotransferase elevation does not correlate well with the severity of ALD.[11] Thus, diagnosing ALD might be clinically challenging as there is no reliable diagnostic "biomarker" that predict and confirm patients at the risk of ALD. The definitive diagnosis of ALD is liver biopsy as "gold standard". But there are several drawbacks such as invasive with pain, costly procedure. Most importantly, liver biopsy is not a practical and efficient tool for screening ALD in large cohorts of individuals. Therefore, the novel approaches and high-throughput analysis are necessary to monitor and predict global fluctuation in multiple biochemical forms, with the purpose of demonstrate intrinsic molecular mechanisms and associated biomarkers, which can promote the advance diagnosis in the preclinical and clinical, and propose new therapeutic strategies when treatment is likely to be most effective.

Metabolomics is an emerging platform that allows to profile entire endogenous metabolites in biological systems and monitor their fluctuations related to a genetic, biological or environmental perturbation.[13] As a new promising "omics" in systems biology, metabolomics provides a useful tool for discovering novel molecular signature and understanding

biochemical pathways to improve disease diagnosis, prognosis and therapy. In the recent years, metabolomics approach has been extensively employed to offers a unique view of the metabolic phenotype and phenotypic perturbations associated with many diseases.[14-17] To date, the increasing number of studies have employed metabolomics approach in the study of ALD in animals to elucidate potential diagnostic and prognostic biomarkers of ALD. Thus, the aim of the present review is to summarize the metabolic biomarkers and metabolic pathways associated with ALD described in recent metabolomic studies.

Metabolomics Analytical Techniques: the Metabolite Hunters

Metabolomics is an emerging bioanalytical platform that is capable of characterizing and quantifying global small-molecule metabolites (defined as the metabolome) present in biological samples.[18] Endogenous metabolites are intermediates or end-products of biochemical reactions in a biological system. Thus, there is no doubt that these metabolites are present in relatively large concentration ranges and exhibit the high diversity of chemical structures.[19] The large-scale analyses of metabolites require high level and intensive analytical apparatus with great peak capability and high detection sensitivity. High-resolution mass spectrometry (MS) coupled with either liquid chromatography (LC) or gas (GC) chromatography, as well as high-resolution nuclear magnetic resonance (NMR) spectroscopy, are the primarily analytical approaches in metabolomics study of ALD. NMR is a widely used high-throughput approach that is based on the magnetic properties of the atomic nucleus (e.g., 1H, 13C, or 31P), NMR analyzes the signal caused by the behavior of molecules when their nuclei spin orientations are modified after the exposure to a magnetic field and resonance frequencies.[20,21] NMR technique, including ^{1}H-NMR and ^{31}P-NMR, were early used for biomarker discovery in ALD.[22-23] NMR lacks the sensitivity for detection of low abundance metabolites with limits of detection on the order of 10 μM or

a few nmol at high fields using new cryoprobes.[21] However, NMR is highly quantitative and reproducible, which is extremely important for multivariate statistical analysis of metabolomics data.[21] More importantly, NMR is a non-destructive analysis with minimal preparation of the samples and do not need separation or ionization steps.[21] MS is a powerful tool to investigate molecular structure according to their mass-to-charge (*m/z*) ratio for both qualitative and quantitative analysis in metabolic profiling study. Coupled with several chromatographic technologies, such as GC, LC and CE, MS-based metabolomics were significantly broadened its applicability.[24] Furthermore, many types of mass analyzers were available for interfacing with LC, the most commonly-used mass analyzers is a Quadrupole Time - of - Flight (Q-TOF) mass spectrometer in metabolomics study of ALD.[25,26] Additionally, tandem MS,[27] ion trap-TOF MS[27] and Fourier transform ion cyclotron resonance mass spectrometer (FT-ICR MS)[28] have been also employed for biomarker discovery of ALD. Compared to NMR, MS analysis is more sensitive and selective, and allow to detect the broader metabolites although the sample preparation and chromatographic separation for MS analysis is time consuming.[24] Recently, by combining the high sensitivity and specificity of MS with highly quantitative and reproducible of NMR, multi-platform analytical methodologies, have been used to hunt the more comprehensive metabolic profile with different performance characteristics in ALD.[28,29] The typical workflow of an untargeted metabolomic study is illustrated in **Figure 1**, by comparing the metabolome of multiple biological groups, such as healthy and disease states, the molecular signatures or the metabolites of interest that are significantly altered are highlighted using unsupervised and supervised multivariate statistical analysis, and further identified according to the information of their chemical structures that provided by analytical platform. These metabolites are then subjected to functional interpretation and pathway analysis through pathway analysis tools including Ingenuity Pathways Analysis and MetaboAnalyst.
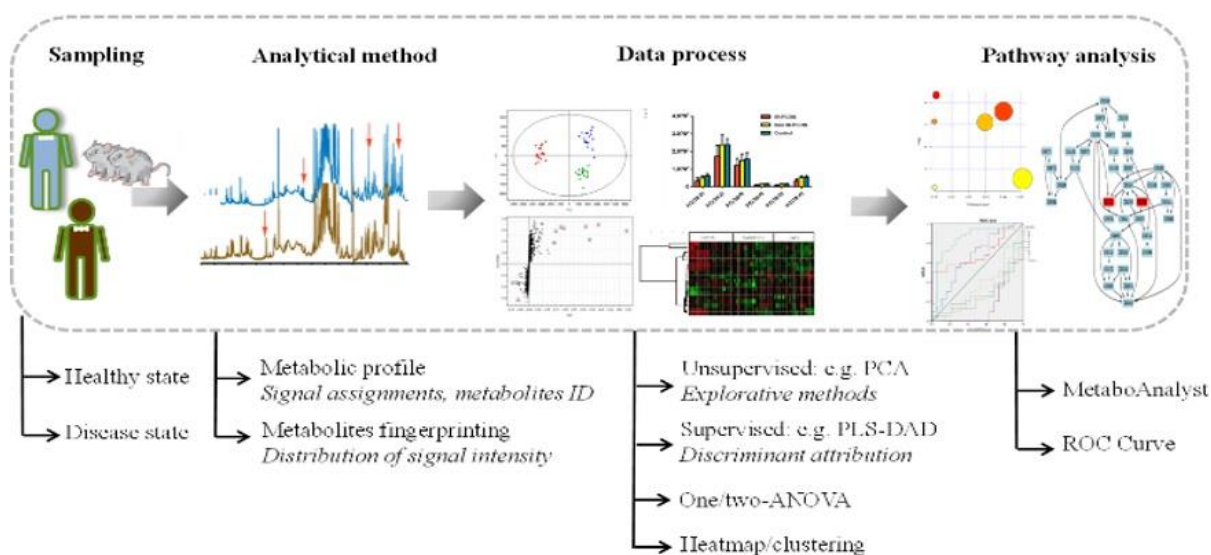

Figure 1. The Workflow of a metabolic study.

Metabolomics Study on ALD

Identification of early and unique biomarkers is beneficial to adequate screening diagnostics of ALD. Numbers of biomarker metabolites for monitoring and prediction of ALD have been reported. Metabolomics discovery of ALD biomarkers may reveal underlying mechanism and facilitate to diagnosis individuals at risk. Metabolite profiles of tissues or body fluids samples are considered as key indicators of pathological or physiological states, may support more comprehensive view of etiological pathways, and increase the possibility of identifying biomarkers of ALD. Loftus et al.[27] had investigated nonpolar metabolite profiles in liver samples both from alcohol-treated rats and mice. LC-MS was used to quantify main metabolite classes, including fatty acyls, fatty acid ethyl esters, glycerolipids, and phosphatidylethanol. Significant metabolites, such as eicosapentaenoic acid, octadecatrienoic acid, ethyl arachidonate, ethyl docosahexaenoic acid, ethyl linoleate, ethyl oleate and phosphatidylethanol homologues were all up-regulated by alcohol administration in both mouse and rat livers. It is worth noting that phosphatidylethanol homologues are regarded as potential biomarkers may use in monitoring and detecting alcoholic patients during the treatment. The study clearly showed a marked disturbance in fatty acid synthesis and glycerophospholipid metabolism in alcohol-treated mice and rats.

Lipids-related metabolites are link closely to pathogenesis and aggravation of ALD. The fluctuation of lipid metabolites in plasma and liver could perform as a signature to represent the progression of ALD. A whole comprehensive lipid metabolic map of ALD was constructed by NMR analysis.[23,30] Male Fischer 344 rats were detected and principal component analysis and cluster analysis of NMR data of lipid metabolome in ethanol-fed vs. control rats, showed a significant difference in both plasma and liver tissue. As a result, hepatic fatty acids and triglycerides were both increased by ethanol administration, while phosphatidylcholine decreased. But in plasma, both free fatty acids and phosphatidylcholine decreased. Of note, over-accumulation of lipids in liver steatosis was accompanied by mild inflammation and oxidative stress during long ethanol exposure. Results imply that liver and plasma lipid profiles may supply a potential approach for detecting early stage of ALD by analyzing the plasma lipid profile.

In a study, 1H and 31P NMR was developed for the liver tissue based dose-dependent hepatic lipid metabonomic profiling of ALD. Principal component analysis (PCA) of NMR result showed that enhanced clustering by gradual separation of alcohol dehydrogenase (ADH)-deficient (ADH−) deer mice groups from their pair-fed control groups and corresponding hepatic ADH-normal (ADH+) deer mice groups, after fed 1, 2 or 3.5% ethanol daily for 2 months. The data indicated that alcohol dose and hepatic ADH deficiencies are two major factors related to initiation and progression of ALD. It also suggested that characterization of individual lipid entities and associated metabolic pathways altered in deer mouse model after different durations of ethanol feeding could be important

to explore mechanism and identify potential biomarker candidates of early stage ALD.[31]

Urinary metabolomes were analyzed to expand understanding of the metabolic indicators of ALD pathogenesis and progression. It proposed that urinary compounds including n-acetylglutamine, n-acetylglycine, and taurine, which could be used as novel non-invasive biomarker, have a remarkable increase in the ALD models when compared with the normal. The findings suggest that alcohol exposure greatly impairs the glutathione metabolism, which might be contributed to ALD.[28] A comprehensive metabolic map of intragastric feeding model ALD was constructed by UHPLC-TOFMS analysis. A panel of metabolite markers, such as taurine, arginine, hydroxyproline, 5-oxoproline, n-Acetylglutamine, 5-hydroxy-tryptophan, 2-amino-2-deoxy-d-gluconate, revealed robust differences between profiles from control and alcohol-treated animals from both species, which indicated great disturbance in glutathione, lycerophosphocholine and tryptophan metabolisms.[32]

Identification of genetic background independent ALD biomarkers is especially crucial for improving screening and diagnosis, due to genetic background have an intimate relationship to the development and prognosis of ALD. Manna et al. had recently reported ALD-associated urinary metabolites, based on C57BL/6 (B6) and 129/SvJ (129S) Ppara-null mouse models, along with their wild-type counterparts. The observation of urinary metabolic signatures of alcohol administration in Ppara-null mice, that is, 4-hydroxyphenylacetic acid, ethylsulfate, 4-hydroxyphenylacetic acid sulfate, pimelic acid, adipic acid, xanthurenic acid, and taurine, were all dependent on genetic background, as well as indole-3-lactic acid and phenyllactic acid were remark elevated, strongly reflected the biochemical information associated with early stages pathogenesis of ALD. These metabolites provided promising candidates for pathology-specific noninvasive biomarkers for early stages of ALD.[33] Using Q-TOF-MS technology and Cyp2e1-null mouse model, Shi et al.[26] performed urinary metabolomics to attain time- and dose-dependent shifts in the chemical composition of urine. Under the biochemical analysis, it identified that N-acetyltaurine, dramatically increases in urine after ethanol consumption, is a novel metabolite of ethanol and a potential biomarker.

Considerable efforts and significant progress have been made for understanding the underlying molecular mechanisms in the development of ALD in rats and mice, but these rodent disease models are generally need more time to maintain and more expensive compared to zebrafish models. As a tractable system, zebrafish models are considered as an alternative option to study diseases mainly because of cost-effectiveness and short generation time. Of note, zebrafish also complement mammalian models of disease. A recent investigation[34] of metabolic profiling of ALD in zebrafish models, using NMR and GC/MS spectra, were identified biomarkers and elucidated underlying pathways related to ALD development

in zebrafish. Finally, a significant separation between alcoholic groups and control was observed, and a set of 11 metabolites were elevated, such as isoleucine, acetate, succinate, choline, while excretion of aspartate, citrate, glycine, tyrosine, glucose, betaine, alanine, and maltose were decreased. It demonstrates the advantages of zebra fish models provide optional insight into finding significant potential biomarkers and revealed that alcohol exposure significantly affects fatty acid metabolism and tricarboxylic (TCA) cycle.

Table 1. Characteristics of studies included in this review.

No.	Analytical methods	Models	Specimen types	Metabolite association	Pathway involved	Ref.
1	HPLC-IT-TOF-MS^n	C57BL/6J mice and Wistar rats	liver	Fatty acyls, fatty acid ethyl esters, glycerolipids, and phosphatidylethanol homologues-related metabolites	Fatty acid synthesis Glycerophospholipid metabolism	27
2	UPLC-Q-TOF-MS	Athymic BALB/c nude mice	serum	Phosphatidylcholines, lysophosphatidylcholines	Glycerophospholipid metabolism	35
3	^{1}H NMR ^{31}P NMR	Fischer rats	plasma, liver	Lipids-related metabolites	Phosphatidylcholine metabolism	30
4	UPLC-Q-TOF-MS	Ppara-null mouse	urine	Indole-3-lactic acid	Tryptophan metabolism	25
5	UPLC-Q-TOF-MS	*Cyp2e1*-null mice	serum, urine	N-acetyltaurine	N-acetyltaurine biosynthesis	26
6	^{1}H NMR ^{31}P NMR	hepatic ADH$^-$ and ADH$^+$ deer mice	liver	Lipids-related metabolites		31
7	FTICR-MS ^{1}H NMR	C57BL/6J mice	urine, liver	Taurine, n-acetylglutamine, n-acetylglycine	Glutathione metabolism	28
8	UPLC-Q-TOF-MS	C57BL/6J mice and Wistar rats	plasma, urine	Glycerophosphocholine, Taurine, Arginine, Hydroxyproline, 5-oxoproline, N-Acetylglutamine, 5-Hydroxy-tryptophan, 2-Amino-2-deoxy-d-gluconate, Xanthurenic acid, 2/4-hydroxyphenylacetic sulphate, Eicosatrienoic acid	Tryptophan metabolism, Glycerophosphocholines metabolism Glutathione metabolism	32
9	UPLC -Q-TOF-MS	C57BL/6 and 129/SvJ Ppara-null mouse	urine	indole-3-lactic acid, phenyllactic acid	Tryptophan metabolism Phenylalanine metabolism	33
10	^{1}H NMR	Sprague-Dawley rats	liver, serum, brain	Lactate, alanine, acetate, β-hydroxybutyrate, acetoacetate, betaine	Energy metabolism TCA cycle Betaine Metabolism	22
11	^{1}H NMR ^{31}P NMR	Fischer rats	plasma, liver	Lipids-related metabolites	Metabolisms of cholesterol, triglycerides and phospholipids	23
12	^{1}H NMR GC-MS	Zebrafish	liver	isoleucine, acetate, succinate, choline, creatine, acetoacetate, 3-hydroxybutyrate, ethyl glucuronide, lactate/pyruvate ratio, fatty acids, cholesterol, citrate, aspartate, tyrosine, glycine, glucose, alanine, betaine, maltose	TCA cycle Fatty acid metabolism	34

CONCLUSION

Metabolomics presents a novel approach to observe and identify the dynamic changes in complicated and multiple biochemical networks over the process of ALD. Metabolomics profiling allows establishment and identification alteration metabolites both in animal models and human samples that associated with ALD biochemical pathways, which correlate with disease severity in body liquid and tissue samples. In addition, it is getting more attractive for the clinical application. Since metabolic turbulence related to ALD develop in the early of clinical symptoms progression, metabolomics by itself or in complement with the currently available approaches for ALD diagnosis could serve as an additional tool to predict the disease progression, increase the accuracy of diagnostic, and monitor the efficacy of therapeutic intervention. However, metabolomics is so immature that could not offer reliable biomarkers indicative of metabolic alterations, compared with other omic approaches, and still needs more advance technologies, appropriate animal models, rigorous quality control standards and further validation, to achieved valuable insights about mechanisms of ALD, prognostic assessment, and diagnostic biomarkers.

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