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Review

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Application of Metabolomics to Epidemiological Studies of Atherosclerosis and Cardiovascular Disease

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Metabolomics has developed as a powerful tool for investigating the complex pathophysiology underlying atherosclerosis and cardiovascular disease. Many epidemiological studies have applied this technique to accurately and comprehensively assess the effects of environmental factors on health outcomes, which used to be a perpetual challenge. Metabolites are defined as small molecules which are intermediate products of metabolic reactions catalyzed by numerous enzymes occurring within cells. Consequent to both genetic variation and environment, they allow us to explore the gene–environment interactions and to gain a better understanding of multifactorial diseases like cardiovascular disease. This review article highlights the findings of well-known prospective cohort studies around the world that have utilized metabolomics for a wide range of purposes, including biomarker discovery, improving cardiovascular risk prediction and early disease diagnosis, and exploring detailed mechanisms of disease onset and progression. However, technical challenges still exist in applying them clinically. One limitation is due to various analytical platforms that are used based on the judgment of each study; comparative assessments among different platforms need to be conducted in order to correctly interpret and validate each data externally. Secondly, metabolite levels obtained in most high-throughput metabolomics profiling studies are often semiquantitative rather than fully quantitative concentrations, which makes it difficult to compare and combine results among different studies and to determine the levels for practical use. In 2014, the Consortium of Metabolomics Studies was developed, which is expected to take the lead in overcoming these issues.

Key words: **Metabolomics, Epidemiology, Cardiovascular disease, Atherosclerosis**

Introduction

Omics approach is expected to shed light on the "black box" mechanism of atherosclerosis (AS) and related noncommunicable diseases and their phenotypes (**Fig. 1**). Of those, metabolomics, a comprehensive characterization of small molecule metabolites, such as amino acids, organic acids, nucleic acids, and lipids, can provide an overview of the metabolic status and global pathophysiological changes associated with cellular or biological system. Introducing this high-throughput technology into epidemiologic studies is of value for understanding the consequences of gene–environment interaction and discovering novel biomarkers for early prevention and detection of AS and cardiovascular disease (CVD)[1-4]. Indeed, the American Heart Association released a statement in 2017, addressing the potential impact of metabolomics in cardiovascular health and disease and the current challenges of its clinical applications[5].

In this review, we summarize epidemiological findings of metabolic profiling studies associated with AS and CVD. Results of metabolic profiling related to major atherosclerotic risk factors are also indicated. Lastly, the methodological challenges of metabolomics in epidemiology are mentioned.

Metabolomic Profiling and Prediction of Atherosclerosis and Cardiovascular Diseases

Wurtz *et al.*[6] conducted nuclear magnetic resonance (NMR) metabolomics to three population-based cohorts to identify biomarkers for incident CVD. The National Finnish FINRISK study (n= 7,256; 800 events) was used as a discovery cohort, and the Southall And Brent REvisited (SABRE) study (n= 2,622; 573 events) and British Women's Health and Heart Study (n=3,563; 368 events) were used as replication cohorts. Thirty-three out of the 68 lipids and

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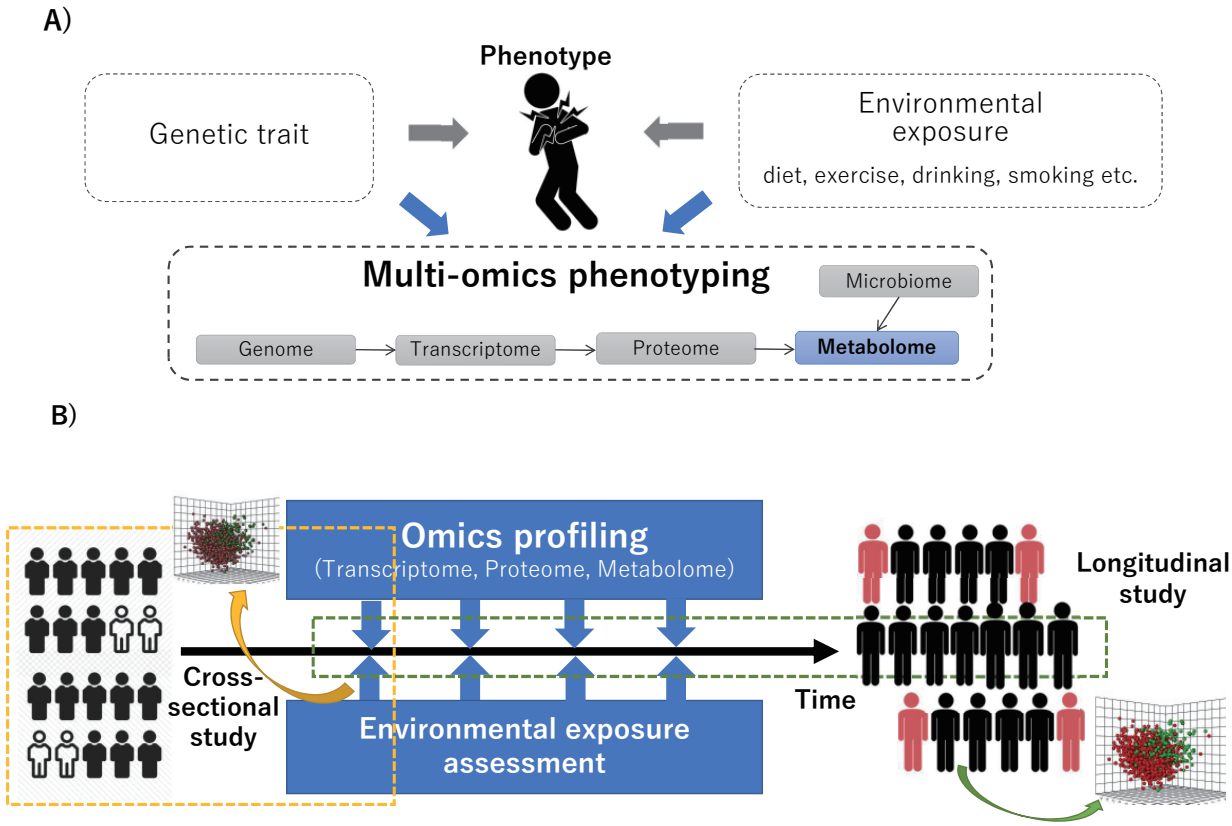

Fig. 1. Metabolome: a representation of an individual's health status (phenotype) and its application to population-based studies

(A) Among multiple omics approaches, metabolomics, the systematic study of metabolome, deals with the final downstream products of gene transcription, enabling us to identify the "black box" of mechanisms behind genetic variations, environmental factors, and health status.
(B) There are mainly cross-sectional and longitudinal studies in epidemiological research when applying metabolomics. Comparisons at a single time point (cross-sectional studies) need to be interpreted with caution because they may be severely confounded by lifestyle, socioeconomic factors, or baseline health status, even in large-scale studies. Longitudinal studies, such as prospective cohort studies, require time and are often quite expensive, but is preferred when handling health outcomes which involve aging. Also, a large number of participants are recommended for robust biomarker findings regarding complex outcomes like cardiovascular health.

metabolites were significantly associated with incident CVD during the 15-year follow-up in the FINRISK study after adjusting for age, sex, blood pressure, smoking, diabetes, and medication in addition to multiplicity adjustment of statistical comparison ($P<$ 0.0007). In the meta-analyses with all three cohorts, 4 metabolites, i.e., phenylalanine, monounsaturated fatty acid (MUFA), omega-6 fatty acids, and docosahexaenoic acid (DHA), were associated with future cardiovascular events. They indicated that cardiovascular risk prediction was improved by incorporating these 4 metabolites along with the established risk factors.

Vaarhorst *et al.*[7] conducted a case-cohort analysis with 79 cases who developed coronary heart disease (CHD; myocardial infarction, unstable angina (UA) pectoris, coronary artery bypass graft, or percutaneous transluminal coronary angioplasty) during follow-up (median 8.1 years) and 565 individuals in a randomly-selected subcohort within the Monitoring Project of Chronic Disease Risk Factors from 1993 to 1997. Metabolite score derived from NMR-based plasma profiling including lipid fractions, glucose, valine, ornithine, glutamate, creatinine, glycoproteins, citrate, and 1,5-anhydrosorbitol, was associated with the incidence of CHD independent of traditional risk factors.

While NMR is fast and inexpensive, mass spectrometry (MS) has been used for measurement platform for in-depth investigation of various metabolites. With three Swedish population-based cohorts among the elderlies (ULSAM, TwinGene, and PIVUS), Ganna *et al.*[8] performed a 10-year follow-up of 1,028 participants in the discovery cohort to examine the association between incident CHD and baseline metabolomic profiling with ultra-performance liquid chromatography (UPLC)/MS-based non-targeted metabolomics with two replication cohorts ($n=1,670$ and 970, respectively). Four plasma or serum lipid

metabolites, lysophosphatidylcholine 18:1(LPC 18:1), LPC 18:2, monoglyceride 18:2 (MG 18:2), and sphingomyelin 28:1 (SM 28:1), were associated with incident CHD independent of main cardiovascular risk factors. They not only found that these metabolites moderately improved risk reclassification beyond traditional risk factors but also suggested the association between CHD-related SNPs and some LPCs.

Another lipidomics profiling study was conducted in the Italian population-based Bruneck study to better predict future development of CVD (i.e., incident fatal and nonfatal myocardial infarction, ischemic stroke, and sudden cardiac death) with 135 lipids from eight lipid classes, phosphatidylcholine (PC), lysophosphatidylcholine (LPC), cholesterol ester (CE), sphingomyelin (SM), phosphatidylserine (PS), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), and triacylglycerol (TAG), determined by MS-based shotgun lipidomics[9]. Twenty-eight lipids were significantly associated with incident CVD with a 10-year follow-up of 702 participants. Interestingly, the authors compared these lipid species with lipids previously found to be associated with advanced atherosclerotic plaques[10], and indicated that a broad overlap was observed such as PC (38:3). Then, three lipids, TAG (54:2), CE (16:1), and PE (36:5), were determined to be significantly associated with incident CVD with a Cox proportional hazard model. Further analysis revealed that the addition of these 3 lipids to conventional risk factors (age, sex, diabetes mellitus, smoking, systolic blood pressure, total and high-density lipoprotein (HDL) cholesterol) could improve risk discrimination and classification significantly in a risk prediction model.

Some patient-based studies have also been conducted. Shah *et al.*[11] reported the result of predictivity of baseline metabolomic profiling for cardiovascular events among patients with CHD who underwent catheterization in the Measurement to Understand the Reclassification of Disease of Cabarrus and Kannapolis Cardiovascular (MURDOCK CV) study. They intended to evaluate "clinomic" profiles, an integration of molecular profiles with clinical data to better classify risks for clinical cardiovascular events in the study. Coupled with conventional analysis (low-density lipoprotein (LDL) and HDL cholesterol, triglyceride, glucose, total ketones, β-hydroxybutyrate, and total free fatty acid), 45 acylcarnitines and 15 amino acids were measured using a targeted MS-based approach with tandem MS. A total of 2,023 patients were enrolled in this analysis, and 5 of 13 metabolite factors were independently associated with mortality: medium-chain acylcarnitines, short-chain dicarboxylacylcarnitines, long-chain dicarboxylacylcarnitines, branched-chain amino acids (BCAA), and fatty acids. Of those, three lipid profiles, i.e., short-chain dicarboxylacylcarnitines, long-chain dicarboxylacylcarnitines, and fatty acids, significantly predicted fatal events independent of standard predictors.

Plasma metabolomic profiling was applied to fasting plasma samples of 45 patients with UA and 43 with AS to determine whether metabolomic profiling could discriminate UA from AS and to discover potential biomarkers of UA[12]. Patients with UA were diagnosed with newly-onset symptoms of angina worsening or occurring with minimal activity and organic stenosis ≥ 50% by angiography in at least one major coronary artery without electrocardiogram changes of ST elevation. AS was defined as luminal irregularities without lesion stenosis >20% in any major coronary artery by angiography. Using rapid resolution liquid chromatography quadrupole time-of-flight mass spectrometry (RRLC-QTOF/MS), three MGs, three phospholipids (phytosphingosine, PC, and phosphatidylglycerol), 3 amino acids and related metabolites (kynurenine, indoxyl sulfate, and p-cresol sulfate), and seven other metabolites including 18-hydroxycorticosterone and 7,8-dihydropteroic acid in plasma significantly differed between the two groups, which were suggested to be pathophysiological biomarkers for the diagnosis of UA. To assess whether metabolic profiles could improve the prediction of AS in preclinical stage, NMR spectrometry was applied to serum samples of 1,595 healthy adults aged 24–39 years from the Cardiovascular Risk in Young Finns Study[13]. As a marker of subclinical AS, intima-media thickness (IMT) was measured twice, in 2001 and 2007, on the posterior wall of the left common carotid artery. After developing a high-throughput NMR measurement platform, the authors measured serum concentrations of seven lipoprotein major lipid fractions, 14 lipoprotein subclasses, and 18 small molecules including tyrosine, histidine, and glutamine in 2001 as the baseline profile. Lipoprotein lipid fractions included total cholesterol, intermediate-density lipoprotein (IDL) cholesterol, LDL cholesterol, HDL cholesterol, total triglycerides, very low-density lipoprotein (VLDL) triglycerides, and IDL triglycerides; lipoprotein subclasses included small to extremely-large VLDLs, IDL, small to large LDLs, and small to very-large HDLs. The study indicated that the incidence of high IMT (≥ 90th percentile) or plaque over 6 years was better predicted by combining risk factors from the Framingham risk score and NMR-determined metabolites (LDL cholesterol, medium HDL, DHA, and tyrosine) than the model consisted only of the factors from the Framingham risk score (age, sex, systolic BP, smoking status, glucose, total- and HDL-

cholesterols). For amino acids, tyrosine and glutamine levels were associated with 6-year incident high IMT independent of baseline lipid measures.

Metabolomic Profiling of Major Risk Factors of AS

Major risk factors of AS, such as elevated blood pressure, dietary intake, obesity, and insulin resistance/diabetes, are also subjects of metabolomics research. Although improvements continue to be made to management guidelines and risk assessment tools for the prevention of atherosclerotic CVD[14], new strategies are in need to identify the risks prior to the appearance of current risk factors. By applying metabolomics to large-scale epidemiologic studies, key biomarkers and novel pathways are rapidly being discovered. Effects of lifestyle factors, such as physical activity and alcohol drinking, which account for primary prevention of AS on human metabolome, are also being studied.

1) Metabolic Profiling of Elevated Blood Pressure

Early work by Holmes *et al.*[15] showed that urinary metabolites are associated with blood pressure levels. They applied NMR spectrometry to 24 hour urine samples from the INTERnational study of MAcro/micronutrients and blood pressure (INTERMAP) Study (n=4,630) and indicated that 24 hour urinary excretion of alanine and hippurate, which might be linked to diet and gut microbial activities, was associated with blood pressure levels. Urinary formate was also significantly associated with blood pressure levels. Interestingly, urinary metabolite excretion patterns differed across four ethnic groups among this study population; 25 discriminatory metabolites identified from 7,100 spectra data were well differentiated in population samples from Japan, China, UK, and USA. East Asian and Western populations were first divided into two different clusters, and then sub-clusters were identified with relation to gender and living area, which may reflect the differences in diets and diet-related major risk factors. Another well-known cohort study, the AS Risk in Communities (ARIC) study, employed metabolomics to identify individual metabolites and their pattern associated with incident hypertension among their black population[16]. Three-hundred forty-four men and women developed hypertension among 896 normotensive blacks aged 45–64 during a 10-year follow-up, and 204 serum metabolites at baseline were measured by non-targeted metabolomics approach with LC-MS and gas chromatography (GC)-MS. After multiple adjustments for various risk factors, baseline 4-hydroxyhippurate, a microbial end-product derived from the fermentation of polyphenols by the microflora in the intestine[17], was associated with an 18% increased risk of hypertension. The interplay between the gut microbiome and blood pressure had also been indicated in this study. After principal component analyses, a metabolite pattern consisting of 16 metabolites, including sex steroids, was also significantly associated with the incident hypertension risk.

2) Effects of Dietary Intake on Metabolome and Health Outcomes via the Gut Microbiome

Food consumption and its impact on health outcomes is an important subject[18], but dietary assessment in large epidemiological studies remains a challenge. Various methods exist, such as dietary recalls or records, and food frequency questionnaires, but each approach has its limitations, and metabolomics is expected to provide a comprehensive picture of what individuals consume. The results of the two studies mentioned in the previous paragraph show that metabolomics can also shed light on the complex relationship between diet and health, as human metabolome includes nutrients derived from food and their related metabolites modified by enteric microbiota. The INTERMAP Study group extensively studied this aspect. In a study comparing 369 African Americans (AA) with 1,190 non-Hispanic white Americans (NHWA) aged 40–59 years[19], systolic and diastolic blood pressure levels were significantly higher in AA both for men (4.7 and 3.4 mmHg, respectively) and women (9.0 and 4.8 mmHg, respectively). The authors conducted NMR-based urinary metabolome analyses which revealed that nine metabolites (creatinine, 3-hydroxyisovalerate, N-acetyls of glycoprotein fragments, dimethylglycine, lysine, N-acetyl neuraminic acid, leucine, dimethylamine, and 2-hydroxyisobutylate) were higher in AA, and four metabolites (trimethylamine, N-methyl nicotinic acid, hippurate, and succinate) were elevated in NHWA, some of which are known as cometabolites of gut microbiome. There were various foods which significantly differed in intakes between AA and NHWA, for example, total and raw vegetables, fresh fruit, processed meats, and eggs. Multiple linear regression analysis was then performed to evaluate how adding those foods and their nutrients (such as vegetable protein, magnesium, urinary potassium, urinary sodium/potassium ratio) to the urinary metabolites as explanatory variables have any impact on blood pressure differences between AA and NHWA. As a result, the inclusion of 11 nutrients and 10 non-nutrient factors into the regression model led to significantly reduced blood pressure differences by 21%–52%. The results of this study might have displayed a part of the complex interaction between dietary intake and gut microbiota modification and its

effect on blood pressure levels.

Another metabolite of current interest in the field of CVD is trimethylamine N-oxide (TMAO). A diet-derived, gut microbial-host cometabolite and its precursor metabolites being choline, betaine, and carnitine, TMAO has been reported to be associated with increased cardiovascular and mortality risk in a wide range of populations[20, 21]. A nested case-control study within the Shanghai Women's Health Study and Shanghai Men's Health Study (n=275 incident CHD, n=275 individually matched controls) demonstrated that urinary TMAO, but not its precursors, was positively associated with the risk of developing CHD among Chinese population, with the odds ratio (OR) for the highest versus lower quartiles of TMAO being 1.91, after adjustment for traditional CHD risk factors[22]. The authors also added that this association was more pronounced among diabetic (OR=6.21) than nondiabetic individuals (OR=1.56). TMAO was strongly associated with deep-fried meat or fish. This study also highlighted not the effect of dietary intake alone, but the importance of interactions between diet, gut microbiota, and host on cardiovascular health.

3) Metabolic Profiling of Obesity and Metabolic Syndrome

A growing body of evidence has shown the intimate relationship between metabolomic profiles and obesity. Elliott *et al.*[23] explored the metabolic signatures of obesity with 24 hour urinary metabolic profiling among the western cohort members of the INTERMAP and identified that 29 and 25 urinary metabolites were associated with body mass index (BMI) in the US population (n=1,880) as a discovery set and in the United Kingdom (UK) population (n=444), respectively. Among those, nine metabolites reflecting five gut microbial co-metabolic pathways were correlated with BMI; trimethylamine, dimethylamine, and dimethylglycine were related to choline metabolism, 4-cresyl sulfate, tyrosine, and 4-hydroxymandelic acid related to tyrosine metabolism, phenylacetylglutamine related to phenylalanine metabolism, hippurate related to benzoic acid production, and 2-hydroxyisobutyrate related to N-butyrate production by the bacterium *Faecalibacterium prausnitzii*. Various metabolites reflecting the citric acid cycle intermediates, skeletal muscle turnover, mitochondrial metabolism, and BCAA metabolism were also found to be significantly related to BMI. Ho *et al.*[24] also discovered that BMI was associated with 69 of 217 metabolites profiled using LC-MS/MS, including aromatic amino acids (tyrosine, phenylalanine) and BCAAs (valine, isoleucine, leucine) among 2,383 participants of the Framingham Heart Study (FHS) Offspring cohort by cross-sectional evaluation as well as assessment of their longitudinal changes in cardiometabolic traits.

Obesity and metabolic disease traits commonly occur together in individuals with metabolic syndrome (MetS). In the above FHS Offspring Study, considerable overlap was observed in metabolite profiles associated with BMI and other metabolic traits, including abdominal adiposity, insulin resistance, and dyslipidemia. However, profiles of fasting glucose and blood pressure traits were relatively distinct, indicating differences of metabolic signatures among various cardiometabolic phenotypes. Their findings also demonstrated the important interaction of obesity status and insulin resistance; bile acid metabolites were strongly associated with insulin resistance among obese but not lean individuals, whereas isoleucine had a stronger association with insulin resistance in lean individuals. A metabolic profiling by Iida *et al.*[25] also indicated that isoleucine and other polar metabolites including BCAAs were associated with MetS among Japanese postmenopausal women, a population with relatively normal BMI with insulin resistant background.

BCAAs are some of the main metabolites that have repeatedly been identified as obesity-related, but their use as biomarkers for obesity should be handled with care since they are also indicative of and possible biomarkers for insulin resistance and diabetes. Wang *et al.*[26] performed a nested case-control study in the FHS Offspring Study, where 201 out of 2,422 normoglycemic individuals developed diabetes after follow-up for 12 years. They discovered that fasting concentrations of the baseline BCAA and aromatic amino acids (phenylalanine and tyrosine) were prognostic for the onset and progress of diabetes in long-term follow-up; 1 standard deviation increment in the above five amino acids was associated with a 57%–102% increase in the risk of future diabetes development. Recently, various studies have performed investigations on BCAA and insulin resistance as well as diabetes risks across different races, and it may be considered that the BCAA levels and its relationship with insulin resistance are race-dependent. In the Insulin Resistance Atherosclerosis Study, a multiethnic cohort consisted of Caucasians, AA, and Hispanics, plasma BCAA levels of 685 nondiabetic individuals were measured by MS method, and the positive relationship with later diabetes risk was found to be more obvious in Caucasians or in the combined Caucasian and Hispanic group but not in AA during its 5-year follow-up[27]. In another prospective study by Tillin T *et al.*[28], South Asian and European male participants from the SABRE study were compared regarding cross-sectional associations between nine serum amino acid concen-

trations, metabolic and obesity traits, and longitudinal associations with incident diabetes during 19 years of follow-up. As a result, South Asian individuals had higher serum concentrations of isoleucine, but weaker correlation with obesity measures, and longitudinal analyses indicated higher diabetes risk for South Asian (35%) subjects compared with European subjects (14%). Although Asians usually hold lower BMI, studies have shown similar alterations in amino acid profile in metabolic disorders[25], or higher susceptibility to elevated BCAA levels and insulin resistance compared with the Western people[29]. The mechanism between BCAA and insulin resistance remains unclear, although previous studies have indicated ectogenic BCAAs intake constituting only a small portion in the onset of insulin resistance and genetic variants of BCAAs catabolism in adipose tissue as important influences. Ongoing basic science investigations are expected to unveil the complex mechanism behind BCAAs and systemic metabolic disturbances.

4) Metabolic Profiling of Lifestyle Factors

Metabolomics is responsive to environmental factors, such as physical activity and drinking habits. A cross-sectional relationship between daily physical activity level and plasma metabolites profiled by capillary electrophoresis-mass spectrometry (CE-MS) was investigated in over 1,000 Japanese male participants of Tsuruoka Metabolomics Cohort Study (TMCS)[30]. Higher levels of physical activity and shorter sitting time were significantly associated with lower concentrations of BCAA and other amino acids after adjustment for confounding factors. The same cohort study has examined the associations of daily ethanol intake with plasma concentration of metabolites profiled by CE-MS method, and identified 19 potential biomarkers of daily alcohol intake and 4 (threonine, guanidinosuccinate and glutamine, and glutamate/glutamine ratio) biomarker candidates of alcohol-induced liver injury[31]. **Fig. 2** illustrates the results of metabolomic profiling among TMCS participants according to their levels of daily alcohol intake. Partial least squares-discriminant analysis (PLS-DA) using 94 metabolites measured by CE-MS showed relevant associations among the metabolic phenotypes with different levels of daily alcohol consumption among men (R2: 0.41 Q2: 0.38) (**Fig. 2A**). Similar results were obtained for women, although the predictive ability of the model was poor due to the limited number of heavy drinkers (R2: 0.18 Q2: 0.14) (**Fig. 2B**). Using the cutoff of PLS-DA variable importance in projection score $>$ 1.5, 14 metabolites were considered the largest contributing variables to the model in men (**Fig. 2C**). The heatmap visualized the different distribution patterns of the 14 metabolites between nondrinkers and heavy drinkers; certain metabolites are lower in concentrations among nondrinkers, whereas the rest are higher compared with those of heavy drinkers (**Fig. 2D**). When plasma concentration levels were compared, all 14 metabolites showed a linear association according to the level of drinking categories (**Fig. 2E**). Results of the above, and similar, studies could provide insight into the mechanisms underlying the protective or atherogenic effects of various lifestyles on human health. Accumulation of such evidence will then lead to a better understanding of mechanisms behind positive and negative lifestyle, which could eventually result in developing personalized approaches to disease prevention.

Methodological Issues on Metabolomics in Cardiovascular Epidemiology

Metabolomics in epidemiological studies is a promising approach to identify biomarkers for prevention, diagnosis, and prognosis of CVD and AS. However, until recently, metabolomics has not been suitable for large-scale epidemiologic applications mainly due to the limited capacity of the analytical platforms for sample throughput and the processing requirements for the enormous amounts of data created. Advances in methodological technologies have enabled the application of metabolite profiling to epidemiological researches, allowing large-scale population-based investigations.

1) Various Analytical Platforms of Metabolomics Measurement

Metabolomics typically involves the measurement of hundreds to thousands of metabolites, and no single platform can currently assay all metabolites comprehensively. To optimize the measurement of broad classes of metabolites, numerous analytical platforms, as well as customized instrumentation and sample extraction protocols, have been developed and used for metabolic profiling. There are two primary platforms: NMR spectroscopy and MS, in which the latter may be coupled with GC, LC, and CE. Each of the platforms has its advantages and weaknesses[32]. NMR can analyse various biofluids without sample preparation, but the sensitivity is relatively poor compared with MS methods, and concentrations of potential biomarkers may be below the limit of detection. The main advantages of using GC-MS are its reliability and relatively low instrument cost and maintenance. On the other hand, it requires sample derivatization to create volatile compounds, and therefore, nonvolatile compounds that do not derivatize and large or thermo-labile compounds may not be

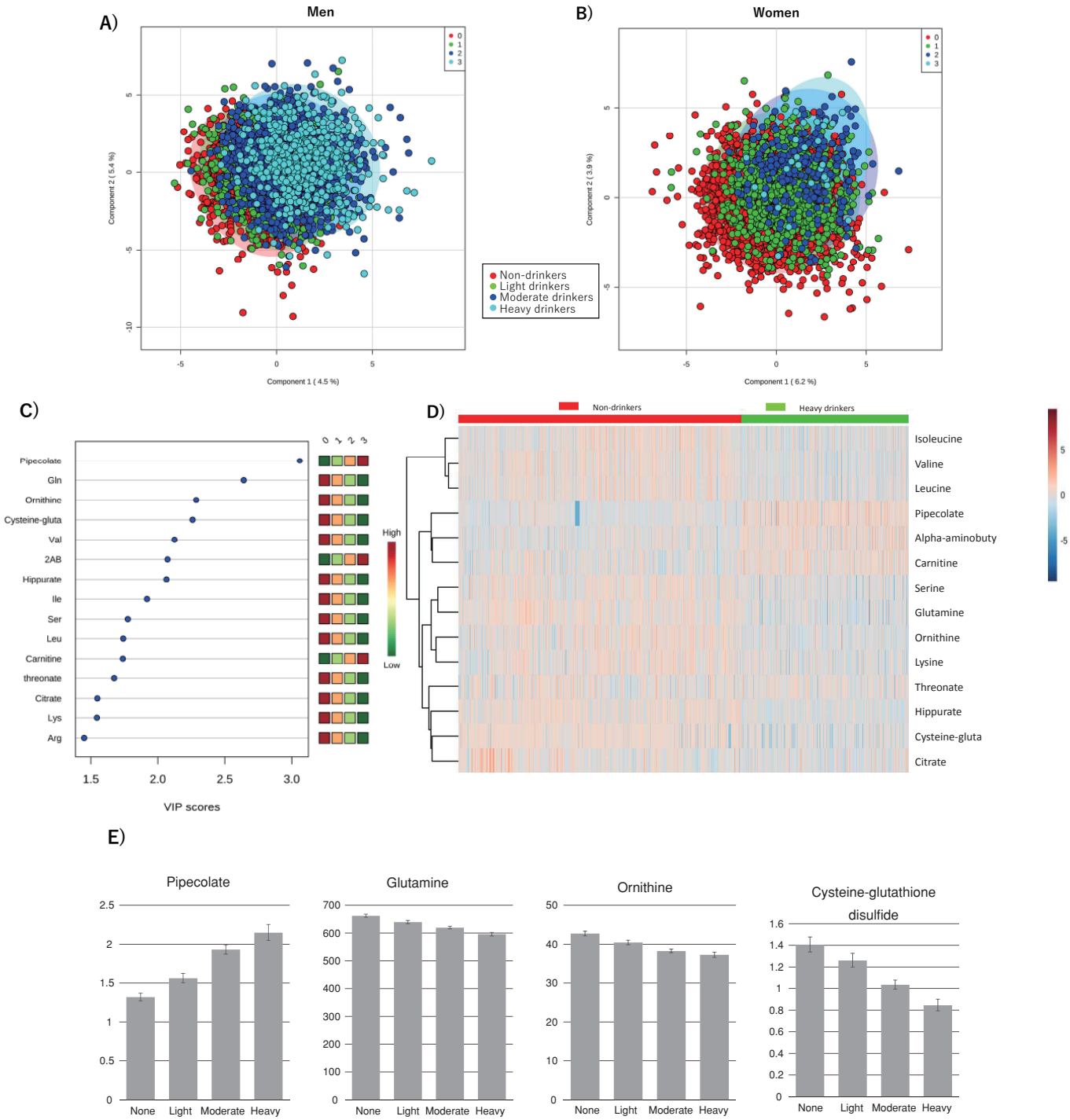

Fig. 2. Discrimination according to the amount of daily alcohol consumption using partial least squares-discriminate analysis (PLS-DA): Tsuruoka Metabolomics Cohort Study

(A)(B) PLS-DA score plots of men (A) and women (B) with no/light/moderate/heavy drinking. Red: no drinking, n=945, 3451 (men, women), less than 1 gram of ethanol per day. Green: light drinking, n=824, 885 (men, women), 1 to less than 20 grams of ethanol per day. Blue: moderate drinking, n=1413, 262 (men, women), 20 to less than 60 grams of ethanol per day. Light blue: heavy drinking, n=569 (men), 60 grams or more of ethanol per day.
(C) Possible biomarkers of heavy drinkers in men. Gln, glutamate; Cysteine-gluta, Cysteine-glutathione disulfide; Val, valine; 2AB, 2-aminobutyrate; Ile, isoleucine; Ser, serine; Leu, leucine; Lys, lysine; Arg, arginine.
(D) Clustering result shown as heatmap. (A)–(D) Data analyzed and images produced by MetaboAnalyst ver 4.0.
(E) Plasma concentrations of the top four contributing metabolites to the PLS-DA model, according to drinking categories in men. Concentrations of metabolites are in μmol/L units; error bars reflect 95% confidence intervals.

observed in the GC-MS analysis[33]. LC-MS is highly applicable to the analysis of a wide range of semi-polar compounds including many secondary metabolites of interest, making it the most widely applied chromatography–MS strategy for analysis of both hydrophilic and hydrophobic metabolites. Although the analysis of charged or very polar metabolites (e.g., sugar phosphates, amino acids) are considered difficult to analyse by LC-MS, strategies such as combining different columns and applying hydrophilic interaction chromatography (HILIC) to obtain good separation of polar compounds have enabled it to cover broader metabolites. CE-MS is performed less frequently for metabolomics than GC- and LC-MS but is a powerful tool regarding the absolute quantification of polar metabolites, including carbohydrates, amino acids, organic acids, and nucleotides, some of which are known as important potential biomarkers for CVD. Another key advantage of CE-MS is its potential for high-throughput metabolite screening on serial injections of multiple samples, which is an advantage when conducting large-scale studies[34]. Major European cohorts such as Cooperative Health Research in the Region of Augsburg (KORA) and TwinsUK registry, and American cohorts including FHS Offspring cohort and ARIC study have conducted metabolic profiling mainly by LC-MS and GC-MS. Population studies such as Finnish cohorts, Estonian Biobank, COMBIBIO, and the INTERMAP Study have applied NMR for their metabolomic analyses. As one of the few large-scale population-based cohort studies in Asia applying metabolomics, TMCS has utilized CE-MS for polar metabolites and LC-MS for lipid metabolites. Although most laboratories use a single analytical approach due to instrumental limitations, parallel application of several techniques may be considered desirable to study the global metabolome.

2) Reliability in Metabolite Measurements in Large-Scale Epidemiological Studies

Metabolites are sensitive not only to pathological alterations such as AS but also to improper sample handling. Accurate quality control and quality assurance are critically important to obtain reliable results, especially in large-scale epidemiological studies where thousands of samples are measured over long periods of time.

One aspect of limiting measurement error and bias in metabolomics can be found in the preanalytical phase, such as sample collection, processing, transport, and storage. In multicenter studies, which are conducted in large-scale epidemiology, it is often difficult to ensure that each institution strictly follows the standard sample preparation protocols. Testing the blood collection tubes, avoiding hemolysis, placing whole blood immediately in ice water, using EDTA plasma, and preferably using non-refrozen biobank samples are some of the recommendations reported for preanalytical phase[35]. Hirayama *et al.*[36] have examined the effects of sampling procedure and storage conditions on the stability of metabolomic profiles both in plasma and serum by CE-MS. They indicated that profiles in plasma showed better stability than those in serum. Additionally, optimal metabolomic profiles were obtained from plasma stored for a shorter period, and shorter clotting times were recommended for serum analyses.

Limiting measurement error and bias in the analytical phase is also critical to ensure reliable data, such as estimating precise disease risks with high statistical power in epidemiological studies. In a study reported by Townsend MK *et al.*[37], plasma samples were donated by a few dozen participants of the Nurses' Health Study and Health Professionals Follow-up Study, some of the largest prospective cohort studies into the risk factors for CVD in women and men, to investigate the reproducibility of metabolomic profiles measured by LC-MS method. Most metabolites, particularly lipids and lipid metabolites, amino acids, and bile acids, were profiled with acceptable laboratory variability and were reproducible over processing delays and within individuals over 1 to 2 years. However, their results also suggested difficulty in evaluating certain metabolites by LC-MS in epidemiologic studies when blood samples were processed after a delay of 24 hours or longer. Harada *et al.*[34] have examined the reproducibility and validity of CE-MS measurements on a much larger scale using the data from the TMCS Asian populations. Over 800 quality control samples for each cation and anion metabolites and plasma samples from more than 8,000 cohort participants were analyzed, with data acquired over a period of 52 months. The authors concluded that the reproducibility of metabolites measured by CE-MS was comparable to other platforms used in other large-scale epidemiological studies. However, since large-scale studies applying metabolomics by CE-MS are still limited, the authors have described the need for external validation.

3) Overcoming Challenges of Metabolomics

In order to facilitate large-scale collaborative research on the human metabolome and to overcome issues similar to the ones mentioned above, a consortium called the Consortium of Metabolomics Studies (COMETS) was developed in 2014 [38]. Being the world's largest consortium of metabolomics cohorts, it comprises 47 prospective studies from Asia, Europe,

North America, and South America as of April 2018, and together includes more than 136,000 participants with blood metabolomics data measured by multiple platforms. Communication among researchers regarding associations that vary between different platforms could improve the quality and consistency of each measurement data, not to mention the distinctive advantage of having a large sample size which enables high-powered statistical analyses. Comparative assessment was conducted by measuring 111 metabolite levels by two leading platforms in split samples; results showed that the values showed high intercorrelations. However, challenges remain for the agreement of absolute concentrations since the measurement units differed between platforms. Metabolite levels obtained in most high-throughput metabolomics profiling studies are often semiquantitative rather than fully quantitative concentrations. Further work is required to establish clinically meaningful concentration thresholds for even the important associations identified in previous studies with CVD and AS.

There are two distinct analytical strategies applied in metabolomics research; targeted and untargeted. Targeted approach involves quantitating a group of defined metabolites with the use of internal standard compounds. Since it has high specificity and accuracy, this method has been widely used, most commonly in MS methods, to analyse and compare multiple metabolites under different physiological states. Untargeted approach is a comprehensive analysis of all the measurable analytes in a sample, including chemical unknowns. Identification of unknown metabolites is greatly limited, making it difficult to effectively identify important biomarkers. Nevertheless, the wide coverage provided by untargeted strategy has a strong potential to identify novel metabolic pathways and disease biomarkers. Since COMETS is currently restricted to analyses based on identified metabolites, it aims to integrate data from unidentified metabolites in the future.

Conclusion

Metabolomics in recent years has received further attention as one of the valuable approaches among other omics approach to unravel the black box mechanism of cardiovascular health and disease. In this review, we highlighted recent findings of metabolomics applied to cardiovascular epidemiology worldwide, offering new insights into the factors that contribute to the complexity of CVD and its underlying process, including the effects of dietary intake on metabolome, gut microbiome, and health outcomes, and metabolic signatures on various risk factors of AS. This review also addressed the current analytical limitations and challenges that need to be overcome, from comparative assessment of different platforms to determination of absolute metabolite concentrations for the establishment of clinically meaningful thresholds, and elucidation of unidentified metabolites using untargeted approach. Each progress made will allow metabolomics research to substantially contribute to the prevention of AS and CVD.

Conflict of Interest

The authors have no conflicts of interest.

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