There is now overwhelming evidence that alterations in lipid metabolism underlie the pathogenesis of atherosclerotic cardiovascular disease (CVD). However, research in this area has focused primarily on abundant plasma lipids such as cholesterol, triglycerides, and oxidized phospholipids that are largely carried on circulating lipoproteins. Circulating levels of abundant lipids can provide valuable information for CVD risk stratification, yet the majority of CVD risk cannot be explained by traditional lipid risk factors. Over the last decade, there have been rapid advances in mass spectrometry–based methods to comprehensively annotate the entire “lipidome,” which includes a large variety of lipid molecular species such as non–lipoprotein-associated lipids and less abundant signaling lipids that affect disease. This new field of lipidomics provides a powerful platform for the identification of potential lipid biomarker signatures predicting disease risk but also provides clues into novel lipid metabolic pathways that may be directly involved in the pathogenesis of disease. At an amazingly rapid pace (Figure 1), lipidomic approaches have yielded valuable information linking specific lipid types with structural and signaling roles in the development of cancer, obesity, diabetes mellitus, hypertension, Alzheimer disease, infectious diseases, and CVD. Undoubtedly, lipidomic approaches provide a powerful discovery platform that has promise both to identify new biomarkers of chronic disease and to provide clues into unrecognized lipid metabolic pathways with potential mechanistic links to disease pathogenesis and untapped therapeutic potential.

In this issue of Circulation, Stegemann and colleagues applied a targeted lipidomic approach to population-based cohorts with documented CVD events. The purposes of this editorial are to summarize the results from this seminal study and to discuss the implications of identifying a lipid signature that predicts CVD risk. To begin, it is important to discuss the broad field of lipidomics and its rapid development over the last decade. Although lipids are among the earliest biological chemical entities studied by scientists, the lipidome, which refers to the complete collection of chemically distinct lipids in a biological sample, is a relatively new term in the scientific literature, appearing after the new millennium. Since then, the field of lipidomics has grown at a rapid pace (Figure 1) and now provides a powerful hypothesis-generating platform that is readily available to the scientific community. Lipidomics typically refers to mass spectrometric identification of individual lipid species, which can annotate the type, elemental composition (molecular weight), and component composition (eg, fatty acid molecular species) of a particular lipid species. Lipidome diversity arises through differences in the type and composition of distinct groups of lipids (eg, sterols, phospholipids, triglycerides [TGs]). Phospholipid composition diversity is governed by the makeup of the polar head group, which determines the class of the lipid (choline, ethanolamine, serine, etc), the covalent nature of the linkage with fatty acid chains (ie, acyl, alkyl [ether lipid], alkenyl [plasmalogen], amide [sphingolipids]), and the individual molecular species of fatty acids, which is influenced by the length of individual aliphatic chains of fatty acids, the amount and position of double bonds, and the regiospecificity of the aliphatic moieties (ie, sn-1, sn-2, position on the glycerol backbone). Mass spectrometry can differentiate among these aspects of complex lipids, although species that are isobaric (possess the same molecular weight) often are more difficult to distinguish without more precise approaches that involve both fragmentation in the mass spectrometer and chromatographic resolution on the front end leading into the instrument, typically through use of high-performance lipid chromatography. Stegemann and colleagues used a targeted analysis that focused on a broad range of known lipids using what they called a shotgun lipidomics approach and relied on a direct lipid extract analysis without chromatographic separation. The shotgun lipidomics methodology used has as its major advantages its simplicity, high sensitivity, ease of sample preparation, and instrument cost (can be done with both low- and high-end mass spectrometers).

However, the methodology used also has several major disadvantages: The type of mass spectrometry used could not identify individual aliphatic species but rather determined total carbon number for fatty acids in a compound; isobaric lipid species are not identified but rather cumulatively analyzed; and as performed, the methodology was only semiquantitative because synthetic isotope–labeled internal standards for individual molecular species were not used. Furthermore, it is important to note that any data generated with lipidomic approaches rely heavily on the way the samples are stored (eg, “freezer burn” is lipid peroxidation, and oxidative modifications and degradation of certain lipids may be particularly problematic unless special precautions are taken to prevent adventitious oxidation during storage [eg, plasmalogens, polyunsaturated fatty acids (PUFAs)]). In addition, the method of extraction can greatly influence the recovery of different forms of lipid.
of lipids (ie, not all lipids extract with the same efficiency, and there is no universal method that works well for all lipid forms). Another factor influencing results is the method of separation (ie, direct infusion versus chromatography-based separation). These issues are key considerations in the interpretation of lipidomic data.

Surprisingly, the application of large-scale lipidomics to large sample sets has only recently been used to understand the pathobiology of CVD. Stegemann and colleagues\textsuperscript{11} performed shotgun lipidomics in the prospective Bruneck Study, which importantly includes incident CVD end-point measures in 685 individuals. The resultant lipidomic analyses detected 135 distinct lipids within the following lipid types and phospholipid classes: cholesteryl ester (CE), TG, phosphatidylcholine (PC), lysophosphatidylcholine, phosphatidylethanolamine, lysophosphatidylethanolamine, phosphatidylserine, and sphingomyelin. Overwhelmingly, 50 plasma lipid species were significantly associated with CVD risk, and 28 of those maintained significance when controlling for multiple comparisons. Interestingly, the majority of the neutral lipid species linked to CVD risk contained primarily saturated fatty acyl (SFA) and monounsaturated fatty acyl (MUFA) chains. Collectively, 3 key lipids (TG-54:2, CE-16:1, and PC-36:5) were identified as most consistently linked to incident CVD. The term TG-54:2 refers to TG species that contain 3 fatty acids and collectively contain 54 carbons and 2 double bonds. It is thus a composite of multiple distinct isobaric molecular species, although the low degree of unsaturation (only 2 double bonds) significantly limits the types of fatty acids available. Similarly, PC-36:5 refers to a glycerophospholipid containing a choline head group (PC) and 2 fatty acids that together contain 36 carbons and 5 double bonds; thus, it represents a composite of numerous isobaric molecular species. CE-16:1 refers to a CE-containing isobaric fatty acid molecular species containing 16 carbons and 1 double bond, the most common being the MUFA palmitoleic acid (9-cis-16:1), although sapienic acid (6-cis-16:1, and 6-trans-16:1) is also found in plasma and low-density lipoprotein (LDL).\textsuperscript{13} Remarkably, measuring the plasma levels of the 3 “signature lipids” identified (TAG-54:2, CE-16:1, and PC-36:5) outperformed standard lipid measures of the Framingham Risk Score (total and high-density lipoprotein cholesterol) in determining risk discrimination and 10-year risk classification. For replication purposes, Stegemann and colleagues\textsuperscript{11} performed additional studies in an independent validation cohort from the TwinsUK study and found a characteristic plasma fatty acid composition similar to that in the Bruneck cohort, that SFA and MUFA levels were more strongly associated with CVD than other PUFAs. Collectively, the lipidomic study performed by Stegemann and colleagues\textsuperscript{11} provides novel insights into a lipid signature containing SFA and MUFA species that is linked to CVD risk.

Unfortunately, the study by Stegemann and colleagues\textsuperscript{11} does not go further to determine the precise molecular species of lipids that comprise TAG-54:2, CE-16:1, and PC-36:5 and drive the associations identified with incident CVD risks. This information would also have been ideally leveraged for the development of more specific quantitative mass spectrometry–based analyses of the precise molecular species involved, which would have ideally then been performed on both primary and validation cohorts. Knowledge of the precise molecular species may also have helped to reveal the underlying mechanism through which the identified SFA- and MUFA-rich lipids are linked to CVD risk. In fact, although this study provides one of the first looks into the CVD risk plasma lipidome, many further studies are required both to define the precise lipid molecular species that drive the associations discovered and to understand the underlying mechanisms behind the associations observed.

Although not expanded on by Stegemann and colleagues,\textsuperscript{11} there is ample evidence supporting a mechanistic link between SFA and MUFA metabolism in the pathogenesis of CVD. In fact, it has long been know that diets enriched in SFA promote CVD, in part by promoting hypercholesterolemia.\textsuperscript{14} In addition to effects on hypercholesterolemia, SFAs have been shown to activate pattern recognition receptors such as the Toll-like receptor 4, thereby promoting vascular inflammation and CVD\textsuperscript{15} (Figure 2). Likewise, MUFA-rich diets promote atherosclerosis in animal models, yet epidemiological studies link MUFA-rich diets like the Mediterranean diet to beneficial effects on CVD.\textsuperscript{17} Although the reasons for this discrepancy between epidemiology and controlled animal studies are unknown, it has been suggested that it can likely be attributed to other dietary constituents in the Mediterranean diet that override the potential proatherogenic MUFA component.\textsuperscript{17} Proatherogenic SFAs and MUFAs can be either derived from dietary sources or made endogenously via the
de novo lipogenesis pathway primarily in the liver (Figure 2). Therefore, interventions limiting dietary SFA and MUFA or those limiting the endogenous production of these fatty acids have potential to reduce or halt the progression of atherosclerosis. It is important to note that most dietary or endogenously synthesized SFA is rapidly converted to MUFA by the action of steroyl-CoA desaturase 1 (SCD1) and that the MUFA products of the SCD1 reaction (16:1 and 18:1) are preferentially esterified into neutral lipids such as TG and CE (Figure 2). As a result, there are much higher levels of MUFAs than SFAs in most mammalian species. In the circulation, the vast majority of SFAs and MUFAs are esterified in complex lipids such as phospholipids, triacylglycerols, and cholesteryl esters. This esterification is dictated enzymatically through the actions of the phosphatidylethanolamine (PE) N-methyltransferase (PEMT) or cytidine diphosphate-choline (CDP-choline) pathways for phospholipids (preference toward esterfying 16:0 and 18:0), acyl-CoA:cholesterol acyltransferase 2 (ACAT2) for CEs (strong preference for esterfying 16:1 and 18:1), and diacylglycerol O-acyltransferase 1/2 (DGAT1 or DGAT2) for triacylglycerols (strong preference for esterfying 18:1). These complex lipids are then packaged into nascent very-low-density lipoproteins (VLDLs) and this lipid cargo also remains in low-density lipoproteins (LDLs) after intravascular metabolism. Circulating LDL can deliver this lipid cargo to the artery wall, where cell types such as lesion macrophage can de-esterify these complex lipids and then rebuild them using similar enzymatic mechanisms. Previous studies have shown that LDL containing monounsaturated CEs (16:1) can be retained preferentially on arterial proteoglycans and thereby promote atherogenesis. Additionally, it has been shown that reorganizing membrane saturated vs unsaturated FAs in macrophages by inhibition of the desaturase SCD1 can alter pattern recognition receptor (PRR) signaling. Additional mechanistic work is necessary to structurally identify the molecular species within 16:1-CE, 54:2-TAG, and 36:5-PE associated with CVD. In addition, the enzymes that control their levels in the circulation and whether they are causally linked to CVD progression or are simply associated with the presence of disease await further clarification. HDL indicates high-density lipoprotein; PC, phosphatidylcholine; and TG, triglyceride.

Figure 2. Proposed mechanistic insights into how cardiovascular disease (CVD)–predicting signature lipids may promote the pathogenesis of atherosclerosis. Within the liver, the saturated fatty acids (FAs; palmitate, 16:0; stearate, 18:0) are produced via de novo lipogenic pathways or derived from dietary sources. After de novo FA synthesis or acquisition from the diet, the saturated FAs (16:0 and 18:0) can be desaturated by the microsomal enzyme steroyl-CoA desaturase 1 (SCD1) to form monounsaturated FAs (palmitoleate, 16:1; oleate, 18:1). Both saturated and monounsaturated FAs are selectively incorporated into certain complex lipid classes such as phospholipids (high level of saturated), triacylglycerols (high levels of 18:1), and cholesteryl esters (CE; high levels of 16:1 and 18:1). The esterification of saturated FA vs monounsaturated FA into these distinct complex lipids is dictated enzymatically through the actions of the phosphatidylethanolamine (PE) N-methyltransferase (PEMT) or cytidine diphosphate-choline (CDP-choline) pathways for phospholipids (preference toward esterfying 16:0 and 18:0), acyl-CoA:cholesterol acyltransferase 2 (ACAT2) for CEs (strong preference for esterfying 16:1 and 18:1), and diacylglycerol O-acyltransferase 1/2 (DGAT1 or DGAT2) for triacylglycerols (strong preference for esterfying 18:1). These complex lipids (16:1-CE, 54:2-TAG, and 36:5-PE) are then packaged into nascent very-low-density lipoproteins (VLDLs), and this lipid cargo also remains in low-density lipoproteins (LDLs) after intravascular metabolism. Circulating LDL can deliver this lipid cargo to the artery wall, where cell types such as lesion macrophage can de-esterify these complex lipids and then rebuild them using similar enzymatic mechanisms. Previous studies have shown that LDL containing monounsaturated CEs (16:1) can be retained preferentially on arterial proteoglycans and thereby promote atherogenesis. Additionally, it has been shown that reorganizing membrane saturated vs unsaturated FAs in macrophages by inhibition of the desaturase SCD1 can alter pattern recognition receptor (PRR) signaling. Additional mechanistic work is necessary to structurally identify the molecular species within 16:1-CE, 54:2-TAG, and 36:5-PE associated with CVD. In addition, the enzymes that control their levels in the circulation and whether they are causally linked to CVD progression or are simply associated with the presence of disease await further clarification. HDL indicates high-density lipoprotein; PC, phosphatidylcholine; and TG, triglyceride.
as CE, TG, and phospholipids, which are tightly associated with lipoprotein carriers. There is also a much smaller pool of circulating free SFA and MUFA complexed to albumin, which is thought to be contributed mostly by adipose tissue lipolysis. The work by Stegemann and colleagues provides a snapshot of this plasma lipidome, which is primarily lipoprotein-associated complex lipids (CE, TG, and PL). A key consideration for the plasma lipidome is that lipid-modifying enzymes dictate circulating lipid levels, and the CVD lipid signature identified (TG-54:2, CE-16:1, and PC-36:5) points toward well-studied enzymatic pathways.

For instance, the link between CE-16:1 and CVD found by Stegemann and colleagues might in retrospect not be surprising given that the enzymatic source of MUFA-enriched CE (acyl-CoA:cholesterol acyltransferase 2 [ACAT2]) has long been linked to atherosclerosis development and continues to be a potential drug target for CVD prevention.16,18 There are only 2 known enzymatic sources of circulating CE in mammals: the intracellular enzyme ACAT2, which is expressed exclusively in enterocytes and hepatocytes where apoB-containing lipoproteins are made, and the plasma enzyme lecithin:cholesterol acyltransferase, which catalyzes a 2-step reaction in which the sn-2 fatty acyl chain of PC is hydrolyzed following transacylation of the released fatty acid to the 3β-hydroxyl group of cholesterol-generating CE and lysocephatidylcholine.18 It is clear that these 2 enzymes have striking substrate preference, with ACAT2 preferring to esterify MUFA (generating CE-16:1 and CE-18:1, which are preferentially carried in apolipoprotein B–containing lipoproteins) and lecithin:cholesterol acyltransferase preferring to esterify PUFA (generating CE-18:2, CE-20:4, and many other PUFA-CEs, which are preferentially carried in high-density lipoprotein particles).16,18 Before the advent of unbiased lipidomics, several previous studies have shown that patients with coronary artery disease have higher MUFA-containing CE (ACAT2-derived) and lower levels of PUFA-containing CE (lecithin:cholesterol acyltransferase-derived) compared with healthy control subjects.16 This well-appreciated connection between MUFA-containing CE species and atherosclerosis development in mouse and humans may be explained in part by the fact the LDL particles enriched in ACAT2-derived CE (16:1 and 18:1) have enhanced ability to bind to arterial proteoglycans and may be preferentially retained in the artery wall more than PUFA-enriched LDL.14 Because there are no known circulating cholesteryl esterases to affect the plasma lipidome, therapeutic targeting of ACAT2 remains an attractive approach for limiting circulating CE-16:1 and CE-18:1 (the major CE species found in atheroma).18

In addition to CE species, circulating TG levels have long been associated with CVD risk. Therefore, the association of a number of TG species (including TG-54:2) with CVD risk by Stegemann and colleagues (Figure 2) is not surprising. Similar to cholesterol esterification, there are 2 dedicated enzymes mediating TG biosynthesis known as acyl-CoA:diacylglycerol acyltransferase 1 and 2 (DGAT1 and DGAT2). However, unlike circulating CE metabolism, circulating TG levels are also affected by intravascular lipase activity through enzymes like lipoprotein lipase and hepatic lipase. Therefore, the circulating TG lipidome is highly regulated but dynamic, and changes with the fasting versus fed state of the individual. Thus, time of sampling relative to the last meal is critical and can significantly affect TG molecular species composition. One study has suggested that DGAT1 knockout mice are protected against atherosclerosis development, primarily because of decreased intestinal cholesterol absorption rates.19 However, whether circulating TGs are actually involved in CVD disease pathogenesis is an intense matter of debate. Some believe that because circulating TGs are carried in the same lipoprotein class (apolipoprotein B containing) as proatherogenic CE, the TGs are simply associated with disease and are not causatively linked to disease pathogenesis. Support for this concept comes from ACAT2 knockout mice, which have no CE packaged into newly secreted very-low-density lipoprotein particles but instead the very-low-density lipoprotein core is filled with TGs. These TG-rich very-low-density lipoproteins completely lack the ability to promote atherosclerosis in mice, supporting the notion that CE is the culprit lipid driving CVD in mice. Hence, it is possible that the TG forms associated with CVD in the study by Stegemann and colleagues are simply associated lipids and not mechanistically linked to disease progression.

The work by Stegemann and colleagues identifies an important link in both SFA- and MUFA-containing lipid species and CVD risk. It is therefore important to consider the enzyme that desaturates SFA to generate MUFA, known as SCD1, and how it relates to the pathogenesis of atherosclerosis. Because of its role in generating the preferred substrate of TG biosynthesis, oleic acid (18:1), SCD1 has been a highly studied target for metabolic diseases such as obesity and diabetes mellitus.15 In fact, many major pharmaceutical companies have at some point had SCD1 inhibitor programs because inhibition or genetic deletion of SCD1 in mice decreases TG biosynthesis and protects against diet-induced obesity and diabetes mellitus.15 However, the role of SCD1 in CVD complicates such efforts. It turns out that either inhibition or genetic deletion of SCD1 causes a markedly advanced atherosclerotic phenotype in mice.16 It is thought that inhibition of SCD1 causes an atypical accumulation of SFA-enriched lipid in the circulation and in the artery wall, where SFA enrichment can enhance Toll-like receptor 4 signaling and vascular inflammation.15 These studies highlight the fact that altering the ratio of SFA and MUFA can dramatically alter macrophage inflammatory responses and associated vascular inflammation, implicating SFA-enriched lipids in the pathogenesis of CVD. Ultimately, mechanistic animal studies for lipid metabolic enzymes (ACAT2, DGAT1, SCD1, etc) are necessary to provide clues into whether certain lipid classes are simply associated with disease or mechanistically linked to disease pathogenesis.

In conclusion, the study by Stegemann and colleagues provides an early glimpse into the plasma lipidome of CVD. It is important to mention that a prominent strength of this study is the application of unbiased lipidomic technology to a prospective cohort with incident CVD end-point data. However, there are several limitations that leave unanswered questions. The shotgun lipidomics methods used do not provide identification among isobaric species, so the specific molecular species that are a part of the CVD risk signature remain unknown. A related issue is whether more accurate quantification of the
molecular species that constitute the signature lipids identified can be validated in independent prospective cohorts with more specific mass spectrometry–based analyses. How does the plasma lipidome change temporally during key phases of atherosclerosis progression or regression? Finally, can the associations observed be leveraged such as through genetic analyses to mechanistically explore the pathways potentially involved for evidence of causal relationships with CVD in humans. In addition to these unanswered questions, it remains extremely important for the field of lipidomics in general to progress from simple descriptive lists of lipids that associate with human disease to mechanistic studies linking identified lipids to the pathogenesis of disease. In other words, lipidomics can provide useful data for the identification of disease biomarkers or candidate species, as was done by Stegmann and colleagues. However, such data should also be used as a hypothesis-generating tool to examine enzymatic pathways that regulate such lipids in an attempt to therapeutically intervene. Moving beyond the provision of descriptive lipidomic lists to mechanistic studies using defined animal model systems represents the next and more challenging step. Indeed, it is an exciting time for the field of lipidomics. We are no longer limited by technology; innovative ideas will be the only limitation moving forward. Undoubtedly, lipidomics provides a rich resource of new information linking metabolism to human disease and will be an important tool for drug discovery in the future.

Acknowledgments

We thank David Schumick for assistance with the preparation of Figure 2.

Sources of Funding

Drs Brown and Hazen are supported through the National Institutes of Health and Office of Dietary Supplements grants R00 HL096166, R01 HL103866, P20 HL113452, P01 HL076491, and P01 HL098055.

Disclosures

None.

References

Seeking a Unique Lipid Signature Predicting Cardiovascular Disease Risk
J. Mark Brown and Stanley L. Hazen

Circulation. 2014;129:1799-1803; originally published online March 12, 2014;
doi: 10.1161/CIRCULATIONAHA.114.009224
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7322. Online ISSN: 1524-4539

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://circ.ahajournals.org/content/129/18/1799

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation is online at:
http://circ.ahajournals.org//subscriptions/