Seeking a Unique Lipid Signature Predicting Cardiovascular Disease Risk

Running title: Brown et al.; Seeking a CVD Lipid Signature

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Journal Subject Codes: Atherosclerosis:[134] Pathophysiology, Atherosclerosis:[90] Lipid and lipoprotein metabolism

Key words: Editorial, atherosclerosis, lipids, triglycerides, cholesterol
There is now overwhelming evidence that alterations in lipid metabolism underlie the pathogenesis of atherosclerotic cardiovascular disease (CVD). However, research in this area has primarily focused 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 including non-lipoprotein associated lipids and less abundant signaling lipids that impact disease. This new field of “lipidomics” provides a powerful platform for 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, lipidomic approaches have yielded valuable information linking specific lipid types with structural and signaling roles in the development of cancer, obesity, diabetes, hypertension, Alzheimer’s disease, infectious diseases, and CVD. Undoubtedly, lipidomic approaches provide a powerful discovery platform that have promise to both identify new biomarkers of chronic disease, and provide clues into unrecognized lipid metabolic pathways with potential mechanistic links to disease pathogenesis and untapped therapeutic potential.

In this issue of Circulation, Stegmann and colleagues apply a targeted lipidomic approach to population-based cohorts with documented CVD events. The purpose of this editorial is to summarize the results from this seminal study and 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. While lipids are among the
earliest biological chemical entities studied by scientist, the concept of 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 turn of the century\textsuperscript{12}. 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 (e.g. fatty acid molecular species) composition, of a particular lipid species. Lipidome diversity arises through differences in the type and composition of distinct groups of lipids (e.g. sterols, phospholipids, triglycerides). Phospholipid composition diversity is governed by the makeup of the polar headgroup, which determines the class of the lipid (i.e. choline, ethanolamine, serine, etc.), the covalent nature of the linkage with fatty acid chains [i.e. 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 (i.e. $sn$-1, $sn$-2, $sn$-3 position on the glycerol backbone). Mass spectrometry can differentiate amongst these aspects of complex lipids, though species which 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/or chromatographic resolution on the front end leading into the instrument, typically through use of high performance lipid chromatography (HPLC). The work by Stegemann and colleagues\textsuperscript{11} employed a targeted analysis that focused on a broad range of known lipids using what they termed 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 employed also has several major disadvantages including: (i) the type of mass spectrometry used could not identify individual aliphatic species, but rather determined total carbon number for fatty acids in a compound; (ii) isobaric lipid species are not identified but rather cumulatively analyzed; and (iii) as performed, the methodology was only semi-quantitative, since synthetic isotope labeled internal standards for individual molecular species were not used. Furthermore, it is important to note that any data generated using lipidomic approaches rely heavily on the way the samples are stored [e.g. “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 (e.g. plasmalogens, polyunsaturated fatty acids)]. In addition, the method of extraction can greatly influence the recovery of different forms of lipids (i.e. 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 includes the method of separation (i.e. direct infusion vs. chromatography-based separation). These issues are key considerations when interpreting lipidomic data.

Surprisingly, the application of large-scale lipidomics to large sample sets has only recently been utilized to understand the pathobiology of CVD. Stegemann and colleagues\textsuperscript{11} performed shotgun lipidomics in the prospective Bruneck Study, which importantly includes incident CVD endpoint measures in 685 individuals. The resultant lipidomics analyses detected 135 distinct lipids within the following lipid types and phospholipid classes: cholesteryl ester (CE), triglyceride (TG), phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), phosphatidylserine (PS),
and sphingomyelin (SM). 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 (SFA) and monounsaturated (MUFA) fatty acyl chains. Collectively, three 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 triglyceride species that contain three fatty acids and collectively contain 54 carbons and 2 double bonds. It is thus a composite of multiple distinct isobaric molecular species, though 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 (phosphatidylcholine) and 2 fatty acids which together contain 36 carbons and 5 double bonds, and thus represents a composite of numerous isobaric molecular species. CE-16:1 refers to a cholesteryl ester containing isobaric fatty acid molecular species containing 16 carbons and one double bond, the most common being the mono-unsaturated fatty acid palmitoleic acid (9-cis-16:1), though sapienic acid (6-cis-16:1, and 6-trans-16:1) is also found in plasma and LDL. 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 HDL cholesterol) in determining risk discrimination and 10-year risk classification. For replication purposes, Stegemann and colleagues performed additional studies in an independent validation cohort from the TwinsUK study, and found similar characteristic plasma fatty acid composition to the Bruneck cohort, that saturated fatty acid (SFA) and monounsaturated fatty acid (MUFA) levels were more strongly associated with CVD than other polyunsaturated fatty acids (PUFA). Collectively, the lipidomic study performed by Stegemann and colleagues provide novel insights into a lipid signature.
containing saturated and monounsaturated fatty acid 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 have been ideally also leveraged for 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 to both define the precise lipid molecular species that drive the associations discovered, and to understand underlying mechanisms behind the associations observed.

Although not expanded on in the work 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 also been shown to activate pattern recognition receptors (PRR) such as the toll-like receptor 4 (TLR4), thereby promoting vascular inflammation and CVD (Figure 2)\textsuperscript{15}. 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{16}. While 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 overrides the potential proatherogenic MUFA component\textsuperscript{16}. Proatherogenic SFA and MUFA
can either be derived from dietary sources, or be 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 the most of dietary or endogenously synthesize SFA is rapidly converted to MUFA by action of steroyl-CoA desaturase 1 (SCD1), and the MUFA products 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 MUFA than SFA in most mammalian species. In the circulation, the vast majority of SFA and MUFA are esterified in complex lipids such 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 mostly contributed 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 towards 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-coenzymeA:cholesterol acyltransferase 2, ACAT2) has long been linked to atherosclerosis development, and continues to be a potential drug target for CVD prevention. There are only two known enzymatic sources of circulating CE in mammals: 1) the intracellular enzyme ACAT2, which is expressed exclusively in enterocytes and hepatocytes where apoB-containing lipoproteins are made, and 2) the plasma enzyme lecithin:cholesterol acyltransferase (LCAT),
which catalyzes a two-step reaction that hydrolyzes the sn-2 fatty acyl chain of PC followed by transacylation of the release fatty acid to the 3β-hydroxyl group of cholesterol generating CE and LPC. It is clear that these two enzymes have striking substrate preference, with ACAT2 preferring to esterify MUFA (generating CE-16:1 and CE-18:1, which are preferentially carried in apoB-containing lipoproteins) and LCAT preferring to esterify PUFA (generating CE-18:2, CE-20:4 and many other PUFA-CE, which are preferentially carried in HDL particles).

Before the advent of unbiased lipidomics, several previous studies have shown that patient with coronary artery disease have higher MUFA-containing CE (ACAT2-derived) and lower levels of PUFA-containing CE (LCAT-derived), when compared to healthy controls. This well-appreciated connection between MUFA-containing CE species and atherosclerosis development in mouse and man, 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 so than PUFA-enriched LDL. Since there are no known circulating cholesteryl esterases to impact 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).

In addition to CE species, circulating TG levels have also long been associated with CVD risk. Therefore, 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 two 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 impacted by intravascular lipase activity through enzymes like lipoprotein lipase (LPL) and hepatic lipase (HL). Therefore, the circulating TG lipidome is...
highly regulated but dynamic, and changes with the fasting vs. fed state of the individual. Thus, time of sampling relative to the last meal is critical and can significantly impact TG molecular species composition. One study has suggested that DGAT1 knockout mice are protected against atherosclerosis development, primarily due to decreased intestinal cholesterol absorption rates\(^1\). However, whether circulating TGs are actually involved in CVD disease pathogenesis is an intense matter of debate. Some believe that since circulating TG are carried in the same lipoprotein class (apoB-containing) as proatherogenic CE, the TG are simply associated with disease and not causatively linked to disease pathogenesis. Support of this concept comes from ACAT2 knockout mice, which have no CE packaged into newly secreted VLDL particles, but instead fill the VLDL core with TG. These TG-rich VLDL completely lack the ability to promote atherosclerosis in mice\(^2\), 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\(^1\) are simply associated lipids and not mechanistically linked to disease progression. The work by Stegemann and colleagues\(^1\) 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 stearoyl-CoA desaturase-1 (SCD1)\(^1\), 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\(^1\). In fact many major pharmaceutical companies have at one point had SCD1 inhibitor programs due to the fact that inhibition or genetic deletion of SCD1 in mice decreases TG biosynthesis and protects against diet-induced obesity and diabetes\(^1\). 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\textsuperscript{15}. 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 TLR4 signaling and vascular inflammation\textsuperscript{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 one prominent strength of this study is the application of unbiased lipidomic technology to a prospective cohort with incident CVD endpoint data. However, there are several limitations that leave unanswered questions: 1) The shotgun lipidomics methods employed do not provide identification amongst isobaric species, so the specific molecular species that are a part of the CVD risk signature remain unknown. Related, whether more accurate quantification of the molecular species that constitute the “signature lipids” identified can be validated in independent prospective cohorts employing more specific mass spectrometry based analyses remains unknown; and 2) How does the plasma lipidome change temporally during key phases of atherosclerosis progression and/or regression? and 3) 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 identification of disease biomarkers or candidate species as was done in the work by Stegemann and colleagues.\textsuperscript{11} 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 provision of descriptive lipidomic lists to mechanistic studies using defined animal model systems is recommended. Indeed, it is an exciting time for the field of lipidomics, where we are no longer limited by technology, and innovative ideas will be the only limitation moving forward. Undoubtedly, lipidomics provides a rich resource of new information linking metabolism to human disease\textsuperscript{3-11}, and will be an important tool for drug discovery moving forward.

**Acknowledgments:** We thank David Schumick for assistance in the preparation of figures.

**Funding Sources:** J.M.B and S.L.H 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.

**Conflict of Interest Disclosures:** None.

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**Figure Legends:**

**Figure 1.** Growth of lipidomic technology over the last decade. Histogram represents the number of publications using lipidomics as a discovery platform. Numbers obtained by using “lipidomics” as a search term in PubMed database.

**Figure 2.** Proposed mechanistic insights into how CVD predicting signature lipids may promote the pathogenesis of atherosclerosis. Within the liver, the saturated fatty acids (palmitate, 16:0 and stearate, 18:0) are produced via de novo lipogenic pathways or derived from dietary sources. Following de novo fatty acid synthesis or acquisition from the diet, the saturated fatty acids (16:0 and 18:0) can be desaturated by the microsomal enzyme stearoyl-CoA desaturase 1 (SCD1) to form monounsaturated fatty acids (palmitoleate, 16:1 and oleate, 18:1). Both saturated and
monounsaturated fatty acids are selectively incorporated into certain complex lipid classes such as phospholipids (high level of saturated), triacylglycerols (high levels of 18:1), and cholesteryl esters (high levels of 16:1 and 18:1). The esterification of saturated vs. monounsaturated fatty acids into these distinct complex lipids is dictated enzymatically through the actions of the phosphatidylethanolamine N-methyltransferase (PEMT) or cytidine diphosphate-choline (CDP-choline) pathways for phospholipids (preference towards esterifying 16:0 and 18:0), acyl-CoA:cholesterol acyltransferase 2 (ACAT2) for cholesteryl esters (strong preference for esterifying 16:1 and 18:1), and diacylglycerol O-acyltransferase 1/2 (DGAT1 or DGAT2) for triacylglycerols (strong preference for esterifying 18:1). These complex lipids (16:1-CE, 54:2-TAG, and 36:5-PE) are then packaged into nascent very low density lipoproteins (VLDL), and these lipid cargo also remain in low density lipoproteins (LDL) following intravascular metabolism. Circulating LDL can deliver this lipid cargo to the artery wall where cell types such as lesional macrophage can de-esterify these complex lipids and then rebuild them using similar enzymatic mechanisms. Previous studies have shown that LDL containing monounsaturated cholesteryl esters (16:1) can preferentially be retained on arterial proteoglycans, and thereby promote atherogenesis. Additionally, it has been shown that reorganizing membrane saturated vs. unsaturated fatty acids 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.
Figure 1
Diet and de novo Lipogenesis

- Saturated FA (16:0, 18:0)
- Monosaturated FA (16:1, 18:1)
- PE MT or CDP- Choline pathways
- Cholesteryl Esters (16:1-CE)
- Triglycerides (54:2-TG)
- Phospholipids (32:1-PC, 36:5-PE)

VLDL
- 16:1-CE
- 54:2 TG
- 36:5-PE

LDL
- 16:1-CE
- 54:2 TG
- 36:5-PE

- Inflammation
- Atherogenesis
- Altered PRR Signaling

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Circulation, published online March 12, 2014;
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

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