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Potential and Caveats of Lipidomics for Cardiovascular Disease

Short title: *Pechlaner et al.; Lipidomics for Cardiovascular Disease*

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Since the seminal publications from the Framingham study in the mid-sixties,[1] the measurement of lipid levels, mainly of total cholesterol, total triglycerides, and LDL and HDL cholesterol, is routine clinical practice for cardiovascular disease (CVD) and lipid-lowering therapy. A more detailed assessment of the lipid composition, that is, the molecular species that constitute the lipid classes is not widely used, mainly due to the caveats of assessing the lipidome. The human lipidome is estimated to include thousands of molecular lipid species with functional diversity. The molecular lipid species within a lipid classes share a modular composition with fatty acids being attached to a common backbone. While a characteristic head group within the backbone defines the lipid class, the diversity of molecular lipid species derives from the conjugated fatty acids.[2] The conjugated fatty acids can differ in their carbon chain length, the number, position, and configuration (cis or trans) of their double bonds, and in the position and type (acyl, alkyl, or vinyl) of linkage to the backbone.

Lipidomics refers to the comprehensive profiling of lipids, facilitated by recent advances in mass spectrometry (MS).[2] The identified molecular lipid species are designated by abbreviations that combine the description of the lipid class with the characteristics of the conjugated fatty acids[3]. For example, a diglyceride (DG) with two fatty acids of 12 and 22 carbon atoms and 0 and 5 double bonds, respectively, both linked by an acyl linkage, can be described either as DG(12:0/22:5), or as DG(36:5). Most MS studies report the numbers of carbon atoms and double bonds either for the individual fatty acids or in total, but do not assign the precise positions of the fatty acids at the backbone or of the double bonds within the individual fatty acids.

Sample extraction is key for lipidomic analysis. Traditionally, a two-phase method has been used. The most popular one is the Folch extraction method with cholorform/methanol.[4]

Methanol precipitates the proteins whilst chloroform ensures effective extraction of a broad range of lipid classes from the precipitated lipoproteins. The two-phase extraction, however, is laborious and not very amenable to automation. Single-phase methods are less time demanding, but concerns have been raised that the lipid extraction might be less uniform and less efficient.[5]

Liquid chromatography (LC) is applicable to a broad range of lipid classes and thus the principal separation method used in lipidomics. Chromatographic separation of the lipid extract enhances sensitivity and specificity by reducing sample complexity, but can also add technical variability.[6] Before MS analysis, the lipids have to be ionized by electrospray ionization. Then, the mass-to-charge ratio of the lipid species can be determined by MS. Next, fragmentation is commonly induced by tandem MS to enable the structural elucidation of the lipid species. For quantitation, stable isotope-labelled standards are spiked into the samples before lipid extraction to account for technical variation. The addition of authentic standards (targeted MS analysis) enhances accuracy of analyte identification and quantitation compared to untargeted MS analysis without authentic standards.[7] Due to economic considerations and lack of availability, a limited set of standards is commonly used, and relative quantitation is achieved by linear extrapolation through response factors.[6]

This issue of *Circulation* features two studies that break new ground in relating lipid species to cardiometabolic outcomes.

Alshehry and co-workers investigated 310 lipid species within 22 lipid classes in almost 4000 subjects with diabetes and found a signature of 42 lipid species associated with incident CVD, cardiovascular death, or both.[8] The lipid signature determined using targeted LC-ESI-tandem MS was dominated by glycerophospholipids and sphingolipids. Addition of select lipid species to conventional risk factors entailed moderate improvements in CVD risk prediction and

replication in independent patient samples was in part successful. Lipids included in the cardiovascular events model were: PC(O-36:1), CE(18:0), PE(O-36:4), PC(28:0), LPC(20:0), PC(35:4), LPC(18:2). Lipids included in the cardiovascular death model were: PC(O-36:1), DG(16:0/22:5), SM(34:1), PC(O-36:5). Alshehry and colleagues integrated their findings for PCs and PEs with underlying enzymatic pathways.[8] Only by considering lipid species in the context of their metabolism can new mechanistic knowledge be generated.

Syme and co-workers,[9] using targeted LC-ESI-MS, report the first lipidomic study conducted in adolescents. In almost 1000 participants, they measured 69 phosphatidylcholines (PC), i.e. members of the glycerophospholipid class, of which 21 were associated with at least one CVD risk factor out of visceral adiposity, blood pressure, insulin resistance, and atherogenic dyslipidemia. PC(16:0/2:0), a platelet-activating factor possessing a 2-carbon acetyl at its sn-2 position, and PC(14:1/0:0), a lysophosphatidylcholine possessing only one fatty acid of 14 carbon atoms, showed the strongest inverse and direct associations with CVD risk factors, respectively. The findings by Syme and colleagues point towards detrimental effects of CVD risk factors on biologically active molecular lipid species, such as PC(16:0/2:0),[9] that may impact on platelet activation.

At a first glance, the difference in the final selection of molecular lipid species is apparent, also with regards to previous lipidomics studies on cardiometabolic disease.[10-15] This is not unexpected given the sources of variability in lipidomics measurements (**Figure 1**). However, there are also consistencies:

A number of studies targeting CVD and diabetes unraveled associations with cholesteryl esters (CE),[13,15] predominantly of species with shorter-chained and more saturated fatty acids, like CE(16:0) and CE(16:1). The relevance of CE is expected to decline with statin therapy.

About 40% of the diabetic patients analyzed by Alshehry et al[8] received lipid-lowering medication. Yet, CE(16:0) still emerged as significantly associated with incident CVD and CE(18:0) was in their final selection.

Triacylglycerols (TG) were part of adverse lipid signatures identified previously.[12-15] Alshehry et al[8] detected a signal of protection for TG(56:6) but no TG species were in their final selection. Potential reasons for this discrepancy are limited variability of TG levels in diabetic dyslipidemia, the use of lipid lowering medication including fibrates, life-style modifying strategies including diet, and the single-phase extraction procedure applied in this and another study, which failed to obtain an association for TG.[8,12] Single-phase extraction methods may result in less efficient extraction of TG.[5]

Lyso-PCs relate to the risk of CVD, diabetes and other metabolic abnormalities in most studies available so far including those published in the current issue of Circulation[7,8] but the selected molecular species and their directionality differ. It remains to be defined whether this reflects biological variation as suggested[8,9] or arises from methodological differences in the measurements.

The perhaps most consistent finding in the published lipidomic studies so far is a general shift in fatty acid chain length across multiple lipid classes in men and women. Species with shorter or more saturated fatty acid substituents confer risk, while species possessing longer or polyunsaturated fatty acids offer protection.[8,13] This is corroborated by a large metabolic profiling study using nuclear magnetic resonance spectroscopy, an entirely different technological platform from MS: plasma monounsaturated fatty acids (MUFAs, which are generated from saturated fatty acids by the action of stearoyl-CoA desaturase-1), were again directly associated with incident CVD, whereas polyunsaturated fatty acids (PUFAs) showed an

inverse association.[16] This is in line with previous research on dietary fatty acids and CVD.[17] It also implicates a role for hepatic *de novo* lipogenesis, the endogenous synthesis of fatty acids from glucose, which is influenced by lifestyle and diet. Hepatic *de novo* lipogenesis produces mostly saturated fatty acids with 16 to 18 carbon atoms and at most a single double bond. Therapeutic inhibitors of *de novo* lipogenesis have been developed and studied in obesity.

A necessary next step towards clinical application of lipidomics is the standardization of the measurement methods. Lipidomics has potential in the context of cardiometabolic disease, but requires methodological standardization and better inter-laboratory reproducibility before its power can be harnessed towards clinical application. Another important consideration is that most plasma lipids are associated with apolipoproteins, and thus associations of lipid species with CVD may partly reflect associations of apolipoproteins with CVD. Apart from apolipoprotein A1 and apolipoprotein B, other apolipoproteins have not been extensively explored in the context of lipidomics measurements to date. It will be interesting to see which of the two postgenomic technologies, lipidomics or proteomics, will deliver new biomarkers with clinical utility for CVD.[18,19]

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Conflict of Interest Disclosures: The authors have filed patents on biomarkers for cardiovascular disease, including molecular lipids.

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Figure Legends

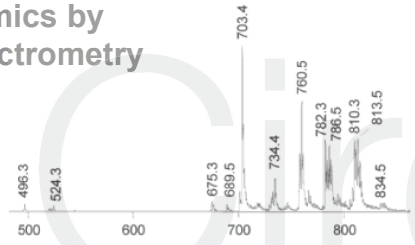
Figure 1. Lipidomics for CVD. Summary of the potential for clinical translation and the current caveats of lipid profiling. Sources of variability include the clinical characteristics, such as medication (heparin administration and lipid lowering therapy), pre-analytical variation (blood sampling and storage) as well as the different methods for lipid extraction, MS measurements and statistical analysis of the multidimensional, highly correlated lipid data. Standardization will be required for any future clinical application of lipidomics profiling for CVD.

Figure 1

Sources of variability

Population & clinical characteristics	Pre-analytical variability	Measurement	Statistical analysis	Random noise
demographic, genetic, lifestyle, comorbidities, medication, disease stage, etc.	blood sampling, storage and processing	extraction method, separation, ionization, mode of MS, use of standards, etc.	high data dimension, variable selection, multicollinearity and multiplicity	True biological variation

Lipidomics by Mass Spectrometry



Myocardial infarction

Stroke

Diabetes & Metabolic syndrome

T2DM

Potential future clinical translation

Improvement of CVD risk prediction and risk stratification of patients	Discovery of disease mechanisms and underlying molecular pathways	Diet and life-style-based modification of lipid quality	Novel therapeutic targets (e.g. change in lipid composition)

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