Lipidomics

Quest for Molecular Lipid Biomarkers in Cardiovascular Disease

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Abstract—Lipidomics is the comprehensive analysis of molecular lipid species, including their quantitation and metabolic pathways. The huge diversity of native lipids and their modifications make lipidomic analyses challenging. The method of choice for sensitive detection and quantitation of molecular lipid species is mass spectrometry, either by direct infusion (shotgun lipidomics) or coupled with liquid chromatography. Although shotgun lipidomics allows for high-throughput analysis, low-abundant lipid species are not detected. Previous separation of lipid species by liquid chromatography increases ionization efficiency and is better suited for quantifying low abundant and isomeric lipid species. In this review, we will discuss the potential of lipidomics for cardiovascular research. To date, cardiovascular research predominantly focuses on the role of lipid classes rather than molecular entities. An in-depth knowledge about the molecular lipid species that contribute to the pathophysiology of cardiovascular diseases may provide better biomarkers and novel therapeutic targets for cardiovascular disease. (***Circ Cardiovasc Genet***. **2014;7:941-954.**)

Cardiovascular diseases (CVDs) are responsible for almost one third of all deaths worldwide. However, the bulk of CVDs cannot be explained by traditional risk factors alone. Many patients have lipid levels within the recommended range.[1] Thus, there is a need for additional biomarkers for early diagnosis and prevention of CVD. With the complete sequencing of the human genome, attention has shifted to postgenomic technologies, including metabolomics. The first metabolomics studies predominantly focused on metabolites that are easier to analyze. In comparison, lipids are less amenable to comprehensive analysis because of the sheer complexity of the lipidome with tens of thousands of different lipid species.[2] Labor-intensive workflows and different instruments are required to interrogate the lipidome. Thus, lipidomics is not widely used as reflected in the limited number of publications when compared with other-omics technologies (Figure 1).

The paucity of lipidomics data is particularly relevant in the context of CVD. Our understanding of the role of lipids in the pathophysiology of CVDs is mainly confined to lipid classes, such as total triglycerides (TG), rather than detailed molecular entities (ie, the type of fatty acids [FA] that are conjugated to the glycerol backbone). Different molecular lipid species exert different biological effects and important information may be missed by sum measurements. It is well established that lipids and oxidized lipids are involved in several inflammatory diseases, including atherosclerosis.[3] A systematic study of the lipidome, including the structural characterization, identification, and quantification of molecular lipid species, will be an important first step to identify the most relevant candidates for CVD. Deciphering their function will require additional detailed studies of their metabolism and biological activity, as well as their tissue distribution.

Lipids in a Nutshell

Lipids are defined as hydrophobic or amphipathic small molecules that originate entirely or, in part, from carbanion-based condensation of thioesters (ketoacyl groups) and carbocation-based condensation of isoprene units (isoprene groups).[4] The polar head group of lipids in combination with the hydrophobic tails provides the basis for the energy-driven formation of lipid bilayers within the aqueous, polar cellular environment. Lipid species play key roles in cellular membranes, in cell signaling, and in cell metabolism. Table 1 gives an overview about the lipid classes, the major subgroups, and examples of their biological function. On the basis of the chemical structure, lipids are divided into 8 main categories:

1. FAs: FAs are circulating as free FAs bound to albumin or form components of complex lipids.
2. Glycerolipids: glycerolipids are esters of glycerol that are mono-, di-, or trisubstituted with FA. TG are stored as energy source and can be released during fasting. In comparison with proteins or carbohydrates (4 kcal/g; 17 kJ/g), the caloric value of lipids (9 kcal/g; 38 kJ/g) is twice as high.
3. Glycerophospholipids: glycerophospholipids are the main membrane components, including phospholipids such as phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, and phosphatidic acid. They are also important for cellular signaling (ie, phosphatidylinositol, phosphatidylinositol monophosphate, phosphatidylinositol bisphosphate). Cardiolipin is a diphosphatidylglycerol lipid and essential for mitochondrial function.

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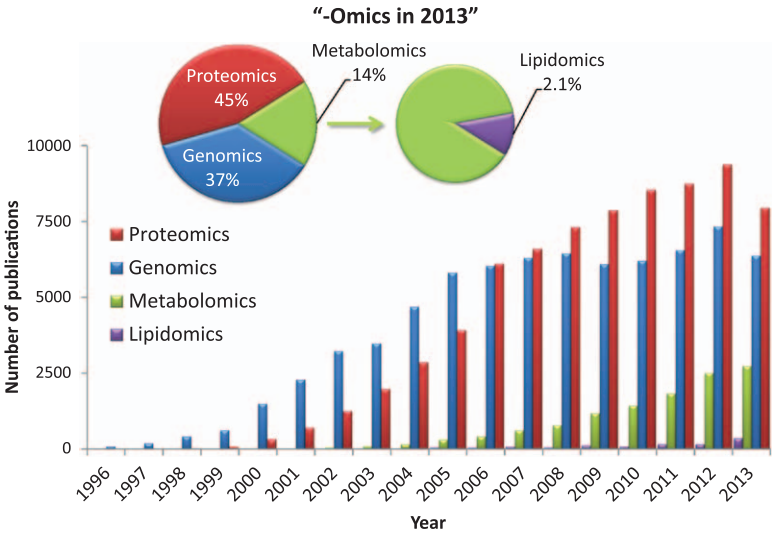

Figure 1. Number of -omics publications found by SciFinder keyword search for proteomics, genomics, metabolomics, and lipidomics, respectively (January 9, 2014). The insert shows the percentage distribution of published articles in 2013 and the under-representation of lipidomics.

4. Sphingolipids: sphingolipid species, such as sphingomyelin, sulfatides, and cerebrosides, are important for myelin function and stability in nervous cells. Ceramides are regulators for cell differentiation and apoptosis.
5. Sterol lipids: sterol lipids, such as cholesterol and cholesteryl esters (CE), are responsible for the membrane fluidity and form steroid hormones (estrogens, testosterones, and cortisoides).
6. Prenol lipids: prenol lipids have diverse functions, such as the regulation of cell and tissue growth and differentiation (vitamin A), antioxidant properties (vitamin E), post-translational modification of proteins (vitamin K), or hormone-like function (vitamin D).
7. Saccharolipids: saccharolipids are lipids linked with a sugar backbone and found in Gram-negative bacteria as precursor of the lipid A component.
8. Polyketides: several polyketides (PK) are used as antibiotics and drugs, whereas the PK aflatoxin is a natural mycotoxin.

Each of these 8 categories contains distinct classes and subclasses that are listed in the LipidMaps database. Examples are shown in Figure 2.

Another classification system of lipids is based on the charge properties in weak acidic pH conditions. It divides lipid species into anionic lipids (cardiolipin, phosphatidylglycerol, phosphatidylinositol phosphatidylserine, phosphatidic acid, sulfatide, their lysolipids, and acyl CoA) carrying ≥1 net charge; charge-neutral, weak anionic lipids (phosphatidylethanolamine, lyso-phosphatidylethanolamine, nonesterified FA and their derivatives, bile acids, and ceramides); and charge neutral, polar lipids (phosphatidylcholine, lysophosphatidylcholine, sphingomyelin, HexCer, acylcarnitine, mono- and diacylglycerol, TG, and cholesterol and its esters).[2] The chemical diversity in the lipid head group (lipid classes), the covalent bond of FA at the sn-1 position of glycerol (lipid subclasses), the alteration in FA chain length, and the number and location of double bonds allow a calculation of the possible combinations for complex lipids. Estimates are in the range of 36 000 molecular lipid species (ie, 6500 species for glycerophospholipids, 3200 species for sphingolipids, and 100 species for sterol lipids), not counting isomers, oxidized lipids, or other covalently modified entities.[2]

Detailed information about nomenclature of lipid species can be found elsewhere.[4–6] Databases, Web-based search engines, protocols, and software tools for lipidomics are available free of charge, as well as commercially (Table 2). A unified lipid nomenclature and classification provide the basis for the development of new methods and a better understanding of the role of lipid species in health and disease.

Preanalytic Considerations

With increasing lipid hydrophobicity (ie, increase of FA chain lengths and degree of saturation), lipid concentration, and solvent polarity, the critical micellar concentration decreases and lipids aggregate more easily (eg, they form micelles and bilayers). Lipids also readily stick to plastic surfaces. Moreover, organic solvents commonly used for lipid extraction, such as chloroform/methanol can extract plasticizers (ie, polyethylene glycol and polypropylene glycol). Such contaminants complicate the interpretation of mass spectrometry (MS) spectra and can suppress the signals from the analytes. Thus, the use of plastic ware must be avoided during sample preparation. Instead, samples should be stored and handled in glass vials.

Solvent composition, polarity, and pH are important factors for not only lipid extraction but also detection. Solid phase extraction is useful for the reduction of polar and nonpolar contaminants and for sample prefractionation before MS reducing matrix effects and thereby enhancing sensitivity.[7] Sample storage and sample preparation are crucial steps that can lead to unexpected losses of lipid species or lipid oxidation. Antioxidants (eg, butylated hydroxytoluene), metal chelators, and reducing agents minimize oxidation of polyunsaturated FAs (PUFA). For long-time sample storage, the sample should be frozen at −80°C under nitrogen and repeated freeze–thaw cycles must be avoided.

Nuclear Magnetic Resonance: Measurement of Lipoprotein Particle Size

High-density lipoprotein-cholesterol (HDL-C) and total cholesterol are routinely measured for CVD risk assessment and

Table 1. Overview of Lipid Classes and Examples of Their Biological Functions

Lipid Category	Biological Function
FA	Major component/precursors of more complex lipid species Energy-source, released from triglycerides during fasting Acetyl-CoA involved in the citric acid cycle (Krebs cycle) Linoleic acid and linolenic acid are considered essential FA Polyunsaturated FA (ω-3-FA, ω-6-FA, and ω-9-FA) play a key role in metabolism
GL	Includes subgroups of monoglycerol, diglycerol, and TG TG as the main storage of FA Seminolipid is a testis-specific sulfoglycolipid
GP	Major component of cellular membrane Phosphatidylcholine (lecithine) and phosphatidylethanolamine as most abundant lipid species in membranes LPC: product of phospholipase Phosphatidylserine found in cellular membranes Phosphatidic acid influences membrane curvature Phosphatidylinositol, phosphatidylinositol monophosphate, and phosphatidylinositol biphosphate mainly in the inner membrane and participate in signaling Phosphatidylglycerol is a pulmonary surfactant Diphosphatidylglycerol (Cardiolipin) important lipid for mitochondrial function
SP	Sphingomyelins are highly concentrated in the membranes of the myelin sheath of neurons Sulfatides play a role in myelin function and stability Ceramides are bioactive lipids in the regulation of apoptosis and cell differentiation Cerebrosides are found in brain and nerve cells
ST	Cholesterol and CE maintain membrane fluidity Bile acids are end products of cholesterol and involved in processing of dietary fat Important role as steroid hormones (ie, estradiol and testosterone) Glucocorticoides play an important role in immune system and inflammation
PR	Fat-soluble vitamins, such as vitamin A, E, and K Derivatives of quinone act as electron acceptors in electron transport chain Isoprenes are produced by many plants
SL	Precursor of the lipid A component in Gram-negative bacteria
PK	Used in pharmacy as antibiotics, antifungals, cytostatics, anticholesteremic, antiparasitics, coccidiostats, animal growth promoters, and natural insecticides Aflatoxin as mycotoxin

CE, cholesteryl esters; FA indicates fatty acids; GL, glycerolipids; GP, glycerophospholipids; LPC, lysophosphatidylcholine; PC, phosphatidylcholine; PK, polyketides; PR, prenol lipids; SL, saccharolipids; SP, sphingolipids; and ST, sterol lipids.

for monitoring a patient's response to lipid-lowering therapy.[8] Low-density lipoprotein cholesterol (LDL-C) is not always measured directly but often calculated according to the Friedewald formula.[9] Nuclear Magnetic Resonance (NMR) provides a rapid method for distinguishing and quantifying a wider range of lipoprotein subclasses. On exposure to a magnetic field, distinct lipoprotein subclasses emit a unique signal that is directly proportional to their concentration.[10,11] In a population-based study of 9399 Finnish men, concentrations of lipoprotein subclasses and lipid composition were altered in subjects with impaired glucose tolerance.[12] Mackey et al[13] reported a stronger association of HDL particle number with CVDs than lipoprotein-cholesterol content. Furthermore, HDL particle number measured by NMR has been implicated as a superior biomarker for residual vascular risk after statin therapy when compared with HDL-C or ApoA-I.[14] Other applications of NMR in lipid research include studies of the structures and cellular functions of lipids, interaction with proteins, peptides, and small molecules, as well as diagnostic strategies by magnetic resonance spectroscopy and magnetic resonance imaging (MRI).[15] NMR provides a useful tool for lipid structure analysis in both solution and solid state; however, it lacks sensitivity and requires more sample when compared with MS.

MS: Measurements of Molecular Lipid Species

MS is the method of choice for identification and quantification of lipid species with high sensitivity and selectivity. Figure 3 provides an overview about the different lipidomics methods:

1. Matrix-assisted laser desorption/ionization[16,17] and other desorption techniques, such as desorption electrospray ionization MS[18] and liquid extraction surface analysis,[19] allow the analysis of lipid species in samples directly without previous separation by liquid chromatography (LC). Desorption techniques are also used for tissue imaging to determine the spatial distribution of lipid species.[20]
2. Electrospray-ionization is a low-energy ionization (soft-ionization) technique, and the most common method for lipid characterization because of its minimal in-source fragmentation.[21] It can be used for direct infusion of lipid extracts (shotgun lipidomics) or coupled with LC (LC-MS).
3. Additional information for lipid characterization is obtained by ion mobility MS. The different mobility of ions is a function of their shape and their size (the collisional cross section), allowing the separation of isomers and isobars (eg, *cis*/*trans* PL isomers, PUFA unsaturation positional isomers).[22]

Separation of Lipid Species

For detection and quantification of low-abundant lipid species, chromatographic separation is required to reduce sample complexity before MS.[23] Otherwise, high abundant compounds suppress the ionization of coeluting low-abundant compounds. This phenomenon is termed ion-supression and can be reduced by LC separation of different lipid species before electrospray-ionization. The reduction of lipid interferences and coionization increases the number of detectable species. However, changes in ionization efficiency can occur during gradient elution caused by lipid enrichment within the same chromatographic peak or by changes in the mobile phase composition. Furthermore, high aqueous content in the mobile phase at the beginning of the gradient elution can cause solubility problems for polar lipids. This has to be taken into consideration for accurate quantification using standards for internal or external calibration. Column memory effects resulting in lipid carryover between chromatographic runs are

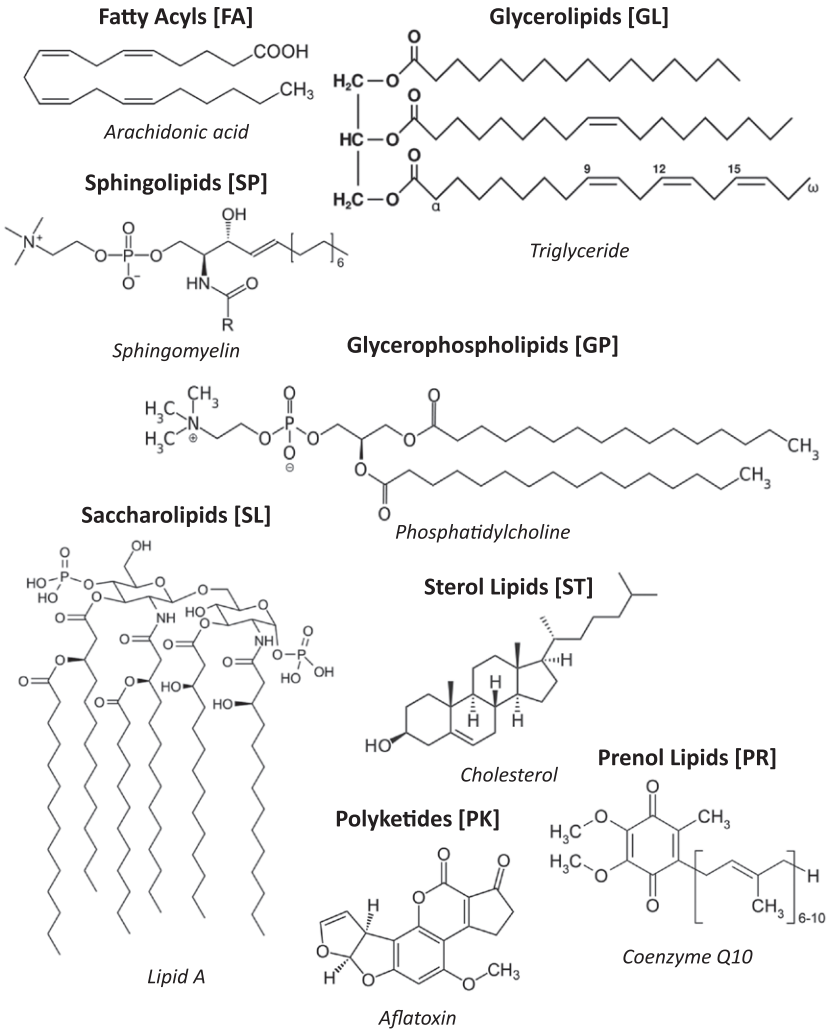

Figure 2. Lipid species can be classified in 8 major lipid classes and several subgroups. Examples for the diversity of lipids representative for the 8 lipid classes are provided. FA indicates fatty acid; GL, glycerolipids; GP, glycerophospholipids; PK, polyketides; PR, prenol lipids; SL, saccharolipids; SP, sphingolipids; and ST, sterol lipids.

another concern. An advantage of chromatographic separation is the possibility to separate isomeric and isobaric compounds (eg, *cis*/*trans* PL isomers, PUFA unsaturation positional isomers), which cannot be distinguished by MS alone.

Normal phase and reversed phase LC are the most common stationary phases for lipid species.

1. Normal phase LC is used for lipid class separation based on their different polarities and dipole moments. The nonpolar mobile phase, however, is less effective in generating an optimal ionization spray.[24]
2. Reversed phase LC is better suited for coupling with electrospray-ionization. It separates lipids according to their hydrophobicity (ie, PLs according to their FA chain length and degree of saturation). The mobile phase consists of an aqueous buffer with organic solvents (methanol, acetonitrile, isopropanol, hexane, etc.) and ion-pairing reagents (eg, ammonium acetate, ammonium formate, formic acid, and acetic acid). It is also suitable for separation of oxidized PL. They elute earlier because of their higher polarity when compared with nonoxidized PL.
3. Alternative chromatographic techniques for lipid separations[25] are hydrophilic interaction LC,[26] supercritical fluid chromatography,[27,28] mixed mode columns and chiral stationary phases,[28] silver ion high performance LC, and gas chromatography. Gas chromatography MS (GC-MS) is mainly used for volatile analytes. Alternatively, analytes must be amenable to chemical derivatization to render them volatile for GC-MS analysis.

Bottom-Up Lipidomics: LC-MS/MS

In bottom-up lipidomics, lipids are analyzed according to their specific fragmentation pattern obtained by tandem MS (MS/MS). In combination with chromatographic separation, it allows accurate quantification of low-abundant lipid species. Triple quadrupole MS combined with LC is the workhorse for targeted measurements of individual metabolites, including lipids. In general, a triple quadrupole MS consists of 3 quadrupoles: 2 of which serve as mass analyzers, whereas the middle one is used as a collision cell. A collision gas produces structure-specific fragment ions. This setup allows different scan options and is commonly used for the identification of individual lipid classes and subclasses based on specific fragmentation behavior of ionized lipid species (Figure 4).

1. The precursor ion (PI) scan mode detects all precursor or parent ions, which produce a selected product ion after fragmentation. This is a powerful method to analyze

Table 2. Overview of Databases and Software Available for Lipidomics

Database/Software	Links to Websites	Comment
AMDMS-SL	www.shotgunlipidomcs.com	A comprehensive freeware package designed for shotgun lipidomics
European Lipidomics Initiative	www.lipidomicnet.org/	European Lipidomics Consortium
LIMSA	www.helsinki.fi/science/lipids/software.html	LIMSA can assist peak finding, integration, and assignment, isotope correction and quantitation with internal standards.
LipidBank	www.Lipidbank.jp	Open-access, Web-based search engine: 7009 lipid species
Lipid MAPS	www.LipidMAPS.org	Open-access, Web-based search engine: 37 566 lipid species entries
Lipid View	www.absciex.com	Data processing tool for the molecular characterization and quantification of lipid species from ESI-MS data
LipidX	www.systemsx.ch	A subset of the systems biology freeware System X
LipidXPLORER	https://wiki.mpi-cbg.de/lipidx/Main_Page	Shotgun MS and MS/MS spectra
MassBank	www.massbank.jp	>40 000 MSn spectra
Metlin	www.metlin.scripps.edu	>240 000 metabolites entries
MZmine2	www.mzmine.sourceforge.net	Open-source project delivering a software for MS data processing, with the main focus on LC-MS data
SphinGOMAP	www.sphingomap.org	Sphingolipid biosynthesis pathway: 450 sphingolipid entries

AMDMS-SL indicates automated lipid identification and quantification by multi-dimensional mass spectrometry-based shotgun lipidomics; DCM, dilated cardiomyopathy; ESI-MS indicates electrospray ionisation-mass spectrometry; EUFAM, European Study of Familial Dyslipidemias; and LC-MS, liquid chromatography-MS; LFAT, liver fat; LIMSA, lipid mass spectrum analysis; LMNA, Lamin A/C gene; MALDI, matrix-assisted laser desorption/ionization; and RADAR, Rosuvastatin and Atorvastatin in different Dosages And Reverse cholesterol transport.

lipid species with common structural motifs and allows distinguishing lipid classes and subclasses within the same spectrum, while simultaneously reducing chemical noise. For example, phosphatidylcholine and sphingomyelin can be detected by their fragment ion with a mass-to-charge ratio (m/z) of 184.1 in positive mode and by their fragment ion with m/z 241.0 in negative mode.

2. The product ion scan mode analyzes the fragment or daughter ions of a selected precursor ion of interest and is a useful tool to study lipid fragmentation patterns and kinetics.
3. Neutral loss (NL) scans detect ions that show a constant mass difference between the 2 mass analyzers. The NL is the offset that can be observed between the precursor mass and the fragment mass for a particular class of compounds. NL scans allow the detection of lipid classes or groups with a specific neutral-loss fragment, such as phosphatidylethanolamines with NL of 141.9 Da and phosphatidylserine with NL of 185.0 Da in positive mode or of phosphatidylserine with a NL of 87 Da in negative ion mode.
4. Selected reaction monitoring provides the highest specificity and sensitivity and is the method of choice in targeted lipidomics for lipid quantitation. The mass analyzers detect a selected precursor ion and its fragment ions, whereby the best transitions between the precursor ion/fragment ion mass pair have to be known or experimentally determined in advance.

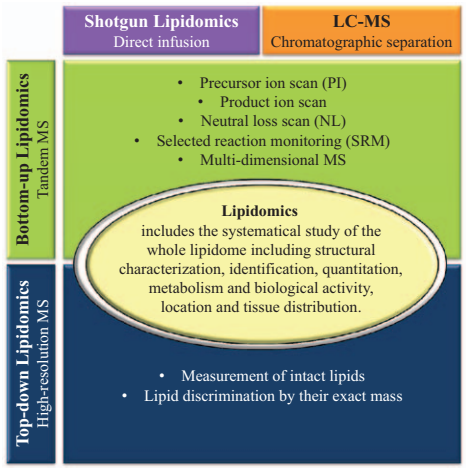

Figure 3. Schematic overview about lipidomic methods (**top**) distinguishing shotgun lipidomics and previous chromatographic separation (liquid chromatography-mass spectrometry [LC-MS]). Both strategies can be used in bottom-up and top-down approaches.

Bottom-Up Shotgun Lipidomics Using a Triple Quadrupole MS

The different scan options of a triple quadrupole MS (PI, NL, and product ion scans) can be combined as inter-related modes in a multidimensional MS-based approach often used in shotgun lipidomics.[2] Shotgun lipidomics simultaneously screens and analyzes lipid species in nonseparated lipid extracts by direct infusion. It detects the head group or neutral-loss–specific fragmentation of different lipid classes and subclasses and uses class-specific standards. Thus, it does not require a priori decisions on which lipid species to measure. The analysis of the lipidome by direct infusion excludes variation by LC and minimizes sample cross-contamination in a high-throughput setting. Shotgun lipidomics, however, allows detection and quantification of only abundant lipid species.[2] For example, polar lipids, such as PL and sulfatides, are easily ionized and readily detected. Ion suppression of low-abundant lipids and of lipids that do not ionize well interferes with accurate quantification. Similarly, aggregated lipids are not ionized efficiently, and the recommended upper limit of the total lipid concentration for shotgun lipidomics is 100 pmol/μL in 2:1

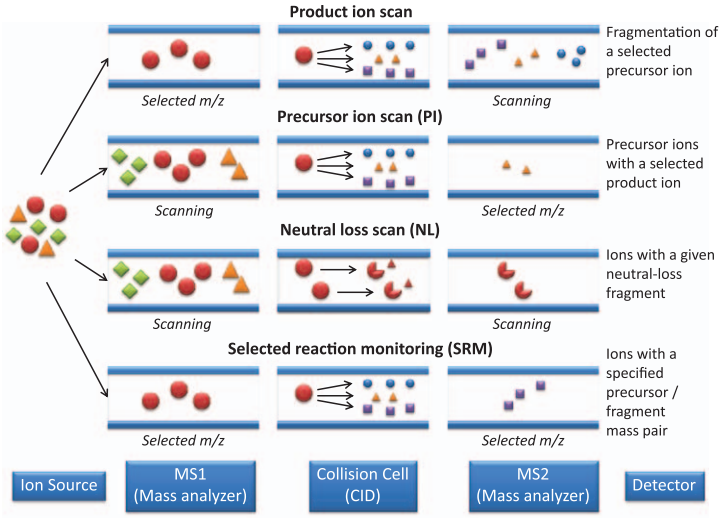

Figure 4. Schematic overview about the different scan options on a triple quadrupole MS. *m/z* indicates mass-to-charge ratio; NL, neutral loss scan; PI, precursor ion scan; and SRM, selected reaction monitoring.

(v/v) chloroform/methanol.[2] Moreover, distinguishing the FA chain lengths, the number of double bonds and the linkage to the glycerol backbone is difficult and requires high-resolution MS and chromatographic separation before MS.

Top-Down Shotgun Lipidomics Using High-Resolution MS

In top-down shotgun lipidomics, the identification and quantification of intact lipid species rely on accurate masses and abundance of their molecular ions, respectively. Shotgun lipidomics provides the long acquisition time necessary for high mass resolution and allows the full mass spectrum scan of lipid ion species. On the basis of MS/MS alone, phosphatidylcholine and sphingomyelin species cannot be distinguished because both lipid species have the same head group and produce fragment ions of *m/z* 184.1 in the PI scan. However, they can be discriminated by their exact mass because of their different heteroatom compositions (NO_8P and N_2O_6P, respectively). Sphingomyelin signals with 2 nitrogen atoms seem at odd *m/z* values, whereas phosphatidylcholine signals occur at even *m/z* values. This nitrogen rule allows a discrimination of the 2 lipid classes. Thus, a top-down lipidomic strategy can discriminate intact lipids on their unique number of N, O, and P atoms in their molecular cations or anions and by their different ionization efficiency in negative and positive modes.

High-resolution MS (including Orbitrap and ion cyclotron resonance instruments) with resolving power >100 000 and a mass accuracy of subparts per million provides new possibilities in the unambiguous identification and accurate quantification of lipid species with similar masses. For example, phosphatidylcholine and phosphatidylserine with 1 additional double bond are isobaric species with the same nominal mass but differ in the exact masses by 0.0726 Da.[29] Hydroxylated lipids with 1 carbon chain unit less and an additional double bound differ by 0.03699 Da and peroxylated lipids with 2 chain units less and 2 double bonds more by 0.07277 Da. Only high-resolution MS can reliably discern such lipid species by exact mass. However, some lipid species, ie, peroxylated lipids and 2× hydroxylated lipids, have the same exact mass and cannot be differentiated even by high-resolution MS. Alternatively, isobaric species can be also grouped by their different ionization capacity in positive and negative modes, such as phosphatidylcholine and phosphatidylethanolamine, which form in the negative mode adducts with acetate or formate anions and deprotonated anions, respectively.[29]

Quantitation of Lipid Species

Quantification can be either relative or absolute. Relative quantification compares the signal intensities of the samples to controls under identical conditions and is widely used in biomarker profiling. Absolute quantification requires an internal or external standard with similar structure to the analyte. An external standard can be used to establish a calibration curve. Using the analyte of interest itself for external calibration offers the advantage of identical molecular structure. External calibration avoids lipid–lipid interferences during ionization and peak overlap in the mass spectrum. Drawbacks of external calibration arise from alterations because of differences in sample preparation, lipid and matrix composition, or different ionization efficiency as a result of the chromatographic conditions and spray instability during electrospray-ionization analysis. Calibration with an internal standard (IS) avoids the problems of sample preparation and can be used also for recovery calculations. The IS should have similar chemical structural properties when compared with the analyte of interest (eg, a stable isotope-labeled analogue). The accurate quantification allows the stoichiometric comparison between different lipid species and also between different samples. Several studies demonstrated that the ionization efficiency and the response factor of low-abundant lipid species are mainly dependent on the polar head group and less affected by the FA chain length and saturation.[30] Thus, in shotgun lipidomics, 1 IS can be used per polar lipid class. Another strategy is based on the response factor as ratio of the calibration slope of a lipid class to the slope of the IS. The absolute concentration can be calculated by the ratio of the lipid species to the IS multiplied by the response factor and the known concentration of the IS.[26] However, 1 IS may not be sufficient for nonpolar lipid classes, such as TG and polar lipids, in concentrations above the critical micellar concentration.[30]

Table 3. Clinical Studies Using Lipidomics in the Context of Cardiovascular Disease, Diabetes Mellitus, and Related Metabolic Disorders

References	Name of Study/ Description	Study/Diseases	Study Size (% Women)	Age, y	BMI, kg/m^2	Sample	Method
Family/population-based studies							
Gonzalez-Covarrubias et al[33]	Leiden Longevity Study	Middle-aged offspring of nonagenarians	1526 (43)	58.8±7.4	25.8±3.6	Citrate plasma	LC-MS
		Spouses	675 (42)	59.4±6.6	25.3±3.6		
Weir et al[38]	SAFHS	Population-cohort	1076 (61)	35.7 (24.7–48.9)	28.5 (24.6–33.0)	Plasma	LC-MS
Kulkarni et al[39]		Hypertension	42 Extended families				
Andersson et al[34]	PIVUS	Entire cohort	942 (49)	70	…	Serum	ELISA; echogenicity of carotid plaques
		Hypertension	678 (52)				
		Heart failure	38 (37)				
		Diabetes mellitus	85 (39)				
		Myocardial infarction	57 (25)				
		Previous stroke	19 (26)				
Stegemann et al[37]	Bruneck Study	Population-based, prospective	<1000 (50)	50–80	…	Plasma	Shotgun lipidomics; ELISA
Tsimikas et al[35]		Cardiovascular events					
Case–control studies: diabetes mellitus							
Rhee et al[41]	Framingham Heart Study	CTRL	189	56±9	30.5±5.0	Plasma	LC-MS
		T2D	189	57±8	30.0±5.5		
Stahlman et al[42,43]	DIWA	CTRL	20 (100)	64	24±3.3	EDTA plasma	Offline LC-MS
		T2D (normolipidemic)	20 (100)		28±2.7		
		T2D+dyslipidemic	20 (100)		32±4.5		
Zhao et al[44]	Lipidomics and gene expression	CTRL	60 (47)	58.5±16.1	30.1±7.3	Peripheral blood	Shotgun lipidomics; gene expression
		T2D	84 (54)	63±13	34.2±8.4		
Sorensen et al[45]	Diabetes Antibody Standardization Program	CTRL	10	<30	…	Plasma and serum	LC-MS
		T1D	10				
Schwartzman et al[46]	Diabetic retinopathy	CTRL	14	…	…	Vitreous	LC-MS
		NPDR (diabetic)	18				
		PDR (diabetic)	12				
Case–control studies: cardiovascular disease							
Sysi-Aho et al[48]	DCM	Asymptomatic LMNA mutation carriers	11 (64)	…	…	Serum	LC-MS; cardiac MRI
		CTRL (LMNA gene)	11 (64)				
		Unrelated patients with DCM	8 (63)				
		CTRL (DCM)	8 (63)				

(*Continued*)

Table 3. Continued

References	Name of Study/ Description	Study/Diseases	Study Size (% Women)	Age, y	BMI, kg/m^2	Sample	Method
Lankinen et al[49]	Acute myocardial infarction or unstable ischemic attack during the previous 3–36 mo	Fatty fish diet	11 (27)	62.1±6.3	26.7±3.1	Plasma	LC-MS; GC
		Lean fish diet	12 (17)	60.7±5.1	27.8±2.1		
		CTRL	10 (10)	60.2±6.4	27.0±2.9		
Graessler et al[50]	Hypertension	Normotensive	51 (0)	51.7 (47.3–56.1)	25.6 (24.7–26.5)	EDTA plasma	Shotgun lipidomics
		Hypertensive	19 (0)	57.3 (50.8–63.9)	27.8 (26.3–29.3)		
Spijkers et al[51]	Hypertension, Ceramide	CTRL	18 (61)	44.1±2.5	27.3±1.3	Plasma	LC-MS
		Stage 1 hypertension	12 (25)	44.1±2.8	26.0±0.9		
		Stage 2+3 hypertension	19 (53)	47.4±2.6	26.8±0.9		
Stegemann et al[52]	Carotid endarterectomy	Atherosclerotic plaque	26	…	…	Plasma; plaques	Shotgun lipidomics
		Plasma	35	69±8.8	25.4		
Case–control studies: metabolic disorders							
Laurila et al[40]	EUFAM family study (n=450)	Low HDL-C	227 (37)	54.6±0.7	27.9±0.6	Plasma	LC-MS
		High HDL-C	223 (41)	54.3±0.7	23.8±0.4		
Yetukuri et al[53]	HDL-C	Low HDL-C	24 (50)	53 (51–53)	27.9 (24.3–31.5)	Serum; EDTA plasma	LC-MS
		High HDL-C	23 (50)	54 (50–60)	22.8 (21.4–24.6)		
Stuebiger et al[54]	FH/combined hyperlipidemia (FCH)	CTRL	7 (67)	15±7	30±8	Plasma	MALDI-MS
		FH/FCH	13 (37)	11±4	19±4		
Case–control studies: dietary and obesity							
Gurdeniz et al.[55]	Effect of TFA intake;	Moderately overweight, normolipidemic, postmenopausal	52 (100)	45–70	25–30	EDTA plasma	LC-MS; ^{1}H-NMR
Kien et al.[47]	FA dietary	High palmitic acid diet	18 (50)	18–40	>18, <30	Serum; muscle tissue	GC
		High oleic acid diet					
Jauhiainen et al.[56]	Lactotripeptide dietary	Placebo	89 (39)	49±5	28.5±3.6	Plasma	LC-MS
		Peptide			27.6±3.6		
Leskinenet al.[57]	Twin pair study	Discordant in weight	Same-sex	See Ref.	See Ref.	Serum; plasma	LC-MS
Pietilainen et al.[58,59]			Twin pairs			Adipose tissue	
Kolak et al.[60]	Obesity (liver fat, LFAT)	Normal LFAT	10 (100)	44±3	…	Adipose tissue	LC-MS
		High LFAT	10 (100)	37±2			
Martin et al.[61]	Obesity	Healthy obese (visceral/ subcutaneous adipose tissue)	40 (100)	25–45	28–40	Plasma	LC-MS; NMR
Case–control studies: drug therapy							
Hu et al[62]	Drug therapy: antihypertensive therapy	CTRL	28 (0)	44.9±5.3	23.9±2.8	Heparin plasma	LC-MS
		Hypertension	30 (0)	44.3±5.6	25.7±2.1		

(*Continued*)

Table 3. Continued

References	Name of Study/ Description	Study/Diseases	Study Size (% Women)	Age, y	BMI, kg/m^2	Sample	Method
Drew et al[63]	rHDL infusion	T2D	13 (0)	52±8.9	31.1±2.0	Plasma	LC-MS
Kaddurah-Daouk et al[64]	Drug therapy: simvastatin	Good response	24 (67)	60.3±13.8	28.5±6.4	Plasma	GC
		Poor response	24 (67)	53.1±12.4	29.1±5.1		
Bergheanu et al[65]	RADAR study: drug therapy	Rosuvastatin	39	66.0 (60–73)	28.3 (26.1–31.2)	Plasma	LC-MS
		Atorvastatin	41	66 (55–73)	28.0 (25.5–30.0)		

CTRL indicates control group; DL, dyslipidemic; FA, fatty acid; FCH, familial combined hyperlipidemia; FH, familial hyperlipidemia; GC, gas chromatography; HDL-C, high-density lipoprotein-cholesterol; LC-MS, liquid chromatography-mass spectrometry; NMR, nuclear magnetic resonance; NPDR, nonproliferative diabetic retinopathy; PIVUS, Prospective Investigation of the Vasculature in Uppsala Seniors; rHDL, reconstituted HDL; SAFHS, San Antonio Family Heart Study; T2D, type 2 diabetes mellitus; T1D, type 1 diabetes mellitus; and TFA, trans FA.

Applications of Lipidomics to Clinical Cohorts

The study of the lipidome and its interactions with metabolism is a new avenue for cardiovascular research. Preclinical models ranging from mice, hamsters,[31] rabbits, and pigs to nonhuman primates have been used to study atherosclerosis although the translation of findings especially from rodents to humans can be challenging. In general, mice are resistant to atherosclerosis. Knockout mice for apolipoprotein E (*apoE*$^{-/-}$) and LDL receptor (*LDLr*$^{-/-}$) develop atherosclerosis, but their atherosclerotic plaques do not display clear evidence of plaque ruptures,[32] which constitute the main cause of myocardial infarction in men. Although preclinical models are important for informing further clinical studies, we focus on the application of lipidomics to clinical cohorts.

To date, few articles applied lipidomics to large patient cohorts. Thus, we include articles not only with lipidomics but also with measurements related to lipids and CVDs.[33–40] A summary of the studies is presented in Table 3. Lipid profiling has shown promising results in diabetes mellitus,[41–47] CVDs,[35,37,38,48–52] metabolic disorders,[40,53,54] dietary habits, and obesity,[47,49,55–61] as well as in determining response to drug therapy.[62–65] Most studies analyzed either blood or tissue samples (ie, diabetic retinopathy,[46] atherosclerotic plaques,[52] muscle,[47] or adipose tissue).[58,59] Predominant methods of choice are LC-MS/MS and shotgun lipidomics. ELISAs have been used for detection of oxidized lipoproteins[34,35] and NMR for profiling of lipoprotein subclasses.[48,55,61]

In a family cohort study exploring the relation of circulating lipids to longevity, a sex-specific lipid signature was associated with familial longevity in women, whereas no significant differences were observed in men.[33] Female middle-aged offsprings of nonagenarians (between 90 and 100 years old) showed a higher ratio of monounsaturated instead of polyunsaturated lipid species and thus the authors suggest that the female plasma lipidome is less prone for oxidative stress. Furthermore, they identified ether phosphatidylcholine and sphingomyelin species as novel longevity biomarkers in women, independent of total TG levels.

The Prospective Investigation of the Vasculature in Uppsala Seniors (PIVUS) study related the plaque size to Framingham risk scores, apoB/apoA1 ratio, oxidized LDL, and other risk factors, such as increased systolic blood pressure and blood glucose levels.[34] The authors describe a significant higher level of baseline conjugated diene LDL among subjects with atherosclerotic plaques and a stepwise increase of oxidized LDL levels with increasing plaque size confirming a role of oxidative stress in plaque formation. However, they gave no insight in the lipid composition.

In the prospective Bruneck study, Tsimikas et al[35] observed that subjects within the highest tertile of oxidized phospholipids on apoB had a higher risk of CVD and stroke. Furthermore, an association between antibodies to oxidized LDL (oxLDL) and apoB-immune complexes and chronic infections was described.[36] The Bruneck Study is also the first example for the application of MS-based lipidomics profiling in the primary preventive setting for CVD. Lipid profiling was conducted by shotgun lipidomics measuring 135 lipid species from 8 different lipid classes (Figure 5). Molecular lipid

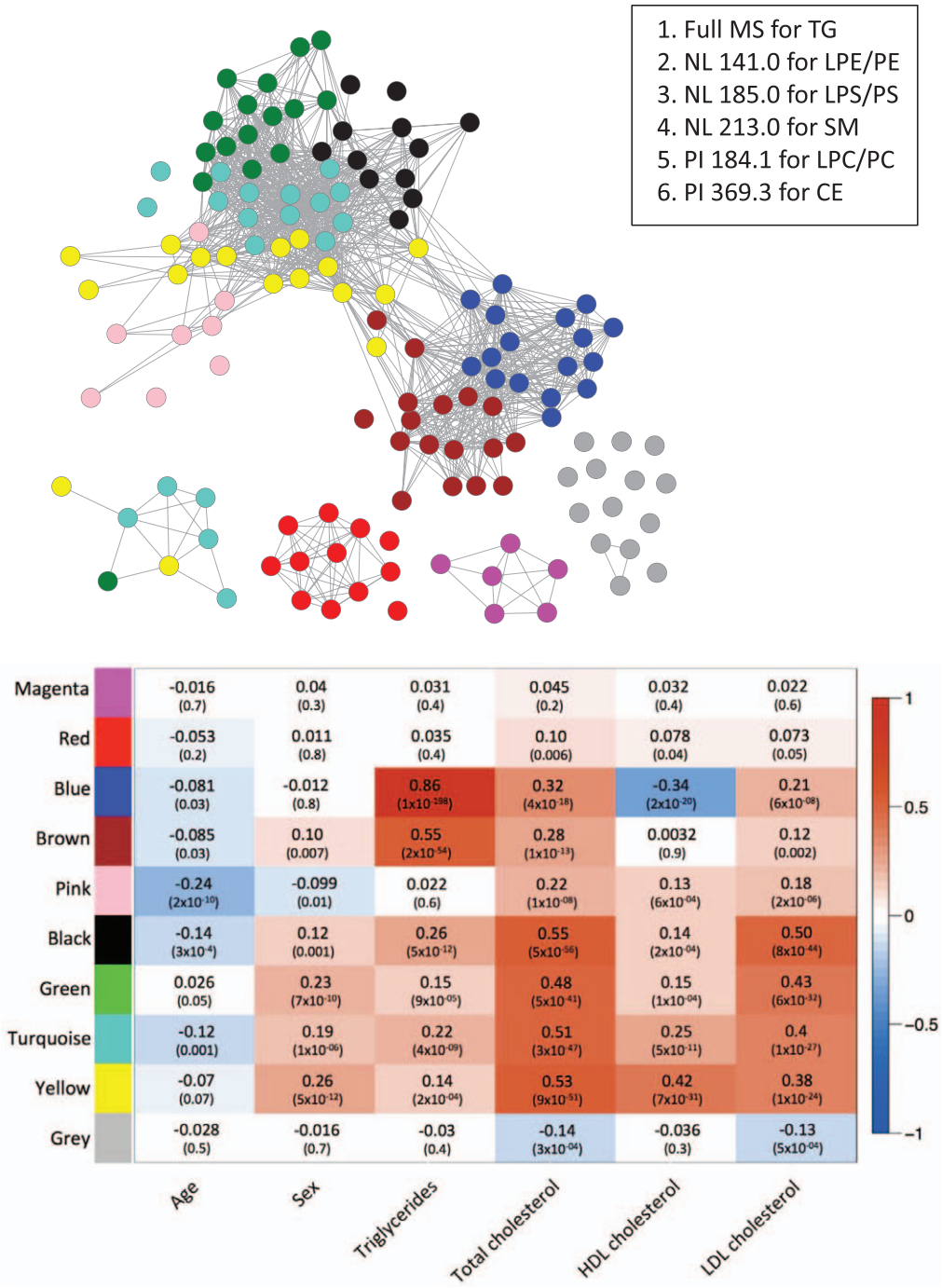

Figure 5. Plasma lipid network analysis and correlations between network modules and traits. The lipid correlation network, thresholded at an adjacency of 0.02 (akin weighted correlation of 0.8), is based on the following spectra: full mass spectrometry (MS) for TG; precursor ion (PI) scan 184.1 for lysophosphatidylcholines LPC and PCs; neutral loss (NL) scan 141.0 for lysophosphatidylethanolamines LPE and PE; NL scan 185.0 for lysophosphatidylserine LPS and PS; NL scan 213.0 for sphingomyelins (SMs); PI scan 369.3 for cholesteryl esters (CE). The colors indicate the modules detected by Topological Overlap Measure, a mathematical metric of putative functional similarity. The modules are described by the first principal component of the species present in the module and can be correlated with external clinical traits. The module trait associations are shown where the colours correspond to the correlation coefficients. The strongest association is shown for the blue module with triglyceride levels (correlation coefficient, 0.86). This is expected, when looking at the module membership, as the blue module contains TG species. Total cholesterol and low-density lipoprotein also have strong associations with the black, green, and turquoise modules (correlation coefficients, 0.38–0.55). Reprinted from Stegemann et al.[37]

species in plasma were then associated with cardiovascular events during a 10-year observation period.[37] Notably, the FA composition of complex lipids revealed a shift in the number of carbon atoms for long-chain FAs that are associated with cardiovascular risk (Figure 6): shorter saturated and monounsaturated FA in both CE and TG were associated with higher cardiovacular risk. Interestingly, these long-chain FAs are derived not only from dietary sources but can be produced via

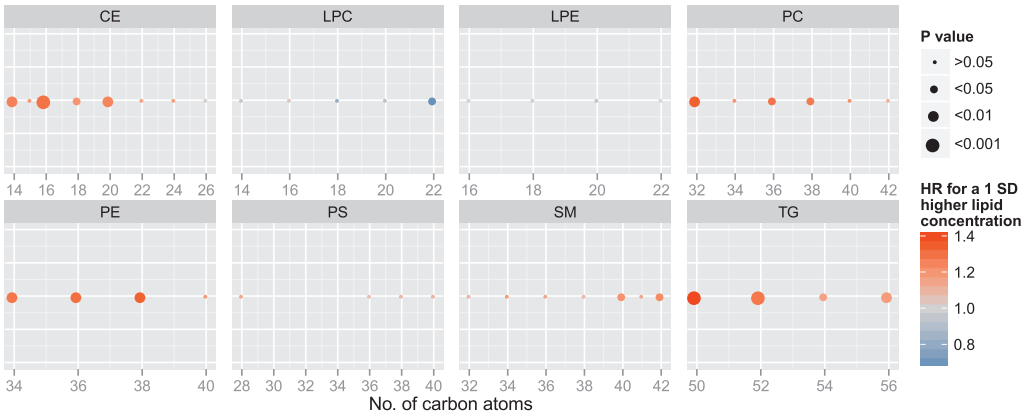

Figure 6. Association of fatty acid chain length to cardiovascular disease risk in different lipid classes. Circle color indicates the magnitude of hazard ratio (HR) and circle size corresponds to the significance level (see legend). HRs are aggregated by carbon number of each lipid class (ie, species comprising alkyl ether linkages or double bonds are not separately depicted for each carbon number). CE indicates cholesteryl esters; LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SM, sphingomyelin; and TG, triglycerides.

de novo lipogenic pathways in the liver.[66] Some of the plasma lipid species associated with cardiovascular events were also found to be enriched in atherosclerotic plaques.[37] Shotgun lipidomics analysis of carotid endarterectomies demonstrated the potential of lipidomics for unravelling the heterogeneity within atherosclerotic plaques, including the liquid extraction surface analysis of tissue sections.[52]

Similarly, in the Framingham Heart Study, lipids of lower carbon number and double-bond content (especially TG) were associated with an increased risk for type 2 diabetes mellitus, whereas the risk decreased with lipids of higher carbon number and double-bond content.[41] Furthermore, a TG signature was associated with insulin resistance and TG (50:0) and TG (58:10) emerged as positive and negative predictors for type 2 diabetes mellitus, respectively.

The San Antonio Family Heart Study (SAFHS) analyzed the lipid profile of 42 extended families at baseline. The comparative measurement of 312 lipids in >1000 individuals revealed CE and TG to be associated with age, sex, and body mass index, whereas ceramides was found to be significantly higher in men and independently associated with age and body mass index.[38] Furthermore, lipid species, such as diglycerols (DGs), in general, and the DG 16:0/22:5 and DG 16:0/22:6 lipid species, in particular, were associated with hypertension.[39]

The combination of genomics, transcriptomics, and lipidomics data in a Finnish cohort highlighted inflammatory pathways, as well as differences in lipid composition that are associated with low HDL-C levels. Ether containing phosphatidylethanolamines (so-called plasmalogens, which are considered to have antioxidative properties) and lysophosphatidylcholine were reduced in HDL particles of subjects with low HDL-C, whereas ceramides, sphingomyelin, and TG were found to be increased.[40]

The Diabetes and Impaired glucose tolerance in Women and Atherosclerosis development study (DIWA) showed that dyslipidemia rather than hyperglycemia is responsible for an altered HDL lipidome.[42] Thereby, lyso-phosphatidylcholine, a product of proinflammatory phospholipases, and palmitate-rich TG and DG were enriched in small HDL particles in dyslipidemic when compared with normolipidemic patients and healthy controls. Furthermore, dyslipidemia was associated not only with increased DG and TG but also with changes in lipid species composition in VLDL and LDL of women with type 2 diabetes mellitus (eg, increased C16:1 and C20:3 in phosphatidylcholine and CE and increased palmitic acid and vaccenic acid in TG), whereby levels of palmitic acid (C16:0) correlate with insulin resistance.[43] Combined analysis of gene expression and lipidomic profiling revealed type 2 diabetes mellitus–associated lipid species and enriched biological pathways in peripheral blood.[44] Diabetes mellitus–specific changes were also shown in vitreous lipid autocoids.[46]

Other lipidomics studies in the context of CVD encompass dilated cardiomyopathy,[48] acute myocardial infarction and unstable angina,[49] hypertension,[38,39,50,51] and carotid atherosclerosis.[52] Patients with dilated cardiomyopathy differed from controls in regard to distinct serum lipidomic profiles dominated by diminished odd-chain TG and lipid ratios related to desaturations.[48] Lipidomics analysis demonstrated that hypertension is accompanied by specific reduction of the content of ether lipids and free cholesterol[50] and marked alterations in vascular sphingolipid biology, such as elevated ceramides levels and signaling.[51] A comparison of patients with low and high HDL-C identified novel *cis*-expression quantitative trait loci variants in the human leukocyte antigen region associated with low HDL-C.[40] Low HDL-C subjects were found to have elevated TG and diminished lyso-phosphatidylcholine and sphingomyelin.[53]

Studies on dietary habits[47,49,55,56] and obesity[57–61] also compared the lipid composition. For example, increased concentrations of TG with long FA chains and ceramides, specifically ceramides (d18:1/24:1), correlated with macrophage infiltration in subcutaneous adipose tissue in subjects with high liver fat content.[60] The authors explain these findings that ceramides could mediate adverse effects of long-chain FA and induce both insulin resistance and inflammation. Studies of same-sex twin pairs discordant in weight allowed the investigation of lipid composition in blood and adipose tissue, as well as of gene expression to physically activity and obesity.[57–59] Obese twin individuals had increased proportions of palmitoleic and arachidonic acid in their adipose tissue, including increased

levels of ethanolamine plasmalogens containing arachidonic acid. Thus, obesity, already in its early stages and independent of genetic influences, is associated with deleterious alterations in the lipid metabolism known to facilitate atherogenesis, inflammation, and insulin resistance.

The effect of drugs, such as of antihypertensive agents,[62] recombinant HDL infusion,[63] and statins,[64,65] on lipid profiles has also been assessed. Other important biomarker studies include oxidation of PUFA. Lipid oxidation is not only an artifact of sample preparation but also generated in vivo either enzymatically mediated by enzymes, such as lipoxygenase, cyclooxygenase, and cytochrome P450, or nonenzymatically by reactive oxygen species, such as hydroxyl radical, nitrogen dioxide, hypochlorite, higher oxidation state of heme, and Fenton-like reactions.[67] Oxidized lipids and oxLDL are known to be involved in several inflammatory diseases, such as atherosclerosis, and are described as cytotoxic, proinflammatory, proliferative, and proatherogenic. The oxidation of PUFA is initiated by the formation of PL peroxyl radicals and hydroperoxides. Oxidation endproducts, such as malondialdehyde, 4-hydroxy-2-nonenal, and 4-oxo-2-nonenal, and other highly reactive aldehyde oxidation products, such as acrolein and isoprostane, are studied as biomarkers of several inflammatory diseases.

Although oxidation-specific epitopes of LDL have been previously measured using conventional ELISAs,[35,37,52] new MS-based approaches for detection of oxidized lipids in oxLDL are being developed. For example, a recent study combined anti-oxLDL antibodies immobilized onto nanoparticle for selective extraction of oxLDL followed by release and sensitive MS detection.[68] However, the detection of oxidized lipids by MS is challenging because of the variety of fragmented and nonfragmented oxidation products formed by additional oxidation, intramolecular cyclization, and rearrangement, and the limited availability of suitable standards.

Conclusions

Cardiovascular risk assessment currently relies on conventional lipid measurements, such as total triglcyerides, total cholesterol, and HDL-C and LDL-C. MS offers an unprecedented opportunity to interrogate the human plasma lipidome further, including thousands of different lipid species plus modifications, oxidations, and fragmentation products. To date, few studies have applied lipidomics to CVD, but molecular lipid species were shown to correlate to cardiovascular events, atherosclerosis, and diabetes mellitus, and response to drug therapy. A wider use of lipidomics profiling will provide new insights into the association of molecular lipids with CVD.

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King's College London has filed a patent on lipid biomarkers for cardiovascular risk prediction.

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