Top-down lipidomics of low density lipoprotein reveal altered lipid profiles in advanced chronic kidney disease

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Abstract This study compared the molecular lipidomic profile of LDL in patients with nondiabetic advanced renal disease and no evidence of CVD to that of age-matched controls, with the hypothesis that it would reveal proatherogenic lipid alterations. LDL was isolated from 10 normocholesterolemic patients with stage 4/5 renal disease and 10 controls, and lipids were analyzed by accurate mass LC/MS. Top-down lipidomics analysis and manual examination of the data identified 352 lipid species, and automated comparative analysis demonstrated alterations in lipid profile in disease. The total lipid and cholesterol content was unchanged, but levels of triacylglycerides and N-acyltaurines were significantly increased, while phosphatidylcholines, plasmenyl ethanolamines, sulfatides, ceramides, and cholesterol sulfate were significantly decreased in chronic kidney disease (CKD) patients. Chemometric analysis of individual lipid species showed very good discrimination of control and disease sample despite the small cohorts and identified individual unsaturated phospholipids and triglycerides mainly responsible for the discrimination. These findings illustrate the point that although the clinical biochemistry parameters may not appear abnormal, there may be important underlying lipidomic changes that contribute to disease pathology. The lipidomic profile of CKD LDL offers potential for new biomarkers and novel insights into lipid metabolism and cardiovascular risk in this disease.—Reis, A., A. Rudnitskaya, P. Chariyavilaskul, N. Dhaun, V. Melville, J. Goddard, D. J. Webb, A. R. Pitt, and C. M. Spickett. Top-down lipidomics of low density lipoprotein reveal altered lipid profiles in advanced chronic kidney disease. J. Lipid Res. 2015. 56: 413–422.

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Chronic kidney disease (CKD) is a serious and increasingly common condition (1). Patients with CKD have a greatly increased risk of CVD, which represents the most common cause of mortality and morbidity in these patients, to the extent that CKD is considered an independent risk factor for CVD (2, 3). In CKD, many conventional risk factors for CVD are prevalent, including hypertension, dyslipidemia, and insulin resistance. Underlying conditions that are typical of CVD also occur, such as heightened inflammatory status, oxidative stress, endothelial dysfunction, and arterial stiffness (3, 4). Consequently, understanding the factors in CKD that could contribute to increased CVD risk is very important.

In CVD there is a clearly established link between dyslipidemia (specifically hypercholesterolemia and hypertriglyceridemia) and atherosclerosis, an underlying pathology of most CVD (5, 6). In view of the clear cardio-renal relationship, there has been considerable interest in the possible contribution of hyperlipidemia to CKD-associated CVD (7, 8). The nature of this lipid imbalance is significantly different to nonrenal-related CVD; in particular, the relationship with cholesterol level is less clear than in
the general population and is dependent on the stage of disease (9, 10). In some patients, total cholesterol and LDL-cholesterol are not elevated, while patients on hemodialysis may even have reduced cholesterol compared with control subjects (11). It is apparent that CKD involves multiple lipid abnormalities, some of which may contribute to increased CVD risk. However, most studies in lipid abnormalities in CKD have focused on lipoprotein profile or on overall lipid classes such as triglycerides. While in many inflammatory diseases, including preeclampsia (12), diabetes (13), rheumatoid arthritis (14), and Crohn’s disease (15), lipidomic studies have identified characteristic lipid signatures that have potential as diagnostic tools, there have as yet been few attempts at profiling individual lipids in CKD. Evidence for an altered phospholipid profile in CKD (16) and a decrease of serum sulfatide (ST) levels in patients with end-stage renal failure (ESRF) (17) have been reported, but otherwise little is known about molecular changes.

Modern lipidomics depends almost entirely on analysis by electrospray MS, as this is able to identify a very wide variety of individual lipid species in several classes. Both shotgun lipidomics, involving direct infusion of the sample into the instrument, and LC/MS are widely used for this purpose (18). Chromatographic separation provides additional information to facilitate lipid identification, and separation of the lipids reduces interference (19). Although with lower-resolution instruments MS/MS is necessary to distinguish lipids of similar mass but different formula, modern high-resolution instruments such as orbitraps offer sufficient mass accuracy that isobaric species can be distinguished, thus allowing classification of lipid analytes and identification of the total number of carbons and double bonds in the acyl chains by a top-down approach (20). It has been demonstrated that this untargeted approach, coupled with principle component analysis, can be used without internal standards for comparative analysis of lipoproteins, owing to the high dynamic range of the orbitrap (21). Similar “semi-quantitative” approaches on a triple quadrupole instrument have also been used for comparative lipidomics in CVD (22, 23). However, MS/MS or MS² is still required for confirmation of individual acyl chain length and double bonds.

We recently demonstrated that a top-down lipidomics approach using LC/MS on a high resolution instrument (Orbitrap Exactive) was able to identify more than 350 individual lipid species or isomeric lipid clusters in normolipidemic LDL (24). The lipids were identified by matching the experimental m/z for the molecular ions to calculated mono-isotopic masses available in lipidomic and metabolic databases. LDL is an important carrier of a wide variety of lipid species within the plasma and reflects systemic changes in lipid metabolism. We hypothesized that the application of this methodology to CKD would identify novel differences in lipid profile at a molecular level between disease and control samples that would enhance understanding of the disease mechanisms and offer potential as diagnostic markers.

MATERIALS AND METHODS

Materials

All chemicals used were of analytical quality and purchased from Sigma-Aldrich (UK) or ThermoFisher (UK) unless stated otherwise. Organic solvents were HPLC-grade and purchased from Fisher Scientific (Loughborough, UK).

Subjects and blood collection

Male CKD patients (stage 4-5) were recruited from the renal outpatient clinic at the Royal Infirmary of Edinburgh following ethical approval by NHS Lothian Research Ethics Committee and gave informed consent as described previously (25). Renal patients were excluded on the basis of renal transplant, dialysis, systemic vasculitis or connective tissue disease, a history of established CVD, peripheral vascular disease, diabetes mellitus, respiratory disease, neurological disease, alcohol abuse, or treatment with an organic nitrate or β-agonist. The causes of kidney disease in patients were autosomal dominant polycystic kidney disease (n = 4), IgA nephropathy (n = 2), reflux nephropathy (n = 3), and neurogenic bladder (n = 1). Smokers and hypercholesterolemic patients were not excluded, but the latter were controlled by statin medication (two individuals in the disease group) and stable on treatment for 3 months prior to inclusion in the study. Subjects refrained from alcohol for at least 24 h, and caffeinated drinks and smoking for at least 12 h before the study.

Blood samples were collected in polypropylene tubes containing EDTA (final concentration, 1 mg/ml of blood); plasma was promptly separated by centrifugation (2,500 g, 20 min, 4°C) and stored in 2 ml aliquots at −80°C in the dark.

Clinical and biochemical measurements

Blood pressure, high sensitivity C-reactive protein, oxidized LDL (OxLDL), and interleukin (IL)-6 were determined as described previously (25). Other parameters [plasma glucose, total cholesterol, triglyceride, lipoproteins, creatinine, and glycated hemoglobin (HbA₁c)] were determined in the hospital biochemistry laboratory by assays validated to Good Laboratory Practice standard.

Plasma samples and LDL separation

LDL was isolated from plasma aliquots essentially as described previously (26). KBr (0.3816 mg) was dissolved in 1 ml of plasma at 4°C and underlaid below 4.1 ml of a deoxygenated EDTA solution, before centrifuging in a Beckman VTi 90 rotor for 2 h at 60,000 rpm to generate a density gradient. LDL formed bands in the density range 1.019–1.060 g/ml. The LDL collected was stored in sterile vials under nitrogen and desalted before determining the cholesterol content using CHOL PAD reagent (Roche Diagnostics), and protein concentration of isolated LDL was determined by the Bradford assay as reported by Yue et al. (27). Purity of isolated LDL was confirmed by polyacrylamide gel electrophoresis (24, 28).

Vitamin E content

Vitamin E content (α-tocopherol) in LDL was determined by reverse-phase chromatography using spectrophotometric detection as described previously (29). The samples and standards were injected randomly in triplicate and area under the curve was plotted against the calibration curves and used to calculate the concentration of vitamin E in the samples (μg/mg protein). Standards (0.1–10 μg/ml), made up in methanol and extracts redissolved in 100 μl methanol, were analyzed in triplicate by injection of 20 μl. The intra-day cross-validation (CV; n = 5) at a concentration of 2.5 μg/ml was
3.1%. Statistical analysis was carried out using an unpaired t-test, with Welch's correction to estimate the P-values.

Electrophoretic mobility of LDL

The particle size of LDL was assessed by 1% agarose gel electrophoresis in barbital buffer as described previously (30). Retardation factors were defined as the distance (cm) traveled by sample/distance (cm) traveled by dye front.

Lipid extraction

LDL lipids were extracted from LDL containing 25 µg protein by the Folch method as described recently (24). The lipid extracts were combined into an amber vial (Supelco), dried under a stream of nitrogen filtered with a 0.22 µm mesh (Millipore), and stored at −70°C until further analysis. Mean recovery (%) of phosphatidylcholine (PC; 13:0/13:0) lipid standard in spiked LDL samples by the Folch method was 103.9 ± 8.6. Similar recoveries were achieved with dehydroepiandrosterone sulfate as a representative of more polar lipid classes, and d5-myristic acid (Sigma Aldrich Chemical Co., UK) as a representative of less polar lipids.

Top-down LC/MS analysis of lipidomic profile

Lipid extracts were solubilized in 100 µL CHCl₃-methanol (1:1, v/v), further diluted in methanol and analyzed by LC/MS essentially as described previously (24). Separation of LDL lipid classes was performed using a Dionex Ultimate 3000 HPLC system (Thermo Scientific, Hemel Hempstead, UK) by injection of 10 µL sample onto a silica gel column (150 mm × 3 mm × 3 µm; HiChrom, Reading, UK) used in hydrophilic interaction chromatography mode (24). Two solvents were used: (A) 20% isopropyl alcohol (IPA) in acetonitrile and (B) 20% IPA in ammonium formate (20 mM). Elution was achieved using the following gradient at 0.3 ml/min: elution at 5% B for 1 min, followed by a rise to 9% B at 5 min, 15% B at 10 min, to 25% B at 15 min, 35% B at 25 min, and from 20 to 40 min a decrease to 5% B. Detection of lipids was achieved in a Orbitrap Exactive Mass Spectrometer (ThermoFisher Scientific Inc., Bremen, Germany) equipped with polarity switching. The instrument was calibrated according to the manufacturer specifications to give an rms mass error <2 ppm. The following electrospray ionization settings were used: source voltage, ±4.50 kV; capillary voltage, 25 V; capillary temperature, 320°C; 50,000 resolution (Full Width at Half Maximum at m/z =500). Three microscans were collected per data point with the injection time limited by either an automatic gain control target ion intensity of 10⁶ or a maximum inject time of 250 ms.

For certain lipids of interest, MS/MS was carried out on an LTQ Orbitrap instrument (ThermoElectron, Hemel Hempstead, UK) controlled by Xcalibur (version 2.0, Thermo Fisher Corporation) in either positive or negative ion modes as appropriate for the best detection of the parent ion. The capillary voltage was set at 4.5 kV, capillary temperature at 275°C, with sheath gas and sweep gas flow rates set at 30 and 10 AU, respectively. Collision energy was set according to the ion of interest, typically between 25 and 35 (arbitrary units).

LC/MS data processing

In the first stage, LC/MS data were analyzed and lipid species identified by manual matching of retention times and accurate mass data to a home-built database and the Human Metabolome project database (HMDB) (31), with identifications based on ions showing a mass error of <5 ppm (and in most cases <2 ppm) to the monoisotopic mass calculated from the theoretical formula. A total of 352 lipids were identified by this approach.

Subsequently, LC/MS data were analyzed by filtering with MZMatch (32) followed by using the XCMS pipeline [XCMS Online version 0.0.83, Scripps Center for Metabolomics, https://xcmsonline.scripps.edu/ (33)] for peak detection, alignment, and isotope annotation as described previously (24). Ions with intensity <5,000 cps were excluded. Integration of features extracted in different samples corresponds to the reported extracted ion chromatogram areas. Peak intensities for the ions identified from individual lipid classes in the data sets were summed and used to evaluate overall differences in disease versus age-matched control groups. Extracted features were included if they were present in >50% of the samples in each group, within 2.5 ppm from the exact monoisotopic mass, and with <5 s retention time deviation. In order to prevent overestimation of the number of lipid species identified, all lipid species detected in positive and negative ion modes were manually cross-referenced. Overall, 142 and 158 individual lipids were identified in positive and negative ion modes, respectively. Isomeric species are reported as one single ion, for instance PC(16:0/18:1), PC(18:1/16:0), PC(16:1/18:0), PC(18:0/16:1), PC(14:0/20:1), and others are expressed as PC(34:1). The data processing steps and number of features or lipids identified at each stage are summarized in supplementary Fig. 1.

Statistical analysis

The merged data set comprising 300 lipids species (supplementary Fig. 1) was further analyzed using partial least squares discriminant analysis (PLSDA) (34, 35). PLSDA calibration models were validated using segmented CV, and optimization of PLSDA models was achieved using the variable importance in projection (VIP) score (36). A VIP cut-off value of 0.8 was repeatedly applied to eliminate less discriminating variables, with a cut-off of 0.85 for the merged set. The final classification model included 48 species detected in the positive mode and 55 in the negative mode. The statistical significance of the classification PLSDA models was assessed using permutation testing with 1,000 permutations (37). Q² was used as quality-of-fit criterion for the permutation test (38). Further details are given in supplementary Methods.

Statistical analysis of clinical and biochemical parameters was conducted using nonparametric t-tests (Mann-Whitney) using two-tailed P-value calculation, and values with P < 0.05 were considered statistically significant.

RESULTS

Evaluation of clinical and biochemical parameters in kidney disease

Baseline measurements of clinical and biochemical parameters for age- and body mass-matched subjects included in this study are summarized in Table 1. Glomerular filtration rate (GFR) was estimated using the Modification of Diet in Renal Disease study equation and confirmed all patients as stage 4 or 5 CKD; they also had significantly increased systolic blood pressure. There were no significant differences in levels of glycated hemoglobin and plasma glucose. The inflammatory marker C-reactive protein was significantly elevated, although IL-6 was not. The levels of total plasma cholesterol and LDL were not altered with CKD, and there was no change in OxLDL. In contrast,
Analysis of LDL from control and CKD samples by manual matching to databases identified more than 300 different lipid species

In order to investigate the lipidome of LDL from control and CKD patients, LDL extracts were analyzed by normal-phase LC/MS in both positive and negative ion modes as

HDL levels showed a significant decrease, and plasma triglycerides were elevated, as expected for patients with CKD and published previously (25). LDL vitamin E content and particle heterogeneity (electrophoretic mobility) were also determined, but there was no statistical difference (Table 1).

### TABLE 1. Clinical biochemistry parameters in plasma for control subjects and CKD patients

| Clinical Parameters                  | Controls     | CKD           | P   |
|-------------------------------------|--------------|---------------|-----|
| n                                   | 10           | 10            | —   |
| Age (years)                         | 47 ± 6       | 44 ± 3        | 0.111|
| BMI (kg/m²)                         | 26 ± 2       | 29 ± 6        | 0.113|
| Smokers/ex-smokers/nonsmokers       | 0/1/9        | 2/2/8         | —   |
| Systolic blood pressure (mm Hg)     | 113 ± 12     | 124 ± 10      | 0.049|
| Diastolic blood pressure (mm Hg)    | 72 ± 11      | 78 ± 6        | 0.103|
| Mean arterial pressure (mm Hg)      | 85 ± 11      | 93 ± 7        | 0.065|
| Pulse pressure (mm Hg)              | 42 ± 6       | 46 ± 7        | 0.189|
| Plasma glucose (mg/dl)              | 5.1 ± 0.5    | 4.8 ± 0.4     | 0.231|
| HbA1c (% of Hb)                     | 5.3 ± 0.4    | 5.6 ± 0.5     | 0.117|
| Serum creatinine (mg/dl)            | 85 ± 11      | 460 ± 179     | <0.0001|
| MDRD eGFR (ml/min/1.73 m²)          | 91.2 ± 14.1  | 14.8 ± 5.3    | <0.0001|
| High sensitivity C-reactive protein (µg/ml) | 1.2 ± 1.5 | 4.2 ± 3.5 | 0.027|
| IL-6 (pg/ml)                        | 9.6 ± 10.5   | 7.9 ± 8.7     | 0.713|
| Total cholesterol (mg/dl)           | 5.1 ± 0.8    | 4.5 ± 0.8     | 0.130|
| Triglycerides (mM)                  | 1.0 ± 0.3    | 1.8 ± 0.7     | 0.004|
| HDL (mM)                            | 1.4 ± 0.5    | 1.0 ± 0.2     | 0.020|
| LDL (mM)                            | 4.8 ± 0.7 (n = 9) | 4.2 ± 0.8 | 0.091|
| OxLDL (U/l)                         | 56 ± 18      | 51 ± 12       | 0.475|
| LDL vitamin E (µg/mg protein)       | 2.43 ± 0.540 | 2.39 ± 0.524 | 0.65 |
| LDL particle size (nm)              | 0.24 ± 0.02  | 0.24 ± 0.03   | 0.387|

Values are given as mean ± SD. Significance (P values) was calculated using a two-tailed Student’s t-test, and statistically significant differences are indicated by bold typeface. MDRD, Modification of Diet in Renal Disease.

**Analysis of LDL from control and CKD samples by manual matching to databases identified more than 300 different lipid species**

In order to investigate the lipidome of LDL from control and CKD patients, LDL extracts were analyzed by normal-phase LC/MS in both positive and negative ion modes as

![Typical normal-phase LC/MS chromatograms of normolipidemic LDL lipid extract (Control, top) and LDL extract from CKD patients (Disease, bottom) in positive (+ve, left) and negative (−ve, right) ion mode. Lipid extracts were prepared according to the Folch method, and chromatograms were normalized to relative intensity (%). Labeled peaks are triacylglycerides and cholesteryl esters (TAG + CE), phosphatidylglycerols, phosphatidylethanolamines (PE), PCs (PC), and SMs (SM). The insets depict a zoomed region for the elution of cholesterol sulfate (CS), N-acyltaurines (NAT), ceramides (Cer), and fatty acids (FA) in negative ion mode.](http://www.jlr.org/content/suppl/2014/11/25/jlr.M055624.DC1.html)
reported previously (24). Representative chromatograms for control and CKD samples in each mode are shown in Fig. 1. The various chromatographic peaks observed correspond to the elution of different lipid classes. Some lipid species were observed in both positive and negative ionization modes, as indicated by the appearance of peaks with the same retention times, notably in the retention time ranges 2–3.5 and 20.5–22.5 min. It can be seen that there were no gross changes in the profile of either positively or negatively charged lipids between sample types, although some minor changes in intensity of chromatographic peaks eluting with retention times shorter than 5 min were apparent.

All features (ions) detected in the chromatograms using XCMS software were recorded by retention times, accurate mass/charge measurement, and intensity, to generate a combined list of 1,619 distinct features. Manual matching of the experimental values with theoretical databases was carried out to identify lipid species, as well as manual cross-checking for multiple adducted forms of some lipids (e.g., [MH]+, [MNa]+, or [M+NH₄]⁺ in positive ion mode; [M-H]⁻ and [M+HCOO]⁻ in negative ion mode) to avoid duplication, and isomeric species are reported as one single ion. A summary list corresponding to 352 individual lipid and lipid-related species covering 18 lipid classes or subclasses was compiled (Table 2), which were very similar to those reported previously in healthy volunteers (24). The full list of lipid species detected is given in supplementary Table 1.

Comparative analysis showed changes in CKD LDL lipid classes including CS, STs, Cer’s, and lysolipid ratios

In order to investigate the hypothesis that changes in molecular composition of LDL occur in CKD, an automated analysis of the LC/MS data using the XCMS and MZMatch platform was undertaken. This procedure identified 300 lipid features, 142 in positive ion and 158 in negative ion mode, which after manual cross-checking for removing adducts and duplicates corresponded to approximately two-thirds of the lipid species identified by manual analysis. The smaller number of positive identifications results from several factors, including the exclusion of ions with intensity <5,000 cps, the requirement that peaks were present in >50% of samples in either group, and the ability of the program to adjust for minor differences in retention times.

The intensities of ions identified in this data set were summed to provide an estimate of the total lipid intensity and the lipid intensity in each of the identified classes, following a previously published procedure (24). The variability of total lipids extracted (extraction repeatability and analytical reproducibility) between replicates

TABLE 2. List of lipid classes and subclasses identified in LDL using dual polarity detection in a high-resolution mass spectrometer by manual analyte identification using the HMDB

| Lipid Classes                          | Number of Molecular Ions Identified | Formula                                      | Adducts Searched and Ionization Mode |
|----------------------------------------|-------------------------------------|---------------------------------------------|--------------------------------------|
| Glycerolipids                          | 72                                  | CₙHₘNO₆ ([M+NH₄]⁺)                          |                                      |
| TAGs                                    | 72                                  | CₙHₘO ([M-H⁺]⁺)                             | (M⁻)                                |
| Sterols and steroids                    | 14                                  | CₙHₘO₄S ([M⁻])                             | ([M-H⁻])                             |
| Cholesterol                            | 4                                   | CₙHₘNO₂ ([M+NH₄]⁺)                         | ([M⁺]/([M-H⁻])                        |
| CSs                                     | 8                                   | CₙHₘO₄ ([M+NH₄]⁺)                         | ([M⁺]/([M-H⁻])                        |
| CEs                                     | 8                                   | CₙHₘO₄ ([M+NH₄]⁺)                         | ([M⁺]/([M-H⁻])                        |
| Steroid conjugates                     | 1                                   | CₙHₘO₄ ([M+NH₄]⁺)                         | ([M⁺]/([M-H⁻])                        |
| FAs                                     | 24                                  | CₙHₘO₂ ([M⁺])                             | ([M⁺])                                |
| Free FAs                               | 24                                  | CₙHₘO₂ ([M⁺])                             | ([M⁺])                                |
| Sphingolipids                           | 96                                  | CₙHₘNO₆P ([M⁻])                           | ([M⁻])                                |
| SMs                                     | 41                                  | CₙHₘNO₅ ([M⁻])                            | ([M⁻])                                |
| Cers                                    | 15                                  | CₙHₘNO₈ ([M⁻])                            | ([M⁻])                                |
| Hexosyl-Cer’s                          | 8                                   | CₙHₘNO₈ ([M⁻])                            | ([M⁻])                                |
| Lactosyl-Cer’s                         | 5                                   | CₙHₘNO₁₅ ([M⁻])                          | ([M⁻])                                |
| Acidic glycosphingolipids (STs)        | 27                                  | CₙHₘNO₁₅ ([M⁻])                          | ([M⁻])                                |
| Glycerophospholipids                   | 140                                 | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| PCs                                     | 15                                  | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| Phosphatidylglycerols                  | 6                                   | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| PEs                                     | 47                                  | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| Diacyl-PE                               | 15                                  | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| pPE                                    | 32                                  | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| LPEs                                    | 5                                   | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| PCs                                     | 63                                  | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| Diacyl-PC                               | 41                                  | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| pPC                                     | 22                                  | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| LPCs                                    | 4                                   | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| Lipid-related compounds                 | 6                                   | CₙHₘO₆P ([M⁻])                           | ([M⁻])                                |
| Prenols                                 | 1                                   | CₙHₘO₈ ([M⁺]/([M-H⁻])                      |
| NATs                                    | 5                                   | CₙHₘO₈ ([M⁺]/([M-H⁻])                      |
| Total                                   | 352                                 |                                             |                                      |

LPC, lyso-phosphatidylcholine; LPE, lyso-phosphatidylethanolamine; pPC, plasmanyl/plasmenyl phosphatidylcholine; pPE, plasmanyl/plasmenyl phosphatidylethanolamine.
in the samples of control and disease group was <10%, thus enabling this approach to be used for comparisons of lipid content between samples for any one lipid class, although it cannot be used for comparisons between lipid classes owing to differences in ionization efficiencies.

The total lipid in LDL remained essentially unaltered in the CKD patient group compared with the age-matched control group (Fig. 2A), but changes in the intensity of a number of lipid classes were observed in LDL. There was an observed statistically significant increase in TAGs (Fig. 2B), in agreement with the clinical data in Table 1, whereas the content of PCs decreased significantly in the CKD group (Fig. 2C). There were no changes in the intensities of total cholesterol and SMs, or in the total levels of PEs (data not shown), although the contribution of PE containing a vinyl ether linkage to the total PE pool was significantly lower in CKD samples (Fig. 2C). Interestingly, the total content of lysolipids (LPC + LPE) in LDL in disease patients was similar when compared with age-matched controls (Fig. 2F), but the ratio of lyso-lipid types (LPC/LPE) showed a significant decrease in LDL from CKD samples (Fig. 2F). In addition, changes in CKD were observed in four other lipid class that constitute minor components of LDL; specifically, the content of Cer’s (Fig. 3A), CS (Fig. 3B), and STs (Fig. 3D) decreased significantly in LDL from CKD patients, whereas a significant increase was observed in the content of NATs (Fig. 3D). Thus, in addition to changes in commonly assessed lipid class triglycerides, CKD patients show changes in both abundant and minor lipid components of LDL that are not apparent simply from standard lipoprotein assessment.

**Statistical analysis discriminated control and CKD LDL lipidomes based on individual species of unsaturated phospholipids and triglycerides**

For a full statistical analysis of the MS data and to identify the contribution of individual lipid species to differences between the control and CKD samples, PLSDA was used. The classification summary for three optimized models calculated using lipid profiles measured in positive and negative ion modes and the merged (+ve plus –ve mode) data set are shown in Table 3. Q^2 values, which are a measure of the ability of the model to predict correctly the class, lie between 0.79 and 0.82. Values of 0.5 are often classed as acceptable, and 0.8 as good, for PLSDA analysis of data sets with a limited number of classes. The power of the discrimination is further illustrated by the PLSDA score plot for the merged data (Fig. 4), which shows the samples from control and CKD cluster together, and that the classes are well separated from each other within the plot. Similar statistical results were obtained for all three data sets, with a correct classification rate (percentage of samples assigned to the correct class) between 94% and 98%, sensitivity (true positive rate, a measure of the proportion of positive correctly assigned) between 0.93 and 0.97, and specificity (true negative rate, a measure of the proportion of negatives correctly assigned) between 0.96 and 1 for the CV data, though slightly better results were obtained using merged data (Table 3). The closer the values are to 1 the better the quality of the model. The ions that contributed most strongly to the discrimination can be determined from their contribution to the PLSDA model and were unsaturated lipid species from the most abundant lipid classes, namely PC, TAG, and PE; the complete set of discriminating ions is indicated in supplemen-

![Box plots showing changes in major lipids in the disease group (n = 10) against the age-matched control group (n = 10). Samples were analyzed in triplicate (n = 3), and statistical analysis was carried out using the Mann-Whitney test to estimate the P-values. Differences were considered statistically significant at P < 0.05. Plots are total lipids detected (A), TAGs (B), total PC (C), ratio of pPE to total PE (D), total LPC and LPE (E), and ratio of LPC to LPE (F).](http://www.jlr.org/content/suppl/2014/11/25/jlr.M055624.DC1.html)
Lipid profiles in chronic kidney disease

To test the hypothesis that molecular information about LDL lipidomic profile would reveal novel details of dyslipidemia in CKD, we used a top-down lipidomic approach that allows a comparative analysis of the LDL lipid profile from age-matched controls and patients with stage 4/5 CKD. This identified significant differences in LDL lipidome of CKD patients. Multivariate analysis by PLSDA showed very good discrimination of the control and disease data sets, with a combination of positively and negatively charged lipids providing the best discrimination. The lipid species that contributed most were specific isomeric clusters from the abundant lipid classes, namely PCs (decreased), triglycerides (increased), and pPEs (decreased as a proportion of total PE), as these contain a large number of individual lipids, many of which are known to be present at relatively high levels in LDL.

However, minor lipid subclasses also showed significant differences in CKD and control LDL, specifically CSs, STs, and Cer’s, which were lower in CKD LDL, and NATs, which increased in CKD LDL, compared with control. Thus, although the clinical lipoprotein profile only showed an increase in triglycerides and a decrease in HDL, detailed molecular analysis identified several lipid classes and subclasses that are altered in LDL, and within these classes, changes in several molecular lipid species.

Some of the molecular changes observed in the LDL lipidome in CKD are linked to atherogenic mechanisms and therefore could explain the increased risk of CVD in these patients. PCs are phospholipids present in the surface monolayer of LDL and are important for the conformation of ApoB-100, macrostructure of LDL, and its correct interaction with LDL receptors. Interestingly, the overall decreased PC observed in this study was apparently not sufficient to cause major structural changes in the LDL, as there was no significant change in the size of the LDL particles in CKD. The decrease in PCs observed is in agreement with a previous report on levels of PC in plasma of ESRF patients, and also with the observation that PCs are lost in urine of patients with CKD.

The lower levels of STs and CSs in LDL of CKD patients are in agreement with some previous studies. STs are anionic glycosphingolipids that are known to have

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**DISCUSSION**

CKD is associated with increased risk of CVD, and dyslipidemia contributes to this increased risk. However, studies in lipids in CKD have mainly focused on lipoprotein balance or measured total levels of major classes. To test the hypothesis that molecular information about LDL lipidomic profile would reveal novel details of dyslipidemia in CKD, we used a top-down lipidomic approach that allows a comparative analysis of the LDL lipid profile from age-matched controls and patients with stage 4/5 CKD. This identified significant differences in LDL lipidome of CKD patients. Multivariate analysis by PLSDA showed very good discrimination of the control and disease data sets, with a combination of positively and negatively charged lipids providing the best discrimination. The lipid species that contributed most were specific isomeric clusters from the abundant lipid classes, namely PCs (decreased), triglycerides (increased), and pPEs (decreased as a proportion of total PE), as these contain a large number of individual lipids, many of which are known to be present at relatively high levels in LDL.

However, minor lipid subclasses also showed significant differences in CKD and control LDL, specifically CSs, STs, and Cer’s, which were lower in CKD LDL, and NATs, which increased in CKD LDL, compared with control. Thus, although the clinical lipoprotein profile only showed an increase in triglycerides and a decrease in HDL, detailed molecular analysis identified several lipid classes and subclasses that are altered in LDL, and within these classes, changes in several molecular lipid species.

Some of the molecular changes observed in the LDL lipidome in CKD are linked to atherogenic mechanisms and therefore could explain the increased risk of CVD in these patients. PCs are phospholipids present in the surface monolayer of LDL and are important for the conformation of ApoB-100, macrostructure of LDL, and its correct interaction with LDL receptors.

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**TABLE 3. Summary of classification of plasma from CKD patients and controls using lipids profiles measured in either positive or negative ion modes, or the merged data set**

| Data Set       | Correct Classification, % | Sensitivity, % | Specificity, % |
|----------------|---------------------------|----------------|----------------|
|                | $Q^2$ Cal CV              | Cal CV         | Cal CV         |
| Lipids positive| 0.83 100 94              | 1 0.93         | 1 0.96         |
| Lipids negative| 0.81 100 96              | 1 0.96         | 1 0.96         |
| Merged data    | 0.79 100 98              | 1 0.97         | 1 1            |

Results for calibration (Cal) and CV are shown.

$Q^2 = 1 - \frac{\sum(y_i - \bar{y})^2}{\sum(y_i - \bar{y})^2}$

Correct classification – percentage of cases assigned to correct classes.

Sensitivity = True Positives / (True Positives + False Negatives).

Specificity = True Negative / (True Negatives + False Positives).
Fig. 4. PLSDA scores plot for merged data (lips detected in positive and negative ion mode). Numbers adjacent to the symbols are patient sample codes.

antithrombotic effects, although opposing effects have also been reported under certain experimental conditions (17, 45). Within LDL, they confer negative charge at the particle surface and have been suggested to act as endogenous ligands for chemokines (46) and some selectins (47). Thus, it is clear that they have complex effects within the cardiovascular system and decreased levels could upset delicately balanced processes such as thrombosis and LDL-endothelial cell interactions. Hu et al. (17) reported that STs were the only factor that discriminated control from ESRF groups in their study, and that they have promise as biomarkers for CKD. We observed a decrease in the level of Cer’s in LDL from CKD patients; however, as increased sphingomyelinase activity and free Cer’s are thought to be linked to atherogenesis (48, 49), this does not appear to contribute to the proatherogenic profile of CKD. Finally, it was also noted that there was a significant increase in N-acyltaurines (NATs) in LDL from CKD patients. NATs are well known as components of mammalian nervous tissue and are involved in signaling neuronal events (50), and they have only recently been detected in LDL (24). In neuronal ischemia these lipids have been reported to show protective and anti-inflammatory effects (51), but they can also induce apoptosis of macrophages (52). As yet, their role in lipoproteins and potential atherogenic contribution has not been elucidated.

A limitation of this study is that only patients with severe kidney disease and age-matched controls were compared, so, in the absence of intermediate stages, limited conclusions about the role of these changes in disease progression can be drawn. Also, the CKD patients studied here had minimal comorbidity with no current clinically apparent CVD, despite the high risk of developing CVD. Although in this cohort the levels of high sensitivity C-reactive protein were high, indicating some inflammation, IL-6 and measures of oxidative stress (OxLDL) were low. These findings differ from some previous studies (3, 4) where both inflammation and oxidative stress were substantially increased in CKD and contribute significantly to its accelerated vascular pathology. Our data suggest that uremia is not itself prooxidant, and this feature may be driven largely by comorbidity. However, it is unclear whether uremia or increased inflammation is responsible for the LDL lipidomic changes or results from them. These changes in CKD patients are unlikely to be the consequence of lipid-lowering drugs, as only two patients were on statin therapy and none were on fibrate therapy. A full understanding of the role of LDL lipidomics in CVD risk in CKD patients would require a prospective study with comparison of intermediate stages of CKD, as well as CKD with and without CVD complications.

A limitation of the top-down lipidomic methodology is that some features in the mass spectra remain unidentified, and it is not possible to discriminate some individual lipids within isomeric clusters, so further differences could be present that are not reported here. It should be noted that as internal standards were not used, the analysis is comparative and semiquantitative. Such approaches have been reported previously (21–23) and have the advantage of avoiding the high expense of labeled compounds, although they do not allow the fully quantitative analysis that can be achieved with use of isotope-labeled internal standards (53). Finally, although, bootstrapping of the test data gave very good sensitivity and selectivity for discrimination, demonstrating the robustness of the analysis, further analysis on a validation data set would be desirable as the next stage of study.

In summary, using an LC/MS approach, we report the first comprehensive top-down lipidomic signature of LDL in kidney disease patients with normocholesterolemia. Patients with stage 4/5 CKD demonstrated significant changes in the lipodome of their LDL compared withagematched controls, and multivariate analysis gave very good discrimination of control and disease samples, despite the small cohorts used (n = 10). These findings illustrate the point that although the clinical biochemistry parameters may not appear abnormal, there may be important underlying lipidomic changes that contribute to disease pathology. The lipidomic profile of CKD LDL offers potential for
new biomarkers and novel insights into lipid metabolism and cardiovascular risk in this disease. Further work with early disease stages is now warranted to enable the relationship of these lipids with disease severity and cardiovascular events to be correlated.

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