Abstract

Hemodialysis (HD) and peritoneal dialysis (PD) are the primary means of managing end stage renal disease (ESRD). However, these treatment modalities are associated with the onset of coagulation abnormalities. Effective management of coagulation risk among these patients requires the identification of surrogate markers that provide an early indication of the coagulation abnormalities. The role of sphingolipids in the manifestation and prediction of coagulation abnormalities among dialysis patients have never been investigated. Herein, we report the first instance of an in depth investigation into the sphingolipid changes among ESRD patients undergoing HD and PD. The results reveal distinct differences in terms of perturbations to specific sphingolipid biosynthetic pathways that are highly dependent on the treatment modality. Our studies also demonstrated strong correlation between specific sphingolipids and coagulation parameters, such as HexCer(d18:1/26:0) and maximal amplitude (MA), SM(d18:1/24:1) and tissue factor pathway inhibitor, and sphingosine 1-phosphate d18:1 and FX (Spearman ρ of 0.93, 0.89, and −0.89, respectively). Furthermore, our study revealed the potential for using HexCer(d18:1/22:0), HexCer(d18:1/24:0), and HexCer(d18:1/26:0) (r² = 0.71, 0.82, and 0.63, respectively) and coagulation parameter MA (r² = 0.7) for successful diagnosis of differential coagulopathies among ESRD patients undergoing HD, providing an opportunity toward personalized disease management.—Contaifer, D., Jr., D. E. Carl, U. O. Warncke, E. J. Martin, B. M. Mohammed, B. Van Tassell, D. F. Brophy, C. E. Chalfant, and D. S. Wijesinghe. Unsupervised analysis of combined lipid and coagulation data reveals coagulopathy subtypes among dialysis patients. J. Lipid Res. 2017. 58: 586–599.

Dialysis is the primary treatment for patients with end stage renal disease (ESRD). While this treatment modality has made significant advances over the past years, ESRD patients still have unacceptably high rates of mortality (1). Indeed, CVD is the primary cause of mortality among patients with ESRD, accounting for about 45% of all deaths (2). Complicating this is the fact that ESRD patients...
present paradoxical pathology of coagulation; a pro-coagulant state is observed while they are also at significant risk of increased bleeding (3). The pro-coagulant profile is a prevalent problem on the arteriovenous graft site (4) leading to complications in performing the hemodialysis (HD) treatment and places these patients at risk of general vascular thrombosis. Therefore, the hemostatic balance in ESRD patients receiving dialysis modalities is often a guessing game. Thus, bleeding abnormalities associated with ESRD are difficult to assess due to both the overlapping pro-coagulant and pro-bleeding profiles (5).

We have previously investigated the coagulopathy associated with ESRD in a single-center cross-sectional study of HD and peritoneal dialysis (PD) patients (6). This study identified a pro-coagulant phenotype among the dialysis patients. Furthermore, this pro-coagulant profile was observed to be more prominent among the PD patients. However, the coagulation parameters used in this study were unable to identify significant determinants of the pro-coagulant risk among the HD population because of the inclusion of subjects with high and low thrombotic potentials. This suggests that coagulation parameters alone are insufficient to determine the pro-coagulant risk among the study population and that additional data are required.

In terms of the identification of coagulopathies, lipids have been relatively understudied despite the fact that many lipids are involved in coagulation cascades (7). Furthermore, to date, no studies have investigated the role of sphingolipids, specifically, with respect to the coagulation potential of ESRD patients undergoing dialysis. Given that the coagulation parameters alone were insufficient to discriminate the pro-coagulant potential among the cohort of dialysis patients in our previous study, and the fact that the lipids have a close association with coagulation, led us to investigate the variability of the lipidome and especially the sphingolipidome as an additional orthogonal measurement to delineate between pro-coagulant dialysis subpopulations. The data demonstrate a clear distinction in the sphingolipid profiles of the different coagulation phenotypes among the dialysis patients and the feasibility of using a limited subset of combined sphingolipids and coagulation parameters as a potential biomarker for identifying patient subpopulations susceptible to clotting events.

MATERIALS AND METHODS

Study design, setting, and patient selection

A single-center cross-sectional pilot study was designed to characterize differences in biochemical, cellular, and functional coagulation parameters in patients receiving maintenance HD and PD. Fifty subjects were enrolled in the study, including: 10 healthy volunteers who served as a baseline reference; 20 subjects receiving thrice-weekly maintenance HD; and 20 subjects receiving maintenance continuous cycling PD. The continuous cycling PD regimen consisted of four 2 h exchanges nightly and one 6 h dialysis exchange daily. Each HD patient received thrice-weekly 4 h high-flux HD sessions using a Fresenius Optiflux 180 dialyzer (Fresenius Medical Care North America, Waltham, MA). Of the 20 HD patients, 18 had arteriovenous grafts and 2 had tunneled central venous catheters because of multiple arteriovenous graft failures. Only patients that had a full dataset for coagulation parameters and lipidomic variables were used in the statistical analysis strategy, resulting in 9 healthy volunteers, 19 HD patients, and 17 PD patients.

All subjects received recombinant human erythropoietin as a standard-of-care anemia treatment. Subjects were excluded if they had any recent trauma or surgery (<7 days), active bleeding or a known bleeding disorder (for example, von Willebrand disease, hemophilia), active thrombosis or known thrombotic tendency (for example, antithrombin III, protein C, or protein S deficiency), cirrhosis or other liver abnormality, active cancer, thrombocytopenia (platelets <100 × 109/l), or concurrent use of fish oil or antiplatelet or antithrombotic medications. The VCU Institutional Review Board approved the study before subject enrollment, and the study itself was conducted in accordance with the Declaration of Helsinki. All subjects provided written informed consent before study commencement. Upon enrollment of subjects into the study, demographics, laboratory chemistry parameters, and coagulation parameters were recorded.

Blood sampling and processing

Blood (approximately 25 ml) was collected through a 15-gauge needle into a syringe: 5 ml was injected into each of four 3.2% sodium citrate tubes, and 5 ml was injected into a serum separator tube. In HD patients, the blood samples were drawn immediately before dialysis to avoid interference with heparin administration. Sodium citrate tubes were treated with 180 μl heparinase before sample processing to avoid potential heparin contamination. All blood samples were assayed within 2 h of collection for coagulation parameters.

Coagulation proteins

Coagulation proteins [tissue factor (TF), TF pathway inhibitor (TFPI), and von Willebrand factor (vWF)] were assessed by ELISA using commercially available kits (Imubind tissue factor, Imubind total tissue factor pathway inhibitor, vWF kit; American Diagnostics, Stamford, CT). Prothrombin fragments 1+2 (F1+2) and thrombin-antithrombin III complex (TAT) were analyzed using standard ELISA techniques [Enzymogost F1+2 (monoclonal) and TAT micro; Siemens Healthcare Diagnostics, Marburg, Germany]. Fibrinogen, factor VII coagulant activity, and factor X activity were performed using the standard one-stage clotting assay (STaR4 hemostasis analyzer; Diagnostica Stago, Parsippany, NJ). All assays were performed according to the manufacturer’s instructions and were run in duplicate. The average of the duplicate runs is reported.

Platelet function and coagulation assays

Flow cytometric analysis was performed using citrated whole blood according to current standards from the European Working Group on Cell Analysis (8). To identify platelets and their activation status, CD41a conjugated with PE-Cy5 (mouse anti-human; BD Pharmingen, Franklin Lakes, NJ) and CD62p conjugated with phycoerythrin [PE (mouse anti-human; BD Pharmingen)] were used, respectively. A portion of each whole-blood specimen was treated with 0.005 ml ADP as a marker of platelet activation and the response measured by the percentage of CD62p expression (CD62p+ADP). Soluble P-selectin levels were analyzed using ELISA technique (Invitrogen Corporation, Camarillo, CA). Adhesion of platelets under high flow conditions was determined from time to occlusion of a small orifice by use of the PFA-100 (Dade International, Miami, FL) with exposure to collagen/ADP and collagen/EPI.

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In vitro coagulation monitoring was performed to determine platelet function and the dynamics of blood viscoelasticity during clotting. The whole-blood clotting parameters [platelet contractile force (PCF), clot elastic modulus (CEM), and force onset time] were measured using the Hemodyne hemostasis analysis system (Hemodyne, Richmond, VA). The PCF is the force produced by platelets during clot retraction, and it is, therefore, a measure of platelet function during clotting. Thromboelastography was performed using a TEG 5000 thrombelastograph hemostasis analyzer system (Haemoscope, Niles, IL), and the reaction time (R) (measure of time to clot initiation), kinetic time (K) (measure of clot propagation time), and maximal amplitude (MA) (measure of clot firmness) were reported. All analytic procedures were completed using methods previously described in the literature (9–11). Assays were run in duplicates, and the average of the runs is reported.

**MS of lipids**

Sphingolipids were analyzed as previously described (12). Briefly, a 50 μl aliquot of each of the study plasma samples was spiked with 500 pmol of each sphingolipid internal standard mixture (Avanti), and lipids were extracted via a modified Bligh-Dyer protocol. The resultant mixture was sonicated to disperse aggregates, and followed by incubation for 6 h at 48°C. Following incubation, the extracts were transferred to a new glass tube, dried down, and reconstituted in methanol (600 μl) by sonicating, and incubated at 48°C for 15 min. The reconstitution in methanol and incubation at 48°C are new additions to our previously published method (13) and were incorporated to the existing method to ensure proper solubilization of the long-chain sphingolipids. Extracted lipids were separated using a Kinetix C18 column (50 × 2.1 mm, 2.6 μ) (Phenomenex) on a Shimadzu Nexera ultra-performance LC (UPLC) system and eluted using a linear gradient (solvent A, 58:41:1 CH₃OH/water/HCOOH and 5 mm ammonium formate; solvent B, 99:1 CH₃OH/HCOOH and 5 mm ammonium formate, 20–100% B in 3.5 min and at 100% B for 4.5 min at a flow rate of 0.4 ml/min at 60°C). Electrospray ionization tandem mass spectroscopy using a QTRAP 6500 instrument (SCIEX) was used to detect and quantify sphingolipids. Individual sphingolipids were monitored using precursor → product multiple-reaction monitoring (MRM) pairs. The parameters for detection of sphingolipids via the QTRAP 6500 mass spectrometry system are as follows: curtain gas, 30; CAD, high; ion spray voltage, 5,500 V; source temperature, 500°C; gas 1, 40; gas 2, 60; declustering potential, 80 V. Collision energies varied per transition. MRM transitions monitored together with the collision energies for the precursor product pairs are provided in Supplemental Table S1.

Eicosanoids were analyzed as previously described by us (12, 14, 15). Quantitative analysis of the lipids in the ethanolic extracts was carried out using UPLC ESI-MS/MS, as described with minor modifications (12, 16, 17). Briefly, to 200 μl of plasma, LCMS grade ethanol containing 0.05% BHT and 10 ng of each internal standard was added (1 ml). The samples were mixed using a bath sonicator followed by incubation overnight at −20°C for lipid extraction. Following incubation, the insoluble fraction was precipitated by centrifuging at 12,000 g for 2 min and the supernatant was transferred into a new glass tube. The lipid extracts were then dried under vacuum and reconstituted in LCMS grade 50:50 ethanol/dH₂O (100 μl) for eicosanoid quantitation via UPLC ESI-MS/MS analysis. A 14 min reversed-phase LC method utilizing a Kinetex C18 column (100 × 2.1 mm, 1.7 μm) and a Shimadzu UPLC was used to separate the eicosanoids at a flow rate of 500 μl/min at 50°C. The column was first equilibrated with 100% solvent A [acetonitrile:water:formic acid (20:80:0.02, v/v/v)] for 2 min and then 10 μl of sample was injected. Solvent A (100%) was used for the first 2 min of elution. Solvent B [acetonitrile:isopropanol (20:80, v/v)] was increased in a linear gradient to 25% solvent B to 3 min, to 30% by 6 min, to 55% by 6.1 min, to 70% by 10 min, and to 100% by 10.1 min. Solvent B (at 100%) was held until 13 min, then was decreased to 0% by 13.1 min and held at 0% until 14 min. The eluting eicosanoids were analyzed using a hybrid triple quadrupole linear ion trap mass analyzer (SCIEX 6500 QTRAP®) via MRM in negative-ion mode. Eicosanoids were monitored using species-specific precursor → product MRM pairs. The mass spectrometer parameters used were: curtain gas, 30; CAD, high; ion spray voltage, −3 500 V; temperature, 500°C; gas 1, 40; gas 2, 60; declustering potential, collision energy, and cell exit potential were optimized per transition.

**Statistical analysis**

The statistical strategy is illustrated in Fig. 1. To observe pattern of recognition inside the HD and PD groups, a hierarchical two-way clustering analysis was used separately for the individual datasets. Clustering is a multivariate technique of grouping rows together in a dataset that shares similar values whose values are close to each other relative to those of other clusters. Hierarchical clustering is a process that starts with each point in its own cluster, next the two clusters that are closest together are combined into a single cluster until there is only one cluster containing all the points. This type of clustering permits detection of subgroups that otherwise would be disguised as large variations within a group. The data were then regrouped separately by the identified clusters for the individual datasets. Significant outliers were identified and excluded using Grubbs’ test (GraphPad Prism software). Due to the small sample number, we could not assume the normality of the populations and the clusters were evaluated using nonparametric Wilcoxon-Kruskal-Wallis test, as appropriate. Dunn’s test with Bonferroni adjustment for post hoc multiple-comparison testing was used. The level of significance was recorded when P < 0.05. Spearman’s correlation was used to evaluate association between coagulation and lipoyd parameters. Only the highest Spearman’s coefficients were considered for each pair of variables in the identified pathophenotype for HD and PD patients. Logistic regression was used to find predictors and the area under the curve (AUC) of the receiver operating characteristics assessed the accuracy of the predictors. The predictors with P ≤ 0.001, normalized Rsquare ≥ 0.6, and AUC ≥ 0.8 were selected as the best predictors. Discriminant analysis tested the ability of the best predictors to discriminate the major pathophenotypes, while bootstrap validation for logistic regression and discriminant analysis was used to decrease bias associated with using a small sample of patients as the training set. The predictors were considered as putative biomarkers when their r² values and percent of misclassification were confirmed by the bootstrap validation. All analyses were done with JMP Pro statistical software version 12 (SAS Institute, Cary, NC).

**RESULTS**

Unsupervised statistical analysis of the combined lipid and coagulation data identify distinct phenotypic subtypes among dialysis patients

With respect to coagulopathies among dialysis patients, lipids are heavily understudied entities. Yet there is evidence for their involvement in the coagulation process (8, 18, 19). In order to ascertain whether combined lipid and coagulation parameters could identify dialysis patients (Table 1) that have a greater susceptibility for clotting events, we employed a specific statistical analysis workflow (Fig. 1) using combined coagulation and plasma lipid data (supplemental Tables S2, S3). Cluster analysis of HD patients and their controls
Fig. 1. Statistical analysis workflow for the study. A sequential statistical analysis approach was undertaken to determine significant features capable of classifying the coagulation phenotypes. Clinical and lipidomic data were consolidated into a single data matrix subjected to cluster analysis. The individual clusters identified were used in a nonparametric comparison and the statistically significant variables were selected for further analysis. The coagulation parameters were used to identify the underlying pathophenotypes and the lipidomic variables were used to create the metabolic networks corresponding to the pathophenotypes. The correlation between lipids and coagulation parameters were evaluated to indicate association with the coagulation cascade. The best predictors of the pathophenotype were then identified via a logistic regression with each lipid tested as an individual predictor. The AUC from the receiver operating characteristic (ROC) plot was
TABLE 1. Subject demographics and clinical characteristics

| Characteristics       | Control (n = 10) | PD (n = 20) | HD (n = 20) |
|-----------------------|-----------------|-------------|-------------|
| Age (years)           | 38.7 ± 13.1     | 45.3 ± 11.3 | 50.3 ± 11.9 |
| Gender [n (%)]        |                 |             |             |
| Male                  | 7 (70)          | 10 (50)     | 8 (40)      |
| Female                | 3 (30)          | 10 (50)     | 12 (60)     |
| Weight (kg)           | 70.0 ± 12.3     | 78.3 ± 19.8 | 85.5 ± 25.6 |
| Race [n (%)]          |                 |             |             |
| AA                    | 2 (20)          | 14 (70)     | 16 (80)     |
| Ca                    | 8 (80)          | 5 (25)      | 4 (20)      |
| Others                | 0               | 1 (5)       | 0           |
| Co-morbidities [n (%)]|                 |             |             |
| HTN                   | 0               | 19 (95)     | 19 (95)     |
| DM                    | 0               | 4 (20)      | 7 (35)      |
| CVD                   | 0               | 1 (5)       | 10 (50)     |
| Current smoker        | 0               | 3 (15)      | 3 (15)      |
| Davies score          | —               | 1.5 ± 0.9   | 1.9 ± 1.2   |
| Blood pressure (mmHg) |                 |             |             |
| Systolic BP           | —               | 143.5 ± 21.6| 144.1 ± 18.1|
| Diastolic BP          | —               | 86.5 ± 12.2 | 83.3 ± 13.2 |
| EPO dose (U/kg/week)  | —               | 156.4 ± 127.0| 210.5 ± 146.7|
| Etiology of ESRD      |                 |             |             |
| HTN                   | —               | 6 (30)      | 7 (35)      |
| DM                    | —               | 4 (20)      | 7 (35)      |
| PKD                   | —               | 1 (5)       | 1 (5)       |
| HIV                   | —               | 3 (15)      | 0           |
| Glomerular            | —               | 3 (15)      | 3 (15)      |
| Congenital            | —               | 1 (5)       | 1 (5)       |
| Unknown               | —               | 2 (10)      | 1 (5)       |
| Years on dialysis     | —               | 2.8 ± 1.7   | 5.2 ± 4.2   |
| BUN (mg/dl)           | —               | 37.6 ± 13.8 | 50.4 ± 18.6 |
| SCr (mg/dl)           | —               | 11.0 ± 3.9  | 9.9 ± 3.6   |
| Calcium (mg/dl)       | —               | 8.6 ± 0.8   | 8.7 ± 0.9   |
| Phosphorus (mg/dl)    | —               | 5.8 ± 1.9   | 4.9 ± 1.5   |
| PTH (pg/ml)           | 593 (126–1,021) | 350 (76–940)|             |
| Albumin (g/dl)        | 3.5 ± 0.4       | 3.7 ± 0.5   |             |
| KT/V                  | 2.1 (1.9–2.3)   | 1.6 (1.5–1.9)|           |
| Ferritin (ng/ml)      | 595 (227–975)   | 734 (475–973)|           |

A total of 50 subjects were enrolled for the current study comprised of 10 control patients, 20 PD patients, and 20 HD patients. The data are presented as percent, mean ± SD, or median (IQR). AA, African American; Ca, Caucasian; HTN, hypertension; DM, diabetes mellitus; BP, blood pressure; EPO, erythropoietin; PKD, polycystic kidney disease; HIV, human immunodeficiency virus; BUN, blood urea nitrogen; SCr, serum creatinine; PTH, parathyroid hormone.

CVD is defined as a documented history of myocardial infarction, persistent angina, arrhythmia, heart failure, or coronary intervention procedure.

Blood pressure readings for the HD group were measured predialysis.

Our cluster analysis of HD (Fig. 2A) revealed that patients fall into two categories (Table 2). Both clusters (HDC1 and HDC2) demonstrated significantly elevated levels of sphingosine 1-phosphate (S1P) d18:1 and S1P d18:0 when compared with their normal controls. In addition, the HDC1 group demonstrated significant elevation of SM(d18:1/24:1) and a decrease of HexCer(d18:1/16:0) and SM(d18:1/20:0) when compared with normal controls. On the other hand, HDC2 demonstrated a significant elevation in Cer(d18:1/24:1), Cer(d18:1/24:1), and SM(d18:1/18:1), as compared with their normal controls, while at the same time demonstrating decreased plasma levels of HexCer(d18:1/20:0), HexCer(d18:1/22:0), HexCer(d18:1/24:0), HexCer(d18:1/26:1), and HexCer(d18:1/26:0). These distinct differences among the sphingolipids indicate a central deregulation of the sphingolipid metabolic network between HDC1 and HDC2, as compared with their normal controls (COC) (Fig. 3A, B).
Sphingolipids predict coagulopathy among dialysis patients

Fig. 2. Unsupervised statistical analysis of combined lipid and coagulation data enable the discrimination between dialysis pathophenotypes. Plasma sphingolipid and eicosanoid content was combined with measured coagulation parameters and was subjected to unsupervised statistical interrogation via hierarchical cluster analysis. A: Hierarchical cluster analysis of the HD population together with their control counterparts results in three distinct groups, COC, an adjacent cluster of HD patients (HDC1), and another distal cluster of HD patients (HDC2). B: Hierarchical cluster analysis of the data from the PD population together with their control counterparts revealed two distinct clusters, with one main cluster with patients and one cluster with healthy control subjects mixed with some patients. The cluster with healthy control subjects mixed with some patients was labeled CMC and the cluster with only the patients was labeled as PDC1. The data is represented as a heat map with patients’ profiles arranged vertically and coagulation and lipid features arranged horizontally. The lower the linkage in the hierarchical tree, the more similar the feature (red = higher values, blue = lower values).
Furthermore, investigating each of these clusters in depth (Table 2) revealed that both HDC1 and HDC2 demonstrated significantly lower RBC and hemoglobin levels and elevated levels of F1+2, TF, TFPI, vWf, and fibrinogen. The HDC1 patients demonstrated a pro-bleeding (Fig. 4) pathophenotype characterized by high MA, CEM, and IQR. As was observed among the HD patients, there were significant lipid modulations among the ESRD patients undergoing PD that separated into the PDC (Table 3). Similar to the patients undergoing HD, these patients demonstrated significantly elevated levels of S1P d18:1 and S1P d18:0 (Fig. 3C). As in the HD pro-bleeding patients, SM(d18:1/16:0) was significantly decreased. Otherwise, as in the HD pro-coagulant patients, SM(d18:1/18:1) and SM(d18:1/24:1) were significantly elevated compared with the controls. The PDC also presented elevation of CD62p+ADP and elevation of HexCer(d18:1/22:0) and SM(d18:1/22:0), and elevation of HexCer(d18:1/22:0) (Fig. 3C). Consistent with an inflammatory state, it was found that the thromboxane 2 (TXB2) level as well as CysLTE4 were also elevated. Furthermore, PD patients in the PDC (Table 3) demonstrated elevated levels of F1+2, TF, TFPI, and vWf similar to the HD patients. They also presented significantly higher platelet counts and a high level of thrombin-prothrombin complex. The pro-coagulant pathophenotype (Fig. 4) was characterized by high PCF, CEM, MA, and low K. The levels of sP-selectin were also elevated, which is indicative of platelet activation (Table 3). Taken together, the data indicate the presence of at least two distinct disease pathophenotypes among the PD patients, where those that fall into the PDC category demonstrate significant disruptions to their coagulation and lipid metabolic processes.

### Extrinsic coagulation pathway is strongly affected in ESRD patients under dialysis treatment

Our cluster analysis, so far, demonstrated that significant metabolic and coagulation abnormalities are apparent in ESRD patients undergoing PD that separated into the PDC.
Fig. 3. Significant differences are observed among the sphingolipid metabolic networks between the different patient clusters. Sphingolipid fold changes observed between the different clusters of patients were mapped onto sphingolipid metabolic networks. A: HDC1 that presented the pro-bleeding pathophenotype compared with control patients. B: HDC2 that presented a pro-coagulant pathophenotype compared with control cluster. C: PDC1 that demonstrated a pro-coagulant pathophenotype compared with controls. The node sizes are proportional to the fold change, with nodes with a dashed border indicating a statistically significant fold change difference ($P < 0.05$) and those with no dashed border indicating a statistically nonsignificant fold change difference between the clusters under investigation compared with the control group.
among ESRD dialysis patients (Fig. 2). In agreement with these findings, modulation in the levels and activity of the main coagulation proteins were also observed to be significantly altered for the dialysis patients compared with their controls. Elevation of TF, TFPI, vWF, F1+2, TAT, and fibrinogen and a decrease of FX are responsible for the overt pro-coagulant or pro-bleeding state in ESRD, as detected in our results. These factors, involved in the extrinsic coagulation pathway (Fig. 5), are important markers that help to define the underlying pathophenotypes in ESRD patients (Fig. 4). Also, activation assays detected modulation in platelets. Platelets were detected as responsive to activation in pro-coagulant patients in HD as well as in PD, but failed to show an expected response to activation in pro-bleeding patients in HD (Tables 2, 3).

### Significant correlations were observed between the coagulation parameters and the sphingolipids

Our data, so far, have demonstrated that the sphingolipids and coagulation factors, when clustered together, result in the successful separation of HD patients into pro-bleeding and pro-coagulant phenotypes (Figs. 2A, 3). This indicates that there is a close linkage between the lipidome and the coagulation cascade. With respect to the pro-coagulant patients (HDC2) (Fig. 6A; Table 4), the data revealed a very strong positive correlation between SM(d18:1/24:1) and TFPI (Spearman’s correlation value of 0.89). Very strong positive correlations (Spearman correlation values >0.8) were also observed between HexCer(d18:1/26:1) and TF, HexCer(d18:1/26:0) and MA, as well as SM(d18:1/18:1) and Coll/ADP. Among the pro-coagulant patients, strong negative correlations were also observed between and Cer(d18:1/24:1) and fibrinogen as well as S1P d18:1 and FX (Spearman correlation values less than −0.8). A few significant correlations were also observed in the case of pro-bleeding HD patients (HDC1) (Fig. 6B; Table 4). These include positive correlations between S1P d18:1 and TFPI (Spearman’s correlation of 0.78) as well as SM(d18:1/22:0) and K (Spearman’s correlation of 0.67). Negative correlations were observed between HexCer(d18:1/26:0) and F1+2 as well as SM(D18:1/16:0) and Coll/ADP (Spearman’s correlation of −0.65 and −0.60, respectively). With respect to pro-coagulant PD patients (PDC) (Fig. 6C; Table 4), the data revealed strong positive correlations between SM(d18:1/24:1) and TFPI as well as S1P d18:1 and vWF (Spearman’s correlation values of 0.74 and 0.71, respectively). Taken together our data indicate a significant linkage between the sphingolipid metabolism and aberrant coagulation processes accompanying ESRD patients undergoing dialysis. This indicates the possibility of using the sphingolipids as surrogate biomarkers for dysregulations in the coagulation cascade among these patients.

### Sphingolipids can be used as predictors of coagulation abnormalities among HD patients

Considering that the major causes of mortality among ESRD patients undergoing dialysis are thrombotic events,
it is very desirable to find predictor parameters to be used as biomarkers of coagulation abnormalities. Our data indicated that HD patients present very high correlation between coagulation parameters and lipids (Fig. 6; Table 4). Importantly, the predictor analysis demonstrated MA as the best coagulation predictor and HexCer(d18:1/22:0), Cer(d18:1/20:0) as the best predictor and HexCer(d18:1/26:0) as the best biomarker of coagulation abnormalities. Our data indicated that HD patients present very high correlation between coagulation parameters and lipids (Fig. 6; Table 4).

**DISCUSSION**

The goals of our study were: a) to identify the subtypes of coagulopathies among ESRD patients undergoing dialysis; and b) to identify testable biomarkers for those coagulopathy subtypes to affect better targeted treatments. It is well-established that ESRD patients undergoing dialysis exhibit many abnormalities in their hemostatic response, resulting in an elevated risk of both thrombotic (20) and bleeding events (9). Additionally, the major cause of mortality among dialysis patients is the development of thrombosis (10). Clustering of lipid data together with coagulation data provided the required power to separate the coagulopathy subtypes among the dialysis patients. Furthermore, this approach also highlighted the correlations between these two orthogonal data types. As such, this statistical approach also resulted in new information with respect to changes in the lipidome among HD and PD patients, as well as between different coagulation subtypes within these groups. The fact that the pathophenotypes detected in this study closely matched clinical coagulopathy states speaks to the success of our study and the statistical approach undertaken.

**Analysis strategy was able to match close clinical assessment of coagulopathy**

Our analysis strategy (Fig. 1), so far, has demonstrated the ability to separate distinct disease subtypes when lipid and coagulation data are clustered together. Once the analysis was completed, additional data were requested from the study designers in order to verify the findings of the study. The clinical data revealed that out of the 12 HD patients identified as belonging to HDC1, 67% belonged to patients requiring less than three interventional procedures due to vascular access thrombosis within the last 12 months. On the other hand, out of the seven patients belonging to HDC2, 71% had three or more intervention procedures due to vascular access thrombosis within the last 12 months. Taking into account that coagulation abnormalities giving rise to vascular access thrombosis is a very dynamic process, the study data indicate a very strong ability to discriminate patients who have vascular access thrombosis issues due to coagulation abnormalities among the HD population.

**TABLE 3.** PDC and control patients were compared for coagulation and lipidomic results

| Parameters | Control (n = 9) | PDC (n = 10) |
|------------|----------------|-------------|
| **Coagulation results** | | |
| Platelets (×10⁷/mm³) | 208.0 (157.8–268.3) | 250.9 (218.0–335.3) * |
| Fib (mg/dl) | 279.7 (250.1–314.2) | 581.7 (479.8–736.0) * |
| FII (pmol/L) | 147.4 (107.1–176.3) | 355.7 (266.0–401.4) * |
| TP (mg/ml) | 36.6 (34.2–54.4) | 136.6 (108.5–155.9) * |
| TF (mg/ml) | 90.0 (81.4–119.3) | 527.4 (457.3–591.0) * |
| tWF (U/ml) | 0.8 (0.6–1.1) | 2.0 (1.7–2.9) * |
| TAT (µg/ml) | 2.8 (2.0–3.5) | 5.7 (3.4–8.1) * |
| PCF (µg/d) | 6.9 (6.2–8.0) | 15.9 (10.9–16.3) * |
| CEM (µg/cm²) | 25.8 (22.7–29.9) | 41.1 (35.3–53.5) * |
| K (min) | 2 (1.7–2.3) | 12 (1.0–4.0) * |
| MA (mm) | 64.2 (60.9–68.3) | 74.8 (72.1–78.3) * |
| sP-selectin (ng/ml) | 22.1 (19.9–27.5) | 32.7 (26.4–52.2) * |
| **FX (%)** | 116.7 (101.5–125.4) | 99.7 (81.7–99.4) * |

**Lipidomic results**

| Lipid | Control (n=9) | PDC (n=10) |
|-------|---------------|------------|
| S1P | 412.6 (335.5–716.1) | 1201.5 (930.9–1525.9) * |
| CER | 76.5 (48.8–117.5) | 172.0 (150.3–313.1) * |
| Cer(d18:1/20:0) (pmol/ml) | 66.8 (54.8–89.5) | 103.7 (84.6–197.4) * |
| Cer(d18:1/24:1) (pmol/ml) | 389.9 (358.8–470.3) | 762.6 (589.3–1056.5) * |
| SM(d18:1/16:0) (pmol/ml) | 12.574.0 (12.189.3–13.800.3) | 11.171.9 (9.884.3–12.505.2) * |
| SM(d18:1/18:1) (pmol/ml) | 3951.1 (3427.0–4656.2) * |
| SM(d18:1/24:0) (pmol/ml) | 6927.4 (5025.3–8389.3) | 10111.8 (9353.5–12890.0) * |
| SM(d18:1/26:0) (pmol/ml) | 786.2 (589.3–1056.5) * |
| HexCer(d18:1/24:0) (pmol/ml) | 3566.5 (3282.0–4269.6) | 5751.2 (4742.5–6845.7) * |
| TXB2 (pg/ml) | 0.4 (0.1–1.5) | 2.9 (2.1–9.6) * |
| CysLTE4 (pg/ml) | 0.03 (0.01–0.05) | 0.07 (0.04–0.10) * |

Only parameters with a statistically significant difference between control and PDC (P < 0.05) are presented. Data presented as median and IQR.

*Significantly different from control.
indicating an elevated clot firmness and a fast completeness. These measurements attest to the structural stability and functionality of the platelets in these patients and give emphasis to the activated state of platelets and elevation coagulation markers. A subset of PD patients was observed to cluster together with the control patients, indicating a distinct subclass among the PD population. Additional studies need to be undertaken to identify any underlying commonalities that lead to the separation of this subset of PD patients.

Our data also indicate an accumulation of the bioactive molecules, S1P d18:1 and S1P d18:0, in the plasma of ESRD patients undergoing dialysis. A previous study in mice indicated that S1P provided liver and kidney protection through a selective activation of S1P d18:1 receptors (11). S1P d18:1 is a well-known signaling molecule and is involved in signaling in several different diseases and inflammatory processes (21). It has also been linked to renal disease in animal models. It is known to act as an extracellular ligand binding to at least five specific cell surface receptors that were identified so far (22). The observation that S1P d18:1 is significantly deregulated among dialysis patients may provide an explanation for the greater biochemical disruptions among these patients. Furthermore, a related study (23) of the relationship between sphingolipid metabolism and coagulopathy demonstrated that sphingosine and sphinganine, but not ceramide or S1P d18:1, downregulated thrombin generation on platelet surfaces in vitro. This finding suggests that the in vitro results could reflect an in vivo mechanism. Hence, chronic inflammation could cause S1P d18:1 and S1P d18:0 to increase, which would reduce the anticoagulant activity and thereby add to a pro-coagulant pathophenotype.

Newly generated thrombin further activates coagulation, stimulating the release of S1P d18:1 from platelets. The released S1P d18:1 could stimulate cells together with thrombin to produce TF to generate more thrombin. Thus, thrombin and S1P d18:1 might work synergistically to stimulate cells and induce a pro-inflammatory environment.

PDC patients with pro-coagulant pathophenotype demonstrated elevated levels of TXB2. TXB2 is an eicosanoid produced by activated platelets that stimulates activation of new platelets and increases platelet aggregation. Platelets are believed to be an important source for extracellular S1P (24), and the presence of significant values of TXB2 could suggest an explanation for the elevated levels of S1P, at least in PD patients. Although platelets lack the ability to have de novo synthesis of S1P, Ulrich et al. (25) have demonstrated that platelets store S1P in the intracellular space and release it extracellularly after stimulation by protein kinase C activators. The implied relationship between S1P d18:1 and TXB2 demonstrated elevated of TXB2. TXB2 is an eicosanoid produced by activated platelets that stimulates activation of new platelets and increases platelet aggregation. Platelets are believed to be an important source for extracellular S1P (24), and the presence of significant values of TXB2 could suggest an explanation for the elevated levels of S1P, at least in PD patients. Although platelets lack the ability to have de novo synthesis of S1P, Ulrich et al. (25) have demonstrated that platelets store S1P in the intracellular space and release it extracellularly after stimulation by protein kinase C activators. The implied relationship between S1P d18:1 and TXB2 was also described by Ulrich. In that study, acetylsalicylic acid and two structurally unrelated reversible cyclooxygenases suppressed platelet S1P release. Also S1P d18:1 release was elevated by a TXB2 receptor agonist and inhibited by the TXB2 receptor antagonist, ramatroban.
Fig. 6. Correlational analysis reveals distinct and strongly linked co-relationships between the coagulation parameters and the plasma sphingolipid content. Correlation matrix heat maps for HD pro-coagulant pathophenotype (A), HD pro-bleeding pathophenotype (B), and PD pro-coagulant pathophenotype (C) between the statistically significant lipids and coagulation variables. Red represents a positive correlation, blue represents a negative correlation, and the color intensity corresponds to the strength of the correlation.

and by inhibitors of ABC transport. Furthermore, thrombin-induced release of S1P d18:1 was attenuated in platelets from TXB2 receptor-deficient mice. These published findings indicate that TXB2 synthesis and TXB2 receptor activation mediate S1P release after thrombin receptor activation. These findings are in line with the pro-coagulant pathophenotype observed in the HDC2 and PDC1 patients, but also could be a phenomenon leading to factor consumption and platelet unresponsiveness, as seen on HDC1 patients, as the next step on the coagulopathy of ESRD.

Besides the constant presence of elevated S1P d18:1 among ESRD patients, there appears to be a pattern of decrease in short- and medium-chain SM and an increase in some of the long-chain SM species among patients progressing from the pro-coagulant to the pro-bleeding pathophenotype. Elongation of long-chain fatty acids depends upon the utilization of saturated and monounsaturated C20 and C22 CoA and has been demonstrated to be essential for the production of C24 sphingolipids (26). Longer chain SM species were also found on PD patients, together with an elevation in long-chain ceramides, suggesting that their presence is also a constant lipid modulation on ESRD pathology.

The decrease of glycosphingolipids found in pro-coagulant patients under HD (HDC2) seems to be an exclusive characteristic for this group. By the implied correlation with coagulation variables observed in our study, this down modulation could indicate an involvement with the hyper-coagulopathy phenotype on chronic HD treatment. The stimulus for this coagulopathy state would be different, considering the different dialysis port access. Glycosphingolipids are generally found on the extracellular face of cellular membranes and function to maintain stability of the membrane and to facilitate cell-cell interactions (27). The depletion of these glycosphingolipids is remarkable and could be indicative, in itself, of alterations in cell function and signaling, and should be further investigated.

It should be noted that an argument could be made that if the sphingolipids are directly associated with coagulation, then this association should be reflected with respect coagulation abnormalities irrespective of the mode of dialysis. However, consideration must be given to the alteration of the whole-body small metabolite homeostasis that varies as a function of the mode of dialysis. In this context Choi et al. (28) demonstrated that the dialysis modality did, in fact, affect the circulating metabolite profile. As such, our observation of the discriminant ability of the lipid component with respect to coagulation abnormalities specifically among the HD patients is not out of the norm.

Two possible limitations of our study are the relatively small sample size available for the investigation and the challenge of closely matching the controls to the chronic kidney disease patients. With respect to the limitation regarding small sample size, it should be noted that, especially in human studies, this is not uncommon. In fact, there are many instances where such studies have led to
the identification of valuable and informative candidate biomarkers bearing high significance to the relevant disease state (29–34). In our study, this limitation of relatively small sample size is addressed via an established validation design geared toward minimizing the bias and risk of overfitting. Bootstrapping permutation used in this approach allowed the creation of a theoretically expanded population of samples via resampling from the original study population, thereby enabling the pertinent statistical test to be replayed to test the validity of the discovered model (35). One potential result of this small sample size is also the co-clustering of some of the PD patients with the control patients, which, in the absence of any other information, we categorized as an unknown phenotype. A much larger sample size may lead to the resolution of this co-clustered subset of patients, indicating the need for further studies with a larger cohort of patients for resolution of this particular paradigm. With respect to the second limitation of our study, it should be noted that due to multiple complications and comorbidities among ESRD patients, close matching of controls to the disease state is a significant challenge. As such, identification of the required numbers of non-ESRD controls with the same complications and comorbidities as our ESRD patient cohort, and then enrolling them to obtain a very closely matched patient-control cohort was beyond the available resources and scope of the current study. However, our primary goal in this study was to investigate potential means of identifying coagulation abnormalities among ESRD patients irrespective of any comorbidities present. In this regard, our data clearly indicate the potential for using combined coagulation and lipid data for the stratification of ESRD patients undergoing dialysis with respect to their coagulation status, and make a case for an expanded study to clinically validate these findings.

In conclusion, the segregation of the coagulopathy subtypes among HD and PD patients using combined lipid and coagulation variables demonstrate the applicability of this system’s pathobiology approach to understanding the complex phenotypes in chronic conditions. The pathophenotypes detected and coagulation assay results are consistent with the literature and with the actual clinical records of the patients. The lipid modulation associated with the extrinsic coagulation pathway provides novel information that could advance the understanding of the metabolic imbalances that are responsible for the coagulopathies in these patients. Finally, our study has identified a limited number of molecules that are strongly correlated with the coagulopathy subtypes and warrant further investigation as possible diagnostic/prognostic biomarkers for guiding clinical treatment.

### Table 4

| Coagulation Parameter | Lipid       | Spearman ρ | P   |
|-----------------------|-------------|------------|-----|
| **HD pro-coagulant**  |             |            |     |
| TFPI                  | SM(d18:1/24:1) | 0.89       | 0.007|
| TF                    | HexCer(d18:1/26:1) | 0.89 | 0.007|
| MA                    | HexCer(d18:1/26:0) | 0.93 | 0.003|
| FX                    | S1P d18:1 | -0.89 | 0.007|
| Fibrinogen            | Cer(d18:1/24:1) | -0.82 | 0.023|
| Coll/ADP              | SM(d18:1/18:1) | 0.82 | 0.023|
| **HD pro-bleeding**   |             |            |     |
| TFPI                  | S1P d18:1 | 0.78 | 0.003|
| K                     | SM(d18:1/22:0) | 0.67 | 0.017|
| Coll/ADP              | SM(d18:1/16:0) | -0.6 | 0.04|
| F1+2                  | HexCer(d18:1/26:0) | -0.65 | 0.022|
| **PD pro-coagulant**  |             |            |     |
| TFPI                  | SM(d18:1/24:1) | 0.74 | 0.001|
| vWf                   | S1 d18:1 | 0.71 | 0.002|

Only parameters with Spearman ρ above or below ±0.6 and P<0.05 are presented.

### Table 5

| Parameter          | Training Samples | Bootstrap Samples* |
|--------------------|------------------|--------------------|
|                    | Logistic Regression (r²) | LDA (%)  | Logistic Regression (r²) | LDA (%)  |
| MA                 | 0.70             | 5.26              | 0.75 (0.64–0.83) | 3.80 (1.32–6.50) |
| HexCer(d18:1/22:0) | 0.71             |                   | 0.74 (0.65–0.82) |                   |
| HexCer(d18:1/24:0) | 0.82             |                   | 0.87 (0.79–0.92) |                   |
| HexCer(d18:1/26:0) | 0.63             |                   | 0.67 (0.55–0.77) |                   |

Only predictors with P<0.001, normalized r² ≥ 0.6, and AUC ≥ 0.8 (data not shown) were selected. Data for bootstrap are presented as median and IQR.

*Internal validation with bootstrap of 1,000 replacement.

**Normalized r² using Nagelkerke correction.

*Misclassification rate.
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