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A Pre-transplant Blood-based Lipid Signature for Prediction of Antibody-mediated Rejection in Kidney Transplant Patients

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Author Contributions

G.G. participated in study design, collecting the data, and writing and revising the manuscript.

J.S. participated is analyzing data, writing and revising the manuscript.

S.B. and N.G.K. participated in the generation of data.

P.K, A.K, M.L. and D.K participated in editing the manuscript.

D.C. participated in the generation and analysis of data and writing and revising the manuscript.

D.S.W. participated in study design, collecting the data, analyzing the data, writing, and revising the manuscript

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ABSTRACT

There is a lack of biomarkers for pre-kidney transplant immune risk stratification to avoid over- or under-immunosuppression. Since the circulating lipidome is integrally involved in inflammation, we hypothesized that the lipidome may provide biomarkers that are helpful in the prediction of antibody-mediated rejection. We used mass spectrometry to detect the plasma lipidome in samples collected over 1 year post-kidney transplant from a prospective, observational cohort of adult kidney transplant recipients (KTR), classified in two groups, one with antibody mediated rejection (AMR) and the other with stable graft function (SC). We used linear discriminant analysis to generate predictive models of rejection. A 'lipid-only' model generated from samples taken on day of transplant (T1) revealed a seven lipid classifier (lysophosphatidylethanolamine and phosphatidylcholine species) with misclassification rate of 8.9% [AUC = 0.95 (95% CI = 0.84-0.98), R^2 = 0.63]. A clinical model [(using donor specific antibody (DSA) and panel reactive antibody (PRA)] was inferior with a misclassification rate of 15.6% [AUC = 0.82 (95% CI = 0.69-0.93), R^2 = 0.41]. A combined model using four lipid classifiers and DSA improved the AUC further to 0.98 (95% CI = 0.89-1.0, R^2 = 0.83) with a misclassification of only 2.2%. The polyunsaturated phospholipid subspecies that discriminated the two groups were much lower in the AMR group when compared to the SC group. While the lipidomic profile changed significantly among SC patients on serial sampling post-transplant, such changes were not seen in AMR patients. After taking serial lipidomic changes overtime in SC patients in to account, the AMR group still showed sustained decreased levels of specific lipids at the time of AMR. These findings suggest that a lack of anti-inflammatory polyunsaturated phospholipids could identify patients at a higher risk of AMR at the time of transplant.

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INTRODUCTION

The complex biochemistry of human biological systems has been operationally separated into a set of large molecular categories. The metabolome, as it is termed, includes four classes of biologically active molecules that consist of proteins and amino acids, carbohydrates and sugars, nucleic acids (both DNA and RNA), and lipids. The full lipid profile that encompasses the complete set of lipid molecules in a human is termed the lipidome. The general term lipid describes a very large, ubiquitous and diverse class of molecules that have a structural and functional role in biological systems. Lipids are an integral structural component of cell membranes, play a significant role in energy storage, are involved in a variety of signaling pathways and intersect in the complex biochemistry of the other classes of compounds in the metabolome(1). Furthermore, by altering the properties of cellular membranes, the lipidome also has the ability to influence membrane mediated events such as enzyme association with membranes required for some catalytic events. Since first characterized in 2002, alterations of the lipidome have been intensely studied in a variety of conditions(2). Distinct lipid profiles have been identified in the normal state and in a variety of pathologic conditions and in response to specific therapeutic interventions(3–7).

Renal allograft transplantation is the treatment of choice for End Stage Renal Disease (ESRD). In the United States, a shortage of suitable organ donors and resultant organs available for transplant, creates a marked supply and demand discrepancy leaving many patients on the waiting list for prolonged periods of time(8). If evidence based risk stratification could occur pre-transplant then more effective and tailored immunosuppressive strategies could be designed to minimize the risk of rejection and infection post-transplant. Current immunosuppression protocols have resulted in a marked decrease in T-cell mediated rejection, at the cost of long term immunosuppression with its resultant adverse effects including susceptibility to

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92 opportunistic infections, graft damage, and metabolic complications such as hypertension,
93 diabetes, and lipid abnormalities which predispose to cardiovascular disease(9,10). However,
94 current immunosuppression protocols are not as effective in suppressing antibody mediated
95 rejection (AMR), which is a major cause of graft loss(10).

96 At the present time standardized immunosuppression protocols rather than individualized
97 immunosuppression is the routine practice for kidney transplantation, because suitable pre-
98 transplant risk stratification biomarkers that can predict future transplant rejection are not
99 available for clinical practice. It was previously thought that donor specific antibodies and the
100 degree of sensitization might serve as stratification tools, but they have been shown to be
101 inadequate predictors of future rejection (11). Thus, there is an unmet need for biomarkers that
102 could allow for better initial risk stratification while enhancing the benefits/risks of
103 immunosuppression therapy for individual patients.

104

105 **MATERIALS AND METHODS:**

106 **Patient Selection**

107 The Virginia Commonwealth University Institutional Review Board (IRB) approved this
108 study. Patients were selected from a prospective observational cohort of a single-institution adult
109 kidney transplant center in the United States. The study population consisted of 16 consecutive
110 patients who developed antibody-mediated rejection within 2 years of kidney transplant and 29
111 stable control (SC) patients who did not develop rejection at any point of post-transplant follow-
112 up. Serial plasma samples were collected and stored at Time 1 (T1 - pre-transplant), Month 6
113 (T2) and Month 12 (T3) and then yearly for all patient's post-transplant as part of an IRB
114 approved biobank protocol at our institution. For the AMR group, serum samples were drawn at
115 the time of transplant (T1), at rejection (T2) and at the end of successful therapy (T3).

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The SC patients were selected based on the retrospective observation during the period of the study for stable renal function, with no episodes of rejection, with known adherence to the immunosuppressive regimen, and with a sufficient volume of samples at the appropriate time points for lipid research assays. A minimum follow-up of 2 years was mandated to be a candidate for inclusion in the study. Pediatric kidney recipients and multi-organ transplant recipients were excluded.

At our institution all patients received a standardized immunosuppression induction protocol using anti-thymocyte globulin (Thymoglobulin, Genzyme, Cambridge, MA) with a total of 6 mg/kg over four consecutive days beginning in the operating room. Maintenance immunosuppression included a combination of tacrolimus, mycophenolate mofetil and prednisone tapered to 5 mg/day. Highly sensitized patients received 6 sessions of pre-emptive plasmapheresis with intravenous immunoglobulin (IVIG; 100mg/kg) based upon a pre-specified protocol as reported by us previously (12).

Indication biopsies were performed for acute allograft dysfunction defined as a rise in creatinine >20% above baseline, serum creatinine nadir ≥2.0 mg/dL post-transplant; or delayed graft function >21 days post-transplant. Surveillance biopsies were performed in patients with a positive flow-cytometric crossmatch (T or B >100 mean channel shifts) and/or presence of pre-formed donor-specific antibody [DSA; >5000 mean fluorescence intensity (MFI)] at 1 month and 6-months post-transplant. Biopsies were graded based upon the Banff criteria (13). Patients with AMR were treated with 6-9 sessions of plasmapheresis with intravenous immunoglobulin (IVIG; 100 mg/kg) in conjunction with intravenous methylprednisolone 500 mg administered once daily for 3 days. In selected cases, additional drug therapy with rituximab or bortezomib was instituted based upon clinical response.

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The details of antibody testing performed at our center have been described previously (14). Briefly, pre-transplant complement-dependent cytotoxicity (CDC) assays and three-color flow-cytometric cross matching (FCXM) were performed for all patients at the time of transplant. Donor-specific antibodies (DSA) were analyzed using the Luminex platform (Immucor Platform, San Diego, CA) with the use of an HLA phenotype panel (Lifematch Class I and Class II ID, Gen-Probe) and a single-antigen panel (Single Antigen Beads, Immucor Platform). Results of bead assays were measured as MFI. For highly sensitized patients an MFI of >5,000 and for de-novo kidney transplant recipients an MFI >10,000 was considered unacceptable for routine transplantation. Calculated Panel Reactive Antibody (cPRA) was determined using the OPTN calculator from the following url:

https://optn.transplant.hrsa.gov/resources/allocation-calculators/cpra-calculator/

Lipidomic Analysis

Serial serum samples were stored at -80ºC prior to research use. Upon initiation of experiments, samples were prepared for analysis using an HILIC-based UPLC ESI-MS/MS method. 50 µL of plasma was added to 750 µL of MTBE (methyl-tertiary butyl ether), containing 20 µL of SPLASH internal standards (SPLASH LIPIDOMIX Mass Spec Standard – Avanti 330707), and 160 µL of water. After centrifugation for 2 minutes at 12,300 rpm, 350 µL of supernatant was transferred to auto sampler vials and dried under vacuum. Dried extracts were re-suspended using 110 µL of a methanol:toluene (10:1, v/v) mixture containing CUDA (12-[[(cyclohexylamino) carbonyl] amino]-dodecanoic acid) at a final concentration of 50 ng/ml.

Samples were analyzed on a QTRAP 6500+, with Shimadzu Nexera UPLC. Analytes were separated on a Waters BEH HILIC 1.7 µm 2.1x150 mm column (column temperature = 30°C). Mobile phase A: 10 mM ammonium acetate (pH 8) in 95% ACN (acetonitrile). Mobile

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phase B: 10 mM ammonium acetate (pH 8) in 50% ACN. Gradient (B%) ramps from 0.1 to 20 in 10 mins; rises to 98 at 11 min, keeps for 2 mins, then drops back to 0.1 and maintains for 3 mins.

Statistical Analysis

A comparison t-test analysis (FDR=0.05) was used to select group differences on the day of transplant. Mean values for each lipids class were obtained by sum and average. Linear Discriminant Analysis with regularized correction (RLDA) models for lipids and clinical parameters were created with a stepwise forward method (Fig. 1). Regression performance was estimated with R^2, misclassification error and area under the ROC Curve (AUC). Estimates were validated with bootstrap coefficient interval (Fig. 1). Predictors combined model was cross validated with Random Forest method, and the misclassification out-of-bag error (OOB error) was estimated and compared to the RLDA error for validation (Fig. 1). Changes over time were also estimated using the sparse partial least square method and separation of the groups was validated with a permutation test. A t-test was used to compare two time points within a group and for comparing different groups at matched time points. Data was analyzed with JMP Pro 13 and MetaboAnalyst 3.0. The statistical workflow is depicted in Fig. 1.

RESULTS:

Demographic comparison of the two groups prior to transplantation is shown in Table 1. Patients in the AMR group were more likely to be female, re-transplants and had a higher degree of sensitization (higher cPRA) and presence of donor specific antibody (higher DSA) at the time of transplant. They were also more likely to have hyperlipidemia. There were no differences noted for age, race, weight, years on dialysis, type of dialysis, delayed graft function, or the presence or absence of diabetes mellitus.

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A comparison of phospholipid (PL) classes at T1 revealed relative concentration differences between SC and AMR (Fig. 2). The concentration of phosphatidylcholine (PC) was significantly diminished in AMR, while there was a trend for an increased concentration of lysophosphatidylcholine (LPC). The AMR group also showed a significantly lower concentration of phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE), plasmanylethanolamine (PE-O), and plasmenylethanolamine (PE-P). Although not statistically significant, there was also lower concentration of Phosphoglycerol (PG), lysophosphatidylglycerol (LPG), and sphingomyelin (SM). The activity of phospholipase A_2 (PLA_2) as a signal of increased metabolism was assessed by the ratio of PL to lysophospholipids (LPL). The AMR group showed decreased ratios of PC/LPC and PE/LPE indicating higher activity of PLA_2 at T1. PL degradation, evident for PE, was higher in the AMR group compared to the SC group. .

Combined lipid and clinical parameters allow for the prediction of rejection on the day of transplant (T1).

Preliminary data demonstrated that there are significant differences in the pre-transplant lipidome between SC and AMR. This led to the hypothesis that the T1 lipidome or some combination of the lipidome and clinical parameters could provide insight into the risk of future transplant rejection, enabling better risk stratification for kidney transplant recipients. To investigate this possibility, a stepwise regularized linear regression was deployed using models of lipids alone, clinical data alone, and a merged lipid and clinical data to test for prediction accuracy (Table 2). The analysis identified seven distinct lipids that discriminated between AMR and SC with 8.9% of the events misclassified [Area under receiver operating characteristic curve (AUC) =0.95 (95%CI=0.84-0.98), R2=0.63 (95%CI=0.4-0.8)]. A clinical model using cPRA and

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DSA was inferior with 15.6% of the events misclassified, AUC=0.80 (95%CI=0.66-0.90), R2=0.36 (95%CI=0.16-0.57). Still using a stepwise selection approach, a combined model determined with 4 lipids plus DSA further reduced the misclassification events to 2.2% (Fig. 3), and the AUC improved to 0.97 (95% CI=0.88-1.0), R2=0.81 (95%CI=0.49-0.96).

Further comparison of the four lipids predictors of kidney rejection showed that these lipids are significantly decreased in AMR compared to the SC group. In the PC (18:0 /20:4) plot, it is possible to notice the presence of outliers in both groups (Fig. 4A). Random Forest method was used for statistical validation with 500 bootstrap samples, and the mean decrease accuracy test was used estimate the importance of each predictor to the validation model (Fig. 4B). The result revealed that DSA is the more important clinical biomarker of AMR at T1, and together with LPE (16:0) and PC (18:0/20:4) can discriminate AMR with a very low error (2.2%). The statistical validation also revealed that exclusion of LPE (22:6) and LPE (20:4) in the model would have a minimal effect on the misclassification error. Although in the RLDA modeling training, using the entire study population, the addition of these two lipids takes the model estimation from R^2=0.75 to R^2=0.81.

Serial analyses of the lipidome over the course of one year identify time dependent lipid changes among patients with a favorable transplant outcome, but no differences among graft recipients with non-favorable outcomes.

Following the identification of the lipid differences at T1 and their ability to predict graft rejection in association with measured clinical parameters, we wished to investigate how the lipidome changes over time in patients with a favorable transplant outcome (SC). To achieve this end, serial lipid profiles were analyzed from samples collected at Day 0, 6 months and 12 months post-transplant (Fig. 5). A sPLSDA analysis of the data revealed a statistically significant

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alteration in the metabolic profile at 6 months post-transplant compared to the day of transplant (Fig. 5A). However, for the subsequent times from 6 months to 12 months, there was no significant change in the lipidomic profile. This finding suggests that stabilization of the lipid changes after transplant is associated with the achievement of improved kidney function and possibly a reduced milieu of inflammation (Fig. 5B). The data was subjected to validation using the permutation test (Fig. 5C) and showed a statistically significant metabolic difference (p= 0.034) from T1 to 6 months after transplantation.

Further investigation of the lipid differences between T1 and T2 identified 19 lipids that represent the relevant time dependent alterations in the lipidome that had statistically significant elevations at T2 compared to T1 in the SC group. (Fig. 6). A majority of these lipids changes are LPC, with a few PC, one PE-O, two PE-P, and one PG.

Following the identification of the longitudinal lipid trajectory among patients with favorable transplant outcomes, we investigated the trajectory of the lipidome pre-transplant to post transplant one year, among the patients with non-favorable outcomes (AMR) (Fig. 7). sPLSDA analysis of the data reveal that there was no significant alteration in the lipid profile at pre-rejection and post-rejection compared to T1 (Fig. 7a). While a slight change was observed from T1 to post-rejection (Fig. 7B), validation analysis using permutation testing demonstrated this difference to be non-significant (p=0.869) (Fig. 7C). These findings indicate that in contrast to patients with a favorable transplant outcome (SC), patients with non-favorable transplant outcomes (AMR) demonstrated no change in the lipid profile observed pre-transplant over time.

Significant post-transplant lipid differences were observed between Stable Controls vs. those with Antibody-mediated Rejection

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As our data revealed that there were significant T1 vs T2 lipid differences between SC, but not in AMR, we further investigated the data to identify the exact differences in the lipidome between SC and AMR at T2. Any differences identified would indicate an alteration in the lipid metabolic environment at the time of rejection that would distinguish AMR from SC. Since there were no significant differences between T2 and T3 for SC group we chose to use SC at T2 (6 months post-transplant) to compare with AMT at T2 (time of AMR). The analysis revealed a panel of 13 lipids that were found to differentiate the two groups at T2 (Fig. 8). As noted previously, these 13 lipids were again comprised of LPE and PC species containing monounsaturated and polyunsaturated fatty acids, except for LPE (16:0). This data further confirms the presence of a sustained lipid metabolic difference between SC and AMR over time that distinguish these two groups of patients.

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DISCUSSION:

In this first study, we report novel data that the lipidome could be used to identify kidney transplant patients with a higher risk of antibody-mediated rejection at the time of transplant. In addition, for the first time we demonstrate that combining lipidomic and clinical data to create a model merging the presence of donor-specific antibody and lipids (a reduction of each of the four identified lipid biomarkers, one PC and three LPE species) can discriminate AMR with minimal error even at the time of transplant. Statistical validation suggests that DSA, LPE (16:0) and PC (18:0/20:4) are putative biomarkers that should be further tested in a prospective clinical study. These biomarkers could indicate a state of increased inflammation associated with chronic kidney disease and hemodialysis in selected groups of patients compared with others(15).

Modulation of phospholipids (PL) in chronic kidney disease (CKD) is well described in the literature. In a study of CKD among rats, Zhao *et al.* identified that PC, PE, LPC, LPE and triacyclglycerides (TG) steadily decreased as the pathology progressed over time (16). Braun *et al* described that the aged kidney from adult wild-type mice expresses significant decreases of PC, PE, PG, SM, phosphatidylserine (PS), and Ceramides, suggesting that change in PL metabolism is associated with CKD (3). Kobayashi *et al.* reported an elevation of LPE 20:4 in the plasma of adenine-induced CKD rats when comparing with control animals(17). In a human study comparing healthy controls and CKD patients, Reis *et al.* found that the content of total PC and Ceramides were decreased along with the ratio of LPC/LPE(18). In a study comparing patients with CKD progression compared to control patients, Afshinnia *et al.* reported that CKD progression was associated with lower Cholesteryl ester (CE), diacylglycerols (DG), PC, plasmenylcholine (PC-P), PE-P, and phosphatidic acid (PA), and elevated PE and monoacylglycerols (MAG)(19). This finding suggests that patients with CKD progression with a decrease of longer acyl chains and polyunsaturated lipids might benefit from the effects

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of polyunsaturated fatty acid supplementation, as some previous studies have suggested(20,21). In our study, although both groups represent patients who had CKD progression, the SC group had higher PC and LPE than the AMR group and a trend for lower LPC suggesting that subpopulations with varying degrees of inflammatory milieu might exist with the CKD population. This would be consistent with the real-life observation of patients who have varying degrees of risk of rejection.

LPC has being associated with pro-inflammatory effects(22), but there is not much information about the effects of LPE. Some studies suggest that LPE could have a possible protective effect over inflammation. Schober *et al.* demonstrated that LPE generation from PE oxidation is primarily due to PLA_2 activity rather than by hypochlorous acid generated by myeloperoxidase, while LPC can be generated from both processes(23). The dual effect of PLA_2 is well known by its pro-inflammatory action in hydrolysis of PC to produce LPC promoting atherogenesis, as well as its anti-inflammatory action in hydrolysis of platelet-activating factor (PAF) and oxidized PLs(24). This suggest that processes that are not directly related to oxidative stress generate LPE in CKD patients. The activity of PLA_2 in our study was assessed by the ratio of PL to LPL. The AMR group had a higher PLA_2 activity, especially for degradation of PE to produce LPE. The PC/LPC ratio, as an inflammatory marker is also indirectly represented by the increased activity of PLA_2 in inflammatory diseases(25,26).

It has been reported that *in vitro* LPE induces activation of the mitogen-activated protein kinase (MAPK) cascade, an intracellular signal transduction pathway that controls growth, proliferation, differentiation, motility, stress response, and has a survival along with ananti-apoptotic effects(27). Also LPE increases intracellular Ca^{2+} through a Lysophosphatidic acid (LPA) G-protein-coupled receptor (GPCR)(28). Oral administration of LPE in rats with zymosan A-induced peritonitis demonstrated a vast anti-inflammatory action. In that study LPE-

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containing polyunsaturated fatty acids administration inhibited plasma leakage by diminishing the formation of LTC_4, inhibited the leukocyte extravasation into the peritoneum, decreased formation of potent chemotactic factors such as LTB4 and 12-HETE, lowered IL-1β, IL-6, TNF-α, and augmented IL-10(29).

Our results suggest that the lack of anti-inflammatory protection in patients on the day of transplant is a risk for future rejection. No relevant changes occurred for the AMR group until the onset of rejection, confirming that the metabolic profile at T1 predicting AMR persisted after transplantation. Accordingly, over time comparison of SC and AMR showed that the difference in LPE and PC levels were sustained after 6 months representing the metabolic difference between rejection and non-rejection. The presence of monounsaturated and polyunsaturated fatty acids in PL is also an indication that their low plasma content is a risk factor for kidney health (30). In contrast, the elevation of LPC, PC, PE-O, PE-P, and PG after 6 months in SC group imply that restauration of PL content is the result of successful transplantation. Indeed, some studies have shown that elevation of polyunsaturated fatty acids present a lower risk of developing end-stage renal disease (31), as well as higher survival rates after kidney transplantation(32).

There are some limitations to our study. Demographic comparisons between the SC and AMR groups at T1 revealed that female gender, re-transplant, cPRA, DSA, and hyperlipidemia were statistically more likely to be present in the AMR group. Moreover, we found DSA as the strongest predictor of AMR. These findings are consistent with Dunn *et al.* who reported that DSA and female gender were risk factors for AMR (33). Thus, the two groups could have been inherently different biochemically. Future larger studies with an increased sample size would be need to confirm this preliminary study. Our finding of hyperlipidemia in AMR group could be linked to the fact that hyperlipidemia is the most common form of dyslipidemia, a common

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complication in CKD patients, associated with the decline in kidney function, hypertriglyceridemia, low HDL, and low or normal LDL (34).

CONCLUSION:

Our study for the first time identifies the pre-transplant, post-transplant, and pre-rejection lipid differences that distinguish kidney transplant patients with favorable transplant outcomes (SC) and a major cause of non-favorable transplant outcomes (AMR). We further demonstrate that unlike SC patients that demonstrate a dynamic longitudinal lipid change, AMR patients maintain a relatively unchanging lipid profile over time with respect to the measured lipids. In addition, we demonstrate for the first time the feasibility of risk stratification of kidney transplant patients on the day of transplant about the possibility of prediction for future AMR. Following prospective validation in a larger cohort, these findings have the potential to alter the current paradigm of pre- and post-transplant monitoring. Treatment of these patients with an evidenced based risk stratification strategy could vastly improve the success of kidney transplantation.

DISCLOSURE

There are no conflicts of interest to report for any of the authors.

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367

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368 **FIGURE LEGENDS, TABLES AND FIGURES**

369

370 **Fig. 1: Statistical analysis workflow for the study.** After data filtering and normalization, a
371 statistical workflow based on Regularized Linear Discriminant Analysis (RLDA) and Sparse
372 Partial Least Square Discriminant Analysis (sPLSDA) was applied. Candidate variables were
373 selected by t-test with a False Discover Rate (FDR) =0.05. RLDA at T1 identified lipid
374 biomarkers that predicted AMR. Predictive models using lipids, clinical parameters, and the
375 combination of both markers were analyzed using a forward stepwise regression. . Bootstrap and
376 Random Forest were used as internal validation. sPLSDA at three different time points was used
377 to identify and compare metabolic changes indicative of AMR. A permutation test was then used
378 for validation.

379

380 **Fig. 2: Significant differences are observed among phospholipids at T1 between SC and**
381 **AMR.** A) **The** AMR group showed a significantly lower concentration of PC, PE, and LPE
382 (phospholipids). There was a trend towards higher levels of LPC (lipophopholipids) in AMR. B)
383 PLA_2 activity, an indicator of phospholipid degradation to produce LPL was assessed by the
384 ratio of PL to LPL. A lower value suggests higher activity as shown by PC/LPC and PE/LPE
385 ratios in AMR. Suspected outliers are indicated by open circles in the box plots. Green rectangles
386 represent AMR and the red rectangles represent SC. * indicates significant differences with
387 $p<0.05$.

388

389 **Fig. 3: The RLDA model generated using four lipids and DSA demonstrate good**
390 **separation between AMR and SC groups**. The RLDA plot shows the clear separation of the
391 patients in the two groups based on the Mahalanobis distance. This method determines whether

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the selected predictors can separate the distinct categories and reveals the presence of outliers in the AMR and SC groups. Blue dots among the red dots indicates the one misclassified patient identified in the predictive model. Internal ellipse indicates the 95% confidence region containing the true mean of the group. External ellipse indicates the region estimated to contain 50% of group' population.

Fig. 4: Predictors of AMR on the day of transplant and Random Forest statistical validation. A) Box plot of normalized concentrations shows that the AMR group has lower concentrations of the lipids predictors. Suspected outliers are represented as open circles that appear outside the whiskers. The validation method showed that the prediction model could discriminate SC and AMR at T1 with 0.022 OOB error. The mean Decrease Accuracy method shows that DSA is the more important predictor, followed by LPE (16:0) and PC (18:0/20:4) and they independently could be used as biomarkers. The analysis also reveals that when considering these predictors as biomarkers, the inclusion of LPE (20:4) and LPE (22:6) does not add any predictive power, and rather must be used to compose the RLDA model. * indicates significant differences with $p<0.01$.

Fig. 5: The lipidome of SC demonstrate clear differences between T1 and T2 but no differences between T2 and T3. A) The graphical distribution of T1 (shown in red), T2 (shown in green), and T3 (shown in blue) indicates that there is no difference between 6 months and 1-year post-transplant, after a metabolic shift from T1 to T2. B) The lipid difference is highlighted by the change in the first 6 months. C) Permutation test was performed as a validation test to evaluate the statistical significance of the PLS-DA model separation from T1 to T2 ($p=0.034$). Ellipses represent the 95%CI for each time point.

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Fig. 6: Specific lipids characterize the difference between T1 and T2 among SC patients. The levels of the 19 different lipids that are significantly elevated 6 months after transplantation are mostly comprised from the LPC class containing both unsaturated and saturated fatty acids. PCs, PE-O, PE-P and PG are also elevated after 6 months. * indicates significant differences with $p<0.01$.

Fig. 7: Contrary to SC patients, no statistically significant difference was observed in the T1 and T2 lipidome of AMR patients. A) The graphical distribution of T1 (shown in red), T2 (shown in green), and T3 (shown in blue) indicates that there is no difference over time, although a slight metabolic shift could be detected from T1 to post-rejection. B) The plot of the slight metabolic difference from T1 to T2 highlights the overlap of the 95% CI of the two time points. C) Permutation test was performed as a validation test and shows that this difference in the PLS-DA model separation from T1 to T2 is not statistically significant ($p=0.869$). Ellipses represent the 95% CI of each time point.

Fig. 8: Specific lipids demonstrate significant differences between SC and AMR at T2. The metabolic changes observed at T1 were sustained 6 months after transplant with lower LPE and PC species in AMR group. Except for LPE (16:0) all lipids contained monounsaturated and polyunsaturated fatty acids. SC group shown in red. AMR group shown in green. * indicates significant differences with $p<0.01$.

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441 **Table 1 – Demographic Characteristics of the Patient Cohort -** Categorical variables were
442 analyzed with the Fisher's exact test; Continuous data is presented as a mean of the group ±
443 standard deviation and is analyzed by t-test. SD: Standard deviation; cPRA: calculated panel
444 reactive antibody; DSA: donor specific antibody; GRF: glomerular filtration rate.

Characteristic	SC	AMR	p-value
N	29 (100%)	16 (100%)	
Female Gender	4 (14%)	11 (69%)	0.005*
Age, years (Mean±SD)	47±11	50±9	0.45
African-American Race	17 (59%)	13 (81%)	0.19
Pre-transplant Diabetes	10 (34%)	8 (50%)	0.35
Pre-transplant hyperlipidemia	7 (29%)	16 (100%)	0.04*
Weight at Transplant, kg (Mean±SD)	85±21	82±14	0.6
Years on dialysis (Mean±SD)	2.9±1.9	4.3±4.1	0.26
Mode of dialysis			
Hemodialysis	19 (65%)	13 (81%)	0.49
Peritoneal Dialysis	4 (14%)	2 (12%)	
Preemptive transplant	6 (21%)	1 (7%)	
Re-transplant	4 (14%)	9 (56%)	0.001*
cPRA, % (Mean±SD)	9.8 (±29.4)	40.8(±45.8)	0.023*
DSA	1 (3%)	8 (50%)	<0.001*
Kidney Donor Profile Index, %	52±27	54 ±32	0.89
Delayed Graft Function	13 (45%)	7 (44%)	1.00
GFR at 6 months post-transplant*	67±22	61±23	0.37
GFR at 12 months post-transplant*	68±19	58±22	0.11

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448 **Table 2 – Predictors of Rejection at the Time of Transplant -** Bootstrap validation with 95%
449 Confidence intervals is included for RLDA estimates and area under the curve (AUC)**.** cPRA:
450 Calculated Panel Reactive Antibody; DSA: donor specific antibodies; GFR: Estimated
451 glomerular filtration rate (mL/min/1.73m2); SC: Stable Controls; AMR: Antibody-mediated
452 Rejection; *statistically significant.

Model	Predictors	R^2	Misclassification	AUC
Only lipids	PC (16:0/22:6) PC (18:0/20:4) PC (18:1/20:4) LPE (16:0) LPE (16:1) LPE (20:4) LPE (22:6)	0.63 (0.40 – 0.80)	8.9% (3.3 – 18.6)	0.95 (0.84 – 0.98)
Only clinical	cPRA DSA	0.36 (0.16 – 0.57)	15.9% (7.4 – 29.2)	0.80 (0.66 -0.90)
Merged models	PC (18:0/20:4) LPE (16:0) LPE (20:4) LPE (22:6) DSA	0.81 (0.49 – 0.96)	2.3% (0.1 – 12.1)	0.97 (0.88 – 1.00)

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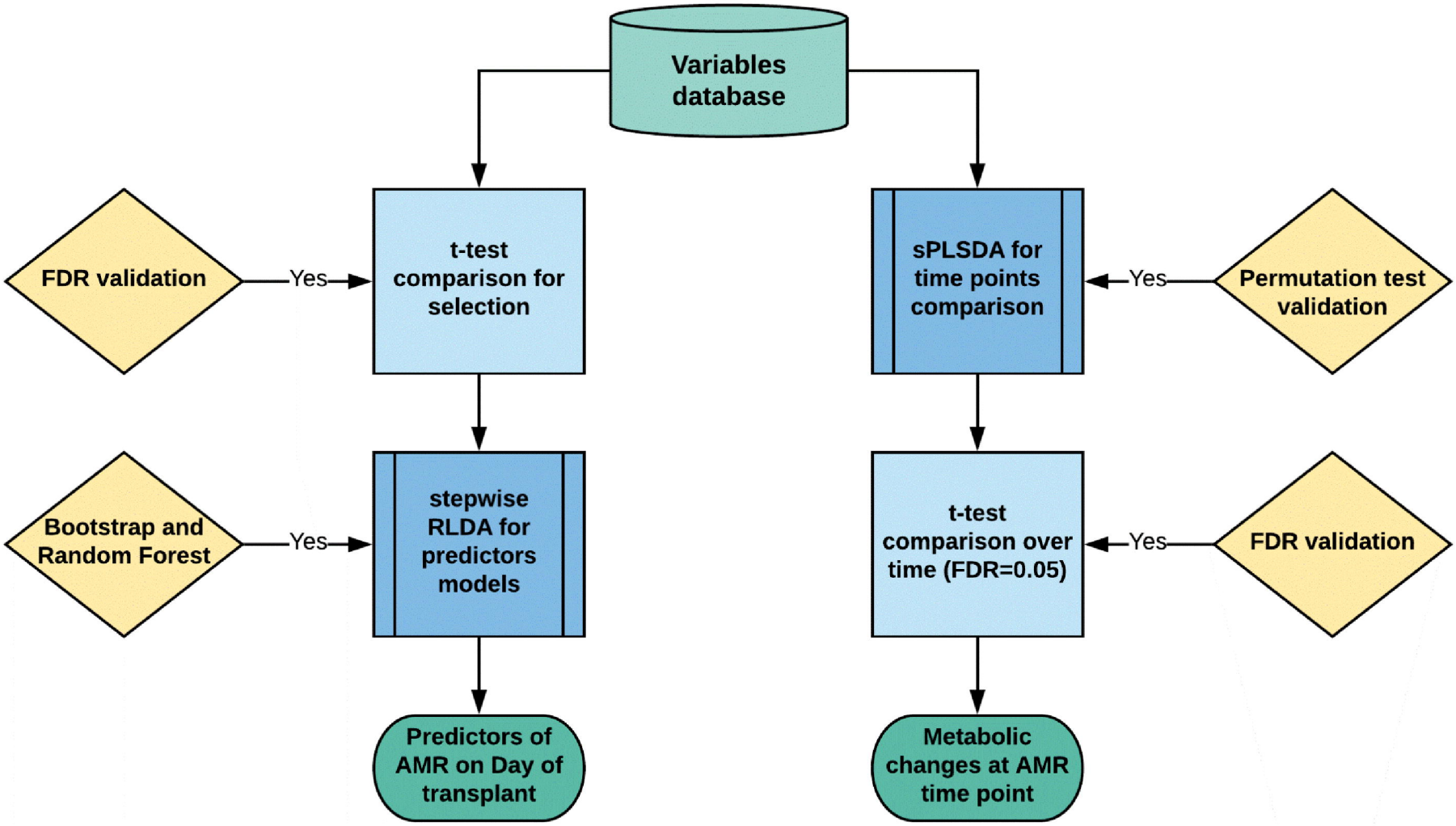
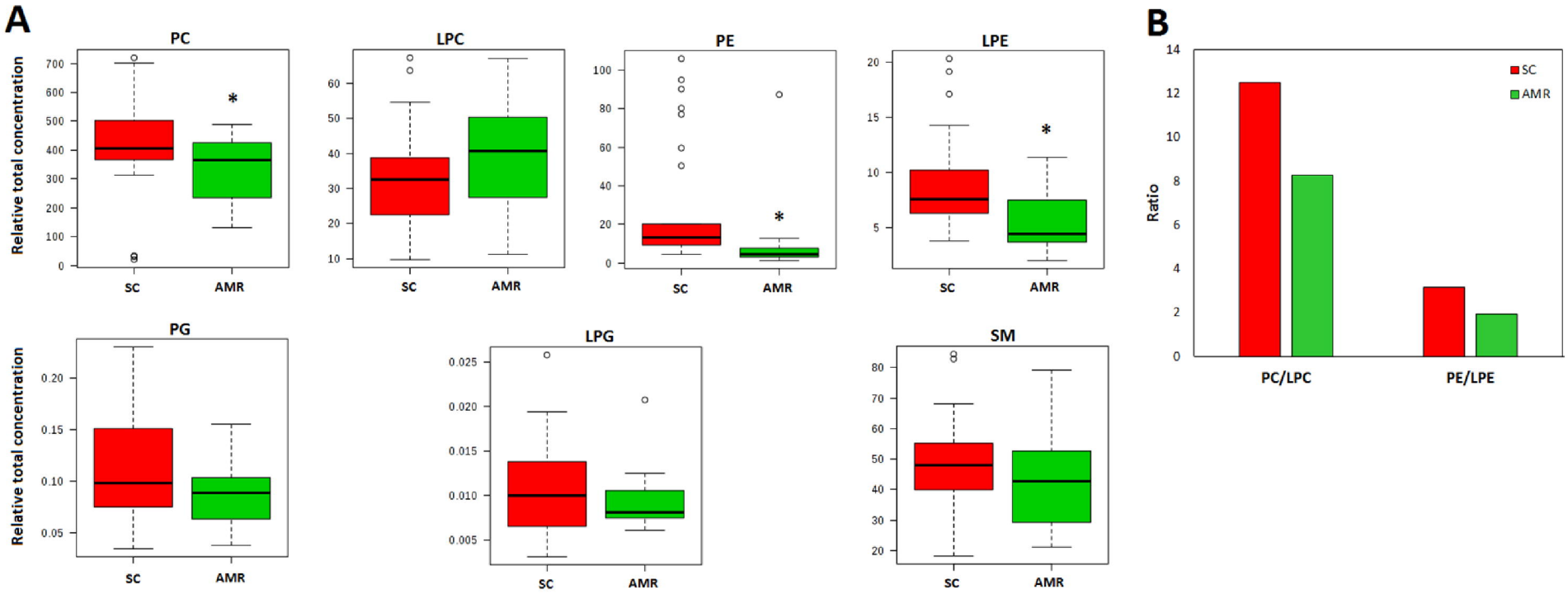

Variables database
FDR validation
Yes
t-test comparison for selection
sPLSDA for time points comparison
Yes
Permutation test validation
Bootstrap and Random Forest
Yes
stepwise RLDA for predictors models
t-test comparison over time (FDR=0.05)
Yes
FDR validation
Predictors of AMR on Day of transplant
Metabolic changes at AMR time point


A
PC
LPC
PE
LPE
PG
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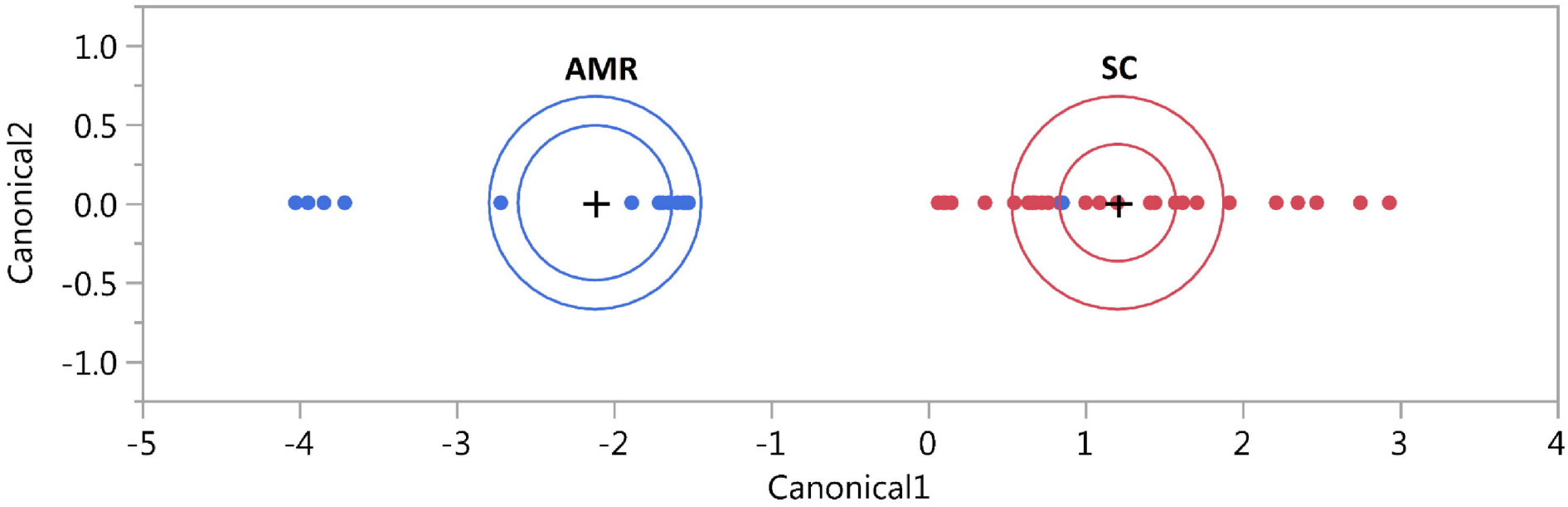
SM
Relative total concentration
Relative total concentration
SC
AMR
B
Ratio
SC
AMR
PC/LPC
PE/LPE


AMR
SC
1.0
0.5
0.0
-0.5
-1.0
Canonical2
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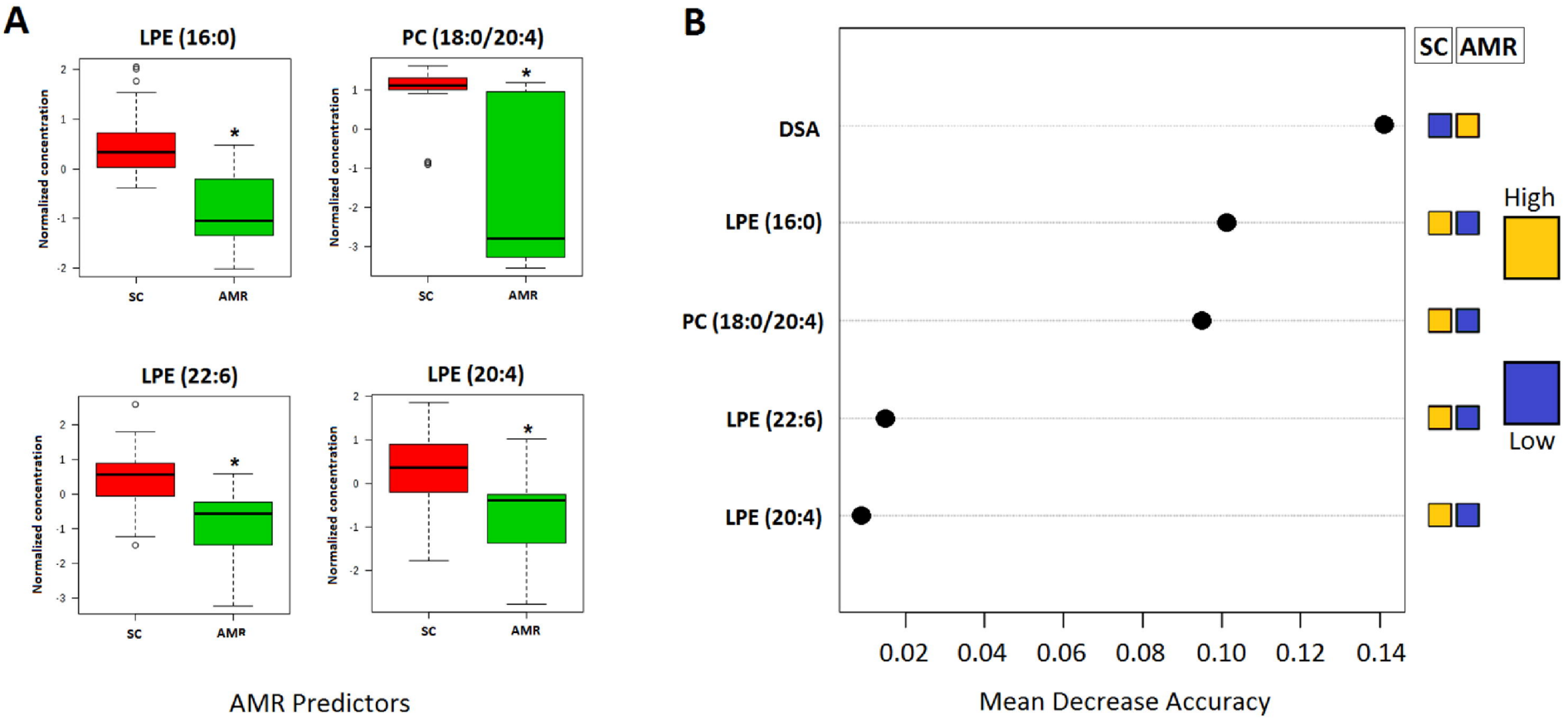
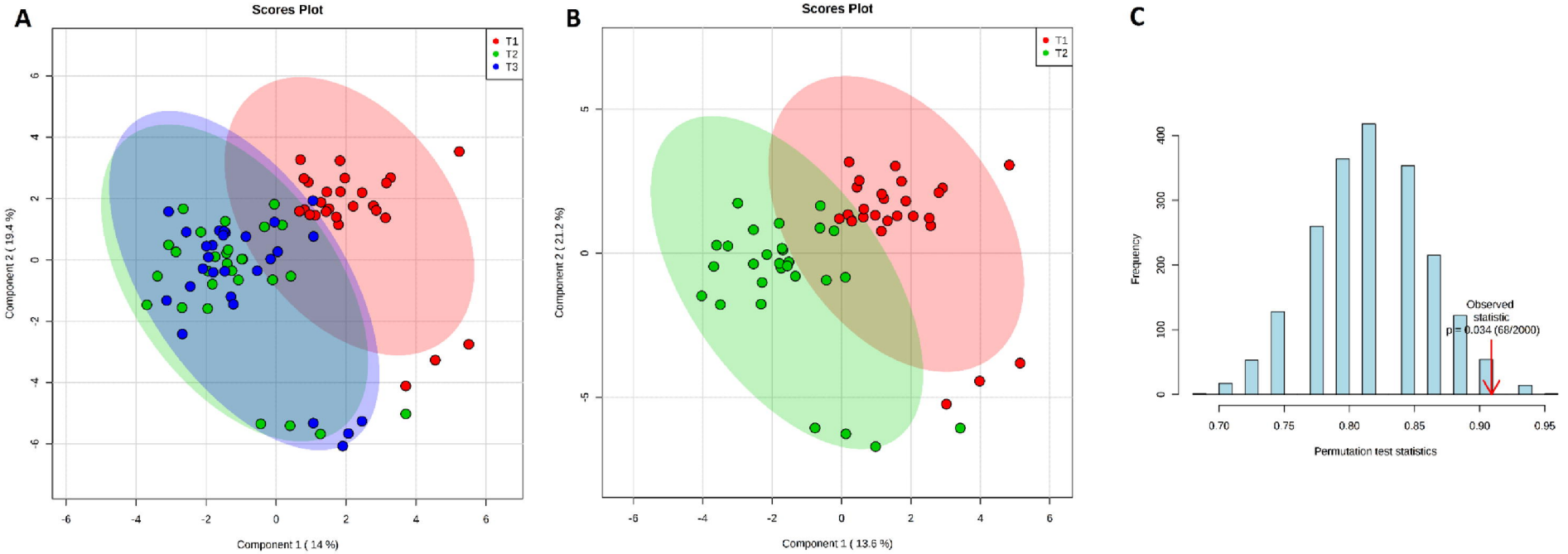
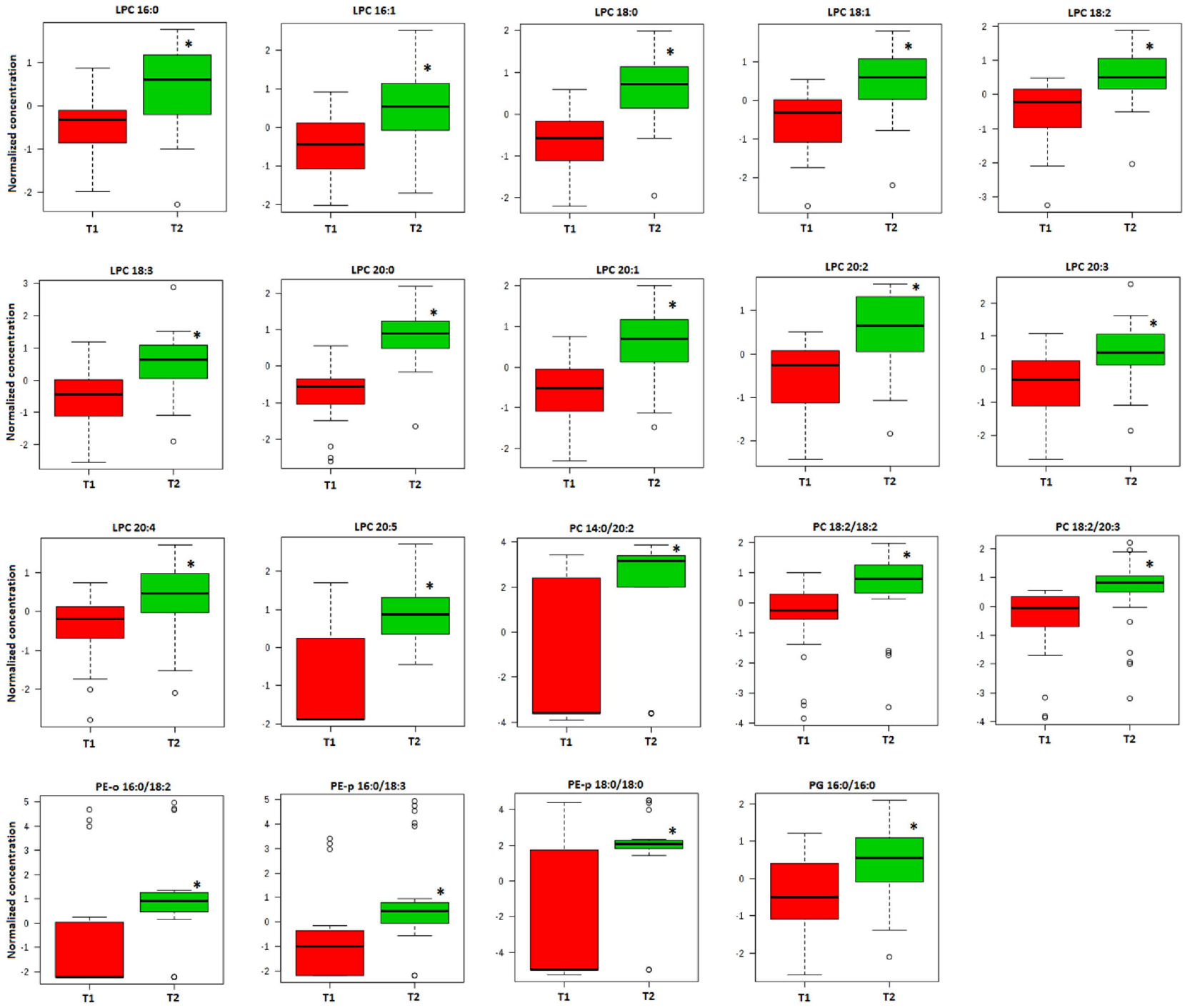
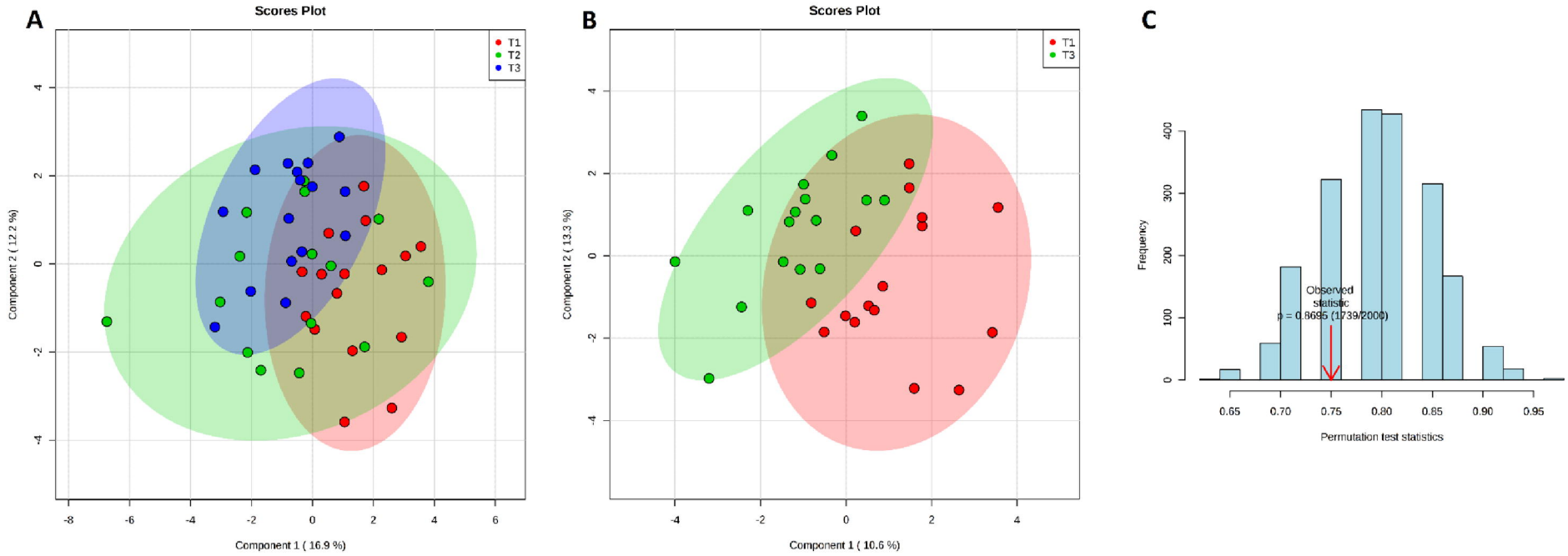
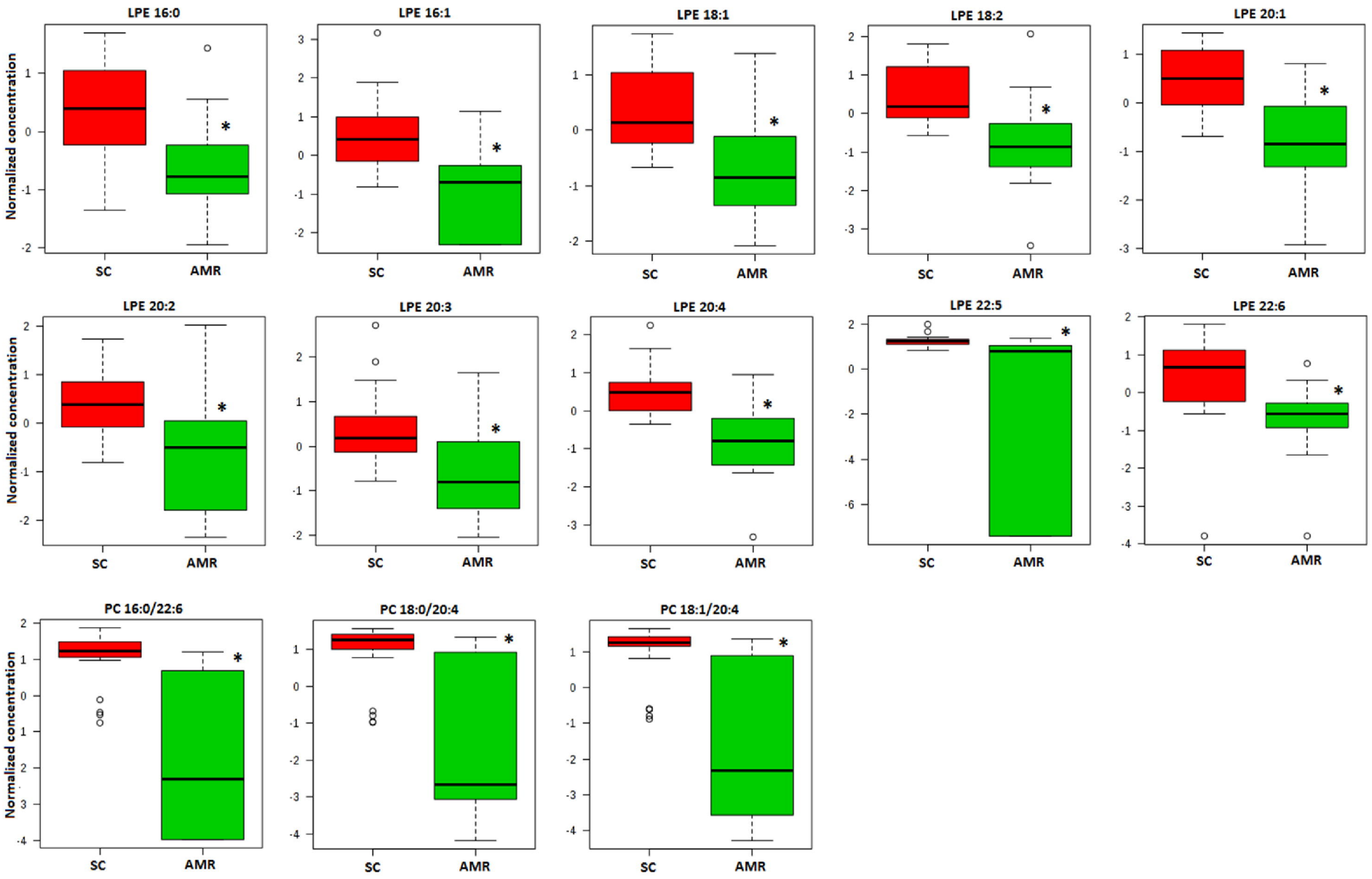
-4
-3
-2
-1
0
1
2
3
4
Canonical1


A
LPE (16:0)
PC (18:0/20:4)
LPE (22:6)
LPE (20:4)
Normalized concentration
SC
AMR
AMR Predictors
B
SC
AMR
DSA
LPE (16:0)
PC (18:0/20:4)
LPE (22:6)
LPE (20:4)
High
Low
0.02
0.04
0.06
0.08
0.10
0.12
0.14
Mean Decrease Accuracy


A
Scores Plot
T1
T2
T3
Component 2 (19.4 %)
Component 1 (14 %)
B
Scores Plot
T1
T2
Component 2 (21.2 %)
Component 1 (13.6 %)
C
Frequency
Observed statistic
p = 0.034 (68/2000)
Permutation test statistics


LPC 16:0
LPC 16:1
LPC 18:0
LPC 18:1
LPC 18:2
LPC 18:3
LPC 20:0
LPC 20:1
LPC 20:2
LPC 20:3
LPC 20:4
LPC 20:5
PC 14:0/20:2
PC 18:2/18:2
PC 18:2/20:3
PE-o 16:0/18:2
PE-p 16:0/18:3
PE-p 18:0/18:0
PG 16:0/16:0
Normalized concentration
T1
T2


A
Scores Plot
T1
T2
T3
Component 2 (12.2 %)
Component 1 (16.9 %)
B
Scores Plot
T1
T3
Component 2 (13.3 %)
Component 1 (10.6 %)
C
Frequency
Observed statistic
p = 0.8695 (1739/2000)
Permutation test statistics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LPE 16:1
LPE 18:1
LPE 18:2
LPE 20:1
LPE 20:2
LPE 20:3
LPE 20:4
LPE 22:5
LPE 22:6
PC 16:0/22:6
PC 18:0/20:4
PC 18:1/20:4
Normalized concentration
SC
AMR
*