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

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15 **Short Title:** Lipidomics in Kidney Transplants
16

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34 **Author Contributions**

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

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

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

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

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

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

41 the manuscript

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44 **ABSTRACT**

45 There is a lack of biomarkers for pre-kidney transplant immune risk stratification to avoid over-
46 or under-immunosuppression. Since the circulating lipidome is integrally involved in
47 inflammation, we hypothesized that the lipidome may provide biomarkers that are helpful in the
48 prediction of antibody-mediated rejection. We used mass spectrometry to detect the plasma
49 lipidome in samples collected over 1 year post-kidney transplant from a prospective,
50 observational cohort of adult kidney transplant recipients (KTR), classified in two groups, one
51 with antibody mediated rejection (AMR) and the other with stable graft function (SC). We used
52 linear discriminant analysis to generate predictive models of rejection. A 'lipid-only' model
53 generated from samples taken on day of transplant (T1) revealed a seven lipid classifier
54 (lysophosphatidylethanolamine and phosphatidylcholine species) with misclassification rate of
55 8.9% [AUC = 0.95 (95% CI = 0.84-0.98), R^2 = 0.63]. A clinical model [(using donor specific
56 antibody (DSA) and panel reactive antibody (PRA)] was inferior with a misclassification rate of
57 15.6% [AUC = 0.82 (95% CI = 0.69-0.93), R^2 = 0.41]. A combined model using four lipid
58 classifiers and DSA improved the AUC further to 0.98 (95% CI = 0.89-1.0, R^2 = 0.83) with a
59 misclassification of only 2.2%. The polyunsaturated phospholipid subspecies that discriminated
60 the two groups were much lower in the AMR group when compared to the SC group. While the
61 lipidomic profile changed significantly among SC patients on serial sampling post-transplant,
62 such changes were not seen in AMR patients. After taking serial lipidomic changes overtime in
63 SC patients in to account, the AMR group still showed sustained decreased levels of specific
64 lipids at the time of AMR. These findings suggest that a lack of anti-inflammatory
65 polyunsaturated phospholipids could identify patients at a higher risk of AMR at the time of
66 transplant.

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68 **INTRODUCTION**

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

84 Renal allograft transplantation is the treatment of choice for End Stage Renal Disease
85 (ESRD). In the United States, a shortage of suitable organ donors and resultant organs available
86 for transplant, creates a marked supply and demand discrepancy leaving many patients on the
87 waiting list for prolonged periods of time(8). If evidence based risk stratification could occur
88 pre-transplant then more effective and tailored immunosuppressive strategies could be designed
89 to minimize the risk of rejection and infection post-transplant. Current immunosuppression
90 protocols have resulted in a marked decrease in T-cell mediated rejection, at the cost of long
91 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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116 The SC patients were selected based on the retrospective observation during the period of
117 the study for stable renal function, with no episodes of rejection, with known adherence to the
118 immunosuppressive regimen, and with a sufficient volume of samples at the appropriate time
119 points for lipid research assays. A minimum follow-up of 2 years was mandated to be a
120 candidate for inclusion in the study. Pediatric kidney recipients and multi-organ transplant
121 recipients were excluded.

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

129 Indication biopsies were performed for acute allograft dysfunction defined as a rise in
130 creatinine >20% above baseline, serum creatinine nadir ≥2.0 mg/dL post-transplant; or delayed
131 graft function >21 days post-transplant. Surveillance biopsies were performed in patients with a
132 positive flow-cytometric crossmatch (T or B >100 mean channel shifts) and/or presence of pre-
133 formed donor-specific antibody [DSA; >5000 mean fluorescence intensity (MFI)] at 1 month and
134 6-months post-transplant. Biopsies were graded based upon the Banff criteria (13). Patients with
135 AMR were treated with 6-9 sessions of plasmapheresis with intravenous immunoglobulin (IVIG;
136 100 mg/kg) in conjunction with intravenous methylprednisolone 500 mg administered once daily
137 for 3 days. In selected cases, additional drug therapy with rituximab or bortezomib was instituted
138 based upon clinical response.

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139 The details of antibody testing performed at our center have been described previously
140 (14). Briefly, pre-transplant complement-dependent cytotoxicity (CDC) assays and three-color
141 flow-cytometric cross matching (FCXM) were performed for all patients at the time of
142 transplant. Donor-specific antibodies (DSA) were analyzed using the Luminex platform
143 (Immucor Platform, San Diego, CA) with the use of an HLA phenotype panel (Lifematch Class I
144 and Class II ID, Gen-Probe) and a single-antigen panel (Single Antigen Beads, Immucor
145 Platform). Results of bead assays were measured as MFI. For highly sensitized patients an MFI
146 of >5,000 and for de-novo kidney transplant recipients an MFI >10,000 was considered
147 unacceptable for routine transplantation. Calculated Panel Reactive Antibody (cPRA) was
148 determined using the OPTN calculator from the following url:
149 https://optn.transplant.hrsa.gov/resources/allocation-calculators/cpra-calculator/
150

151 **Lipidomic Analysis**

152 Serial serum samples were stored at -80ºC prior to research use. Upon initiation of experiments,
153 samples were prepared for analysis using an HILIC-based UPLC ESI-MS/MS method. 50 µL of
154 plasma was added to 750 µL of MTBE (methyl-tertiary butyl ether), containing 20 µL of
155 SPLASH internal standards (SPLASH LIPIDOMIX Mass Spec Standard – Avanti 330707), and
156 160 µL of water. After centrifugation for 2 minutes at 12,300 rpm, 350 µL of supernatant was
157 transferred to auto sampler vials and dried under vacuum. Dried extracts were re-suspended
158 using 110 µL of a methanol:toluene (10:1, v/v) mixture containing CUDA (12-
159 [[(cyclohexylamino) carbonyl] amino]-dodecanoic acid) at a final concentration of 50 ng/ml.
160 Samples were analyzed on a QTRAP 6500+, with Shimadzu Nexera UPLC. Analytes
161 were separated on a Waters BEH HILIC 1.7 µm 2.1x150 mm column (column temperature =
162 30°C). Mobile phase A: 10 mM ammonium acetate (pH 8) in 95% ACN (acetonitrile). Mobile

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163 phase B: 10 mM ammonium acetate (pH 8) in 50% ACN. Gradient (B%) ramps from 0.1 to 20 in
164 10 mins; rises to 98 at 11 min, keeps for 2 mins, then drops back to 0.1 and maintains for 3 mins.
165

166 **Statistical Analysis**

167 A comparison t-test analysis (FDR=0.05) was used to select group differences on the day
168 of transplant. Mean values for each lipids class were obtained by sum and average. Linear
169 Discriminant Analysis with regularized correction (RLDA) models for lipids and clinical
170 parameters were created with a stepwise forward method (Fig. 1). Regression performance was
171 estimated with R^2, misclassification error and area under the ROC Curve (AUC). Estimates were
172 validated with bootstrap coefficient interval (Fig. 1). Predictors combined model was cross
173 validated with Random Forest method, and the misclassification out-of-bag error (OOB error)
174 was estimated and compared to the RLDA error for validation (Fig. 1). Changes over time were
175 also estimated using the sparse partial least square method and separation of the groups was
176 validated with a permutation test. A t-test was used to compare two time points within a group
177 and for comparing different groups at matched time points. Data was analyzed with JMP Pro 13
178 and MetaboAnalyst 3.0. The statistical workflow is depicted in Fig. 1.
179

180 **RESULTS:**

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

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187 A comparison of phospholipid (PL) classes at T1 revealed relative concentration
188 differences between SC and AMR (Fig. 2). The concentration of phosphatidylcholine (PC) was
189 significantly diminished in AMR, while there was a trend for an increased concentration of
190 lysophosphatidylcholine (LPC). The AMR group also showed a significantly lower
191 concentration of phosphatidylethanolamine (PE), lysophosphatidylethanolamine (LPE),
192 plasmanylethanolamine (PE-O), and plasmenylethanolamine (PE-P). Although not statistically
193 significant, there was also lower concentration of Phosphoglycerol (PG),
194 lysophosphatidylglycerol (LPG), and sphingomyelin (SM). The activity of phospholipase A_2
195 (PLA_2) as a signal of increased metabolism was assessed by the ratio of PL to lysophospholipids
196 (LPL). The AMR group showed decreased ratios of PC/LPC and PE/LPE indicating higher
197 activity of PLA_2 at T1. PL degradation, evident for PE, was higher in the AMR group compared
198 to the SC group. .
199
200 **Combined lipid and clinical parameters allow for the prediction of rejection on the day of**
201 **transplant (T1).**
202 Preliminary data demonstrated that there are significant differences in the pre-transplant
203 lipidome between SC and AMR. This led to the hypothesis that the T1 lipidome or some
204 combination of the lipidome and clinical parameters could provide insight into the risk of future
205 transplant rejection, enabling better risk stratification for kidney transplant recipients. To
206 investigate this possibility, a stepwise regularized linear regression was deployed using models
207 of lipids alone, clinical data alone, and a merged lipid and clinical data to test for prediction
208 accuracy (Table 2). The analysis identified seven distinct lipids that discriminated between AMR
209 and SC with 8.9% of the events misclassified [Area under receiver operating characteristic curve
210 (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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211 DSA was inferior with 15.6% of the events misclassified, AUC=0.80 (95%CI=0.66-0.90),
212 R2=0.36 (95%CI=0.16-0.57). Still using a stepwise selection approach, a combined model
213 determined with 4 lipids plus DSA further reduced the misclassification events to 2.2% (Fig. 3),
214 and the AUC improved to 0.97 (95% CI=0.88-1.0), R2=0.81 (95%CI=0.49-0.96).

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

226

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

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

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235 alteration in the metabolic profile at 6 months post-transplant compared to the day of transplant
236 (Fig. 5A). However, for the subsequent times from 6 months to 12 months, there was no
237 significant change in the lipidomic profile. This finding suggests that stabilization of the lipid
238 changes after transplant is associated with the achievement of improved kidney function and
239 possibly a reduced milieu of inflammation (Fig. 5B). The data was subjected to validation using
240 the permutation test (Fig. 5C) and showed a statistically significant metabolic difference (p=
241 0.034) from T1 to 6 months after transplantation.

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

246 Following the identification of the longitudinal lipid trajectory among patients with
247 favorable transplant outcomes, we investigated the trajectory of the lipidome pre-transplant to
248 post transplant one year, among the patients with non-favorable outcomes (AMR) (Fig. 7).
249 sPLSDA analysis of the data reveal that there was no significant alteration in the lipid profile at
250 pre-rejection and post-rejection compared to T1 (Fig. 7a). While a slight change was observed
251 from T1 to post-rejection (Fig. 7B), validation analysis using permutation testing demonstrated
252 this difference to be non-significant (p=0.869) (Fig. 7C). These findings indicate that in contrast
253 to patients with a favorable transplant outcome (SC), patients with non-favorable transplant
254 outcomes (AMR) demonstrated no change in the lipid profile observed pre-transplant over time.
255

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

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

269

270

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271 **DISCUSSION:**

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

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

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

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

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

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

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

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

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343 complication in CKD patients, associated with the decline in kidney function,
344 hypertriglyceridemia, low HDL, and low or normal LDL (34).
345

346 CONCLUSION:

347 Our study for the first time identifies the pre-transplant, post-transplant, and pre-rejection
348 lipid differences that distinguish kidney transplant patients with favorable transplant outcomes
349 (SC) and a major cause of non-favorable transplant outcomes (AMR). We further demonstrate
350 that unlike SC patients that demonstrate a dynamic longitudinal lipid change, AMR patients
351 maintain a relatively unchanging lipid profile over time with respect to the measured lipids. In
352 addition, we demonstrate for the first time the feasibility of risk stratification of kidney
353 transplant patients on the day of transplant about the possibility of prediction for future AMR.
354 Following prospective validation in a larger cohort, these findings have the potential to alter the
355 current paradigm of pre- and post-transplant monitoring. Treatment of these patients with an
356 evidenced based risk stratification strategy could vastly improve the success of kidney
357 transplantation.
358

359 DISCLOSURE

360 There are no conflicts of interest to report for any of the authors.
361

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365 and does not necessarily represent the official views of the National Institutes of Health. This work also
366 received support via a Young Investigator Award from SCIEX for clinical lipidomic research (DSW).

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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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392 the selected predictors can separate the distinct categories and reveals the presence of outliers in
393 the AMR and SC groups. Blue dots among the red dots indicates the one misclassified patient
394 identified in the predictive model. Internal ellipse indicates the 95% confidence region
395 containing the true mean of the group. External ellipse indicates the region estimated to contain
396 50% of group' population.

397

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

408

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

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416

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

422

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

431 **Fig. 8: Specific lipids demonstrate significant differences between SC and AMR at T2.** The
432 metabolic changes observed at T1 were sustained 6 months after transplant with lower LPE and
433 PC species in AMR group. Except for LPE (16:0) all lipids contained monounsaturated and
434 polyunsaturated fatty acids. SC group shown in red. AMR group shown in green. * indicates
435 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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454

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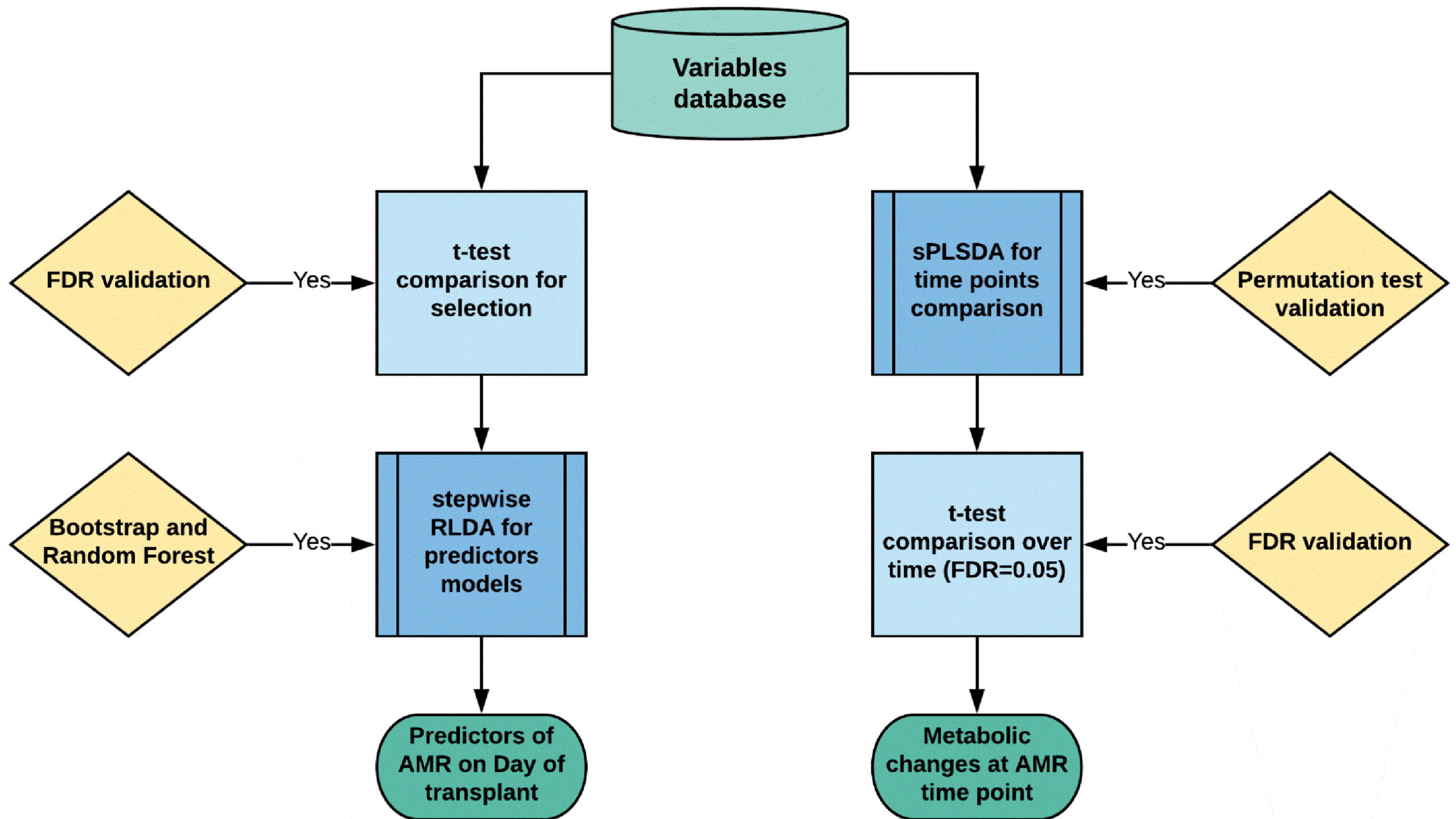
Variables database
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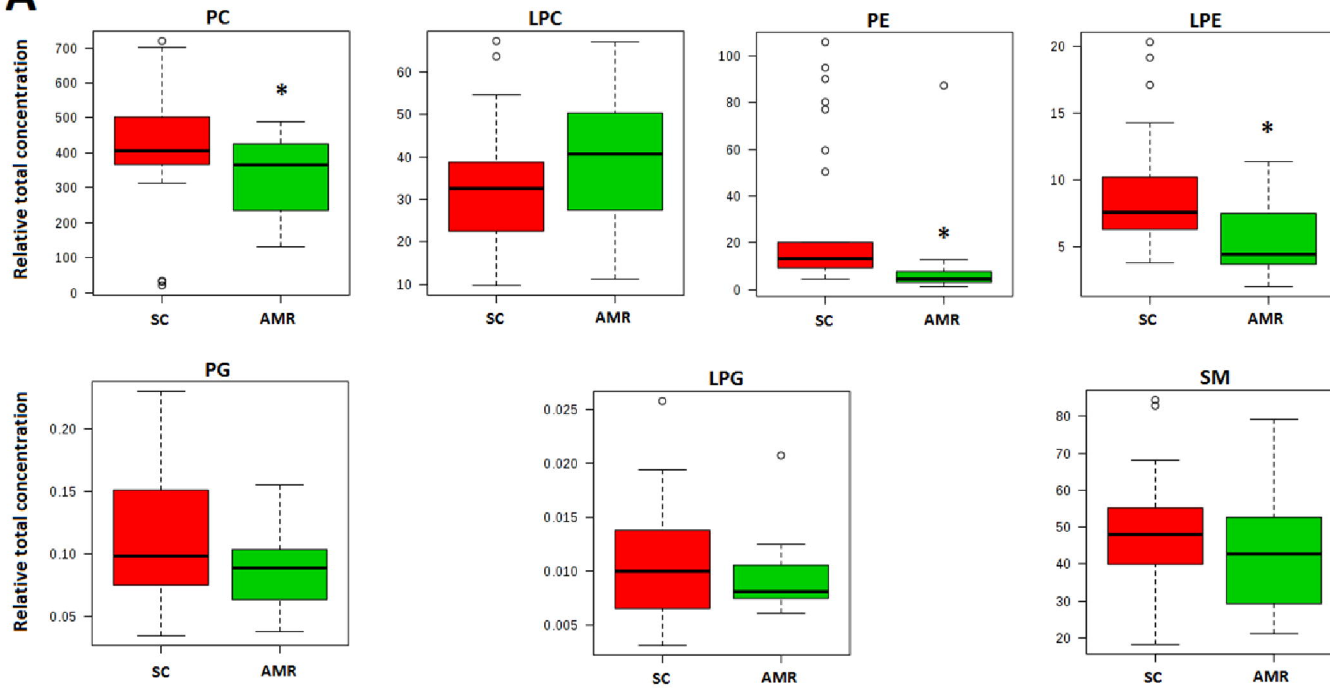
sPLSDA for time points comparison
FDR validation
Yes
Yes
Permutation test validation
stepwise RLDA for predictors models
t-test comparison over time (FDR=0.05)
Bootstrap and Random Forest
Yes
Yes
FDR validation
Predictors of AMR on Day of transplant
Metabolic changes at AMR time point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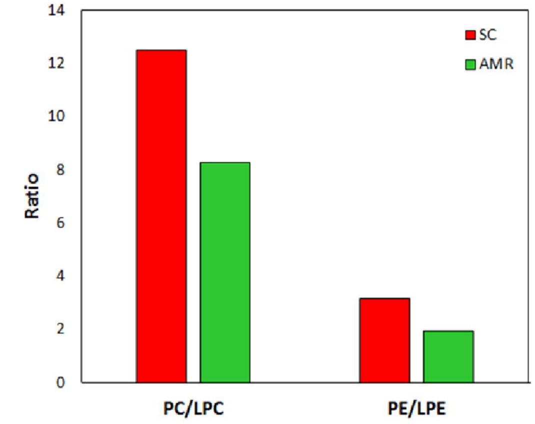
PC
LPC
PE
LPE
PG
LPG
SM
Relative total concentration
SC
AMR
*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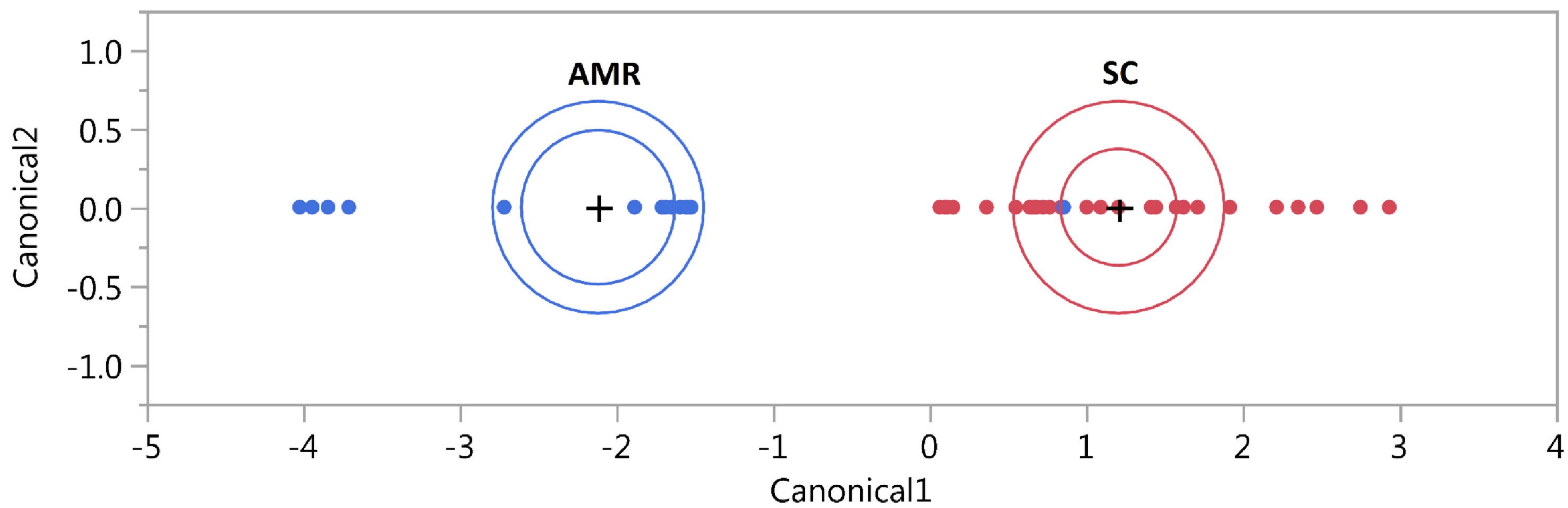
SC
AMR
PC/LPC
PE/LPE


AMR
SC
Canonical2
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-3
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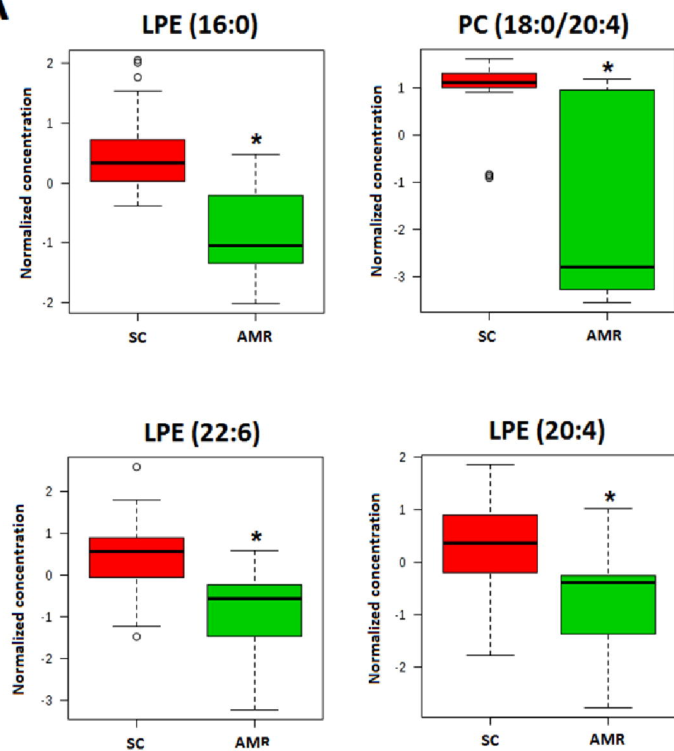
-1
0
1
2
3
4
Canonical1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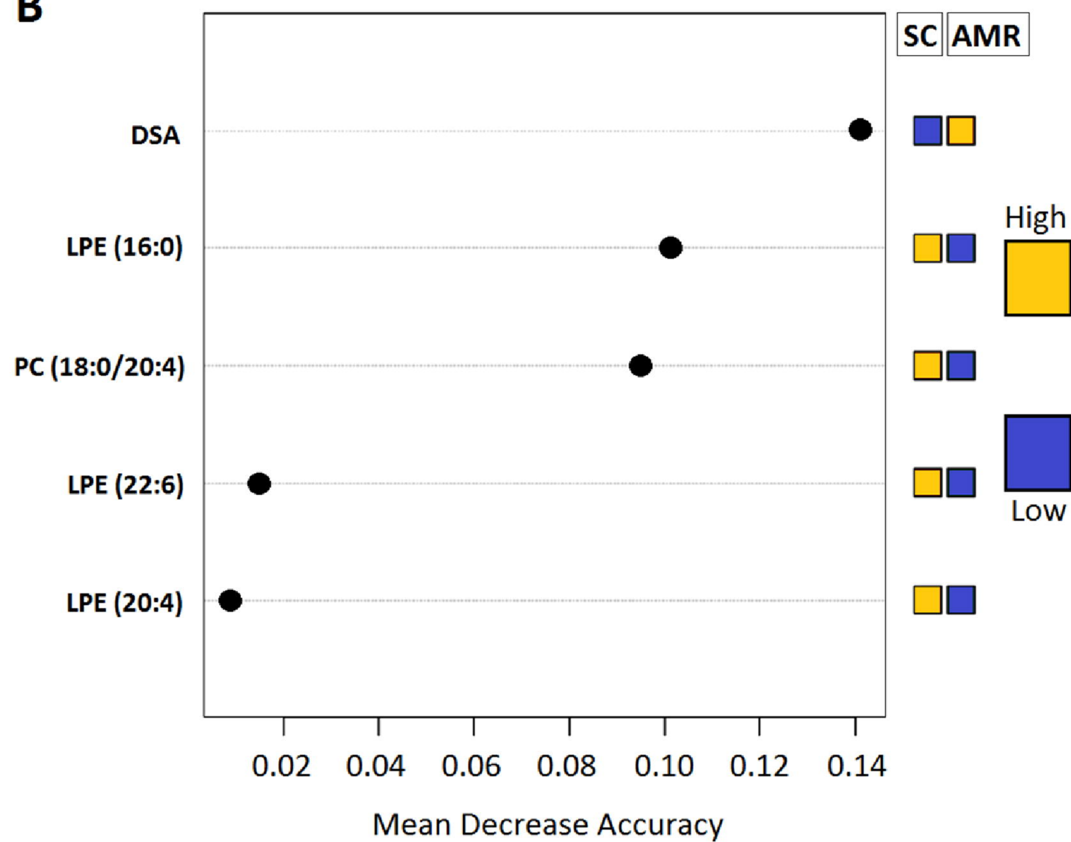
PC (18:0/20:4)
LPE (22:6)
LPE (20:4)
Normalized concentration
SC
AMR
AMR Predictors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SC
AMR
DSA
LPE (16:0)
PC (18:0/20:4)
LPE (22:6)
LPE (20:4)
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Low
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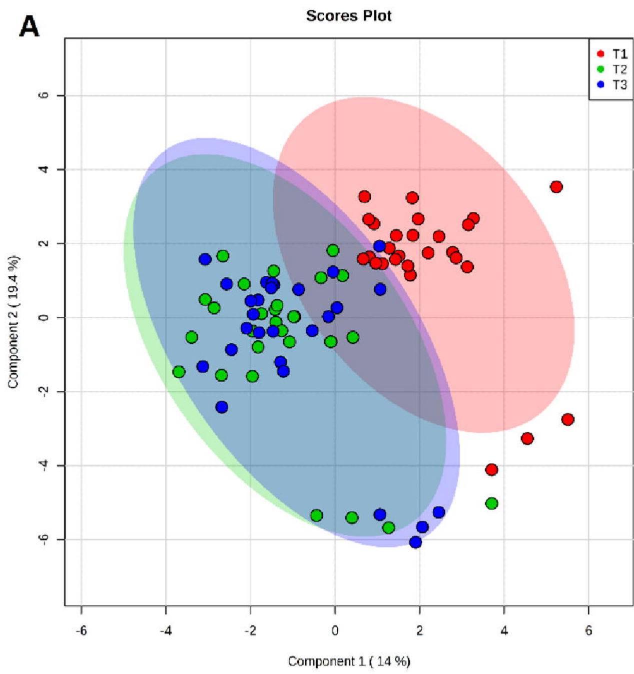
0.06
0.08
0.10
0.12
0.14
Mean Decrease Accuracy

A



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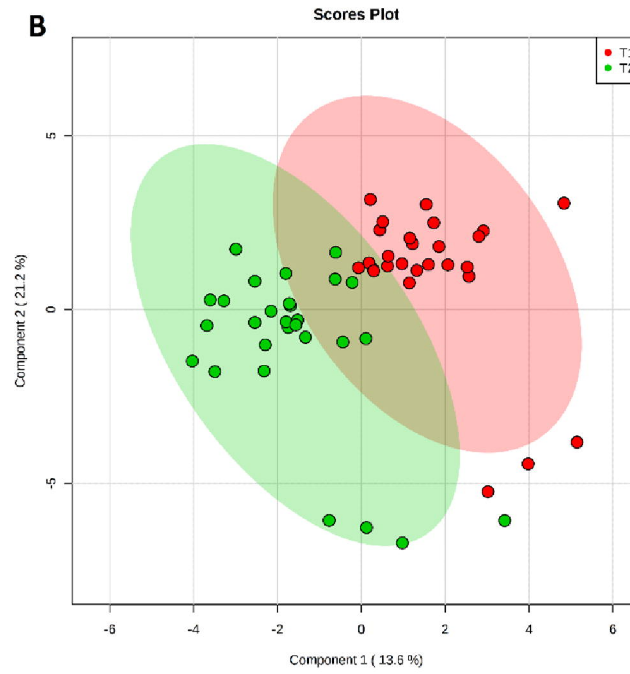

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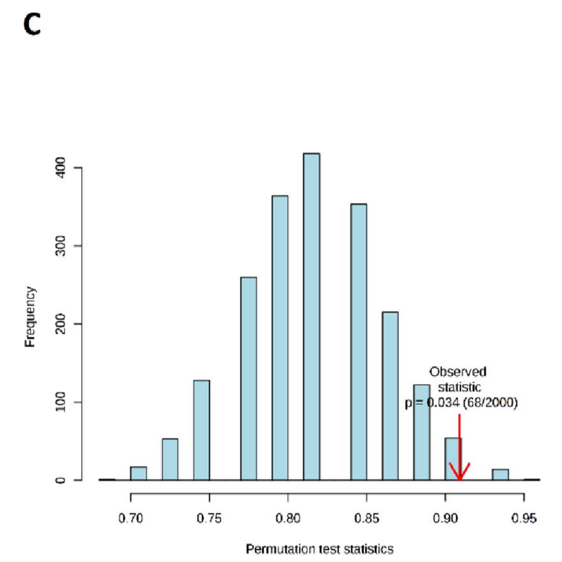
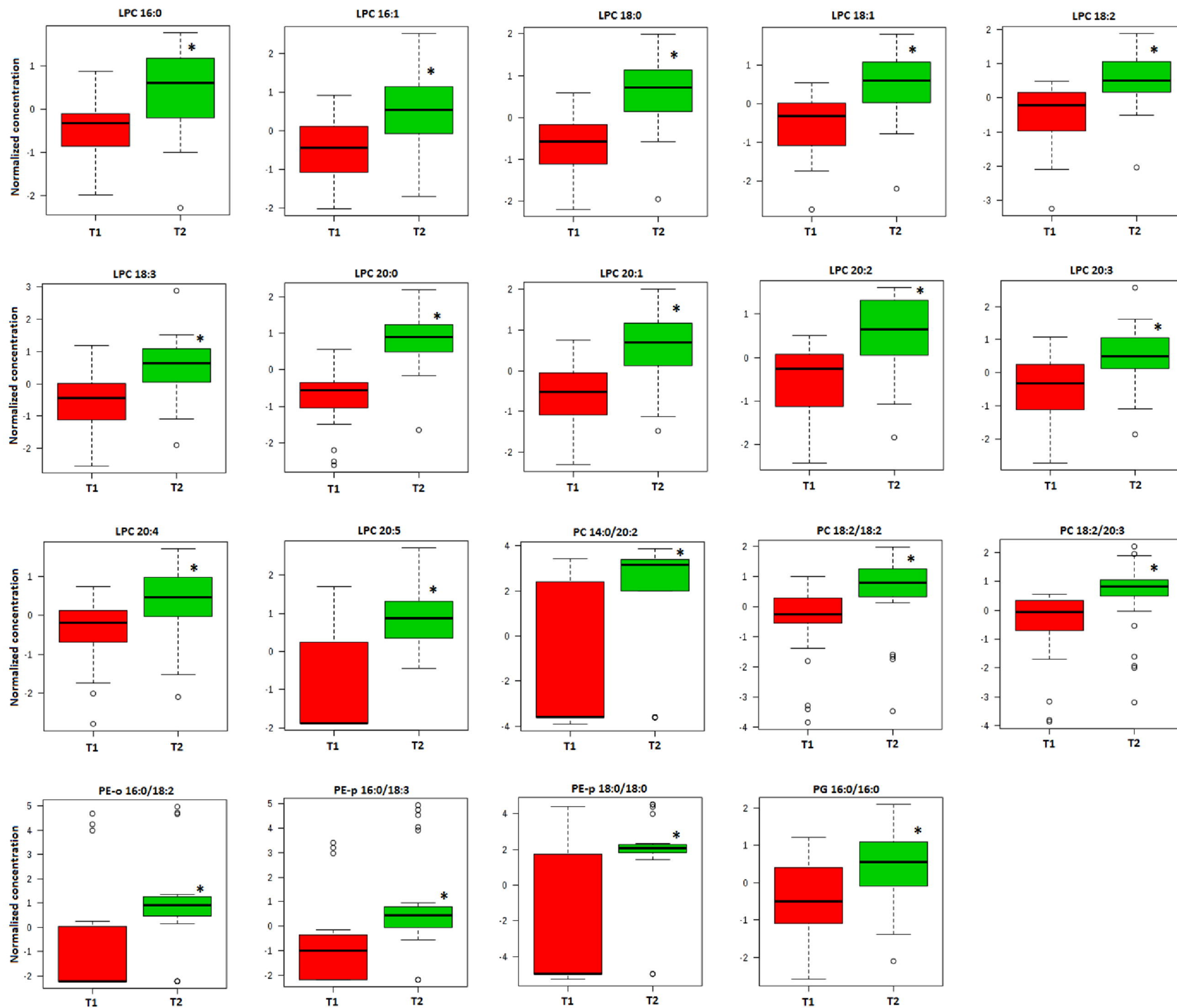
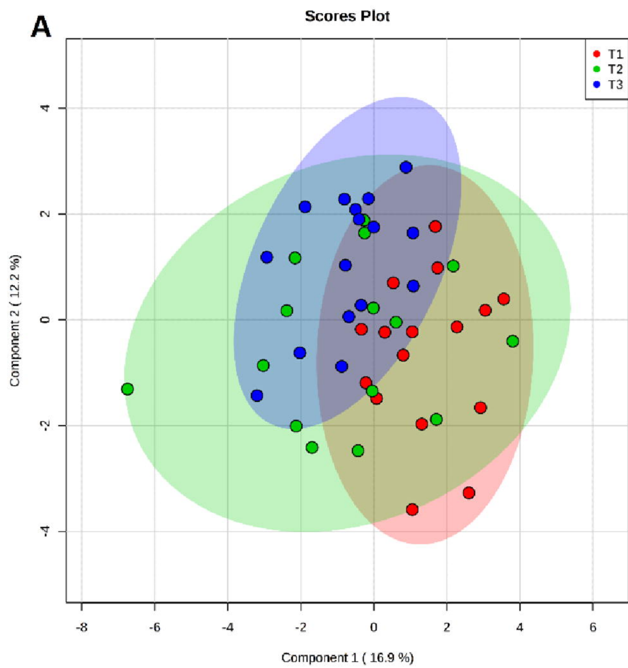
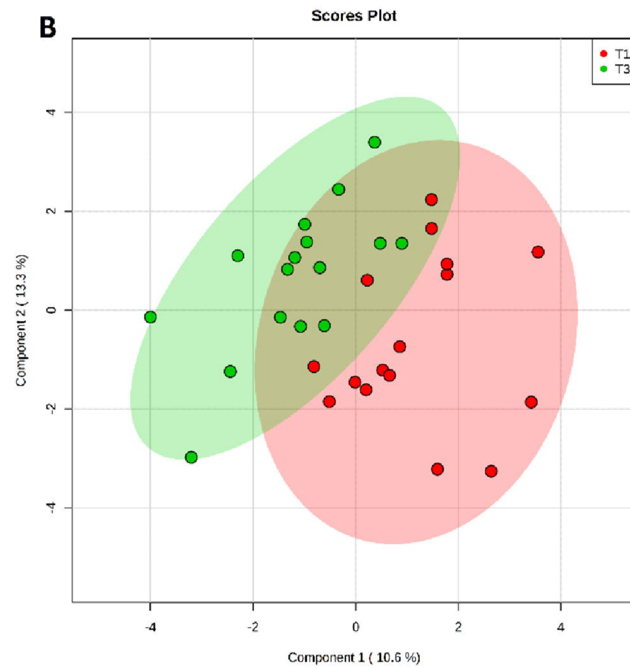
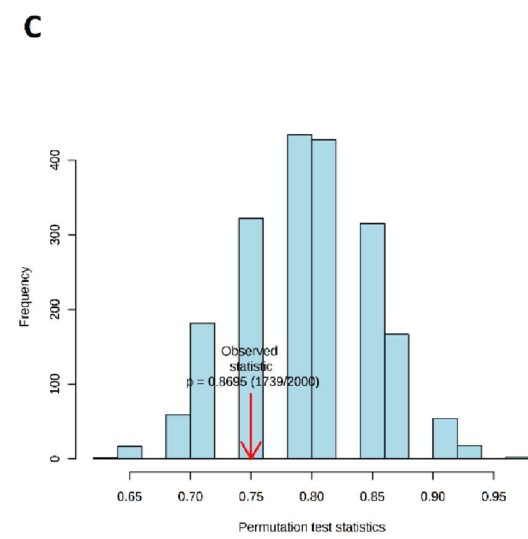


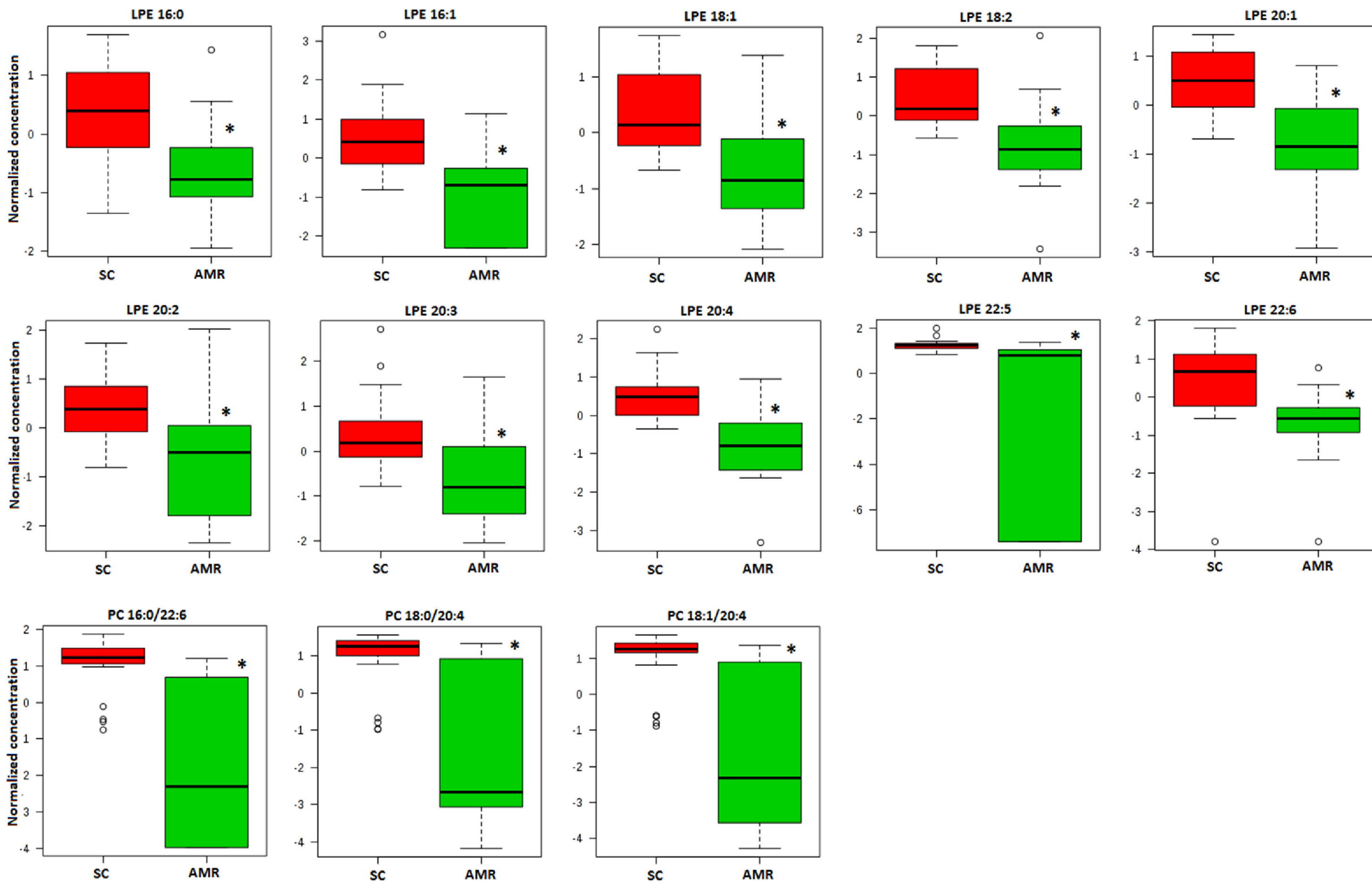


LPC 16:0
LPC 16:1
LPC 18:0
LPC 18:1
LPC 18:2
LPC 18:3
LPC 20:0
LPC 20:1
LPC 20:2
LPC 20:3
LPC 20:4
LPC 20:5
PC 14:0/20:2
PC 18:2/18:2
PC 18:2/20:3
PE-o 16:0/18:2
PE-p 16:0/18:3
PE-p 18:0/18:0
PG 16:0/16:0
Normalized concentration
T1
T2
*


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
Observed
statistic
p = 0.8695 (1739/2000)
Frequency
Permutation test statistics


LPE 16:0
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
*