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Corresponding Author:	Barbara Bojko Uniwersytet Mikolaja Kopernika w Toruniu Bydgoszcz, kujawsko-pomorskie POLAND
Corresponding Author's Institution:	Uniwersytet Mikolaja Kopernika w Toruniu
Corresponding Author E-Mail:	bbojko@cm.umk.pl
Order of Authors:	Barbara Bojko
	Iga Stryjak
	Natalia Warmuzińska
	Kamil Łuczykowski
	Matyas Hamar
	Peter Urbanellis
	Emilia Wojtal
	Marek Masztalerz
	Markus Selzner
	Zbigniew Włodarczyk
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1 **TITLE:**
2 Using a Chemical Biopsy for Graft Quality Assessment
3
4 **AUTHORS:**
5 Iga Stryjak[1*], Natalia Warmuzińska[1*], Kamil Łuczykowski[1], Matyas Hamar[2], Peter Urbanellis[2],
6 Emilia Wojtal[3], Marek Masztalerz[3], Markus Selzner[2,4], Zbigniew Włodarczyk[3], Barbara Bojko[1]
7
8 [1]Department of Pharmacodynamics and Molecular Pharmacology, Faculty of Pharmacy,
9 Nicolaus Copernicus University in Toruń, Collegium Medicum in Bydgoszcz, Poland
10 [2]Multi Organ Transplant Program, Department of Surgery, Toronto General Hospital,
11 University Health Network, Toronto Canada
12 [3] Department of Transplantation and General Surgery, University Hospital, University of
13 Nicolaus Copernicus Torun, Bydgoszcz, Poland
14 [4]Department of Medicine, Toronto General Hospital Toronto, Toronto, Canada
15
16 [*]These authors contributed equally to this work
17
18 i.stryjak@cm.umk.pl
19 n.warmuzinska@cm.umk.pl
20 k.luczykowski@cm.umk.pl
21 mahamar@gmail.com
22 peter.urbanellis@queensu.ca
23 terenia4@interia.pl
24 masztalerz@cm.umk.pl
25 Markus.Selzner@uhn.ca
26 wlodarczyk@cm.umk.pl
27 bbojko@cm.umk.pl
28
29 **CORRESPONDING AUTHOR:**
30 Barbara Bojko
31 bbojko@cm.umk.pl
32

33 **KEYWORDS**:
34 transplantation, kidney, chemical biopsy, metabolomic, lipidomic, solid phase
35 microextraction (SPME), liquid chromatography coupled with mass spectrometry (LC-MS)
36

37 **SUMMARY:**
38 The protocol presents utilization of the chemical biopsy approach followed by comprehensive
39 metabolomic and lipidomic analysis for quality assessment of kidney grafts allocated for
40 transplantation.
41

42 **ABSTRACT:**
43 Kidney transplantation is a life-saving treatment for a large number of people with end-stage
44 renal dysfunction worldwide. The procedure is associated with an increased survival rate
45 and greater quality of patient's life when compared to conventional dialysis. Regrettably,
46 transplantology suffers from a lack of reliable methods for organ quality assessment. Standard
47 diagnostic techniques are limited to macroscopic appearance inspection or invasive tissue

48 biopsy, which do not provide comprehensive information about the graft. The proposed
49 protocol aims to introduce solid phase microextraction (SPME) as an ideal analytical method
50 for comprehensive metabolomics and lipidomic analysis of all low molecular compounds
51 present in kidneys allocated for transplantation. The small size of the SPME probe enables
52 performance of a chemical biopsy, which enables extraction of metabolites directly from the
53 organ without any tissue collection. The minimum invasiveness of the method permits
54 execution of multiple analyses over time: directly after organ harvesting, during its
55 preservation, and immediately after revascularization at the recipient's body. It is
56 hypothesized that the combination of this novel sampling method with a high-resolution mass
57 spectrometer will allow for discrimination of a set of characteristic compounds that could
58 serve as biological markers of graft quality and indicators of possible development of organ
59 dysfunction.
60
61 **INTRODUCTION:**
62 According to the United States' Organ Procurement and Transplantation Network, there were
63 94,756 patients waiting for kidney transplants in the US in 2019; while in Europe in 2018, that
64 number was 10,791. Every ten minutes, someone is added to the national transplant waiting
65 list in the U.S., and it is estimated that 20 people die each day waiting for a transplant[1,2].
66 Kidney transplantation is a life-saving treatment for a large number of people suffering with
67 end-stage renal dysfunction worldwide. The procedure is associated with increased survival
68 rate and greater quality of life when compared to conventional dialysis.
69
70 However, transplantation faces many serious problems, such as organ shortage or lack
71 of effective tools for organ quality assessment. The standard protocols are limited to
72 macroscopic appearance inspection or invasive tissue biopsy, which do not provide
73 comprehensive information regarding the quality of the graft. While a visual appraisal allows
74 for identification of tumors visible to the eye, anatomical abnormalities, or extensive damage
75 to the grafts, this approach is very subjective, varying in its effectiveness according to the
76 experience of the observers. Biopsy, on the other hand, can provide valuable information
77 regarding pre-existing renal disorders, and is thus considered to be a method of objective and
78 evidenced value in determining graft outcomes. However, the biopsy procedure is not free of
79 flaws; there is the risk of potential complications such as bleeding and an additional 4-5 hours
80 of sample preparation is required, which significantly prolongs the cold ischemic time.
81 Therefore, especially in Europe, the use of direct tissue analysis is limited to expanded criteria
82 donors (ECD) and donors after circulatory death (DCD)[3,4].
83
84 Metabolomics and lipidomics have been recently recognized as promising approaches to
85 attaining a better understanding of the changes in biochemical pathways occurring during
86 organ preservation. Metabolomic and lipidomic profiling enables monitoring of immediate
87 responses of the system to sudden environmental changes related to organ removal with
88 subsequent consequences: ischemia, oxidative stress, or inflammatory responses[5,6,7,8]. The
89 kidney is an organ that is largely associated with metabolic processes, thus measurements of
90 metabolites and lipids concentrations may permit identification of potential organ quality
91 biomarkers and enable better predictions of graft outcome.
92
93 Given the above complications and limitations associated with current organ quality
94 assessment methods, a less invasive diagnostic solution is needed for quick and complex organ

quality assessment. Solid phase microextraction (SPME) complies with these requirements as a minimally invasive analytical method that enables coverage of a broad spectrum of metabolites and lipids. The technique is based on the insertion of a thin (~200 µm), biocompatible, titanium-nickel alloy probe covered with a selective extraction phase into the examined organ for a short time. It should be emphasized that SPME prevents protein extraction, and hence enables metabolism inhibition already at the stage of sample collection, which is a significant advantage over alternative methods. Moreover, the miniaturization of the device allows for the execution of repetitive and simultaneous analyses of few structures of the organ[9,10,11].

PROTOCOL:

All animals received humane care in compliance with the ''Principles of Laboratory Animal Care'' formulated by the National Society for Medical Research and the ''Guide for the Care of Laboratory Animals'' published by the National Institute of Health, Ontario, Canada. The Animal Care Committee of the Toronto General Research Institute approved all studies. The research involved human subjects was approved by the Bioethical Committee at Collegium Medicum in Bydgoszcz Nicolaus Copernicus University in Torun.

NOTE: Obtain approval from appropriate ethical boards. Remember to always wear safety gloves. Do not touch the extraction phase of SPME probes. The use of deactivated glass vials is recommended for lipidomic analyses.

1.   Preparation of probes

1.1.   Prepare titanium-nickel alloy probes (40 mm length; 0.2 mm diameter) coated with 7 mm mix-mode sorbent. The number of probes depends on the time points targeted during the entire procedure and the number of replicates (3 is recommended per time point).

NOTE: The length and type of extraction phase may be adjusted based on the mode of study, the polarity of metabolites, and the sample matrix.

1.2.   Prepare a cleaning mixture composed of 2:1 chloroform:methanol (v/v). Pipet 1.0 mL of the solution to each 2.0 mL glass vial and place one probe, previously pierced through the cap, in each vial.

NOTE: Before use, clean all probes to remove contaminating particles.

1.3.   Put the vials on the agitator and set the agitation speed at 1,200 rpm. After 45 min, stop the device and rinse the coatings with LC-MS grade water.

1.4.   As coatings must undergo a preconditioning step to activate them, prepare a preconditioning mixture composed of 1:1 methanol:water (v/v). Pipet 1.0 mL of the solution to each 2.0 mL glass vial and place one probe, previously pierced through the cap, in each vial.

1.5.   Put the vials on the vortex agitator and set the agitation speed at 1,200 rpm.

142   1.6.   After 60 min, stop the agitator and rinse the coatings with LC-MS grade water.

144   1.7.   Sterilize probes according to the standard surgical equipment sterilization protocol.

146   **2.   Extraction**

148   2.1.   Open the sterile package right before sampling to ensure a sterile environment.

150   2.2.   Insert two probes directly into the kidney cortex for 10 min at each time point. The
151   entire length of the coating must be covered by the tissue matrix; no specific angle is required,
152   but ca. 90 deg is usually used.

154   NOTE: The entire medical procedure follows standard protocols in given institution. No
155   modification concerning SPME sampling is considered. The procedure involves the six
156   following sampling time points:
157   a)   before kidney resection, in vivo from donor;
158   b)-e) after 1 h, 3 h, 5 h, 7 h of kidney perfusion, ex vivo in organ chamber;
159   f) after reperfusion, in vivo from recipient.

161   2.3.   Retract the probes by pulling it out from the tissue and then rinse coatings with LC-
162   MS grade water to clear any remaining blood from the coating surface. Rinse away from the
163   surgical site, and immediately after removal of the probes.

165   **3.   Transport and storage**

167   3.1.   Place probes in separate vials and close them.

169   3.2.   Place vials in a Styrofoam box filled with dry ice or in liquid nitrogen for transport.

171   3.3.   Store samples in a freezer (-80 °C), or immediately commence the desorption step.

173   **4.   Desorption**

175   4.1.   Prepare desorption solutions composed of 80:20 acetonitrile:water (v/v) for
176   metabolomic analysis, and 1:1 isopropanol:methanol (v/v) for lipidomic analysis.

178   4.2.   Pipet 100 µL of the solution to inserts placed into 2.0 mL labeled vials and place one
179   probe, previously pierced through the cap, in each vial.

181   4.3.   Put the vials on the vortex agitator and set the agitation speed at 1,200 rpm for 120
182   min.

184   4.4.   Remove probes from the vials. Obtained extracts are now ready for instrumental
185   analysis.

187   **5.   LC-MS analysis**

189
190

191 NOTE: Liquid chromatography (RP, HILIC) coupled with high resolution mass spectrometry and
192 an orbitrap mass analyzer were used for this study. For metabolomic analysis parameters, go
193 to step 5.2. For lipidomic analysis parameters, go to step 5.6.

194

195 5.2.   Use a pentafluorophenyl (PFP) column (2.1 mm x 100 mm, 3 μm) for reversed phase
196 separation.

197

198 5.2.1.   Set the flow rate to 300 μL/min, and autosampler and column temperatures to 4 °C
199 and 25 °C, respectively.

200

201 5.2.2.   Prepare mobile phases according to the following proportions: mobile phase A:
202 water:formic acid (99.9:0.1, v/v), and mobile phase B: acetonitrile:formic acid (99.9:0.1, v/v).
203 Set the mobile phase flow according to the following parameters: starting mobile phase flow
204 (0-3 min): 100% A followed by a linear gradient to 10% A (3-25 min), ending with isocratic flow
205 of 10% A until 34 min, followed by 6 min of column re-equilibrium time.

206

207 5.3.   For HILIC separation, use a HILIC column (2.0 mm x 100 mm, 3 μm, 200A). Set the flow
208 rate to 400 μL/min.

209

210 5.3.1.   Prepare mobile phases according to the following proportions: mobile phase A:
211 acetonitrile:ammonium acetate buffer (9:1, v/v, effective salt concentration 20 mM), mobile
212 phase B: acetonitrile:ammonium acetate buffer (1:1, v/v, effective salt concentration 20
213 mM).

214

215 5.3.2.   Set the mobile phase flow according to the following parameters: starting mobile
216 phase flow (0-3 min) at 100% A, hold for 3.0 min and then ramp to 100% B within 5 min.
217 Hold with 100% B until 12 min, followed by 8 min of column re-equilibrium time.

218

219 5.4.   Set the scan range to 85-1000 m/z. Set HESI ion source parameters in positive
220 ionization mode to: spray voltage 1,500 V, capillary temperature 300 °C, sheath gas 40 a.u.,
221 aux gas flow rate 15 a.u., probe heater temperature 300 °C, S-Lens RF level 55%.

222

223 5.5.   Run QC samples composed of 10 μL of each analyzed sample (regularly, every 10-12
224 samples) to monitor instrument performance.

225

226 5.6.   For reversed phase separation, use a C18 column (2.1 mm x 75 mm, 3.5 μm).

227

228 5.6.1.   Set the flow rate to 200 μL/min, and autosampler and column temperatures to 4 °C
229 and 55 °C, respectively. Prepare mobile phases according to the following proportions:
230 mobile phase A: H_2O:MeOH (60:40, v/v), 10 mM ammonium acetate and 1 mM acetic acid;
231 mobile phase B: IPA:MeOH (90:10, v/v), 10 mM ammonium acetate and 1 mM acetic acid.

232

233 5.6.2.   Set the mobile phase flow according to the following parameters: 0-1 min (20% B), 1-
234 1.5 min (20-50% B), 1.5-7.5 min (50-70% B), 7.5-13 min (70-95% B), 13-17 min (95% B), 17-
235 17.1 min (95-20% B), 17.1-23 min (20%).

236

237   5.7.   For HILIC separation, use a HILIC column (100 x 2.1 mm, 3 μm).

238

239   5.7.1. Set the flow rate to 400 μL/min, and autosampler and column temperatures to 4 °C
240   and 40 °C, respectively.

241

242   5.7.2. Prepare mobile phases according to the following proportions: mobile phase A: ACN;
243   mobile phase B: 5 mM ammonium acetate in water. Set the mobile phase flow according to
244   the following parameters: 0-2 min (96% B), 2-15 min (96-80% B), 15-15.1 min (80-96% B), 15.1-
245   21 min (96% B).

246

247   5.8.   Set HESI ion source parameters in positive ionization mode to spray voltage 3,500 V,
248   capillary temperature 275 °C, sheath gas 20 a.u., aux gas flow rate 10 a.u., probe heater
249   temperature 300 °C, S-Lens RF level 55%.

250

251   5.9.   Run QC samples composed of 10 μL of each analyzed sample (every 10-12 samples) to
252   monitor instrument performance.

253

254   5.10.   Perform data acquisition in software compatible with the instrument.

255

256   **6.   Data analysis**

257

258   6.1.   Perform data processing, putative identification, and statistical analysis with the use
259   of software dedicated to untargeted metabolomics and lipidomics analysis.

260

261   NOTE: Principal components analysis (PCA) and box-whisker plots can be obtained to visualize
262   data structure.

263

264   **REPRESENTATIVE RESULTS:**
265   Sample collection was performed according to the SPME method described above, using
266   probes coated with a 7 mm mixed-mode extraction phase. Sampling was conducted directly
267   from the graft tissue (in vivo before transplantation), ex vivo in kidney chamber after 1 h, 3 h,
268   5 h, and 7 h of perfusion, and in vivo after revascularization in the recipient. Untargeted
269   metabolomic and lipidomic analyses were carried out with the use of liquid chromatography
270   (RP, HILIC) coupled with high resolution mass spectrometry, with the mass analyzer set in
271   positive ionization mode. To assess data quality and attain general insights regarding the
272   results, data was subjected to principal component analysis (PCA). As shown in **Figure 1**, QC
273   samples formed a tight cluster, confirming the quality of the analyses. The studied groups
274   exhibit relatively good separation, allowing for visualization of differences in metabolic and
275   lipidomic profiles before and after transplantation, as well as during organ perfusion (**Figure
276   2**). SPME sampling permits metabolic profiling of the organ over time. Box-whisker plots of
277   selected metabolites serve to exemplify alterations in metabolite levels throughout the
278   experiment (**Figure 3**). The wide spectrum of extracted features separated on both RP and
279   HILIC columns is shown in **Figure 4**.

280

281   **Figure 1. Principal component analysis showing clustering of quality control samples for
282   metabolomic reversed-phase (A), HILIC (B) and lipidomic reversed-phase (C), HILIC (D)**

283 **analyses.** The quality control is necessary in untargeted analysis to monitor any possible
284 changes induced by instrumental instability. Tight cluster of QC samples ensures that all
285 changes observed are of biological origin.
286
287 **Figure 2. Principal component analysis showing metabolomic (A) and lipidomic (B)**
288 **differences between particular sampling points.** Untargeted profiling of kidney with SPME-
289 LC-HRMS enables to observe biochemical differences in organs at subsequent steps of their
290 preservation.
291
292 **Figure 3. Box-whisker plots of selected compounds showing alterations in metabolites (A,**
293 **B) and lipids (C, D) during the peritransplant period.** After selection and identification of
294 discriminating compounds box-whisker plots enables to monitor changes in levels of these
295 compounds at subsequent steps of the protocol and compare the metabolites levels in organs
296 subjected to different preservation protocols or harvested from different donors e.g. heart
297 beating donors or donors after cardiac death.
298
299 **Figure 4. Ion map (m/z versus retention time) of features obtained by LC-MS analysis with**
300 **(A) reversed-phase and (B) HILIC separation.** The plot enables to estimate overall number of
301 features obtained with the proposed protocol (from extraction to detection) and assess
302 analyte coverage based on the polarity.
303
304 **DISCUSSION:**
305 Evaluation of organ quality remains a large challenge for physicians, who must make quick
306 informed decisions regarding whether a given organ is viable for transplantation or whether
307 it must be discarded. Multiple factors, such as donor age, duration of ischemia, and infections
308 and inflammatory processes, can affect the long-term graft outcome. While diverse methods
309 have been developed to date to diagnose kidney allograft function, histopathological
310 inspection remains the gold standard in that matter[3,4,12]. Although the biopsy procedure can
311 yield significant information regarding pre-existing donor disease and vascular changes, it is
312 not free of flaws. Sampling errors associated to interobserver variability and sampling of
313 insufficient glomeruli for comprehensive information regarding organ function remain typical
314 concerns in this regard. Moreover, specimen preparation brings some issues such as an
315 incomplete appraisal of the graft in case of frozen sections, and extension of procedure time
316 for paraffin sectioning. However, the increased risk of hemorrhage, which may appear acutely
317 as microscopic or gross hematuria, is the major life-threatening complication associated with
318 the biopsy procedure. For this reason, the number of allowable biopsies is strictly limited in
319 transplantation procedures, a factor that hampers the capture of dynamic changes and time
320 series analyses via this method[12,13,14]. The benefits of a histological analysis must be weighed
321 against the risks associated with the methodology. The value of histological findings is
322 indisputable, but they do not explain the molecular mechanisms of the aberrations.
323
324 Metabolomics and lipidomics are the youngest domains of the "-omics" scientific family. The
325 complete set of low molecular (<1,200 Da) human metabolites and lipids connected within a
326 metabolic network is defined as a human metabolome. The genome remains relatively
327 constant throughout its lifetime, with slight modifications caused by mutations occurring
328 infrequently. The metabolome is the product of gene expression, which is highly sensitive to
329 changes in all biological processes as well as environmental factors. The dynamic nature of

330 metabolites and lipids makes them perfect indicators of current organ condition[7,8,15,16]. The
331 SPME method proposed in the abovementioned protocol enables detection of changes
332 occurring in the organ during its preservation, starting from organ removal from the donor's
333 body till revascularization at the recipient's. The small diameter of the probe (~200 μm)
334 provides minimal invasiveness and allows for several samplings from the same organ without
335 causing any damage to the tissue. Conducting studies using kidney, as the most frequently
336 transplanted organ, allows for a better understanding and further characterization of the
337 metabolic pathways responsible for declining the quality and function of grafts. The possibility
338 of monitoring modifications over time certainly is an important advantage of the technique as
339 compared to conventional invasive methods such as biopsy. The currently presented analysis
340 identified altered concentrations of various groups of lipids and metabolites, especially of
341 essential amino acids, purines, purine nucleosides, and glycerophospholipids. These results
342 are consistent with previous tissue analysis reports[5,6,17,18,19,20]. To date, the majority of
343 scientific reports utilizing metabolomics or lipidomics to explain processes inducing
344 complications after transplantation or ischemia/reperfusion injury (IRI) phenomena have
345 been limited to analysis of biofluids[21,22,23].
346
347 Each clinical application requires optimization of the sampling protocol to ensure that the
348 performance of the analytical method meets expected criteria. In this regard, the benefit of
349 utilizing SPME is the possibility of adjusting conditions for various experimental designs. The
350 variety of accessible extraction phases provides a broad spectrum of extracted metabolites
351 with diversified polarities. At the same time, this might be considered as a limitation of the
352 method due to the fact that each sorbent provides selectivity towards specific features and
353 does not extract all compounds present in the sample matrix. It should be noted that SPME
354 coatings extract only via free molecules, and simply do not interact with a bound fraction of
355 the analyte. The biocompatibility of the coatings does not introduce toxicity to the tissue while
356 restraining the extraction of large molecules such as proteins; as a consequence, the
357 enzymatic processes are inhibited already at the stage of sample collection and the presence
358 of artefacts is minimized, which is a great advantage over alternative sampling methods. The
359 length of the coating influences the efficiency of extraction (i.e., the length of the coating
360 designates the surface area and the extraction phase volume); thus, longer coatings yield
361 higher recoveries. On the other hand, shorter coatings enable higher spatial resolution. For
362 reliable results, it is crucial to submerge the probe to the exact same depth of kidney cortex.
363 Insertion too deep causes the risk of entering the kidney medulla. The time of the extraction
364 is also proportional to the extraction efficiency. Therefore, selection of optimum extraction
365 time is one of the most critical steps in SPME method development. The accuracy of time
366 measurement provides the highest repeatability. In biological applications such as the one
367 discussed, there is always a compromise between the sensitivity and repeatability of the
368 analytical protocol and the restrictions of the medical procedure. While equilibrium extraction
369 provides the highest sensitivity, for safety reasons, pre-equilibrium conditions are often used
370 in such applications, as extraction time should not affect the total duration of the surgery. The
371 efficiency of desorption is determined by the time of the process and the composition of the
372 desorption solvent, which should be compatible with the mobile phase used for
373 chromatographic separation.
374
375 One of the major requirements for diagnostic instrumentation used for intra-surgical
376 assessments is time of analysis. Current attempts are being made to develop a rapid tool for

in vivo SPME extraction coupled directly to a mass spectrometer via microfluidic open interface (MOI)[24] or coated blade spray (CBS)[25]. Such approaches would allow for the disclosure of analytical results in real or close to real time. The use of such methods for pre-intervention analyses of metabolic and lipidomic profiles could enhance the decision-making process during transplantation procedures, enabling the best possible personalized approach and fast response in case of organ failure.

As a summary, it is hypothesized that the proposed protocol will enable attainment of full metabolic and lipidomic profiles of kidney grafts, which in turn would provide a comprehensive assessment of organ quality and characterization of the processes responsible for ischemia-reperfusion injury. The novelty of the project includes the utilization of solid-phase microextraction (SPME), offering low invasive sampling of living systems, in combination with one of the most innovative technologies available for metabolomics and lipidomics analysis (e.g., the Orbitrap high resolution mass spectrometer). SPME combines sample collection, extraction, and quenching of metabolites in one step, therefore making it a perfect tool for rapid analysis. It is expected that this protocol will help to answer questions related to what pre-transplant conditions of the kidney are responsible for delayed organ function or its dysfunctions after transplantation, as well as to how the graft preservation protocol influences the biochemistry of the organ. Such knowledge would not only have significant impact on the prevention of possible complications related to transplantation, but may help to improve current graft preservation protocols, minimizing loss of viable transplant tissue as well as loss of life. The proposed solution will open the door to further investigations in this field, including the validation of specific potential biomarkers and improvement of therapeutic outcomes in transplantology.

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472

Figure 1
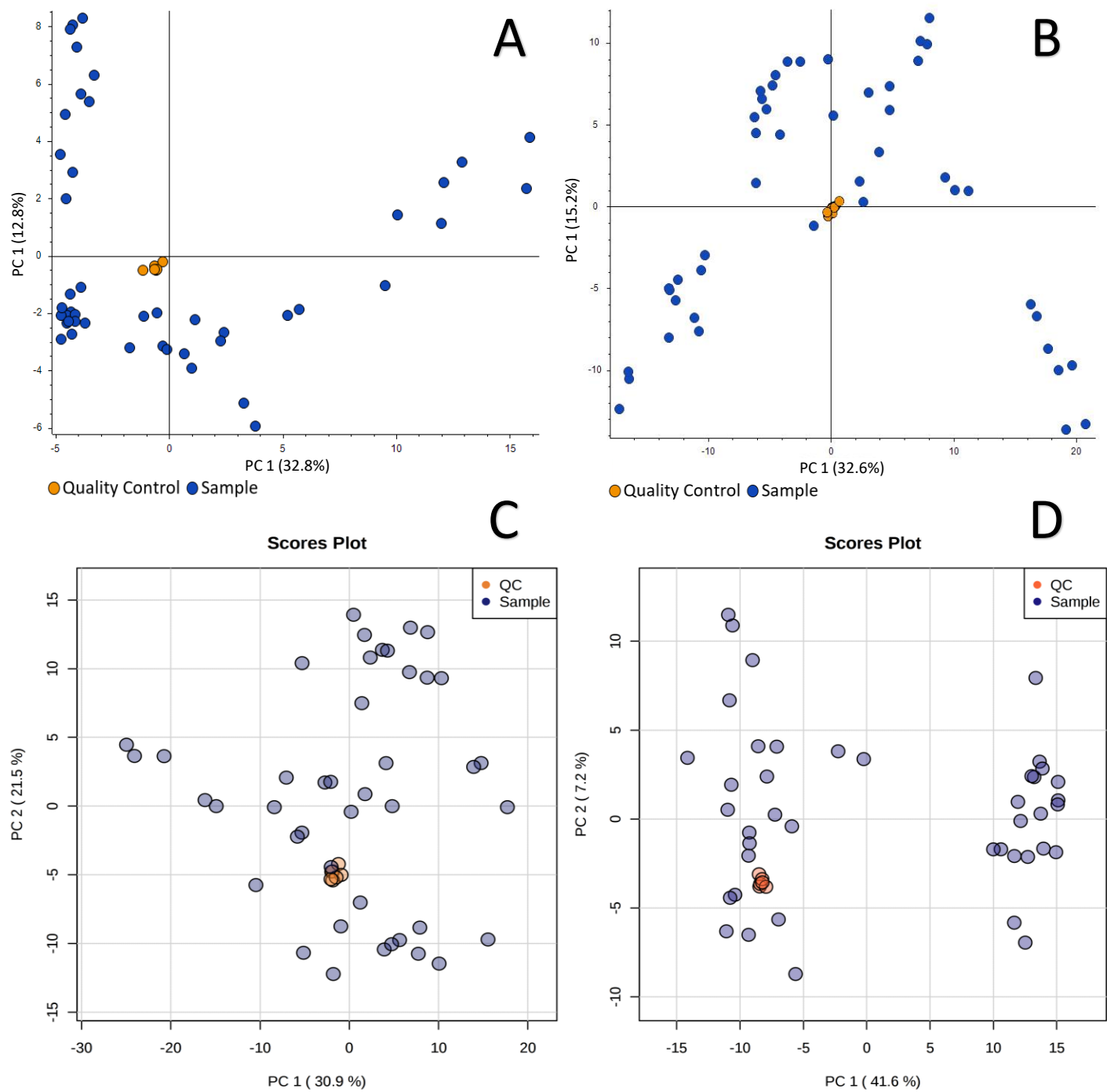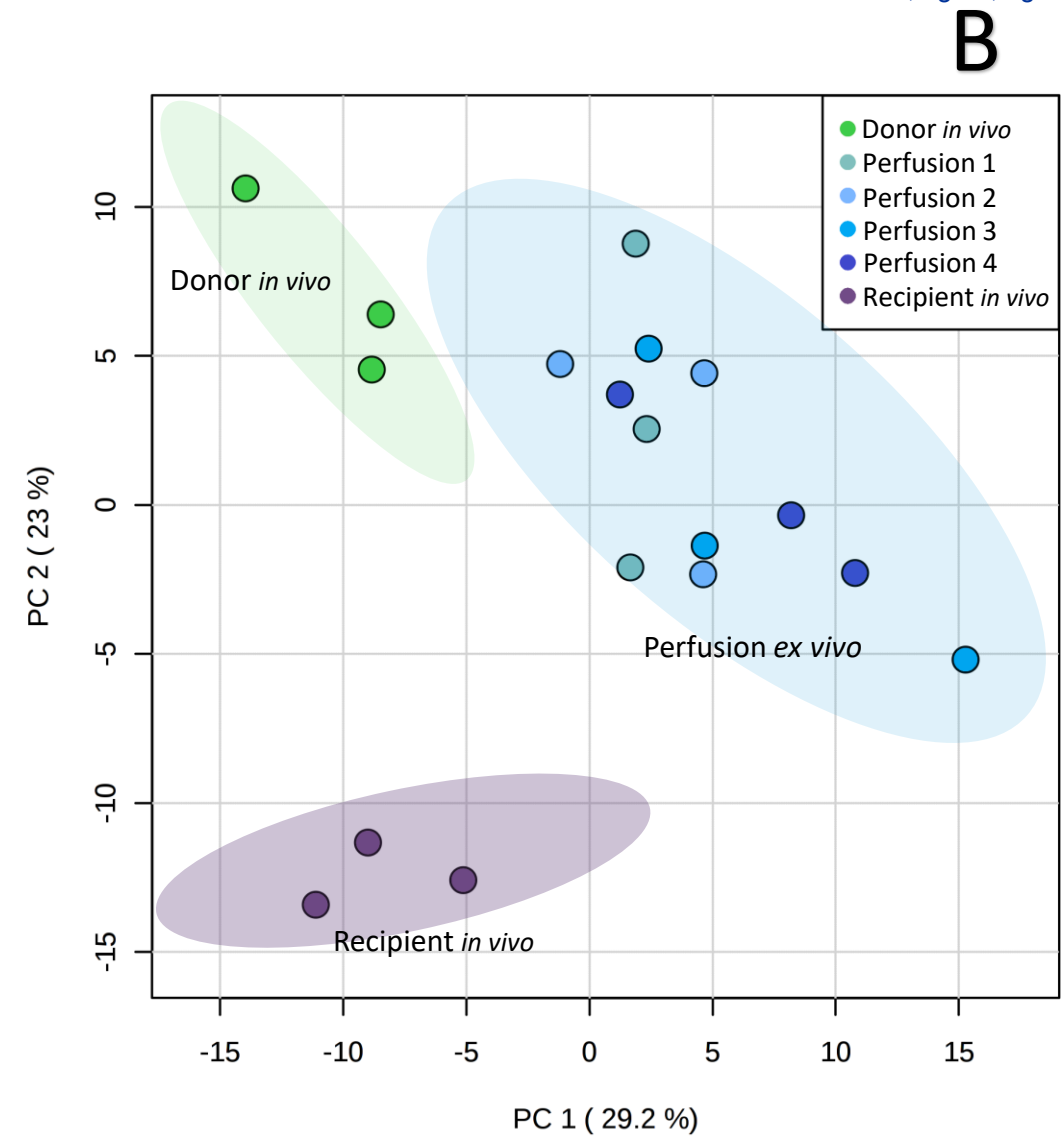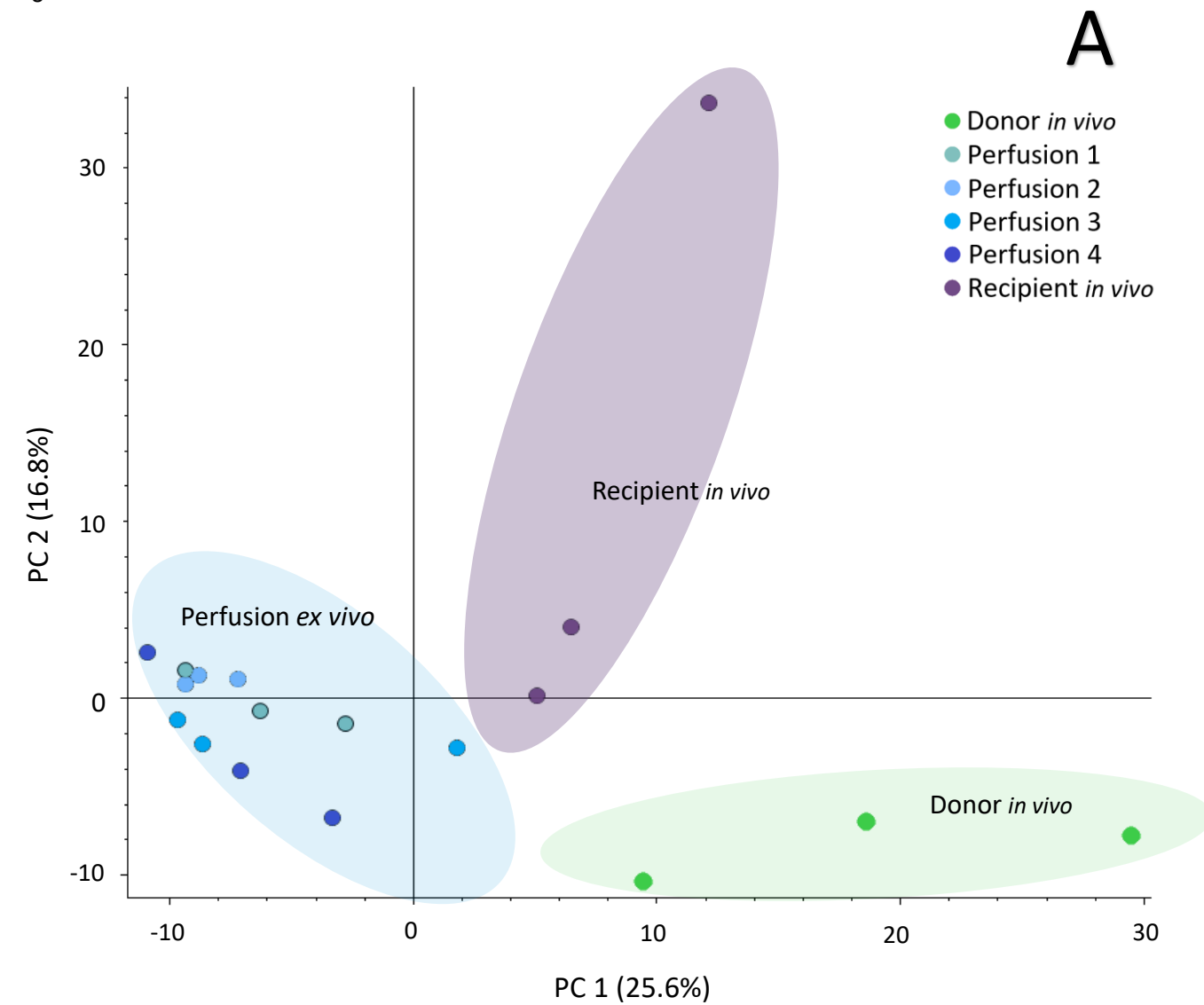
Figure 1

Figure 2
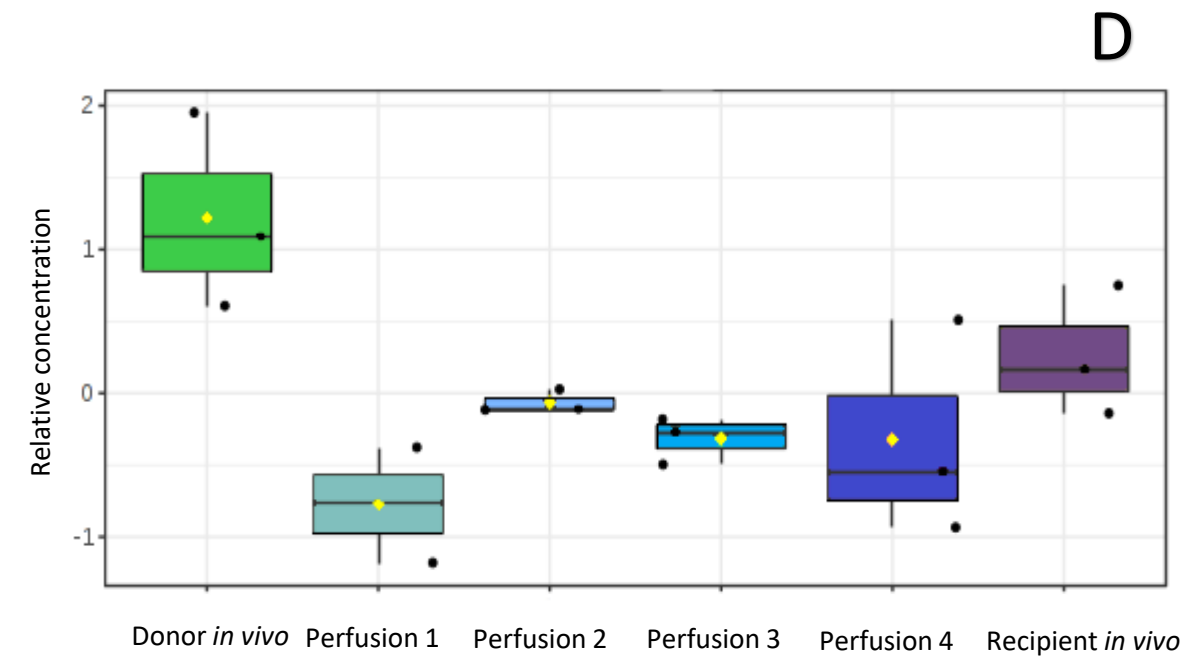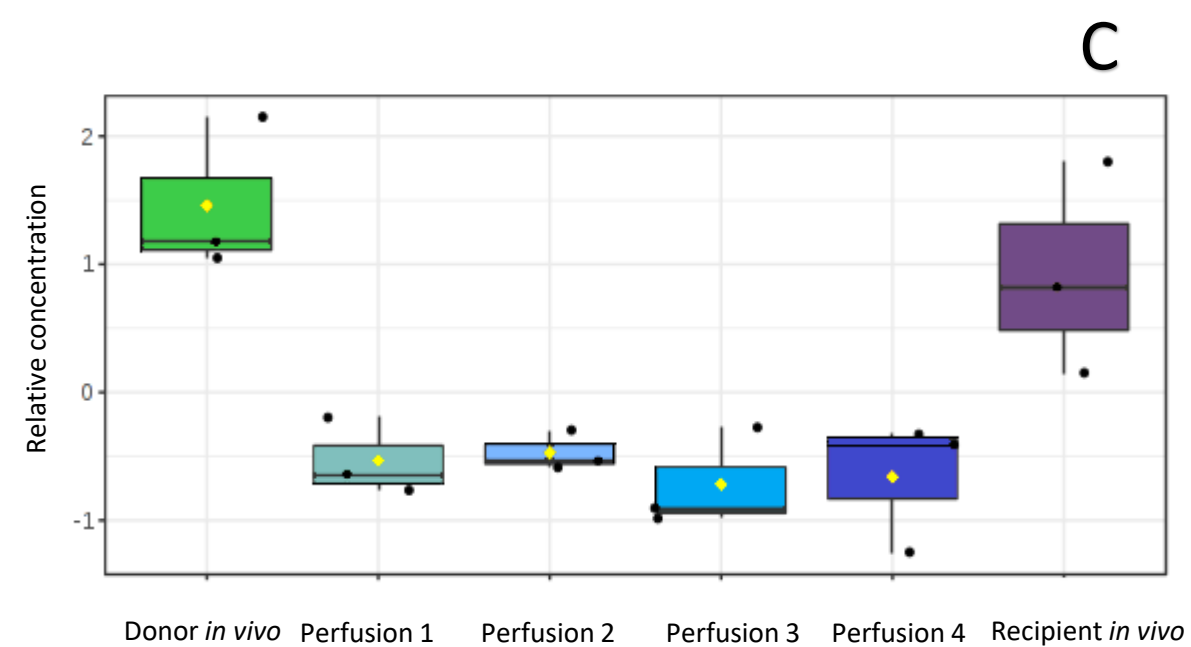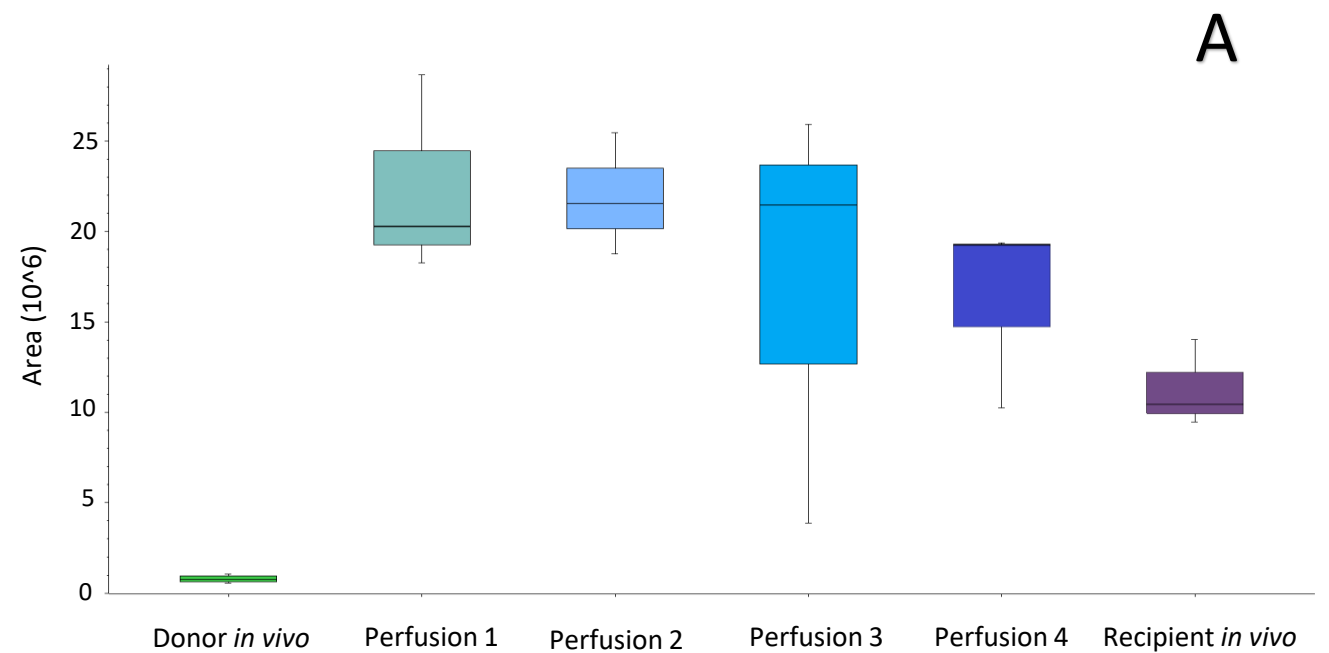
Figure 2

Figure 3

Figure 4

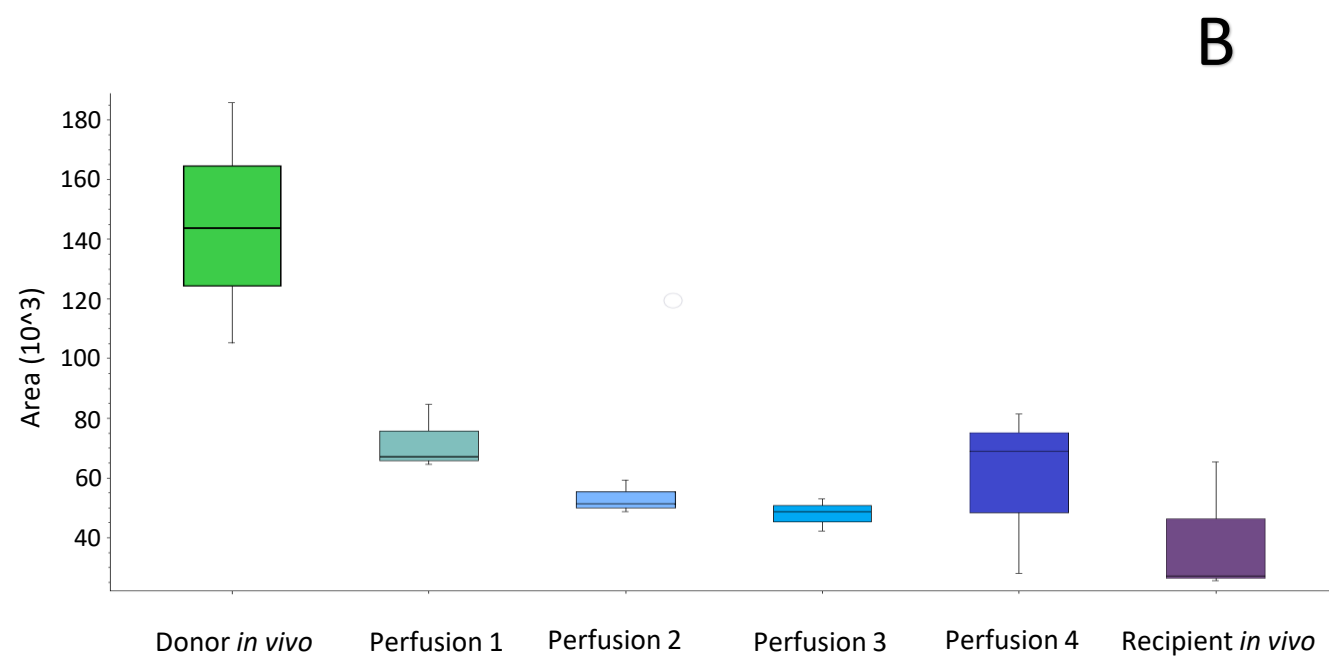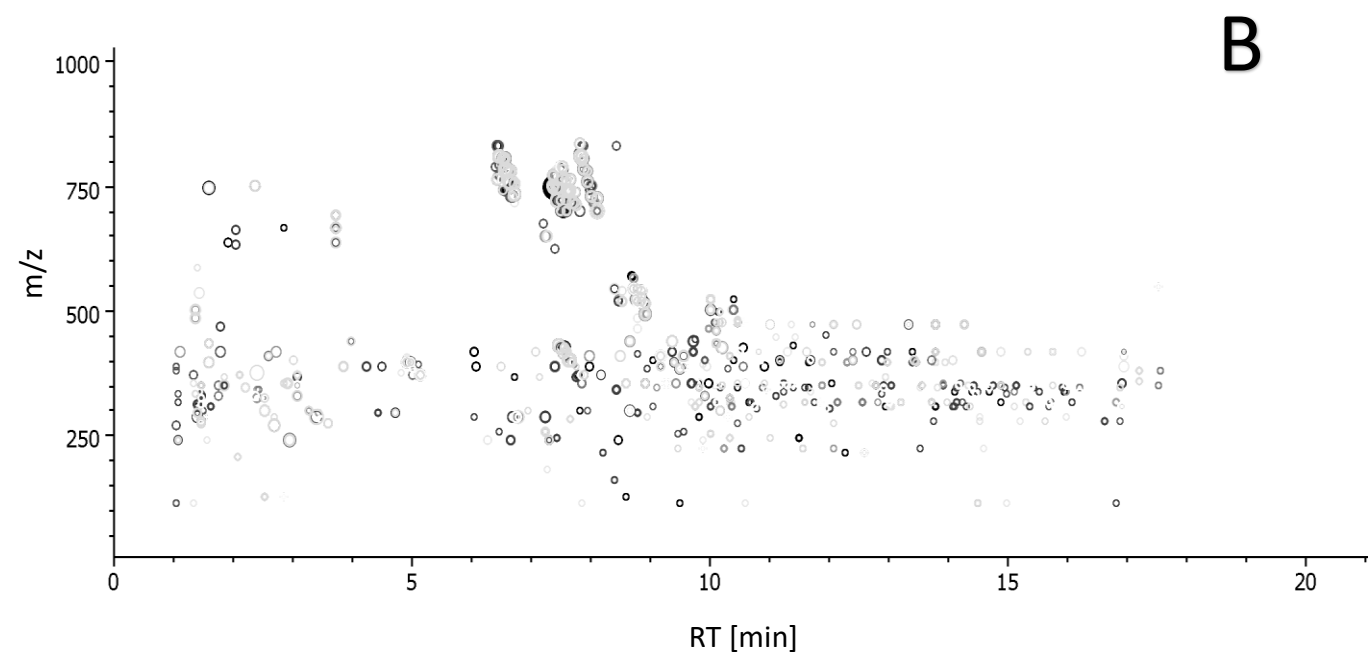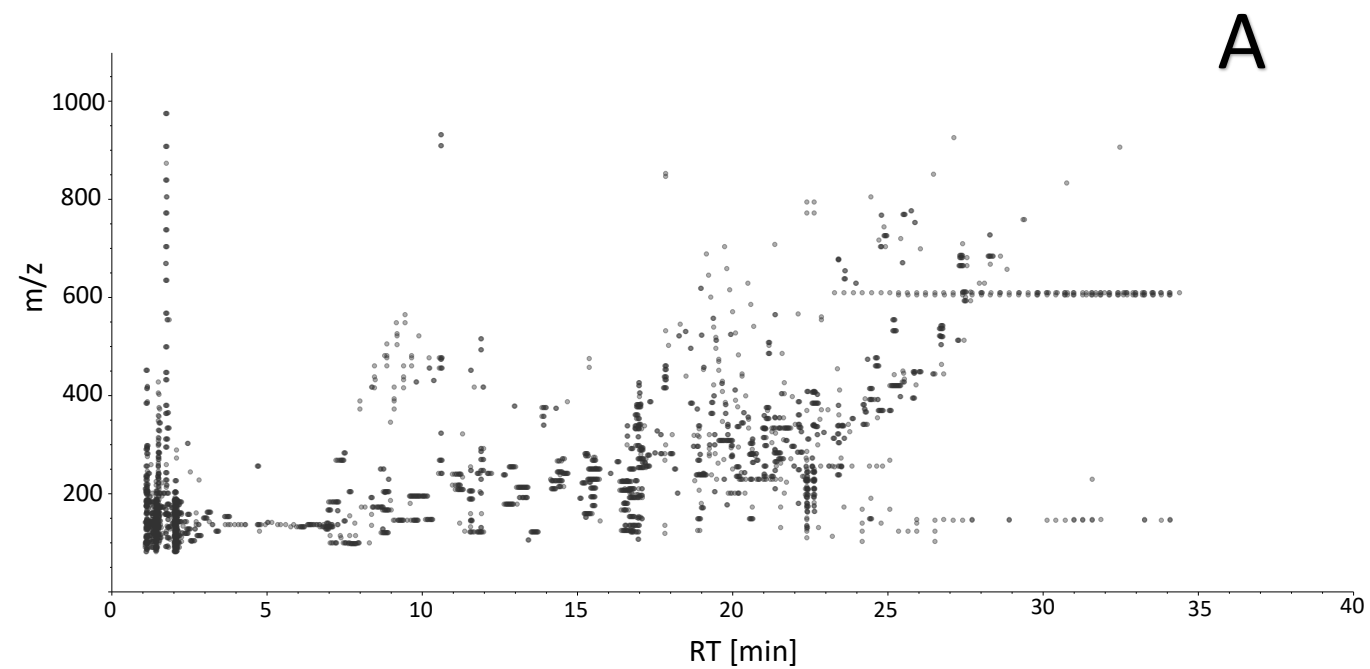| Name of Material/Equipment	Company	Catalog Number	Comments/Description
Acetic acid	Merck	5330010050	Mobile phase additive
Acetonitrile	Alchem	696-34967-4X2.5L	HPLC solvent
Ammonium acetate	Merck	5330040050	Mobile phase additive
BENCHMIXER XL MULTI-TUBE VORTEXER	Benchmark Scientific	BV1010	Vortex mixer
Caps	Perlan Technologies	5183-2076	Blue scrw tp, pre-slit PTFE/Si spta, 100PK
Chloroform	Merck	1024441000	
Discovery HS F5 Supelguard Cartrid	Merck	567570-U	HPLC guard column
Discovery HS F5, 2.1 mm x 100 mm	Merck	567502-U	HPLC column
Formic acid	Alchem	497-94318-50ML	Mobile phase additive
Glass vials	Perlan Technologies	5182-0714	
HILIC Luna 3 µm, 200A, 100 x 2.0 mm	Shim-Pol	PHX-00D-4449-B0	HPLC column
HILIC SecurityGuard Cartridge, 3 µm, 4 x 2.0 mm	Shim-Pol	PHX-AJ0-8328	HPLC guard column
Isopropanol	Alchem	231-AL03262500	HPLC solvent
Methanol	Alchem	696-34966-4X2.5L	HPLC solvent
Nano-pure water	Merck	1037281002	HPLC solvent
Q Exactive Focus hybrid quadrupole-Orbitrap MS	Thermo Scientific	Q Exactive Focus	Mass Spectrometer
SeQuant ZIC-cHILIC 3µm,100Å 100 x 2.1 mm	Merck	1506570001	HPLC column
SeQuant ZIC-HILIC Guard Kit 20 x 2.1 mm	Merck	1504360001	HPLC guard column
SPME LC fiber probes, mixed mode	Supelco	prototype fibers	
UltiMate 3000 HPLC systems	Thermo Scientific	UltiMate 3000	HPLC system
Vial inserts (deactivated)	Perlan Technologies	5181-8872	
XSelect CSH C18 3.5µm 2.1x75mm	Waters	186005644	HPLC column
XSelect CSH C18 VanGuard Cartridge 3.5µm, 2.1x5mm	Waters	186007811	HPLC guard column

Department of Pharmacodynamics and Molecular Pharmacology

Dr. A. Jurasza 2 , 85-089 Bydgoszcz, POLAND
tel. +48 52 585-35-64, 585-35-26;
e-mail: bbojko@cm.umk.pl

NICOLAUS COPERNICUS UNIVERSITY IN TORUŃ
Faculty of Pharmacy
Collegium Medicum in Bydgoszcz

Bydgoszcz, 18/01/2020

Dear Editor,

On behalf of all authors I would like to thank for consideration of our manuscript to be published in JoVe. Based on the Editor's and Reviewers' comments we have prepared a revision of our manuscript. Manuscript has been corrected by English editor and all the comments and suggestions were addressed.

Editorial Comments:

• The manuscript will benefit from thorough language revision as there are a number of grammatical errors throughout. Please thoroughly review the manuscript and edit any errors.

Reply: The manuscript was corrected by English editor (native speaker).

• Title: Please avoid the split title, i.e., remove the colon.

Reply: The title has been modified

• Protocol Language: Please ensure that ALL text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. "Do this", "Measure that" etc.) Any text that cannot be written in the imperative tense may be added as a "Note", however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.
1) Step 5.3 (lines 145-161) must be re-written. Please also split up this steps into 4 steps.
2) Step 5.4 (lines 145-161) must be re-written. Please also split up this steps into 4 steps.

Reply: The protocol language has been modified according the Editor's comments

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Some examples:
1) Please include an ethics statement before your numbered protocol steps indicating that the protocol follows the guidelines of your institutions human research ethics committee.
2) 1.1.: What kind of probes are these? Describe the material and all relevant specifications (e.g., size, diameter etc)
3) 1.2: Which fibers? What are their specifications? how many fibers?
4) 2.2: Please briefly describe the surgical setting, preparation steps, sterile draping, anesthesia, surgical tools used etc. How is the kidney exposed? How exactly as the probes

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NICOLAUS COPERNICUS
UNIVERSITY
IN TORUŃ
Faculty of Pharmacy
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inserted? Is this a laparoscopic procedure? What is the anatomical insertion site? What is the depth and angle of insertion? How are the fibers retracted? Is the rinsing done away from the surgical site? Are the fibers exposed to air (drying) before the cleaning step?
5) 3.1: Unclear, please describe in detail.
6) 6.1: Cite references for each database.
7) 6.2: What kind of data processing and statistical analysis is to be performed? Please add reference citations.

Reply: Details to the SPME protocol were added. No information about the transplantation protocol itself was incorporated because it strictly depends on the given hospital/institute and any modifications or adaptation with regard to SPME sampling is necessary or recommended.

• Protocol Numbering: Please add a one-line space after each protocol step.

Reply: Numbering was applied

• Protocol Highlight: Please highlight ∼2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps.
1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.
2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.

Reply: We have highlighted fragments of the protocol for video recording based on the instructions from the Editor

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Reply: Discussion has been modified accordingly

• Figures:
1) Fig 1: Increase font size.
2) Fig 2, 3: Match axis tick sizes for consistency between the panels. Place the panel labels at the upper right corners.
3) Fig 3 A,B: axis labels and y-axis ticks are too tiny.
4) Fig 4A: Axis ticks and labels are too tiny.

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NICOLAUS COPERNICUS
UNIVERSITY
IN TORUŃ
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Reply: Figures have been modified based on Editor's comment

• Figure/Table Legends: Please expand the legends to adequately describe the figures/tables. Each figure or table must have an accompanying legend including a short title, followed by a short description of each panel and/or a general description.

Reply: Figures legends have been expanded

• References:
1) Use superscripted citation numbering (without brackets).
2) Please make sure that your references comply with JoVE instructions for authors. Citation formatting should appear as follows: (For 6 authors or less list all authors. For more than 6 authors, list only the first author then et al.): [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage, doi:DOI (YEAR).]
3) Please spell out journal names.

Reply: The references have been modified accordingly

• Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are nano-pure, Styrofoam, (Supelco Discovery HS, Luna HILIC, (Phenomenex, Waters, XSelect CSH, SeQuant ZIC-cHILIC, Xcalibur software v. 4.0., Compound Discoverer, Lipid Search 4.1, MetaboAnalyst 4.0, Q-Exactive Focus, etc.
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.
2) Please remove the registered trademark symbols TM/R from the table of reagents/materials.

Reply: The trademark symbols, company brand names and other commercial language have been removed from the text of the manuscript

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)"

Department of Pharmacodynamics and Molecular Pharmacology

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NICOLAUS COPERNICUS
UNIVERSITY
IN TORUŃ
Faculty of Pharmacy
Collegium Medicum in Bydgoszcz

section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Comments from Peer-Reviewers:

Reviewer #1:

Manuscript Summary:
The manuscript entitled "Chemical biopsy: a new tool for graft quality assessment" presents a detailed and well-thought-out experimental procedure for mapping the in-vivo metabolome of organs for transplant. The experiments are very well designed and the results of the study are carefully interpreted, resulting in a very strong application of a new in-vivo sampling technique.

Major Concerns:
1. Abstract and several times in the article: since 'lipidomics' is a subset of metabolomics, it is erroneous to say that the authors performed both lipidomics and metabolomics

Reply: Indeed, lipidomics is technically the subset of lipidomics, but because of the complexity of lipidome and analytical requirements related to lipids analysis lipidomics has been separated from metabolomics in recent years and currently is most often considered sepatately.
Please see some references below:
- **Cajka T., Fiehn O. Toward Merging Untargeted and Targeted Methods in Mass Spectrometry-Based Metabolomics and Lipidomics. Anal. Chem. 2016, 88, 524**
- **Yu K., Astarita G. New Frontiers for Mass Spectrometry in Lipidomics. LCGC Europe 2012, 25, 154**
- **Wenk M.R. Lipidomics: New Tools and Applications. Cell 2010, 143, 888**

Minor Concerns:
1. Line 123: the area/location of/no the kidney should be mentioned
Reply: More details were added
2. Lines 157-161: instrument-specific parameters are mentioned, but the actual MS instrument has not been specified
Reply: Instrument specification is available in the Table of materials associated with the manuscript. Based on the journal rules no brand names or trademarks can be present in the text of the manuscript
3. Line 186: coating length is different from what was previously mentioned
Reply: Thank you for this note. The mistake has been corrected.
4. Figure 3: unclear what the y axis is
Reply: The figure has been improved
5. Figure 4: the two sections A and B look very different (not content, but the symbols and font size)
Reply: The figure has been improved

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UNIVERSITY
IN TORUŃ
Faculty of Pharmacy
Collegium Medicum in Bydgoszcz

The authors would like to thank both Reviewers for their positive reviews and constructive comments. Below we present detail reply to individual comments:

Reviewer #2:

Manuscript Summary:

Chemical biopsy: a new tool for graft quality assessment is an interesting paper and I suggest to publish it.

The novelty of the project includes the utilization of solid-phasemicroextraction (SPME) offering a low invasive sampling of living systems in combination with the innovative technology used for metabolomics and lipidomics analysis.

There is huge need to increase the number of potential donors but what is also very important to prolong the transplant survival.

The "chemical biopsy" has the potential to create a biological markers which may help clinicians in their routine practise.

Major Concerns:
No

Minor Concerns:
No

We would like to add authors' contribution to the final version if manuscript is accepted for publication, but provided template does not include specific section for that. Therefore I submitted list of contributions to the publication office asking to add it in appropriate section during final edition.

We hope the manuscript will meet the requirements of JoVE journal and will be considered for publication in current version.

Sincerely yours,
Barbara Bojko, PhD, D.Sc.
Associate Professor